Treatment of Microbial Infections with NTBC (OTT ID 1133)

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Treatment of Microbial Infections with NTBC

2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione

Current problems with antimicrobials

- Drug resistant fungi and bacteria
- Lack of success with single drug treatments for infection
- Fungi are difficult to treat and infections may recur
- Low spectrum activity of commercial drugs
- Toxicity of current commercial drugs
- Unfavorable interactions of current drugs with other medications
- Decreased investment in creation of novel antimicrobials

Solutions and Advantages of NTBC

- Excellent toxicity profile in humans
- Can be used orally or topically
- Bacterial infections and systemic and topical fungal infections
- Great potential for use in combination with current antimicrobials
- More stable in humans; longer half-life after oral ingestion
- Less time to market; already FDA approved
- Potential for treatment of animal infections in veterinary or livestock practices

Market Potential and Intellectual Property

Market

- According to the CDC, over 70% of hospital bacterial infections are resistant to one or more classes of antibacterial drugs
- In the U.S. alone these infections result in $4.5 billion in excess healthcare costs
- The antibacterial market is expected to reach $43.8 billion in 2016 (Visiongain)
- Revenues for systemic antifungal drugs are predicted to approach $6 billion by 2014 (Datamonitor)
- Antibiotic resistance in fungi and bacteria has become increasingly prevalent leading to a lack of success with single drug treatments

Intellectual Property

- A notice of allowance has been received for U.S. Utility Patent Application 12/720,381; Publication US2010-022793 A1

Seeking Partner for Research and Development

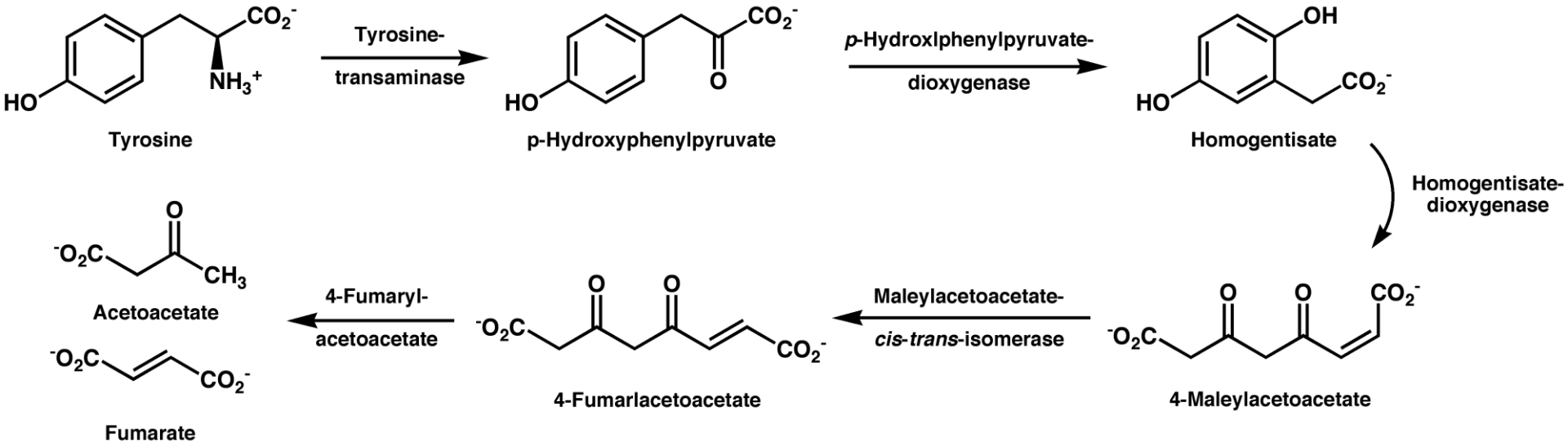
Next Steps

- **Describe next experiments that will be carried out…i.e. cell culture testing**
- **Testing of supernatants**
- **Further clinical isolates**

Funding Required

- **$100,000 in support for one year for a student or post-doc carrying out the experiments and research supplies**

Tyrosine catabolism has been adapted to a variety of functions by living organisms



Other Molecules derived from Tyrosine catabolism pathway

- Antibiotics such as vancomycin
- Redox co-factors in mycobacteria and methanogens
- Pigment production in eubacteria such as melanin (from HmgA)
- Redox cofactors in plants

NTBC Specifically Inhibits HPPD

- **Originally developed as an herbicide, NTBC is FDA approved to treat Type I Tyrosinemia which is caused by a deficiency of the enzyme fumarylacetoacetate hydrolase**

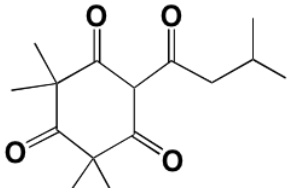
- **Fumarylacetoacetate hydrolase catalyzes the final step in the degradation of tyrosine - fumarylacetoacetate to fumarate, acetoacetate and succinate**

- **NTBC is also a potential therapeutic for alkaptonuria and hawkinsinuria which are also disorders of tyrosine metabolism**

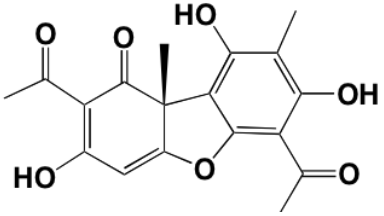
A Number of HPPD Inhibitors are Sold as Herbicides

Natural Products - allelopathics

LEPTOSPERMONE



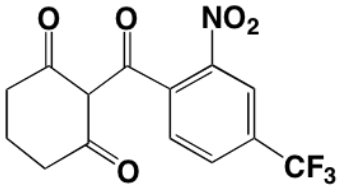
USNIC ACID



Drug

SWEDISH ORPHAN INTERNATIONAL AB

NTBC



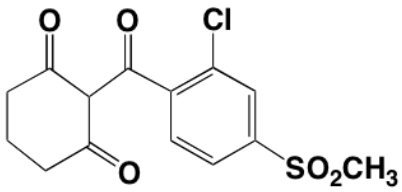
Herbicides

ESSENCHEM

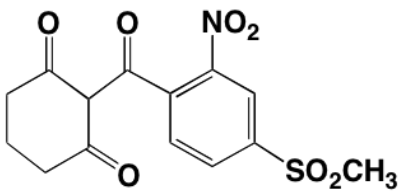
syngenta

Aventis

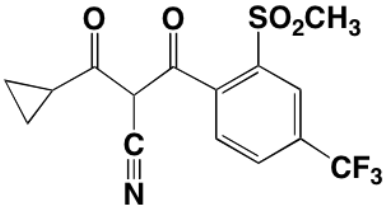
SULCOTRIONE



MESOTRIONE



ISOXAFLUTOLE



The Moran Laboratory has Extensively Studied the Kinetics of NTBC Inhibition

- **NTBC binds exclusively to the Fe(II) form of HPPD**
- **NTBC completely suppresses dioxygen reactivity and exhibits biphasic binding kinetics (UV spectroscopy)**
- **A minimal kinetic model for NTBC association with the HPPD.Fe(II) complex involves an initial weak pre-equilibrium and two metal centered binding events**
- **HPPD inhibitors bind very tightly to HPPD and HMS**

M. Kavana and G. R. Moran (2003) *Biochemistry*, 42, 10238-10245.
J. Brownlee, *et al.* (2004) *Biochemistry* 43, 6370-6377.
M.L Neidig, *et al.* (2005) BBRC 338, 206-214

Evidence that NTBC May be a Treatment for Infection

Observations

- Injury often results in infections that leave pigment in the skin
- Pyomelanin is linked to virulence in bacteria and fungi
- The pathogenic fungus *Cryptococcus neoformans* has been found to melanize in tissue (Nosanchuk et al. 1999. Mol. Cell. Biol. 19(1): 745)

Bacteria and Fungi that produce pyomelanin

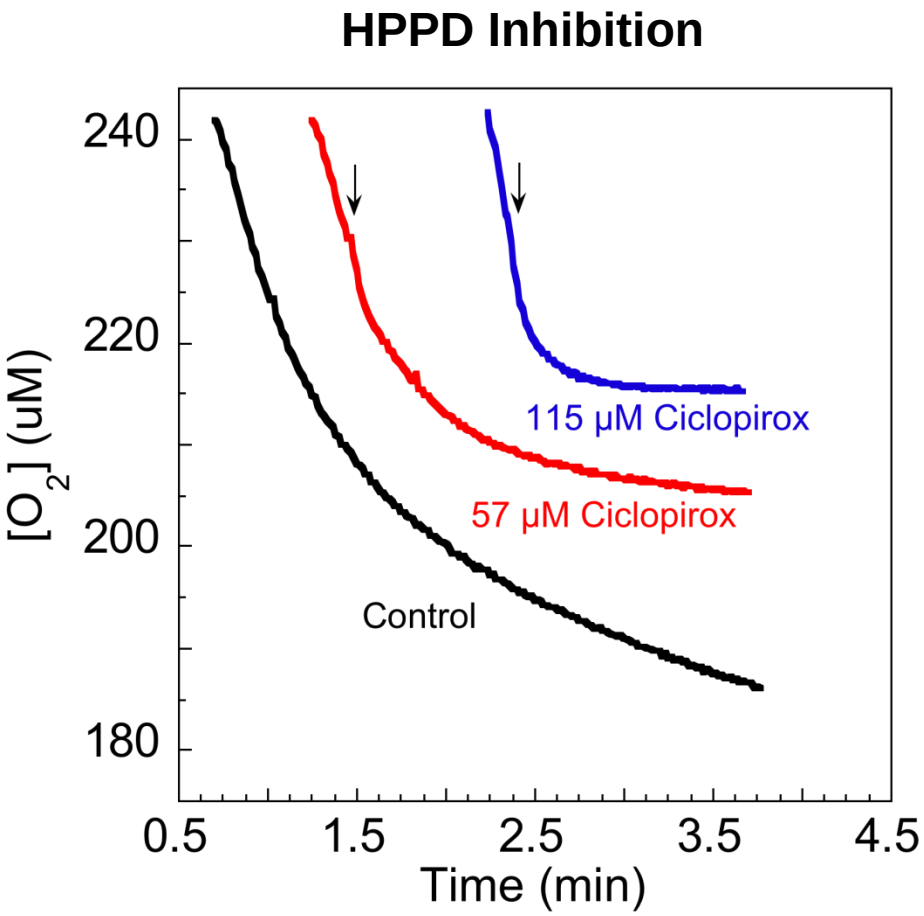
- *Cryptococcus neoformans* –lung infection; fungal meningitis
- *Aspergillus fumigatus* –pathogenic in immuno-compromised people
- *Paracoccidioides brasiliensis* -most important South American systemic mycosis
- *Vibrio cholerae* –Cholera; acute intestinal infection; through contaminated water
- *Psuedomonas aeruginosa* –opportunistic hospital pathogen (cancer, cystic fibrosis, burn patients); forms biofilms
- *Legionella pneumophila* –Legionnaires' disease; pneumonia
- *Burkholderia cenocepacia* –opportunistic pathogen (cystic fibrosis); plant disease

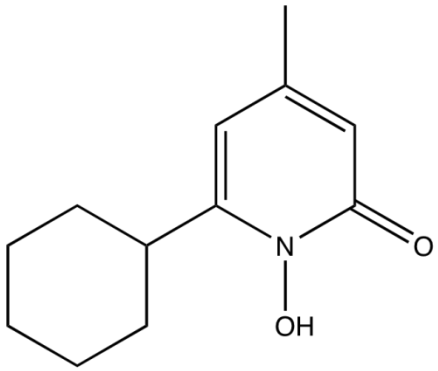
4-HPPD is Overexpressed in an Infectious Fungi During Differentiation

- **Paracoccidioides basiliensis is a thermodimorphic fungi associated with systemic mycosis in South America**
- **Infection occurs through inhalation of the fungal propagules which transform into the yeast parasitic form once in the pulmonary epithelium**
- **The mycelium to yeast transition is essential for infection**
- **Studies revealed that 4-HPPD is highly overexpressed during this transition, and NTBC was able to inhibit growth and differentiation of the pathogenic yeast phase of the fungus *in vitro***

Nunes et al. 2005. Eurkaryotic Cell. 4(12): 2115.

Cyclopirox, a Well Known Antifungal, is also an HPPD Inhibitor





Cyclopirox

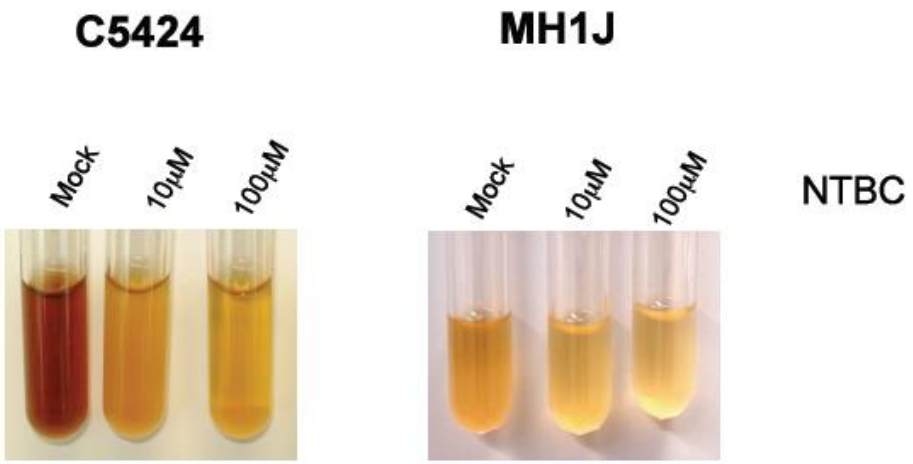
Usnic Acid has been Used as an Antibiotic

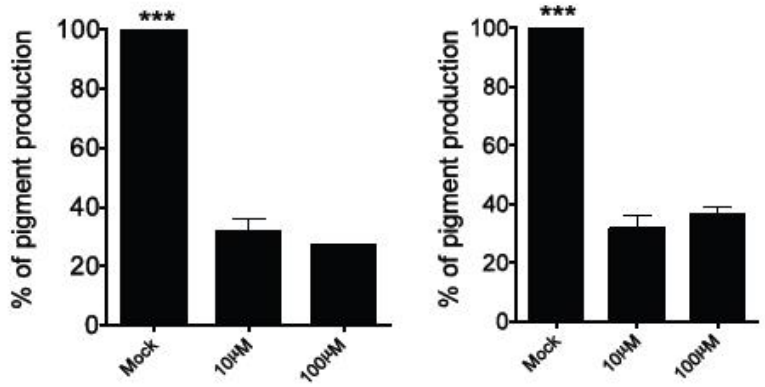
- **Usnic acid is a natural product HPPD inhibitor from lichen that has been utilized as an antibiotic for bacteria and pathogenic fungi**

- **Usnic acid is effective against gram positive bacteria including *Mycobacterium tuberculosis, Staphylococcus, Streptococcus, and Pneumococcus*, as well as some pathogenic fungi**

http://en.wikipedia.org/wiki/Usnic_acid

NTBC Decreases Pigment Production in *B. cenocepacia*



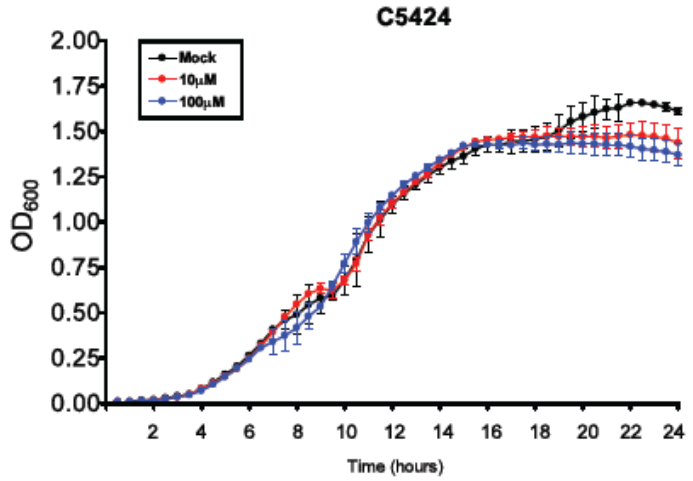


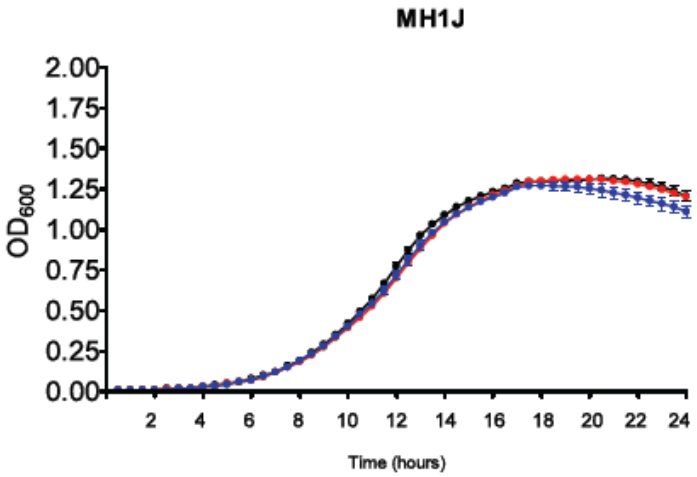
- Strains C5424 and MH1J were grown for 24hr
- Cultures were centrifuged and pigment in supernatant quantified at OD 597 nm; N=2

- **NTBC has a significant inhibitory effect on pigment production in *B. cenocepacia***
- ***B. cenocepacia*** **is a common pathogen seen in cystic fibrosis and nosocomial infections**

NTBC Does Not Effect Bacterial Growth and Viability

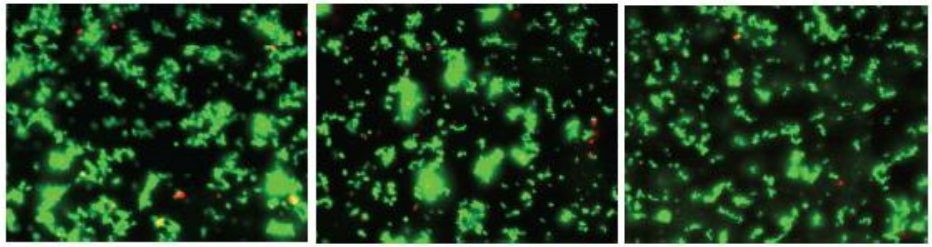
Growth Curve





LIVE/DEAD Assay for Viability

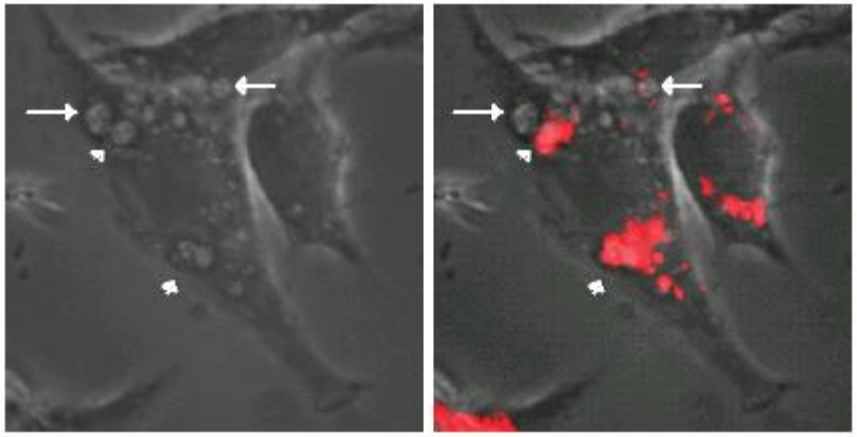
(red indicates dead cells)



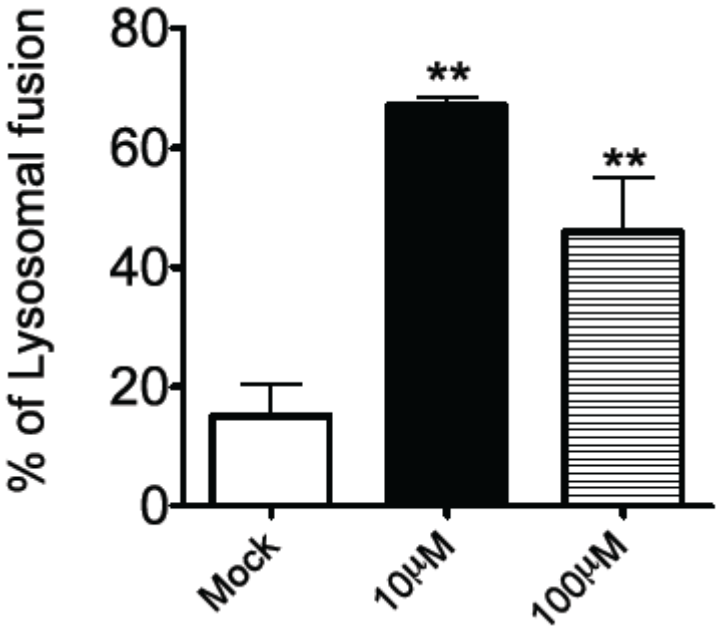
10 μM NTBC **100μM NTBC** **Control**

***Reduction of pigment production with NTBC treatment is not due to a loss in viability or change in growth kinetics**

Reduced Pigment Production Does Not Delay Phagolysosomal Fusion in Macrophages



Bacteria were grown with or without NTBC for 24hr; Macrophages were infected with bacteria and stained with LysoTracker Red-99 which accumulates in lysosomes



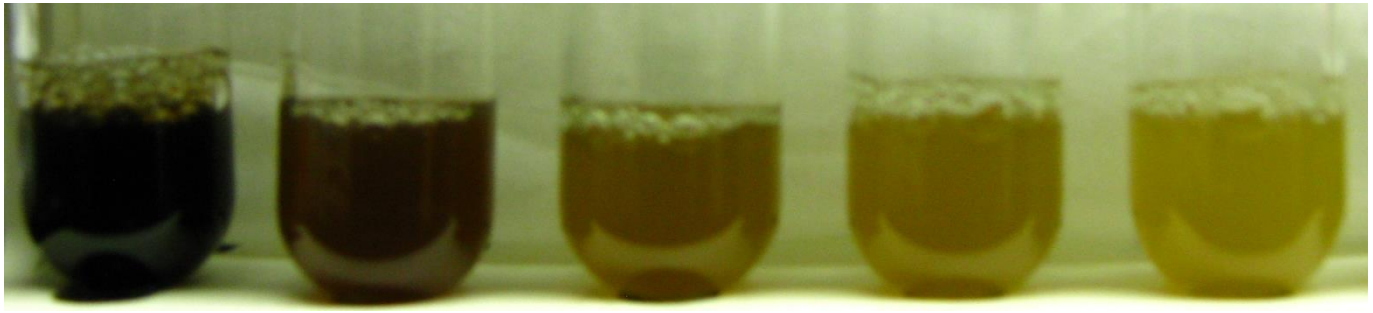
- **Previous studies have shown that *B. cenocepacia* C5424 and MH1J can delay phagolysosomal fusion**
- **This property is lost in an *hppD* mutant**
- **Treatment with NTBC and loss of pigment reduces the ability to delay phagolysosomal fusion in RAW 264.7 macrophages**

NTBC and Pyomelanin-Producing *Pseudomonas aeruginosa*

- **In Cystic Fibrosis (CF) lung infections, approximately 5% of *Pseudomonas aeruginosa* isolates produce pyomelanin, an extracellular reddish-brown pigment that provides protection from oxidative stress and contributes to infection persistence**
- **Pyomelanin is derived from the tyrosine catabolism pathway and is produced when homogentisic acid (HGA) is secreted from the cell, auto-oxidized, and self-polymerized**
- **(NTBC) irreversibly binds to HppD, which synthesizes HGA**
- **We propose that NTBC treatment of bacterial cells will inhibit pyomelanin production and increase sensitivity to oxidative stress**
- **Pyomelanin producing strains of bacteria have been found to be more resistant to oxidative stress, leading to increased persistence of infection**

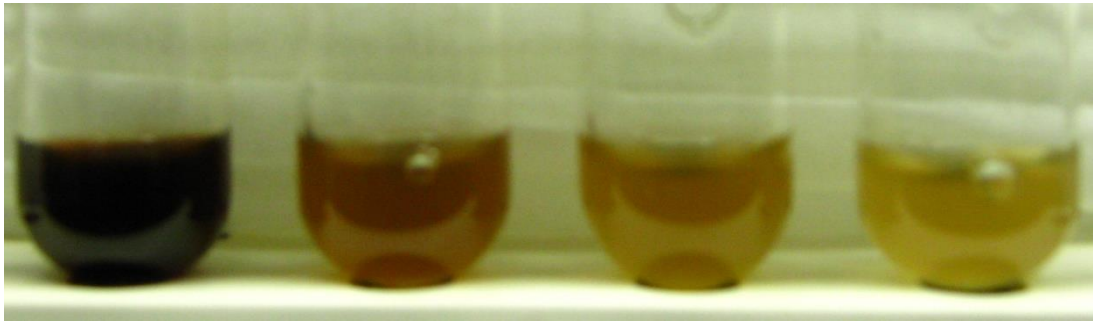
Inhibition of Pyomelanin with NTBC Treatment

0 μM 50 μM 100 μM 200 μM 300 μM



Treatment of *hmgA::tn* with increasing concentrations of NTBC.

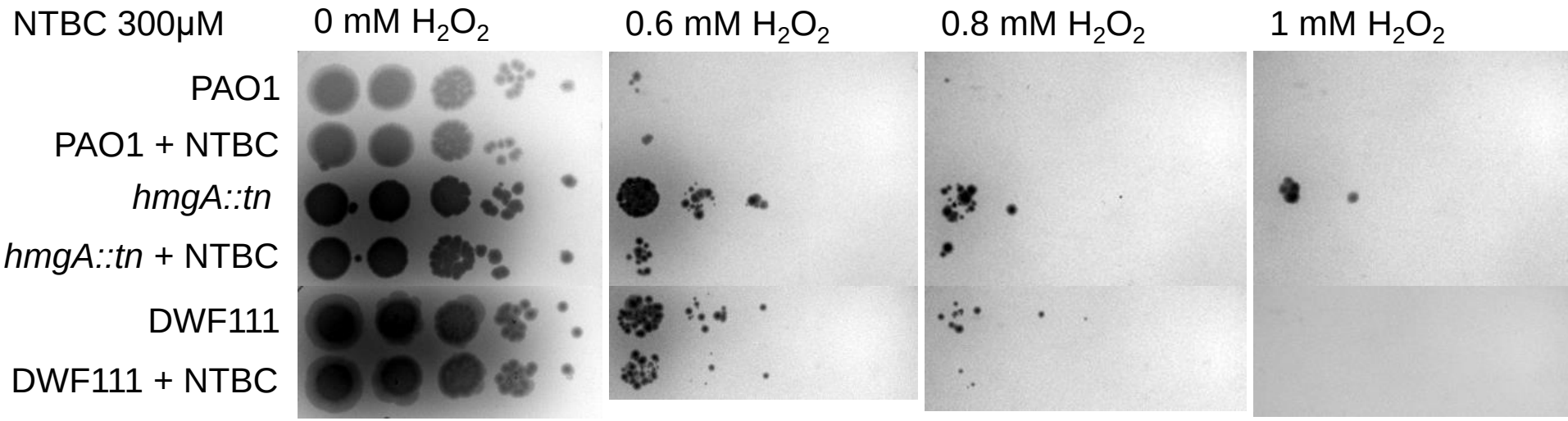
0 μM 300 μM 600 μM 900 μM



Treatment of DWF111 with increasing concentrations of NTBC.

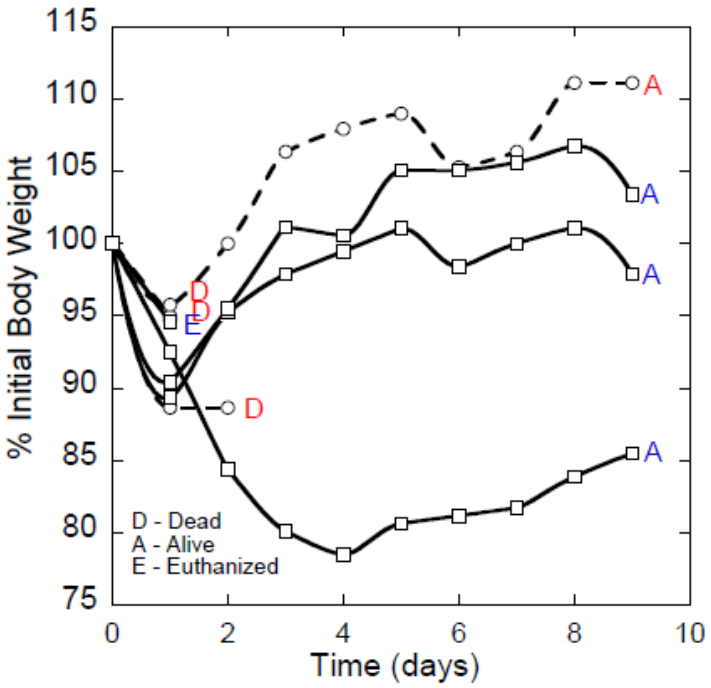
Treatment of laboratory and clinical strains of *P. aeruginosa* with NTBC. DWF111 requires a 3x higher concentration of NTBC than *hmgA::tn* to abolish pyomelanin production.

NTBC Treatment Increases Sensitivity to H2O2



- **Cells were grown overnight in either LB or LB+NTBC, adjusted to the same OD_{600}, serial diluted 10-fold, and spotted on LB plates containing the indicated concentrations of H_2O_2.**
- **PAO1 and *hmgA::tn* strains were incubated 24 hours and DWF111 strains were incubated 45 hours (due to slow colony growth) at 37°C.**
- ***hmgA::tn* exhibits an approximately 10-fold increase in sensitivity to H_2O_2 induced oxidative stress with NTBC treatment. DWF111 also exhibits an increase in sensitivity to oxidative stress with NTBC treatment**

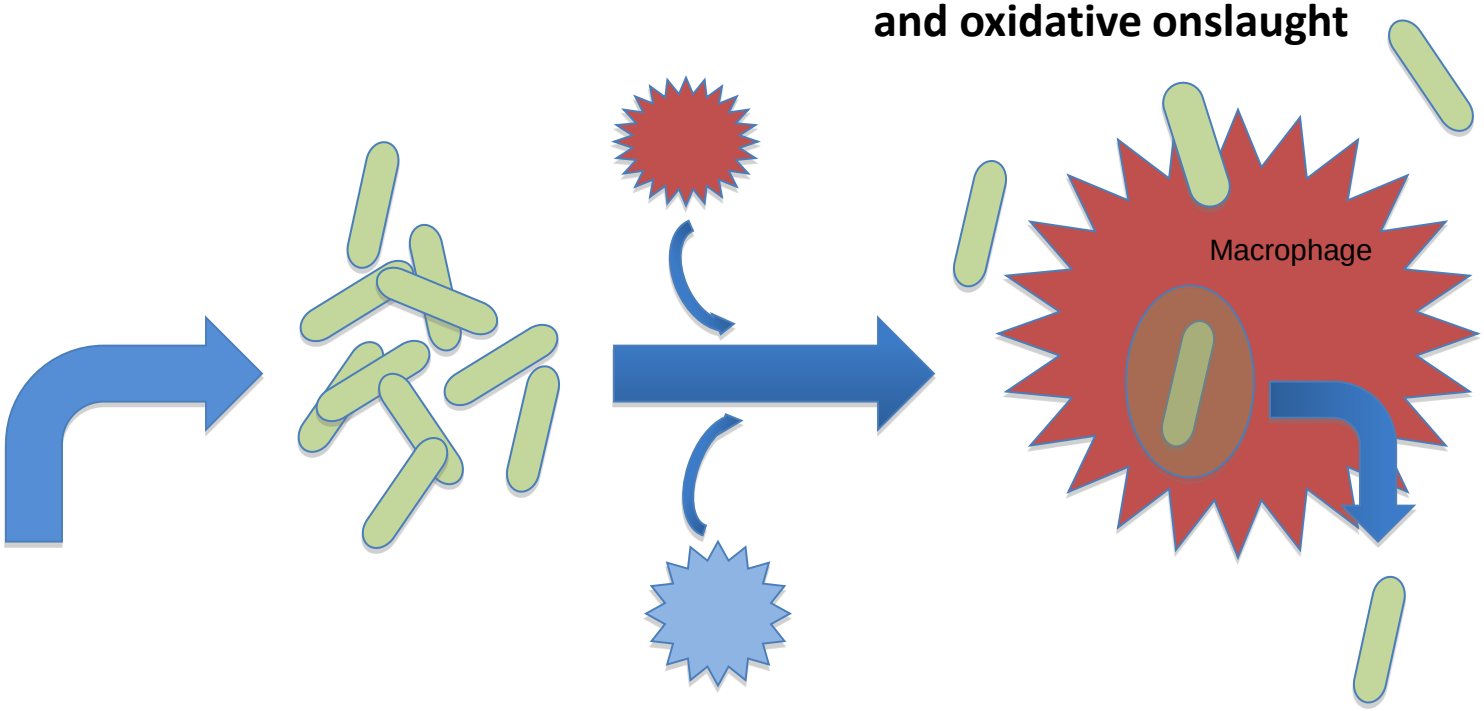
NTBC protects Mice Infected with *P. aeruginosa*



Dashed lines = bacteria only
Solid lines = bacteria and NTBC

- **8-week old C57BL/6 mice were injected intraperitonally with 3.2 x 10^8 colony forming units of *Psuedomonas aeruginosa***
- **Half were treated with 1mg/kg NTBC**
- **3 of the 4 NTBC treated mice survived while 3 of the 4 untreated mice died within 2 days of infection**

Model for the Mode of Action: Enhancing the Immune Response



Phagocytosis and oxidative onslaught

Pyomelanin absorbs oxidative substances and promotes microorganism survival

Macrophage and neutrophil migration

NTBC undermines the ability of the microorganism to survive inside phagocytic vessicle by stemming the production of pyomelanin

Summary

- NTBC decreases pigment production in *B. cenocepacia* and *P. aeruginosa* but does not affect bacterial growth and viability
- Data shows that NTBC aids in phagolysosomal fusion in macrophages and protects mice from death by infection with *Pseudomonas aeruginosa*
- NTBC treatment increase sensitivity of pyomelanin producing *P. aeruginosa* to hydrogen peroxide
- Potential use in the treatment of bacterial infections that rely upon pyomelanin to colonize the host - as either an adjunct to other treatments or as stand-alone antibacterial.
- Indications that it may be effective against dimorphic fungi and possibly generally against pathogenic fungi

Work in Progress

- **Determine the effects of NTBC treatment on biofilms subjected to H_2O_2 oxidative stress.**
- **Test NTBC treated and untreated cells against additional classes of antibiotics to determine MICs.**
- **Quantify the amount of HGA produced by *hmgA::tn* and DWF111.**
- **Determine the cytotoxic effects of pyomelanin against a murine macrophage-like cell line.**
- **Assay bacterial survival during phagocytosis with and without NTBC treatment.**

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