Effects of *Pseudomonas aeruginosa* pyocyanin and 1-hydroxyphenazine on intracellular calcium, mitochondrial function, and viability in human nasal epithelial cells

Joel C. Thompson[1], Yobouet Ines Kouakou[1], Zoey A. Miller[1], Nithin D. Adappa[1], James N. Palmer[1], and Robert J. Lee[1,2,3]

Department of Otorhinolaryngology[1], Department of Physiology[2], University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104, USA.
[3]Correspondance to: rjl@pennmedicine.upenn.edu (R.J.L.)

Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that produces phenazine metabolites pyocyanin and 1-hydroxyphenazine that have been suggested to have detrimental effects on mitochondrial function and reactive oxygen species (ROS) production. Prior studies have suggested activation of Ca^{2+} signaling by pyocyanin in an airway cell line. Ca^{2+} is tightly linked to both normal mitochondrial function as well as mitochondrial ROS and apoptosis during mitochondrial Ca^{2+} overload. We found that pyocyanin but not 1-hydroxyphenazine induced both cytosolic and mitochondrial Ca^{2+} increases. Despite this, both pyocyanin and 1-hydroxyphenazine decreased in cell viability in RPMI 2650 nasal carcinoma cells but not in primary human nasal epithelial cells (HNECs) at 24 hours. However, in both RPMI 2650 and HNEC, mitochondrial membrane potential acutely decreased after treatment with either pyocyanin or 1-hydroxyphenazine. Our data suggest that Ca^{2+} signaling is not required for acute effects of 1-hydroxyphenazine or pyocyanin on mitochondrial function. The greater sensitivity of RPMI 2650 cells to pyocyanin-induced and 1-hydroxyphenzine-induced cytotoxicty compared with primary cells suggests that these compounds might have some applicability in treating nasal squamous carcinoma or other types of head and neck squamous carcinoma.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen which primarily infects immunocompromised individuals such as burn victims or cancer patients, and causes fatal respiratory infections in patients with cystic fibrosis.[1,2] The prevalence of multidrug-resistant Pseudomonas aeruginosa has increased in recent years, making it a significant public health threat in these patient populations[3]. Understanding the interactions of *P. aeruginosa* with host cells may aid the development of new therapies that might circumvent antibiotic resistance[4]. We previously studied activation of bitter taste receptors (taste family 2 receptors or T2Rs) by *P. aeruginosa* acylhomoserine lactone (AHLs) and quinolone molecules involved in quorum sensing[5]. *P. aeruginosa* AHL 3-oxo-C12HSL induces apoptosis in epithelial cells through activation of bitter receptor[6], which causes mitochondrial Ca^{2+} overload and excessive mitochondrial reactive oxygen species production that inhibits the ubiquitin proteasome pathway[7].

P. aeruginosa synthesizes multiple other toxic virulence factors[8], including phenazine metabolites pyocyanin and 1-hydroxyphenazine, before and during biofilm development.[1,9] Concentrations of phenazines in late-stationary cultures or biofilms can reach between 100-300 μM[10]. Pyocyanin is a “redox-active” compound that has been reported to induce cellular apoptosis through superoxide production.[1,2,11] The exact mechanisms are unclear, but likely involve mitochondrial dysfunction. Pyocyanin has been suggested to have potential anti-cancerous effects via activation of apoptosis, and also has potential anti-fungal and anti-bacterial properties.[1,2] Our goal was to further study host-pathogen interactions mediated by pyocyanin and 1-hydroxyphenazine both because of their roles in infection as well as their potential utility as apoptosis-inducing cancer therapeutics. We also sought to determine if phenazines activated bitter taste receptors, as observed with AHLs, to understand if these molecules shared similar mechanisms of apoptosis induction.

In A549 lung cancer cells, pyocyanin induced a cytosolic Ca^{2+} response in the presence and absence of extracellular Ca^{2+}, suggesting endoplasmic reticulum (ER) Ca^{2+} release,[10] which would fit with activation of a GPCR. Pretreating cells with SERCA pump inhibitor thapsigargin diminished Ca^{2+} responses to pyocyanin.[10] Other studies have shown that pyocyanin and 1-hydrpoxyphenazine inhibit ciliary beating in human nasal epithelial cells[12,13,14], as we have also observed with activation of T2R1 in nasal epithelial cells (lupulone paper). It is unknown of if pyocyanin activation of Ca^{2+} is GPCR related, and if excessive mitochondrial Ca^{2+} influx is linked to mitochondrial dysfunction and epithelial damage. Since only one study has investigated the effects of pyocyanin and no studies have examined 1-hydroxyphenazine in

Ca^{2+} signaling, these compounds warrant more investigation, including in primary nasal epithelial cells. Defining the effects of these molecules on airway epithelial cells will lead to insight into the context of Ca^{2+} signaling in host-pathogen interactions in airway epithelial cells.

Materials and Methods

Cell culture

RPMI 2650 (ATCC CCL-30) and NCI-H520 (ATCC HTB-182) cells were from ATCC (Manassas, VA, USA), while UM-SCC-47 hereafter referred to as SCC-47 (Millipore SCC071) was from MilliporeSigma (St. Louis, MO, USA) and all were cultured as described[15]. Cells were cultured in Minimal Essential Medium with Earle's salts and L-glutamine (Corning; Glendale, AZ, USA) with 10% (v/v) fetal bovine serum (FBS) (MedSupply Partners; Atlanta, GA, USA), 1% (v/v) penicillin/streptomycin (PenStrep) mix (Gibco; Gaithersburg, MD, USA), and 1% (v/v) MEM nonessential amino acids (Gibco). Cell media was changed twice per week and cells were passaged between 70-80% confluence.

Human nasal epithelial cells (HNEC) were extracted via cytology brushings which occurred during sinus surgery, as previously described[16]. Brushes were obtained from patients undergoing sinonasal surgery for CRS or other procedures (e.g., trans-nasal approaches to the skull base for tumor resection) with institutional review board (IRB) approval (protocol #800614) and with written informed consent. All work was done in accordance with The University of Pennsylvania guidelines for use of residual clinical material, the U.S. Department of Health and Human Services code of federal regulation Title 45 CFR 46.116, and the Declaration of Helsinki. Inclusion criteria were patients ≥18 years of age undergoing sinonasal surgery. Exclusion criteria included use of antibiotics, oral corticosteroids, or anti-biologics (e.g. Xolair) within one month of surgery or history of systemic inheritable disease (e.g., granulomatosis with polyangiitis, cystic fibrosis, systemic immunodeficiencies). Individuals ≤18 years of age, pregnant women, and cognitively impaired persons were excluded.

HNECs were isolated from nasal brushings by washing 5 times with DMEM/F12 media containing 5% (v/v) FBS, 1% (v/v) PenStrep, and 0.125μg/mL amphotericin B (Gibco) referred to as Culture Media (CM). Cells were centrifuged at 1200 rpm for 5 mins, then combined in 5mL CM and strained through a 70μm mesh strainer (Fisher). Cells were centrifuged again using the same settings, and were resuspended in PneumaCult Ex-Plus medium containing 1% (v/v) PenStrep, 0.125μg/mL amphotericin B, 10μM ROCK inhibitor Y-27632 (Tocris; Bristol, UK), and 1μM DMH-1 (Tocris) referred to as Growth Media (GM). HNECs in the expansion phase were cultured in T-25 flasks, had their media changed 2-3 times per week, and were passaged between 70-80% confluence.

Reagents and Solutions

Hanks buffered balanced solution (HBSS) was used in cell imaging experiments which contained 137mM NaCl, 5mM KCl, 400μM KH2PO4, 300μM Na2HPO4, 5.6mM glucose, 1.8mM

CaCl2, 1.5mM MgCl2, 20mM HEPES, pH 7.4. Crystal Violet (CV) solution was used in cell viability assays which contained 0.1% CV in DI water with 10% acetic acid. Pyocyanin and 1-hydroxyphenazine were from Cayman Chemical.

Live cell imaging of cytosolic Ca^{2+}, mitochondrial Ca^{2+}, and PKC activity

HNECs or RPMI 2650 cells were loaded with 5μM Fluo-4 AM (AAT-bioquest; Pleasanton, California, USA) in HBSS containing 5.6mM glucose for 1 hour in the dark at room temperature (RT). Cytosolic Ca^{2+} was imaged using a Nikon Eclipse TS100 microscope (20x 0.75 NA objective), FITC filters (Chroma Technologies), QImaging Retiga R1 camera (Teledyne; Tucson, Arizona, USA), MicroManager software, and XCite 120 LED Boost (Excelitas Technologies; Mississauga, Canada).

For mitochondrial Ca^{2+} or PKC activity, HNEC or RPMI 2650 were transfected with CMV-Mito-LAR-Geco1.2 (Addgene Plasmid #61245;[17]) or CMV-ExRaiCKAR (Addgene Plasmid #118409) respectively, using Lipofectamine 3000 reagents (ThermoFisher Scientific) 48 hours prior to imaging. Mitochondrial Ca^{2+} was imaged with an Olympus IX-83 microscope (20x 0.75 NA objective), TRITC filters (Chroma Technologies), Orca Flash 4.0 sCMOS camera (Hamamatsu, Tokyo, Japan) and MetaFluor (Molecular Devices; Sunnyvale, CA USA). PKC activity was imaged as described above with the Olympus IX-83 microscope system, but with TRITC and FITC filters (Chroma Technologies).

Mitochondrial membrane potential, superoxide production, cell viability, and apoptosis

HNEC and RPMI 2650 cells were loaded for 15 minutes at RT in the dark with Tetramethylrhodamine ethyl ester (TMRE) and Hoechst dye prepared as described in the Multi-Parameter Apoptosis Assay Kit (Cayman Chemical; Ann Arbor, MI, USA). Cells were imaged with TRITC filters (Chroma Technologies) as described above with the Olympus IX-83 microscope system.

For JC-1 mitochondrial membrane depolarization, HNEC and RPMI 2650 cells were loaded for 15 minutes at 37°C and 5% CO_2 with 1.5μM JC-1 dye (Cayman Chemical). Imaging occurred as described above with the Olympus IX-83 microscope system, but with TRITC and FITC filters (Chroma Technologies) and MetaMorph (Molecular Devices). For monitoring superoxide production, HNEC and RPMI 2650 cells were loaded for 30 minutes at 37°C and 5% CO_2 with 0.5μM MitoSOX Red dye (Life Technologies; Carlsbad, CA, USA). Imaging occurred as decribed above from JC-1 imaging.

For crystal violet cell viability assays, HNEC and RPMI 2650 were treated, in their respective culture medias, for 24-hours. Cells remaining on plates were stained with the CV solution described above, then washed with DI water, and allowed to dry for 6-24 hours at RT.

Stains were dissolved in 30% acetic acid in DI water and absorbance was measured in a Spark 10M Multimode Microplate Reader (Tecan; Manndeorf, Switzerland) at 590 nm.

For caspase 3 and 7 assays, RPMI 2650 were loaded with CellEvent dye (ThermoFisher Scientific), used to measure caspases 3 and 7, according to the manufacturer's instructions at 37°C and 5% CO_2. Cells were treated with pyocyanin, 1-hydroxyphenazine or lupulone during loading with CellEvent dye, and imaged after 21 hours of treatment. Imaging occurred as described above with the Olympus IX-83 microscope system, with FITC filter (Chroma Technologies).

Statistical Analysis

All data were analyzed for statistical significance using unpaired, two-tailed t-tests (2 comparisons) or one-way ANOVA (more than 2 comparisons) in GraphPad PRISM (San Diego, CA, USA). Dunnett's and Bonferroni post tests were used for one-way ANOVA when appropriate. In each of the figures, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and no statistical significance (N.S). All data points represent the mean ± SEM.

Results

Pyocyanin and 1-hydroxyphenazine are produced from the same precursor, with the ratio of pyocyanin and 1-hydroxyphenzine production dictated by the balance of PhzM and PhzS enzymatic activity (Figure 1). We first investigated the ability of these molecules to increase cytosolic Ca^{2+} signaling by imaging primary human nasal epithelial cells (HNECs) and RPMI 2650 nasal squamous carcinoma cells loaded with Ca^{2+} indicator dye Fluo-4 pyocyanin induces Ca^{2+} responses in both cell types, while 1-hydroxyphenazine did not (Figures 2A-D). Similarly, pyocyanin, but not 1-hydroxyphenazine induced elevations of mitochondrial Ca^{2+} in both RPMI 2650 and HNECs (Figures 2E-H). Mitochondrial Ca^{2+} was not significantly decreased when various mitochondrial inhibitors were used and treated with pyocyanin in RPMI 2650 (Figures 2E-F). Futhermore, only pyocyanin increased protein kinase C (PKC) activity in RPMI 2650 (Figures 3A-B) and this activation of cytosolic Ca^{2+} by pyocyanin was completely diminished through phospholipase C (PLC) inhibiton (Figures 3C-D). These data could suggest activation of GPCRs by pyocyanin as a part of its cellular effects in RPMI 2650.

Despite the differential effects on Ca^{2+} signaling and PKC activation, mitochondrial membrane potential drastically decreased within minutes after treatment with either pyocyanin or 1-hydroxyphenazine in both TMRE (single wavelength) or JC-1 (ratiometric) mitochondrial membrane indicators (Figure 4). Pre-treating RPMI 2650 with BAPTA, a potent Ca^{2+} chelator, did not diminish mitochondrial membrane depolarization (Figure 4G-H), indicating that this depolarization is separate of Ca^{2+} signaling.

However, only pyocyanin showed a strong increase in superoxide production in RPMI 2650 and seen especially in HNECs (Figures 5A-D). Further pre-treatment with BAPTA, similar to results from Figure 4, showed no significant decrease superoxide production in RPMI 2650 as a result of pyocyanin treatment after 2 hours, although BAPTA pre-treatment did slightly hinder 1-hydroxyphenazine superoxide production after 2 hours in the same cell line (Figures 5E-H).

Regardless, both pyocyanin and 1-hydroxyphenazine decreased cell viability in squamous carcinoma cell lines RPMI 2650, SCC-47, and NCI-H520, but not in HNECs from either cystic fibrosis (CF) or non-CF individuals at 24 hours, shown by crystal violet staining (Figure 6). HNECs from three individual patients (non-CF) and three CF individuals appeared to be resistant to the cytoxic effects of pyocyanin and 1-hydroxyphenazine.

Interestingly, neither pyocyanin nor 1-hydroxyphenazine induced caspase 3 and 7 production over a 21-hour period (Figure 7). Although this has been shown before in human monocyte-derived macrophages[18], in the context of this data and in new cell types, this

suggests caspase 3/7 production is not necessary for the decrease in cell viability and superoxide production seen before.

Taken all together, these data suggest that acute Ca^{2+} signaling is not required for reductions in mitochondrial function, as both Ca^{2+}-activating pyocyanin and Ca^{2+}-independent 1-hydroxyphenazine decrease mitochondrial function. Likewise, the acute reductions in mitochondrial function coupled with superoxide production may not be the only mechanism for the decreased cell viability in cells exposed to *P. aeruginosa* phenazines, as similar depolarization was observed in both HNECs and squamous cell carcinomas but only the cancer cells died. The exact mechanism(s) of cell death with pyocyanin and 1-hydroxyphenazine remain to be determined.

Discussion

P. aeruginosa plays a key role in lower airway infections in CF lung disease[19] and upper airway infections in CF-related chronic rhinosinusitis[20]. Pyocyanin and 1-hydroxyphenazine are *P. aeruginosa* metabolites produced during P. aeruginosa infection. A myriad of effects have been reported for pyocyanin in airway epithelial cells, including reduced ciliary beating [21,22], goblet cell metaplasia[23] and IL-8 production[24]. Pyocyanin has been proposed to have toxic effects on mitochondrial by redox cycling with NADPH, generating superoxide (O(2)*), hydrogen peroxide (H(2)O(2)), and hydroxyl radical (HO*), which may directly oxidize glutathione[25] and enhances NFκB activation during co-stimulation with TLR5 agonist flagellin[26]. Oxidative stress production by pyocyanin has been proposed to inhibit Cl- secretion via the cystic fibrosis transmembrane conductance regulator (CFTR)[27] and inhibit the dual oxidase (DUOX)-thiocyanate (SCN^-)-lactoperoxidase (LPO) antimicrobial defense system[28]. Pyocyanin-induced oxidative stress has also been proposed to underly the goblet cell metaplasia and mucus hypersecretion[29].

Better understanding the effects of pyocyanin and 1-hydroxyphenazine on nasal epithelial cell mitochondrial function is important for understanding host-pathogen interactions during P. aeruginosa infection. We started investigating Ca^{2+} signaling, as pyocyanin has been linked to Ca^{2+} elevations [10 11]. While Ca^{2+} is essential for oxidative phosphorylation and other mitochondrial metabolic pathways, excessive Ca^{2+} influx into the mitochondria in nasal epithelial cells can cause mitochondrial Ca^{2+} overload and excess ROS production [7,15]. We hypothesized that Ca^{2+} would be involved in mitochondrial toxicity observed with both pyocyanin and 1-hydroxyphenazine.

Here, we found that only pyocyanin induces Ca^{2+} responses, both cytosolic and mitochondrial. 1-hydroxyphenazine had no detectible effects on Ca^{2+}. Despite this, both pyocyanin and 1-hydroxyphenazine displayed decreases in cell viability in RPMI 2650s but not in HNEC at 24 hours. However, in both RPMI 2650 and HNEC, mitochondrial membrane potential drastically decreased after treatment with either pyocyanin or 1-hydroxyphenazine. Our data suggest that the decrease in mitochondrial function and cell viability induced by both pyocyanin and 1-hydroxyphenazine is thus not likely through a T2R/Ca^{2+}-dependent pathway.

Other unknown targets of pyocyanin, and likely 1-hydroxyphenazine, exist in airway epithelial cells. In bronchail epithelial cells, pyocyanin induces IL-8 synthesis partially via TLR signaling and the aryl hydrocarbon receptor but also through unknown pathways[30]. Pyocyanin also can activate the EGFR-PI3K-AKT/MEK1/2-ERK1/2 MAPK pathway to increase nuclear Nrf-2[31], though these pathways are typically pro-survival pathways rather than cell death pathways.

Notably, an early study reported that while 1-hydroxyphenazine and pyocyanin both inhibited mitochondrial respiration in intact cells, only 1-hydroxyphenzine inhibited respiration of isolated liver mitochondria[32]. These authors hypothesized that inhibition of mitochondrial function by pyocyanin may require intracellular demethylase conversion of pyocyanin to 1-hydroxyphenazine. Early studies have also reported that 1-hydroxyphenazine can interact with ubiquinone-cytochrome b within the mitochondrial electron transport chain[33,34]. It may indeed be that the Ca^{2+} responses observed here are due to pyocyanin activation of an unknown target, possibly a GPCR, while the mitochondrial depolarization and reduced viability of cancer cells we observe here requires intracellular conversion of pyocyanin to 1-hydroxyphenazine.

The greater sensitivity of RPMI 2650 cells vs primary cells suggests these compounds might have some applicability in treating nasal squamous carcinoma or other types of head and neck squamous carcinoma. Future experiments will evaluate ER and nuclear Ca^{2+} and test inhibitors of GPCR pathways to determine if a GPCR underlies the pyocyanin-induced Ca2+ response. We will also test the relationship of Ca^{2+} to ROS production. Differentiated air-liquid interface models will test effects of these compounds in mature epithelial cells, including Ca^{2+}, ciliary responses, and mitochondrial membrane potential changes. We will also identify if there are Ca^{2+}-dependent innate immune effects (e.g., increased cytokine transcription) caused by pyocyanin to better understand host-pathogen interactions during *P. aeruginosa* infections.

Acknowledgements:

This study was supported by National Institutes of Health grants HL168060 and AI167971 to R.J.L. We thank M. Victoria (University of Pennsylvania) for excellent technical assistance.

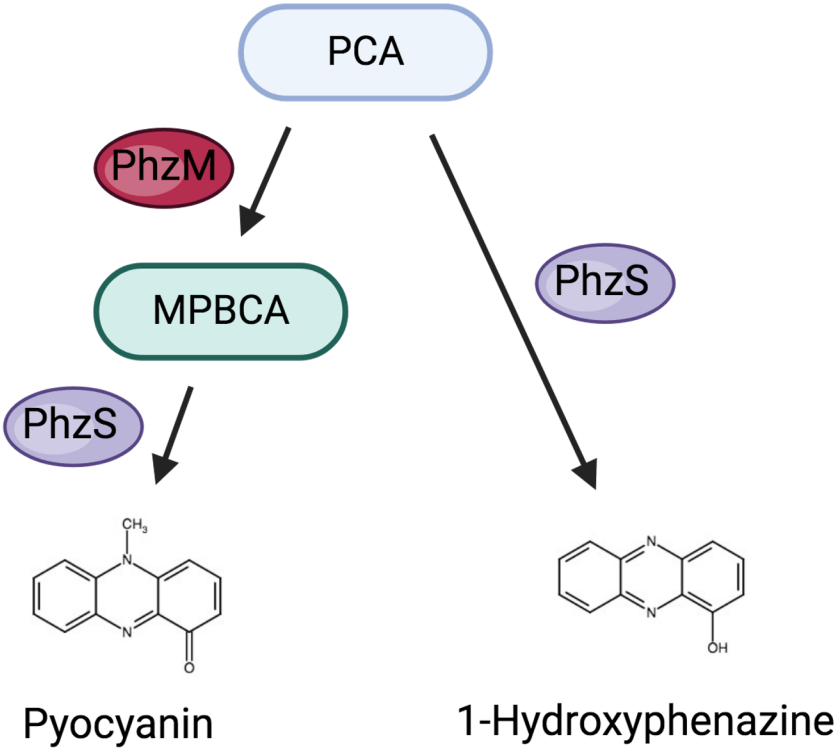


Figure 1. Synthesis of pyocyanin and 1-hydroxyphenazine in *P. aeruginosa*. Phenazine-1-carboxylicacid (PCA) precursor is converted to 5-methylphenazine-1-carboxylic acid betain (MPBCA) by the methyltransferase PhzM[35], which is converted to pyocyanin by hydroxylase PhzS[36]. PhzS, in the absence of PhzM, or build up in PCA, directly converts PCA to 1-hydroxyphenazine[37].

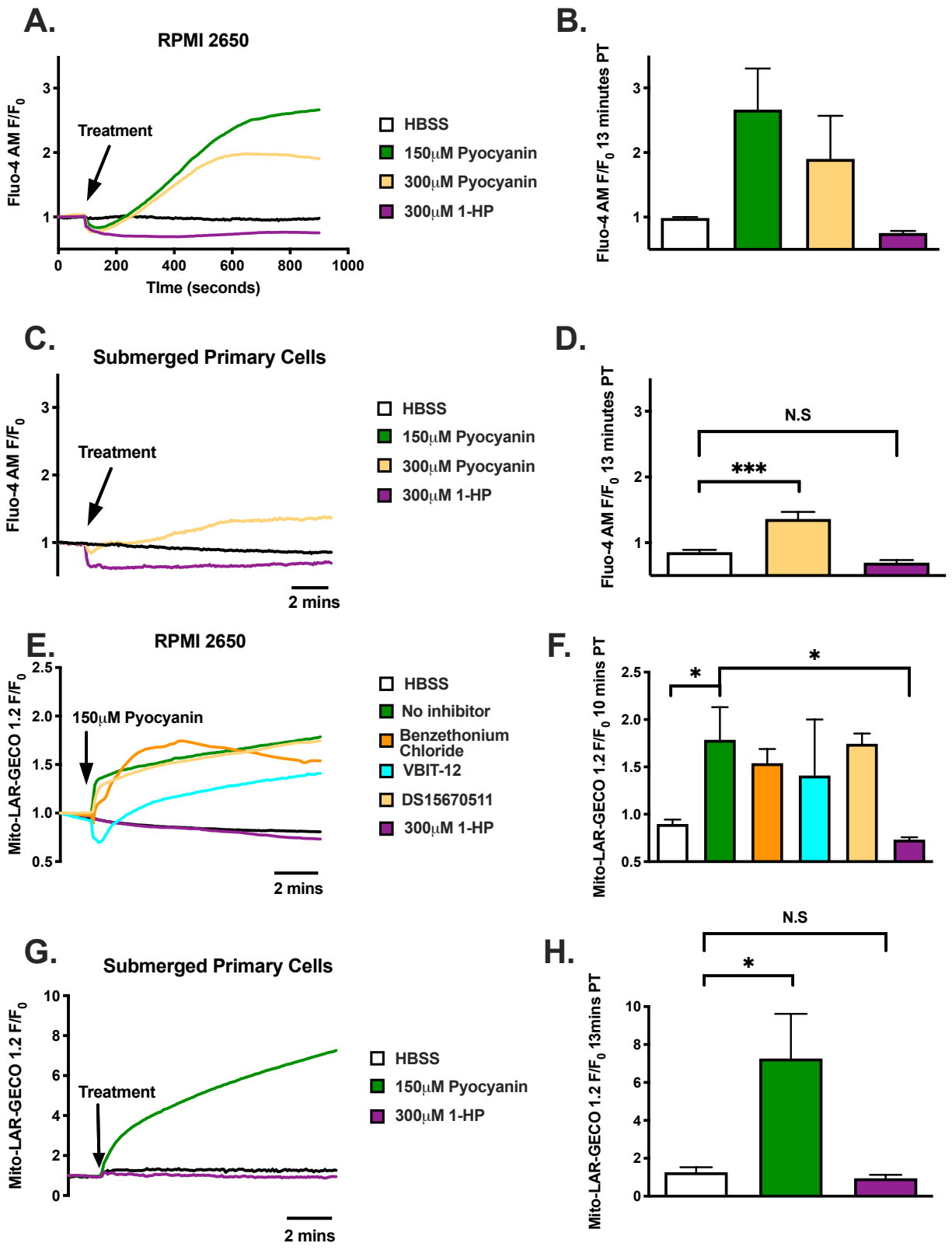


Figure 2. Pyocyanin induces cytoplasmic and mitochondrial Ca^{2+} responses in RPMI 2650 and primary nasal cells while 1-hydroxyphenazine does not. A). Average traces of Fluo-4

intracellular Ca^{2+} responses in RPMI 2650 nasal squamous carcinoma cells. Responses were induced by 150 μM, 300 μM Pyocyanin (Pyo) but not 300 μM 1-Hydroxyphenazine (1-HP), which are concentrations that naturally occur in *Pseudomonas* biofilms. **B).** Intracellular Ca^{2+} (mean ± SEM) 13 minutes post treatment in RPMI 2650. **C).** Average traces of intracellular Ca^{2+} in submerged primary nasal epithelial cells. Responses were induced by 300 μM Pyo but not 300 μM 1-HP. **D).** Intracellular Ca^{2+} (mean ± SEM) 13 minutes post treatment in submerged nasal epithelial cells. **E).** Average traces of mitochondrial Ca^{2+} in RPMI 2650's transfected with Mito-LAR-GECO 1.2. Responses were induced by 150 μM Pyocyanin (Pyo) but not 300 μM 1-hydroxyphenazine (1-HP). This was not significantly different after pre-treatment with mitochondrial Ca^{2+} uniporter inhibitors 10 μM benzethonium chloride (Pyo w/BenzCl), or 10 μM DS16570511 (Pyo w/DS16), or with voltage dependent anion chanel (VDAC) inhibitor 50 μM VBIT-12 (Pyo w/VBIT-12) HBSS and 1-HP treated wells were not pre-treated with any inhibitor and serve as controls **F).** Mitochondrial Ca^{2+} (mean ± SEM) 10 minutes post treatment in RPMI 2650s. **G).** Average traces of mitochondrial Ca^{2+} in submerged primary nasal epithelial cells transfected with Mito-LAR-GECO 1.2. **H).** Mitochondrial Ca^{2+} (mean ± SEM) 13 minutes post treatment in submerged primary nasal epithelial.

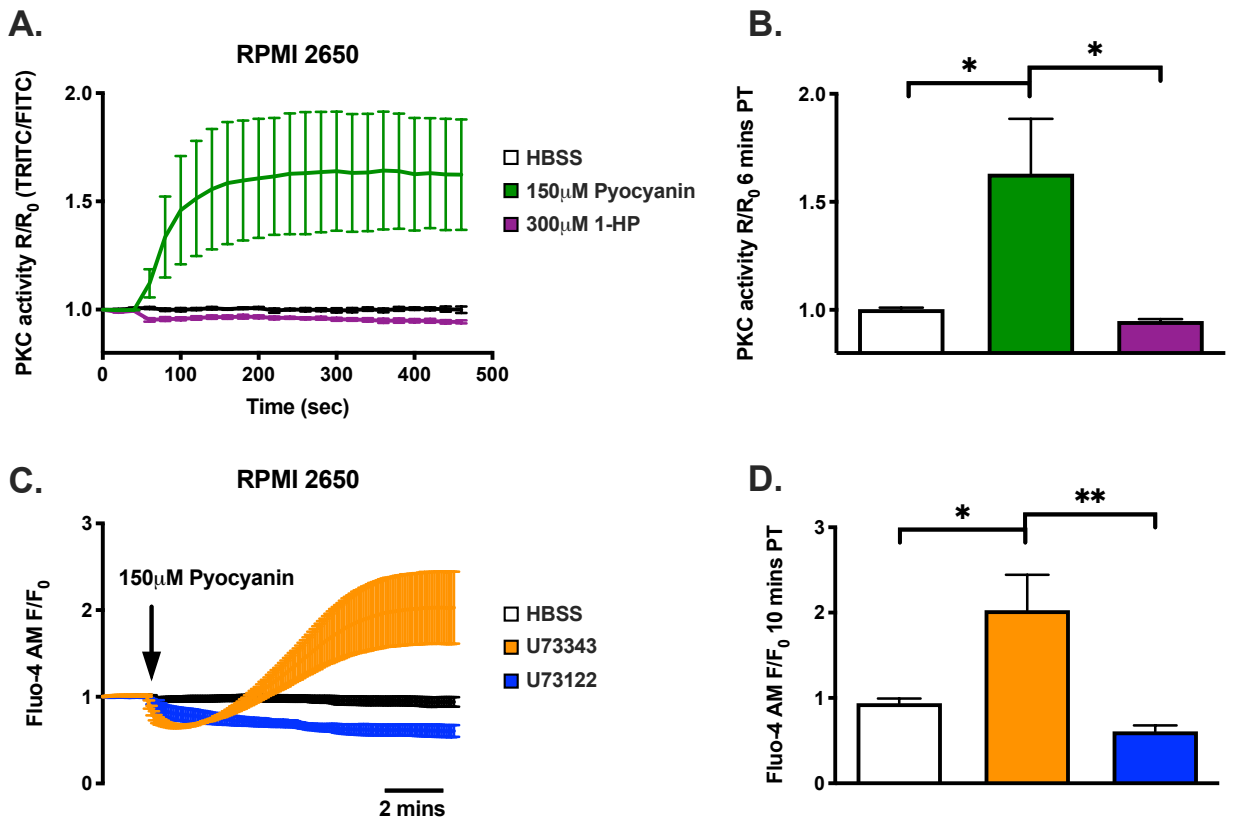


Figure 3. Pyocyanin induces PKC activation while 1-hydroxyphenazine does not and pyocyanin activation is diminished duirng PLC inhibition in RPMI 2650 A). Average traces, with error bars, of PKC activity in RPMI 2650s transfected with ExRai-CKAR. Responses were induced by 150 μM Pyocyanin (Pyo) but not 300 μM 1-hydroxyphenazine (1-HP). **B).** PKC activity (mean ± SEM) 6 minutes post treatment in RPMI 2650s. **C).** Average traces, with error bars, of Fluo-4 intracellular Ca^{2+} responses in RPMI 2650 that were pre-treated 1 hour before with U73122 PLC inhibitor vs U73343 inactive control. 150 μM pyocyanin induced a calcium response in the inactive control, but no response in the active PLC inhibitor. **D).** Intracellular Ca^{2+} (mean ± SEM) 10 minutes post treatment in RPMI 2650.

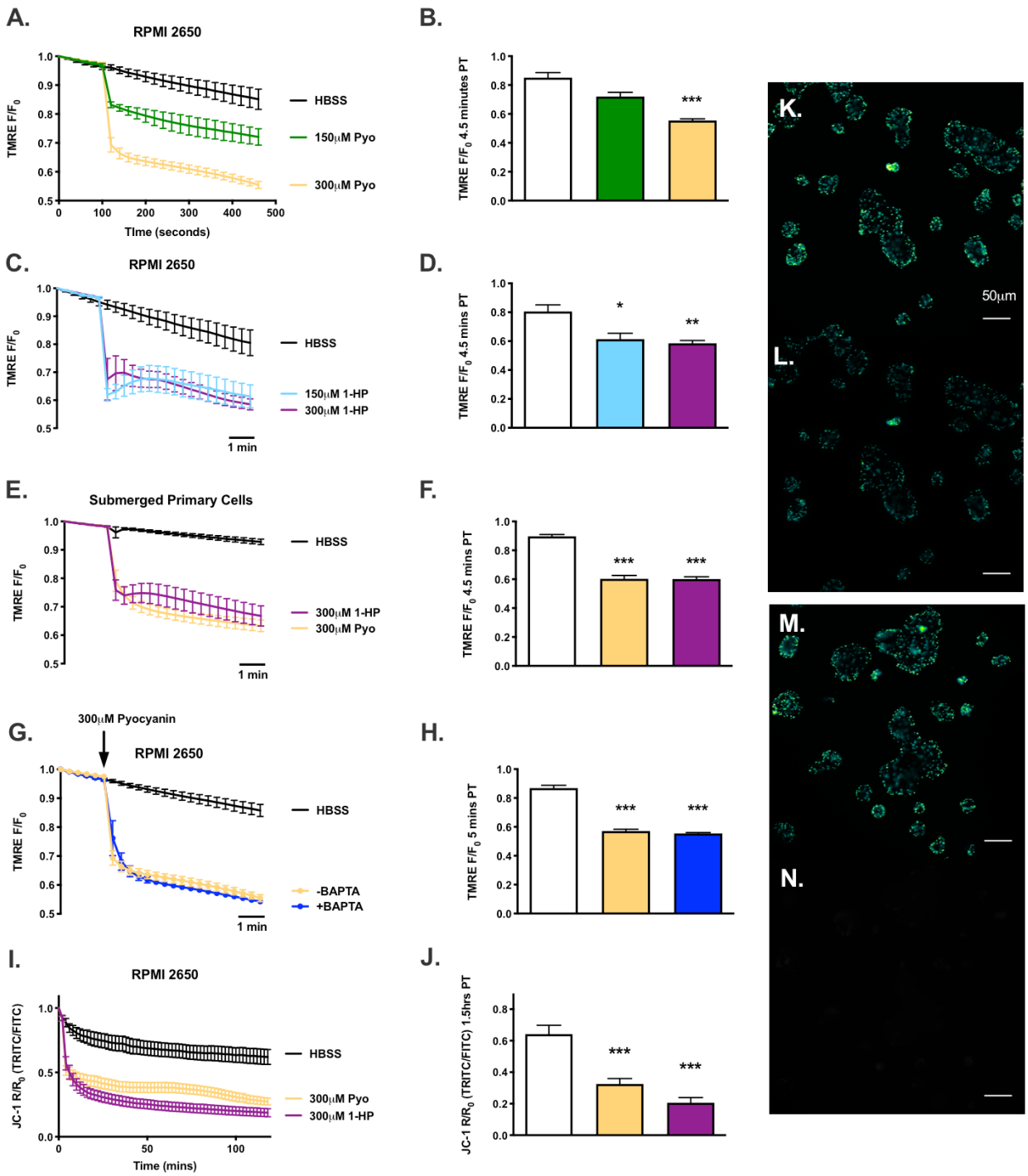
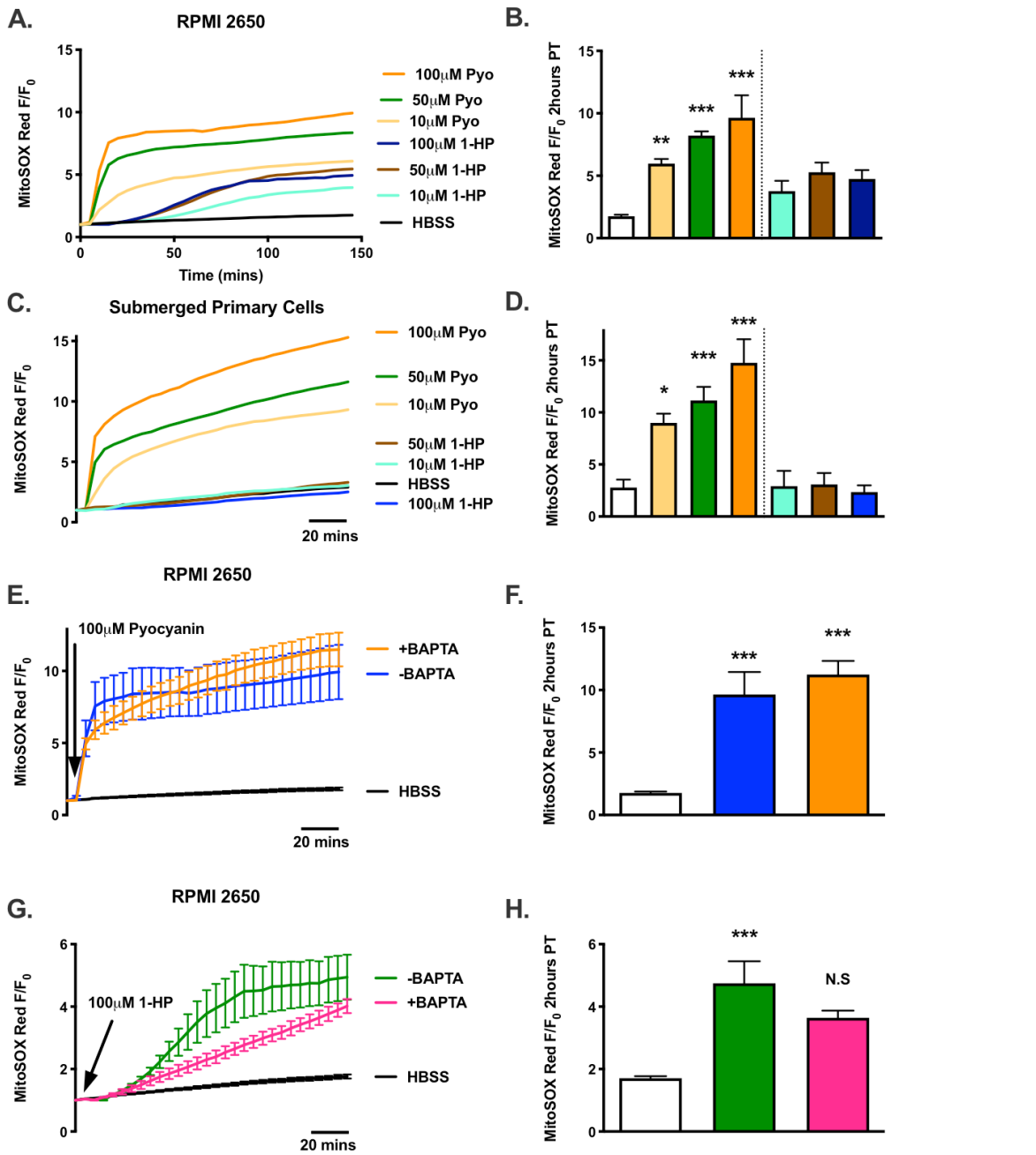


Figure 4. Acute mitochondrial depolarization with pyocyanin and 1-hydroxyphenazine. **A).** Average traces of tetramethylrhodamine ethyl ester (TMRE) dye fluorescence over time and **B).** mean ± SEM after 4.5 minutes in RPMI 2650 after treatment with HBSS, 150 μM, and 300 μM pyocyanin. A reduction in TMRE fluorescence reflects a loss of mitochondrial membrane potential. **C).** Average traces of TMRE and **D).** mean ± SEM after 4.5 minutes in RPMI 2650

after treatment with HBSS, 150 μM, and 300 μM 1-hydroxyphenazine**. E).** Average traces of TMRE dye and **F).** mean ± SEM after 4.5 minutes in submerged primary cells (HNEC) after treatment with HBSS, 300 μM pyocyanin, and 300 μM 1-hydroxyphenazine **G).** Average traces of TMRE pretreated with or without (+/-) BAPTA calcium chealator and **H).** mean ± SEM after 5 minutes in RPMI 2650 after treatment with HBSS, and 300 μM pyocyanin. **I).** Average traces of JC-1 dye fluorescence over time and **J).** mean ± SEM after 1.5 hours in RPMI 2650 after treatments with 300 μM pyocyanin, and 300 μM 1-hydroxyphenazine. Similar to TMRE, the reduction in JC-1 R/R_0 fluorescence indicates a loss of mitochondrial membrane potential. **K and M).** Representative images of JC-1 loaded RPMI 2650 (just TRITC) before treatment with either HBSS or 300 μM pyocyanin respectively and 1.5 hours post treatment with **L).** HBSS or **N).** 300 μM pyocyanin. Images were captured on a 20x objective and TRITC/FITC filters. All scale bars are 50 μm.


A.
RPMI 2650
MitoSOX Red F/F0
Time (mins)
100μM Pyo
50μM Pyo
10μM Pyo
100μM 1-HP
50μM 1-HP
10μM 1-HP
HBSS
B.
MitoSOX Red F/F0 2hours PT
C.
Submerged Primary Cells
20 mins
D.
E.
100μM Pyocyanin
+BAPTA
-BAPTA
F.
G.
100μM 1-HP
H.
N.S

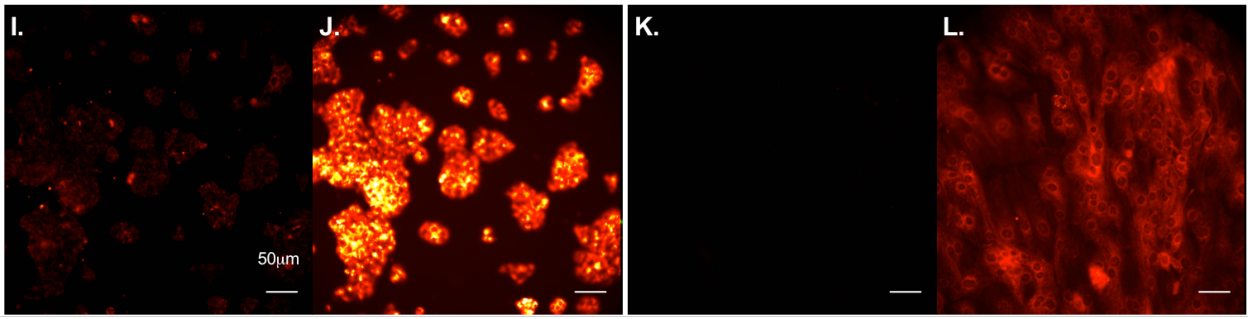

I.
J.
K.
L.
50μm

Figure 5. Pyocyanin and 1-hydroxyphenazine induce superoxide production in RPMI 2650 and but only pyocyanin induces superoxide production in primary nasal cells. A). Average traces of MitoSOX Red fluorescence over time and **B).** mean ± SEM after 2 hours in RPMI 2650 after treatement with HBSS, 100 μM pyocyanin, 50 μM pyocyanin, 10 μM pyocyanin, 100 μM 1-hydroxyphenazine, 50 μM 1-hydroxyphenazine, or 10 μM 1-hydroxyphenazine. An increase in MitoSOX Red fluorescnce indicates an increase in superoxide production. **C).** Average traces of MitoSOX Red fluorescence over time and **D).** mean ± SEM after 2 hours in submerged primary nasal epithelial cells after treatement with HBSS, 100 μM pyocyanin, 50 μM pyocyanin, 10 μM pyocyanin, 100 μM 1-hydroxyphenazine, 50 μM 1-hydroxyphenazine, or 10 μM 1-hydroxyphenazine. **E and G).** Average traces of TMRE pretreated with or without (+/-) BAPTA calcium chealator and **F and H).** mean ± SEM after 2 hours in RPMI 2650 after treatment with HBSS, **F).** 300 μM pyocyanin, or **H).** 300 μM 1-hydroxyphenazine. Representative images of MitoSOX Red loaded **I-J).** RPMI 2650 and **K-L).** submerged primary nasal epithelial cells **I and K).** at baseline, and **J and L).** 2 hours post treatment with 100 μM pyocyanin. Images were captured on a 20x objective and TRITC filter. All scale bars are 50 μm.

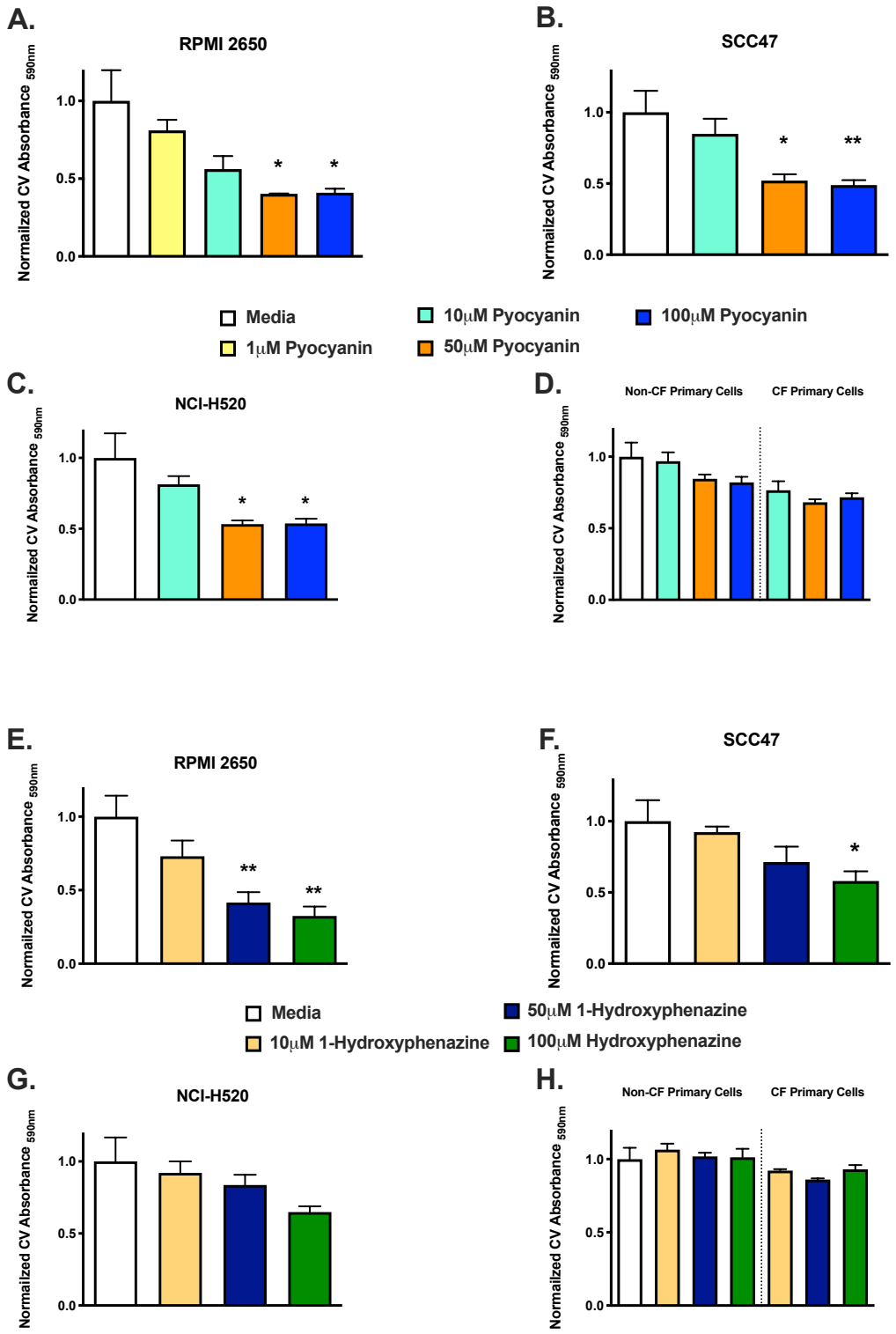


Figure 6. Pyocyanin and 1-hydroxyphenazine reduce viability of RPMI 2650 nasal carcinoma cells but not primary nasal epithelial cells. A-D). Crystal Violet (CV) absorbance in **A).** RPMI 2650s, **B).** SCC-47, **C).** NCI-H520, and **D).** submerged primary nasal epithelial cells

(non-CF vs CF), all after 24 hr treatment with media, 100 μM, 50 μM, 10 μM, or 1 μM pyocyanin (only RPMI 2650)**.** A decrease in CV absorbance indicates a reduction in cell numbers and thus cell viability. RPMI 2650, SCC-47, and NCI-H520 are all squamous cell carcinomas originating from the nasal septum, lateral tongue, and lung mass respectively. **E-H).** Crystal Violet (CV) absorbance in **E).** RPMI 2650 **F).** SCC-47, **G).** NCI-H520, and **H).** submerged primary nasal epithelial cells (non-CF vs CF),, all after 24 hr treatment with media, 100 μM, 50 μM, or 10 μM 1-hydroxyphenazine.

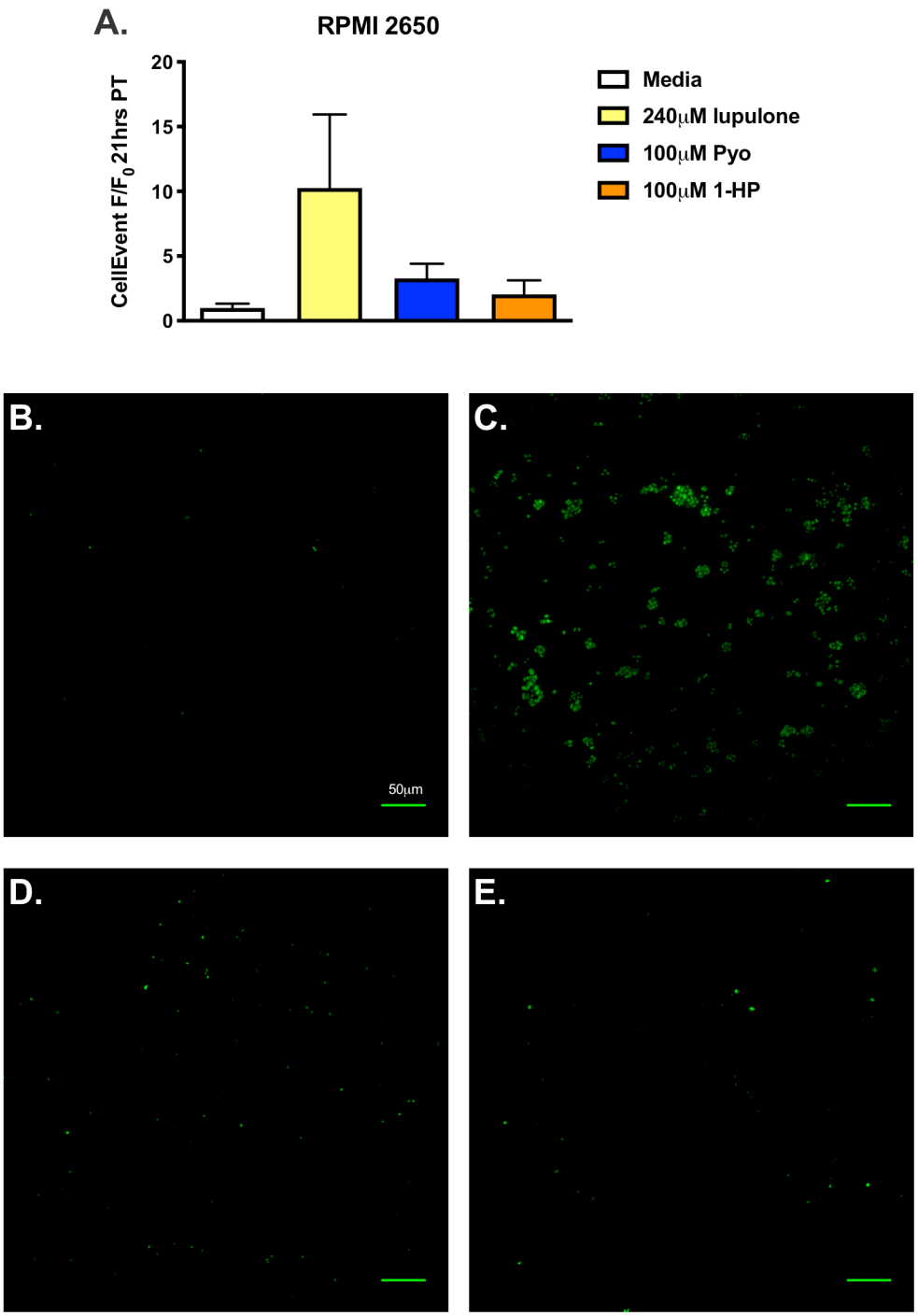


Figure 7. Pyocyanin and 1-hydroxyphenazine do not increase caspase 3 and 7 production significantly in RPMI 2650. A). CellEvent fluorescnce in RPMI 2650 after 21 hour treatment with media, 240 μM lupulone, 100 μM pyocyanin, or 100 μM 1-hydroxyphenazine. An increase in CellEvent fluorescence indicates an increase in caspase 3 and 7 production. **B-E).** Representative images of RPMI 2650 treated with **B).** media, **C).** 240 μM lupulone, **D).** 100 μM

pyocyanin, or **E).** 100 μM 1-hydroxyphenazine, all at 21 hours. Images were captured on a 20x objective and FITC filter. All scale bars are 50 μm.

References

1 Mudaliar, S. B. & Bharath Prasad, A. S. A biomedical perspective of pyocyanin from Pseudomonas aeruginosa: its applications and challenges. *World J Microbiol Biotechnol* **40**, 90, doi:10.1007/s11274-024-03889-0 (2024).

2 Kang, J., Cho, Y. H. & Lee, Y. Pyocyanin and 1-Hydroxyphenazine Promote Anaerobic Killing of Pseudomonas aeruginosa via Single-Electron Transfer with Ferrous Iron. *Microbiol Spectr* **10**, e0231222, doi:10.1128/spectrum.02312-22 (2022).

3 Horcajada, J. P. *et al.* Epidemiology and Treatment of Multidrug-Resistant and Extensively Drug-Resistant Pseudomonas aeruginosa Infections. *Clin Microbiol Rev* **32**, doi:10.1128/CMR.00031-19 (2019).

4 Pang, Z., Raudonis, R., Glick, B. R., Lin, T. J. & Cheng, Z. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies. *Biotechnol Adv* **37**, 177-192, doi:10.1016/j.biotechadv.2018.11.013 (2019).

5 Kouakou, Y. I. & Lee, R. J. Interkingdom Detection of Bacterial Quorum-Sensing Molecules by Mammalian Taste Receptors. *Microorganisms* **11**, doi:10.3390/microorganisms11051295 (2023).

6 Miller, Z. A. *et al.* Pseudomonas aeruginosa metabolite 3-oxo-C12HSL induces apoptosis through T2R14 and the mitochondrial calcium uniporter. *bioRxiv*, 2024.2010.2024.620094, doi:10.1101/2024.10.24.620094 (2024).

7 Miller, Z. A. *et al.* Lidocaine induces apoptosis in head and neck squamous cell carcinoma through activation of bitter taste receptor T2R14. *Cell Rep* **42**, 113437, doi:10.1016/j.celrep.2023.113437 (2023).

8 Qin, S. *et al.* Pseudomonas aeruginosa: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. *Signal Transduct Target Ther* **7**, 199, doi:10.1038/s41392-022-01056-1 (2022).

9 Sinha, S. *et al.* Generation of Reactive Oxygen Species Mediated by 1-Hydroxyphenazine, a Virulence Factor of Pseudomonas aeruginosa. *Chem Res Toxicol* **28**, 175-181, doi:10.1021/tx500259s (2015).

10 Denning, G. M., Railsback, M. A., Rasmussen, G. T., Cox, C. D. & Britigan, B. E. Pseudomonas pyocyanine alters calcium signaling in human airway epithelial cells. *Am J Physiol* **274**, L893-900, doi:10.1152/ajplung.1998.274.6.L893 (1998).

11 Li, T. *et al.* Pyocyanin induces NK92 cell apoptosis via mitochondrial damage and elevated intracellular Ca(2). *Innate Immun* **25**, 3-12, doi:10.1177/1753425918809860 (2019).

12 Wilson, R. *et al.* Measurement of Pseudomonas aeruginosa phenazine pigments in sputum and assessment of their contribution to sputum sol toxicity for respiratory epithelium. *Infect Immun* **56**, 2515-2517, doi:10.1128/iai.56.9.2515-2517.1988 (1988).

13 Munro, N. C. *et al.* Effect of pyocyanin and 1-hydroxyphenazine on in vivo tracheal mucus velocity. *J Appl Physiol (1985)* **67**, 316-323, doi:10.1152/jappl.1989.67.1.316 (1989).

14 Wilson, R. *et al.* Pyocyanin and 1-hydroxyphenazine produced by Pseudomonas aeruginosa inhibit the beating of human respiratory cilia in vitro. *J Clin Invest* **79**, 221-229, doi:10.1172/JCI112787 (1987).

15 McMahon, D. B. *et al.* The bitter end: T2R bitter receptor agonists elevate nuclear calcium and induce apoptosis in non-ciliated airway epithelial cells. *Cell Calcium* **101**, 102499, doi:10.1016/j.ceca.2021.102499 (2022).

16 Kouakou, Y. I. *et al.* Hops bitter beta-acids have antibacterial effects against sinonasal Staphylococcus aureus but also induce sinonasal cilia and mitochondrial dysfunction. *Int Forum Allergy Rhinol*, doi:10.1002/alr.23487 (2024).

17 Wu, J. *et al.* Red fluorescent genetically encoded Ca2+ indicators for use in mitochondria and endoplasmic reticulum. *Biochem J* **464**, 13-22, doi:10.1042/BJ20140931 (2014).
18 Bianchi, S. M. *et al.* Impairment of apoptotic cell engulfment by pyocyanin, a toxic metabolite of Pseudomonas aeruginosa. *Am J Respir Crit Care Med* **177**, 35-43, doi:10.1164/rccm.200612-1804OC (2008).
19 Ribeiro, C. M. P. *et al.* Revisiting Host-Pathogen Interactions in Cystic Fibrosis Lungs in the Era of CFTR Modulators. *Int J Mol Sci* **24**, doi:10.3390/ijms24055010 (2023).
20 Kang, S. H., Dalcin Pde, T., Piltcher, O. B. & Migliavacca Rde, O. Chronic rhinosinusitis and nasal polyposis in cystic fibrosis: update on diagnosis and treatment. *J Bras Pneumol* **41**, 65-76, doi:10.1590/S1806-37132015000100009 (2015).
21 Kanthakumar, K. *et al.* Mechanisms of action of Pseudomonas aeruginosa pyocyanin on human ciliary beat in vitro. *Infect Immun* **61**, 2848-2853, doi:10.1128/iai.61.7.2848-2853.1993 (1993).
22 Jackowski, J. T. *et al.* Effects of P. aeruginosa-derived bacterial products on tracheal ciliary function: role of O2 radicals. *Am J Physiol* **260**, L61-67, doi:10.1152/ajplung.1991.260.2.L61 (1991).
23 Hao, Y. *et al.* Pseudomonas aeruginosa pyocyanin causes airway goblet cell hyperplasia and metaplasia and mucus hypersecretion by inactivating the transcriptional factor FoxA2. *Cell Microbiol* **14**, 401-415, doi:10.1111/j.1462-5822.2011.01727.x (2012).
24 Denning, G. M. *et al.* Pseudomonas pyocyanin increases interleukin-8 expression by human airway epithelial cells. *Infect Immun* **66**, 5777-5784, doi:10.1128/IAI.66.12.5777-5784.1998 (1998).
25 O'Malley, Y. Q., Reszka, K. J., Spitz, D. R., Denning, G. M. & Britigan, B. E. Pseudomonas aeruginosa pyocyanin directly oxidizes glutathione and decreases its levels in airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **287**, L94-103, doi:10.1152/ajplung.00025.2004 (2004).
26 Schwarzer, C., Fu, Z., Fischer, H. & Machen, T. E. Redox-independent activation of NF-kappaB by Pseudomonas aeruginosa pyocyanin in a cystic fibrosis airway epithelial cell line. *J Biol Chem* **283**, 27144-27153, doi:10.1074/jbc.M709693200 (2008).
27 Schwarzer, C. *et al.* Oxidative stress caused by pyocyanin impairs CFTR Cl(-) transport in human bronchial epithelial cells. *Free Radic Biol Med* **45**, 1653-1662, doi:10.1016/j.freeradbiomed.2008.09.011 (2008).
28 Rada, B., Lekstrom, K., Damian, S., Dupuy, C. & Leto, T. L. The Pseudomonas toxin pyocyanin inhibits the dual oxidase-based antimicrobial system as it imposes oxidative stress on airway epithelial cells. *J Immunol* **181**, 4883-4893, doi:10.4049/jimmunol.181.7.4883 (2008).
29 Hao, Y., Kuang, Z., Xu, Y., Walling, B. E. & Lau, G. W. Pyocyanin-induced mucin production is associated with redox modification of FOXA2. *Respir Res* **14**, 82, doi:10.1186/1465-9921-14-82 (2013).
30 Roussel, L., LaFayette, S., Nguyen, D., Baglole, C. J. & Rousseau, S. Differential Contribution of the Aryl-Hydrocarbon Receptor and Toll-Like Receptor Pathways to IL-8 Expression in Normal and Cystic Fibrosis Airway Epithelial Cells Exposed to Pseudomonas aeruginosa. *Front Cell Dev Biol* **4**, 148, doi:10.3389/fcell.2016.00148 (2016).
31 Xu, Y. *et al.* Pseudomonas aeruginosa pyocyanin activates NRF2-ARE-mediated transcriptional response via the ROS-EGFR-PI3K-AKT/MEK-ERK MAP kinase signaling in pulmonary epithelial cells. *PLoS One* **8**, e72528, doi:10.1371/journal.pone.0072528 (2013).

32 Stewart-Tull, D. E. & Armstrong, A. V. The effect of 1-hydroxyphenazine and pyocyanin from Pseudomonas aeruginosa on mammalian cell respiration. *J Med Microbiol* **5**, 67-73, doi:10.1099/00222615-5-1-67 (1972).
33 Armstrong, A. V. & Stewart-Tull, D. E. The site of the activity of extracellular products of Pseudomonas aeruginosa in the electron-transport chain in mammalian cell respiration. *J Med Microbiol* **4**, 263-270, doi:10.1099/00222615-4-2-263 (1971).
34 Armstrong, A. V., Stewart-Tull, D. E. & Roberts, J. S. Characterisation of the Pseudomonas aeruginosa factor that inhibits mouse-liver mitochondrial respiration. *J Med Microbiol* **4**, 249-262, doi:10.1099/00222615-4-2-249 (1971).
35 Parsons, J. F. *et al.* Structural and functional analysis of the pyocyanin biosynthetic protein PhzM from Pseudomonas aeruginosa. *Biochemistry* **46**, 1821-1828, doi:10.1021/bi6024403 (2007).
36 Jayaseelan, S., Ramaswamy, D. & Dharmaraj, S. Pyocyanin: production, applications, challenges and new insights. *World J Microbiol Biotechnol* **30**, 1159-1168, doi:10.1007/s11274-013-1552-5 (2014).
37 Mavrodi, D. V. *et al.* Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from Pseudomonas aeruginosa PAO1. *J Bacteriol* **183**, 6454-6465, doi:10.1128/JB.183.21.6454-6465.2001 (2001).