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Antimicrobial Agents and Chemotherapy: Article

Exploiting interkingdom interactions for the development of small molecule inhibitors of *Candida albicans* biofilm formation

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Running Head: Hydroxy Alkylquinolone signals target *Candida* biofilm.

FJR and JPP contributed equally to this work.

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Abstract

A rapid decline in the development of new antimicrobial therapeutics has coincided with the emergence of new and more aggressive multidrug resistant pathogens. Pathogens are protected from antibiotic activity by their ability to enter an aggregative biofilm state. Therefore, disrupting this process in pathogens is a key strategy for the development of next generation antimicrobials. Here we present a suite of compounds, based on the *Pseudomonas aeruginosa* 2-heptyl-4(1H)-quinolone (HHQ) core quinolone interkingdom signal structure, that exhibit non-cytotoxic anti-biofilm activity towards the fungal pathogen *Candida albicans*. In addition to providing new insights into what is a clinically important bacterial-fungal interaction, the capacity to modularize the functionality of the quinolone signals is an important advance in harnessing the therapeutic potential of signaling molecules in general. This provides a platform for the development of potent next generation small molecular therapeutics targeting clinically relevant fungal pathogens.

Introduction

With the ever increasing emergence of antibiotic resistant pathogens and the lack of new antibiotics coming to market, we are entering a 'post-antibiotic era' (1-3). This realization has underpinned a global initiative to identify new and innovative approaches to infection management. As such, targeting virulence as a potential strategy for developing new anti-microbial drugs has been the focus of several research initiatives (4-11). In principle, suppressing virulence behavior and locking pathogens in a vegetative non-biofilm forming lifestyle renders them less infective and more susceptible to conventional antibiotics (4, 12). While some success has been achieved against bacterial pathogens (6, 10, 13-19), less focus has been placed on fungal infections which nevertheless continue to cause serious complications and mortality in patients (8, 20-22). Indeed, despite the medical and economic damage caused by fungal biofilms, there remains an urgent and largely unmet need for the identification of compounds able to specifically and selectively target and inhibit this mode of growth in clinically relevant fungal pathogens (23).

The predominant nosocomial fungal pathogens, which include *Candida* spp., *Aspergillus* spp., and *Fusarium* spp., are difficult to diagnose and cause high morbidity and mortality, even following antifungal therapy (21). *Candida albicans* causes a variety of complications ranging from mucosal disease to deep seated mycoses, particularly in immunocompromised individuals (21, 24). Along with other fungal and yeast pathogens, *C. albicans* are known to form structured communities known as biofilms on medical devices either pre- or post-implantation leading to recurring infections and in some cases death (25, 26). Once established in the biofilm phase, *C. albicans* presents a significant clinical problem with current treatment options severely limited

by the intrinsic tolerance of fungal biofilms to antimycotics (20, 27, 28). Recent combination therapies incorporating antibacterial and antifungal agents have provided some success (29). However, as with all antibiotic based strategies, reports of resistance continue to emerge (27), and biofilms themselves are considered a breeding ground for the emergence of antibiotic resistant strains, effectively hastening the onset of the perfect storm whereby the rapid decline in new antibiotic production has been met by an equally rapid increase in multidrug resistant organisms (1). Thus the need to consider new anti-infective strategies that do not target essential processes in the target organism. While blocking biofilms in these organisms' remains a major clinical challenge (26, 30), exploiting our increased understanding of microbial signaling networks to control virulence and biofilm behavior is one innovative approach with significant potential.

Many sites of infection are colonized by communities of mixed fungal and bacterial organisms, and several layers of communication impact significantly on the dynamics and flux of these populations (31, 32). For example, *C. albicans* is known to co-exist with *Pseudomonas aeruginosa* in the cystic fibrosis (CF) lung, and interkingdom communication between the two organisms has previously been reported (16, 33). The Pseudomonas Quinolone Signal (PQS), 2-heptyl-3-hydroxy-4-quinolone, and its biological precursor 2-heptyl-4-quinolone (HHQ) are important virulence factors produced by *P. aeruginosa*. Structurally, PQS and HHQ differ by the presence of a hydrogen at C3 in HHQ and a hydroxyl group in PQS, giving rise to the increased interest in modulating this position to assign biological function to the structure of these molecules (34-37). Previously, we have shown that HHQ, but not PQS, suppresses biofilm formation of *C. albicans* (10). In response, *C. albicans* produces farnesol which has been shown

to modulate PQS production in *P. aeruginosa* (33). As both PQS and HHQ promote virulence and pathogenicity of *P. aeruginosa* (38, 39), their utility as an anti-*Candida* treatment falls short of being a viable anti-fungal treatment. However, the amenability of these small molecules to chemical modification provides an opportunity to develop compounds with specificity of function.

The transcriptional data and microscopic imaging described in this study have implicated components of the cell wall as key factors in the response of *C. albicans* to *P. aeruginosa* alkylhydroxyquinolone (AHQ) signaling. Furthermore, the biological activity of each class of analogue in bacterial, fungal and host systems provides a new insight into the possible interkingdom role of AHQs, particularly in a clinical setting such as the CF lung where all three systems co-exist. From a translational perspective, lead HHQ analogues were identified with four key features: (1) potent anti-biofilm activity towards *C. albicans*, (2) selective non-cytotoxicity towards mammalian cell lines, (3) non-agonistic and (4) potentially antagonistic to the virulent pathogen *P. aeruginosa*. Several analogues retained the significant potency of the parent HHQ molecule against *C. albicans* biofilm formation, whilst simultaneously becoming inactive in *P. aeruginosa* quorum sensing. This suggests that these molecules have the potential to be further optimized for use as anti-*Candida* infectives without the concomitant limitation of *P. aeruginosa* virulence augmentation.

Materials and Methods

C. albicans stock maintenance and culturing conditions.

C. albicans strain SC5314 was sub-cultured from 15% (v/v) glycerol stocks at -80ºC onto Yeast Peptone Dextrose (YPD) medium [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose] and incubated at 30°C overnight.

P. aeruginosa stock maintenance and culturing conditions.

P. aeruginosa strains, PAO1 and *pqsA*$^{-}$ mutant, containing the chromosomally inserted *pqsA-lacZ* promoter fusion on plasmid pUC18-mini-Tn7, were maintained on Luria Bertani (LB) agar plates, supplemented with carbenicillin (200 µg/ml) and X-gal (40 µg/ml), and incubated at 37°C overnight. Single colonies were inoculated into LB broth (20 ml), supplemented with carbenicillin (200 µg/ml), and incubated at 37°C, shaking at 180 rpm overnight. For subsequent experiments, the OD_{600nm} was recorded and a starting OD_{600nm} of 0.02 was inoculated into fresh LB broth, supplemented with carbenicillin (200 µg/ml) and incubated at 37°C, shaking at 180 rpm.

Structural modification of HHQ

The synthesis of HHQ, PQS (40, 41) and other HHQ-based analogues (36, 37) was carried out via previously described methods. Novel compounds and compounds which required modified syntheses are described *vide infra* and in the supporting information (**Supplemental Data**).

Thin Layer Chromatographic (TLC) analysis.

Silica TLC plates, activated by soaking in 5% (w/v) K_2HPO_4 for 30 min were placed in an oven at 100ºC for 1 hr (42). Analogues (5 µl, 10 mM) were spotted approximately 1 cm from the bottom. The spots were dried and the plate placed in a mobile phase comprising 95:5

dichloromethane:methanol. The plate was viewed under UV light when the mobile phase had run 5 cm below the top of the plate.

Biofilm formation, quantification, and visualization.

C. albicans biofilm formation was carried out in 96 well plates, as previously described (43). Seeding densities for all subsequent experiments (n=3) were OD_{600nm} 0.05. Biofilm formation was measured as previously described using a semi-quantitative tetrazolium salt, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H tetrazolium-5-carboxanilide inner salt (XTT) reduction assay (44). Experiments were repeated at least three times, with at least eight technical replicates. Visualization of biofilm formation was performed on glass coverslips in 6-well plates using Confocal Scanning Laser Microscopy (CSLM). All images were captured using the Zeiss HBO-100 microscope illuminating system, processed using the Zen AIM application imaging program and converted to JPGs using Axiovision 40 Ver. 4.6.3.0. A minimum of three independent biological repetitions were carried out.

Viable colony biofilm assay

C. albicans biofilms, supplemented with analogues and parent compounds, were grown in 6-well plates and incubated overnight at 37°C. Briefly, *C. albicans* Yeast Nitrogen Base (YNB) cultures were measured at OD_{600nm}, diluted to 0.05 in YNB-NP supplemented with analogues, plated onto 6-well plates and incubated for 1 hr at 37^{o}C. Media was removed, wells were washed twice with sterile PBS and supplemented with fresh YNB-NP with analogues. Plates were incubated overnight at 37°C after which media was removed and wells washed with sterile PBS. For serial dilutions, biofilms were cell-scraped into 1 ml PBS, vortexed, and serially diluted into sterile

PBS. Serial dilutions were plated (100 µl) onto YPD agar and incubating overnight at 37°C. Colonies were counted and recorded the next day.

C. albicans growth curves

Overnight *C. albicans* cultures grown in YNB were diluted to 0.05 in YNB supplemented with analogues. Cultures (200 µl) were added to each well of a 100 well plate and grown for a 24 hr period on a Bioscreen C spectrophotometer (Growth Curves USA).

RNA isolation and qRT-PCR transcriptional analysis.

Overnight *C. albicans* cultures were diluted to 0.05 at OD_{600nm} in either YNB or YNB-NP (Difco). YNB cultures were supplemented with methanol whereas YNB-NP cultures were supplemented with either 100 µM HHQ or the methanol volume equivalent. Cultures were grown at 37ºC with agitation (180 rpm) for 6 hr after which they were centrifuged at 4000 rpm, supernatants discarded and pellets frozen at -20ºC until processing. RNA was isolated using the MasterPure Yeast RNA purification kit (Cambio Ltd, Cambridge UK) according to manufacturer's specifications, and was quantified using a ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Genomic DNA was enzymatically removed using Turbo DNA-free DNase (Ambion), and samples were confirmed DNA free by PCR. RNA was converted to cDNA using random primers and AMV reverse transcriptase (Promega) according to manufacturer's instructions. qRT-PCR was carried out using the Universal ProbeLibrary (UPL) system (Roche) according to manufacturer's specifications, and samples were normalized to *C. albicans* actin transcript expression (*ACT1*). A full list of primers and UPL probes used in this study is detailed in Supplemental Table 1.

Phenazine extraction.

P. aeruginosa strains were cultured as described above for 24 hr, with the addition of analogues (100 µM), and pyocyanin was extracted as described previously (45). Cultures were centrifuged at 4000 rpm for 10 minutes and the cell free supernatant (5 ml) removed. Chloroform (3 ml) was added, and mixed by vortex. After centrifugation at 4000 rpm for 5 min, the lower aqueous phase was transferred to 0.2 M HCl (2 ml). Samples were mixed by vortex and centrifuged at 4000 rpm for 5 min to separate the phases. An aliquot of the top phase (1 ml) was removed and spectrophotometrically analyzed at OD_{570nm}. Phenazine production was calculated using the following formula: OD_{570nm} x 2 x 17.072 and the units expressed in µg/ml.

Promoter fusion based expression analysis.

Promoter fusion analyses were performed in a 96-well format, with β-galactosidase activity measured as described previously (46). Briefly, overnight cultures of wild-type *PAO1 pqsA-lacZ* (pLP0996) and mutant strain PAO1 *pqsA*$^{-}$ *pqsA-LacZ* were diluted to OD_{600nm}=0.02 in LB. Analogues at 100 µM final concentration were added, mixed, aliquoted into 96 well plates and incubated overnight at 37ºC with shaking. The next day, OD_{600nm} values were recorded in a plate reader. Aliquots of cells (0.02 ml) were permeabilized [100 mM dibasic sodium phosphate (Na_2HPO_4), 20 mM KCl, 2 mM $MgSO_4$, 0.8 mg/mL CTAB (hexadecyltrimethylammonium bromide), 0.4 mg/mL sodium deoxycholate, 5.4 µL/mL beta-mercaptoethanol] and added to substrate solution [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mg/mL o-nitrophenyl-β-D-Galactoside (ONPG), 2.7 µL/mL β-mercaptoethanol]. The kinetics of color development was monitored and the reactions were stopped using 1M $NaCO_3$. OD_{420nm} were recorded as above.

Miller units were calculated using the following equation; 1000 x [OD_{420nm}/(OD_{600nm}) x 0.02 ml x reaction time (min)].

Cytotoxicity Assay.

Lactate dehydrogenase (LDH) release from a panel of mammalian cells was assayed as a measure of cytotoxicity using an LDH colorimetric kit (Roche) according to manufacturers' instructions (36). Briefly, IB3-1 lung epithelial cells, A549 human lung adenocarcinoma epithelial cells, DU-145 human prostate cancer cells and HeLa cervical cancer cells were seeded onto 96 well plates and treated with methanol (control) and analogues. Following 16 hr incubation at 37°C and 5% CO_2, supernatants were removed and added to catalyst reaction mixture in a fresh plate and further incubated at 37°C and 5% CO_2 for 30 min to allow for color development. After this period, the plate was analyzed on an ELISA plate reader at OD_{490nm}. Cytotoxicity was expressed as a percentage of cells treated with 0.1% (v/v) Triton (100% cytotoxicity).

Statistical analysis.

All graphs were compiled using GraphPad Prism (version 5.01) unless otherwise stated. All data were analyzed using built-in GraphPad Prism (version 5.01) functions as specified. The level of significance was set at $p = 0.05$ (*) and post-hoc comparisons between groups were performed using the Bonferroni multiple-comparison test.

Results

Key modifications of the quinolone framework retain anti-biofilm activity towards *C. albicans*

The HHQ molecule has previously been shown to suppress biofilm formation in *C. albicans* at concentrations from 10 – 100 μM (2.47 – 24.7 μg/ml) independent of any effects on the growth of planktonic cells (10). Previous structure function analysis of the activity of the quinolone framework had implicated the C-3 position as a key component of interspecies anti-biofilm activity (36). We undertook further modification of the HHQ parent molecule with the aim of developing viable anti-biofilm compounds to target *C. albicans*. These compounds were incorporated into a larger collection of alkylquinolone analogues, systematically modified at different positions on the molecule and classified on the basis of their substitutions relative to the parent framework HHQ (**Table 1**).

The suite of analogues was first tested to establish their potency as anti-biofilm compounds against *C. albicans* using an optimized XTT assay, a commonly used quantitative method to assess *Candida* biofilm mass and growth (47). As previously described (10), HHQ significantly suppressed biofilm formation when compared to untreated and methanol treated cells, whereas PQS appeared to induce biofilm formation (**Figure 1**). When all analogues were similarly screened by XTT assay, several had similar anti-biofilm activity to HHQ [**1** and **2** (class I; modified at C-3)], **3, 4**, **6**, **7**, **9** (class II; modified anthranilate ring), and **12** (class III; modified alkyl chain) (**Figure 2a**). These analogues were diverse members of classes I, II, and III

suggesting that several components of the HHQ framework contribute to anti-biofilm activity of the parent compound. A number of substitutions led to intermediate anti-biofilm activity, including **5**, **8**, **10**, **11**, (class II; modified anthranilate ring) and **15** (class V; modified anthranilate ring and alkyl chain length), while some analogues had completely lost the ability to suppress *C. albicans* biofilm formation e.g. **13** (class IV; modified C-4), and the class V compounds **14**, **16**, and **17** (**Figure 2a**). While modification of the C-3 position to produce PQS led to loss of anti-biofilm activity (**Figure 1**), incorporation of an –N-NH_2 moiety (**2**) at the 3-position or substitution of C-3 with NH (**1**), did not affect the ability to suppress *C. albicans* biofilm formation (**Figure 2a**). Addition of Cl at the C6 and C8 positions of the anthranilate ring (**6** and **7**) also did not lead to loss of anti-biofilm activity. In contrast, the introduction of considerable steric bulk with the addition of an *n*-hexyl alkyl chain at C6 of the anthranilate ring (**5**), or elaboration of the aromatic group as with the naphthyl compound (**8**), resulted in compounds with significantly less potent anti-biofilm activity relative to HHQ. These data suggest an exquisite level of specificity for the interaction between HHQ and the *C. albicans* biofilm intracellular machinery. Modification of the C2 alkyl chain from *n*-heptyl (HHQ) to *n*-nonyl C9 (**12**) did not affect anti-biofilm activity, while parallel modification of the anthranilate ring resulted in a complete loss, as with the Class V compounds **16** or **17**. Modifying the C-4 position (C=O to C=S) (**13**), the quinolone thiol exhibited an increase in XTT activity ($P<0.05$) relative to controls (**Figure 2a**), comparable to the increase observed in the presence of PQS (**Figure 1**). Previously, we have shown that HHQ elicits a dose-dependent reduction in *C. albicans* biofilm formation (10). In order to determine if this also applied to the analogues that retain anti-biofilm activity, dose response analysis of selected compounds **1**, **2**, **4**, **6**, and **12**

representing classes I, II, and IV, was undertaken. This revealed compound specific responses, with 10 µM of compounds **2**, **4**, and **12** being sufficient to elicit a statistically significant reduction in biofilm formation (**Figure 2b**). All five compounds reduced biofilm formation when applied at 50 µM and 100 µM. To further confirm the anti-biofilm activity of the lead compounds, viable colony counts were performed on selected analogues using the maximum 100 µM compound dose. This confirmed the outputs from the XTT assays; all analogues, along with HHQ, significantly reduced viable biofilm cells in comparison to the control (**Figure S1a**). Importantly, the anti-biofilm activity was found to be independent of planktonic growth, which was unaffected in the presence of selected compounds (**Figure S1b**).

Microscopic staining reveals structural changes in *C. albicans* biofilms

The formation of biofilms in bacteria, yeast and fungi is a highly ordered process involving multicellular behavior and has been defined in several stages (22). Confocal microscopy combined with intracellular staining was used to assess structural integrity and cellular morphology of *C. albicans* incubated on coverslips. Biofilms were individually stained with each of the dyes and multiple fields of view were visualized to accurately represent the effect of the analogues. The biofilm observed for methanol and untreated controls displayed all the characteristics of a typical *C. albicans* biofilm and were classified as wild-type (**Figure 1**). Calcofluor, Concanavalin A and FUN-1 staining revealed a uniform distribution of chitin/cellulose and cell wall mannosyl/glucosyl residues indicative of viable wild-type morphology (**Figure 1**). Those analogues identified by XTT assay as causing impaired biofilm

formation (**1, 2**, **3**, **4**, **6**, **7**, **9**, **12**, and **15)** exhibited markedly disrupted structures when grown on coverslips and were classified as atypical morphologies (**Figure 3**). Cells treated with class I analogues were found to be largely compromised in their biofilm forming capabilities and were classified as morphologically atypical. Biofilms produced with both **1** and **2** were significantly distorted, displaying a spindle-like phenotype. Hyphae were short in length, and predominantly displayed yeast cell types rather than hyphal structures. Other structure disrupting analogues were from Class II, III and V, suggesting that specific modifications on the anthranilate ring and alkyl chain variation do not significantly affect the anti-biofilm activity compared to the parent compound (**Figure 3**).

Some analogues, including those that exhibited intermediate activity in the XTT assay, did not alter the biofilm structure, with **5**, **11**, **13**, **14** and **16** all placed into the wild-type morphology group. Biofilms formed in the presence of **13** showed hyper-production of short hyphae, creating a dense mycelial network (**Figure S2**). The remaining analogues from Class II; **8** and **10** (**Figure S1**) caused significant biofilm disruption with fragmented hyphae, stunted vegetative growth and considerably large cell debris fields. Cells incubated with the Class V molecule **17** induced a severely compromised phenotype (**Figure S2**) where debris fields comprising yeast cells and blastospores characterized the structural phenotype.

Enhanced gene transcript expression of *HWP1*, *ECE1*, *ALS3*, *IHD1* and the uncharacterized open reading frame *ORF 19.2457* provides a molecular mechanism for alkyl quinolone activity towards *C. albicans*

In addition to providing new insights into the interkingdom relationship between these important pathogens, there has recently been a strong emphasis placed on ligand receptor interactions and the need to provide molecular mechanisms for the action of any potential therapeutic compound (48). We previously implicated *TUP1* in the HHQ-mediated suppression of biofilm formation in *C. albicans* suggesting a role for the cell-wall in this interaction (10, 16). More recently, several reports have shown changes in expression of cell-wall associated genes linked to biofilm formation in this organism (16, 20, 28, 49-51). These included a cohort of eight genes that are proposed to constitute the core filamentous response network; namely *ALS3*, *ECE1*, *HGT2*, *HWP1*, *IHD1*, *RBT1*, *DCK1*, and the gene of unknown function open reading frame *orf19.2457* (51). Therefore, transcript expression of a cohort of genes implicated in cell wall biogenesis, hyphal development, biofilm formation and other related functions that were previously shown to be upregulated during the morphological transition from yeast to filamentous growth was investigated (**Table S1**) (16, 51). The housekeeping gene *ACT1* was chosen for normalization based on previous biofilm studies (52). We observed that several transcripts were hyper-expressed in a HHQ dependent manner; specifically, *HWP1*, *ECE1*, *ALS3*, *IDH*1 and the as yet uncharacterized open reading frame *(ORF) 19.2457* (**Figure 4**). The remaining transcripts (*CPH1*, *EFB1*, *ESS1*, *RBT1*, *TUP1*, *BCR1*, *DCK1*, and *HGT2*) yielded expression patterns similar to control cells (**Figure S3**). It was perhaps somewhat surprising that, while treatment of *C. albicans* with *P. aeruginosa* supernatants has previously been shown to downregulate expression of the *RBT1*, *RBT5* and *RBT8* genes (16), expression of *RBT1* was unaltered in the presence of HHQ (**Figure S3**). Taken together, these data suggest that HHQ induces a specific subset of cell wall proteins in *C. albicans*. Further work is needed to identify the upstream

components of this response, although *in silico* screening of *C. albicans* genome sequences has ruled out the presence of an obvious PQS receptor (unpublished data).

Lead compounds display reduced cytotoxic activity towards specific mammalian cell lines

Evaluating the cytotoxicity of synthetic compounds is crucial in the context of developing targeted and highly optimized molecular therapeutics that are benign to human cellular physiology and ideal for use in a clinical environment. In previous work, we showed that analogue **1** was significantly less cytotoxic than HHQ, with an 80% reduction in LDH release relative to the parent compound (36). Therefore, the suite of analogues was tested for *in vitro* cytotoxicity towards IB3-1 airway epithelial cells. Class I analogues exhibited reduced cytotoxicity to IB3-1 cells with **2** displaying approximately 34% toxicity (**Figure 5a**). Several class II analogues (**4**, **6**, and **9**) exhibited reduced cytotoxicity relative to IB3-1 cells treated with HHQ, with **7** not reaching statistical significance. The class III analogue **12** was comparable to HHQ. Of the analogues that did not retain anti-biofilm activity, **5, 8, 10** and **11** exhibited variable cytotoxicity to IB3-1 cells whereas **13** exhibited considerably reduced cytotoxicity to IB3-1 cells (**Figure S4**). Finally, **16** exhibited very low levels of cytotoxicity, while **17** was reduced relative to HHQ treated cells. Compound **15** was the most toxic killing approximately 91% of all cells (**Figure 5a**).

In order to achieve a more comprehensive understanding of the selective toxicity of the lead compounds, several additional cell lines were tested (**Figure 5b**). LDH release assays were performed in A549, DU145, and HeLa cell lines in the presence of 100 μM of the lead

compounds revealed distinct cytotoxicity profiles, with **1** and **9** consistently proving the least cytotoxic of the compounds tested. Compounds **4** and **6** exhibited reduced cytotoxicity in DU145 cells (although not statistically significant) but were comparable to HHQ in both the A549 and HeLa cell lines, while compound **2** exhibited increased cytotoxicity relative to HHQ in DU145 cells (**Table 2**). These data suggest that cell-specific cytotoxicity analysis will need to be performed prior to the introduction of these compounds in an applied setting.

HHQ analogues display a spectrum of agonist activity towards *P. aeruginosa* virulence.

Taken together, compounds **1**, **4**, **6**, and **9** pass both the first and second criteria described above, i.e. they retain anti-biofilm activity towards *C. albicans* while exhibiting reduced selective cytotoxicity towards specific host cell lines. However, both HHQ and PQS are co-inducers of the virulence associated LysR-Type Transcriptional Regulator; PqsR (41). The structural moieties that underpin the interaction between HHQ/PQS and PqsR remain to be fully characterized, although recent studies have reported diverse classes of PqsR antagonist (53-55), and implicated the hydrophobic pocket situated within the PqsR protein (56). Therefore, in order to assess whether the lead compounds could elicit a virulence response from *P. aeruginosa*, phenazine production and *pqsA* promoter activity (57) were monitored in a *pqsA* mutant where the capacity to produce native HHQ and PQS had been lost.

Both HHQ and PQS restored phenazine production in the *pqsA*- strain (**Figure 6A**). In contrast, the majority of analogues did not restore phenazine production in this strain, with the notable exception of compound **9**. Several analogues from different classes did partially restore

phenazine production in the mutant background, including **10**, **12**, and **17** (**Figure 6** and **Figure S5**). None of the analogues interfered with phenazine production in the wild-type PAO1 strain, suggesting that they are ineffective as PQS antagonists (**Figure S5**).

Similarly, while some degree of PqsR agonist activity was observed in the presence of compounds **6**, **9**, **10**, **12**, **13**, and **17**, only HHQ and PQS significantly induced promoter activity. All other analogues did not influence promoter activity in this system (**Figure 6B** and **Figure S5**). Somewhat surprisingly, antagonistic activity towards *pqsA* promoter activity was not observed, with almost all analogues failing to significantly suppress *pqsA* promoter activity in the wild-type strain (**Figure S5**). The relative ineffectiveness of these analogues as PQS antagonists may in part be due to hydroxylation of HHQ analogues (H at C3) to PQS analogues (OH at C3), thus establishing the non-antagonistic behavior explained by a recent report by Lu and colleagues, where the action of PqsH rendered anti-PQS compounds ineffective through bioconversion (55).

Discussion

Current antimicrobial therapies tend to be non-pathogen-specific and there is evidence to suggest that the availability of relatively non-toxic broad-spectrum therapies has contributed to the emergence of resistance among both targeted and non-targeted microbes (58, 59). Consequently, there is an urgent need to innovate new options for the targeted prevention of microbial infection while avoiding the inevitable emergence of resistance that is the hallmark of broad spectrum antibiotic therapies (59, 60). Increasingly, industry, academia and regulatory bodies have become interested in single-pathogen therapies to treat highly resistant or totally resistant bacterial

pathogens, rightly viewed as an area of high unmet need (61-63). Exploiting interkingdom communication networks, and the mode of action of the chemical messages or signals employed therein, offers us a powerful platform from which to deliver on this.

Previously we had shown that the HHQ interkingdom signal molecule from *P. aeruginosa* could suppress biofilm formation in *C. albicans* at concentrations ranging from 10 - 100 μM (2.47 – 24.7 μg/ml) (10). This suppression occurred independent of any growth limitation in planktonic cells, and morphogenesis on spider media was also found to be unaffected (10). The design and subsequent analysis of a suite of analogues based on the core HHQ quinolone framework has led to the identification of several lead compounds that retain anti-biofilm activity towards *C. albicans* but exhibit significantly reduced cytotoxicity towards IB3-1 epithelial cells when compared with the parent HHQ molecule. The selective cytotoxicity of the lead compounds together with the dose dependent anti-biofilm effects will be key considerations in determining the cell line specific therapeutic index of lead analogues as part of the ongoing development of these compounds. Furthermore, unlike HHQ, these lead compounds are now inactive towards the *P. aeruginosa* PqsR quorum sensing system, a critical requirement for their potential future development as anti-biofilm therapeutics. In addition, the ability to generate hydrochloride salts of the compounds ((36) and data not shown) suggests that solubility of future therapeutics based on these scaffolds will not be a bottleneck. Several strategies have been proposed for the implementation of anti-biofilm compounds as clinical therapeutics to target *C. albicans* biofilm infections (64). As the HHQ analogues possess anti-biofilm, but not anti-*Candida* activity, they would disrupt the formation of biofilms but not likely remove the planktonic cells that remain at the site of infection. Therefore, combination with conventional anti-fungal compounds would be

405 required for effective clearance. Alternatively, where the potency of the anti-biofilm activity can
406 be synthetically enhanced through further derivatization, clearance by the immune system might
407 also be realistic.
408 The molecular mechanisms through which AHQs and the lead compounds identified in this study
409 disrupt the formation of biofilms by *C. albicans* remains to be fully elucidated. Previously we
410 have shown that HHQ does not affect adhesion, but rather impacts directly on the subsequent
411 developmental stages in a TUP1-dependent manner (10). In this study we have shown that the
412 expression of several cell-wall associated genes is increased in response to HHQ during the
413 switch to hyphal growth. These genes have previously been implicated in the formation of *C.*
414 *albicans* biofilms and have been shown to exhibit increased levels of expression during the
415 hyphal transition (50, 51, 65). Therefore, anti-biofilm compounds might be expected to suppress
416 this induction rather than enhance it. However, five of the target genes tested exhibited an
417 increase in expression relative to control cells under inducing conditions. This may be a
418 reflection of the previous observation that HHQ interferes with the later stages of biofilm
419 development (10). Alternatively, this hyper-expression phenotype may affect the capacity of the
420 cell to engineer a community based biofilm. Future studies will focus on elucidating the
421 pathways through which *C. albicans* perceives and responds to challenge with HHQ with the aim
422 of identifying potential therapeutic targets.
423 Further work using defined *in vivo* models of biofilm and infection will be required to progress
424 the development and evaluation of these small molecules as anti-biofilm compounds. Models are
425 now available for the investigation of infections involving medical devices such as vascular
426 catheters, dentures, urinary catheters, and subcutaneous implants, as well as mucosal biofilm

infections (66). The ongoing development of cell-based or animal models to study *in vivo* infections (66-69), whether as single pathogen or co-culture systems (70), has provided a well-equipped tool-kit for the pre-clinical assessment of these AHQ-based compounds.

Conclusions

In this study, we have functionalized the important microbial signaling molecules HHQ and PQS in order to exploit their interkingdom role to control biofilm formation in *C. albicans*. In addition to deciphering further insights into the molecular mechanism through which these chemical messages elicit a biofilm suppressive response from *C. albicans*, the bioactivity of several lead compounds has provided a viable platform for the development of next generation therapeutics. Crucially, some of these compounds are non-toxic to mammalian cells and have been rendered incapable of activating *P. aeruginosa* virulence systems, thus highlighting their potential utility as an effective therapy combatting human infection.

Author Contributions

FJR, GPM and FOG conceived and designed the investigation. FJR, JPP, LG, and DW performed the biological experimentation, while RC, RS, and EOM conducted the chemical synthesis. FJR, JPP and FOG wrote the manuscript and all authors read and edited the final draft.

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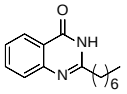
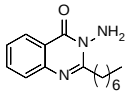
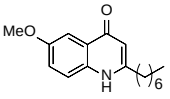
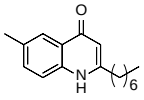
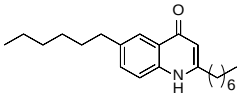
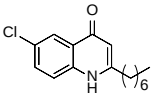
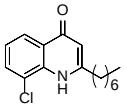
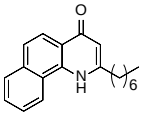
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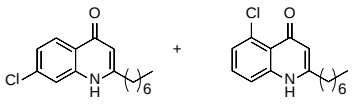
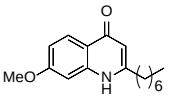
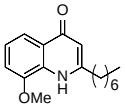
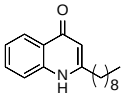
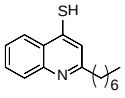
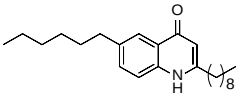
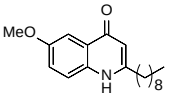
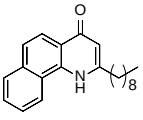
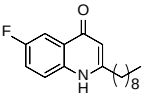
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643 **Table 1. Compound Data**

Compound	Structure	Yield [%][a]	MW	Rf[b]	Class[c]
1		76	244.3	0.319	I
2*		3	259.3	0.907	I
3		34	273.4	0.252	II
4		31	257.4	0.286	II
5		35	327.5	0.504	II
6		21	277.8	0.403	II
7		19	277.8	0.630	II
8		46	293.4	0.294	II

9*		6	277.8	0.361	II
10		33	273.4	0.261	II
11		12	273.4	0.504	II
12		23	271.4	0.395	III
13*		48	259.4	1	IV
14		16	355.6	0.538	V
15		28	301.4	0.286	V
16		51	321.5	0.462	V
17		16	289.4	0.504	V

644

[a] % yields are isolated yields over all steps

[b] TLC on silica plates with Dichloromethane:MeOH (95:5) mobile phase

[c] Class I – modified C-3; Class II – modified anthranilate ring; Class III – modified alkyl chain; Class IV – modified C-4; Class V – modified anthranilate ring and alkyl chain.

* New compounds synthesized in this study

Table 2. Selective Toxicity Index of Lead Compounds

Compound	**Selective Toxicity Index**			
	IB3-1	A549	DU145	HeLa
HHQ	**	***	**	****
PQS	*	**	*	*
1	*	*	*	*
2	**	****	****	****
4	*	****	**	****
6	**	***	*	****
9	*	**	*	**
12	**	***	**	****

% Toxicity * (0-25), ** (26-50), *** (51-75), **** (76-100)

Figure Legends

Figure 1. *C. albicans* biofilms are altered in the presence of HHQ. Filamentous *C. albicans* biofilms grown in the presence of PQS and HHQ (100 μM) were assessed structurally by confocal microscopy and metabolically using the XTT biofilm assay. Data (means ± SEM) are representative of three independent biological experiments and are presented relative to the untreated control. Two-tailed paired student's t-test was performed by comparison of *C. albicans* in the presence of HHQ and PQS with *C. albicans* treated with methanol or ethanol (*, p-value ≤ 0.05).

Figure 2. Decoration of HHQ exhibits variable biofilm activity against *C. albicans.* (a) A panel of HHQ derivatized analogues incubated with filamentous *C. albicans* and screened for biofilm formation using the metabolic XTT biofilm assay. Data is presented as OD_{492nm} spectrophotometric output normalized to the untreated control, and is representative of at least three independent biological replicates, with error bars representing SEM. (**b**) Dose-dependent XTT analysis of selected anti-biofilm compounds applied at 10, 50, and 100 μM. Data is the average of at least two independent biological replicates, each constituting eight technical replicates. Statistical analysis of both datasets was performed by one-way ANOVA with Bonferroni corrective testing, and is presented relative to the MeOH control; * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

688 **Figure 3. Microscopic analysis reveals altered biofilm structures.** Analogues that lead to
689 reduced *C. albicans* biofilm formation in the XTT assay (**1**, **2**, **3**, **4**, **6**, **7**, **9**, **12**, and **15**) exhibit
690 compromised biofilm structures. Filamentous *C. albicans* biofilm in the presence of analogues
691 (100 μM) was stained for chitin and cellulose (calcofluor; blue), lectins which binds to sugars,
692 glycolipids and glycoproteins (concanavalin A; green) and live-dead cells (FUN-1; red).

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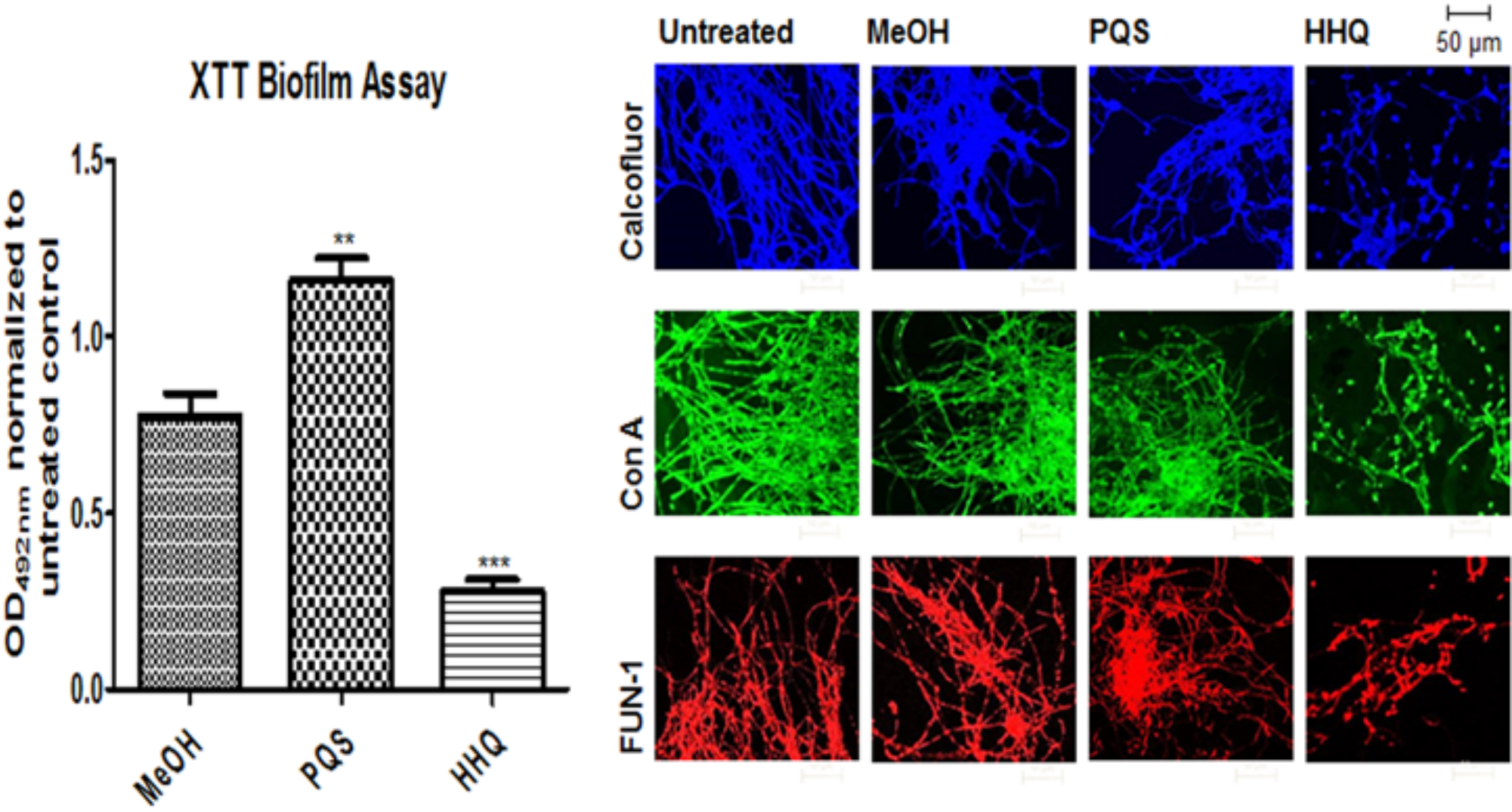
694 **Figure 4. Hyphal pathway genes are hyper-expressed in response to HHQ.** Transcript
695 expression analysis (Real Time RT-PCR) of a panel of biofilm genes was assessed in *C. albicans*
696 grown in YNB-NP (filamentous inducing media) in 100 μM HHQ for 6 hours at 37°C. All data
697 was normalized to a housekeeper gene (*ACT1*). Error bars represent SD of three independent
698 biological replicates. Two-tailed paired student's t-test was performed by comparison of HHQ
699 treated cells with methanol control in YNB-NP inducing medium (*, p-value ≤ 0.05).

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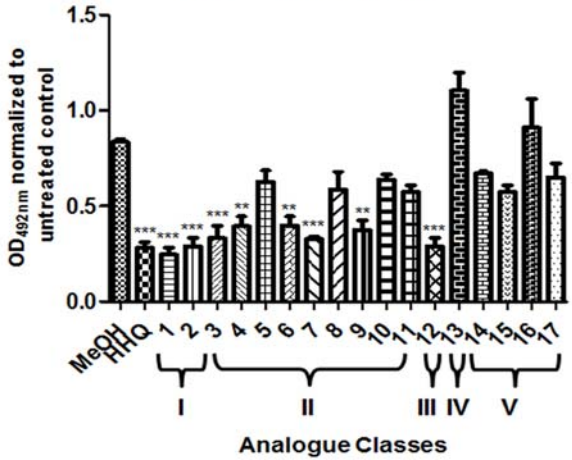
701 **Figure 5. Cytotoxicity towards specific mammalian cell lines is reduced in lead compounds.**
702 **(a)** Cytotoxicity, measured as a percentage of total lactate dehydrogenase (LDH) released from
703 IB3-1 cells treated with 0.1% Triton X-100 (100% cytotoxicity), was significantly reduced in the
704 presence of several lead compounds. Data (means ± SEM) are representative of three
705 independent biological experiments. **(b)** Selected lead compounds were tested against A549,
706 DU145, and HeLa cell lines. Data represents four independent biological replicates and all
707 datapoints are normalized to Triton X-100 as above. A one-way ANOVA was performed with

Bonferroni corrective testing on all datasets and comparison relative to MeOH control is presented; * $p \leq 0.05$, and *** $p \leq 0.001$.

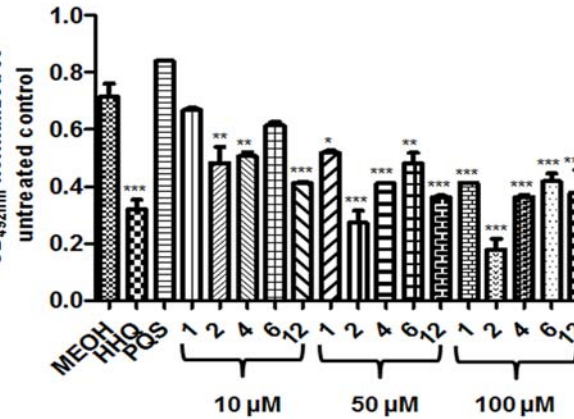
Figure 6. Influence of HHQ analogues on PQS-dependent virulence phenotypes in *P. aeruginosa*. (**a**) Phenazine production and (**b**) *pqsA-lacZ* promoter activity quantified in a PAO1 *pqsA* mutant in the presence of HHQ, PQS and lead compounds. Data is presented as mean +/- SEM and is representative of at least three independent biological replicates. A one-way ANOVA was performed with Bonferroni corrective testing and statistical significance relative to the MeOH control is presented; ** $p \leq 0.01$ and *** $p \leq 0.001$.

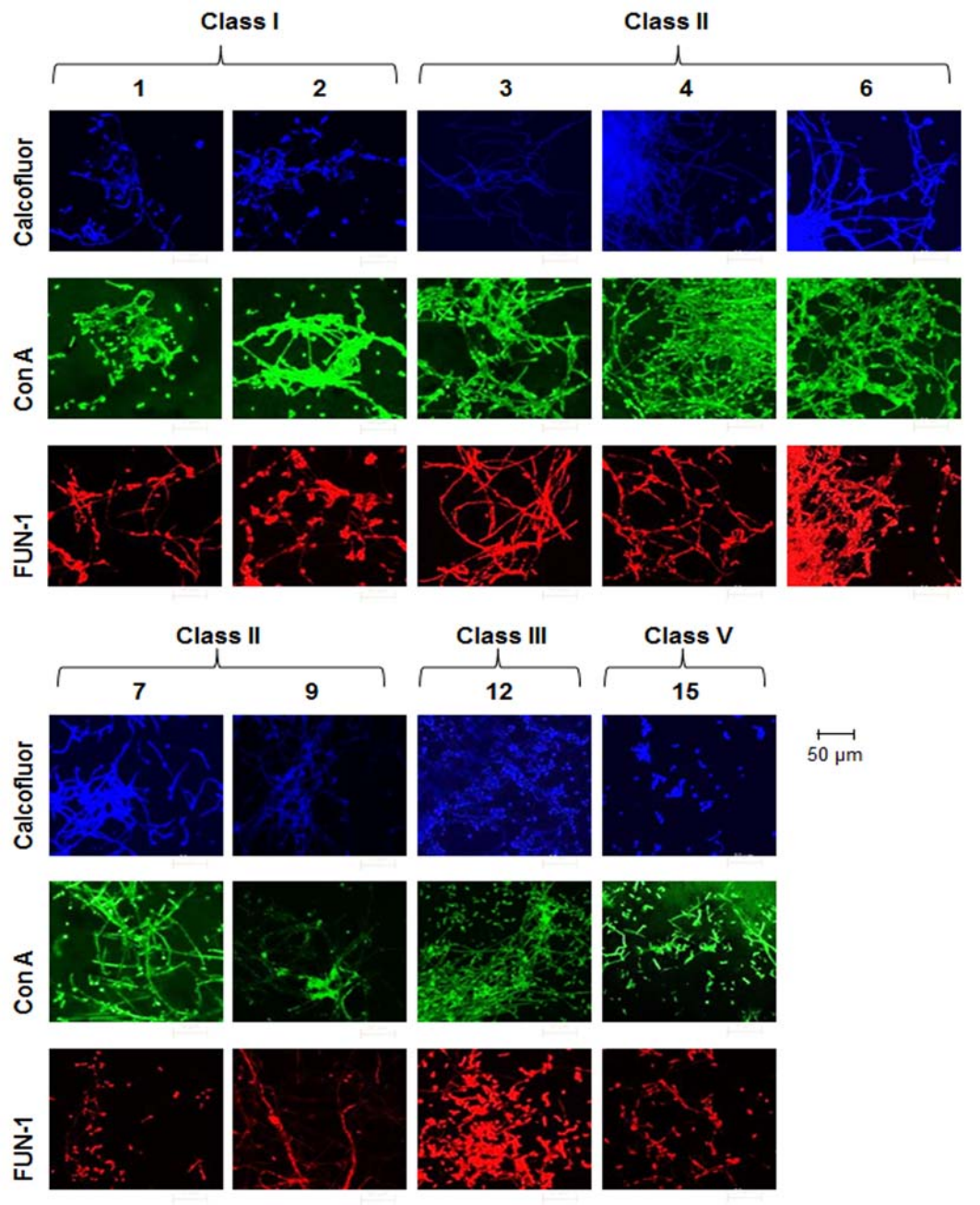

XTT Biofilm Assay
OD492nm normalized to untreated control
1.5
1.0
0.5
0.0
**
***
MeOH
PQS
HHQ
Untreated
MeOH
PQS
HHQ
50 µm
Calcofluor
Con A
FUN-1

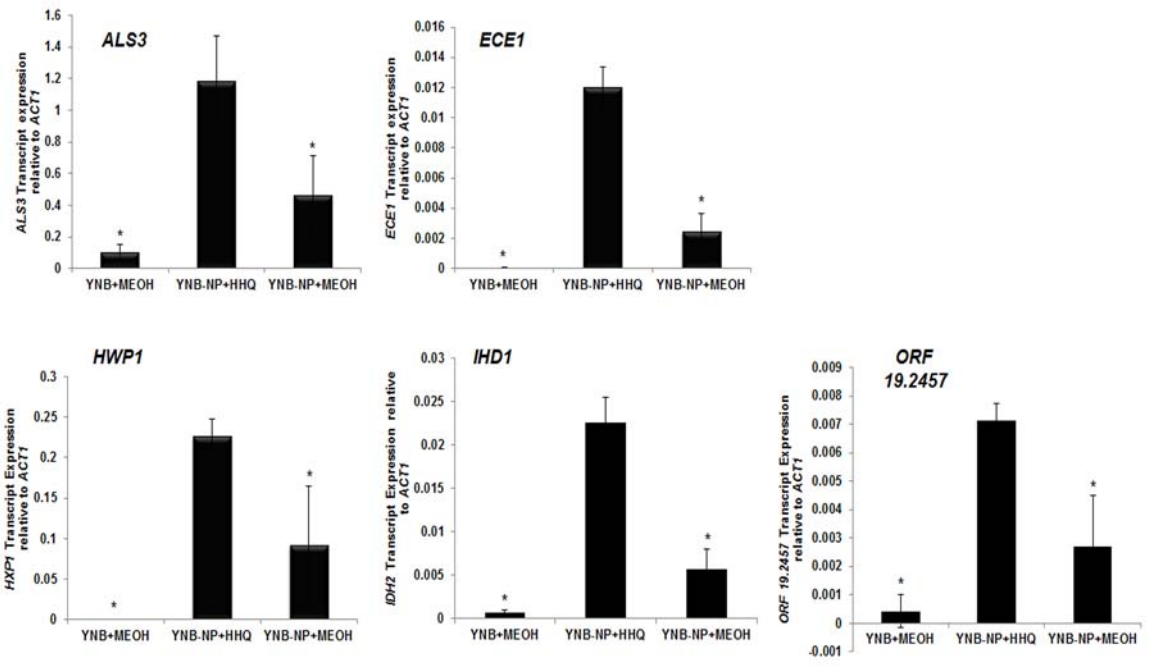
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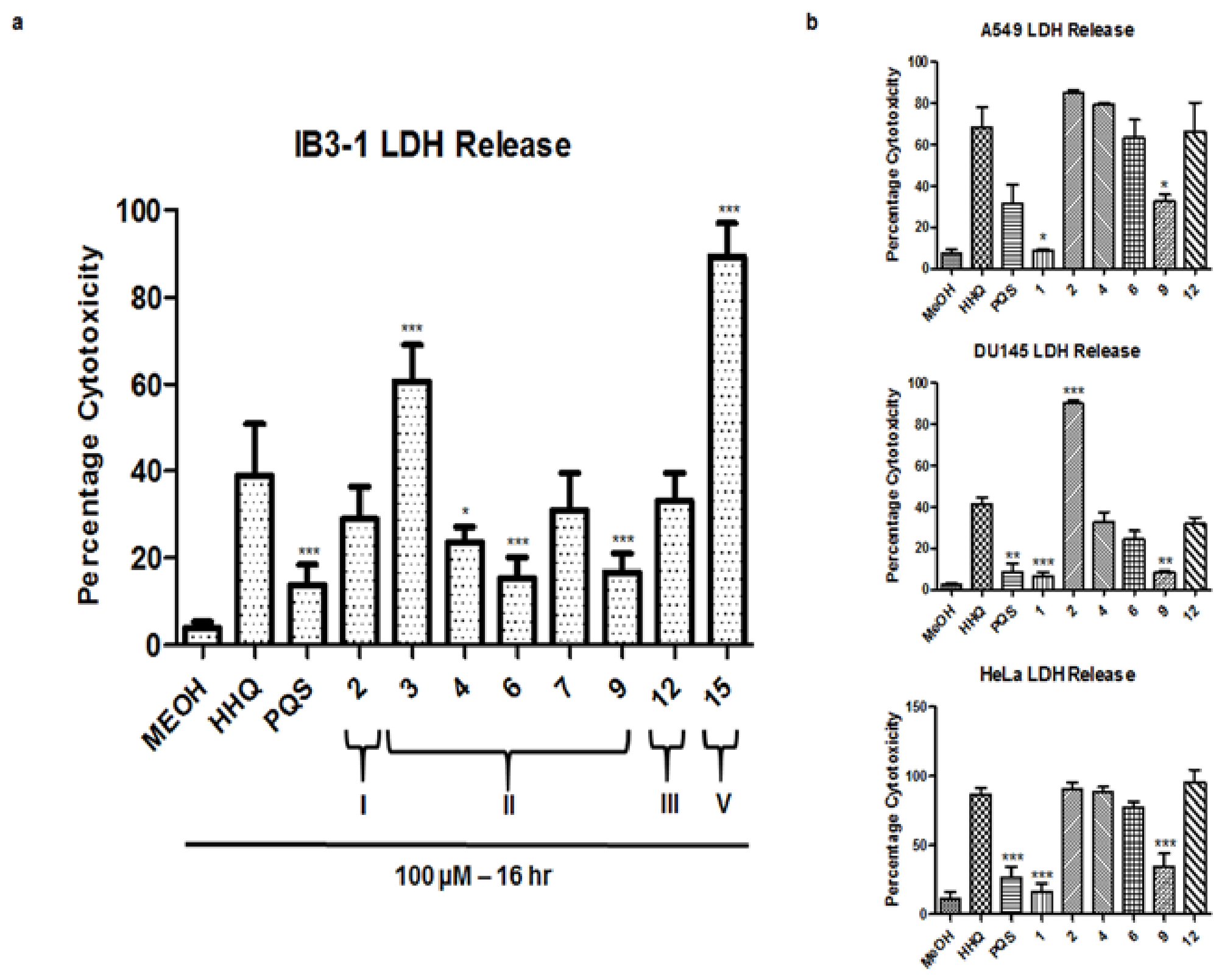

XTT Biofilm Assay
OD492nm normalized to untreated control
1.5
1.0
0.5
0.0
MeOH
HHQ
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
I
II
III
IV
V
Analogue Classes

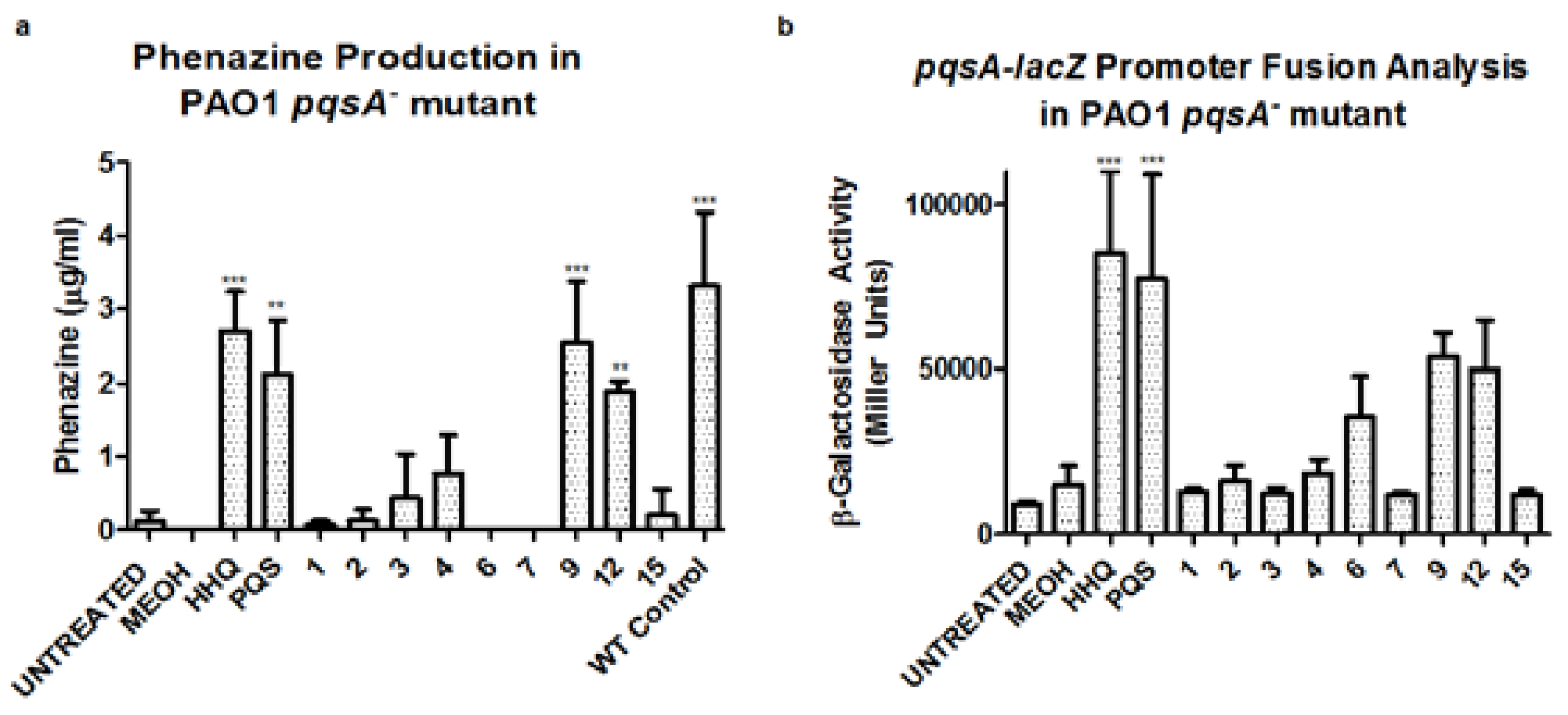
b


XTT Dose Response Biofilm Assay
OD492nm normalized to untreated control
1.0
0.8
0.6
0.4
0.2
0.0
MEOH
HHQ
POS
1 2 4 6 12
10 µM
50 µM
100 µM


Class I
Class II
1
2
3
4
6
Calcofluor
Con A
FUN-1
Class II
Class III
Class V
7
9
12
15
50 µm
Calcofluor
Con A
FUN-1


ALS3
ALS3 Transcript expression relative to ACT1
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
YNB+MEOH
YNB-NP+HHQ
YNB-NP+MEOH
ECE1
ECE1 Transcript expression relative to ACT1
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
YNB+MEOH
YNB-NP+HHQ
YNB-NP+MEOH
HWP1
HXP1 Transcript Expression relative to ACT1
0.3
0.25
0.2
0.15
0.1
0.05
0
YNB+MEOH
YNB-NP+HHQ
YNB-NP+MEOH
IHD1
IDH2 Transcript Expression relative to ACT1
0.03
0.025
0.02
0.015
0.01
0.005
0
YNB+MEOH
YNB-NP+HHQ
YNB-NP+MEOH
ORF 19.2457
ORF 19.2457 Transcript Expression relative to ACT1
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
-0.001
YNB+MEOH
YNB-NP+HHQ
YNB-NP+MEOH


a
IB3-1 LDH Release
Percentage Cytotoxicity
100
80
60
40
20
0
MEOH
HHQ
PQS
2
3
4
6
7
9
12
15
I
II
III
V
100 µM – 16 hr
b
A549 LDH Release
DU145 LDH Release
HeLa LDH Release
Percentage Cytotoxicity
MeOH
HHQ
PQS


a
Phenazine Production in
PAO1 pqsA- mutant
Phenazine (µg/ml)
0
1
2
3
4
5
***
**
**
***
***
UNTREATED
MEOH
HHQ
PQS
1
2
3
4
6
7
9
12
15
WT Control
b
pqsA-lacZ Promoter Fusion Analysis
in PAO1 pqsA- mutant
β-Galactosidase Activity
(Miller Units)
0
50000
100000
***
***
UNTREATED
MEOH
HHQ
PQS
1
2
3
4
6
7
9
12
15