The effect of *Pseudomonas*-released quinolones on the mTOR pathway and phagocytic bacterial killing

By
Stephen Lanahan

Senior Honors Thesis
Biology
University of North Carolina at Chapel Hill

10/25/16

Approved:

Amy Shaub Maddox,
Thesis Advisor
Ann Matthysse, Reader
Melinda Beck, Reader

Acknowledgements

I would like to acknowledge the following individuals for help throughout the creation of this thesis:

Dr. Matthew Wolfgang
Dr. Cindy Gode
The rest of the Wolfgang Lab
Dr. Amy Shaub Maddox
Dr. Bob Duronio

I would also like to acknowledge the following sources of funding throughout my undergraduate research career:

National Institutes of Health
Cystic Fibrosis Foundation
UNC's Summer Undergraduate Research Fellowship

Abstract

Cystic fibrosis (CF) patients are immunocompromised and often suffer from polymicrobial infections involving *Pseudomonas aeruginosa,* an opportunistic bacterial pathogen. *P. aeruginosa* pathogenicity is influenced by the secretion of bacterial compounds such as quinolones. *P. aeruginosa* secretes at least 50 different quinolone-based molecules. Although two of these, PQS and HHQ, are signals for a bacterial quorum sensing system, there are no known roles for the majority of the quinolones. A third quinolone molecule, HQNO, is secreted at high levels in culture. Preliminary data using macrophages suggest that HQNO inhibits respiratory burst, the process by which eukaryotic cells kill phagocytosed bacteria with reactive oxygen. Respiratory burst is known to be regulated by the mammalian target of rapamycin pathway (mTOR). We hypothesize that HQNO inhibits mTOR and thus the ability of macrophages to kill bacteria such as *P. aeruginosa* and the secondary pathogen *Burkholderia multivorans*. We used western blotting to demonstrate that in HQNO-treated mouse embryonic fibroblasts (MEFs), phosphorylation of the mTOR inhibitor AMPK was increased, and phosphorylation of the downstream mTOR effector, S6 ribosomal protein was reduced, in a time and dose dependent manner. Next, we tested whether bacterial killing by RAW 264.7 macrophages depended on HQNO. We found that HQNO treatment decreased killing of *P. aeruginosa* and *B. multivorans* by RAW macrophage cells. HQNO more strongly suppresses the ability of macrophages to kill *B. multivorans* than *P. aeruginosa*. Thus, the production of *P. aeruginosa* quinolones may protect secondary pathogens, such as *B. multivorans*, during polymicrobial infection in CF patients.

Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disorder caused by a mutation of the CFTR gene. Defective CFTR results in the build up of thick mucus in the airways, pancreas, and other mucus secreting organs. CF affects approximately 70,000 people worldwide. Currently, the median life expectancy for individuals with CF in the United States is 40 years old; approximately half the normal life expectancy. CF leaves the host susceptible to frequent and chronic lung infection by opportunistic pathogens [1]. *Pseudomonas aeruginosa* is the most common pathogen that infects CF patients.

Pseudomonas aeruginosa is a gram-negative mucosal bacterium that often infects immunosuppressed and immunocompromised individuals [2]. The production and secretion of small molecules play an integral role in the pathogenicity of many bacteria. Previous studies have shown that *P. aeruginosa* releases at least 50 different quinolone-like molecules. Although two of these, PQS and HHQ, are signals for a quorum sensing system (cell-to-cell signaling), there are no known roles for the majority of these quinolones in *Pseudomonas*. A third quinolone molecule, HQNO, is secreted at high levels in culture. PQS, HHQ, and HQNO have all been detected in the sputum of CF patients when infected with *P. aeruginosa* [3][4].

While *P. aeruginosa* is the primary pathogen in the CF lung, CF airway infections are polymicrobial and often involve the acquisition of other secondary pathogens including *Burkholderia* species. It is believed that the polymicrobial nature of CF airway infections contributes to a rapid decline in lung function [5]. *Burkholderia* species, unlike *P. aeruginosa*, are highly susceptible to killing by reactive oxygen released during respiratory burst, which is a process that delivers reactive oxygen species into a pathogen containing vacuole [6]. Previous research in the Wolfgang lab suggests that HQNO inhibits respiratory burst in macrophages. We hypothesize that *P. aeruginosa* produced HQNO inhibit the ability of macrophages to kill *Burkholderia multivorans,* thus facilitating polymicrobial infection in CF.

The primary host enzyme that generates reactive oxygen is NADPH oxidase [7]. The synthesis of NADPH oxidase and thus respiratory burst is regulated through the mammalian Target of Rapamycin pathway (mTOR) [8]. The mTOR pathway plays a central role in controlling a wide variety of important activities in the innate and adaptive immune

system [9][4]. mTOR is controlled by a number of protein kinases, including AMP-activated protein kinase (AMPK) [10]. AMPK is a kinase naturally active when there is a high AMP to ATP ratio in the cell, which usually occurs when cells are metabolically active [11]. To summarize, an active mTOR pathway leads to the phosphorylation of the S6 ribosomal protein and AMPK is a mTOR inhibitor when phosphorylated. We hypothesize HQNO causes dysregulation of host mTOR signaling and thus decreases the killing ability of macrophages.

The role that HQNO plays on the host innate immune response is not known. The goal of our study was to understand the impact that HQNO has on the ability of macrophages to kill bacteria. Western blotting was used to show that HQNO dysregulates the mTOR pathway. Macrophages were observed to be less efficient at killing *P. aeruginosa* and *B. multivorans* when treated with HQNO. A better understanding of the effects of HQNO may lead to treatment options that can combat the negative consequences this molecule.

Materials and Methods

Cell Culture and Bacterial Strains

Mouse Raw 264.7 macrophages (RAW) and immortalized 10.1 Mouse Embryonic Fibroblasts (MEF) cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (Sigma-Aldrich) and 1% Penicillin-Streptomycin (Life-Technologies).

Bacterial Killing Assay

RAW macrophages were plated onto 24 well plates at a density of 250,000 cells per well and incubated for 24 hours. Cells were treated with 1 μM Torin1 (Cayman Chemical), a known mTOR inhibitor, or 1 or 10 μM HQNO (Cayman Chemical). Approximately 16 hours after treating, the cells were primed with 100 ng/ml *E. coli* lipopolysaccharide (Sigma) and 20 ng/ml mouse interferon gamma (Peprotech) for one hour. Next, the cells were infected with *Burkholderia multivorans* in media lacking antibiotics to achieve an MOI of 1. The plates were spun down in the centrifuge at 500 relative centrifugal force (rcf) for five minutes to ensure contact between the bacteria and the RAW macrophages. The plates were incubated for one hour to allow for phagocytosis. The media was then aspirated and media containing 100 μg of kanamycin per mL of media was added. At each designated

time, one set of wells for each condition were lysed and plated. The cells were lysed using 0.01% Triton and plated on tryptic soy agar plates at 10 fold dilutions. Plates were left at 37°C and then colonies were counted after they became visible. Data at each time point are expressed as percent of bacteria killed compared to time zero for a given condition. The same protocol was carried out using *P. aeruginosa* using gentamicin instead of kanamycin.

Western Blot

MEF or RAW cells are plated onto 24 well plates at a density of 250,000 cells per well and incubated. 24 hours later, the cells were treated with Torin1 or HQNO. Depending on the experiment, cells may or may not have been primed using the same concentration of interferon gamma and LPS previously described. Next, 50 µL of Radio Immuno Precipitation Assay buffer (Boston BioProducts) with (1%) Halt Protease and Phosphatase Inhibitor Cocktail 100x (Thermo Fisher Scientific) was added to each well. The samples were then transferred to 1.5 ml Eppendorf tubes. 6x Loading buffer with 5% beta-mercaptoethanol was added to the samples. The mixture was boiled for 5 minutes and then proteins were separated on a 12% SDS-polyacrylamide gel. The SDS gel was run for about an hour, followed by transfer of proteins to a nitrocellulose membrane. The transfer was run for another hour. Next, LI-COR Odyssey Blocking Buffer was used to block overnight. The membrane was washed 3 times for five minutes using PBS with (1%) Tween-20. Rabbit Anti-phospho-S6 ribosomal protein and rabbit anti-phospho-AMPK primary antibodies (Cell Signaling Technologies) were diluted in PBS-Tween 1:2,000 added to the membrane incubated overnight. The membrane was washed 3 times for 5 minutes with PBS-Tween and IRDye Goat Anti-Rabbit 800CW (LICOR) secondary antibody diluted 1:10,000 in PBS tween was added for an hour. Lastly, the membrane was washed three times and then imaged with a LI-COR Odyssey Imaging System. Blots were similarly probed for total AMPK and total S6 ribosomal protein except the goat-anti-mouse-680LT secondary antibody was used to visualize S6 ribosomal protein.

Results

mTOR Inhibition

The innate immune response, specifically the production of reactive oxygen species, is regulated by the mTOR pathway. As a first step to determine if HQNO alters the host

immune response, we assessed whether mTOR is inhibited in cells treated with HQNO. To do this western blotting was used to view the presence of phosphorylated AMPK and S6. AMPK is an upstream mTOR inhibitor when phosphorylated and S6 is a protein downstream of the mTOR pathway that becomes phosphorylated when mTOR is active. We hypothesized that HQNO inactivates the mTOR pathway due to the phosphorylation of AMPK.

Figure 1 illustrates an increase in AMPK phosphorylation as the dosage of HQNO increases. This figure also shows that phosphorylation of S6 protein is dose-dependent. AMPK seems to be phosphorylated at as little as 1 μM HQNO, but phosphorylation of S6 starts to become inhibited at 2.5 μM HQNO. Figure 2 indicates that AMPK is phosphorylated as early as one hour after MEF cells are treated with HQNO. The blots also display that although AMPK is phosphorylated quickly, S6 phosphorylation starts to become inhibited around 6 hours post treatment.

Bacterial Killing Assay

Respiratory burst is a host immune response that uses reactive oxygen species to kill bacteria. Since HQNO treatment led to considerable mTOR inhibition, we next wanted to determine if HQNO could alter the killing ability of macrophages. To view this, we set up bacterial killing assays using both *B. multivorans* and *P. aeruginosa* and RAW macrophage cell lines.

Initially, we chose *B. multivorans* as a candidate bacteria for the killing assay because it is known to be susceptible to respiratory burst. Macrophages that were not treated with HQNO killed approximately 60% of intracellular *B. multivorans* in one hour (Figure 3). This killing efficiency dropped to approximately 20% for 1 uM HQNO and to about 0% killing for 10 μM HQNO conditions. Torin, a known inhibitor of mTOR, was used as a control. Torin treatment resulted in approximately 5% of bacteria killed. These results demonstrate that bacterial killing is impaired in cells treated with HQNO concentrations of 1 and 10 μM and reduced killing is likely to be mediated by mTOR inhibition (Figure 3).

We also wanted to view the effect of HQNO on the host response to *P. aeruginosa*. Untreated macrophages killed approximately 60% of intracellular *P. aeruginosa* in 30 minutes. When treated with 1 and 10 μM HQNO, macrophages killed approximately 35%

and 10% of intracellular *P. aeruginosa*, respectively. Lastly, treating the cells with Torin did not result in a major decrease in bacterial killing.

Discussion

We studied the effect of *P. aeruginosa* released HQNO on the mTOR pathway and the killing ability of host immune cells. Initially, western blot analysis was used to determine if HQNO dysregulates the mTOR pathway. After a 16-hour incubation, the phosphorylation of the S6 ribosomal protein, and thus mTOR, is inhibited at HQNO concentrations above 2.5 μM (Figure 1). We determined that as the concentration of HQNO increased, the strength of inhibition increased. Furthermore, HQNO was seen to lead to phosphorylation of AMPK at every concentration (Figure 1). Although the bands for phosphorylated AMPK were poorly resolved, the blots were still analyzed. These results will be repeated with new antibodies.

After discovering that HQNO inhibits mTOR in MEF cells, we next wanted to determine the kinetics at which this process occurred. HQNO at a high concentration (10 μM) results in the phosphorylation of AMPK beginning at one hour (Figure 2). However, disruption of S6 phosphorylation does not start until 6 hours post-treatment with 10 μM HQNO (Figure 2). This could be due to a threshold of phosphorylated AMPK being necessary for the full inhibition of the mTOR pathway. Western blots will be quantified using ImageJ in future experiments. Additional experiments showed that HQNO also inhibits mTOR in bone marrow derived macrophages (data not shown). In these additional experiments, tubulin levels were probed for to ensure similar total protein levels in samples (data not shown).

Since it is known that an active mTOR pathway leads to respiratory burst, we investigated if treating cells with HQNO was sufficient to hinder the bacterial killing ability of RAW macrophages. RAW cells ability to kill *B. multivorans* and *P. aeruginosa* is substantially reduced when treated with HQNO. Torin treatment led to decreased killing of *B. multivorans*, but its effect on *P. aeruginosa* was not as strong. This could be due to immune responses, other than respiratory burst, targeting *P. aeruginosa*. For example, it is known that the NLRP3 and NLRC4 inflammasome target *P. aeruginosa* in addition to respiratory burst. As such, it is possible that HQNO treatment may inhibit additional pathways not directly regulated by mTOR. This would explain why Torin might inhibit

killing of some bacteria more than others. Interestingly, J774a.1 macrophage cell lines did not show the same trends in bacterial killing as in RAW macrophages (data not shown). This may be due to a cell-line specific abnormality. These experiments will be also completed in bone marrow derived macrophages to reinforce our conclusions. Bone marrow derived macrophages are not immortalized and behave more like healthy cells found in live animals. In sum, we hypothesize that bacterial killing is in part reduced due to mTOR inhibition and diminished respiratory burst.

To strengthen our hypothesis that HQNO is decreasing bacterial killing due to decreased respiratory burst, we will investigate the effect of HQNO treatment on respiratory burst directly. Levels of respiratory burst can be measured by using a Dihydrorhodamine 123 (DHR) assay. This assay directly measures respiratory burst by using a dye that becomes fluorescent only when internalized by a cell in the presence of reactive oxygen species. Completing a DHR assay in immune-stimulated macrophages during HQNO, Torin, and untreated conditions will allow us to determine if respiratory burst is inhibited. We will also be exploring upstream AMPK activators via western blot since many different kinases are known to regulate AMPK [12]. Probing for the phosphorylated versions of these upstream proteins will allow us to determine what protein(s) phosphorylate AMPK, allowing us to further define the mechanism of HQNO action. So far, no candidate molecules have been able to recover mTOR activity and bacterial killing ability without substantial negative consequences (data not shown). In the future, we will continue our pursuit to determine the molecular mechanism of mTOR inhibition through *P. aeruginosa* quinolones so that treatments can be provided to CF patients chronically infected with this bacterium.

Figures

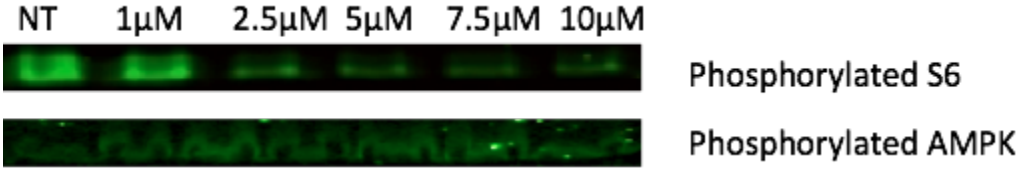

Figure 1: Treatment of MEF cells with HQNO results in decreased phosphorylation of S6 protein and increased phosphorylation of AMPK. MEFs were treated for 16 hours with the indicated concentration of HQNO or not treated. Western blots of cell lysates were probed with

anti-phospho-S6-ribosomal protein or anti-phospho-AMPK antibodies as indicated.

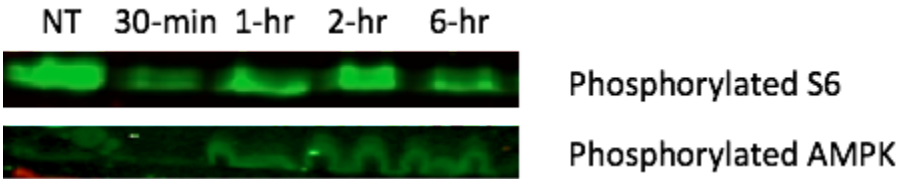

Figure 2: Kinetics of HQNO-dependent phosphorylation of S6 ribosomal protein and AMPK. All MEFs were treated with 10 μM HQNO. MEFs were treated for different durations of time or were left untreated. Western blot for cell lysate was probed with anti-phospho-S6-ribosomal protein or anti-phospho-AMPK antibodies as indicated.

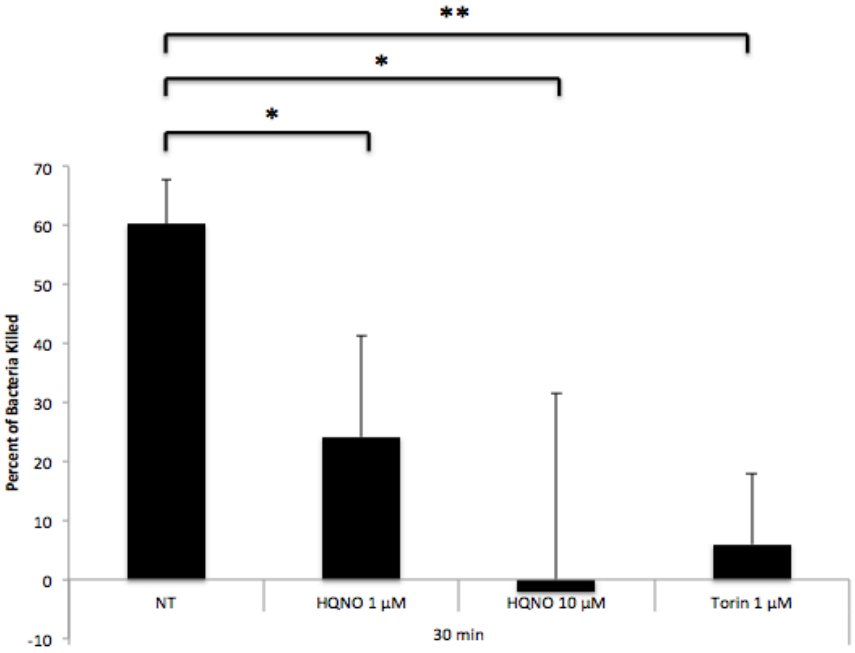

Figure 3: The ability of macrophages to kill *B. multivorans* was dampened by HQNO. RAW macrophages were treated for 16 hours with indicated concentration of HQNO or Torin. After phagocytosis and killing of extracellular bacteria with kanamycin, killing of phagocytosed bacteria was assessed at 30 minutes. This figure depicts a single killing assay with three sets of cells per condition, but is representative of two more replicates. Error bars show standard deviation. HQNO 1μM, 10μM, and Torin 1μM treated conditions had p-values of 0.025, 0.039, and 0.002 respectively.

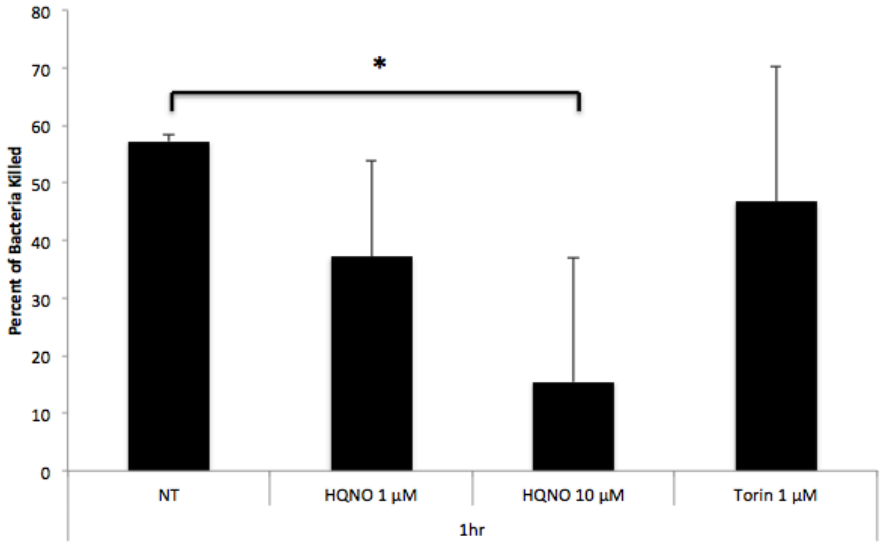

Figure 4: The ability of macrophages to kill *P. aeruginosa* was dampened by HQNO. RAW macrophages were treated for 16 hours with indicated concentration of HQNO or Torin. After phagocytosis and killing of extracellular bacteria with kanamycin, killing of phagocytosed bacteria was assessed at 1 hour. This figure depicts a single killing assay with three sets of cells per condition, but is representative of one more replicate. Error bars show standard deviation. HQNO 10µM treated condition had a p-value of 0.039.

References

[1] C. F. F. About Cystic Fibrosis. at <https://www.cff.org/What-is-CF/About-Cystic-Fibrosis/>

[2] Iglewski, B. H. in *Medical Microbiology* (ed. Baron, S.) (University of Texas Medical Branch at Galveston, 1996). at <http://www.ncbi.nlm.nih.gov/books/NBK8326/>

[3] Collier, D. N. *et al.* A bacterial cell to cell signal in the lungs of cystic fibrosis patients. *FEMS Microbiol. Lett.* **215,** 41–46 (2002).

[4] Barr, H. L. *et al.* Pseudomonas aeruginosa quorum sensing molecules correlate with clinical status in cystic fibrosis. *Eur. Respir. J.* **46,** 1046–1054 (2015).

[5] Sibley, C. D., Rabin, H. & Surette, M. G. Cystic fibrosis: a polymicrobial infectious disease. *Future Microbiol* **1,** 53–61 (2006).

[6] Sousa, S. A. *et al.* Virulence of Burkholderia cepacia complex strains in gp91phox−/− mice. *Cellular Microbiology* **9,** 2817–2825 (2007).

[7] Stanton, R. C. Glucose-6-Phosphate Dehydrogenase, NADPH, and Cell Survival. *IUBMB Life* **64,** 362–369 (2012).

[8] Soliman, G. A. The Role of Mechanistic Target of Rapamycin (mTOR) Complexes Signaling in the Immune Responses. *Nutrients* **5,** 2231–2257 (2013).

[9] Slauch, J. M. How does the oxidative burst of macrophages kill bacteria? Still an open question. *Mol Microbiol* **80,** 580–583 (2011).

[10] Thomson, A. W., Turnquist, H. R. & Raimondi, G. Immunoregulatory functions of mTOR inhibition. *Nat. Rev. Immunol.* **9,** 324–337 (2009).

[11] Richter, E. A. & Ruderman, N. B. AMPK and the biochemistry of exercise: Implications for human health and disease. *Biochem J* **418,** 261–275 (2009).

[12] Lizcano, J. M. *et al.* LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *EMBO J* **23,** 833–843 (2004).