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14. ABSTRACT

Purpose: *Pseudomonas aeruginosa* (PA) and *Staphylococcus aureus* (SA) are among the "ESKAPE organisms, and can cause severe and potentially fatal healthcare-acquired infections (HAI) such as pneumoni The rise of these "superbugs" threatens our ability to treat severe HAI, and has awoken us to the urgent need f new antibacterial strategies. With the near absence of new classes of antibiotics, adjuvants that render bacter more susceptible to existing antibiotics may broaden the spectrum of available drugs and overcome resistance

The purpose of our project is to explore the use of the bacteria-derived small molecules 4-hydroxyl-2-alky quinolines (HAQ) as adjuvants to potentiate the activity of conventional bactericidal antibiotics. We hypothesi that HAQ potentiate the bactericidal activity of conventional antibiotics and can be used as adjuvants to tre infections recalcitrant to antibiotics. The objective of this proposal is to characterize the *in vitro* and *in vi* adjuvant activity of HAQ used in combination with bactericidal antibiotics against major bacterial pathoge implicated in HAI.

Specific aim 1: To determine the *in vitro* bactericidal and anti-biofilm activity of HAQ (purified HAQ extrac and/or synthetic compounds) used in combination with bactericidal antibiotics against major HAI bacteri pathogens.

Specific aim 2. To test whether HAQ used in combination with conventional bactericidal antibiotics a tolerated and improve bacterial eradication and mortality in murine models of severe PA and SA infections.

Major findings: HAQ containing extracts significantly potentiate the *in vitro* bactericidal activity of sever classes of bactericidal antibiotics in multiple bacterial species. Purified and fractionated HAQ+ extracts sugge that C7-HHQ is the most bioactive, but other HAQ molecules likely also contribute to the adjuvant activit Synthetic C7-HHQ has moderate adjuvant activity but is not as bioactive as the crude extract. Studies to furth characterize and validate the adjuvant activity of HAQ+ extracts against a more comprehensive set of *aeruginosa* and *S. aureus* isolates, as well as additional ESKAPE organisms are under way. In vivo studies ha been redesigned to test an alternative subcutaneous abscess model of *P. aeruginosa* and *S. aureus* infections.

Significance: The adjuvant activity of HAQ is completely novel and highly unusual as it potentiates multip classes of bactericidal antibiotics and different bacterial species. This could lead to the development of powerf new adjuvants that will extend the life of current drugs and greatly speed up the development of combinatic antibacterial regimens effective against drug-resistant and difficult to treat infections. This will improve t outcome and survival of military personnel, wounded soldiers, veterans and their families afflicted by bacteri infections.

15. SUBJECT TERMS
bacterial infection; antibiotic; drug discovery; antibacterial adjuvant therapy; pneumonia; Pseudomonas aeruginos Staphylococcus aureus;

16. SECURITY CLASSIFICATION OF:

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1. INTRODUCTION:

2. The purpose of our project is to explore the use of the bacteria-derived small molecules 4-hydroxyl-2-alkyl-quinolines (HAQ) as adjuvants to potentiate the activity of conventional bactericidal antibiotics. The objective of this proposal is to determine whether HAQ potentiate the bactericidal activity of conventional antibiotics and can be used as adjuvants in combination with bactericidal antibiotics to treat infections against major bacterial pathogens implicated in HAI that are recalcitrant to antibiotics.

3. KEYWORDS:

bacterial infection; antibiotic; drug discovery; antibacterial adjuvant therapy; pneumonia; Pseudomonas aeruginosa; Staphylococcus aureus; ESKAPE pathogens, antibiotic resistance

4. ACCOMPLISHMENTS:

The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The major goals for the project are presented in the table below. All major tasks were completed as proposed. The SOW was modified and updated in the Annual report submitted in July 2017.

Major Aims/Tasks	SOW-defined Timeline (Months)	Site 1 Nguyen-Radioch	Site 2 Deziel	Completion (%)
Aim 1. To determine the in vitro anti-bacterial and anti-biofilm activity of HAQ used in combination with bactericidal antibiotics against major HAI bacterial pathogens.				
Major Task 1. Preparation of PA extracts and synthetic HAQ				
Subtask 1.1 Preparation of purified PA extracts	1-3	x		100
Subtask 1.2 Synthesis of HAQ	1-3		x	100
Major Task 2. Determination of the adjuvant activity of HAQ in combination with different classes of bactericidal antibiotics				
Subtask 2.1 Testing of antimicrobial activity of HAQ-antibiotic combinations against P.a.	4-12	x		100
Subtask 2.2 Testing of antimicrobial activity of HAQ-antibiotic combinations against S.a.	4-12	x		100
Major Task 3. Determination of HAQ activity against PA and SA biofilms				
Subtask 3.1 Test of biofilm inhibitory concentration and biofilm bactericidal kill kinetics	10-18	x		100
Major Task 4. Validate the HAQ adjuvant activity against ESKAPE pathogens				
Subtask 4.1 Testing of antimicrobial activity of HAQ-antibiotic combinations against other drug susceptible ESKAPE pathogens	10-18	x		100
Aim 2. To test whether HAQ used in combination with conventional bactericidal antibiotics improve bacterial eradication and mortality in murine models of severe PA and SA infections.				
Major Task 1. Determine the toxicity of HAQ in mice				
Subtask 1.1 In vitro toxicity studies using RBC lysis and LDH release assays in HeLa cells	6-9	x		100
Subtask 1.2 Dose escalation studies of HAQ extract and synthetic compound by IT administration	6-9	x		100
Subtask 1.3 Sample analyses	6-9	x		100
Major Task 2. Investigate the adjuvant activity of HAQ in sub-lethal P.a. murine skin abscess				
Subtask 2.1 P.a. Skin Abscess	9-18	x		100
Subtask 2.2 Sample analyses	9-18	x		100
Major Task 3. Investigate the adjuvant activity of HAQ in sub-lethal S.a. murine skin abscess				
Subtask 2.1 S.a. skin abscess	9-18	x		100
Subtask 2.2 Sample analyses	9-18	x		100

Abbreviations:

CF	cystic fibrosis
CA-MRSA	community acquired methicillin resistant *Staphylococcus aureus*
EPS	extracellular polysaccharide
HAQ	hydroxyl alkyl quinolone
Ex	Ethyl acetate extract
$Ex_{\Delta SR}$	Ethyl acetate extract derived from PAO1 ΔSR
$Ex_{\Delta SR\ \Delta pqsA}$	Ethyl acetate extract derived from PAO1 ΔSRΔpqsA
HAQ+ Ex	HAQ containing ethyl acetate extract
HAQ- Ex	HAQ deficient ethyl acetate extract
C1-HHQ	C7-HHQ analog having a C1 alkyl side-chain
C3-HHQ	C7-HHQ analog having a C3 alkyl side-chain
C5-HHQ	C7-HHQ analog having a C5 alkyl side-chain
C7-HHQ	2-heptyl-4-quinolone (≥98% from Sigma)
C9-HHQ	2-nonyl-4-quinolone (HNQ), C7-HHQ analog having a C9 alkyl side-chain
HPLC	high pressure liquid chromatography
HQNO	2-heptyl-4-quinolinol 1-oxide
LCMS	liquid chromatography mass spec
MBC	minimum bactericidal concentration
MIC	minimum inhibitor concentration
MSSA	methicillin sensitive *Staphylococcus aureus*
C1-PQS	PQS analog having alkyl side-chain length of 1
P.a	*Pseudomonas aeruginosa*
PQS	Pseudomonas Quinolone Signal
S.a	*Staphylococcus aureus*
SpM	Stationary Phase Spent Media
$SpM_{\Delta SR}$	Stationary Phase Spent Media derived from PAO1 ΔSR
$SpM_{\Delta SR\Delta A}$	Stationary Phase Spent Media derived from PAO1 ΔSRΔpqsA
SR	Stringent Response
ΔSR	PAO1 *relA spoT* deletion mutant strain
ΔSRΔpqsA	PAO1 *relA spoT pqsA* deletion mutant strain
VC	Vehicle Control

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

SPECIFIC AIM 1

Previous studies from our lab had shown that the *relA spoT* mutant, also named the stringent response mutant (ΔSR) of *Pseudomonas aeruginosa* PAO1 was highly susceptible to bactericidal antibiotics compared to its isogenic wild-type parent (Nguyen *et al* Science 2011). Importantly, we observed that HAQ molecules, a family of approximately 56 related molecules with 3 related core structures, were produced at high concentrations in culture supernatants of the ΔSR mutant, and were capable of sensitizing bacteria to subsequent bactericidal antibiotics. This antibiotic potentiating activity was abrogated upon disruption of *pqsA*, an anthranilate-Coenzyme A ligase required for HAQ biosynthesis. This led us to propose that one or more HAQ molecule(s) sensitize bacteria to bactericidal antibiotics and may be used as an adjuvant to the tolerance of susceptible bacteria by sensitizing them.

MAJOR TASK 1: PREPARATION OF PA EXTRACTS AND SYNTHETIC HAQ

SUBTASK 1.1: PREPARATION OF PURIFIED PA EXTRACTS

- **Preparation of HAQ containing culture supernatants.**

We generated *P. aeruginosa* culture supernatants (also referred to as spent media or SpM) from strain ΔSR to generate $SpM_{\Delta SR}$ which contains HAQ, and from strain ΔSR*ΔpqsA* to generate $SpM_{\Delta SR\Delta A}$ which does not contain HAQ. We confirmed that adjuvant activity of SpM as shown in Figure 1, where only $SpM_{\Delta SR}$ potentiated gentamicin killing of *P. aeruginosa*, but not $SpM_{\Delta SR\Delta pqsA}$.

- **Adjuvant activity bioassay with *E. coli* indicator strain**

We first developed an "adjuvant activity" bioassay to measure the antibiotic potentiating effects of HAQs using a highly sensitive "indicator" *E. coli* strain (*E. coli relA spoT* mutant) grown to stationary phase and challenged with gentamicin. Adjuvant activity is evidenced by an increase in gentamicin bactericidal activity when it is combined with the HAQ adjuvant (Figure 2).

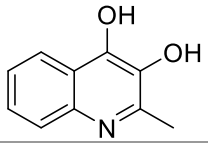
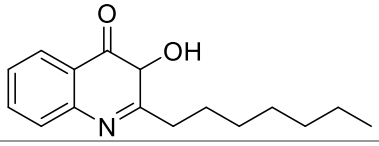
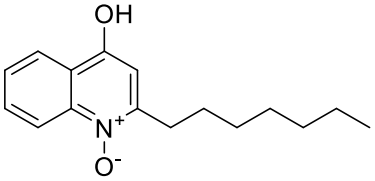
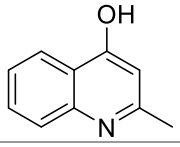
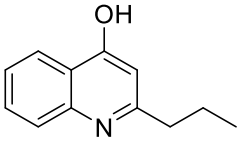
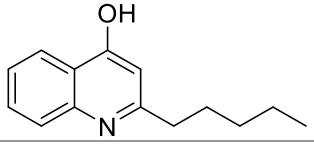
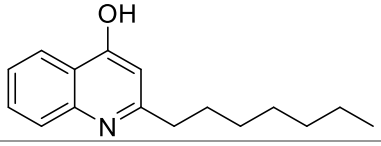
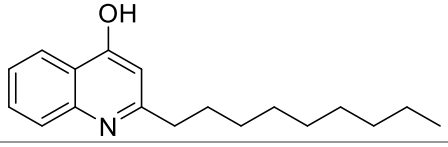
- **Optimization of *P. aeruginosa* cultures for HAQ recovery**

We tested several growth conditions and identified the one associated with the highest yield of HAQ molecule(s) and adjuvant activity to be cultures of the PAO1 ΔSR strain, grown for 24 h in 100 mL of TSB (Wisent) in 1 L flask with vigorous shaking/aeration at 250 r.p.m at 37 ^{o}C. This was determined by testing the SpM in the adjuvant activity bioassay described above and LC/MS measurements of HAQ.

- **Quantification of HAQ in SpM by LC/MS**

We quantified HAQ levels in $SpM_{\Delta SR}$ by LC/MS. LCMS quantification of HAQs showed a recovery of 1.2±0.42, 0.7±0.4 and 11.8±4.1 µg/mL for C7-HHQ, PQS and HQNO respectively. The predominant HAQs identified in the $SpM_{\Delta SR}$ crude extract are shown in Table 1.

Table 1. Structures of hydroxy-alkyl quinolones.

Name	Structure	Chemical Name
C1-PQS		2-methyl-3,4-quinolinediol
PQS		2-heptyl-3-hydroxyquinolin-4(3*H*)-one
HQNO		2-heptyl-4-hydroxyquinoline 1-oxide
C1-HHQ		4-Hydroxy-2-methylquinoline
C3-HHQ		2-propylquinolin-4-ol
C5-HHQ (PQ)		2-pentylquinolin-4-ol
C7-HHQ		2-heptylquinolin-4-ol
C9-HHQ (HNQ)		2-nonylquinolin-4-ol

- **Preparation of crude HAQ extracts from SpM**

We then carried out an ethyl acetate extraction of the $SpM_{\Delta SR}$ to generate crude extracts ($Ex_{\Delta SR}$). All batches of $SpM_{\Delta SR}$ were first confirmed to increase gentamicin killing by at least 2 log(10) prior to ethyl acetate extraction.

- **Quantification of HAQ in $Ex_{\Delta SR}$ by LC/MS**

Following evaporation of the $Ex_{\Delta SR}$ by rotovap, the crude extract was dissolved in methanol and subjected to LC/MS analysis. We found significant variability between different batches of $Ex_{\Delta SR}$ with yields of C7-HHQ 2.1±1.9 mg/mL, PQS 0.7±0.7 mg/mL, HQNO 4.8±3.8 mg/mL and C9-HHQ 0.7±0.9 mg/mL, as well as smaller concentration of the less common HAQs (Figure 3A).

- **Comparison of the HAQ profiles and adjuvant activity in different *P. aeruginosa* mutants**

We also tested the crude extract of mutants carrying mutations in the *pqsL* gene (responsible for synthesis of HQNO) and *pqsH* (responsible for synthesis of PQS). LCMS analysis showed that $Ex_{\Delta SR}$ contained high concentrations of HQNO, while $Ex_{\Delta SR\Delta H\Delta L}$ isolated from strain PAO1 ΔSRΔpqsHΔpqsL produced no HQNO and only the C7-HHQ congeners (Figure 3A). Comparisons of the different extracts suggested that HQNO and PQS likely did not contribute to the adjuvant activity (Figure 3B), and that the yield of C7-HHQ was highest in $Ex_{\Delta SR\Delta H\Delta L}$ compared to $Ex_{\Delta SR}$ (6.9±2.4 vs 2.1±1.9 mg/mL).

- **Fractionation and purification of $Ex_{\Delta SR}$ by HPLC and HAQ measurements by LC/MS**

We purified the crude extracts, using $Ex_{\Delta SR}$ to generate fractionated HAQ+ extracts, and $Ex_{\Delta SR\Delta A}$ to generate HAQ- control extracts. The $Ex_{\Delta SR}$ and $Ex_{\Delta SR\Delta A}$ were fractionated by HPLC using a semi-preparative reverse phase C18 column, generating 45 fractions each. LC/MS analysis of individual fractions was performed to quantify the predominant HAQ (Figure 4).

Accomplishments and Conclusions:
We optimized the HAQ production in *P. aeruginosa* cultures, developed a sensitive *E. coli* bioassay to measure adjuvant activity, generated and fractionated HAQ+ containing extracts, and quantified HAQs in all fractions by LC/MS.

SUBTASK 1.2: SYNTHESIS OF SYNTHETIC HAQs

Our goal was to obtain synthetic and purified compounds of all HAQs produced by *P. aeruginosa* and detected in bioactive extracts. Since C7-HHQ was the dominant HAQ in our bioactive $Ex_{\Delta SR\Delta H\Delta L}$ extract, we also synthesized different HHQ congeners which share the same quinolone ring but have different length side chains compared to C7-HHQ. We expected that these HHQ congeners would allow us to test whether the quinolone ring moiety was the bioactive component.

- **Commercial synthetic HAQ**

Synthetic C1-HHQ was purchased from Sigma (Figure 5B) and C1-PQS were purchased from Aces Pharma (Product #77881), C7-HHQ from Sigma (C7-HHQ Product # SML0747: ≥98%: Figure 5A), and HQNO from Cayman Chemical (HQNO Product # 15159: ≥98%: Figure 6).

- **Custom synthesis of HAQ**

Synthetic C3-HHQ (3g, 87%), C5-HHQ (3g, 99%), C9-HHQ (1g, 97%) and PQS (100 mg, 95%) were synthesized by the Deziel lab (site 2). Synthesis of C3-HHQ, C5-HHQ, and C9-HHQ was done following a 4 step process with a final cyclization step with an approximate yield of %50. Their structures were confirmed by proton NMR (Figure 7) and LC/MS (Figure 8).

Accomplishments and Conclusions:
We obtained synthetic compounds of all major HAQ and HHQ congeners for subsequent testing. Synthetic C7-HHQ and its C3-, C5- and C9- congeners in addition to PQS, were synthesized in-house while C1-HHQ, C1-PQS and HQNO were obtained commercially.

MAJOR TASK 2

DETERMINATION OF THE ADJUVANT ACTIVITY OF HAQ IN COMBINATION WITH DIFFERENT CLASSES OF BACTERICIDAL ANTIBIOTICS

SUBTASK 2.1: TESTING OF HAQ-ANTIBIOTIC COMBINATIONS AGAINST P.A.

- **Optimization of the *P. aeruginosa* adjuvant activity bioassay**

To optimize the reproducibility of the adjuvant bioassay, we used $Ex_{\Delta SR\Delta A}$ (HAQ- crude extract) as negative controls, normalized the $Ex_{\Delta SR}$ to an equivalent of 100 µM C7-HHQ as determined by LC/MS and added $Ex_{\Delta SR}$ extracts 2h prior to antibiotic challenge.

- **Minimal inhibitory concentration (MIC) of bactericidal antibiotics**

Since we planned to test HAQ in combination with various bactericidal antibiotics, we first determined the MIC for gentamicin, ofloxacin, meropenem and colistin in the *P. aeruginosa* panel. As shown in Table 2, all strains tested were within the susceptible range. It is important to note that the HAQs potentiate the bactericidal activity of conventional antibiotics against phenotypically tolerant bacteria. Phenotype tolerance is not inheritable and does not affect the minimal inhibitory concentrations (MIC) to antibiotics.

	MIC (µg/mL)			
Strain	Gentamicin	Ofloxacin	Meropenem	Colistin
PAO1 WT	2	4	1	4
PAO1 ΔSR	2	4	2	4
PAO1 ΔSRΔpqsA	2	4	1	4
CI-28 Mucoid	2	0.25	N/A	2
CI-28 ΔSR	2	0.25	N/A	2
CI-29 ΔSR	4	4	N/A	4

Table 2. MIC of bactericidal antibiotics against *P. aeruginosa* strains

- **Adjuvant activity of $Ex_{\Delta SR}$ in combination with different classes of bactericidal antibiotics against *P. aeruginosa***

We confirmed that the HAQ+ extract $Ex_{\Delta SR}$ potentiates the bactericidal activity of gentamicin (Figure 9A) and ofloxacin (Figure 9B) compared to antibiotic alone, while the HAQ- crude extract $Ex_{\Delta SR\Delta A}$ had no adjuvant activity.

- **Adjuvant activity of $Ex_{\Delta SR}$ in different drug susceptible *P. aeruginosa* strains**

We validated the adjuvant activity of $Ex_{\Delta SR}$ on different *P. aeruginosa* strains, namely the PAO1 lab strain and two *P. aeruginosa* cystic fibrosis clinical isolates CI-29 (Figure 10). We observed adjuvant activity with all wild-type strains, but the magnitude of effect was lower that with the ΔSR strains and differed from strain to strain. Notably, we observed significant potentiation of gentamicin killing of CI-28, a mucoid clinical isolate from a cystic fibrosis patient which are typically highly recalcitrant to antibacterial therapy.

- **Adjuvant activity of $Ex_{\Delta SR}$ in drug resistant *P. aeruginosa* strains**

In order to test whether $Ex_{\Delta SR}$ adjuvant activity could overcome genotypic resistance, we created two antibiotic resistant strains, namely PAO1 ΔSR::*aaC1* which carries transposon with the *aaC1* gentamicin resistance cassette (MIC ≥128 μg/mL), and a lab-evolved spontaneous PAO1 ΔSR ofloxacin-R mutant (MIC 128 μg/mL) generated through stepwise selection. As shown in Figure 11A and 11B respectively, $Ex_{\Delta SR}$ did not enhance killing of 50 μg/mL gentamicin nor of 50 μg/mL ofloxacin in these resistant mutants, indicating that $Ex_{\Delta SR}$ adjuvant activity is insufficient to overcome high level ofloxacin and *aaC1*-mediated genotypic drug resistance.

- **Adjuvant activity of $Ex_{\Delta SR}$ fractions**

As described in Subtask 1.1 the $Ex_{\Delta SR}$ and $Ex_{\Delta SR\Delta pqsA}$ was separated in 45 fractions by HPLC. Pooled fractions were tested using the *E.coli* adjuvant bioassay: $Ex_{\Delta SR}$ F1-45 and F21-45 were active (Figure 12A) but not $Ex_{\Delta SR}$ F1-20 nor the $Ex_{\Delta SR\Delta pqsA}$ F1-45 (Figure 12B).

LC/MS quantification of HAQ in each fractions from the $Ex_{\Delta SR}$ pool F21-45 indicated that fractions F22, F25, F26, F28, F30, F33, F35 and F36 contained C7-HHQ, HQNO, PQS and HNQ and other less abundant HAQs (Table 4). Each fraction was individually tested using the *E.coli* adjuvant bioassay at 1:100 dilution in combination with 50 μg/mL gentamicin (Figure 12 C&D) for 30 min and 60 minutes respectively. We identified two peaks of activity Peak I and Peak II centered on fraction 26 and 35 respectively.

- **Molecular characterization of bioactive fractions**

In Peak I, the two most active fractions F25 and F26 (Figure 12C&D) contained the highest amount of C7-HHQ (Figure 4). C7-HHQ represented 83% of the chemical material in F25 as measured by signal intensity, and the remainder of the chemical material was an uncharacterized molecule with m/z of 258. F26 had a higher concentration of C7-HHQ compared to F25, but at a purity of only 60%. The remainder of the chemical material in F26 also contained m/z 258 (17%) and HQNO (8.5%).

In Peak II, F35 contained numerous molecular species including C7-PQS (27.7%), C9-HHQ (25.5%), m/z 270 (16.5%), m/z 274 (13.5%) and m/z 286 (16.5%) (Table 4). F36 contained C7-PQS in greater amount and purity (39%) than F35, in addition to C9-HHQ (25.5%) and m/z of 270, 274 and 286. Since the adjuvant activity of F36 is lower than F35 (Figure 12C&D), this suggested that C7-PQS was likely not responsible for Peak II activity, a conclusion also supported by our comparison of the extracts (see Figure 3B and 13A&B) from $Ex_{\Delta SR}$ with $Ex_{\Delta SR\Delta H}$ and $Ex_{\Delta SR\Delta H\Delta L}$ (the latter two not containing any PQS).

Based on the analysis of the bioactive fractions, adjuvant activity is best correlated with the presence of C7-HHQ and to a lesser extent C9-HHQ. This suggests that C7-HHQ and C9-HHQ are the most likely bioactive HAQ molecules.

	***Purity HPLC purified fractions* LCMS (m/z)**									
Fraction	**230**	**HHQ**	**258**	**HQNO**	**PQS**	**270**	**C9-HHQ**	**274**	**286**	**Total**
22	91%									91%
25		83%	14%							97%
26		60%	17%	8.5%						85.5%
28		29%	7%	55%						91%
30		12%	24%	50%				8%		94%
33		5%	18%	27%				50%		99%
35					27.7%	16.5%	25.5%	13.5%	16.5%	99.7%
36					39%	6%	11%	15.5%	23.4%	94.9%

Table 4. LCMS quantification of ExΔSR HPLC purified fractions

- **Correlation between C7-HHQ content and adjuvant activity of extracts derived from different *P. aeruginosa* mutants**

As part of Subtask 1.1, we had generated extracts from *P. aeruginosa* mutants which carried inactivating mutations in the HAQ biosynthetic genes *pqsH* (PAO1 ΔSRΔpqsH) and *pqsL* (PAO1 ΔSRΔpqsL), as well as *pqsE* (PAO1 ΔSRΔpqsE) involved in HAQ signaling. LC/MS quantification of the $SpM_{\Delta SR\Delta H}$, $SpM_{\Delta SR\Delta L}$ and $SpM_{\Delta SR\Delta E}$ extracts determined that the C7-HHQ concentration of each extract was 67.1, 37.7 and 17.4 μM respectively.

To determine whether the extract's adjuvant correlated with C7-HHQ concentration, we tested the three $SpM_{\Delta SR\Delta H}$, $SpM_{\Delta SR\Delta L}$ and $SpM_{\Delta SR\Delta E}$ extracts against *P. aeruginosa* PAO1 ΔSR in combination with ofloxacin (Figure 13A) and against *S. aureus* ATCC 29213 in combination with gentamicin (Figure 13B). As shown in (Figure 13C), the correlation between C7-HHQ concentration in extracts and the rate of antibiotic killing was excellent, with R^2 of 0.98 for the *S. aureus* and 0.93 for *P.aeruginosa*.

- **Adjuvant activity of synthetic HAQs against *P. aeruginosa***

Based on the results described above, we tested the adjuvant activity of synthetic HAQ in combination with ofloxacin and gentamicin against the *P. aeruginosa* PAO1 WT and ΔSR strains. We used HAQ concentrations within ~1.5 X physiological concentrations and solubility in aqueous solution. 100 μM C7-HHQ enhanced gentamicin (Figure 14A) and ofloxacin (Figure 14B) killing of PAO1 ΔSR compared to antibiotic alone, but minimal adjuvant activity in combination with 50 μg/mL gentamicin was observed for killing of PAO1 WT (Figure 14C). These results are in line with results using $Ex_{\Delta SR}$ extracts (Figure 10A) where we observed modest adjuvant activity of $Ex_{\Delta SR}$ + gentamicin against PAO1 WT strain.

We tested all other synthetic HAQ and found no adjuvant activity in combination with 50 μg/mL gentamicin against PAO1 ΔSRΔA and WT: 100 μM PQS (Figure 15 B/D), 100 μM HQNO (Figure

15A/C). Surprisingly, 100 µM C9-HHQ (HNQ) had no adjuvant activity in combination with 50 µg/mL gentamicin against PAO1 ΔSR or *E. coli* ΔSR (Figure 16: P=0.2). C7-HHQ and PQS have C7-alkyl side chain and are the most abundant members of their respective families. To determine whether the quinoline ring structure was bioactive and sufficient for the adjuvant activity, we also tested non-alkylated PQS and C1-HHQ analogues. Neither showed any adjuvant activity with ofloxacin (Figure 17), suggesting that the alkyl-chain was required for C7-HHQ activity.

- **Minimal inhibitory concentration (MIC) of HAQ in *P. aeruginosa***

In order to determine whether HAQs may have direct antibacterial activity, we determined the MIC by microbroth dilution in a panel of 6 *P. aeruginosa* strains (3 strains derived from PAO1 and 3 clinical isolates). The $Ex_{\Delta SR}$ crude extract (~50 µg/mL C7-HHQ) and synthetic C7-HHQ and HQNO showed no significant antibacterial activity against any of the tested strains, as demonstrated by a MIC >128 µg/mL (Table 5). This was corroborated using standard antibiotic disk diffusion assays where no significant zone of clearance was observed (data not shown). PQS, on the other hand, had a MIC of 2-4 µg/mL against the mucoid *P. aeruginosa* clinical isolate CI-28 but >128 µg/mL in all other *Pseudomonas* strains.

	MIC (µg/mL)			
Strain	C7-HHQ	PQS *	HQNO	ExΔSR
PAO1 WT	128	>128	128	>50
PAO1 ΔSR	>128	>128	>128	>50
PAO1 ΔSRΔpqsA	>128	>128	>128	N/A
CI-28 Mucoid	>128	2 (16)	>128	N/A
CI-28 ΔSR	>128	4 (128)	>128	N/A
CI-29 ΔSR	>128	>128	>128	N/A

Table 5. Minimum inhibitory concentration (MIC) of HAQs against *P. aeruginosa*. All assays were done in CAMHB medium (n=4 replicates) following CLSI approved guidelines.

- **Minimal bactericidal concentration (MBC) of synthetic HAQ**

MBC were tested and no HAQ showed significant bactericidal activity (Table 6), with the exception of weak activity of PQS against the CI-28 mucoid strain. We noted that MBC for C7-PQS against *Pseudomonas* were 8-32 fold higher than the MIC, indicating that C7-PQS was bacteriostatic in nature. This may be due to its iron chelation effect that can limit bacterial growth.

	MBC (µg/mL)			
Strain	**C7-HHQ**	**C7-PQS ***	**HQNO**	**ExΔSR**
PAO1 WT	>128	>128	>128	>50
PAO1 ΔSR	>128	>128	>128	>50
PAO1 ΔSRΔpqsA	>128	>128	>128	N/A
CI-28 Mucoid	>128	16	>128	N/A
CI-28 ΔSR	>128	128	>128	N/A
CI-29 ΔSR	>128	>128	>128	N/A

Table 6. Minimum bactericidal concentration (MBC) of HAQs against *P. aeruginosa*. All assays were done in CAMHB medium following CLSI approved guidelines.

- **Synergy in HAQ-antibiotic combination using the checkerboard assays**

MIC synergy of combinations of synthetic C7-PQS, C7-HHQ and HQNO and extracts of $Ex_{\Delta SR}$ with gentamicin or ofloxacin were performed using a checkerboard assay. The highest achievable concentration of $Ex_{\Delta SR}$ was ~50 µg/mL C7-HHQ due to solubility limitations. Synergy testing of *Pseudomonas aeruginosa* included PAO1 WT, PAO1 ΔSR, mucoid CI-28, it's isogenic ΔSR mutant and CI-29 ΔSR. MICs for both gentamicin and ofloxacin were found to be unchanged in the presence of $Ex_{\Delta SR}$, C7-PQS, C7-HHQ and HQNO making the combination indifferent for all tested strains and combinations (Table 7).

	Fractional Inhibitory Concentration Index (FIC)		
Strain	**Adjuvant**	**Gentamicin**	**Ofloxacin**
PAO1 WT	C7-HHQ	Indifferent	Indifferent
	C7-PQS	Indifferent	Indifferent
	HQNO	Indifferent	Indifferent
	$Ex_{\Delta SR}$	Indifferent	Indifferent
PAO1 ΔSR	C7-HHQ	Indifferent	Indifferent
	C7-PQS	Indifferent	Indifferent
	HQNO	Indifferent	Indifferent
	$Ex_{\Delta SR}$	Indifferent	Indifferent
PAO1 ΔSRΔpqsA	C7-HHQ	Indifferent	Indifferent
	C7-PQS	Indifferent	Indifferent
	HQNO	Indifferent	Indifferent
	$Ex_{\Delta SR}$	Indifferent	Indifferent
CI-28 Mucoid	C7-HHQ	Indifferent	Indifferent
	C7-PQS	Indifferent	Indifferent
	HQNO	Indifferent	Indifferent
	$Ex_{\Delta SR}$	N/A	N/A
CI-28 ΔSR	C7-HHQ	Indifferent	Indifferent
	C7-PQS	Indifferent	Indifferent
	HQNO	Indifferent	Indifferent
	$Ex_{\Delta SR}$	N/A	N/A
CI-29 ΔSR	C7-HHQ	Indifferent	Indifferent
	C7-PQS	Indifferent	Indifferent
	HQNO	Indifferent	Indifferent
	$Ex_{\Delta SR}$	N/A	N/A

Table 7. Fractional Inhibitory Index for Combinations of Bactericidal Antibiotics and Synthetic HAQs against *P. aeruginosa*.

Accomplishment and Conclusions:
We demonstrated significant in vitro adjuvant activity in stationary phase time kills for HAQ+ containing extracts when tested in combination with bactericidal antibiotics gentamicin, ofloxacin

and colistin against laboratory and clinical isolates of *Pseudomonas aeruginosa*. We successfully fractionated HAQ+ extracts and identified two peaks containing three bioactive fractions. LC/MS analysis showed that C7-HHQ and C9-HHQ were the most abundant molecules in the bioactive fractions and their concentrations correlated best with the adjuvant activity. Synthetic C7-HHQ partially recapitulates the adjuvant activity of HAQ+ extracts but to a lesser potency. No activity was identified with any other synthetic HAQ.

When tested by MIC and MBC assays, neither synthetic C7-HHQ, HQNO nor $Ex_{\Delta SR}$ extract had direct anti-bacterial activity in MIC broth microdilution assays against either laboratory or clinical isolates of *P.a.*. PQS was found to have an intermediate MIC against the mucoid clinical isolate CI-28 but was not found to potentiate the activity of either tested antibiotic in FIC assays. Furthermore when combinations were tested in MIC synergy studies, no adjuvant activity was observed for extract or synthetic HAQ.

Together these results suggested that the HAQ adjuvant activity was specific for stationary phase bacteria and its adjuvant activity is only measurable in time-kill assays where C7-HHQ enhances bactericidal activity of gentamicin, ofloxacin and colistin.

SUBTASK 2.2: TESTING OF HAQ-ANTIBIOTIC COMBINATIONS AGAINST *S. aureus*.

- ***S. aureus* strains**

We chose four representative *S. aureus*, namely one methicillin sensitive reference ATCC 29213 and strain, and three methicillin resistant strains (USA100 and USA300, and ATCC 43300). The USA100 and USA300 are two well characterized epidemic community acquired MRSA strains.

- **Minimal inhibitory concentration (MIC) of bactericidal antibiotics**

MICs for *S. aureus* was also performed for antibiotics gentamicin, ofloxacin, meropenem, oxacillin and vancomycin (Table 8). Four isolates including an MSSA, MRSA USA100, USA300 and ATCC 43300 were tested in CAMHB (n=4 replicates) following CLSI approved guidelines. The well characterized strain ATCC 29213 was, as expected, sensitive to the 5 test antibiotics. The oxacillin MIC for all 3 MRSA strains was between 16-32 µg/mL. *S. aureus* USA100 also had an ofloxacin MIC of 8 µg/mL while *S. aureus* ATCC 43300 had a gentamicin MIC of 128 µg/mL which has been reported elsewhere previously. Vancomycin was effective against all 4 strains regardless of methicillin resistance status as expected.

	MIC (µg/mL)				
Strain	**Gentamicin**	**Ofloxacin**	**Meropenem**	**Oxacillin**	**Vancomycin**
MSSA ATCC 29213	2	0.25	0.125	0.125	1
MRSA USA 100	2	8	2	16	1
MRSA USA 300	2	1	0.5	32	1
MRSA ATCC 43300	128	0.25	1	16	1

Table 8. MIC of bactericidal antibiotics against *S. aureus* strains

- **Adjuvant activity of $Ex_{\Delta SR}$ against methicillin sensitive *S. aureus***

The $Ex_{\Delta SR}$, $Ex_{\Delta SR\Delta A}$, $Ex_{\Delta SR\Delta E}$, $Ex_{\Delta SR\Delta H}$ and $Ex_{\Delta SR\Delta L}$ extracts were tested for adjuvant activity in combination with gentamicin against methicillin sensitive *S. aureus* ATCC 29213 (Figure 13B & Figure 18). As done with *P. aeruginosa*, *S. aureus* was grown to stationary phase in planktonic cultures. Bacteria were pre-incubated with $Ex_{\Delta SR}$ to an equivalent concentration of 100 μM C7-HHQ. Viable CFU counts were measured at defined time points to determine the antibiotic killing rate. As with *P. aeruginosa*, we observed that the adjuvant effect of C7-HHQ in potentiating the rate of gentamicin killing is dose-dependent with an R^2= 0.98 (Figure 13C).

- **Adjuvant activity of $Ex_{\Delta SR}$ against methicillin resistant *S. aureus* (MRSA) strains**

Initial tests examined if $Ex_{\Delta SR}$ could potentiate gentamicin killing in USA100 and USA300 CA-MRSA. Indeed addition of combination of extract and gentamicin showed significant activity over gentamicin alone for both isolates (Figure 19A). We therefore tested whether $Ex_{\Delta SR}$ could potentiate oxacillin killing and overcome methicillin resistance in the MRSA strains USA100 and USA300. As shown in Figure 19C, $Ex_{\Delta SR}$ had significant adjuvant activity against both USA100 and USA300 when used in combination with oxacillin where an additional 2-2.5 log kill over oxacillin alone at 24h is observed. However, this adjuvant activity required very high concentrations of oxacillin that would not be clinically achievable. It should be noted that stationary phase cultures are extremely tolerant to β-lactam antibiotics such as oxacillin. Even meropenem showed minimal bactericidal activity against USA100 and USA300 in our assays (Figure 19B). However, $Ex_{\Delta SR}$ potentiated meropenem killing by a modest additional 1-1.5 log over meropenem alone at 24h.

- **Adjuvant activity of synthetic HAQs against *S. aureus***

Synthetic C7-HHQ, C7-PQS and HQNO were tested against *S. aureus* MSSA (ATCC 29213) and CA-MRSA USA100 and USA300 for adjuvant activity in combination with gentamicin. Similar to results in *P. aeruginosa,* 100 μM C7-HHQ potentiated gentamicin killing against MSSA (Figure 20A) and to a lesser extent USA300 (Figure 20B). Moreover, C7-HHQ also showed a dose-dependent adjuvant activity when tested at concentrations of 50 to 200 μM in combination with 50 μg/mL gentamicin Figure 21A). A linear regression analysis showed R^2 correlation of 0.88 between C7-HHQ concentration and bacterial killing (Figure 21B). It is worth noting that under our test conditions gentamicin alone had minimal bactericidal in our killing assays, and C7-HHQ alone had none.

Synthetic C7-PQS showed no adjuvant activity against MSSA when used in combination with gentamicin (Figure 22A). HQNO had a very modest adjuvant activity in combination with gentamicin (Figure 22B). This is consistent with previous studies elsewhere that have reported that HQNO has antibacterial activity against several Gram positive bacteria.

- **Minimal inhibitory concentration (MIC) of HAQ in *S. aureus***

MIC assays showed that C7-HHQ, HQNO and crude $Ex_{\Delta SR}$ extract had no direct anti-bacterial activity against *Staphylcoccous aureus* with MICs above the highest concentration tested (Table 9). C7-PQS, however, had MICs of 4 and 8 μg/mL against *S. aureus* MSSA and MRSA USA300 respectively. C7-PQS activity is likely tied to its function as an iron scavenging molecule which could potentially reduce the growth of *Staphylococcus* due to iron limitation.

	MIC (µg/mL)			
Strain	**C7-HHQ**	**PQS ***	**HQNO**	**ExΔSR**
***S.aureus* MSSA (ATCC 29213)**	>128	4 (8)	>128	>50
***S.aureus* MRSA USA300**	>128	8 (128)	>128	N/A

Table 9. Minimum inhibitory concentration (MIC) of HAQs against *S. aureus*.

- **Minimal bactericidal concentration (MBC) testing of HAQ in *S. aureus***

C7-HHQ, HQNO and the $Ex_{\Delta SR}$ extract did not have any measurable bactericidal activity when tested against *S.aureus* MSSA or MRSA. The MBC of PQS was 2 and 16 fold higher than its MIC in *S. aureus* MSSA and MRSA USA300 (Table 10), suggesting bacteriostatic activity in the MRSA USA300 strain, and potential bactericidal activity against MSSA ATCC 29213. As mentioned previously PQS has significant iron scavenging activity at this concentration and likely impairs the growth of *S. aureus*.

	MBC (µg/mL)			
Strain	**C7-HHQ**	**PQS ***	**HQNO**	**ExΔSR**
***S.aureus* MSSA (ATCC 29213)**	>128	8	>128	>50
***S.aureus* MRSA USA300**	>128	128	>128	N/A

Table 10. Minimum bactericidal concentration (MBC) of HAQs against *S. aureus*.

- **Synergy in synthetic HAQ-antibiotic combination using the checkerboard assays**

MIC synergism against *S. aureus* MSSA ATCC 29213 was examined between combinations of $Ex_{\Delta SR}$ and gentamicin or ofloxacin using a checkerboard assay. For this strain of *S. aureus*, the MICs decreased by 16 and 4 fold for gentamicin and $Ex_{\Delta SR}$ respectively indicating that the combination was synergistic (ΣFIC=0.3125). Similarly combinations of HHQ and gentamicin led to a 4 fold decrease in MICs for both compounds (ΣFIC=0.5) again demonstrating a synergistic effect (Table 11). All other adjuvant combinations with gentamicin and ofloxacin were found to be indifferent for both strains of *S. aureus*.

	Fractional Inhibitory Concentration Index (FIC)		
Strain	**Adjuvant**	**Gentamicin**	**Ofloxacin**
***S.aureus* MSSA (ATCC 29213)**	HHQ	Synergy	Indifferent
	PQS	Indifferent	Indifferent
	HQNO	Indifferent	Indifferent
	$Ex_{\Delta SR}$	Synergy	Indifferent
***S.aureus* MRSA USA300**	HHQ	Indifferent	Indifferent
	PQS	Indifferent	Indifferent
	HQNO	Indifferent	Indifferent
	$Ex_{\Delta SR}$	N/A	N/A

Table 11. Fractional Inhibitory Index for Combinations of Bactericidal Antibiotics and Synthetic HAQs against *S. aureus*.

Accomplishments and Conclusion:
We demonstrated that HAQ+ containing extracts had significant *in vitro* adjuvant activity in combination with gentamicin and ofloxacin against a stationary phase MSSA isolate of *Staphylococcus aureus*. Remarkably, HAQ+ containing extracts also potentiated oxacillin killing against the MRSA isolate USA 300 which is normally resistant to this methicillin derivative. This adjuvant activity also correlated best with H7-HHQ in bioactive fractions. Synthetic C7-HHQ partially recapitulated the adjuvant activity of HAQ+ extracts in time kill assays of stationary phase *S. aureus*.

Among synthetic HAQ and with MIC based assays, only C7-PQS had a modest anti-bacterial activity and C7-HHQ showed a modest synergy with gentamicin against the MSSA strain.

MAJOR TASK 3

SUBTASK 3.1: BIOFILM TESTING

- **Effect of HAQs in inhibition of *P. aeruginosa* biofilm formation**

Experiments were done to examine the impact of $Ex_{\Delta SR\Delta H\Delta L}$ (which contains C7-HHQ, but no PQS or HQNO) and synthetic C7-HHQ on *P. aeruginosa* biofilm formation of PAO1 ΔSR and *S. aureus* MSSA ATCC 29213 biofilms.

We first estimated the effect on biofilm growth using a standard 96 well microtiter assay. Briefly, HAQ ($Ex_{\Delta SR\Delta H\Delta L}$ or synthetic C7-HHQ, or methanol control) was added at the time of inoculation. Bacterial cells were incubated in polystyrene 96-wells for attachment, followed by removal of non-attached cells and incubation in LB medium under static condition for biofilms growth for 6h (immature biofilms) and 24h (mature biofilms). The biofilm biomass was quantified with crystal violet staining. Early biofilm formation (at T=6h) was inhibited by 55% when treated with $Ex_{\Delta SR\Delta H\Delta L}$ (adjusted to 100 μM C7-HHQ content) compared to MeOH controls (Figure 23A). Mature biofilms (T=24h) grown in the presence of $Ex_{\Delta SR\Delta H\Delta L}$ for 24h, however, had identical biomass compared to those treated with methanol. Early biofilm growth at 6 h was unaffected by the presence of 100 μM synthetic C7-HHQ compared to the DMSO (Figure 23B: P=0.8) while the biomass of mature (T=24h) was reduced by 25% compared to the DMSO treated biofilms (Figure 23B: P=0.008).

Next, in order to directly quantify viable biofilm bacteria, we used a colony biofilm assay where bacteria form biofilms on polycarbonate filters. Briefly 5 μL of overnight planktonic bacterial cultures were spotted onto sterile polycarbonate filters laid on solid LB agar medium. Biofilms were grown in the presence of 100 μM C7-HHQ or DMSO, and at 6 h or 24 h, viable biofilm cells were dispersed, serially diluted and enumerated by colony forming unit plate counts. As shown in Figure 23C, there was no difference in colony biofilm growth at either 6 h or 24 h. The difference between the 96-well and colony biofilm models suggest that the effect of C7-HHQ may be due to reduced initial attachment of bacteria onto the polystyrene surface. While a small yet significant effect is observed at 24 h for biofilm grown in polystyrene plates in the presence of C7-HHQ, no difference was observed for coupon grown biofilms under C7-HHQ challenge. The discrepancy is likely due to a decreased biomass (EPS) in the crystal violet plate assay as viable cell counts are not specifically measured.

is likely due to a decreased biomass (EPS) in the crystal violet plate assay as viable cell counts are not specifically measured.

- **Adjuvant activity of HAQ in potentiating biofilm killing *P. aeruginosa* biofilms**

We tested whether the $Ex_{\Delta SR\Delta H\Delta L}$ extract or C7-HHQ potentiated the killing activity of ofloxacin against *P. aeruginosa* biofilms. We used the colony biofilm model described above in order to quantify viable biofilm cells. Biofilms were grown for 24h on LB agar medium, then challenged with $Ex_{\Delta SR\Delta H\Delta L}$ extract or 100 µM C7-HHQ in combination with 30 µg/mL ofloxacin for 4h. First, both $Ex_{\Delta SR\Delta H\Delta L}$ extract and 100 µM C7-HHQ alone had a small effect on biofilm viability (Figure 24A&B: P=0.0013 and P=0.0004 respectively). While the difference is statistically significant on viability the difference is unlikely to be biologically relevant. Second, both $Ex_{\Delta SR\Delta H\Delta L}$ extract and 100 µM C7-HHQ in combination with ofloxacin had a small but statistically significant impact on biofilm viability (Figure 24A and 24B: P=0.0015 and P=0.012 respectively) compared to ofloxacin alone. Unexpectedly, however, the combination against biofilms in both cases was less active than ofloxacin alone.

Next we tested whether a pre-conditioning of biofilms with C7-HHQ might enhance ofloxacin killing. Coupon biofilms were grown on LB-agar either in the presence of 100 µM C7-HHQ or methanol for 24 h, followed by challenge with 30 µg/mL ofloxacin +/- 100 µM C7-HHQ. All conditions showed similar killing (Figure 24C), indicating that a pre-conditioning by C7-HHQ was ineffective at increasing ofloxacin killing. Indeed, while growth of the biofilm in the presence or absence of 100 µM C7-HHQ for 24 h had no impact on viable cell counts (P=0.99) there was a small decrease in sensitivity to ofloxacin challenge of biofilms grown in the presence of C7-HHQ independent of its presence during ofloxacin challenge. This difference, however, did not reach statistical significance (P=0.18 and P=0.21 respectively).

- **Adjuvant activity of HAQ in inhibition of *S. aureus* biofilm formation.**

We planned to carry out experiments on *S. aureus* MSSA ATCC 29213 biofilms in a manner similar to *P. aeruginosa*. Unfortunately, the *S. aureus* 29213 was unable to form robust biofilms in the 96-well assay. This led us to test multiple different media and found that tryptic soy broth (TSB) supported the best biofilm growth for *S.aureus,* albeit to a much lower biomass than with *P. aeruginosa*.

Experiments were done to examine the impact of $Ex_{\Delta SR\Delta H\Delta L}$ on *S. aureus* biofilm grown in 96-well plates and demonstrated that $Ex_{\Delta SR\Delta H\Delta L}$ (adjusted to 100 µM C7-HHQ content) had no effect on early biofilm formation (6h), but a small and statistically significant decrease in 24h biofilm (Figure 25A: P=0.01). Biofilm growth +/- 100 µM C7-HHQ showed no differences at either 6 h or 24 (Figure 25B: P=0.99 and P=0.38 respectively). Results were similar with coupon biofilms, with identical viable biofilm cell counts in the presence or absence of 100 µM C7-HHQ, at both 6 h and 24 h biofilms grown (Figure 25C) on polycarbonate coupons. It is worth noting that although the biofilm biomass assesses by crystal violet staining is lower at 6h compared to 24h (Figure 25B), the corresponding viable CFU counts is actually slightly higher (Figure 25C).

- **Adjuvant activity of HAQ in potentiating biofilm killing *S. aureus* biofilms**

Challenge of 24 h *S. aureus* colony biofilms with 30 µg/mL ofloxacin +/- 100 µM C7-HHQ led to identical killing, indicating that C7-HHQ had no effect in potentiating ofloxacin killing *S. aureus* colony biofilms (Figure 25D).

Accomplishments and Conclusion:
We used two different biofilm models of *Pseudomonas aeruginosa* and *Staphylococcus aureus* and found no significant activity by HAQ on either organism: there was no inhibition of biofilm growth nor any potentiation of biofilm killing by ofloxacin.

MAJOR TASK 4. VALIDATE AGAINST ESKAPE PATHOGENS AND E. COLI

Since we have demonstrated that our HAQ extract and synthetic C7-HHQ potentiate bactericidal antibiotics against *P. aeruginosa* and *S. aureus*, our goal was to validate the key findings in other clinically relevant ESKAPE pathogens. Although the main focus of this task was to test ESKAPE organisms, we have also tested *E. coli* because it was highly susceptibility to the HAQ adjuvant activity and served as an indicator strain (Subtask 1.1).

We focused on the following ESKAPE organisms' *A. baumanii*, *K. pneumoniae* and *E. faecalis*. We tested the *Acinetobacter baumannii* AB5075 (*A.b.*), a well characterized virulent isolate, as well as a multidrug resistant clinical isolate of *Klebsiella pneumoniae* (*K.p.* CI). We included two reference *E. faecalis* strains, a susceptible one (ATCC 29212) and one with VanB type vancomycin resistance (ATCC 51299).

SUBTASK 4.1: TESTING THE ANTIMICROBIAL ACTIVITY OF HAQ-ANTIBIOTIC COMBINATIONS AGAINST OTHER ESKAPE PATHOGENS

- **Minimal inhibitory concentration (MIC) of bactericidal antibiotics against *E. coli***

MICs for *E.coli* was also performed for antibiotics gentamicin, ofloxacin, and colistin (Table 12). Two isolates including an *E.coli* WT (MG1655) and *E.coli* ΔSR were tested in CAMHB (n=4 replicates) following CLSI approved guidelines. As anticipated, both strains were susceptible to all antibiotics.

	MIC (µg/mL)		
Strain	**Gentamicin**	**Ofloxacin**	**Colistin**
***E.coli* WT (MG1655)**	2	0.125	0.5
***E.coli* ΔSR**	2	0.125	0.5

Table 12. MIC of bactericidal antibiotics against *E. coli*

- **MIC of HAQ and $Ex_{\Delta SR}$ against *E. coli***

Since *E. coli* is highly susceptible to the adjuvant effects of HAQ and was used as an indicator strain, we tested the anti-bacterial activity of HAQ and $Ex_{\Delta SR}$ extract using the MIC assay (Table 13). Similar to *P. aeruginosa*, C7-HHQ, HQNO, and crude $Ex_{\Delta SR}$ extract had no direct anti-bacterial activity against wild-type or ΔSR *E.coli* with undetectable MIC at achievable concentrations. PQS showed activity with MICs of 4 and 1 µg/mL against WT and ΔSR *E. coli* strains respectively, which is comparable to its effect on the *P. aeruginosa* CI-28 strain.

	MIC (µg/mL)			
Strain	**C7-HHQ**	**C7-PQS ***	**HQNO**	**ExΔSR**
***E.coli* WT (MG1655)**	>128	4	>128	>50
***E.coli* ΔSR**	>128	1	>128	>50

Table 13. Minimum inhibitory concentration (MIC) of HAQs against *E. coli.*

- **Minimal bactericidal concentration (MBC) of HAQ against *E. coli***

E. coli MBCs were virtually identical to their MICs (Table 14). It is likely, similar to previously discussed, that the PQS activity is tied to its iron scavenging activity, which would likely impair the growth of the *E. coli* strains.

	MBC (µg/mL)			
Strain	**C7-HHQ**	**C7-PQS ***	**HQNO**	**ExΔSR**
***E.coli* WT (MG1655)**	>128	4	>128	>50
***E.coli* ΔSR**	>128	2	>128	>50

Table 14. Minimum bactericidal concentration (MBC) of HAQs against *E. coli.*

- **Synergy in HAQ-antibiotic combination using the checkerboard assays**

MIC synergism against *E. coli* wild type and *E. coli* ΔSR was examined between combinations of $Ex_{\Delta SR}$ or synthetic HAQs and gentamicin or ofloxacin using a checkerboard assay. All combinations that included ofloxacin for both *E. coli* wild type and *E. coli* ΔSR were found to be indifferent. Similarly all synthetic HAQs when combined with gentamicin were indifferent towards both *E. coli* strains. Surprisingly, however, 50 ug/mL $Ex_{\Delta SR}$ increased the gentamicin MIC by 4 fold for both strains, suggesting an antagonistic to gentamicin activity.

	Fractional Inhibitory Concentration Index (FIC)		
Strain	**Adjuvant**	**Gentamicin**	**Ofloxacin**
***E.coli* WT (MG1655)**	HHQ	Indifferent	Indifferent
	PQS	Indifferent	Indifferent
	HQNO	Indifferent	Indifferent
	$Ex_{\Delta SR}$	Antagonistic	Indifferent
***E.coli* ΔSR**	HHQ	Indifferent	Indifferent
	PQS	Indifferent	Indifferent
	HQNO	Indifferent	Indifferent
	$Ex_{\Delta SR}$	Antagonistic	Indifferent

Table 15. Fractional Inhibitory Index for Combinations of Bactericidal Antibiotics and Synthetic HAQs against *E.coli*

- **MIC of bactericidal antibiotics against ESKAPE organisms**

We measured the MICs of the major bactericidal antibiotics used against the Gram-negative *A. baumanii* and *K. pneumoniae*, including gentamicin, ofloxacin, meropenem and colistin (Table 16). *A. baumanii* AB5075 showed multidrug resistance to gentamicin (MIC >128 µg/mL), ofloxacin (MIC 32 µg/mL) and meropenem (MIC 64 µg/mL) and "intermediate" susceptibility to colistin (MIC 8 µg/mL). The *K. pneumoniae* clinical isolate was susceptible to meropenem (MIC 1 µg/mL) but highly resistant to both gentamicin and ofloxacin (MIC 128 µg/mL) and "intermediate" resistant to colistin (MIC 8 µg/mL).

	MIC (µg/mL)			
Strain	**Gentamicin**	**Ofloxacin**	**Meropenem**	**Colistin**
*A. **baumanii*** **AB5075**	>128	32	64	8
K. pneumoniae **Cl**	>128	128	1	8

Table 16. Minimum inhibitory concentration (MIC) of HAQs against Gram-negative ESKAPE Pathogens.

- **MIC of HAQ and $Ex_{\Delta SR}$ against ESKAPE Pathogens *A. baumanii* and *K. pneumoniae***

Both *Acinetobacter* and *Klebsiella* HAQs MICs were unmeasurable (Table 17) with values of at least 128 µg/mL. The *A. baumanii* is multidrug resistant, including to meropenem. MBCs were not calculated due to the absence of measurable MICs.

	MIC (µg/mL)			
Strain	**C7-HHQ**	**PQS ***	**HQNO**	$Ex_{\Delta SR\Delta H\Delta L}$
*A. **baumanii*** **AB5075**	>128	>128	>128	>50
K. pneumoniae **Cl**	>128	128	>128	>50

Table 17. Minimum inhibitory concentration (MIC) of HAQs against Gram-negative ESKAPE Pathogens.

- **Synergy in HAQ-antibiotic combination using the checkerboard assays**

MIC synergism against *A. baumanii* AB5075 and a clinical isolate of *K. pneumoniae* was examined between combinations of $Ex_{\Delta SR}$ or synthetic HAQs and colistin, meropenem or ofloxacin using a checkerboard assay. Neither strain was found to have any synergistic effect with either $Ex_{\Delta SR}$ extract or synthetic HAQs and any of the tested antibiotics (Table 18).

	Fractional Inhibitory Concentration Index (FIC)			
Strain	**Adjuvant**	**Colistin**	**Ofloxacin**	**Meropenem**
A. ***baumanii*** **AB5075**	HHQ	Indifferent	Indifferent	N/A
	PQS	Indifferent	Indifferent	N/A
	HQNO	Indifferent	Indifferent	N/A
	$Ex_{\Delta SR\Delta H\Delta L}$	Indifferent	Indifferent	N/A
K. pneumoniae **CI**	HHQ	Indifferent	N/A	Indifferent
	PQS	Indifferent	N/A	Indifferent
	HQNO	Indifferent	N/A	Indifferent
	$Ex_{\Delta SR\Delta H\Delta L}$	Indifferent	N/A	Indifferent

Table 18. Fractional Inhibitory Index for Combinations of Bactericidal Antibiotics and Synthetic HAQs against Gram-negative ESKAPE Pathogens.

We tested the *E.faecalis* strains ATCC 29212 and vancomycin-R *E. faecalis* ATCC 51299 against 3 different classes of antibiotics, namely gentamicin, ofloxacin, and vancomycin (Table 19). The *E. faecalis* ATCC 29212 was susceptible to all 3 drugs but the ATCC 51299 strain was highly resistant to gentamicin (MIC >256 µg/mL) and vancomycin (MIC 16-32 µg/mL) as expected.

	MIC (µg/mL)		
Strain	**Gentamicin**	**Ofloxacin**	**Vancomycin**
E.faecalis ATCC 29212	4	4	2
E.faecalis ATCC 51299	>256	4	16-32

Table 19. MIC of bactericidal antibiotics against other ESKAPE organisms.

- **Synergy in HAQ-antibiotic combination using the checkerboard assays**

Testing of *E. faecalis* with gentamicin, ofloxacin or vancomycin (considered bacteriostatic against *Enterococcus*) in combination with either synthetic HAQs or $Ex_{\Delta SR\Delta H\Delta L}$ extract showed indifferent for all combinations tested (Table 20).

	Fractional Inhibitory Concentration Index (FIC)			
Strain	**Adjuvant**	**Gentamicin**	**Ofloxacin**	**Vancomycin**
E.faecalis ATCC 29212	HHQ	Indifferent	Indifferent	Indifferent
	PQS	Indifferent	Indifferent	Indifferent
	HQNO	Indifferent	Indifferent	Indifferent
	$Ex_{\Delta SR\Delta H\Delta L}$	Indifferent	Indifferent	Indifferent
E.faecalis ATCC 51299	HHQ	Indifferent	Indifferent	Indifferent
	PQS	Indifferent	Indifferent	Indifferent
	HQNO	Indifferent	Indifferent	Indifferent
	$Ex_{\Delta SR\Delta H\Delta L}$	Indifferent	Indifferent	Indifferent

Table 20. Fractional Inhibitory Index for Combinations of Bactericidal Antibiotics and Synthetic HAQs against *Enterococcus faecalis*

- **Adjuvant activity of HAQ against *Klebsiella pneumoniae, Acinetobacter baumannii* and *E. faecalis.***

Since the $Ex_{\Delta SR\Delta H\Delta L}$ had greatest adjuvant activity in *P. aeruginosa*, we tested it in the *K.p.* CI and AB5075 in the time kill assays of planktonic stationary phase bacteria as done with *P. aeruginosa*, *S. aureus* and *E. coli*. We focused on combinations with colistin and meropenem for *K.p.* CI which had MICs of 8 and 1 µg/mL respectively. Overnight cultures of *K.p.* CI were pre-challenged with $Ex_{\Delta SR\Delta H\Delta L}$ for 2h, then challenged with 50 µg/mL of colistin or 500 µg/mL of meropenem. Bacterial killing was quantified by plating of viable CFU. The colistin + $Ex_{\Delta SR\Delta H\Delta L}$ combination demonstrated significantly greater killing activity than colistin alone (Figure 26A), with differences which were greatest in the first 6h. The $Ex_{\Delta SR\Delta H\Delta L}$ did not enhance meropenem killing, as killing was similar between the [meropenem + $Ex_{\Delta SR\Delta H\Delta L}$] and meropenem treatments (Figure 26B). We would like to note that β-lactams show very limited to no bactericidal activity in our time kill assays even when isolates have low MICs, as this assay tests highly drug tolerant cells.

For AB5075 *A.b.* we focused on combinations of $Ex_{\Delta SR\Delta H\Delta L}$ with colistin and ofloxacin, and antibiotics were tested at concentrations of 20 µg/mL for colistin and 200 µg/mL of ofloxacin for *A.b*. Colistin alone showed no bactericidal activity, while addition of $Ex_{\Delta SR\Delta H\Delta L}$ significantly potentiated colistin killing, with the [$Ex_{\Delta SR\Delta H\Delta L}$ + colistin] combination sterilizing the *A.b*. culture within 2h (Figure 26C). The MIC for ofloxacin was 32 µg/mL and 200 µg/mL ofloxacin alone showed no significant bactericidal activity against tolerant AB5075 *A.b.* We also observed potentiation of ofloxacin killing by $Ex_{\Delta SR\Delta H\Delta L}$ (Figure 26D). Interestingly $Ex_{\Delta SR\Delta H\Delta L}$ extract by itself had an early impact on viable bacteria but by 24 h this effect was no longer apparent (Figure 26D).

We tested the adjuvant activity $Ex_{\Delta SR\Delta H\Delta L}$ against two strains of *E. faecalis*, and focused on the combination of $Ex_{\Delta SR\Delta H\Delta L}$ with ofloxacin which had a MICs of 4 µg/mL in both isolates. Overnight planktonic cultures of *E.f.* 29212 and *E.f.* 51299 were pre-challenged for 2 h with $Ex_{\Delta SR\Delta H\Delta L}$ followed by challenge with 10 µg/mL ofloxacin. Ofloxacin alone showed modest bactericidal activity against both 29212 and 51299 strains with a reduction of 2.16 and 1.5 log10 respectively (Figure 27A&B) after 6 h. Ofloxacin in combination with $Ex_{\Delta SR\Delta H\Delta L}$ was slightly more effective in both strains (Figure 27A&B: P=0.04 and P=0.02).

Accomplishment and Conclusions:
HAQ+ extract potentiated killing of *A. baumanii* by colistin, and to a more modest degree in *K. pneumonia*. A more modest adjuvant effect was observed with ofloxacin, and no adjuvant activity was noted with meropenem. Notably, the adjuvant activity was only measurable in time kill assays of stationary phase cells. HAQ+ extract had adjuvant activity with ofloxacin against *E. faecalis*.

None of the synthetic HAQs (HHQ, PQS and HQNO) or extract $Ex_{\Delta SR\Delta H\Delta L}$ had detectable MICs against the ESKAPE pathogens *A. baumanii*, *K. pneumonia*e and *E. faecalis* tested. All combinations of HAQs (synthetic HAQs or HAQ+ extract) with ofloxacin, meropenem or colistin showed indifference using the FIC testing (checkerboard) method.

SPECIFIC AIM 2. TO TEST WHETHER HAQ USED IN COMBINATION WITH CONVENTIONAL BACTERICIDAL ANTIBIOTICS IMPROVE BACTERIAL ERADICATION IN SEVERE PA AND SA INFECTIONS

MAJOR TASK 1. DETERMINE THE TOXICITY OF HAQ

SUBTASK 1.1: MAMMALIAN CELL TOXICITY

The $Ex_{\Delta SR}$ extract contains a mixture of all bioactive HAQ, including the most potent one C7-HHQ. We thus reasoned that preliminary testing of the $Ex_{\Delta SR}$ and $Ex_{\Delta SR\Delta H\Delta L}$ extracts would provide a composite measure of potential toxicity to mammalian cells, and tested $Ex_{\Delta SR}$ concentrations identical to those used in our adjuvant activity assays. We used a standard cytotoxicity assay used toxicology, namely the lactate dehydrogenase (LDH) release assay using HeLa cells, the most commonly used human cell line, and the red blood lysis assay as a measure of potential damage to mammalian cell membranes.

- **Cytotoxicity in HeLa cells by LDH release assay**

HeLa cells were grown to ~70% confluence in 96 well tissue culture treated plates (~50,000 cells/well) in DMEM medium. At the time of challenge, $Ex_{\Delta SR}$ was added to 100 µL fresh media + 10% FBS at 37 ^{o}C in 5% CO_2, and MeOH was used as the vehicle control (VC). After 2h incubation, the culture supernatant was assayed for LDH release and compared to a Triton X100 treated positive control to estimate the % cytotoxicity. We performed these assays with two independent batches of $Ex_{\Delta SR}$ and observed no significant increase in LDH release with either $Ex_{\Delta SR}$ or VC compared to the untreated cells (Figure 28A).

- **Cytotoxicity in red blood by lysis assay**

Human red blood cells (hRBCs) were isolated from human blood according to standard procedures and incubated with $Ex_{\Delta SR\Delta H\Delta L}$ extract or MeOH (extract vehicle control) in 500 µl of erythrocyte buffer (130 mM NaCl, 20 mM Tris-HCl, pH 7.4) at room temperature. The $Ex_{\Delta SR\Delta H\Delta L}$ extract was adjusted to 100 µM C7-HHQ content. After 2h incubation, the % hemolysis was measured by absorption at 405 nm with a microplate reader (Tecan M100pro). In these conditions, $Ex_{\Delta SR\Delta H\Delta L}$ did not cause significant hemolysis compared to MeOH control (Figure 28B).

SUBTASK 1.2: MOUSE TOXICITY

Given that we observed no overt in vitro toxicity to HeLa cells nor hemolysis attributable to HAQ, we proceeded with the testing of HAQ for *in vivo* tolerability. C7-HHQ was the synthetic HAQ with the most potent adjuvant activity against *P. aeruginosa* and *S. aureus* in our in vitro assays, leading us to continue the in vivo testing with this compound.

We initially planned to administer the HAQ through the intra-tracheal route. Our in vitro data suggested that we should aim for at least C7-HHQ 100 µM which we found to be its maximal solubility limit in aqueous solution. The maximum volume of intra-tracheal antibiotic installation was found to be 25 µL. Above this volume the likelihood of suffocation in the mice increased significantly. Furthermore the vehicle chosen to dissolve C7-HHQ (5% Tween-80, 5% PEG_{400}, 5% DMSO) was found to be too viscous and was more problematic for the mice. Reduction of

either Tween-80 or PEG_{400} significantly reduced the solubility of the HHQ in solution. This led us to conclude that intra-tracheal administration of C7-HHQ was not feasible at this stage and opted instead for intra-peritoneal (i.p.) and subcutaneous (s.c.) administration. In order to maximize the possibility of achieving sufficiently high HHQ concentrations following i.p. or s.c. we determined the solubility of HHQ in its vehicle and a maximum injection volume of 400 µL and 50 µL respectively. For a 25 g mouse this would allow us to safely achieve a 12.5 mg/kg i.p. dose.

- **C7-HHQ Toxicity following i.p. administration**

We tested a total of n=14 wild-type C57B6 mice (6-8 weeks, avg weight 23.8±3.6 g) across two experiments by administrating a single i.p dose of 12.5 mg/kg C7-HHQ (7 mice: 5 males/2 females) in a total volume of 400 µL vehicle (5% Tween-80, 5% PEG_{400}, 5% DMSO) and were compared to a vehicle control group (7 mice: 5 males/2 females). At 24h after C7-HHQ treatment, mice were sacrificed for blood collection, peritoneal lavage and necropsy. Organs (liver, spleen, lung harvest) were inspected and no noticeable abnormality was noted following which they were preserved in 4% formalin for later optional analysis following completion of the blood/biochemical if abnormalities were observed. Mice were monitored for 24h and no clinical adverse effects were noted (weight, clinical body score). Blood analyses included complete blood count and differential, biochemistry (including electrolytes, renal and liver function tests). In most mice sufficient blood volume to obtain individual biochemical and hematological results, but samples from several pairs of mice in both the HHQ group and vehicle control the samples were pooled for the biochemical analysis (Table 21&23). All hematological values are from individual mice (Table 22&24). Results were nearly all within the normal range and no significances differences between the C7-HHQ and control groups were observed. A peritoneal lavage was performed using 5 mL ice cold PBS and analyzed by cytospin and Kwik-Diff staining. The total and differential cell count were similar identical between the C7-HHQ and control groups (Figure 29).

Animal ID	Units	**Normal Values**	HHQ-1839*	HHQ-1840*	HHQ-1841**	Vehicle-1842*	Vehicle-1843*	Vehicle-1844*
Laboratory number		**Mouse**	**17-0091**	**17-0092**	**17-0093**	**17-0094**	**17-0095**	**17-0096**
Total Protein	g/L	**36-66**	57	52	59	48	33	50
Albumin	g/L	**25-48**	29	27	31	22	15	25
Albumin/Globulin ratio			1.0	1.1	1.1	0.8	0.8	1.0
Glucose	mmol/L	**5.0-10.7**	17.1	17.6	17.8	15.3	6.5	15.0
BUN Urea	mmol/L	**6.4-10.4**	8.80	9.72	10.95	12.24	5.80	9.67
Creatinine	µmol/L	**18-71**	13	13	<4	13	<4	12
Total Bilirubin	µmol/L	**2-15**	24	17	29	14	15	19
ALT	U/L	**28-132**	62	55	47	72	60	58
AST	U/L	**59-247**	147	123	157	107	270	127
Alkaline Phosphatase	U/L	**62-209**	107	97	100	87	30	89
CK	U/L	**68-1070**	518	463	895	473	3405	1322

Cholesterol	mmol/L	**0.93-2.48**	3.53	2.86	2.85	2.53	<1.29	2.62
Sodium	mmol/L	**124-174**	152	151	150	154	151	149
Potassium	mmol/L	**4.6-8.0**	9.43	9.56	9.76	9.05	6.63	9.35
Chloride	mmol/L	**92-120**	107	108	110	113	122	110
Calcium	mmol/L	**1.47-2.35**	2.92	2.73	2.79	2.67	1.52	2.58
Phosphorus	mmol/L	**1.97-3.26**	QNS	3.41	3.10	3.23	4.60	3.57
Magnesium	mmol/L	**0.33-1.60**	QNS	1.41	1.39	1.37	0.69	1.39

Table 21. Blood biochemistry of 6 male C57B6 mice following a single dose of 12.5 mg/kg C7-HHQ administered via *i.p.*.

Animal ID	HHQ-1839	HHQ-1840	HHQ-1841	Vehicle-1842	Vehicle-1843	Vehicle-1844
Laboratory number	**17-0085**	**17-0086**	**17-0087**	**17-0088**	**17-0089**	**17-0090**
WBCs $x10^9$/L	11.8	9.2	12.1	9.0	13.0	11.4
RBCs $x10^{12}$/L	9.79	8.59	9.67	8.60	7.41	8.20
hemoglobin g/L	152	141	149	143	126	137
hematocrit L/L	0.442	0.392	0.442	0.394	0.336	0.369
MCV fL	45	46	46	46	45	45
MCH pg	15.6	16.5	15.4	16.6	17.0	16.7
MCHC g/L	345	361	338	362	374	370
platelets x 10^9/L	819	823	655	1082	839	929
neutrophils %	27.0	20.0	60.0	28.0	42.0	22.0
lymphocytes %	73.0	80.0	40.0	70.0	58.0	77.0
monocytes %	0.0	0.0	0.0	0.0	0.0	0.0
eosinophils %	0.0	0.0	0.0	2.0	0.0	1.0
neutrophils x 10^9/L	3.19	1.84	7.26	2.52	5.46	2.51
lymphocytes x 10^9/L	8.61	7.36	4.84	6.30	7.54	8.78
monocytes x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00
eosinophils x 10^9/L	0.00	0.00	0.00	0.18	0.00	0.11
band x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00
metamyelocyte x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00
myelocyte x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00
promyelocyte x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00
Other (1) %	0.00	0.00	0.00	0.00	0.00	0.00
blast	0.00	0.00	0.00	0.00	0.00	0.00

Table 22. Hematological counts of 6 male C57B6 mice following a single dose of 12.5 mg/kg C7-HHQ administered via *i.p.*

Animal ID	Units	**Values**	HHQ-1857/1858	Vehicle-1859/1860	HHQ-1861/1862	Vehicle-1863/1864
Laboratory number		**Mouse**	**17-0230***	**17-0231****	**17-0232****	**17-0233****
Total Protein	g/L	**36-66**	56	61	58	58
Albumin	g/L	**25-48**	29	34	31	30
Albumin/Globulin ratio			1.1	1.2	1.1	1.1
Glucose	mmol/L	**5.0-10.7**	13.8	18.3	15.1	15.3
BUN Urea	mmol/L	**6.4-10.4**	7.5	7.3	8.0	7.8
Creatinine	µmol/L	**18-71**	12	5	8	9
Total Bilirubin	µmol/L	**2-15**	18	24	24	23
ALT	U/L	**28-132**	48	58	69	50
AST	U/L	**59-247**	112	142	110	103
Alkaline Phosphatase	U/L	**62-209**	109	112	111	120
CK	U/L	**68-1070**	169	230	168	380
Cholesterol	mmol/L	**0.93-2.48**	2.15	2.53	2.52	2.28
Sodium	mmol/L	**124-174**	152	151	153	154
Potassium	mmol/L	**4.6-8.0**	9.0	10.2	10.7	8.3
Chloride	mmol/L	**92-120**	111	113	110	111
Calcium	mmol/L	**1.47-2.35**	2.63	2.74	2.79	2.69
Phosphorus	mmol/L	**1.97-3.26**	2.63	3.26	4.01	3.03
Magnesium	mmol/L	**0.33-1.60**	1.43	1.48	1.52	1.24

Table 23. Blood biochemistry of 8 C57B6 mice (4 male&4 female) following a single dose of 12.5 mg/kg C7-HHQ administered via *i.p.*.

Animal ID	HHQ-1857	HHQ-1858	Vehicle-1859	Vehicle-1860	HHQ-1861	HHQ-1862	Vehicle-1863	Vehicle-1864
Laboratory number	**17-0222**	**17-0223**	**17-0224**	**17-0225**	**17-0226**	**17-0227**	**17-0228**	**17-0229**
WBCs $x10^9$/L	5.1	5.5	4.5	4.0	5.4	3.8	4.6	7.3
RBCs $x10^{12}$/L	8.86	7.81	7.65	8.60	8.58	8.63	8.05	8.63
hemoglobin g/L	138	134	130	149	147	145	139	146
hematocrit L/L	0.392	0.351	0.344	0.384	0.380	0.383	0.354	0.382
MCV fL	44	45	45	45	44	44	44	44
MCH pg	15.6	17.2	16.9	17.3	17.2	16.9	17.3	16.9
MCHC g/L	353	381	377	389	387	380	393	382
platelets x 10^9/L	807	808	804	511	498	938	1069	940
neutrophils %	9.0	5.0	7.0	12.0	11.0	10.0	15.0	7.0
lymphocytes %	89.0	91.0	92.0	86.0	87.0	89.0	83.0	91.0

monocytes %	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0
eosinophils %	2.0	4.0	1.0	2.0	2.0	0.0	2.0	2.0
neutrophils x 10^9/L	0.46	0.28	0.32	0.48	0.59	0.38	0.69	0.51
lymphocytes x 10^9/L	4.54	5.01	4.14	3.44	4.70	3.38	3.82	6.64
monocytes x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00
eosinophils x 10^9/L	0.10	0.22	0.05	0.08	0.11	0.00	0.09	0.15
band x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
metamyelocyte x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
myelocyte x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
promyelocyte x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Other (1) %	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
blast	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 24. Hematological counts of 8 C57B6 mice (4 male&4 female) following a single dose of 12.5 mg/kg C7-HHQ administered via *i.p.*.

- **C7-HHQ toxicity following s.c. administration**

We tested n=6 (all male, 3 received HHQ and 3 received vehicle alone) wild-type C57B6 mice (6-8 weeks, avg weight 22.7±1.9 g) with a single s.c injection of 6.5 mg/kg (vehicle 5% Tween-80, 5% PEG_{400}, 5% DMSO in total volume 50 µL) and n=6 (3 received HHQ and 3 received vehicle alone) wild-type C57B6 mice (6-8 weeks, avg weight 25 g) 50 mg/kg C7-HHQ (modified vehicle 5% Tween-80, 5% PEG_{400}, 66% DMSO in total volume 50 µL). Although the vehicle for the 6.5 mg/kg dose was identical to that used in the i.p injection, we had to modify the vehicle for the 50 mg/kg dose due to C7-HHQ solubility issues in order to obtain a concentration sufficiently high. Mice were monitored and blood samples analyzed as described for the i.p administration.

With the low C7-HHQ dose (6.5 mg/kg), we observed no adverse events and blood analyses showed no differences between the C7-HHQ and control groups. (Table 25&26) We noted that all mice had elevated levels of glucose, potassium and calcium but these abnormalities were found across both groups indicating that they were not attributable to C7-HHQ.

Animal ID	Units	**Values**	HHQ-19-2	HHQ-19-1	HHQ-19-3	Vehicle-45-1	Vehicle-45-3
Laboratory number		**Mouse**	**17-1419**	**17-1420**	**17-1421**	**17-1422**	**17-1424**
Total Protein	g/L	**36-66**	48	48	52	50	50
Albumin	g/L	**25-48**	24	24	27	25	24
Albumin/Globulin ratio			1.0	1.0	1.1	1.0	1.0
Glucose	mmol/L	**5.0-10.7**	15.9	13.4	14.2	16.0	15.6
BUN Urea	mmol/L	**6.4-10.4**	5.4	6.1	6.7	6.6	6.3
Creatinine	µmol/L	**18-71**	15	16	14	13	13
Total Bilirubin	µmol/L	**2-15**	10	10	11	9	13
ALT	U/L	**28-132**	32	35	40	40	46
AST	U/L	**59-247**	63	110	62	61	124

Alkaline Phosphatase	U/L	**62-209**	129	163	109	142	112
CK	U/L	**68-1070**	67	334	65	71	293
Cholesterol	mmol/L	**0.93-2.48**	2.78	2.31	3.04	2.40	2.49
Sodium	mmol/L	**124-174**	149	153	152	152	153
Potassium	mmol/L	**4.6-8.0**	11.2	9.2	11.4	11.8	12.1
Chloride	mmol/L	**92-120**	109	109	112	108	110
Calcium	mmol/L	**1.47-2.35**	2.79	2.66	2.68	2.84	2.78
Phosphorus	mmol/L	**1.97-3.26**	3.28	3.12	3.14	3.66	3.52
Magnesium	mmol/L	**0.33-1.60**	1.43	1.43	1.43	1.60	1.50

Table 25. Blood biochemistry of 5 C57B6 mice following a single dose of 6.25 mg/kg C7-HHQ administered via *s.c.*.

Animal ID	19-2	19-1*	19-3	45-1	45-2	45-3
Laboratory number	**17-1419**	**17-1420**	**17-1421**	**17-1422**	**17-1423**	**17-1424**
WBCs $x10^9$/L	6.6	8.7	5.2	9.3	11.4	7.3
RBCs $x10^{12}$/L	9.28	8.64	10.03	8.95	9.40	9.77
hemoglobin g/L	138	131	146	136	144	145
hematocrit L/L	0.438	0.411	0.470	0.418	0.451	0.466
MCV fL	47	48	47	47	48	48
MCH pg	14.9	15.1	14.6	15.2	15.3	14.9
MCHC g/L	316	318	311	325	319	311
platelets x 10^9/L	832	624	744	1082	706	913
neutrophils %	13.0	11.0	8.0	10.0	8.0	18.0
lymphocytes %	84.0	85.0	87.0	86.0	89.0	79.0
monocytes %	0.0	0.0	0.0	0.0	0.0	0.0
eosinophils %	3.0	4.0	5.0	4.0	3.0	3.0
neutrophils x 10^9/L	0.86	0.96	0.42	0.93	0.91	1.31
lymphocytes x 10^9/L	5.54	7.40	4.52	8.00	10.15	5.77
monocytes x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00
eosinophils x 10^9/L	0.20	0.35	0.26	0.37	0.34	0.22
band x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00
metamyelocyte x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00
myelocyte x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00
promyelocyte x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00
Other (1) %	0.00	0.00	0.00	0.00	0.00	0.00
blast	0.00	0.00	0.00	0.00	0.00	0.00

Table 26. Hematological counts of 6 C57B6 mice following a single dose of 6.25 mg/kg C7-HHQ administered via *s.c.*.

With the high C7-HHQ dose (50 mg/kg) and controls, we noted some minor skin irritation in both groups, which resolved within after a few minutes. The complete and differential blood counts were within normal range and similar in both groups except for platelet counts (Table 27). The C7-HHQ group had normal platelet counts while the control group showed significantly reduced levels (710±186 versus 134±135 $x10^9$ /L respectively, normal ~1000 $x10^9$ /L). Biochemical analyses were all within the normal ranges (Table 28) and showed no differences between the groups with the exception of elevated levels of ALT and AST (each ~2x upper limit) in the VC group compared to the C7-HHQ group.

Animal ID	HHQ1-blood	HHQ2-blood	HHQ3-blood	VEHICLE1 - blood	VEHICLE2 - blood	VEHICLE3 - blood
Laboratory number	**17-1934**	**17-1935**	**17-1936**	**17-1937**	**17-1938***	**17-1939***
WBCs $x10^9$/L	6.6	3.5	3.8	2.7	2.6	2.4
RBCs $x10^{12}$/L	9.11	9.10	7.42	8.57	8.72	8.20
hemoglobin g/L	139	139	119	135	133	128
hematocrit L/L	0.434	0.431	0.354	0.411	0.423	0.401
MCV fL	48	47	48	48	49	49
MCH pg	15.3	15.2	16.1	15.7	15.3	15.6
MCHC g/L	321	321	338	328	315	319
platelets x 10^9/L	888	728	516	62	54	290
neutrophils %	5.0	9.0	11.0	5.0	12.0	9.0
lymphocytes %	93.0	85.0	85.0	93.0	88.0	90.0
monocytes %	0.0	0.0	0.0	0.0	0.0	0.0
eosinophils %	2.0	6.0	4.0	2.0	0.0	1.0
neutrophils x 10^9/L	0.33	0.32	0.42	0.14	0.31	0.22
lymphocytes x 10^9/L	6.14	2.98	3.23	2.51	2.29	2.16
monocytes x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00
eosinophils x 10^9/L	0.13	0.21	0.15	0.05	0.00	0.02
band x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00
metamyelocyte x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00
myelocyte x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00
promyelocyte x 10^9/L	0.00	0.00	0.00	0.00	0.00	0.00
Other (1) %	0.00	0.00	0.00	0.00	0.00	0.00
blast	0.00	0.00	0.00	0.00	0.00	0.00

Table 27. Hematological counts of 6 C57B6 mice following a single dose of 50 mg/kg C7-HHQ administered via *s.c.*

Animal ID	Units	**Values**	HHQ-serum	Vehicle-serum
Laboratory number		**Mouse**	**17-1932***	**17-1933***
Total Protein	g/L	**36-66**	58	59
Albumin	g/L	**25-48**	31	31
Albumin/Globulin ratio			1.1	1.1
Glucose	mmol/L	**5.0-10.7**	13.0	16.1
BUN Urea	mmol/L	**6.4-10.4**	7.1	8.4
Creatinine	µmol/L	**18-71**	5	7
Total Bilirubin	µmol/L	**2-15**	31	28
ALT	U/L	**28-132**	74	255
AST	U/L	**59-247**	273	466
Alkaline Phosphatase	U/L	**62-209**	165	117
CK	U/L	**68-1070**	959	1015
Cholesterol	mmol/L	**0.93-2.48**	2.51	3.26
Sodium	mmol/L	**124-174**	149	148
Potassium	mmol/L	**4.6-8.0**	8.5	11.1
Chloride	mmol/L	**92-120**	117	112
Calcium	mmol/L	**1.47-2.35**	2.39	2.64
Phosphorus	mmol/L	**1.97-3.26**	3.71	3.54
Magnesium	mmol/L	**0.33-1.60**	1.50	1.37

Table 28. Blood biochemistry of 6 C57B6 mice (combined samples) following a single dose of 50 mg/kg C7-HHQ administered via *s.c.*

Due to the limited volume of blood, we pooled blood samples from each group. It is therefore not possible to carry out statistical analyses nor to determine whether the abnormal values were due to one or more mouse from the control group. The reasons for the few abnormal results in the control group are unknown but suggest that they are not due to C7-HHQ.

Accomplishments and Conclusion:
The single 6.5 mg/kg s.c. dose or 12.5 mg/kg i.p. dose are well tolerated and show no evidence of causing systemic toxicity. The modified s.c. vehicle control (5% Tween-80, 5% PEG_{400}, 66% DMSO) used with 50 mg/kg s.c. dose *may* be associated with some platelet and liver enzyme abnormalities but the data remains inconclusive since these abnormalities were not seen in the 50 mg/kg s.c. C7-HHQ group. Nonetheless, care was taken to ensure that the mice were carefully monitored when the high dose of C7-HHQ was administered.

MAJOR TASK 2. INVESTIGATE THE ADJUVANT ACTIVITY IF HAQ IN SUB-LETHAL PA MURINE SKIN ABSCESS

SUBTASK 2.1: DEVELOPMENT OF A PA SKIN ASBCESS INFECTION MODEL

Our goal was to develop and validate a murine model of sub-lethal PA skin abscess infection based on a model recently published by Pletzer *et al* (mBio 2017). We used the *P. aeruginosa* strain PAO1 ΔSR which causes a localized non-lethal infection that is relatively refractory to conventional antibacterial treatment and allows testing of antibacterial adjuvants. To facilitate continuous live monitoring of *P. aeruginosa* skin abscess infection, we first engineered a luciferase expressing strain **(**PAO1 ΔSR::PrpsL-lux**)** which carries a chromosomally integrated highly expressed bacterial luciferase cloned from *P. luminescens*. Infected mice could thus be imaged *in vivo* with quantification of *in situ* live bacteria at multiple time points without having to sacrifice animals. While luminescence was a useful means to monitor bacterial burden and track the efficacy of the treatment phase. Ultimately, the effectiveness of antibiotic therapy was confirmed by surgical removal of the abscess tissues and quantitated for viable bacteria. In brief, overnight bacteria were harvested, washed in 1xPBS and resuspended in sterile 1xPBS for an inoculum of $5x10^6$ cells in 50 µL injected s.c. in the lower back adjacent to the hip. Mice were immediately imaged with the Bruker In-Vivo extreme to validate the infection dose *in situ* and randomized to different treatment group.

We tested several inoculum ranging from $1x10^6$ to $2x10^8$ CFU per abscess and determined that the optimal infecting dose to be $5x10^6$ CFU per mouse. This inoculum conferred 100% survival and bacterial persistence (mean $3.5x10^6$ the CFU/abscess at day 7). We also validated the in vivo luminescence imaging for up to 9 days (Figure 30A) and correlated the in vivo luminescence intensity with viable bacterial count obtained by homogenization and plating of the abscess tissues. As shown in Figure 30B, there was an excellent correlation between the luminescence and viable bacteria in the abscess tissues.

SUBTASK 2.2: TESTING OF THE ADJUVANT ACTIVITY OF HAQ IN PA INFECTION

We first used the PAO1 ΔSR::PrpsL-lux, a strain highly susceptible to the C7-HHQ adjuvant effect *in vitro*. Based on our in vitro testing, C7-HHQ was given 2h prior to antibiotics in order to maximize its adjuvant activity.

C7-HHQ + gentamicin: Since gentamicin showed the most potent *in vitro* synergy with HAQ extract and synthetic C7-HHQ, we attempted to test it *in vivo* first. A single low dose of 0.5 or 2 mg/kg gentamicin was injected via i.p. 24 h after infection and no effect was found on the abscess bacteria burden compared to control (saline treatment) at day 3 post-infection (Figure 31). Next, we sought to increase the total antibiotic dose tested by increasing to two doses of 0.5 or 2 mg/kg gentamicin as well as multiple routes via i.p. (Figure 32A) or s.c. (Figure 32B) at 24 and 30 h after infection. Again we found no reduction in abscess bacterial burden compared to control (saline treatment) mice at 2 days (Figure 32A&B). The poor response to gentamicin may not be surprising given previous studies reporting that the drug penetrates poorly within infected tissues resulting in

levels ~30-40% of serum levels (Rubenstein Eur. J. Clin. Microbiol 1982). Based on the limited efficacy of gentamicin in this model, we decided not to further pursue testing with gentamicin.

C7-HHQ + ofloxacin: Since C7-HHQ potentiated ofloxacin *in vitro* and ofloxacin has much better penetration into abscess tissues than do aminoglycosides, we switched to testing C7-HHQ + ofloxacin combinations. We first tested and optimized several ofloxacin treatment regimen in order to establish a partially effective treatment where ofloxacin alone resulted in a modest reduction in the abscess bacterial load. A single 0.5 or 2 mg/kg i.p. dose of ofloxacin had no effect on the abscess bacterial load at day 3 p.i (Figure 33), leading us to further optimize the ofloxacin regimen to multiple daily doses based on the literature and our lab's experience. Antibiotic treatment was initiated 24h after inoculation in order to allow for the establishment of a mature abscess. We thus tested the following regimens: 1) daily 5 mg/kg i.p. ofloxacin +/- 50 mg/kg C7-HHQ s.c. or 2) daily 15 mg/kg i.p. ofloxacin +/- 50 mg/kg C7-HHQ s.c. Neither combination of C7-HHQ + ofloxacin was more effective at decreasing the viable bacterial load compared to ofloxacin alone (Figure 34A: P=0.5 and P=0.6 respectively), and measurements of the abscess lesion surface area were identical in all groups (Figure 34B: P=0.6 and P=0.7 respectively). Next, we tested p.o ofloxacin regimens of daily 2, 10 and 50 mg/kg p.o ofloxacin alone, as well as 1 and 5 mg/kg intra-abscess to identify a dose with partial efficacy. The intra-abscess dose was limited by ofloxacin solubility and limited volume of intra-abscess injection. We found that daily 2 and 10 mg/kg p.o ofloxacin regimens were ineffective at reducing bacterial burden after 4 days (Figure 35A). However, 50 mg/kg p.o and both 1 and 5 mg/kg intra-abscess ofloxacin alone reduced the bacterial load by $\Delta 2.3$, $\Delta 3.4$, and $\Delta 2.7$ $\log_{10}$ respectively compared to the saline control group (Figure 35A&B), leading us to test 50 mg/kg p.o ofloxacin +/- 50 mg/kg C7-HHQ (below).

While the intra-abscess ofloxacin regimens appeared to have a more robust effect in reducing bacterial burden, it proved to be technically challenging to administer multiple doses. For this reason, we chose to pursue the 50 mg/kg p.o. ofloxacin regimen in combination with C7-HHQ.

We also speculated that the tissue penetration of C7-HHQ may be limited and thus set out to test intra-abscess delivery of C7-HHQ and tested daily 50 mg/kg p.o. ofloxacin +/- 50 mg/kg C7-HHQ intra abscess (i.a.). In addition, we changed the timing of treatment and administered it starting at 2h rather than 24h post infection, reasoning that late treatment of a mature biofilm may be too challenging. We thus tested daily 50 mg/kg p.o ofloxacin +/- 50 mg/kg intra-abscess C7-HHQ s.c dosed at 2 and 24 h post infection. We observed a significant reduction in luminescence over two days post infection, without significant difference between both treatment groups (Figure 34C). Viable bacterial burden at day 3 p.i were identical for the two treatment groups and stratification of the groups by sex also showed no differences (Figure 34D).

Accomplishments and Conclusion:
We developed a *P.a.* skin abscess model and used a luminescent strain for *in vivo* monitoring of the bacterial burden. Concentrations of HHQ tested were limited by solubility and volume in each route of administration. We tested synthetic C7-HHQ with ofloxacin in multiple regimens through s.c., i.p. and p.o. route, concentrations and dosing regimen. Unfortunately, we were unable to demonstrate any additive or synergistic activity against the *P.a.* abscesses. Limited studies of gentamicin showed no antibacterial activity in the abscess model and was not further investigated.

MAJOR TASK 3: INVESTIGATE THE ADJUVANT ACTIVITY OF HAQ IN SA INFECTION

SUBTASK 3.1: DEVELOPMENT OF A SA SKIN ASBCESS INFECTION MODEL

A luciferase expressing strain *S. aureus* USA300 isolate was obtained from Roger Plaut from The U.S. Food and Drug Administration Center for Biologics Evaluation and Research (Plaut *et al* Plos One 2013). The infection was carried out in a manner similar to the *P. aeruginosa* infection. We tested several infection doses from $3x10^6$ to $5x10^7$ and ~$5x10^7$ CFU/mouse was chosen because it was associated with the greatest proportion of persistent infection (Figure 36), while most of the lower doses were spontaneously cleared by a high proportion of mice.

The *S.aureus* abscess infection was sub-lethal but required the higher dose due to comparatively low RLU/CFU compared to the *P. aeruginosa* infection and persisted for at least 4 days, while for lower doses bacterial clearance was more rapid. We validated that the viable bacterial load persistent at 4 days (avg $7.5x10^7$ CFU/mL). The *in vivo* luminescence monitoring showed persistent bioluminescence for up to 4 days post-infection (Figure 37A). Although in vivo luminescence was near or below the limit of detection after 4 days, its intensity nonetheless correlated very well (R^2= 0.9173) with viable bacterial counts simultaneously obtained by homogenization and plating of the abscess tissues at day 4 (Figure 37B). We noted that after day 4, although the CFU/abscess was equivalent to the starting dose, the luminescence declined over the same period suggesting that the bacteria were likely less metabolically active and thus producing less light. Thus we chose a 4 day endpoint for the *S.aureus* abscess model.

SUBTASK 3.2: TESTING OF THE ADJUVANT ACTIVITY OF HAQ IN SA INFECTION

We used the lux-tagged *S.aureus* USA 300 strain as described above. C7-HHQ was also given 2h prior to antibiotics in order to maximize its adjuvant activity and we tested the following combinations based on our in vitro results.

C7-HHQ + gentamicin: We first attempted to establish the gentamicin dose that would result in partial reduction of bacterial load. We thus tested two gentamicin doses of 5 or 15 mg/kg i.p. administered at daily 24 and 48 h post infection. Similar to results observed for *Pseudomonas* infections, neither gentamicin doses reduced the bacterial load compared to the saline control group (Figure 38A).

We then tested daily 100 mg/kg i.p. gentamicin (the highest tolerable dose) administered daily 24 and 48 h after infection +/- 50 mg/kg C7-HHQ or vehicle administered intra-abscess, and monitored mice for 3 days. We saw no statistically significant differences in luminescence over time (Figure 38B), nor in bacterial load by CFU plating of the abscess tissues at day 3 (Figure 38C) between the groups. There was a trend towards a smaller lesion size in the [C7-HHQ+gentamicin] group compared to gentamicin alone group, but this difference did not reach statistical significance (Figure 38D: P=0.08)

C7-HHQ + ofloxacin: We considered testing C7-HHQ + ofloxacin combinations but opted not to due to the observed relatively poor synergy in our in vitro assays.

Accomplishments and Conclusion:
An *S.a.* skin abscess model was developed and validated using a bioluminescent *S.a.* USA300 strain. *In vivo* luminescence monitoring was reliable up to 4 days post infection. Gentamicin alone at concentrations of 5 up to 100 mg/kg i.p. was insufficient to reduce *S. aureus* bacterial load in this model, and 50 mg/kg C7-HHQ in combination with 100 mg/kg i.p gentamicin showed no greater antibacterial activity against mature abscess infections. There was a trend towards decreased dermatonecrosis in the combination treatment compared to gentamicin alone, but this did not reach statistical significance.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Dr Geoffrey McKay (research associate) received training in the Deziel lab for LC/MS analysis, and training in animal handling and experimental animal procedures at the RI MUHC.

Dr McKay, Pierre Andre Casgrain and Dan Houle have received training on the Bruker Xtreme in vivo luminescence imaging at the RI MUHC SAIL (Small Animal Imaging Lbs).

Andrea Wang and Tiffany Leung (summer workers) were trained by Dr McKay in microbiological techniques.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not

usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

- Scientific dissemination at conferences: Oral presentation at International Caparica Conference in Antibiotic Resistance in Caparica, Portugal (June 2017).
- Community outreach: Participating mentor at the International Young Scientists Mentorship Program (IYSMP) in Montreal (Oct 2017)

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Nothing to report

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

This project has allowed us to develop and optimize highly sensitive and reproducible *in vitro* assays and animal infection models to test the adjuvant activity of candidate compounds. Although our results did not yield any compounds that display *in vivo* effectiveness, our work has established a successful pipeline that can take crude bacterial extracts, fractionate them into bioactive semi-purified compounds, identify candidate compounds, carry out comprehensive analysis for antibacterial activities and perform proof-of-concept *in vivo* testing. The construction of lux-tagged bacterial strains also allowed to develop and optimize an infection model well suited for testing of antibacterial therapies. The tools and expertise developed through this project will thus be readily transposable to other similar project to advance the discovery of antibacterials.

The discovery of novel and effective antibacterials is notoriously challenging, particularly for those targeting gram-negatives and drug resistant organisms. From our experience, we are poised to test other microbial sources of bioactive antibacterial through a complete suite of pre-clinical studies.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:
- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Our specific Aim 2 was to test the toxicity and efficacy of HAQ in combination with conventional antibiotics to improve bacterial eradication and mortality in murine models of severe *P. aeruginosa* and *S. aureus* infections. As reported in the annual report 2017, we modified the animal model tested and used a subcutaneous abscess model of *P. aeruginosa* and *S. aureus* infections. We also tested several route of administration of antibiotics and C7-HHQ (intra-peritoneal, subcutaneous and intra-abscess) as well as multiple antibiotic regimens (escalating dose, single dose vs daily dose, timing at 4h vs 24h post-infection). These approaches were made to overcome the anticipated problems for in vivo tolerability of intra-tracheal administration of C7-HHQ, problems with low solubility of C7-HHQ in aqueous solution and low bactericidal activity of gentamicin and ofloxacin in reducing bacterial burden in the abscess model.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

In our initial proposal, we planned to use acute models of PA and SA pneumonia, where HAQs are administered through the intra-tracheal route. However, our preliminary *in vitro* to date has revealed several HAQs are poorly soluble in aqueous solution. For example, C7-HHQ, our most promising HAQ to date, has very poor solubility in aqueous solution and requires at least 50% % MeOH or DMSO to achieve concentrations of at least 5 mg/mL.

Given that we are aiming to test *in vivo* doses of up to 50 mg/kg, the intra-tracheal administration of HAQ was no longer feasible optimal for these proof-of concept safety and efficacy studies. We were specifically concerned about the toxicity of DMSO, MeOH (or other carrier) and the excessive volume required to solubilize C7-HHQ. In order to address these problems, we have changed the animal model from an acute pneumonia model to a subcutaneous abscess model caused by either PA or SA, as described above. The subcutaneous infection model allowed up to administer C7-HHQ directly into the wound, this leading to higher tissue concentrations and the use of a wider range of solvents. Furthermore, we also used several solvent vehicles to improve the solubility of C7-HHQ, namely 5% Tween-80/ 5% PEG_{400}/5% DMSO and 5% Tween-80/5% PEG_{400}/66% DMSO.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Nothing to report

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals.

As described in the section above, the animal infection model proposed for Aim 2 Major Task 2 and 3 has been modified.

Significant changes in use of biohazards and/or select agents

Nothing to report

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to report

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title;*

editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to report

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

- Nguyen D. Martins D, McKay G. Targeting bacterial stress responses to overcome antibiotic tolerance. Presentation at the 2nd International Caparica Conference in Antibiotic Resistance, Caparica (Portugal), June 12-16 2017.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following informatin for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name:	*Dao Nguyen*
Project Role:	*PI*
Researcher Identifier	*ORCID ID* 0000-0003-4012-5441
Nearest person month worked:	*1.5*
Contribution to Project:	*Dr Nguyen has supervised all of the work carried out in the Nguyen lab and has coordinated the work done in the Deziel lab.*
Funding Support:	*N/A*
Name:	*Geoffrey McKay*
Project Role:	*Research associate*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*18*
Contribution to Project:	*Geoffrey McKay has carried experiments in Aim 1 (Major Tasks 2, 3, 4), managed administrative and logistical aspects of the lab work (e.g ordering and maintenance of lab supplies, biohazard permit approval) and supervised the work of Andrea and Tiffany.*
Funding Support:	*N/A*
Name:	*Daniel Houle*
Project Role:	*Animal technician*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*9*
Contribution to Project:	*Daniel Houle has been responsible for the development of the animal protocol, IACUC and ACURO approval, and has contributed to all experiments in Aim 2 (Major Tasks 1, 2 and 3).*
Funding Support:	*N/A*

Name:	*Annie Beauchamp*
Project Role:	*Animal technician*
Researcher Identifier	
Nearest person month worked:	*2.5*
Contribution to Project:	*Annie replaced Dan Houle and carried out the end of experiments in Aim 2 (Major Tasks 2 and 3).*
Funding Support:	*N/A*
Name:	*Pierre Andre Casgrain*
Project Role:	*Research assistant*
Researcher Identifier	
Nearest person month worked:	*2*
Contribution to Project:	*Pierre Andre contributed to experiments in Aim 2 (Major Tasks 2 and 3)..*
Funding Support:	*N/A*
Name:	*Andrea Wang*
Project Role:	*Summer worker*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*2*
Contribution to Project:	*Andrea Wang has contributed to experiments in Aim 1 (Major Tasks 3 and 4).*
Funding Support:	*N/A*
Name:	*Tiffany Leung*
Project Role:	*Summer worker*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*2*
Contribution to Project:	*Tiffany has contributed to experiments in Aim 1 (Major Tasks 3 and 4).*
Funding Support:	*N/A*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior

written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed. Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Organization Name: INRS Armand Frappier (Dr Eric Deziel, site 2)
Location of Organization: Laval, QC Canada
Partner's contribution to the project

- *Facilities: LCMS analysis of HAQ*
- *Services: HAQ synthesis*

7. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

8. APPENDICES:

Appendix 1

Final technical report

Discovery award
W81XWH-16-1-0097

January 2019

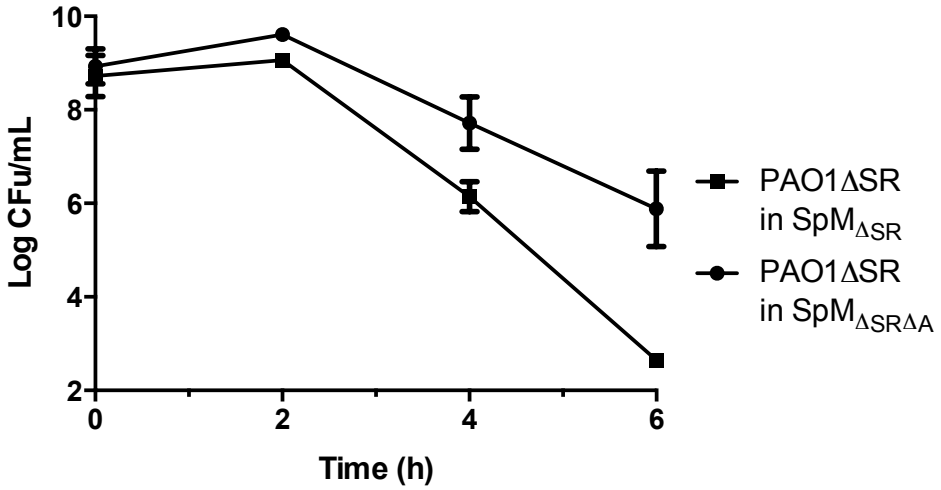


Fig 1. HAQ+ culture supernatants potentiate gentamicin killing of tolerant bacteria. Spent media produced by PAO1 ΔSR ($SpM_{ΔSR}$) but not PAO1 ΔSR ΔpqsA ($SpM_{ΔSRΔA}$) potentiate gentamicin killing of *P. aeruginosa* ΔSR cells. SpM was prepared by centrifugation and filtration of bacterial cultures of indicated strains grown in TSB for 24 h to recover the culture supernatants. Stationary phase cultures of PAO1 ΔSR from overnight cultures were harvested, re-suspended in spent media $SpM_{ΔSR}$ (HAQ+) or $SpM_{ΔSRΔA}$ (HAQ-) at a final OD600~2, then challenged with 50 µg/mL gentamicin for 6 h at 37 °C with shaking (250 RPM). Viable cell counts were measured over time by plating and colony forming units (CFU) counts. The results are presented as mean ± SEM of n=9 biological replicates from 3 independent experiments.

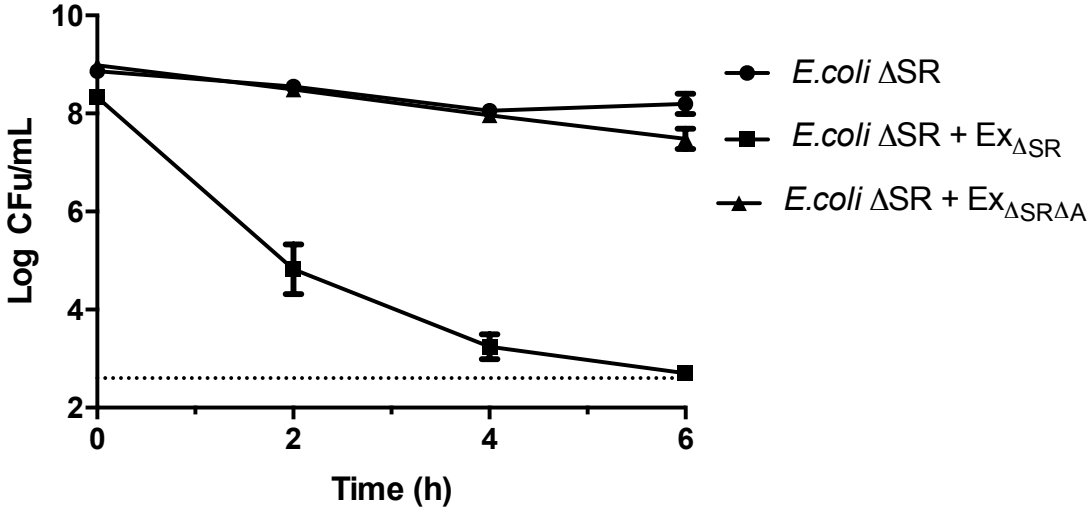


Fig 2. Adjuvant activity bioassay with *E. coli* indicator strain. Extracts of spent media produced by PAO1 ΔSR ($Ex_{ΔSR}$) but not PAO1 ΔSR ΔpqsA ($Ex_{ΔSRΔA}$) sensitize *E. coli* ΔSR to gentamicin. Stationary phase cultures of *E.coli* ΔSR (OD600~3) were pre-incubated for 2 h in the presence of extracts derived from spent media $SpM_{ΔSR}$ ($Ex_{ΔSR}$) or $SpM_{ΔSRΔA}$ ($Ex_{ΔSRΔA}$), while control cells were pre-incubated with methanol alone (solvent control). Bacteria were challenged with 50 µg/mL gentamicin for 6 h at 37 °C with shaking (250 RPM). Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM of n=6 biological replicates from 2 independent experiments.

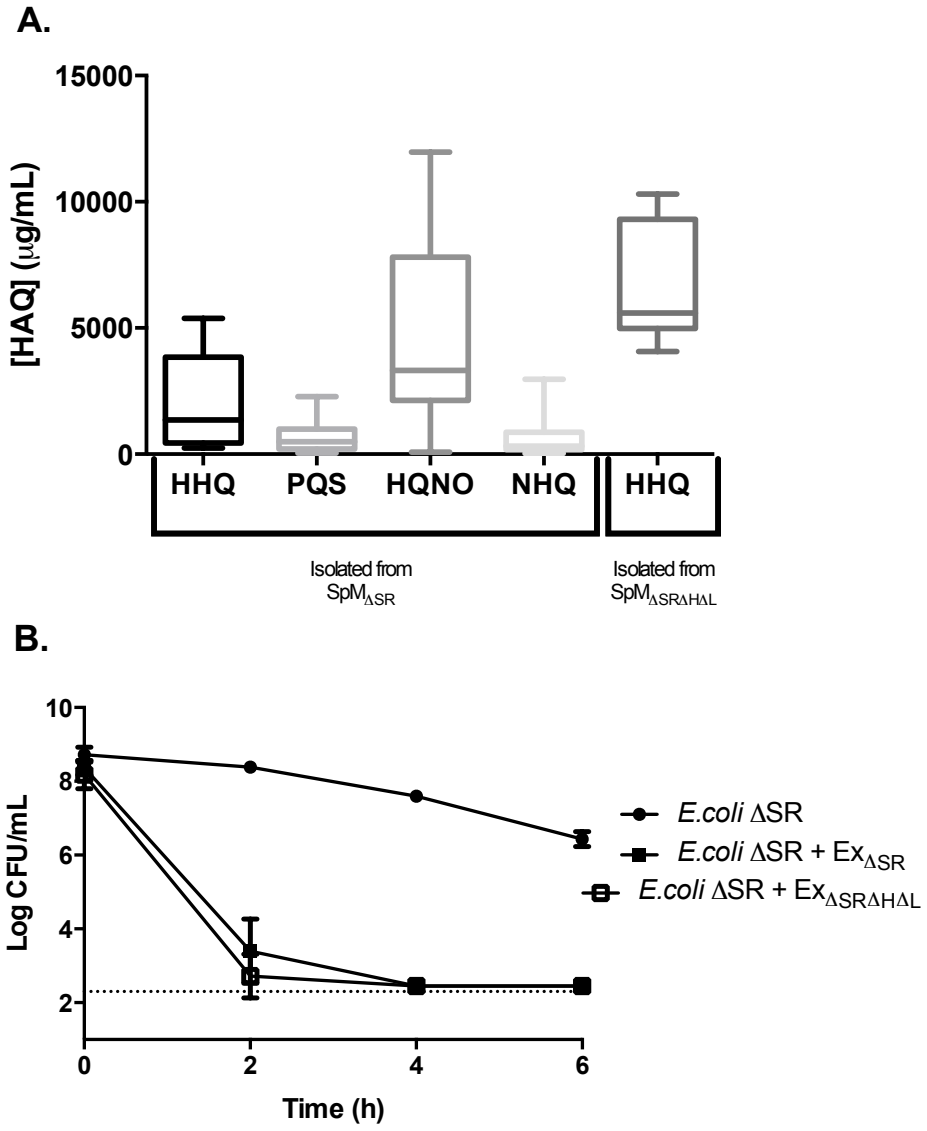


Fig 3. Quantification and comparison of HAQ in Extracts of different *P. aeruginosa* mutants. Characterization of ethyl acetate extracts of SpMΔSR (ExΔSR) and SpMΔSRΔHΔL (ExΔSRΔHΔL) demonstrates significantly different chemical profiles.

(A) LC/MS quantification of C7HHQ, PQS, HQNO and NHQ (C9-HHQ) in Ex$_{\Delta SR}$ and Ex$_{\Delta SR\Delta H\Delta L}$. Data from 9 independent batches (n=9 for Ex$_{\Delta SR}$, n=7 for Ex$_{\Delta SR\Delta H\Delta L}$) are plotted as mean and range.

(B) Extracts of spent media produced by PAO1 ΔSR (Ex$_{\Delta SR}$) and PAO1 ΔSRΔHΔL (Ex$_{\Delta SR\Delta H\Delta L}$) are biologically equivalent at sensitizing *E.coli* ΔSR to gentamicin. Stationary phase overnight cultures of *E.coli* ΔSR were pre-inculated with SpM$_{\Delta SR}$ (Ex$_{\Delta SR}$), SpM$_{\Delta SR\Delta H\Delta L}$ (Ex$_{\Delta SR\Delta H\Delta L}$) or methanol control, then challenged with 50 μg/mL gentamicin for 6 h at 37 °C with shaking (250 RPM) as done in Fig 2. The results are presented as mean ± SEM of n=6 biological replicates from 2 independent experiments.

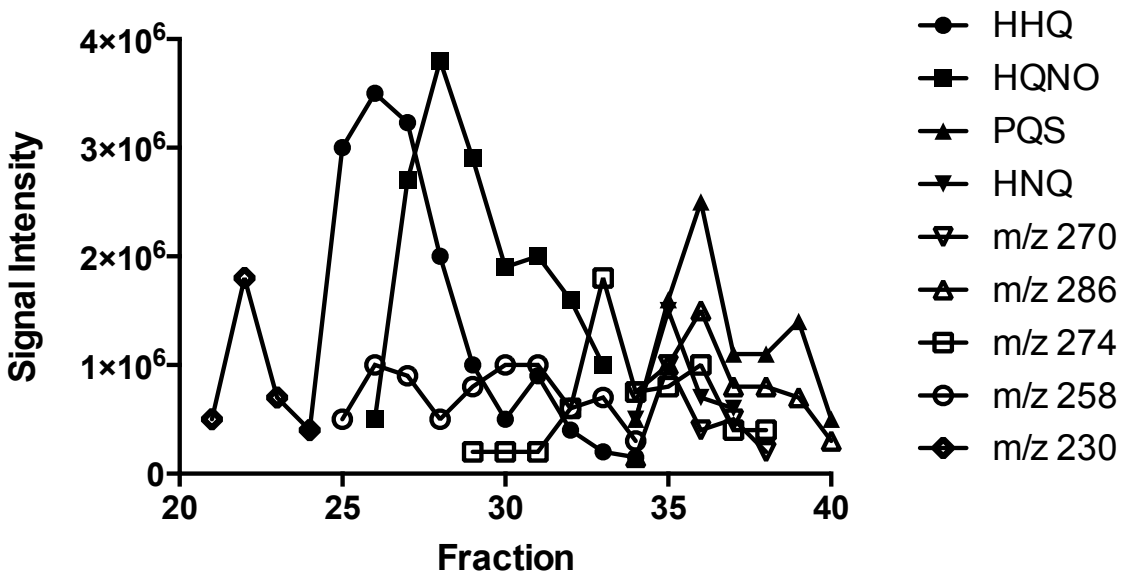


Fig 4. Fractionation and purification of $Ex_{\Delta SR}$ by HPLC and HAQ measurements by LC/MS. Extract was isolated from 2 L of spent media $SpM_{\Delta SR}$ by ethyl acetate extraction followed by drying in a rotary evaporator. The chemical material was resolubilized in 1 mL of LC/MS grade methanol and separated by reverse-phase HPLC (Waters Alliance e2695 separation system, semi-preparative XSelect CSH C18 5 µm 10 × 100 mm column) using a linear gradient of 100% solvent A (0.05% v/w TFA in water) to 50% of solvent B (0.05% v/w TFA in acetonitrile) over 4min, followed by an isocratic run for 1min and a linear gradient to 100% B over 4 min at a flow rate of 4ml/min. Compounds were quantitated and m/z identified by Orbitrap analysis. Fractions were collected and lyophilized.

SIGMA-ALDRICH®
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3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
HHQ - ≥98% (HPLC)

Product Number:	**SML0747**
Batch Number:	**083M4735V**
Brand:	SIGMA
CAS Number:	40522-46-1
Formula:	C16H21NO
Formula Weight:	243.34 g/mol
Storage Temperature:	Store at 2 - 8 °C
Quality Release Date:	30 AUG 2013

Test	Specification	Result
Appearance (Color)	White to Beige	White
Appearance (Form)	Powder	Powder
Elemental Composition C16H21NO	Pass	Pass
Purity (HPLC)	> 98 %	100 %
Identity	Confirmed	Confirmed

Brendan Nye, QC Team Leader
Quality Control
Natick, Massachusetts US

Fig 5A. Certificate of analysis for commercial synthetic HHQ from Sigma-Aldrich.

SIGMA-ALDRICH®

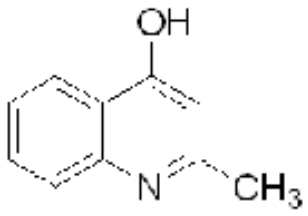
sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Product Specification

Product Name:
4-Hydroxy-2-methylquinazoline - 98.5%

Product Number:	**H43806**
CAS Number:	607-67-0
MDL:	MFCD00006758
Formula:	C10H9NO
Formula Weight:	159.18 g/mol



TEST	Specification
Appearance (Color)	Conforms to Requirements
Yellow to Pink to Tan	
Appearance (Form)	Conforms to Requirements
Powder and/or Lumps	
Infrared spectrum	Conforms to Structure
Melting point by DSC	
Specification: Report Result	
Carbon	74.3 - 76.6 %
Nitrogen	8.5 - 9.1 %
Purity (HPLC)	≥ 98.45 %

Specification: PRD.0.ZQ5.10000014817

Fig 5B. Certificate of analysis for commercial synthetic HHQ from Sigma-Aldrich.

CERTIFICATE of ANALYSIS

HQNO

2-heptyl-4-quinolinol 1-oxide

Item No. 15159 • Batch No. 0492395

Purity Specification: ≥98%
Molecular Formula : C16H21NO2
CAS Number: 341-88-8 Formula Weight : 259.3 Expiry date: 11/3/2018

Tests	Specifications		Results
HPLC	Column	: Gemini C18, 5µ, 4.6 x 250mm	
	Mob. Phase.....	: Methanol:Water:Acetic acid (80:20:0.1)	
	Flow Rate.....	: 1.00 ml/min	Retention Time Actual.: 7.40 min
	Detector UV	: 210 nm	Purity..... : 100.0 %
IR		Conforms with standard	Conforms
Mass	Source Type	: ESI	Expected Actual
		MH+	260.4 260.3
Melting Point	Expected.....	: 153 - 163 ºC	Actual ..... : 154 - 161 ºC
TLC	Mob. Phase.....	: Dichloromethane:Methanol (90:10)	
	Stationary Phase	: Silica Gel	Rf ..... : 0.49
	Visualized With ...	: UV	Purity %..... : 100
UV	λ max	: 330, 242, 214 nm	λ max ..... : 328, 241, 215 nm

Analyzed by: Lauren Cochran

Approved by: Jennifer LaBrecque

WARNING
THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

SAFETY DATA
This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

WARRANTY AND LIMITATION OF REMEDY
Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.

CAYMAN CHEMICAL
1180 EAST ELLSWORTH RD
ANN ARBOR, MI 48108 · USA
PHONE: [800] 364-9897
[734] 971-3335
FAX: [734] 971-3640
CUSTSERV@CAYMANCHEM.COM
WWW.CAYMANCHEM.COM

Fig 6. Certificate of analysis for commercial synthetic HQNO from Cayman Chemicals.

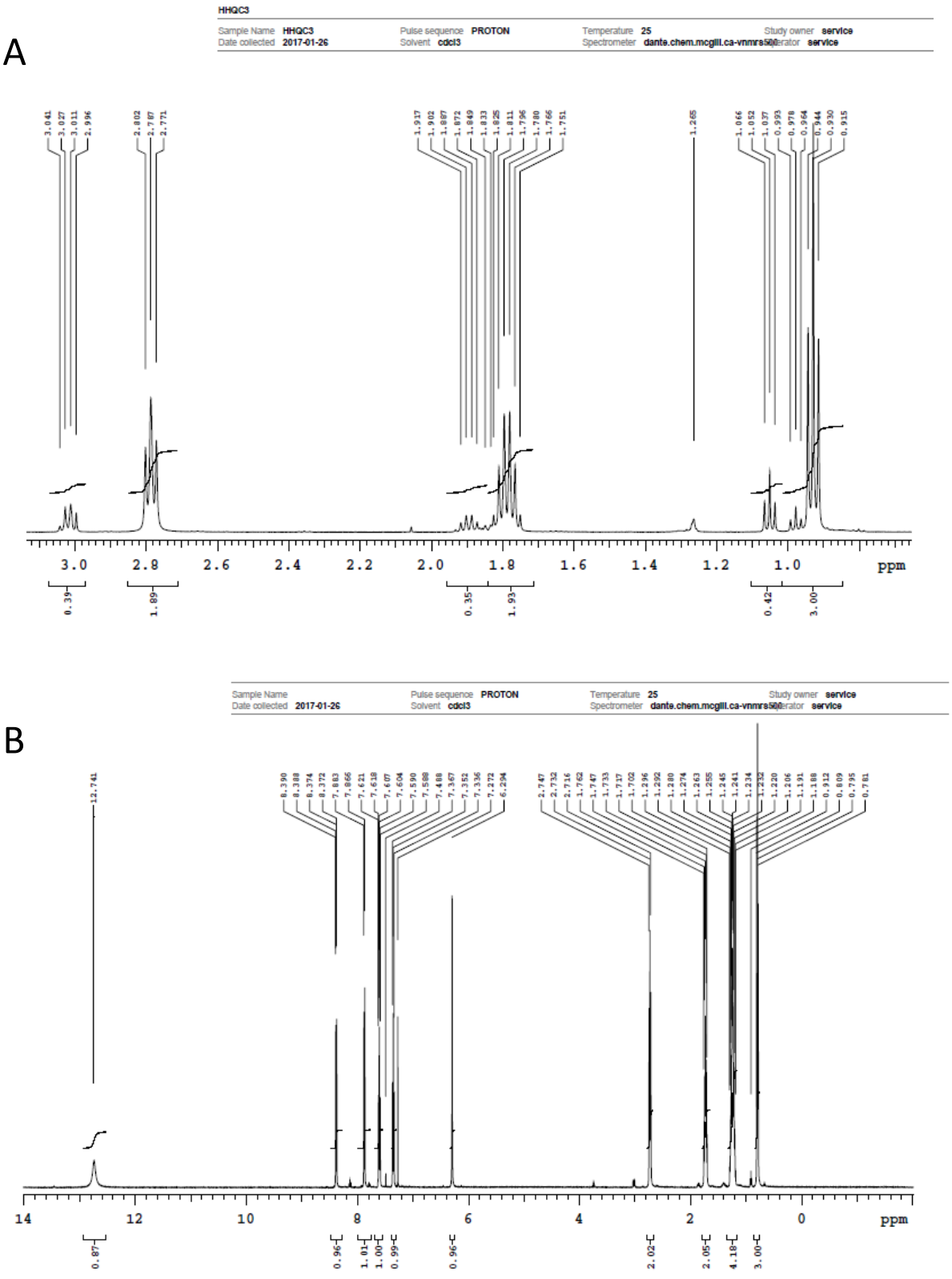


Fig 7. The ^{1}H NMR spectra of synthetic C3-HHQ and C5-HHQ. Synthetic purified C3-HHQ (A) and C5-HHQ (B) were characterized by proton NMR.

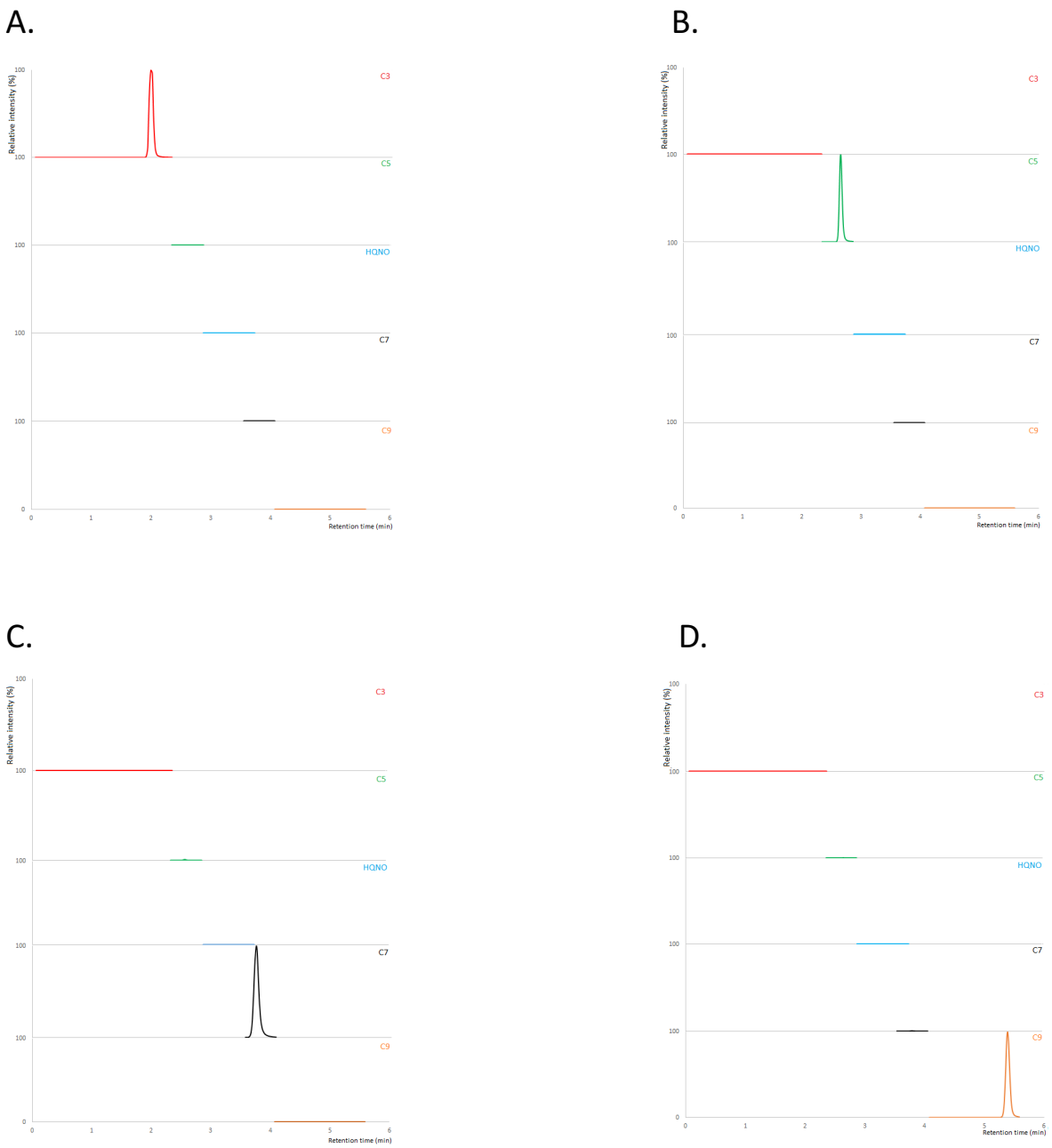


Fig 8. LC/MS chromatogram of C3-HHQ, C5-HHQ, C7-HHQ and C9-HHQ. Samples of C3-, C5-, C7- and C9-HHQ were dissolved in 1:1 methanol:H_2O at 0.1 ppm. Samples (10 µL) were injected, separated on an Agilent C8 reverse phase column and subjected to MRM analysis. Samples were run in triplicate and a representative chromatogram is presented for **(A)** C3-HHQ, **(B)** C5-HHQ, **(C)** HHQ from Sigma and **(D)** C9-HHQ.

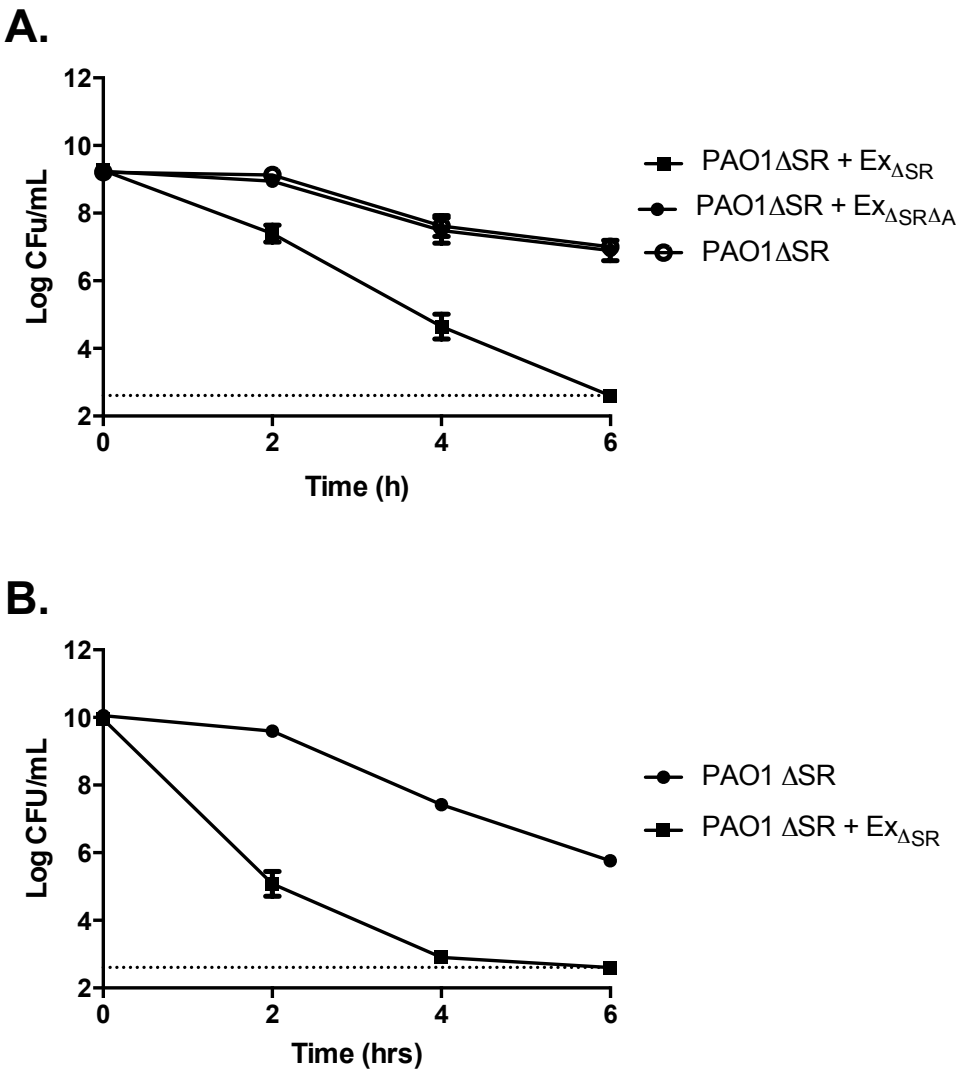


Fig 9. Adjuvant activity of $Ex_{ΔSR}$ in combination with different classes of bactericidal antibiotics against *P. aeruginosa*. Extracts from ΔSR ($Ex_{ΔSR}$) but not ΔSR ΔpqsA ($Ex_{ΔSRΔA}$) sensitize *P. aeruginosa* ΔSR to distinct classes of bactericidal antibiotics. Stationary phase cultures of PAO1 ΔSR (OD600~3) were pre-incubated $Ex_{ΔSR}$ (HAQ+) and compared to the HAQ- extract $Ex_{ΔSRΔA}$ or methanol control as done in Fig 2. Bacteria were then challenged with **(A)** 50 µg/mL gentamicin or **(B)** 5 µg/mL ofloxacin for 6 h at 37 °C with shaking (250 RPM. Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM of n=18 biological replicates from 6 independent experiments.

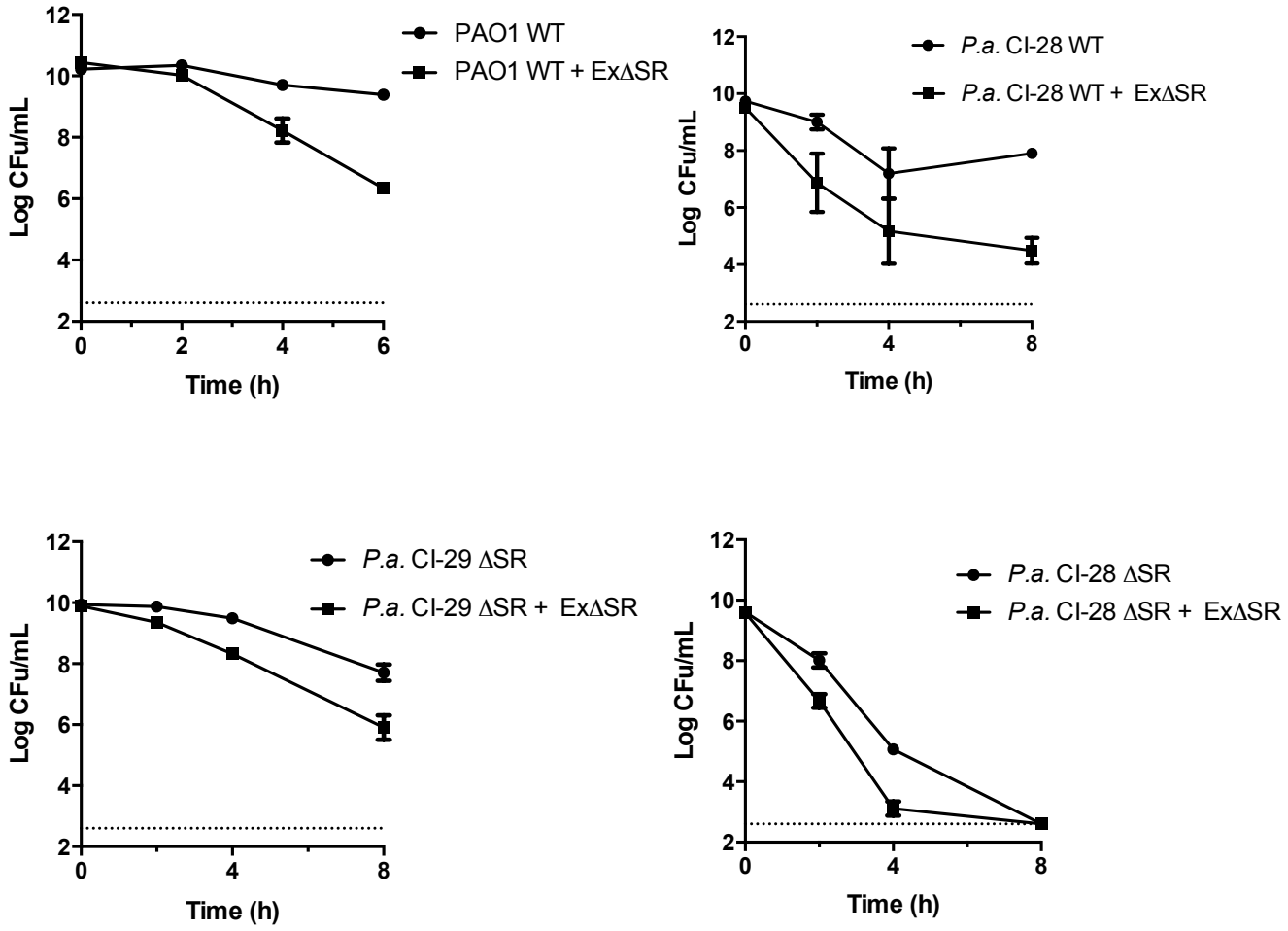


Fig 10. Adjuvant activity of $Ex_{\Delta SR}$ in different drug susceptible *P. aeruginosa* strains. The $Ex_{\Delta SR}$ extracts potentiate gentamicin killing of *P. aeruginosa* wild type (WT) and clinical isolates (CI). The killing assays were done as in Fig 1. Bacteria were pre-incubated with $Ex_{\Delta SR}$ or methanol control, then challenged with 50 µg/mL gentamicin for 6 h at 37 °C with shaking (250 RPM). Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM of n=9 biological replicates from 3 independent experiments.

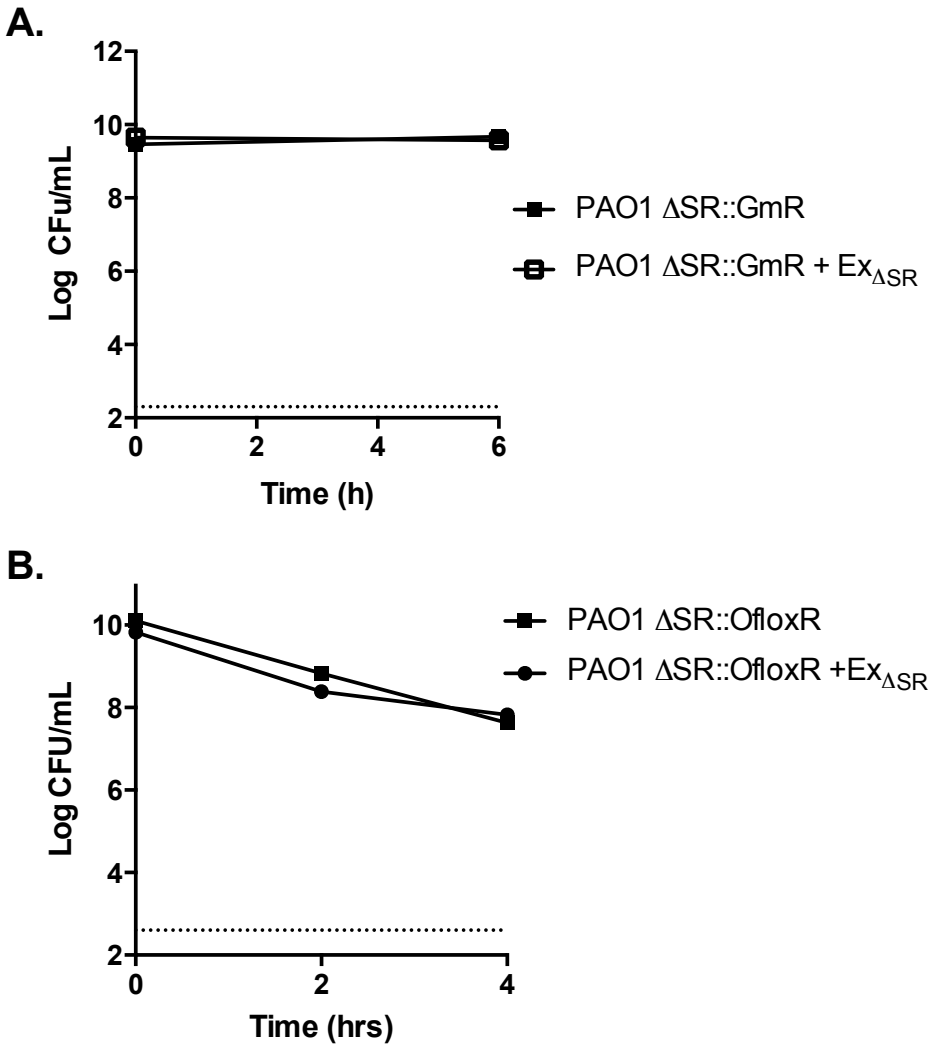


Fig 11. $Ex_{\Delta SR}$ does not potentiate gentamicin and ofloxacin in drug resistant *P. aeruginosa* strains. We tested the gentamicin resistant (Gm-R) strain PAO1 ΔSR::*aaC1* and the ofloxacin resistant strain PAO1 ΔSR-OfloxR. The killing assays were done as in Fig 2. Bacteria were pre-incubated with $Ex_{\Delta SR}$ or methanol control, then challenged with **(A)** 50 μg/mL gentamicin or **(B)** 50 μg/mL ofloxacin for 6 h at 37 °C with shaking (250 RPM). Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SD of n=3 biological replicates, representative of 3 independent experiments.

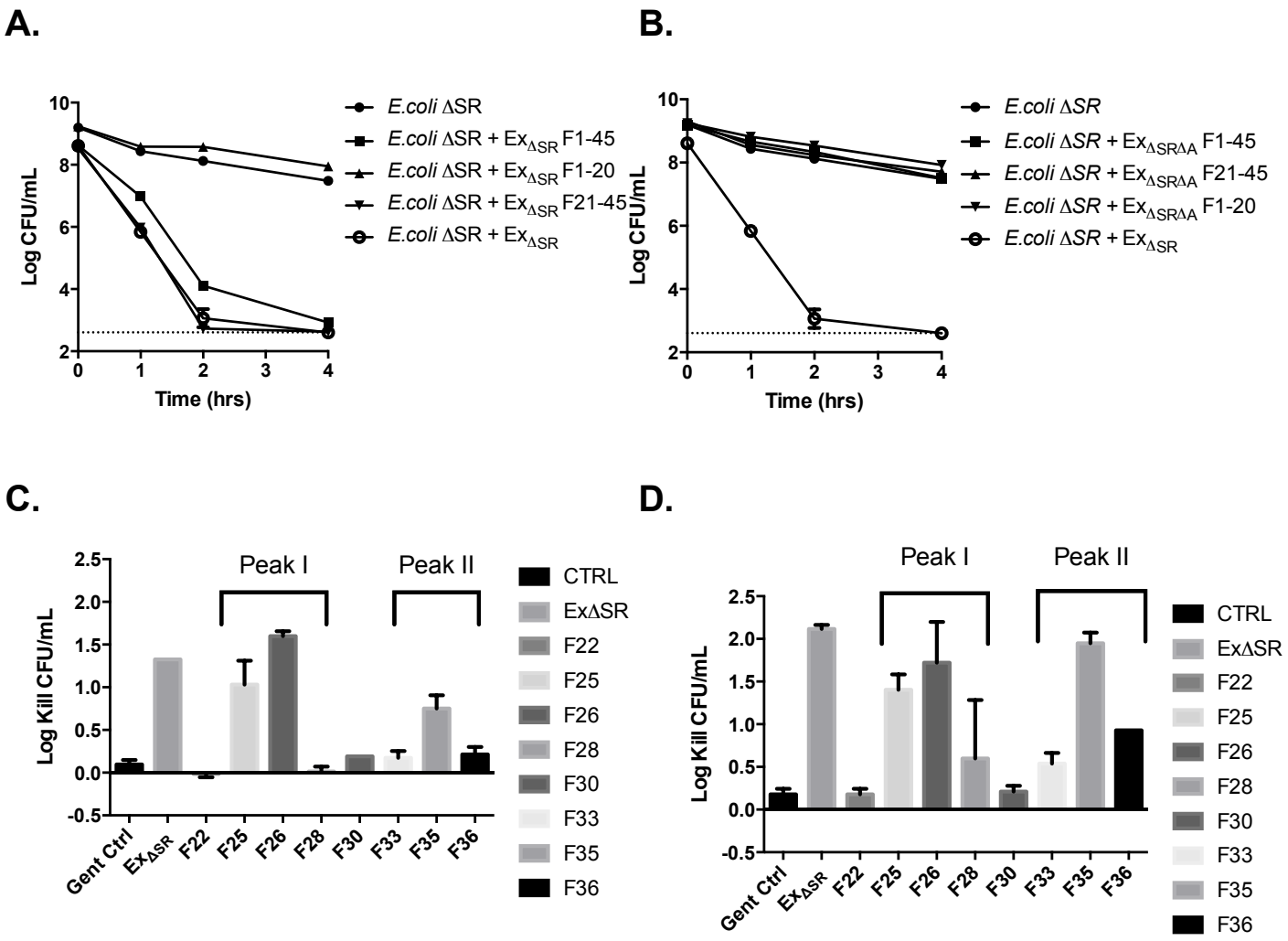


Fig 12. Adjuvant activity of $Ex_{\Delta SR}$ fractions. HPLC fractionation of **(A)** $Ex_{\Delta SR}$ retains adjuvant activity against the indicator strain *E.coli* ΔSR but not **(B)** $Ex_{\Delta SR\Delta A}$. The killing assays were done as in Fig 2. Bacteria were pre-incubated with pooled $Ex_{\Delta SR}$ or $Ex_{\Delta SR\Delta A}$ fractions. or methanol control, then challenged with 50 µg/mL gentamicin for 4 h. With testing of the individual fractions (C & D), two peaks I and II that retain adjuvant activity in combination with gentamicin against the indicator strain *E.coli* ΔSR. Bacteria were pre-incubated with the specified fractions or methanol control for 2 h, then challenged with 50 µg/mL gentamicin for **(C)** 30min or **(D)** 60 min at 37 °C with shaking (250 RPM). Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM of n=6 biological replicates from 2 independent experiments.

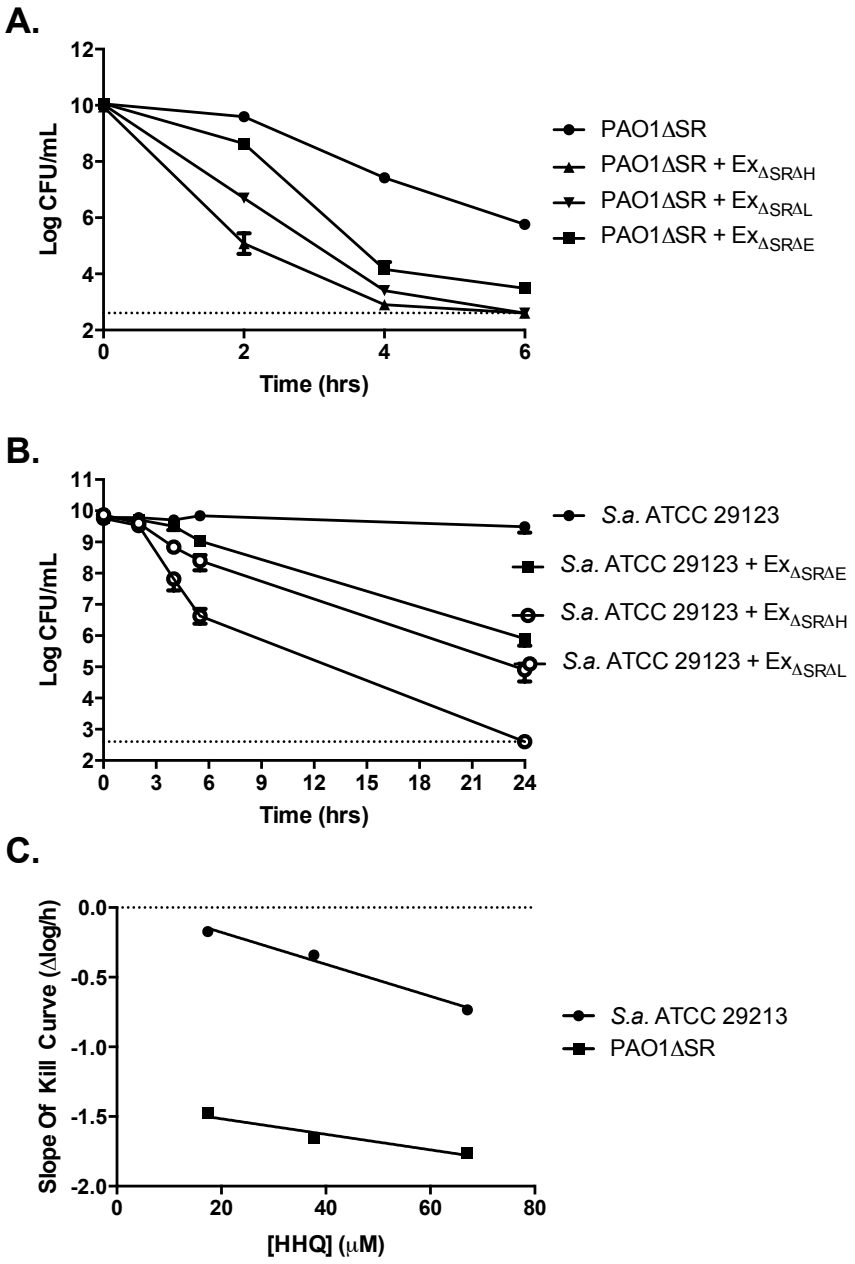


Fig 13. Adjuvant activity of extracts derived from different HAQ mutants and correlation between HHQ content and adjuvant activity. Different HAQ mutants generated extracts with different HAQ profiles, namely *Ex$_{ΔSRΔH}$* (67.1 µM C7-HHQ, no PQS, HQNO+), *Ex$_{ΔSRΔL}$* (37.7 µM C7-HHQ, PQS+, no HQNO) *and Ex$_{ΔSRΔE}$* (17.4 µM C7-HHQ, PQS+, HQNO+). The killing assays were done as in Fig 2. Stationary phase **(A)** *P. aeruginosa* PAO1 ΔSR and **(B)** *S. aureus* MSSA (ATCC 29213) were pre-incubated for 2h with the different extracts or methanol control, then challenged with 5 µg/mL ofloxacin or 50 µg/mL gentamicin respectively for 6h at 37°C with shaking (250 RPM). Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM. **(C)** The rate of bacterial killing was highly correlated with the extract C7-HHQ concentration as determined by LC/MS. The correlation was determined by linear regression and plotted versus C7-HHQ concentration.

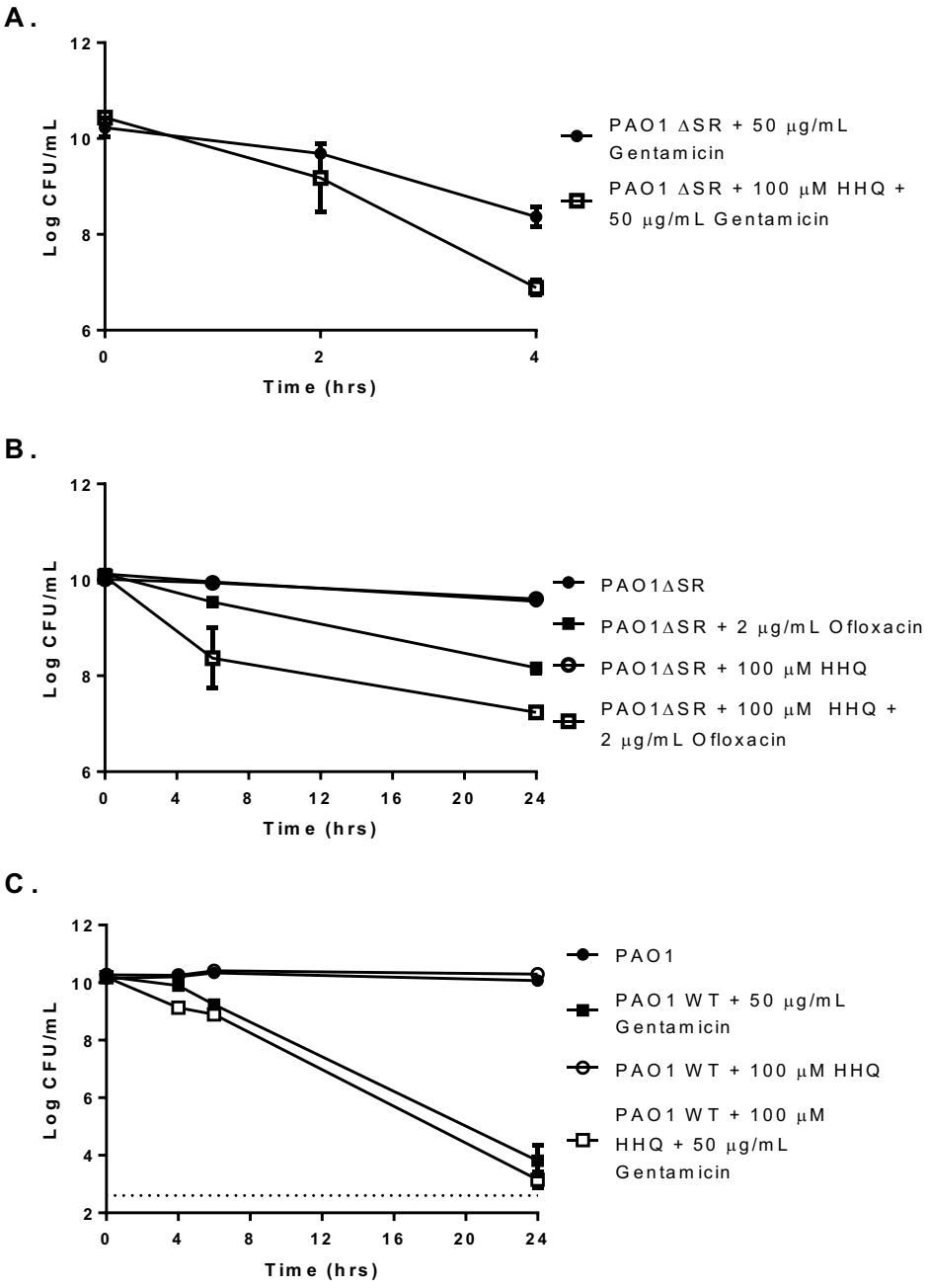


Fig 14. Adjuvant activity of synthetic C7-HHQ against *P. aeruginosa*. C7-HHQ sensitizes *P. aeruginosa* ΔSR to distinct bactericidal antibiotics, but has minimal adjuvant effects on wild-type PAO1 (WT) cells. The killing assays were done as in Fig 2. Bacterial cells were pre-incubated for 2 h with 100 μM synthetic C7-HHQ or DMSO controls, then challenged with (A) 50 μg/mL gentamicin **(B)** 2 μg/mL ofloxacin or **(C)** 50 μg/mL gentamicin for 24 h at 37 °C with shaking (250 RPM). Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM of n=6 biological replicates from 2 independent experiments.

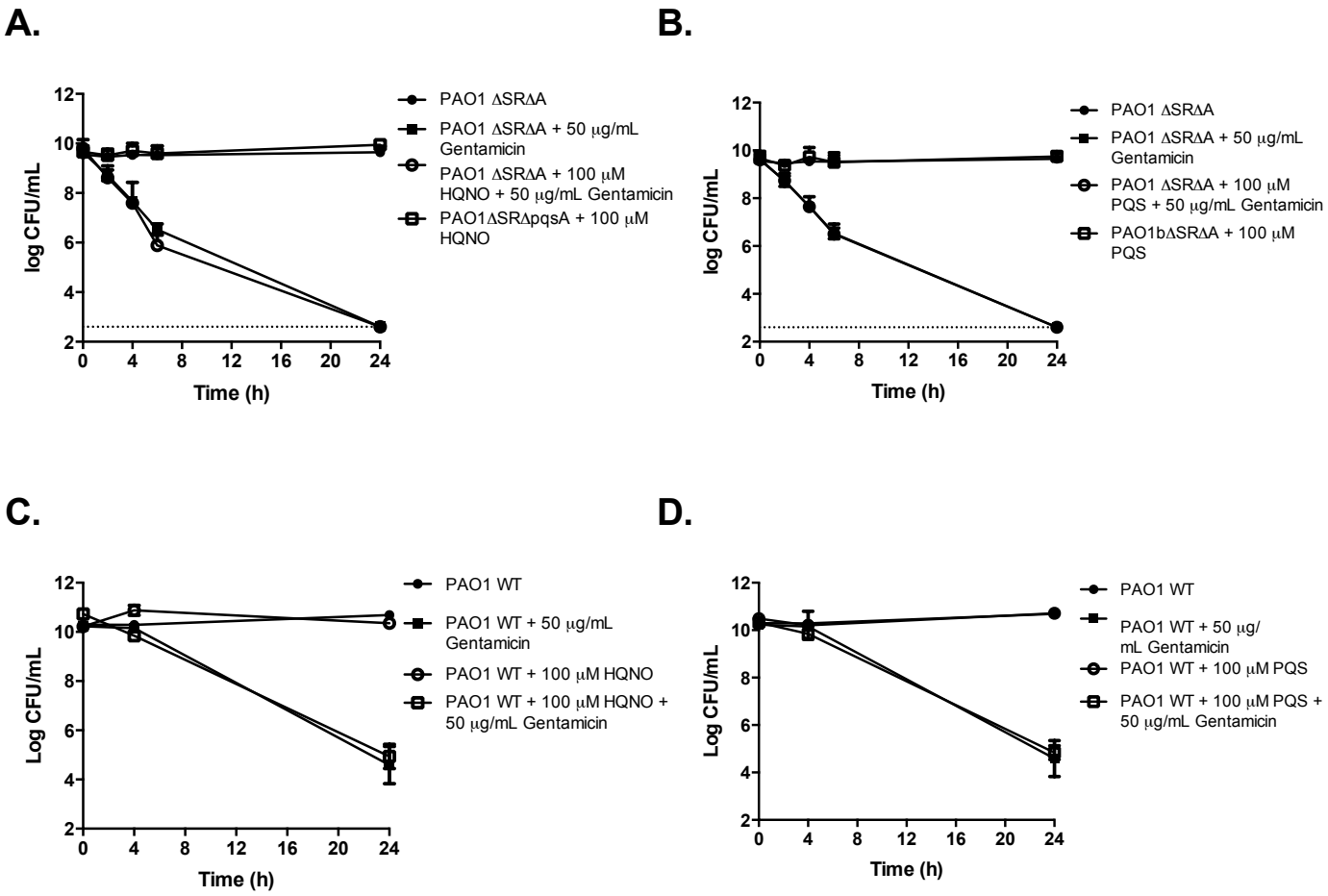


Fig 15. Synthetic C7-PQS and HQNO show no adjuvant activity against *P. aeruginosa.* Neither C7-PQS nor HQNO potentiated gentamicin killing against PAO1 ΔSR or WT. The killing assays were done as in Fig 2. Bacterial cells were pre-incubated for 2 h with 100 μM synthetic 100 μM C7-PQS **(B&D)**, 100 μM HQNO **(A&C)** or DMSO controls, then challenged with 50 μg/mL gentamicin for 24 h at 37ºC with shaking (250 RPM). Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM of n=6 biological replicates from 2 independent experiments.

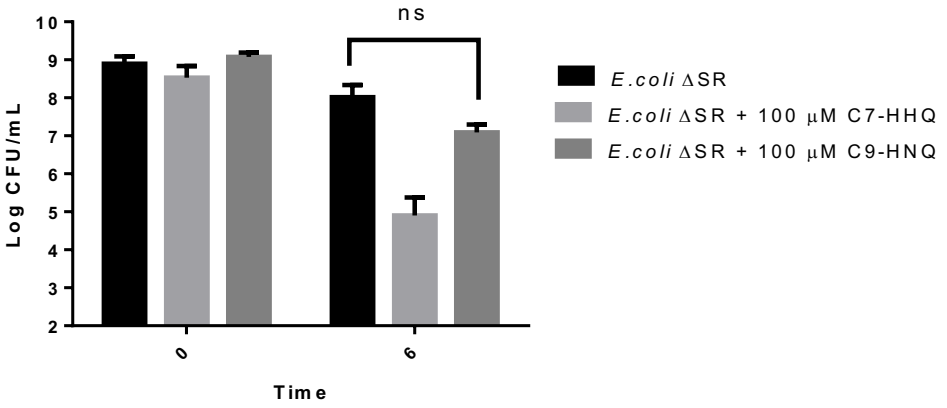


Fig 16. Synthetic C7-HHQ show adjuvant activity against *E. coli* but not C9-HHQ . C7-HHQ potentiated gentamicin killing against *E.coli* ΔSR but not C9-HHQ. The killing assays were done as in Fig 2. Bacterial cells were pre-incubated for 2 h with either 100 μM HHQ, 100 μM C9-HHQ or methanol controls, then challenged with 50 μg/mL ofloxacin for 6 h at 37 ºC with shaking (250 RPM). Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM of n=9 biological replicates from 3 independent experiments.

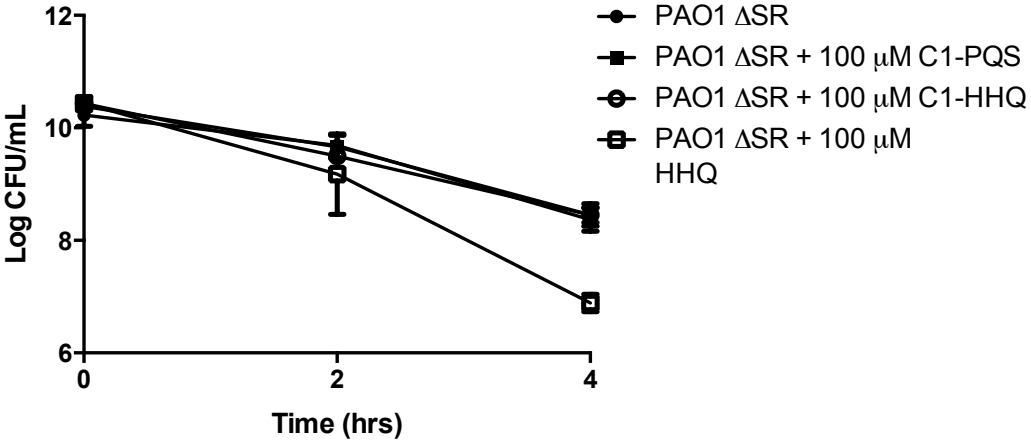


Fig 17. Synthetic C1-PQS and C1-HHQ show no adjuvant activity against *P. aeruginosa.*
Neither C1-PQS nor C1-HHQ potentiated gentamicin killing of PAO1 ΔSR. The killing assays were done as in Fig 2. Bacterial cells were pre-incubated for 2 h with 100 μM synthetic 100 μM C1-PQS, 100 μM C1-HHQ, 100 μM C7-HHQ or DMSO controls, then challenged with 50 μg/mL gentamicin for 4 h at 37ºC with shaking (250 RPM). Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SD of n=3 biological replicates from representative experiment.

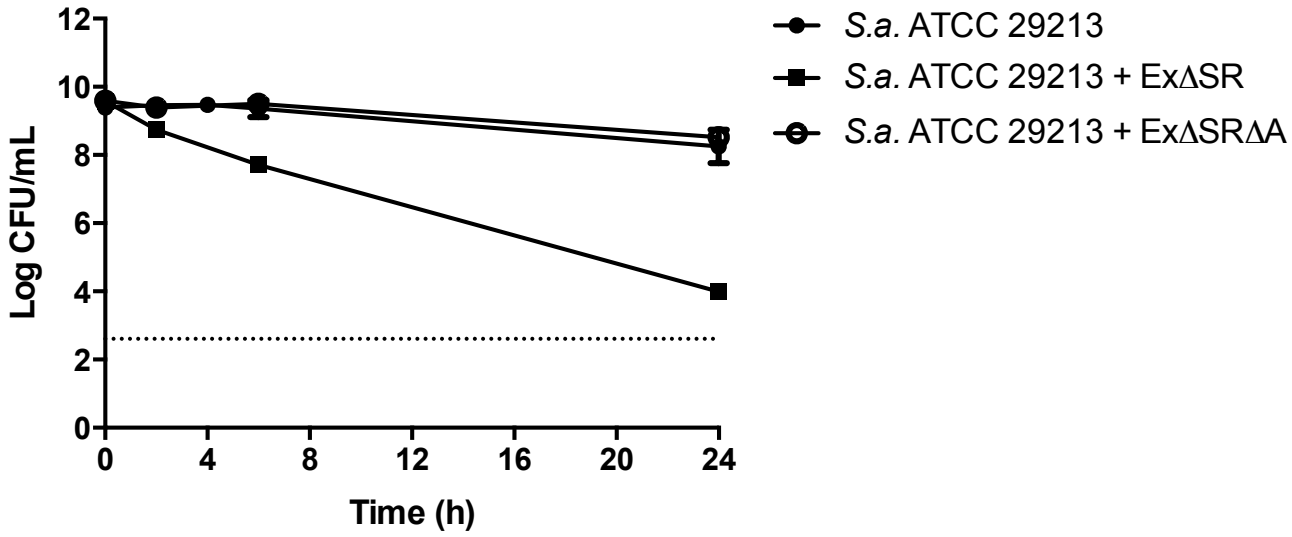


Fig 18. Adjuvant activity of HAQ+ extracts against *S. aureus.* The HAQ+ extract $Ex_{\Delta SR}$ potentiated gentamicin killing of *S. aureus* ATCC 29213 but not the HAQ- extracts $Ex_{\Delta SR\Delta A}$. The killing assays were done as in Fig 2. Bacterial cells were pre-incubated for 2 h with $Ex_{\Delta SR}$, $Ex_{\Delta SR\Delta A}$ or methanol controls, then challenged with 50 µg/mL gentamicin for 24 h at 37 °C with shaking (250 RPM). Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM of n=9 biological replicates from from 3 independent experiments.

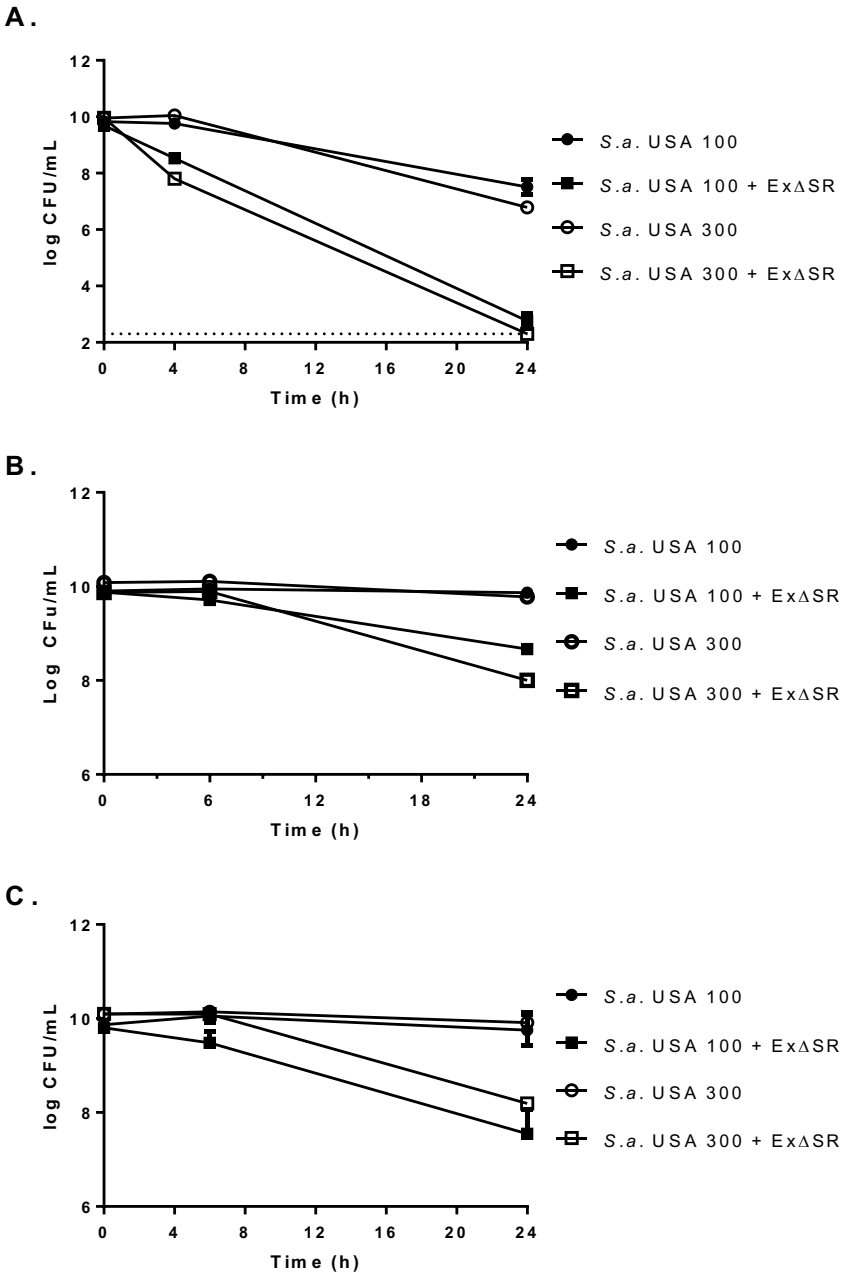


Fig 19. Adjuvant activity of $Ex_{\Delta SR}$ in combination with different classes of bactericidal antibiotics against methicillin resistant *S. aureus* (MRSA) strains. The HAQ+ extracts $Ex_{\Delta SR}$ potentiated killing of *S. aureus* CA-MRSA USA100 and USA300 by gentamcin, meropenem and oxacillin. The killing assays were done as in Fig 2. Bacterial cells were pre-incubated for 2 h with $Ex_{\Delta SR}$, or methanol controls, then challenged with **(A)** 50 µg/mL gentamicin, **(B)** 300 µg/mL meropenem and **(C)** 800 µg/mL oxacillin for 24 h at 37 ^{o}C with shaking (250 RPM). Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM of n=6 biological replicates from from 2 independent experiments.

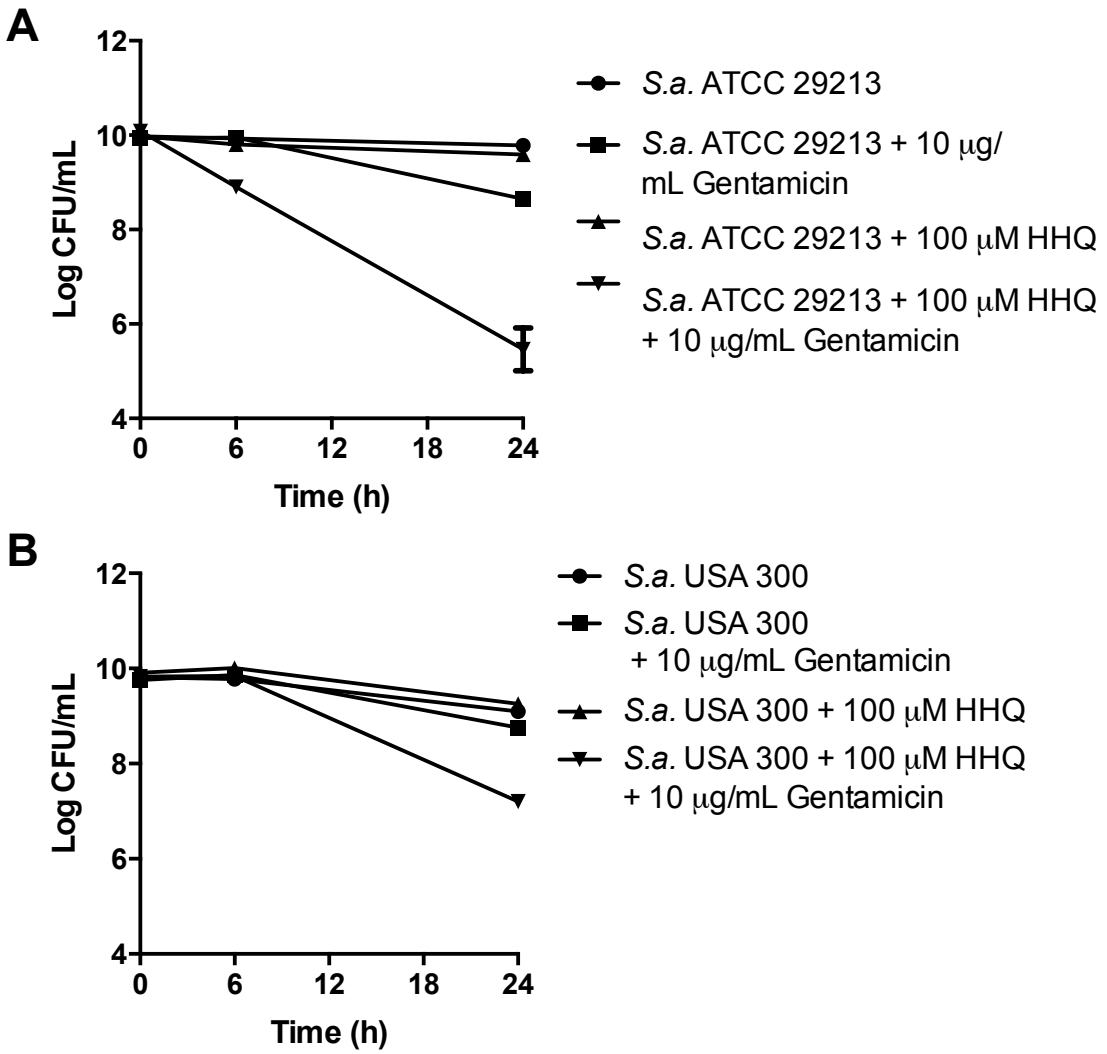


Fig 20. Adjuvant activity of synthetic C7-HHQ against *S. aureus*. C7-HHQ potentiated gentamicin killing of *S. aureus* MSSA **(A)** and CA-MRSA USA300 **(B)**. The killing assays were done as in Fig 2. Bacterial cells were pre-incubated for 2 h with with 100 μM HHQ or DMSO controls, then challenged with 10 μg/mL gentamicin for 24 h at 37 °C with shaking (250 RPM). Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM of n=6 biological replicates from from 2 independent experiments.

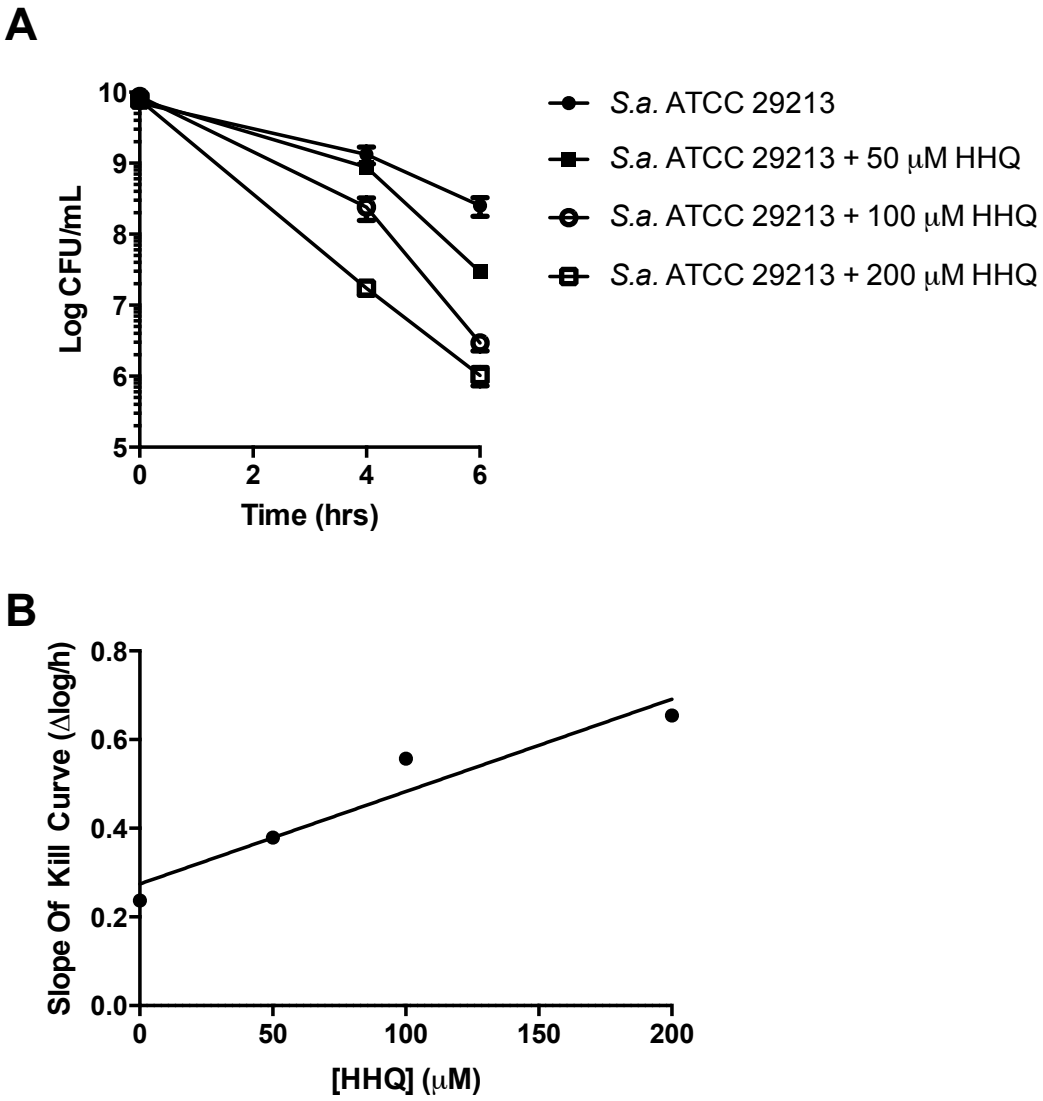


Fig 21. Adjuvant activity of synthetic C7-HHQ against *S. aureus* is dose dependent. C7-HHQ potentiates gentamicin killing of *S. aureus* MSSA (ATCC 29123) in a concentration-dependent manner. **(A)** The killing assays were done as in Fig 2. Bacterial cells were pre-incubated for 2 h with 50 μM, 100 μM or 200 μM C7-HHQ or DMSO control, then challenged with 50 μg/mL gentamicin for 24 h at 37 °C with shaking (250 RPM.) in a 96 well plate and cell viability was monitored over time by plating and colony forming units (CFU) count. Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM of n=6 biological replicates from from 2 independent experiments. **(B)** The rate of bacterial killing is correlated with C7-HHQ concentrations as measured by linear (R^2=0.88).

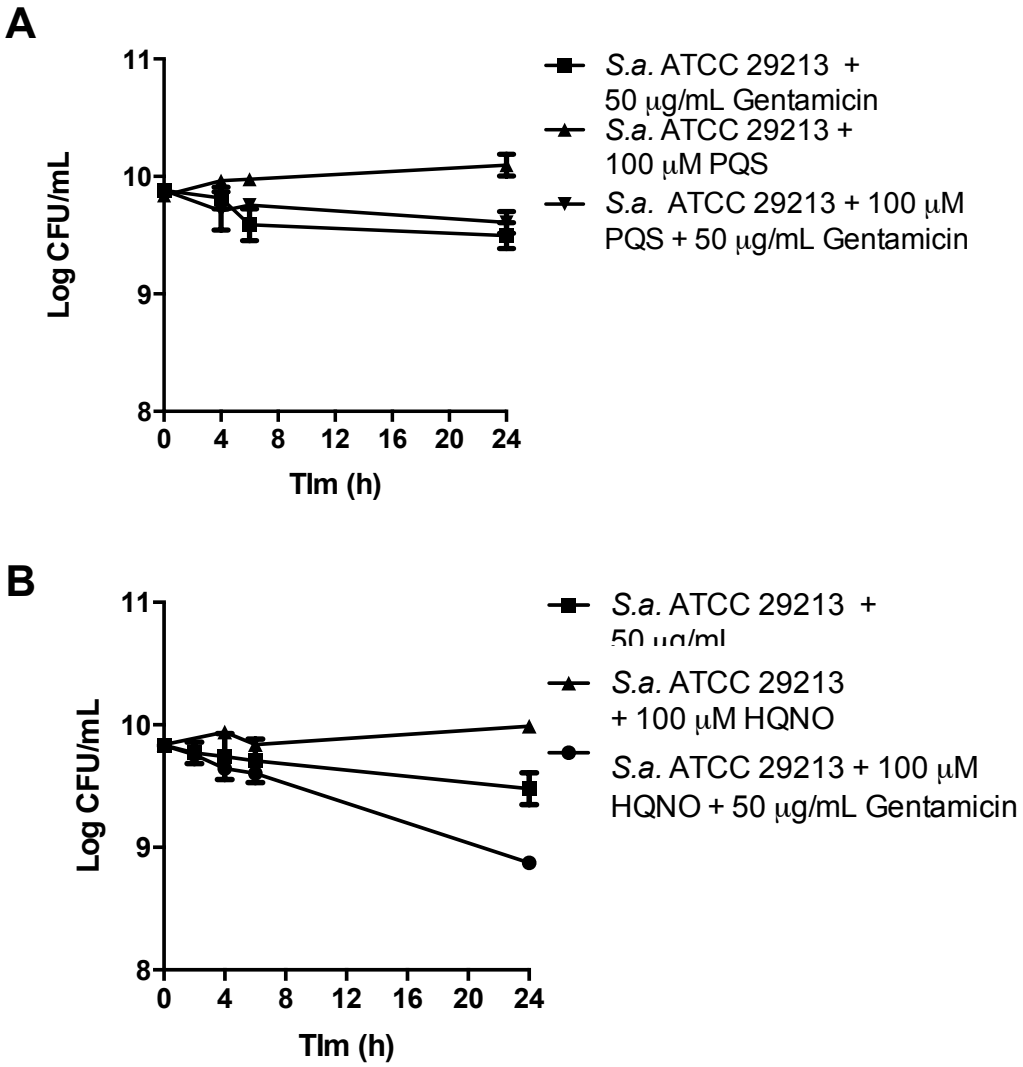


Fig 22. Synthetic C7-PQS and HQNO have no adjuvant activity against *S. aureus.* C7-PQS and HQNO do not potentiate gentamicin killing of *S. aureus* MSSA. The killing assays were done as in Fig 2. Bacterial cells were pre-incubated for 2 h with with **(A)** 100 μM C7-PQS, **(B)** 100 μM HQNO or DMSO control, then challenged with 50 μg/mL gentamicin for 24 h at 37 °C with shaking (250 RPM). Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM of n=6 biological replicates from from 2 independent experiments.

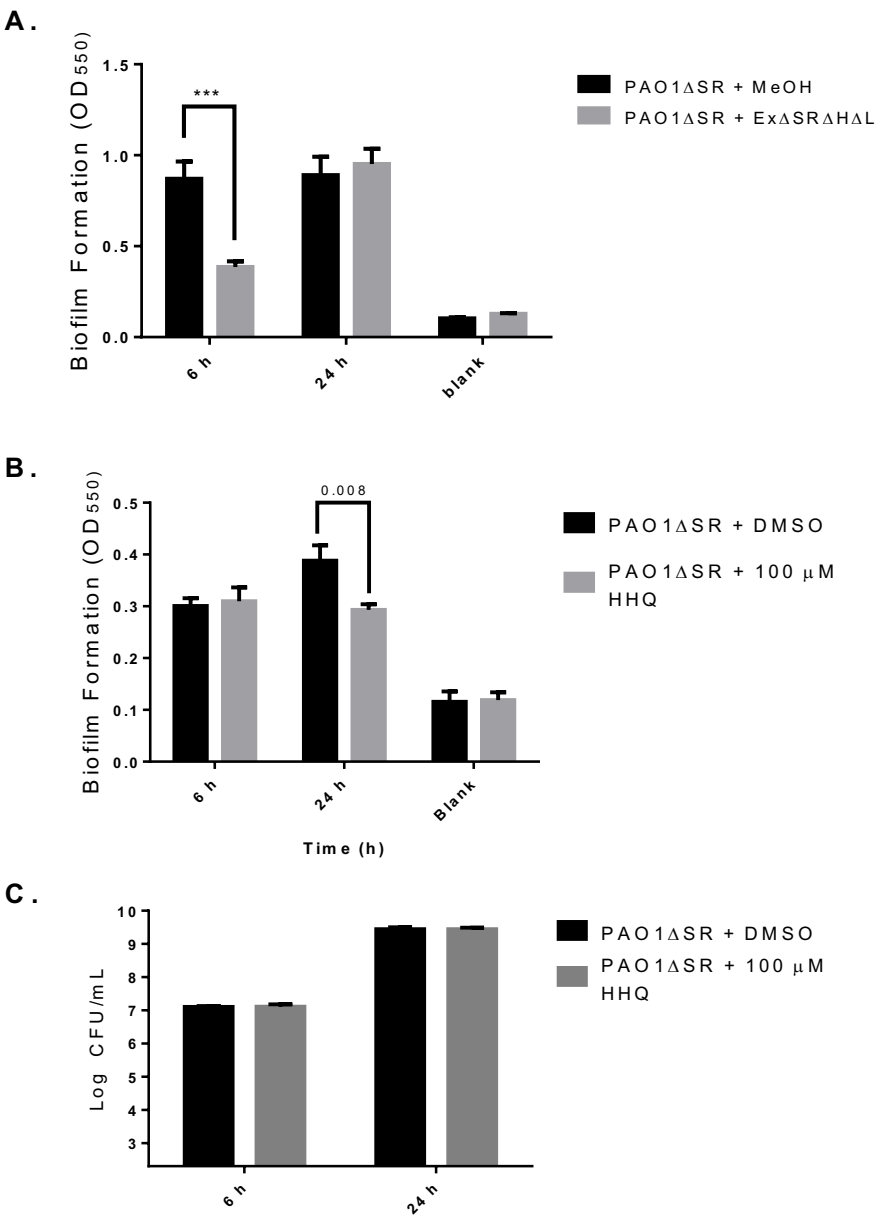


Fig 23. HAQ+ extract and synthetic C7-HHQ have a modest inhibitory effect on *P. aeruginosa* biofilm formation. Bacterial cells (PAO1 ΔSR 1x10^6 CFU/well) were used as inoculum for biofilm formation in a 96 well static biofilm model. Biofilms were grown in LB medium in the presence of **(A)** HAQ+ extract $Ex_{\Delta SR\Delta H\Delta L}$, **(B)** 100 µM C7-HHQ or control statically at 37 ^oC without shaking for 6h and 24h. Biofilm biomass was quantified by staining with 1% crystal violet using established protocols and measurement at OD_{550}. The results are presented as mean ± SEM of n=18 biological replicates from from 2 independent experiments.

(C) Biofilms were grown on polycarbonate filters as colony biofilms. After inoculation of 2.5x10^5 CFU/filter, biofilms were grown statically on LB agar plates supplemented with 100 µM HHQ or DMSO controls at 37 ^oC for 6h and 24h. At each time point, biofilm cells were resuspended in PBS and viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM from n=4 biological replicates.

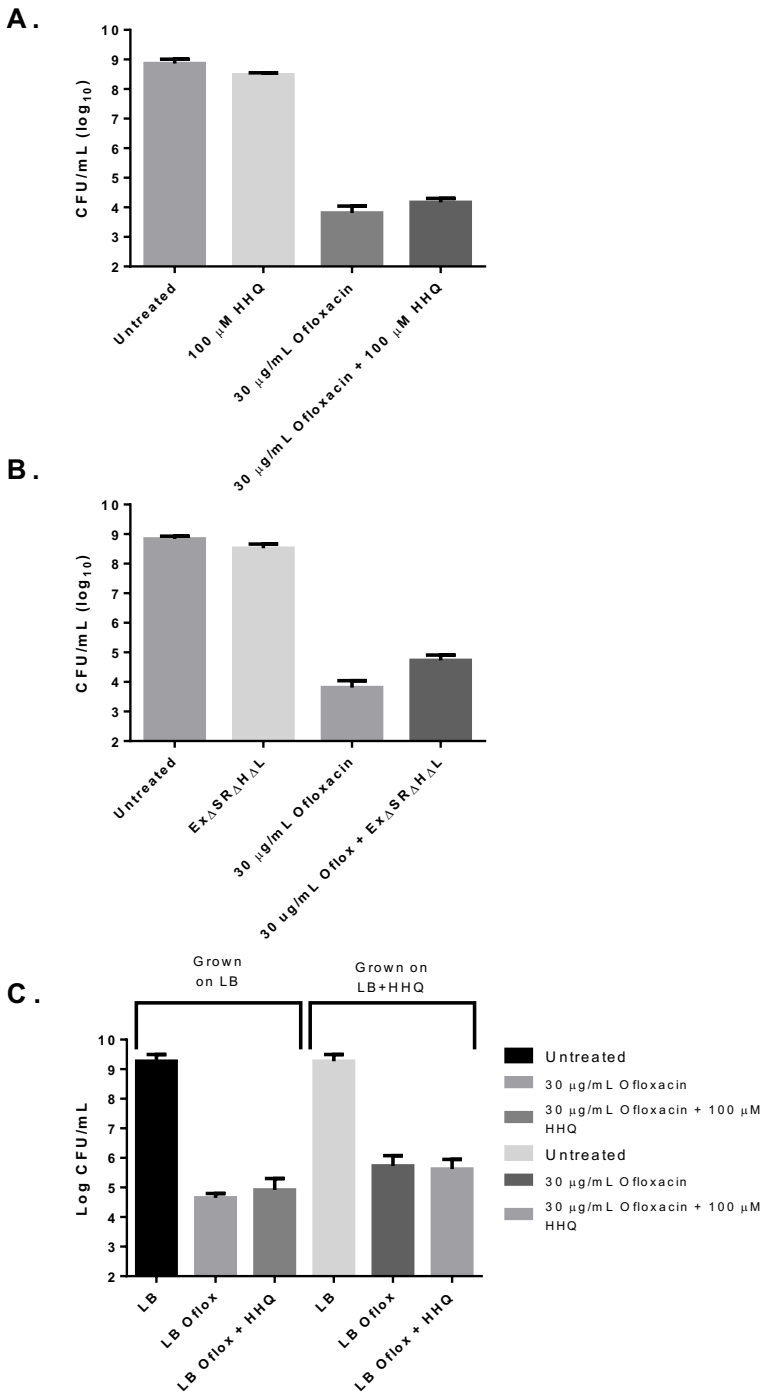


Fig 24. HAQ+ extract and synthetic C7-HHQ do not potentiate killing of *P. aeruginosa* biofilms. PAO1 ΔSR was grown as colony biofilms on polycarbonate filters as done in Fig 23 on LB agar for 24h, then challenged with for 4 h at 37 °C on LB agar containing 30 µg/mL ofloxacin and **(A)** 100 µM synthetic C7-HHQ or **(B)** HAQ+ extracts $Ex_{\Delta SR\Delta HA}$. For **(C)**, biofilms were grown on LB +/- 100 µM synthetic C7-HHQ then challenged with 30 µg/mL ofloxacin +/- 100 µM synthetic HHQ for 4 h. The results are presented as mean ± SEM from n=4 biological replicates from a representative experiment.

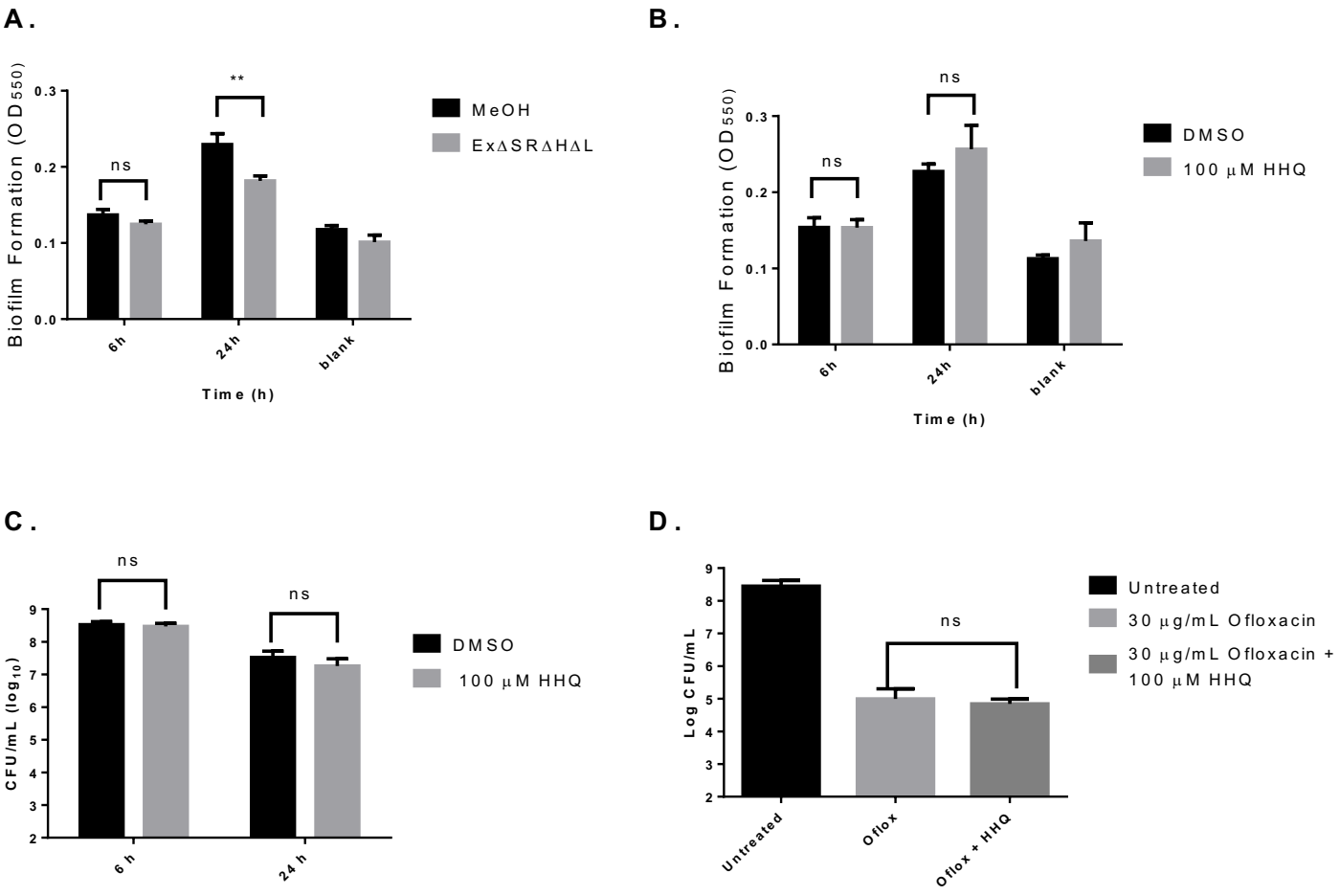


Fig 25. HAQ+ extract and synthetic C7-HHQ do not have a significant or consistent inhibitory effect on *S. aureus* biofilm formation. Bacterial cells (*S. aureus* ATCC 29213 $1x10^6$ CFU/well) were used as inoculum for biofilm formation in a 96 well static biofilm model. Biofilms were grown in LB medium in the presence of **(A)** HAQ+ extract $Ex_{\Delta SR\Delta H\Delta L}$, **(B)** 100 µM C7-HHQ or control statically at 37 °C without shaking for 6h and 24h. Biofilm biomass was quantified by staining with 1% crystal violet using established protocols and measurement at OD_{550}. The results are presented as mean ± SEM of n=36 biological replicates from 2 independent experiments.

(C) Biofilms were grown on polycarbonate filters as colony biofilms. After inoculation of $2.5x10^5$ CFU/filter, biofilms were grown statically on LB agar plates supplemented with 100 µM HHQ or DMSO controls at 37 °C for 6h and 24h. At each time point, biofilm cells were resuspended in PBS and viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM from n=6 biological replicates from 2 independent experiments.

(D) Colony biofilms were grown on LB agar for 24 h then challenged with 30 µg/mL ofloxacin +/- 100 µM synthetic HHQ for 4 h at 37 °C. Viable biofilm cells were measured by plating and CFU counts. The results are presented as mean ± SEM from n=6 biological replicates from 2 independent experiments.

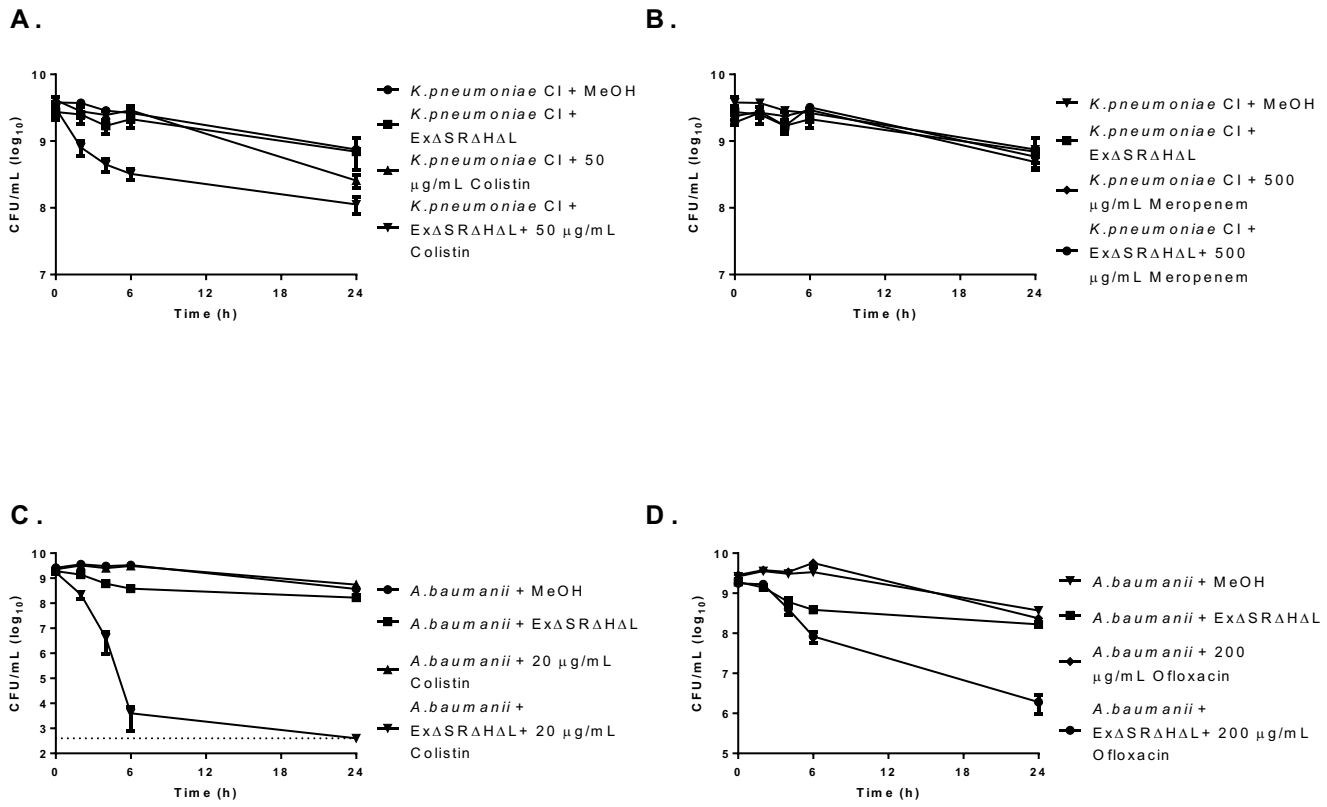


Fig 26. Adjuvant activity of HAQ+ extracts against ESKAPE organisms *Klebsiella pneumoniae* and *Acinetobacter baumanii*. The adjuvant activity of HAQ+ extracts ($Ex_{\Delta SR\Delta H\Delta L}$) in combination with ofloxacin, colistin and meropenem (as indicated) was tested in the ESKAPE pathogens *Klebsiella pneumoniae* (clinical isolate) and *Acinetobacter baumanii* AB5075. The killing assays were done as for Fig 2. Bacterial cells were pre-incubated for 2 h with $Ex_{\Delta SR\Delta H\Delta L}$ or methanol control, then challenged with different antibiotics (as indicated) for 24 h at 37 °C with shaking (250 RPM). Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM of n=6 biological replicates from from 2 independent experiments.

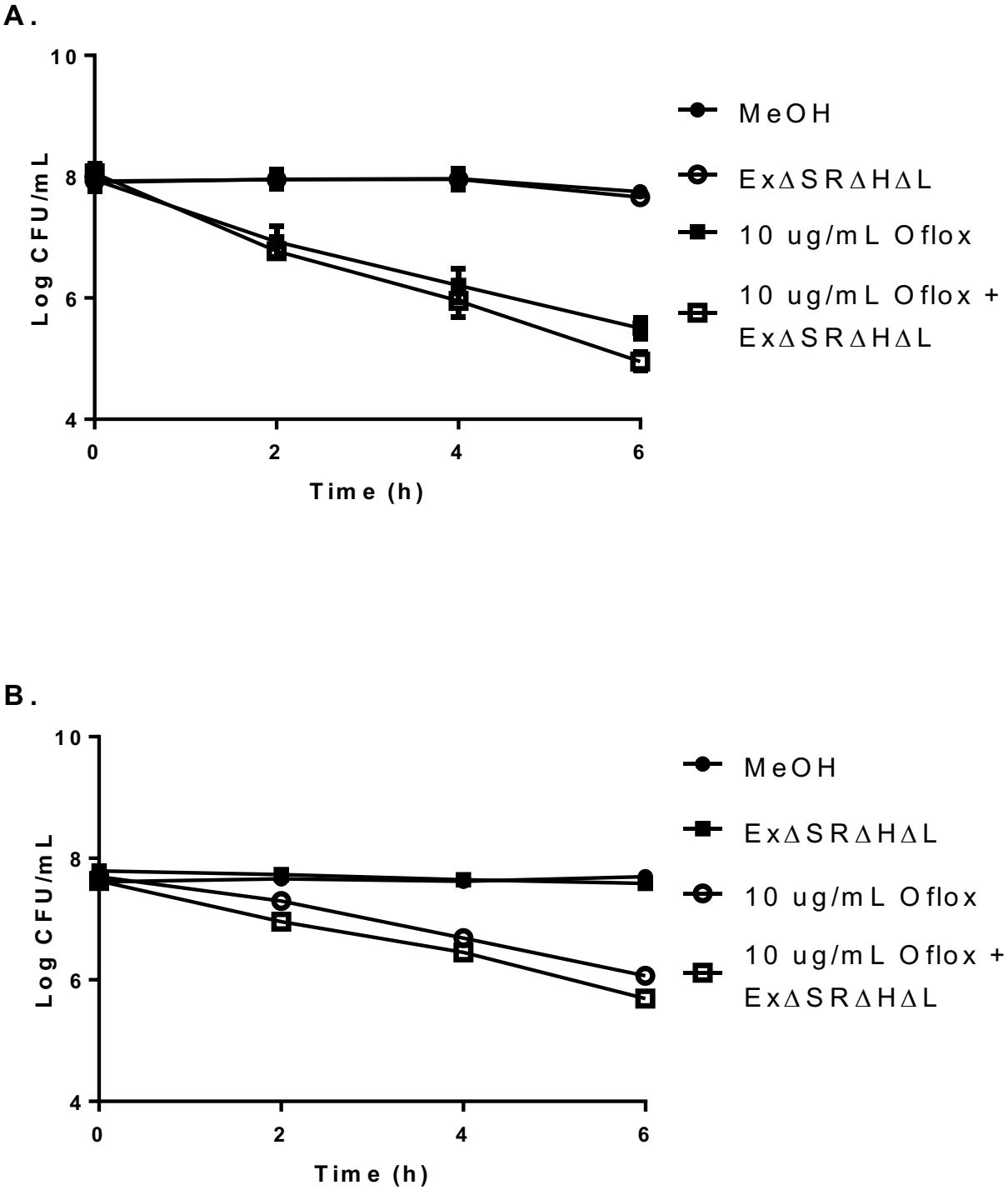


Fig 27. HAQ+ extract does not potentiate ofloxacin killing of *Enterococcus faecalis*. The adjuvant activity of HAQ+ extracts ($Ex_{\Delta SR\Delta H\Delta L}$) in combination with ofloxacin, colistin and meropenem was tested in the ESKAPE pathogens *E. faecalis* **(A)** ATCC 29212 and **(B)** ATCC 51299 as done for Fig 2. Bacterial cells were pre-incubated for 2 h with $Ex_{\Delta SR\Delta H\Delta L}$ or methanol control, then challenged with 10 µg/mL ofloxacin for 6 h at 37 ^{o}C with shaking (250 RPM). Viable cell counts were measured over time by plating and CFU counts. The results are presented as mean ± SEM of n=6 biological replicates from from 2 independent experiments.

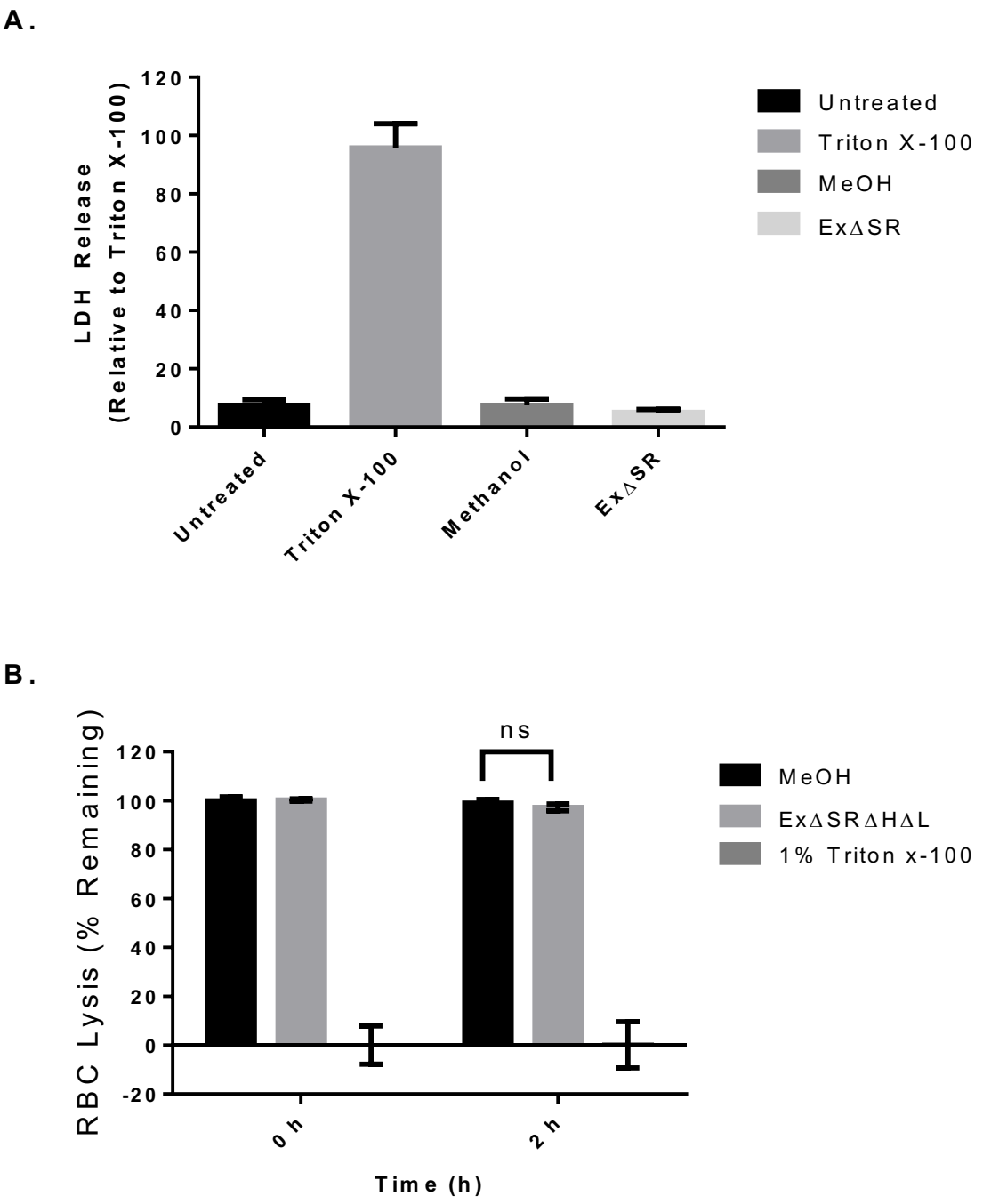


Fig 28. The HAQ+ extract does not cause toxicity to human HeLa cells nor hemolysis. The HAQ+ extract $Ex_{\Delta SR}$ was tested for cytotoxicity to human **(A)** HeLa cells and **(B)** lysis for red blood cells (RBC). HeLa cells were grown to ~70% confluence (~50,000 cells/well) in 96 well tissue culture plates and challenged with $Ex_{\Delta SR}$ (adjusted to 100 µM HHQ as used in time kill assays) for 2h at 37 ^{o}C in 5% CO_2. Controls were untreated or methanol solvent. Following exposure to $Ex_{\Delta SR}$ the media was assayed for LDH release (colorimetric method with OD 490) and compared to 100% release (Triton X-100 lysis of cells). RBC were isolated from human blood and challenged with $Ex_{\Delta SR\Delta L\Delta H}$ (adjusted to 100 µM HHQ), 1% triton x-100 (100% lysis) or MeOH (negative control) for 2 h. Samples were then centrifuged and the supernatant read at OD 405 nm. The results are mean ± SD from n=3 biological replicates form a representative experiment.

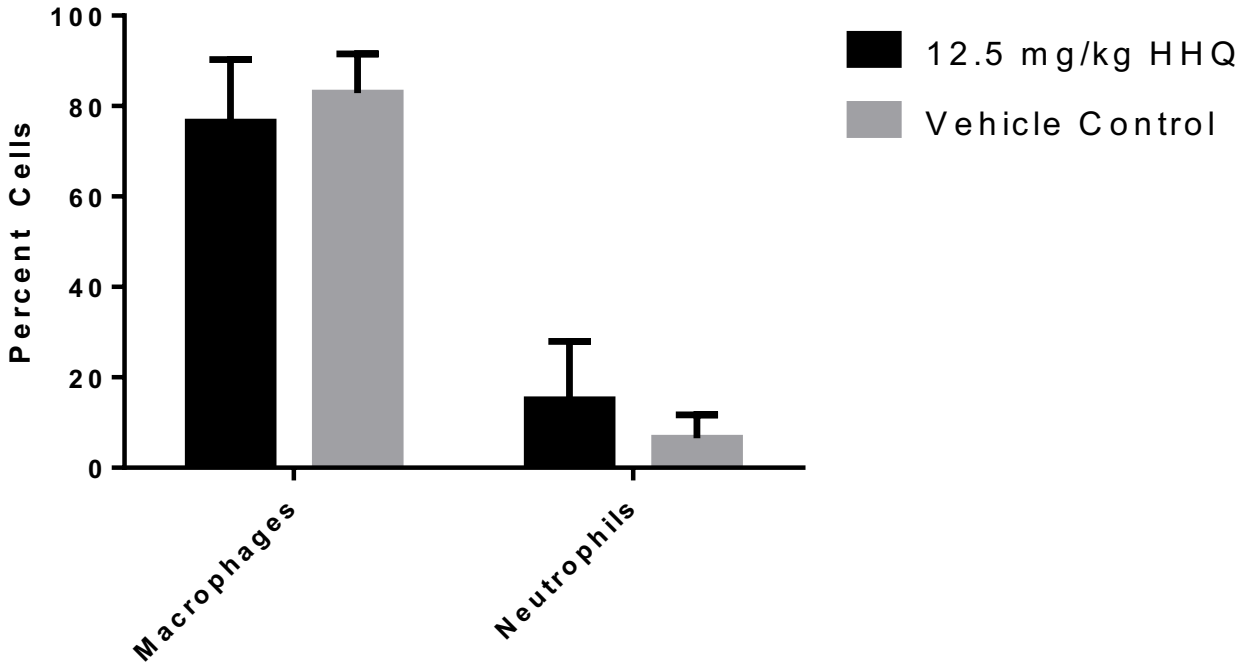


Fig 29. Intraperitoneal administration of synthetic C7-HHQ does not cause increased inflammation. We tested wild-type C57BL/6 mice (6-8 weeks) with a single i.p dose of 12.5 mg/kg C7-HHQ (n=7: 5 males/2 females) in a total volume of 400 µL vehicle (5% Tween-80, 5% PEG_{400}, 5% DMSO) and compared it to the vehicle control group (n=7: 5 males/2 females). Mice were sacrificed after 24h and the peritoneal lavage fluid was analyzed by cytospin and Kwik-Diff staining. The results are presented as mean ± SEM.

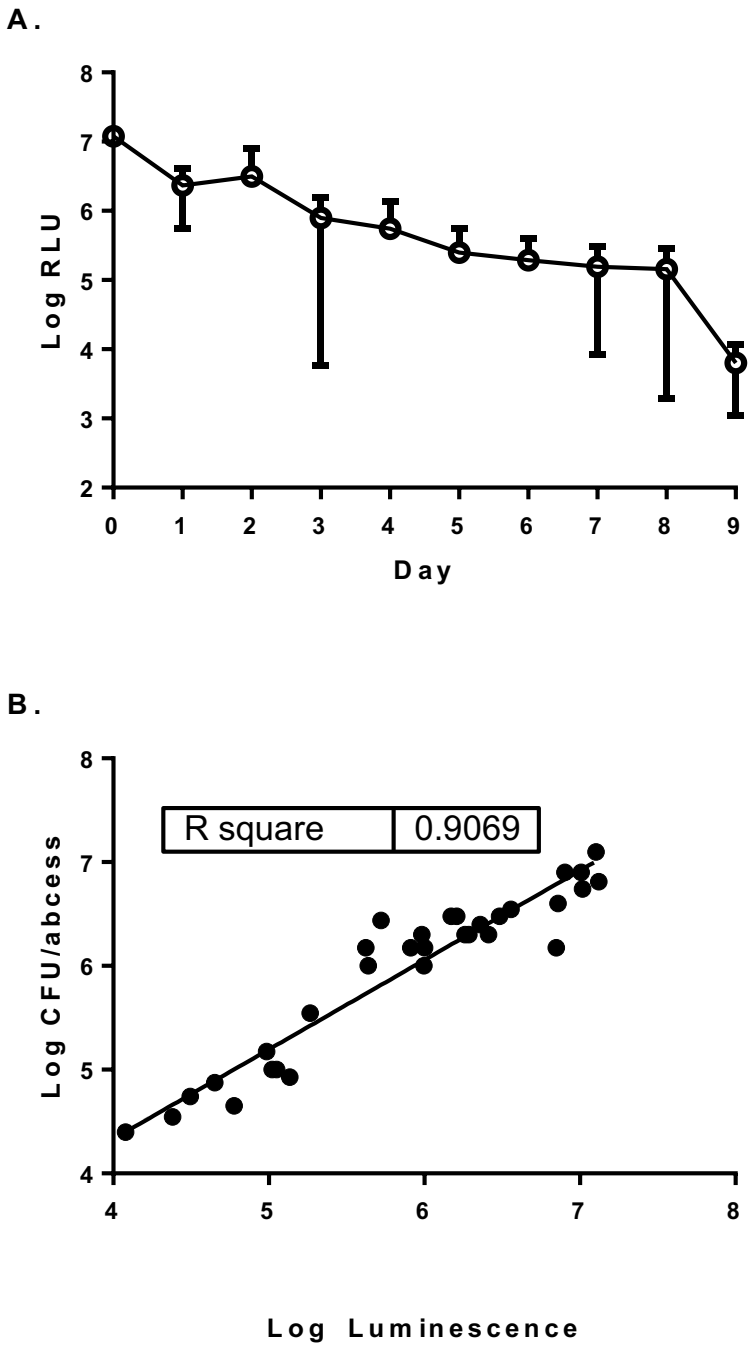


Fig 30. Development of a *P.aeruginosa* skin abscess model. C57BL/6 mice were inoculated with luminescent PAO1 ΔSR in a dorsal abscess model developed **(A)** persistent infection for at least 7 days and **(B)** bioluminescence correlated with viable bacteria. Stationary phase bacteria (PAO1 ΔSR::PrpsL-lux $5x10^6$ CFU in 50 µL PBS) was injected subcutaneously to form an abscess. Mice were imaged immediately after injection and daily using the Bruker In-Vivo Extreme system. In **(A)**, mice were monitored daily for 9 days (n=6). In **(B)**, mice were monitored for 3 days, then euthanized for enumeration of viable bacterial in abscess tissues (n=32) and correlation with in vivo bioluminescence measurement immediately prior to euthanasia.

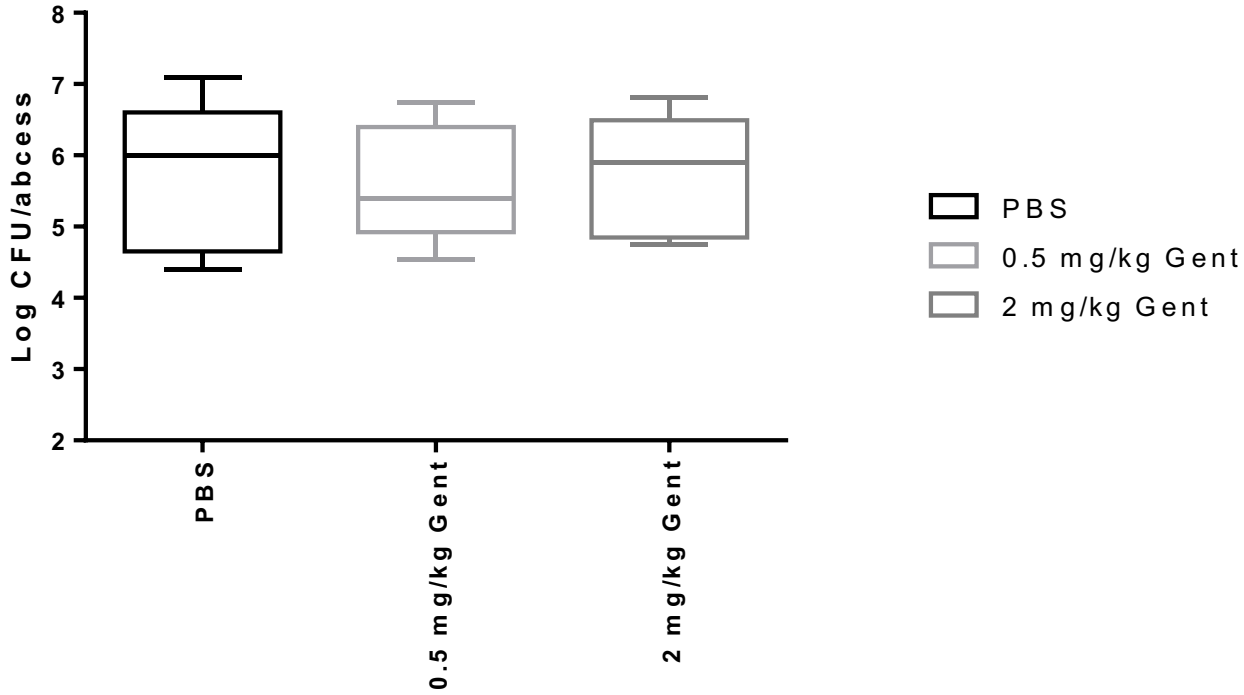


Fig 31. Gentamicin single dose treatment does not reduce abscess bacterial load at Day 3 in a *P.aeruginosa* skin abscess model. C57BL/6 mice were inoculated with luminescent PAO1 ΔSR in a dorsal abscess model. Stationary phase bacteria (PAO1 ΔSR::PrpsL-lux $5x10^6$ CFU in 50 µL PBS) was injected subcutaneously to form an abscess. At 24h p.i, mice were treated with a single i.p. dose of saline, 0.5 mg/kg gentamicin or 2 mg/kg gentamicin. before a single dose of gentamicin was administered i.p. Mice were imaged immediately after injection and daily for 3 days using the Bruker In-Vivo Extreme system. At day 3 p.i, the mice were sacrificed and the abscess tissues were analyzed for viable bacteria. Data is presented in a Bars-Whiskers graph with median, min and max range and upper and lower quartiles.

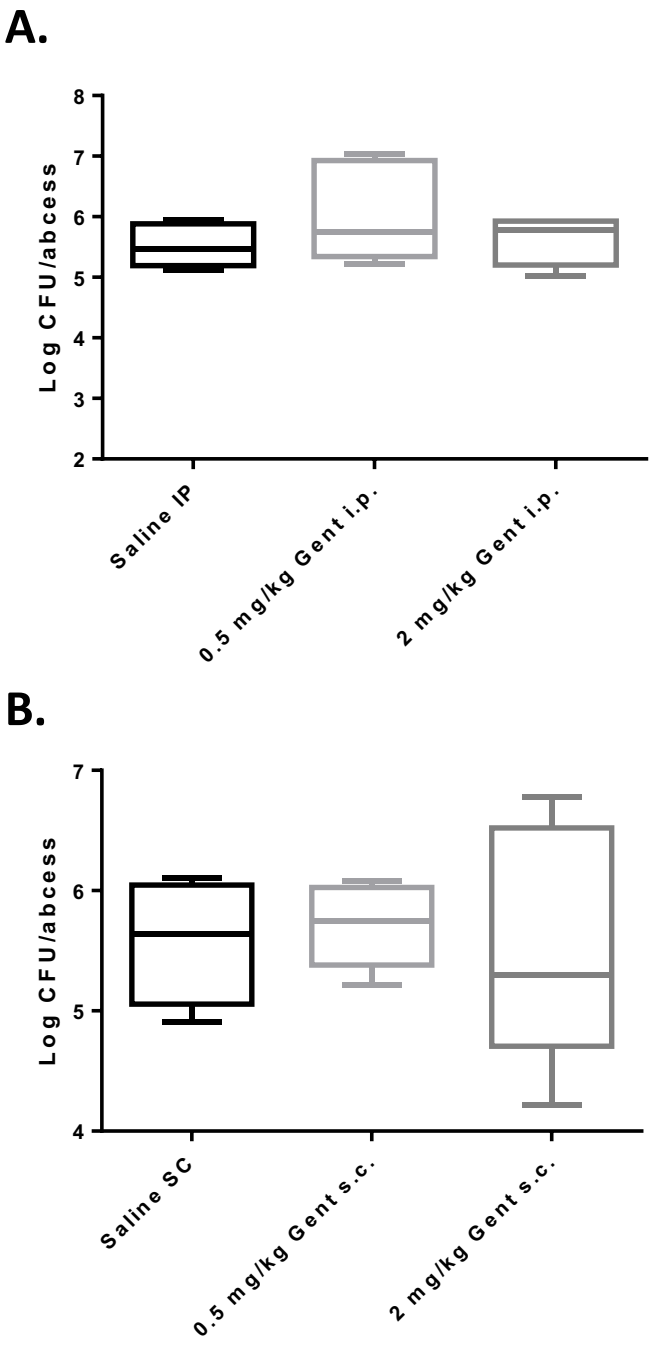


Fig 32. Gentamicin multiple dose regimens does not reduce abscess bacterial load at Day 3 in a *P.aeruginosa* skin abscess model. C57BL/6 mice were infected as done in Fig 31. At 24 h and 30 h post-infection, mice were treated with two doses 0.5 mg/kg, 2 mg/kg gentamicin or saline control by **(A)** i.p. or **(B)** s.c. route. Mice were imaged immediately after infection and daily for 2 days using the Bruker In-Vivo Extreme system. At day 2 p.i, the mice were sacrificed and the abscess tissues were analyzed for viable bacteria. Data is presented in a Bars-Whiskers graph with median, min and max range and upper and lower quartiles.

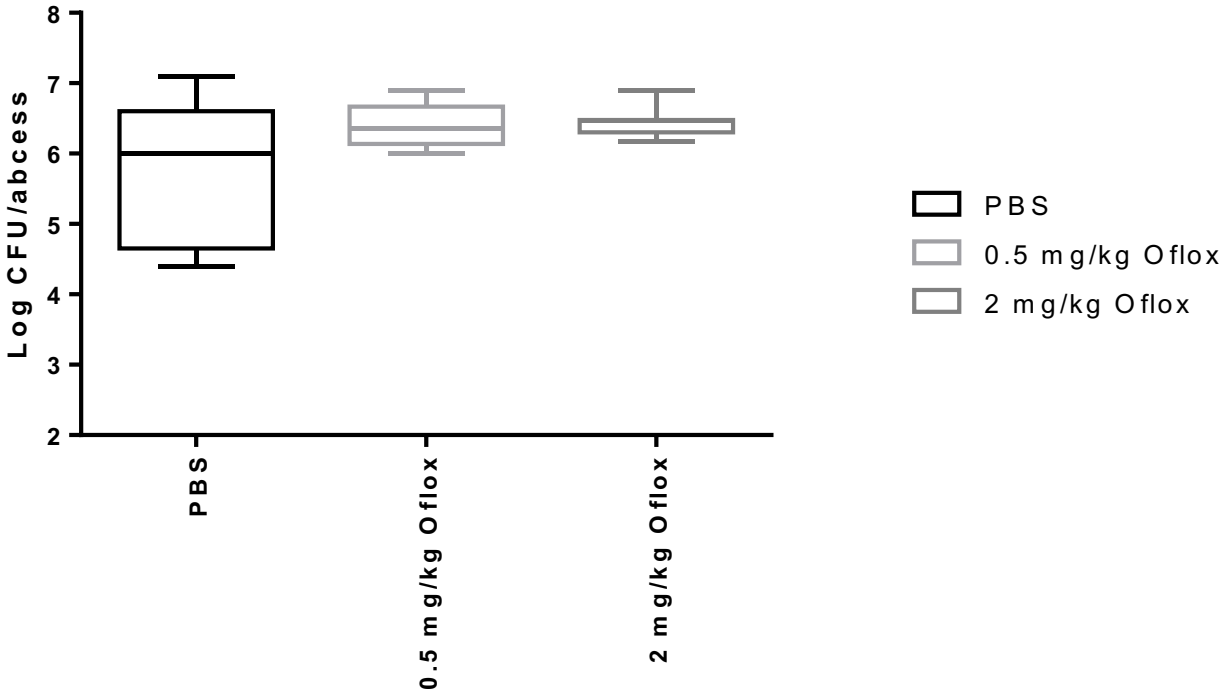


Fig 33. Single dose *i.p.* ofloxacin treatment does not reduce abscess bacterial load at Day 3 in a *P.aeruginosa* skin abscess model. C57BL/6 mice were infected as done in Fig 31. At 24 h after infection, a single dose of 0.5 mg/kg or 2 mg/kg i.p. ofloxacin or saline control was administered. Mice were imaged immediately after infection and daily for 3 days using the Bruker In-Vivo Extreme system. At day 3 p.i, the mice were sacrificed and the abscess tissues were analyzed for viable bacteria. Data is presented in a Bars-Whiskers graph with median, min and max range and upper and lower quartiles.

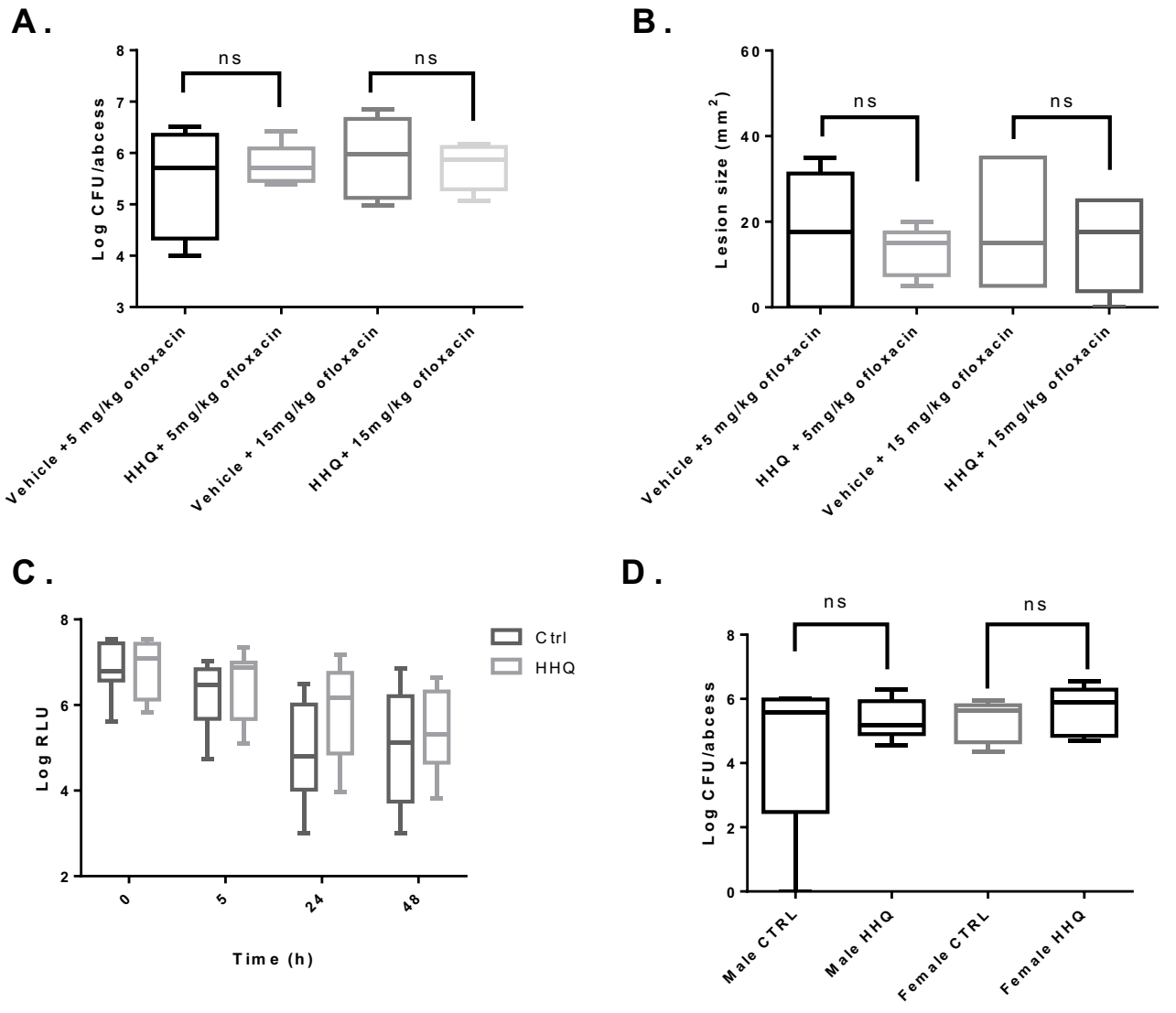


Fig 34. Testing of the adjuvant activity of synthetic C7-HHQ in a *P.a.* abscess infection model. C57BL/6 mice were infected as done in Fig 31. For **(A)** and **(B)**, after 24 h after infection, daily doses of 5 mg/kg or 15 mg/kg i.p ofloxacin +/- 50 mg/kg C7- HHQ were administered. Mice were imaged immediately after infection and daily for 3 days using the Bruker In-Vivo Extreme system. At day 3 p.i, the mice were sacrificed and the abscess tissues were analyzed for **(A)** viable bacteria and **(B)** lesion area was measured.

For **(C)** and **(D)**, two doses of 50 mg/kg p.o ofloxacin +/- 50 mg/kg intra-abscess C7-HHQ were administered at 2 h and 24 h post-infection. At 48 h, mice were were sacrificed and the abscess tissues were analyzed for **(C)** viable bacteria. **(D)** Mice were also stratified by gender.

All Data is presented in a Bars-Whiskers graph with median, min and max range and upper and lower quartiles.

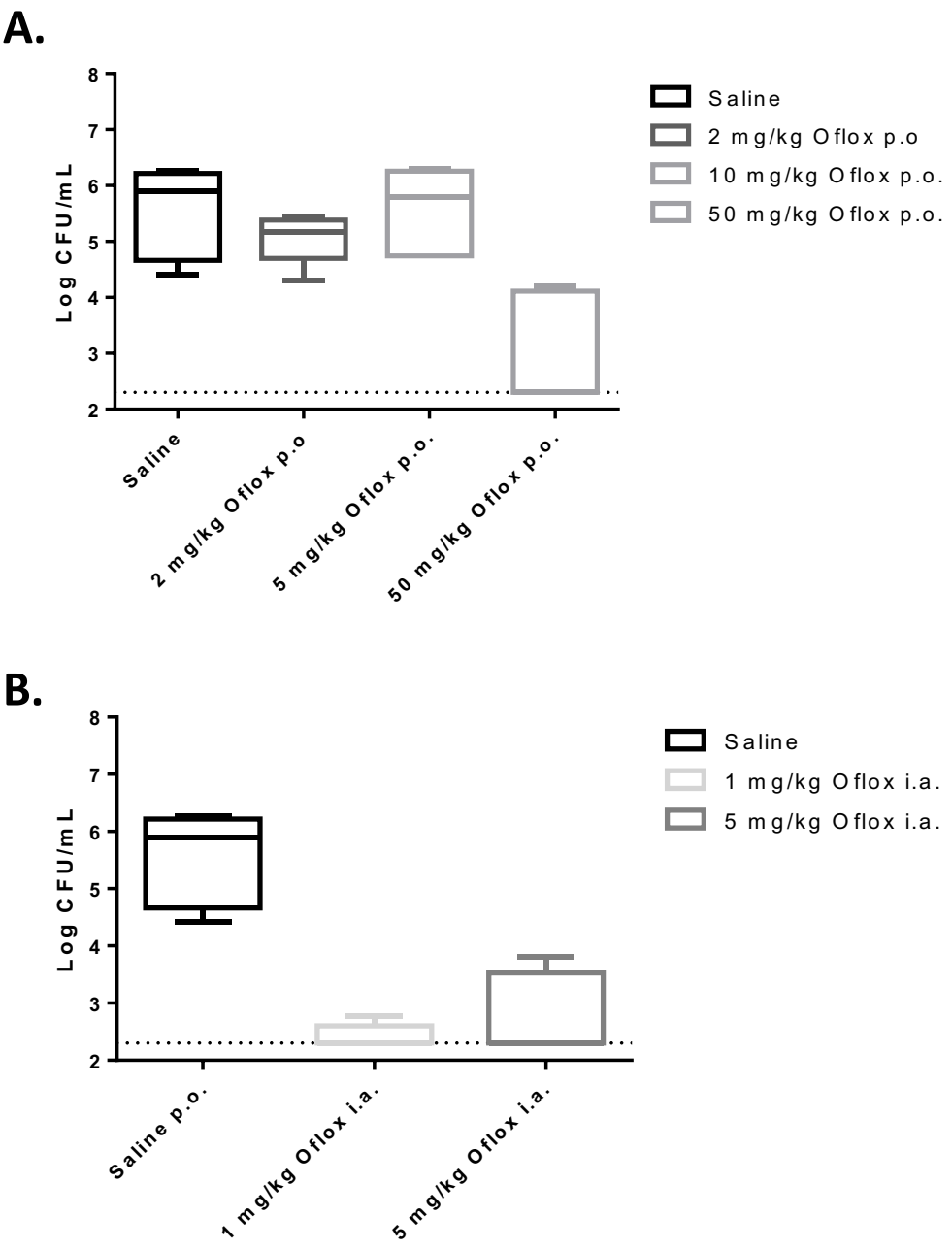


Fig 35. Testing of the adjuvant activity of synthetic C7-HHQ in a *P.a.* abscess infection model. C57BL/6 mice were infected as done in Fig 31. At 24 h after infection, mice were administered **(A)** daily doses of 2 mg/kg, 10 mg/kg and 50 mg/kg p.o ofloxacin. **(B)** daily doses of 1 mg/kg or 5 mg/kg intra-abscess ofloxacin. Mice were imaged immediately after infection and daily for 4 days using the Bruker In-Vivo Extreme system. At day 4 p.i, the mice were sacrificed and the abscess tissues were analyzed for viable bacteria. Data is presented in a Bars-Whiskers graph with median, min and max range and upper and lower quartiles.

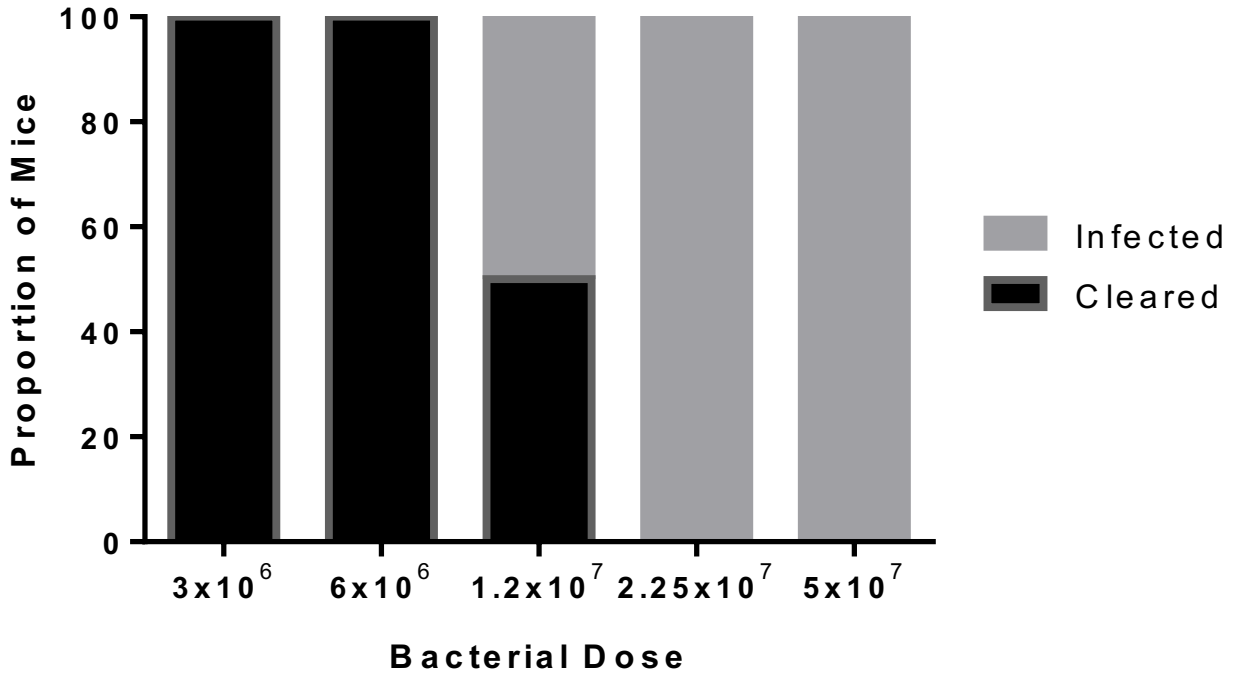


Fig 36. Bacterial dose escalation of a *S. aureus* skin abscess model. C57BL/6 mice were inoculated with lux-tagged *S. aureus* USA 300 in a dorsal abscess model developed. Stationary phase inoculum of *S. aureus* USA300 at $3x10^{6,}$ $6x10^{6,}$ $1.2x10^{7}$, $2.25x10^{7}$ $5x10^{7}$ CFU in 50 µL PBS were injected subcutaneously to form an abscess (n=4 mice per dose). Mice were imaged immediately after injection and daily for 4 days using the Bruker In-Vivo Extreme system. Mice were monitored for 4 days, then euthanized for enumeration of viable bacterial when luminescence fell below the limit of detection.

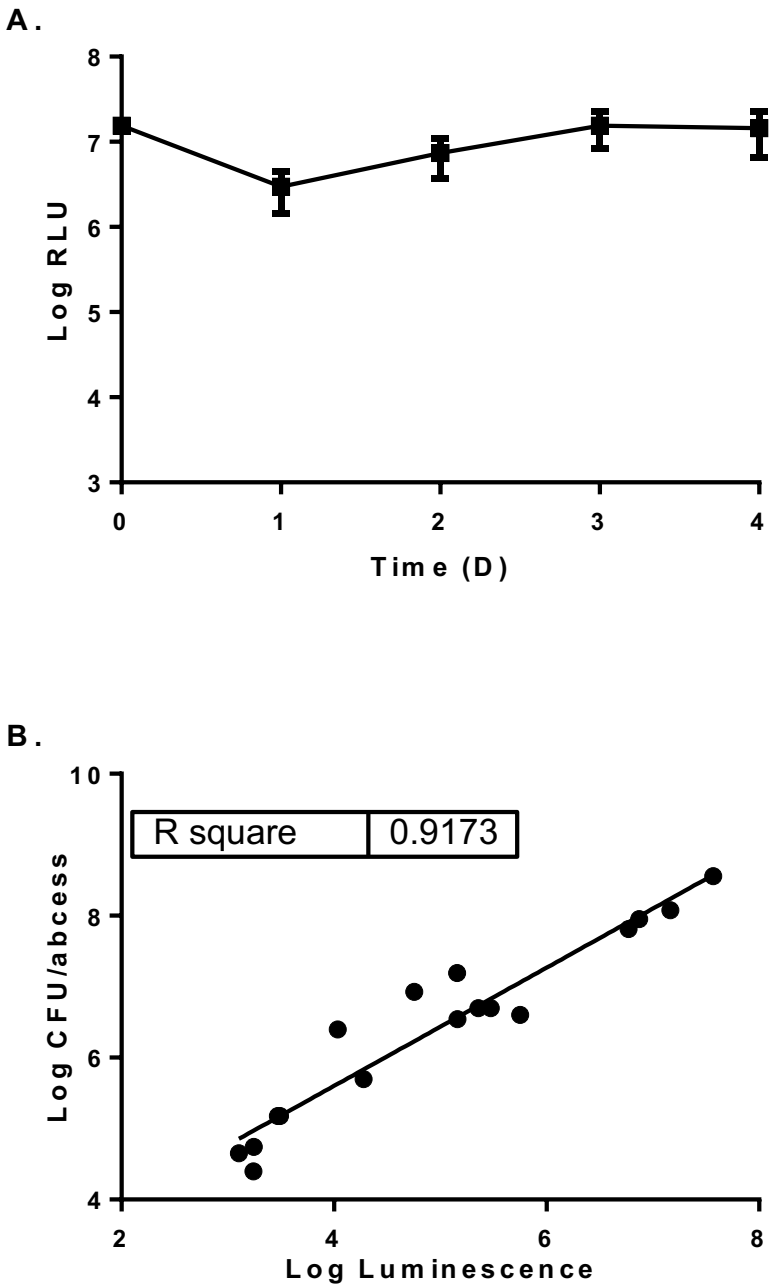


Fig 37. Development of a *S. aureus* skin abscess model. C57B/6 mice were inoculated with lux-tagged *S. aureus* USA 300 in a dorsal abscess model and developed **(A)** persistent infection for at least 4 days. Bioluminescence **(B)** correlated with viable bacteria. In **(A)** stationary phase bacteria (*S. aureus* USA300 at $5x10^7$ CFU in 50 µL PBS) was injected subcutaneously to form an abscess (n=4). Mice were imaged immediately after injection and daily for 4 days using the Bruker In-Vivo Extreme system. In **(B)**, mice were injected with different bacterial doses ($3x10^6$, $6x10^6$, $1,2x10^7$, and $4,5x10^7$), monitored for 3 days, then euthanized for enumeration of viable bacterial in abscess tissues (n=16) and correlation with in vivo bioluminescence measurement immediately prior to euthanasia.

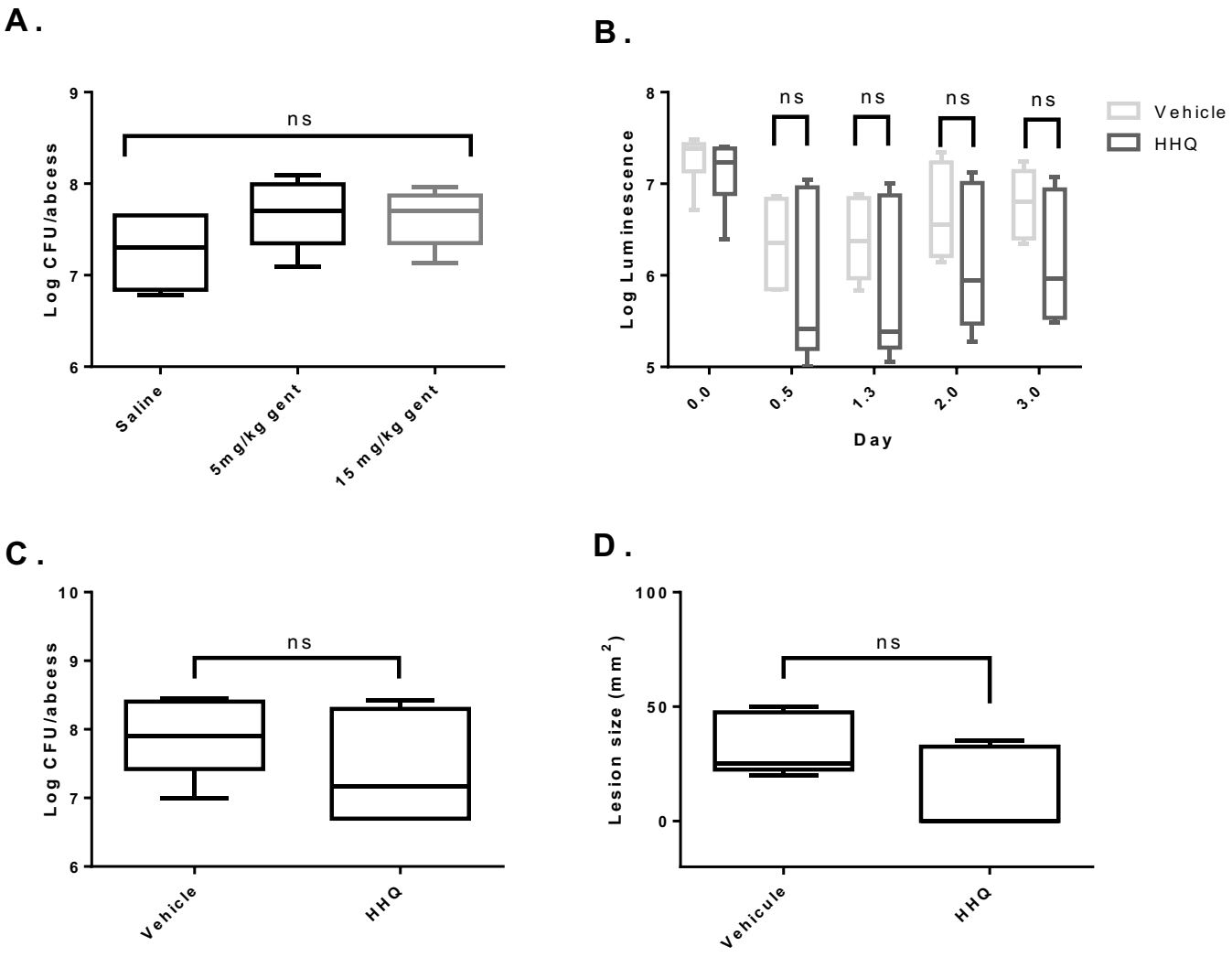


Fig 38. Synthetic C7-HHQ shows no adjuvant activity in a *S.aureus abscess* infection. C57BL/6 mice were inoculated with lux-tagged *S. aureus* USA 300 (at $5x10^7$ CFU in 50 µL PBS) as done in Fig 32. The following treatment were tested: **(A**) 5 and 15 mg/kg i.p gentamicin alone at 24h and 48 h post infection. **(B)** Bioluminescence, **(C)** viable CFU counts and **(D)** skin lesion size at day 3 following 100 mg/kg i.p gentamicin +/- 50 mg/kg s.c C7-HHQ given at 24 and 48 h post infection. Data is presented in a Bars-Whiskers graph with median, min and max range and upper and lower quartiles.

Appendix 2

Final technical report

Discovery award
W81XWH-16-1-0097

January 2019

McGill

June 27, 2017

Animal Certificate

This is to certify that **Dr. Dao Nguyen, RI-MUHC (Glen Site),** currently holds an approved Animal Use Protocol **# 2016-7801** with McGill University and its Affiliated Hospitals' Research Institutes for the following project:

Animal Use Protocol Title: Bacteria derived small molecules as novel antimicrobial adjuvants for treatment of nosocomial and drug resistant bacterial infections - US DoD

Start date: June 1, 2017

Expiration date: June 1, 2018

McGill University and Affiliated Hospitals Research Institutes recognize the importance of animal research in our efforts to further our knowledge of natural processes, diseases and conservation. Research, educational and testing projects are conducted with full commitment to the wellbeing of the animal subjects. In order to limit animal use to meritorious research or educational projects, the institution relies on stringent peer review processes, along with assessment of ethical issues by the Animal Care Committee. McGill University recognizes that the use of animals in research, teaching and testing carries significant responsibilities. The institution will continue to develop and maintain guidelines and regulations, following the high standards established by the Canadian Council on Animal Care. It is committed to conducting the highest-quality research and to providing animals with the best care.

Claude Lalande
Assistant Director, Animal Compliance Office
Office of Vice-Principal (Research and Innovation)
Suite 325, James Administration Building, McGill University
845 Sherbrooke Street West, Montreal, Quebec, Canada H3A 0G4
claude.lalande@mcgill.ca

McGill

June 14, 2018

Animal Certificate

This is to certify that **Dr. Dao Nguyen, Department of Medicine, RI-MUHC (Glen Site),** currently holds an approved Animal Use Protocol **# 2016-7801** with McGill University and its Affiliated Hospitals' Research Institutes for the following project:

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Claude Lalande
Assistant Director, Animal Compliance Office
Office of Vice-Principal (Research and Innovation)
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DEPARTMENT OF THE ARMY

HEADQUARTERS, US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
810 SCHREIDER STREET
FORT DETRICK, MD 21702-5000

REPLY TO
ATTENTION OF

July 12, 2017

Director, Office of Research Protections
Animal Care and Use Review Office

Subject: Review of USAMRMC Proposal Number PR152067, Award Number W81XWH-16-1-0097 entitled, "Bacteria-Derived Small Molecules as Novel Antimicrobial Adjuvants for the Treatment of Nosocomial and Drug-Resistant Bacterial Infections"

Principal Investigator Dao Nguyen
McGill University
Montreal, Canada

Dear Dr. Nguyen:

Reference: (a) DOD Instruction 3216.01, "Use of Animals in DOD Programs"
(b) US Army Regulation 40-33, "The Care and Use of Laboratory Animals in DOD Programs"
(c) Animal Welfare Regulations (CFR Title 9, Chapter 1, Subchapter A, Parts 1-3)

In accordance with the above references, amendment 002 to protocol PR152067 entitled, "Bacteria Derived Small Molecules as Novel Antimicrobial Adjuvants for Treatment of Nosocomial and Drug Resistant Bacterial Infections," IACUC protocol number 2016-7801, Protocol Principal Investigator Dao Nguyen, is approved by the USAMRMC Animal Care and Use Review Office (ACURO) as of 26-SEP-2016 for the use of mice and will remain so until its modification, expiration or cancellation. This protocol amendment was approved by the McGill University IACUC on 09 June 2017.

Required Actions: When updates or changes occur, documentation of the following action or events must be forwarded immediately to ACURO:

- IACUC-approved modifications, suspensions, and triennial reviews of the protocol (All amendments or modifications to previously authorized animal studies must be reviewed and approved by the ACURO prior to initiation.)

- IACUC actions involving this protocol regarding
  a. any noncompliance;
  b. any deviation from the provisions of the Guide for the Care and Use of Laboratory Animals; or
  c. any suspension of this activity by the IACUC

- USDA or OLAW regulatory noncompliance evaluations of the animal facility or program

- AAALAC, International status change

Throughout the life of the award, the awardee is required to submit animal usage data for inclusion in the DOD Annual Report on Animal Use. Please ensure that the following animal usage information is maintained for submission:

- Species used (must be approved by this office)
- Number of each species used
- USDA Pain Category for all animals used

For further assistance, please contact the Director, Animal Care and Use Review Office at (301) 619-2283, FAX (301) 619-4165, or via e-mail: usarmy.detrick.medcom-usamrmc.other.acuro@mail.mil.

NOTE: Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grant Officer can authorize expenditure of funds. It is recommended that you contact the appropriate Contract Specialist or Contracting Officer regarding the expenditure of funds for your project.

Sincerely,

Original Signed

Bryan K. Ketzenberger, DVM, DACLAM
Colonel, US Army
Director, Animal Care and Use
Review Office

Copies Furnished:
Ms. Catherine Henry, US Army Medical Research Acquisition Activity (USAMRAA)
Dr. Shelley C. Jorgensen, Congressionally Directed Medical Research Program (CDMRP)
Mr. Claude Lalande, McGill University
Dr. Susan James, McGill University Health Centre Research Institute

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1- PI Contact Record

Existing contact information will be displayed, based on your login. Information may be added, edited, and saved to this specific protocol application.

Fields marked by a red star * must be completed.

Need help?

Principal Investigator	Nguyen, Dao
Protocol Application Number	2016-7801
Document Type	
PI	Nguyen, Dao
McGill ID	119232965
Classification	Principal Investigator
Phone + Ext	934-1934 42534
Home Phone	
Cell Phone	
Fax	514-933-3962
Email	dao.nguyen@mcgill.ca
Other information	

Table of Contents

2- Protocol Title

If this is for a teaching or training project, please write the course's title and course number, if any.

Copies of Animal Certificates and ALPHA Certificates are attached by the Animal Compliance Office at the bottom of this page once issued and when applicable.

Need help?

Protocol Title

Title
Bacteria derived small molecules as novel antimicrobial adjuvants for treatment of nosocomial and drug resistant bacterial infections - US DoD

Attachments List

File Spec	Description	Created
2016-7801_2_0001_Nguyen 7801 GLEN animal certificate - expiring Jun 2017.pdf	Nguyen 7801 animal certificate Jun 2016-2017	06/27/2017
2016-7801_2_0001_Nguyen 7801 GLEN animal certificate - expiring Jun 2018.pdf	Nguyen 7801 animal certificate Jun 2017-2018	06/27/2017

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3- Goals

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4- Specific Objectives

Need help?

Provide the specific objectives for the coming year (preferably in point form).
•Determine the preliminary toxicity and in vivo tolerability of HAQ (hydroxyl-alkyl-quinolines) •Investigate the adjuvant activity of HAQ in a Pseudomonas aeruginosa cutaneous abscess model. •Investigate the adjuvant activity of HAQ in a severe Pseudomonas aeruginosa pneumonia model. •Investigate the adjuvant activity of HAQ in a Staphylococcus aureus cutaneous abscess model. •Investigate the adjuvant activity of HAQ in a severe Staphylococcus aureus pneumonia model.

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5- Keywords of Procedures

Need help?

Using keywords only, list the procedures used on animals (e.g. euthanasia by anesthetic overdose, anesthesia, breeding colony, injection IP, gavage, drug administration, major survival surgery, behavioural studies).
anesthesia analgesia euthanasia intratracheal infection subcutaneous infection bacterial infection drug administration (injection IP, intratracheal, SC, IM) tissue collection breeding in vivo luminescence imaging

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6- Funding Sources

Based on the boxes checked below, additional screens will appear to further identify the funding sources for this proposal. Complete subsequent pages as appropriate.

Important: for projects that have not been reviewed for scientific merit by the funding source, the investigator must obtain an **internal peer review**. Internal peer reviews can be requested from the Faculty Associate Dean/Institute Director. Only those protocols receiving a positive assessment of scientific merit can be approved. Please attach the peer review letter in the "Attach File" section below.

If you cannot find your grant in the McGill Administered drop down list or you have scenarios not fitting the agency sections, please use the "Department, Startup or Other Funding" section.

NOTE: Sometimes the red dot does not change to green for this section (intermittent issue), continue with the next sections and submit the protocol or amendment as usual. If you are unable, please contact Darwin Support, darwin@mcgill.ca.

Need help?

How will this protocol be funded? Check all applicable boxes	
Agency Funding	Yes
Administered by McGill's Office of Sponsored Research (OSR) ? If cannot find grant in drop-down list, answer "No" here and choose "Yes" for "Department, Startup or Other Funding".	No
Private/Commercial Funding	
Department, Startup or Other Funding (includes OSR administered grants not found in drop-down list)	
Peer Reviewed for the project proposed in this Animal Use Protocol?	Yes

Attachments List

File Spec	Description	Created
2016-7801_2_0001_Nguyen 7801 Glen DoD_HAQ funding decision_jan2016.pdf	US DoD Recommendation for funding = Peer Review	06/27/2017

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Funding Agency List

Add all sources of funding for this protocol.

If you are asked by the committee to supply funding documentation (i.e. grant award letter), please use the attachment section below.

Once all the funding sources are listed in the table, click the "Continue" button to proceed with the protocol application completion.

Need help?

Fund Source/Agency	Grant Number	Fund Title
Department of the Army	US DOD CDMPR PR152067	Bacteria-derived small molecules as novel antimicrobial adjuvants for the treatment of nosocomial and drug-resistant bacterial infections

Attachments List

File Spec	Description	Created

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Agency Funding Source

Funding Source/Agency	Department of the Army
Fund Title (and fund PI name if different from animal protocol PI)	Bacteria-derived small molecules as novel antimicrobial adjuvants for the treatment of nosocomial and drug-resistant bacterial infections
Funding Grant Number, if known	US DOD CDMPR PR152067

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7- Animal Use Location

An offsite location is any location other than facilities at McGill, MNI, MGH, RVH, MCH, Shriners, JGH and Douglas.

If animal work will be conducted at offsite locations, the committee may require additional approval and/or inspections.

Need help?

The next two questions refer to where the animal research work will be done.	
Offsite (non McGill/Affiliated RIs)?	NO
Field Study (wild animals)?	NO

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8- Collaboration

Need help?

Will you be collaborating on this protocol with:	
An outside institution (not McGill, MNI, MGH, RVH, MCH, Shriners, JGH or Douglas affliated)	NO
Another colleague affiliated with McGill, MNI, MGH, RVH, MCH, Shriners, JGH or Douglas	YES

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Inside Collaboration

Inside collaboration refers to another colleague affiliated with McGill, MNI, MGH, RVH, MCH, Shriners, JGH or Douglas.

Need help?

Collaborating PI Name	Danuta Radzioch

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9- Changes in Project's Goals

Need help?

Are the current goals different from those in last year's application? (IF THIS IS A NEW PROJECT, ANSWER NO)	NO

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10- Animal Husbandry and Care

This section pertains to husbandry and care that are to be **different** than what the animal facility would supply or contain; these changes are warranted for the purpose of this project.

Details are to be supplied, and documents can be attached if any, in the next page if answering "Yes".

Need help?

Will this project involve non-standard cages, diet and/or handling or modification to environmental enrichment?	NO

Table of Contents

11- Potential Hazards to Personnel and Animals

Included as potentially hazardous agents are: infectious material (bacteria/viruses/vectors/DNA affecting material), microbial toxins, radioisotopes, toxic and carcinogenic agents as well as human and animal transplantable tumours, tissues and/or cell lines.

Always inform the animal facility Supervisor/Manager prior to commencing a project involving Hazardous Materials. Animal Facility Supervisor/Managers are to post the accidental exposure and spill procedures in the room.

For information about biohazardous agents and the laws applicable to them, visit key links to the Public Health Agency of Canada (PHAC).

It is the responsibility of the investigator to obtain the necessary Biohazard and/or Radiation Safety permits from their site.

Need help?

Will you be using agents potentially hazardous to staff or animals?	YES

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Hazardous Use Information

Attach pertinent documents such as References and/or Certificates for cell line testing. If using a biohazardous agent, please attach the entire certificate when possible if one is issued at your site.

This protocol section is mainly to flag agents that require special precautions and/or certificates and/or testing reports. Details of individual agents is made in section "27- Hazardous Material" which appears once you answer "yes" to the question if using hazardous agents in section "18- Species Information".

Need help?

Infectious agents (bacteria, virus, vector...)?	YES
Radioactive isotope?	NO
Toxic or carcinogenic chemical?	YES
Human/animal transplantable tumours, tissues or cell lines?	NO
If using cell lines according to the SOP #610, have they been tested? If yes, please attach the test certificate. If not, justify.	

Attachments List

File Spec	Description	Created

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12- Species Use Instructions

Note that the rest of the application differs from the older system in segregating information by species.
In the following sections, all information on animal use (procedures, surgeries, drugs administered, etc.) is entered for a specific species. For example, there will be a number of screens for mice, another "set of screens" for rats, etc.

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13- Purpose of Animal Use

Need help?

Purpose of Animal Use Definitions:
1. Studies of a fundamental nature/basic research
2. Studies for medical purposes relating to human/animal diseases/disorders
3. Regulatory testing
4. Development of products/appliances for human/veterinary medicine
5. Teaching and training

Choose below, what describes your project best	
Purpose of Animal Use:	1 Basic research
Service to others?	

Teaching and Training Questions

If Purpose = 5. Teaching and Training, please answer the following questions.

For other purposes (1 to 4), please skip the following section and directly click "Save and Continue".

If a student refuses to work with animals on moral grounds, will an alternative in lieu of using animals be available? (Video, simulation, demonstration)
Will students handle live animals?
If yes, anesthetised animals?
Are wild animals used? (if so, obtain permit if applicable)
How many students are (or are likely to be) enrolled in the course?
Frequency of lab?
What will be the ratio of student / animals used?
What will be the ratio of instructor / students?
Will the procedures be demonstrated to the students?
Is the course "knowledge procedure" based (students learning a procedure)or "product" based (company learning use of a

specific product)?

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14- Transportation of Animals

Must follow the Standard Operating Procedure for Transportation of Live Animals and the Policy on Animals Outside the Animal Facility. Any deviation needs to be justified and approved by the FACC (Committee).

Need help?

Will you be moving animals to other rooms? If yes, answer the question(s) below.	NO
Will transportation be done according to Transportation SOP #501 and/or SOP #518? YES or NO.	YES
If not following the applicable Transportation SOP #501 and/or SOP #518, or to supply more details, please describe transportation:	

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15- Housing Outside Animal Facility

Housing locations other than inside the animal facility must be approved by the FACC.

Need help?

Will animals be housed outside the central facility for more than 24 hours?	NO
If yes, please provide following information:	
Location	
Scientific Justification for housed outside more than 24 hours?	

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16- Use of Human Clinical Areas

Use of human clinical areas for animals will require approval from the FACC and from the administration responsible for the clinical area.

Need help?

Will animals be imaged or treated in Human Clinical areas?	NO

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17- Species List

Choose all proposed species from the drop down list one at a time. If the species is not available, request that it be added by contacting Darwin Support via email (darwin@mcgill.ca). Adding a new species will present you with a series of sections to complete and will return you to this section when finished with that particular species.

IF THIS IS AN AMENDMENT AND YOU WANT TO REMOVE A SPECIES from your list, please contact Darwin Support via email (darwin@mcgill.ca) to request it. This measure is to prevent accidental removal that cannot be undone and requires extensive efforts from the researcher to add back manually.

Once all the species are listed in the table below, click the "Continue" button to proceed with the application completion.

IF THIS IS A RENEWAL, you MUST select the species and click the button "Edit Species", verify if the information is still correct, revise if needed and click the button "Save and Continue". You MUST do this for EACH species. Failure to do so may generate issues with the submission and approval process.

The term "Species" in this form is used as a label to represent a group of animals and is therefore not restricted to the strict definition of the word "Species".

Need help?

Species	# Requested
Mice	716

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18- SPECIES INFORMATION

For the question "Total number of requested animals", note that this is to be the **grand total number of animals for this species** (= number procured + number bred in-house + number from other sources) and includes both experimental and breeding animals.

Need help?

Species	Mice
Sex	Male
Total number of requested animals (# Purchased + # In-house bred + # Other)	716
# of animals housed in the facility/lab at one time?	50
Max # of animals per cage/enclosure/aquaria?	5

Species Activities

Will drugs (other than hazardous or euthanasia agents) be administered on this Species?	Yes
Will animals be euthanized?	Yes
If not, what will be done to animals after the experiment or project is completed?	
Will non-surgical procedures (i.e. injections, blood collection, behavioural tests, imaging etc.) be performed?	Yes
Will surgeries be performed?	No
Will Hazardous Agents be used?	Yes
Breeding will be done?	NO

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19- Justification For Number Requested

Explanation of Animal Usage: BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates.

For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures.

The arithmetic explaining how the total of animals for each species is calculated should be made clear. It helps reviewers to have a summary of subtotals at the end that add up to the grand total stated in the previous section "18 - Species Information". Please breakdown explanations to the strain level.

Note that some funding agencies (such as NIH) are developing policies to require a balance of both sexes in research projects.

An on-line calculator for breeding numbers is available at the Laboratory Animal Science Program's Web site

To help set up a breeding strategy and calculate the costs of generating conditional knockout mice, see the NIH Conditional Allele Mouse Planner (CAMP)

For large and/or complex numbers of animals, a table can be used to explain the numbers requested and attached to this page via the attachment feature at the bottom. A template is available on the UACC site's form section.

If your project is a renewal and involves animals that are not identified with Darwin barcoded cage cards, supply the number of animals used in the protocol's previous year per species. The data is needed in order to submit our annual report to the CCAC.

Need help?

Species Name	Mice

What is your justification for number of animals requested?

2017-18 Annual Review Justification (updated Jun 27 2017)

A) The objective of this project is to test the effectiveness of a new adjuvant compound used in combination with bactericidal antibiotics in treating two types of bacteria that cause severe hospital-acquired infections, namely Pseudomonas aeruginosa and Staphylococcus aureus infections.

B) We have added experiments that will be alternatives to the previously approved ones. The total number of mice has increased by 20 mice in order to do the pilot study for subcutaneous abscess infection (Exp 6).

Experiment 1: To determine the blood and tissue biocompatibility and in vivo tolerability of HAQ.

If HAQ molecules show no significant toxicity using ex vivo red blood cell hemolysis assay and LDH release cytotoxicity assay in

HeLa cells, we will proceed with the following mice experiments. Our aim is to establish the maximal tolerated dose (MTD) for intra-tracheal (IT) administration of HAQ by performing a dose escalation toxicity studies. We plan to test a single IT delivery of the selected HAQ(s) at two doses (expected range 5 to 25 mg/kg) compared with carrier control. We will assess mice 72h after HAQ administration for systemic and pulmonary toxicity and inflammation.

Based on preliminary in vitro results, certain HAQ have poor solubility in aqueous solution and this limits our ability to administer HAQ by the IT route at sufficiently high concentrations. In order to overcome this problem, our alternative will be to establish the maximal tolerated dose (MTD) for subcutaneous (SC) administration of HAQ by performing a dose escalation toxicity studies. We plan to test repeated daily SC injections of the selected HAQ(s) at two doses ranging from 10 to 50 mg/kg for up to 7 days. We will assess mice at the end of the HAQ regimen for local and systemic toxicity and inflammation.

The IT or SC delivery will be chosen based on the solubility of the tested HAQ and expected achievable concentrations. The requested numbers will be sufficient to provide reproducible descriptive data on the potential toxicity and inflammatory effects of HAQ.

Numbers required:
IT group
•4 mice per group x 2 HAQ x 2 doses + 4 controls = 20 mice; Repeat experiment x2 = 40 mice.
•blood differential cell count; plasma inflammatory cytokines; serum biochemistry;
•concurrently, BAL and lung homogenates for cell count differential, lung for histopathology
•all mice for clinical assessment of morbidity and mortality

SC group
•4 mice per group x 2 HAQ x 2 doses + 4 controls = 20 mice; Repeat experiment x2 = 40 mice.
•blood differential cell count; plasma inflammatory cytokines; serum biochemistry;
•concurrently, skin biopsy at injection site for histopathology
•all mice for clinical assessment of morbidity and mortality

Total n=80 mice

Experiment 2: To investigate the adjuvant activity of HAQ in a lethal Pseudomonas aeruginosa pneumonia model

If the HAQ cause no significant toxicity or inflammation in mice, we will assess whether HAQ used in combination with conventional antibiotic treatment improves the outcome in a lethal P. aeruginosa acute pneumonia model. We will compare the highest tolerated dose of [HAQ + antibiotic] vs. [carrier control + antibiotic] using 2 different HAQ. We will also use two different P. aeruginosa strains (one antibiotic susceptible and one resistant). The antibiotic tested will be based on prior in-vitro studies which demonstrate the greatest HAQ potency. We will perform a time course with sampling at 24h, 48h and 72h for each treatment group. Each experiment will be repeated twice for reproducibility.

For these studies, 8 mice per bacterial strain per time point per treatment conditions are required based on the statistical power calculation. This will have sufficient power to detect a 40% difference in survival or a difference of 1.25 log(10) in bacterial load between the two treatment conditions. Each experiment will be repeated twice for reproducibility.

Sample size calculation for a 2-sided comparison n=8: power=0.8, significance=0.05; for survival expected difference= 40%; for bacterial load std= 1, expected difference=1.25.
•N=8 per time point (24h, 48h, 72h) = 24 mice per group
•[2 HAQ + 1 control] = 3 x 24 = 72 mice per infection
•2 P. aeruginosa strains x 2 repeat experiments = 72 x 2 x 2 = 288 mice.

Experiment 3: To investigate the adjuvant activity of HAQ in a Pseudomonas aeruginosa cutaneous abscess model.

In the case of highly hydrophobic HAQ, we will plan to test SC HAQ in a cutaneous abscess model as an alternative to Experiment 2 without additional mice requested. This recently described infection model (Pletzer mBio 2017) has many advantages, including the local rather than systemic administration of antimicrobial compounds, and the ability to perform in vivo luminescence imaging to quantify bacterial burden. In this model, HAQ are administered SC directly into the SC infection site and are not as limited by their solubility in aqueous solution.

If SC injections of HAQ cause no significant toxicity or inflammation in mice, we will assess whether HAQ used in combination with conventional antibiotic treatment improves the outcome in a P. aeruginosa chronic cutaneous abscess model. We will compare the four different antimicrobial regimens and controls. We will also use four different P. aeruginosa strains (antibiotic susceptible vs resistant, invasive vs non-invasive). The antibiotic tested will be based on prior in-vitro studies which demonstrate the greatest HAQ potency. We will perform a time course with in vivo imaging every 24h for up to 7 days. Each experiment will be repeated twice for reproducibility.

Experiment 4: To investigate the adjuvant activity of HAQ in a severe Staphylococcus aureus pneumonia model

We plan to perform a similar set of experiments using a severe S. aureus pneumonia model. The experimental design and power calculations are similar to Experiment 2. For these studies, 8 mice per bacterial strain per time point per treatment conditions are required based on the statistical power calculation. This will have sufficient power to detect a 40% difference in survival or a

difference of 1.25 log(10) in bacterial load between the two treatment conditions. Each experiment will be repeated twice for reproducibility.

Sample size calculation for a 2-sided comparison n=8: power=0.8, significance=0.05; for survival expected difference= 40%; for bacterial load std= 1, expected difference=1.25.
•N=8 per time point (24h, 48h, 72h) = 24 mice per group
•[2 HAQ + 1 control] = 3 x 24 = 72 mice per infection
•2 S. aureus strains x 2 repeat experiments = 72 x 2 x 2 = 288 mice.

Experiment 5: To investigate the adjuvant activity of HAQ in a Staphylococcus aureus cutaneous abscess model.

In the case of highly hydrophobic HAQ, we will plan to test SC HAQ in a cutaneous abscess model as an alternative to Experiment 2 without additional mice requested. This recently described infection model (Pletzer mBio 2017) has many advantages, including the local rather than systemic administration of antimicrobial compounds, and the ability to perform in vivo luminescence imaging to quantify bacterial burden. In this model, HAQ are administered SC directly into the SC infection site and are not as limited by their solubility in aqueous solution.

If SC injections of HAQ cause no significant toxicity or inflammation in mice, we will assess whether HAQ used in combination with conventional antibiotic treatment improves the outcome in a P. aeruginosa chronic cutaneous abscess model. We will compare the four different antimicrobial regimens and controls. We will also use four different P. aeruginosa strains (antibiotic susceptible vs resistant, invasive vs non-invasive). The antibiotic tested will be based on prior in-vitro studies which demonstrate the greatest HAQ potency. We will perform a time course with in vivo imaging every 24h for up to 7 days. Each experiment will be repeated twice for reproducibility.

Experiment 6: Pilot experiment for subcutaneous infection

10 to 20 mice total for P. aeruginosa and S. aureus infections.

Breeding: Although C57B/6 mice are commercially available, we wish to have the option of using mice bred in house in this protocol in order to optimize the use of our animals. When available, mice will be transferred from our other protocols to this one. No additional breeding mice will be used.

Total for all 6 experiments: 80+ 288 + 288 + 20 = 676 + 40 extra = 716

You may attach supporting documentation below

Attachments List

File Spec	Description	Created

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20- Justification For Choice of Species

Need help?

Species Name	Mice
Please justify the need for live animals versus alternate methods (e.g. tissue culture, computer simulation)	
This goal of this study is to discover novel small molecules that will improve antibacterial therapy in the treatment of severe bacterial pneumonia. We will first carry out extensive in vitro studies to characterize the antimicrobial activity of HAQ molecules and the toxicity to mammalian cells using ex vivo cells. Following this, these in vivo studies will provide pre-clinical studies that will establish the tolerability and efficacy of these compounds. Such results are a critical step towards the development of novel antibacterials.	
Describe the characteristics of the species selected that justifies its use in the proposed study (consider characteristics such as body size, species, strain, data from previous studies or unique anatomic/physiological features)	
We choose to use C57BL/6 mice for several reasons: 1) The PI and collaborators have previously developed acute lung infection models using P. aeruginosa. 2) Extensive literature exists on the immune response and susceptibility of C57BL/6 mice to P. aeruginosa. 3) Sufficient literature available on C57BL/6 mice and there is no clear difference in susceptibility to S. aureus infections in different in-bred mice strains.	

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22- Species Strains List

Click the strain name to edit information about that strain.

Once done, you may add another strain if needed. When all are listed, click the "Continue" button to proceed with protocol completion.

Never use double colons, double semicolons or the + sign in strain names.

Need help?

Species Name	Mice	
Strain	**Age**	**Weight**
C57Bl6 wt	6-8 Weeks	20g

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Strain Information

Need help?

Principal Investigator	Nguyen, Dao
Protocol Application Number	2016-7801
Document Type	
Species Name	Mice
Species Strain	C57Bl6 wt
Age	6-8 Weeks
Weight	20g
Source of animals for this strain (specify which vendor, inhouse breeding, wild animal):	Charles River
Why is this strain necessary?	
Is this strain genetically modified (GM)?	No
Background Strain:	
Observed and/or expected phenotype - Include any trait that has affected the breeding, physical ability of the animal to move, eat, drink or result in a decreased lifespan:	
A. Heterozygotes:	n/a
B. Homozygotes:	n/a
Distress - Include information detailing whether the observed and/or expected phenotype will decrease or increase distress in the animal (include plans to monitor or alleviate this distress, frequency of monitoring, and how it may alter the Category of Invasiveness):	n/a

Attachments List

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23- Housing Location List

Need help?

Species Name	Mice
Facility Name	
Glen CL2 Facility	
Glen Housing BDF	
Glen Short-term Housing	

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Housing Location Information

List all the locations where live animals will be housed. It can be an animal facility and/or research laboratory and/or 'other country', etc.

If the location in **not** on the list, please request its addition by email to Darwin Support (darwin@mcgill.ca).

Note that the animal order form will be using the location(s) listed here, so please ensure that it is a complete list.

If a research laboratory, it will need to be inspected prior to its use and yearly thereafter - see information about the ALPHA Certificate.

Need help?

Species Name	Mice
Facility Name	Glen CL2 Facility

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Housing Location Information

List all the locations where live animals will be housed. It can be an animal facility and/or research laboratory and/or 'other country', etc.

If the location in **not** on the list, please request its addition by email to Darwin Support (darwin@mcgill.ca).

Note that the animal order form will be using the location(s) listed here, so please ensure that it is a complete list.

If a research laboratory, it will need to be inspected prior to its use and yearly thereafter - see information about the ALPHA Certificate.

Need help?

Species Name	Mice
Facility Name	Glen Housing BDF

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Housing Location Information

List all the locations where live animals will be housed. It can be an animal facility and/or research laboratory and/or 'other country', etc.

If the location in **not** on the list, please request its addition by email to Darwin Support (darwin@mcgill.ca).

Note that the animal order form will be using the location(s) listed here, so please ensure that it is a complete list.

If a research laboratory, it will need to be inspected prior to its use and yearly thereafter - see information about the ALPHA Certificate.

Need help?

Species Name	Mice
Facility Name	Glen Short-term Housing

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24- Procedures Location List

Need help?

Species Name	Mice
Facility Name	**Room No**
Glen CL2 Facility	1601CL2Unit1
Glen CL2 Facility	1603CL2Unit2
Glen CL2 Facility	1611CL2Unit3
Glen CL2 Facility	1621CL2Unit4
Glen Short-term Housing	7032Surgical3
Glen Short-term Housing	70442Surgical2
Glen Short-term Housing	70443Surgical1

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25- Non-Surgical Procedures List

Indicate the **non-surgical** procedures you will conduct. These can be injections, blood collection, imaging, behavioural tests, anesthesia, analgesia, gavage, etc... If you would like to see a procedure not found in the list below, please request from Darwin Support via email at darwin@mcgill.ca.

You must follow the applicable Standard Operating Procedures(SOPs). If your procedure is different from the SOP, you MUST justify and supply details in section "33- SOP Deviations".

If you are removing a procedure, remember to uncheck the corresponding Activity in the section "35- Personnel" for people who had been assigned to use it.

For assistance, contact the veterinarian.

Need help?

Species Name	Mice
Procedure Name	
Analgesia/sedation	
Anesthesia - Inhalant	
Anesthesia - Injection	
Blood Collection - Cardiac Puncture - terminal	

Injection - Intramuscular
Injection - Intraperitoneal
Injection - Subcutaneous
Imaging
Intratracheal Instillation

If desired procedure(s) is/are not on the list above, please specify the name of the procedure and supply details in section "32- Description of Procedures".	

Attachments List

File Spec	Description	Created

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27- Hazardous Material List

Included as potentially hazardous agents are: infectious material (bacteria/viruses/vectors/DNA affecting material), biological toxins, radioisotopes, toxic and carcinogenic agents, human and animal transplantable tumours as well as tissues and/or cell lines.

If you are removing an agent, remember to uncheck the corresponding Activity in the section "35- Personnel" for people who had been assigned to use it.

For information about biohazardous agents and the laws applicable to them, visit key links to the Public Health Agency of Canada (PHAC).

Need help?

Species Name	Mice

Agent	Type	Hzd. Brought into Facility?
Cyclophosphamide	Toxic Chemical	YES
Pseudomonas aeroginosa	Infectious	YES
Staphylococcus aureus	Infectious	YES
hydroxy-alkyl-quinolones	Toxic Chemical	YES

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Hazardous Material Information

SOPs for the use of some hazardous agents have been created and must be followed, see the list of SOPs for hazardous agents.

Need help?

Species Name	Mice
Hazardous Agent	Cyclophosphamide
Category	Toxic Chemical
Dosage (mg/kg), if applicable	200 mg/kg
Route of administration (check all applicable). If chose 'Other', specify route below.	INTRAPERITONEAL
If route is not on list, please specify other route(s)	
Frequency/Duration	once a day for 2 days prior to infection
If known, specify if doses are lower than the level at which it is considered a hazards for humans:	yes
After administration, will the animals be housed in the animal facility?	YES
After administration, will the animals be housed in the laboratory under supervision of laboratory personnel?	YES
Describe potential health risk(s) to humans or animals:	

If there is an SOP on this hazardous material, it must be followed. - Specify which room(s) will be used to prepare and administer the agent and where the animals will be housed post-administration.

If no SOP on this hazardous material, describe measures that will be used to reduce risk to the environment, the

animals and all research staff and animal facility personnel. Include the following:
A) Specify which room(s) will be used to prepare and administer the agent as well as where the animals will be housed post-administration;
B) If applicable, specify the additional personal protective equipment (PPE);
C) What type of safety cabinet is needed for administration of hazardous agent and for animal husbandry/handling;
D) Describe how bedding and syringes will be disposed of;
E) Explain what is to be done in case of a spill and/or accidental exposure;
F) If known, specify how long the animals and cages will be considered hazardous.

Ensure that special diet, cages or husbandry care information are included in the section "10- Animal Husbandry and Care" if applicable and that housing and procedures rooms before and after administration are listed in the sections "23- Housing Location" and "24- Procedures Location".

If no institutional SOP, please provide documentation that will contain pertinent information about the agent such as publications, MSDS or other institution's SOP.

See attached SOP.

Attachments List

File Spec	Description	Created
2016-7801_2_MICE_818_0001_Cytotoxic drug SOP.docx		06/27/2017

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Hazardous Material Information

SOPs for the use of some hazardous agents have been created and must be followed, see the list of SOPs for hazardous agents.

Need help?

Species Name	Mice
Hazardous Agent	Pseudomonas aeroginosa
Category	Infectious
Dosage (mg/kg), if applicable	10^5 to 10^8 CFU
Route of administration (check all applicable). If chose 'Other', specify route below.	OTHER;SUBCUTANEOUS
If route is not on list, please specify other route(s)	Intratracheal
Frequency/Duration	once
If known, specify if doses are lower than the level at which it is considered a hazards for humans:	Yes
After administration, will the animals be housed in the animal facility?	YES
After administration, will the animals be housed in the laboratory under supervision of laboratory personnel?	YES
Describe potential health risk(s) to humans or animals:	

If there is an SOP on this hazardous material, it must be followed. - Specify which room(s) will be used to prepare and administer the agent and where the animals will be housed post-administration.

If no SOP on this hazardous material, describe measures that will be used to reduce risk to the environment, the animals and all research staff and animal facility personnel. Include the following:
A) Specify which room(s) will be used to prepare and administer the agent as well as where the animals will be housed post-administration;
B) If applicable, specify the additional personal protective equipment (PPE);
C) What type of safety cabinet is needed for administration of hazardous agent and for animal husbandry/handling;
D) Describe how bedding and syringes will be disposed of;
E) Explain what is to be done in case of a spill and/or accidental exposure;
F) If known, specify how long the animals and cages will be considered hazardous.

Ensure that special diet, cages or husbandry care information are included in the section "10- Animal Husbandry and

Care" if applicable and that housing and procedures rooms before and after administration are listed in the sections "23- Housing Location" and "24- Procedures Location".

If no institutional SOP, please provide documentation that will contain pertinent information about the agent such as publications, MSDS or other institution's SOP.

Bacteria will be cultured and handled within a Class II biological safety cabinet within the RI MUHC labs and biohazard animal facility. All invasive procedures (treatment administration, euthanasia and tissue recovery) will be performed within a Class II biosafety cabinet (CL2 holding rooms). After the procedures, infected animals will be housed in CL2 housing units.

Intra-tracheal infection will require a microscope and will be performed outside of a BSC using the following precautions:

1- Perform all handling over plastic-backed absorbent paper (Ex: blue pad).
2- When withdrawing a needle from a stoppered bottle, wrap the needle and bottle cap in a disinfectant-soaked absorbent in order to minimize the generation of aerosols."

Tissues, lung lavage and blood recovered from infected animals will be carried out from the animal facility in sealed containers and manipulated in a Class II biological safety cabinet within the RI MUHC animal facility. Proper attire and rules for the biohazard animal facility will be respected, and all personnel involved will receive proper training. All work done with the infected mice and housing supplies is to be performed using personal protective equipment (masks, sleeves, goggles, gowns, gloves). Good personal hygiene, frequent hand washing and the avoidance of rubbing eyes as a precautionary measure against eye infections. It is unknown how long the infected animals and cages are hazardous. All infected animals, materials and waste will be considered potentially hazardous and will be decontaminated before disposal.

In addition:
-The cages are considered hazardous and should be clearly labelled with "Procedure" cards that include the name of the agent used in this experiment.
-It is strongly recommended that personnel working with rodent be immunized against Tetanus.
-In the case of percutaneous exposure (e.g. needle prick, cut or broken skin) there are some steps to be followed:

1. If the wound is bleeding profusely, request help and attempt to stop the bleeding as quickly as possible;
2. Wash the wound and surrounding area with soap and running water for 10 minutes;
3. Report to the nearest emergency for a medical follow-up;
4. Complete a "REPORT AND ANALYSIS WORK-RELATED INCIDENT/ACCIDENT AND OCCUPATIONAL DISEASE" form and send it to Environmental Health and Safety as soon as possible.

As per discussion with the SAIL platform and EHS, we will follow the SAIL-SOP-03 for transportation of animals, handling of cages and access of the SAIL platform. In addition, we will follow the following procedures

- Prior to imaging, all procedures will be done inside a Class II biological safety cabinet. A dedicated portable anesthesia device and oxygen chamber will be used for all CL2 infected mice, before and after the imaging procedure.
- Once mice are anesthetized, the site of injection will be disinfected with 70% alcohol 70 % and inject bacteria with a sterile syringe and needle. After the bacterial injection, the mice are placed back into their portable dedicated cage and the outside of the cage will be sprayed with Virox. Research personnel will degown and replace their PPE (gown, gloves) with clean ones and move the mice to the SAIL imaging room.
- We will place a Biohazard WARNING sign on the door window to the Xtreme imaging room to indicate to the personnel the ongoing use of Biohazards in the room.
- In the Xtreme imaging room, we will use new clean PPE (gown, glove and mask) and use a protective sheet on the desk, prior to placing the cage on it. The outside of the cage will be sprayed again with Virox. Infected mice (including abscess infection) can be safely handled with PPE in open air for transfer to the imaging unit because they do not generate biohazard aerosols.

1. When anesthetizing mice with injectable Ketamine + Xylazine, we will follow the SAIL-SOP-03 for anesthesia.

2. When anesthetizing mice with isoflurane, we will follow the following procedures:
- For imaging, anesthetized mice will be transferred to the covered imaging tray and placed inside individual nose cone for maintenance of isoflurane anesthesia. The anesthesia outline inside the Xtreme optical imager is equipped with a HEPA filter (changed after each session with CL2 mice) to avoid potential contamination. The imaging tray will be cleaned with Virox between each animal scan. Once the imaging completed, mice will be promptly returned to their cage for recovery or euthanasia in the CL2 holding rooms. At the end of the imaging session, the imaging tray, anesthesia nose cones and all surfaces inside the isoflurane imaging box will be cleaned with Virox, and the used HEPA filter is discarded in the biohazard waste.
- All PPE and materials used during the imaging will be disposed of in a Biohazard (garbage) bin inside the imaging room.

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Hazardous Material Information

SOPs for the use of some hazardous agents have been created and must be followed, see the list of SOPs for hazardous agents.

Need help?

Species Name	Mice
Hazardous Agent	Staphylococcus aureus
Category	Infectious
Dosage (mg/kg), if applicable	10^5 to 10^8
Route of administration (check all applicable). If chose 'Other', specify route below.	OTHER;SUBCUTANEOUS
If route is not on list, please specify other route(s)	intratracheal
Frequency/Duration	once
If known, specify if doses are lower than the level at which it is considered a hazards for humans:	yes
After administration, will the animals be housed in the animal facility?	YES
After administration, will the animals be housed in the laboratory under supervision of laboratory personnel?	YES
Describe potential health risk(s) to humans or animals:	

If there is an SOP on this hazardous material, it must be followed. - Specify which room(s) will be used to prepare and administer the agent and where the animals will be housed post-administration.

If no SOP on this hazardous material, describe measures that will be used to reduce risk to the environment, the animals and all research staff and animal facility personnel. Include the following:
A) Specify which room(s) will be used to prepare and administer the agent as well as where the animals will be housed post-administration;
B) If applicable, specify the additional personal protective equipment (PPE);
C) What type of safety cabinet is needed for administration of hazardous agent and for animal husbandry/handling;
D) Describe how bedding and syringes will be disposed of;
E) Explain what is to be done in case of a spill and/or accidental exposure;
F) If known, specify how long the animals and cages will be considered hazardous.

Ensure that special diet, cages or husbandry care information are included in the section "10- Animal Husbandry and Care" if applicable and that housing and procedures rooms before and after administration are listed in the sections "23- Housing Location" and "24- Procedures Location".

If no institutional SOP, please provide documentation that will contain pertinent information about the agent such as publications, MSDS or other institution's SOP.

Bacteria will be cultured and handled within a Class II biological safety cabinet within the RI MUHC labs and biohazard animal facility. All invasive procedures (treatment administration, euthanasia and tissue recovery) will be performed within a Class II biosafety cabinet (CL2 holding rooms). After the procedures, infected animals will be housed in CL2 housing units.

Intra-tracheal infection will require a microscope and will be performed outside of a BSC using the following precautions:

1- Perform all handling over plastic-backed absorbent paper (Ex: blue pad).
2- When withdrawing a needle from a stoppered bottle, wrap the needle and bottle cap in a disinfectant-soaked absorbent in order to minimize the generation of aerosols."

Tissues, lung lavage and blood recovered from infected animals will be carried out from the animal facility in sealed containers and manipulated in a Class II biological safety cabinet within the RI MUHC animal facility. Proper attire and rules for the biohazard animal facility will be respected, and all personnel involved will receive proper training. All work done with the infected mice and housing supplies is to be performed using personal protective equipment (masks, sleeves, goggles, gowns, gloves). Good personal hygiene, frequent hand washing and the avoidance of rubbing eyes as a precautionary measure against eye infections. It is unknown how long the infected animals and cages are hazardous. All infected animals, materials and waste will be considered potentially hazardous and will be decontaminated before disposal.

In addition:

-The cages are considered hazardous and should be clearly labelled with "Procedure" cards that include the name of the agent used in this experiment.
-It is strongly recommended that personnel working with rodent be immunized against Tetanus.
-In the case of percutaneous exposure (e.g. needle prick, cut or broken skin) there are some steps to be followed:

1. If the wound is bleeding profusely, request help and attempt to stop the bleeding as quickly as possible;
2. Wash the wound and surrounding area with soap and running water for 10 minutes;
3. Report to the nearest emergency for a medical follow-up;
4. Complete a "REPORT AND ANALYSIS WORK-RELATED INCIDENT/ACCIDENT AND OCCUPATIONAL DISEASE" form and send it to Environmental Health and Safety as soon as possible.

As per discussion with the SAIL platform and EHS, we will follow the SAIL-SOP-03 for transportation of animals, handling of cages and access of the SAIL platform. In addition, we will follow the following procedures

- Prior to imaging, all procedures will be done inside a Class II biological safety cabinet. A dedicated portable anesthesia device and oxygen chamber will be used for all CL2 infected mice, before and after the imaging procedure.
- Once mice are anesthetized, the site of injection will be disinfected with 70% alcohol 70 % and inject bacteria with a sterile syringe and needle. After the bacterial injection, the mice are placed back into their portable dedicated cage and the outside of the cage will be sprayed with Virox. Research personnel will degown and replace their PPE (gown, gloves) with clean ones and move the mice to the SAIL imaging room.
- We will place a Biohazard WARNING sign on the door window to the Xtreme imaging room to indicate to the personnel the ongoing use of Biohazards in the room.
- In the Xtreme imaging room, we will use new clean PPE (gown, glove and mask) and use a protective sheet on the desk, prior to placing the cage on it. The outside of the cage will be sprayed again with Virox. Infected mice (including abscess infection) can be safely handled with PPE in open air for transfer to the imaging unit because they do not generate biohazard aerosols.

1. When anesthetizing mice with injectable Ketamine + Xylazine, we will follow the SAIL-SOP-03 for anesthesia.

2. When anesthetizing mice with isoflurane, we will follow the following procedures:
- For imaging, anesthetized mice will be transferred to the covered imaging tray and placed inside individual nose cone for maintenance of isoflurane anesthesia. The anesthesia outline inside the Xtreme optical imager is equipped with a HEPA filter (changed after each session with CL2 mice) to avoid potential contamination. The imaging tray will be cleaned with Virox between each animal scan. Once the imaging completed, mice will be promptly returned to their cage for recovery or euthanasia in the CL2 holding rooms. At the end of the imaging session, the imaging tray, anesthesia nose cones and all surfaces inside the isoflurane imaging box will be cleaned with Virox, and the used HEPA filter is discarded in the biohazard waste.
- All PPE and materials used during the imaging will be disposed of in a Biohazard (garbage) bin inside the imaging room.

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Hazardous Material Information

SOPs for the use of some hazardous agents have been created and must be followed, see the list of SOPs for hazardous agents.

Need help?

Species Name	Mice
Hazardous Agent	hydroxy-alkyl-quinolones
Category	Toxic Chemical
Dosage (mg/kg), if applicable	0.25 to 50 mg/kg
Route of administration (check all applicable). If chose 'Other', specify route below.	OTHER;SUBCUTANEOUS
If route is not on list, please specify other route(s)	intratracheal
Frequency/Duration	once for IT, once daily for SC up to 7 days
If known, specify if doses are lower than the level at which it is considered a hazards for humans:	unknown
After administration, will the animals be housed in the animal facility?	YES
After administration, will the animals be housed in the laboratory under supervision of laboratory personnel?	NO

Describe potential health risk(s) to humans or animals:	

If there is an SOP on this hazardous material, it must be followed. - Specify which room(s) will be used to prepare and administer the agent and where the animals will be housed post-administration.

If no SOP on this hazardous material, describe measures that will be used to reduce risk to the environment, the animals and all research staff and animal facility personnel. Include the following:
A) Specify which room(s) will be used to prepare and administer the agent as well as where the animals will be housed post-administration;
B) If applicable, specify the additional personal protective equipment (PPE);
C) What type of safety cabinet is needed for administration of hazardous agent and for animal husbandry/handling;
D) Describe how bedding and syringes will be disposed of;
E) Explain what is to be done in case of a spill and/or accidental exposure;
F) If known, specify how long the animals and cages will be considered hazardous.

Ensure that special diet, cages or husbandry care information are included in the section "10- Animal Husbandry and Care" if applicable and that housing and procedures rooms before and after administration are listed in the sections "23- Housing Location" and "24- Procedures Location".

If no institutional SOP, please provide documentation that will contain pertinent information about the agent such as publications, MSDS or other institution's SOP.

The preparation of these compounds will be done under a certified chemical hood or biosafety cabinet. Handling of these compounds will be done using standard personal protective equipment, including masks, goggles, gowns and gloves. The compounds will be administered to mice under a certified biosafety cabinet and mice will be housed in the biohazard animal facility post-administration. Syringes and catheters used for the inoculation of the chemical will be disposed in a biohazard sharp containers provided by the animal facility. For the first cage change post-administration, the bedding and cages are considered contaminated. The waste has to be incinerated."The waste will be incinerated.

In the case of an accidental spill, these steps are to be followed: If PPE has become contaminated, remove and replace with clean ones; if eyes have been affected, flush eyes for 15 minutes. Clean the spill area with dry paper towels to absorb compounds then wipe down with 70% EtOH. Dispose of all waste material in the biohazard bin.

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28- Drugs and Other Substances List

Include pre-anesthetic, anesthesic, analgesic agents and ALL other substances, only used on live animals, EXCLUDING hazardous agents and drugs used for euthanasia - these are covered in their own protocol section.

You are expected to follow applicable SOPs for Analgesia, Anesthesia, Controlled Drugs and Substance Administration. Any deviation must be justified and detailed in the section "33 - SOP Deviations".

Consult the Web site for requirements on record keeping, storage and disposal of controlled drugs.

If you are removing a drug, remember to uncheck the corresponding Activity in the section "35- Personnel" for people who had been assigned to use it.

Need help?

Species Name	Mice

Agent	Type	Dosage (mg/kg)	Volume (ml)	Route(s)	Frequency/Duration
Aminoglycosides (e.g. tobramycin ot gentamicin)	Antibiotic	10-100 Mg/kg/day	0.1 mL	INTRAPERITONEAL;SUBCUTANEOUS	Bid Up To 96h
Ampicillin	Antibiotic	20 - 100mg/kg q8hrs	150ul/dose	INTRAMUSCULAR;INTRAPERITONEAL;SUBCUTANEOUS	Q8hrs Up To 96h
Carbapenem (e.g. imipenem, meropenem)	Antibiotic	50-100mg/kg/day	1mL	INTRAPERITONEAL;SUBCUTANEOUS	Bid Up To 96h
Dmso	Other	1 to 25%	up to 0.5 mL	OTHER;SUBCUTANEOUS	Once (intratracheal) Or Once A Day Sc For Up To 7 Days
Isoflurane	Anesthetic	2 to 5%		INHALANT	Up To Once A Day (15-60 Min) For Up To 7 Days
Ketamine-xylazine-acepromazine	Anesthetic	ketamine 100mg/kg, xylazine 10mg/kg, acepromazine	0.05-0.1mL/10g body weight	INTRAPERITONEAL	Once Prior To Intratracheal Instillation

		3mg/kg			
PBS (phosphate buffer saline)	Other		0.5 mL	INTRAPERITONEAL;OTHER;SUBCUTANEOUS	Once To Twice A Day For Up To 7 Days
quinolone (e.g. ofloxacin, ciprofloxacin)	Antibiotic	10-40mg/kg/day	0.1 mL	INTRAPERITONEAL;SUBCUTANEOUS	Bid Up To 96h

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Drug Information

For assistance in determining species-appropriate drugs and dosages, consult Standard Operating Procedures (SOPs), or contact the veterinarian or Animal Health Technician.

If the route of administration that you will be using is not in the choices here, please request its addition via email to darwin.support@mcgill.ca.

Need help?

Species Name	Mice
Drug Name	Ketamine-xylazine-acepromazine
Drug Type, if chose 'Other', specify type on this page.	Anesthetic
Dosage (mg/kg)	ketamine 100mg/kg, xylazine 10mg/kg, acepromazine 3mg/kg
Total volume(ml) per administration	0.05-0.1mL/10g body weight
Route of administration (check all applicable). If chose 'Other', specify route on this page.	INTRAPERITONEAL
Frequency and Duration of Administration	Once Prior To Intratracheal Instillation
Drug Trade Name	
Controlled Substance? If Darwin has answered "Yes", ensure it is kept under double-lock, keep a detailed log of use, dispose of expired drugs and follow the SOP on Controlled Drugs.	Yes

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Drug Information

For assistance in determining species-appropriate drugs and dosages, consult Standard Operating Procedures (SOPs), or contact the veterinarian or Animal Health Technician.

If the route of administration that you will be using is not in the choices here, please request its addition via email to darwin.support@mcgill.ca.

Need help?

Species Name	Mice
Drug Name	Isoflurane
Drug Type, if chose 'Other', specify type on this page.	Anesthetic
Dosage (mg/kg)	2 to 5%
Total volume(ml) per administration	
Route of administration (check all applicable). If chose 'Other', specify route on this page.	INHALANT
Frequency and Duration of Administration	Up To Once A Day (15-60 Min) For Up To 7 Days
Drug Trade Name	
Controlled Substance? If Darwin has answered "Yes", ensure it is kept under double-lock, keep a detailed log of use, dispose of expired drugs and follow the SOP on Controlled Drugs.	

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Drug Information

For assistance in determining species-appropriate drugs and dosages, consult Standard Operating Procedures (SOPs), or contact the veterinarian or Animal Health Technician.

If the route of administration that you will be using is not in the choices here, please request its addition via email to darwin.support@mcgill.ca.

Need help?

Species Name	Mice
Drug Name	Dmso
Drug Type, if chose 'Other', specify type on this page.	Other
Dosage (mg/kg)	1 to 25%
Total volume(ml) per administration	up to 0.5 mL
Route of administration (check all applicable). If chose 'Other', specify route on this page.	OTHER;SUBCUTANEOUS
Frequency and Duration of Administration	Once (intratracheal) Or Once A Day Sc For Up To 7 Days
Drug Trade Name	
Controlled Substance? If Darwin has answered "Yes", ensure it is kept under double-lock, keep a detailed log of use, dispose of expired drugs and follow the SOP on Controlled Drugs.	

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Drug Information

For assistance in determining species-appropriate drugs and dosages, consult Standard Operating Procedures (SOPs), or contact the veterinarian or Animal Health Technician.

If the route of administration that you will be using is not in the choices here, please request its addition via email to darwin.support@mcgill.ca.

Need help?

Species Name	Mice
Drug Name	Ampicillin
Drug Type, if chose 'Other', specify type on this page.	Antibiotic
Dosage (mg/kg)	20 - 100mg/kg q8hrs
Total volume(ml) per administration	150ul/dose
Route of administration (check all applicable). If chose 'Other', specify route on this page.	INTRAMUSCULAR;INTRAPERITONEAL;SUBCUTANEOUS
Frequency and Duration of Administration	Q8hrs Up To 96h
Drug Trade Name	
Controlled Substance? If Darwin has answered "Yes", ensure it is kept under double-lock, keep a detailed log of use, dispose of expired drugs and follow the SOP on Controlled Drugs.	

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Drug Information

For assistance in determining species-appropriate drugs and dosages, consult Standard Operating Procedures (SOPs), or contact the veterinarian or Animal Health Technician.

If the route of administration that you will be using is not in the choices here, please request its addition via email to darwin.support@mcgill.ca.

Need help?

Species Name	Mice
Drug Name	Aminoglycosides (e.g. tobramycin ot gentamicin)
Drug Type, if chose 'Other', specify type on this page.	Antibiotic
Dosage (mg/kg)	10-100 Mg/kg/day
Total volume(ml) per administration	0.1 mL
Route of administration (check all applicable). If chose 'Other', specify route on this page.	INTRAPERITONEAL;SUBCUTANEOUS
Frequency and Duration of Administration	Bid Up To 96h
Drug Trade Name	Aminoglycosides (e.g. tobramycin ot gentamicin)
Controlled Substance? If Darwin has answered "Yes", ensure it is kept under double-lock, keep a detailed log of use, dispose of expired drugs and follow the SOP on Controlled Drugs.	

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Drug Information

For assistance in determining species-appropriate drugs and dosages, consult Standard Operating Procedures (SOPs), or contact the veterinarian or Animal Health Technician.

If the route of administration that you will be using is not in the choices here, please request its addition via email to darwin.support@mcgill.ca.

Need help?

Species Name	Mice
Drug Name	quinolone (e.g. ofloxacin, ciprofloxacin)
Drug Type, if chose 'Other', specify type on this page.	Antibiotic
Dosage (mg/kg)	10-40mg/kg/day
Total volume(ml) per administration	0.1 mL
Route of administration (check all applicable). If chose 'Other', specify route on this page.	INTRAPERITONEAL;SUBCUTANEOUS
Frequency and Duration of Administration	Bid Up To 96h
Drug Trade Name	quinolone (e.g. ofloxacin, ciprofloxacin)
Controlled Substance? If Darwin has answered "Yes", ensure it is kept under double-lock, keep a detailed log of use, dispose of expired drugs and follow the SOP on Controlled Drugs.	

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Drug Information

For assistance in determining species-appropriate drugs and dosages, consult Standard Operating Procedures (SOPs), or contact the veterinarian or Animal Health Technician.

If the route of administration that you will be using is not in the choices here, please request its addition via email to darwin.support@mcgill.ca.

Need help?

Species Name	Mice
Drug Name	Carbapenem (e.g. imipenem, meropenem)
Drug Type, if chose 'Other', specify type on this page.	Antibiotic
Dosage (mg/kg)	50-100mg/kg/day
Total volume(ml) per administration	1mL
Route of administration (check all applicable). If chose 'Other', specify route on this page.	INTRAPERITONEAL;SUBCUTANEOUS
Frequency and Duration of Administration	Bid Up To 96h
Drug Trade Name	Carbapenem (e.g. imipenem, meropenem)
Controlled Substance? If Darwin has answered "Yes", ensure it is kept under double-lock, keep a detailed log of use, dispose of expired drugs and follow the SOP on Controlled Drugs.	

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Drug Information

For assistance in determining species-appropriate drugs and dosages, consult Standard Operating Procedures (SOPs), or contact the veterinarian or Animal Health Technician.

If the route of administration that you will be using is not in the choices here, please request its addition via email to darwin.support@mcgill.ca.

Need help?

Species Name	Mice
Drug Name	PBS (phosphate buffer saline)
Drug Type, if chose 'Other', specify type on this page.	Other
Dosage (mg/kg)	
Total volume(ml) per administration	0.5 mL
Route of administration (check all applicable). If chose 'Other', specify route on this page.	INTRAPERITONEAL;OTHER;SUBCUTANEOUS
Frequency and Duration of Administration	Once To Twice A Day For Up To 7 Days
Drug Trade Name	PBS (phosphate buffer saline)
Controlled Substance? If Darwin has answered "Yes", ensure it is kept under double-lock, keep a detailed log of use, dispose of expired drugs and follow the SOP on Controlled Drugs.	

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29- Euthanasia Methods List

List of SOPs on euthanasia.

Need help?

Species Name	Mice

Method
Isoflurane and CO2 followed by cervical dislocation

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Euthanasia Method Information

You are expected to follow applicable SOPs on euthanasia appropriate for the species involved. Any deviation must be justified in the section "33- SOP Deviations".

If the route of administration that you will be using is not in the choices, please request its addition via email to darwin.support@mcgill.ca.

To remove this agent, return to the euthanasia list of methods, check the box next to the method and click the button "Remove Selected Methods" - remember to uncheck the corresponding Activity in the section "35- Personnel" for people who had been assigned to use it.

Need help?

Species Name	Mice
Euthanasia Method	Isoflurane and CO2 followed by cervical dislocation

If method involve use of drugs, please specify drugs to be used and dosage for each.

Agent	Dose	Administration Route
Isoflurane [Anesthetic]	3-5%	INHALANT
CO2 [Euthanizing Agent]	4-8 L/min	INHALANT

For physical methods of euthanasia without anaesthesia, please justify:

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30- Categories of Invasiveness

The list of non-surgical procedures (section 25) and surgical procedures (section 26) you have previously specified, is listed below to use as a reference to help you determine the Category of Invasiveness.

Species Name	Mice

Type	Procedure
NON SURGICAL	Analgesia/sedation
NON SURGICAL	Anesthesia - Inhalant
NON SURGICAL	Anesthesia - Injection
NON SURGICAL	Blood Collection - Cardiac Puncture - terminal
NON SURGICAL	Injection - Intramuscular
NON SURGICAL	Injection - Intraperitoneal
NON SURGICAL	Injection - Subcutaneous
NON SURGICAL	Imaging
NON SURGICAL	Intratracheal Instillation

The grand total requested for this species you specified in section "18- Species Information" has been copied below as a reference.

Please enter the number of animals requested by Category of Invasiveness, according to the procedures they will undergo.

For a more detailed description of categories of invasiveness, refer to the Canadian Council on Animal Care (CCAC) web site.

Need help?

Total # Requested for Species	716

Category of Invasiveness	# of Animals
D	716

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31- Adverse Effects and Humane Intervention Points

Need help?

Species Name	Mice

A) Specify if you are expecting adverse effects to the animal health or welfare. If so, describe the clinical manifestations in chronological order. B) Based on the clinical manifestations described, determine chronologically the intervention points, i.e., the series of

actions taken to minimize pain or distress. These actions could be support therapy, analgesics, withdrawal of the experiment, euthanasia, etc.

C) If a renewal, state if you have experienced problems and report on the adequacy of the endpoints.

You are expected to follow the Humane Endpoints SOPs #410 and/or #412 and/or #415 when applicable.

In Exp 1, although not expected, mice may develop signs of toxicity due to the HAQ compounds, such as respiratory distress (for intra-tracheal injection), skin irritation (for subcutaneous injection), or general signs of distress. In Exp 2, 3, 4, 5 and 6, animals may develop systemic infections and show signs of distress. In Exp 3 and 5, mice are expected to develop abscess lesions approximately 24-48h after bacterial injection. They are not expected to, but may show signs of distress due severe abscess lesions. A pilot experiment for the subcutaneous abscess infections will be performed first with a report provided to the FACC. Mice are not expected to experience any distress directly related to cyclophosphomide administration but may develop infectious complications.

A humane intervention monitoring log will be used for all experiments. Mice that show signs of respiratory distress, have more than 20% weight loss, or display a level of morbidity of 0 or 1 (as per scale below) will be sacrificed immediately.

Three characteristics are observed to determine the level of morbidity in a mouse: response to stimuli, ease of movement, and condition of fur.

4+ A completely healthy mouse, uninfected - straight from the animal supplier
3+ After infection, responds to stimuli immediately, moves around a lot without stimuli
2+ After infection, responds to stimuli quickly, moves slowly
1 + After infection, does not respond well to stimulus, moves very slowly, some shaking may be seen, some torticollis may be seen.
0 Does not move even with stimuli, some shaking may be seen, coat may be rough, rolling on the longitudinal axis, severe torticollis. This is a moribund mouse and must be euthanized immediately as per SOP 410-01 Humane endpoints.

In addition, we will also monitor the appearance of the abscess for Exp 3 and 5 using the following scale. Mice that show an appearance of 3 or 4 will be immediately euthanized as per SOP 410-01 Humane endpoints.

Abscess appearance:
0 Abscess is healing (usual dry scabbing) or becoming smaller (<1.0cm)
1 Site is red and swollen but not ulcerated (<1.0cm)
2 Site is red and swollen and has a small but dry ulcer (<1.5 cm)
3 Site is ulcerated and moist but no visible pus, animal is scratching or chewing damaging the normal skin around the abscess (<1.5cm)
4 Skin is ulcerated (visible pus), moist wound, animal is self mutilating (scratching or chewing) damaging the normal skin around the abscess (>1.5cm)

It is important to note that bacterial burden alone will not be a sufficient endpoint, as C57BL/6 mice are intrinsically highly resistant to P. aeruginosa and S. aureus and can readily clear bacteria spontaneously from their lungs (up to >1,000,000 bacteria per mice). Given that the purpose of our study is to establish the efficacy of HAQ molecules in the treatment of severe bacterial pneumonia where conventional antibiotics fail to cure infections, our control groups (bacterial infection treated with antibiotics alone without HAQ) are expected to have a high level of morbidity and mortality, as they serve as a surrogate for treatment failure.

Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function (e.g. stress, radiation, steroids, chemotherapeutics, genetic modification of the immune system)? If yes, specify:

We will use cyclophosphamide 2 days prior to IT infection with Staphylococcus aureus. This will immunosupress the mice in order for the infection to be established. Without this, the mice are resistant and will clear all bacteria.

Describe how the monitoring frequency and extent will be increased proportionally to the severity of the clinical manifestations. If it is the responsibility of the animal facility staff, the PI should state it here.

Do not include names and phone numbers of specific people since this is covered in section "35- Personnel".

Frequency of monitoring:
- once a week for non-infected animals.
- daily following administration of cyclophosphamide and HAQ;
- twice daily after subcutaneous bacterial infection,
- at least 3 times per day during the first 72h following intra-tracheal bacterial infection.
- All mice will be weighed daily following infection and/or HAQ administration.

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32- Description of Procedures

In a narrative form, describe the use of the animals in a chronological order for each specific objective, including all interventions from the arrival to the disposal. It is not required to provide details on procedures already described in other sections.

You are expected to follow applicable SOPs, including SOP #531 on Animal Acquisition, Receiving and Acclimation . Any deviation to an SOP requires justification and details to section "33- SOP Deviations".

Need help?

Species Name	Mice

All animals will be purchased from a commercial source or obtained from our in house colonies. Commercially purchased animals will be received, and acclimated as per SOP 531.01 Animal Acquisition, Receiving and Acclimation.

Experiment 1. Blood and tissue biocompatibility and in vivo tolerability of HAQ.
1. Anesthesia: as per SOP 110_03 using ketamine/xylazine/acepromazine cocktail injected IP (SOP 404-01).
2. Non-surgical intra-tracheal (IT) instillation of HAQ compounds (50uL per animal) in order to deliver the compounds directly into the lungs. Once anaesthetized (with no pedal or ocular reflex), the animal is installed under a microscope in the vertical position and held on a restraining board by holding the animal by its upper incisor teeth. The tongue is then gently pulled to the side of the mouth in order to depress the tongue and allow visualization of the vocal chords. A curved 26-G gavage needle is then inserted into the mouth and guided through the pharynx into the trachea to instill the HAQ compounds. This will done under aseptic conditions outside a biosafety cabinet. All handling will be done over plastic-backed absorbent paper (Ex: blue pad). When withdrawing a needle from a stoppered bottle, we will wrap the needle and bottle cap in a disinfectant-soaked absorbent in order to minimize the generation of aerosols.
3. Daily care and evaluation: Animals typically recover completely from anesthesia and IT instillation within 1-2h without any residual adverse effects, and are not expected to suffer pain following the non-surgical IT procedure. They will be monitored twice daily for clinical symptoms for 72h. We do not know what adverse effects these compounds may cause, but literature on compounds of the same chemical family suggest that they are well tolerated in mammals within the dose range we will use. Possible adverse effects could include pulmonary or systemic inflammation.
4. Euthanasia as per SOP 301 at 72h or earlier if clinical endpoints have been reached as per SOP 410 Human interventions points, including weight loss >20%, respiratory distress, a BSC level 1+ or 0 as described below.

Alternative for SC HAQ administration:
1. Skin preparation: The fur on the backs of the mice will be shaved in order to observe possible skin lesions due to HAQ injection.
2. Subcutaneous (SC) injection of HAQ compounds (50uL per animal) will be done repeatedly in approximately the same area in the dorsum of the animal in order to approximate drug administration directly at the abscess site. Injections will be done under aseptic conditions inside a biosafety cabinet to avoid bacterial contamination of the skin. All handling will be done over plastic-backed absorbent paper (Ex: blue pad). Injections will be done daily for up to 7 days. When withdrawing a needle from a stoppered bottle, we will wrap the needle and bottle cap in a disinfectant-soaked absorbent in order to minimize the generation of aerosols.
3. Daily care and evaluation: Animals are not expected to suffer pain following the SC procedure. They will be monitored daily for clinical symptoms for up to 7 days. We do not know what adverse effects these compounds may cause, but literature on compounds of the same chemical family suggest that they are well tolerated in mammals within the dose range we will use. Possible adverse effects could include local or systemic inflammation.
4. Euthanasia as per SOP 301 at 72h or earlier if clinical endpoints have been reached as per SOP 410 Human interventions points, including weight loss >20%, skin lesions causing impaired function or self mutilation, a BSC level 1+ or 0 as described below.

We will use the following scale to determine the level of morbidity in mice (based on previous observation). Three characteristics are observed to determine the BSC level of morbidity in a mouse: response to stimuli, ease of movement, and condition of fur.
• 4+ A completely healthy mouse, uninfected – straight from the animal supplier
• 3+ After instillation of compound, responds to stimuli immediately, moves around a lot without stimuli
• 2+ After instillation of compound, responds to stimuli quickly, moves slowly
• 1+ After instillation of compound, does not respond well to stimulus, moves very slowly, some shaking may be seen, some torticollis may be seen.
• 0 Does not move even with stimuli, some shaking may be seen, coat may be rough, rolling on the longitudinal axis, severe torticollis. This is a moribund mouse and must be euthanized immediately.

Experiment 2. Severe P. aeruginosa pneumonia in immunocompetent mice

1. Anesthesia: as per SOP 110_03 using ketamine/xylazine/acepromazine cocktail injected IP (SOP 404-01).
2. Non-surgical intra-tracheal (IT) instillation of bacteria (P. aeruginosa 10^6 to 10^8 CFU per mice) in combination with HAQ compounds or carrier control (PBS +/- DMSO) in a total volume 50uL per animal. The procedure is performed as described above in Experiment 1.
3. Antibiotics will be given IP within 4h after bacterial infection.
4. Daily care and evaluation: Animals typically recover completely from anesthesia and IT instillation within 1-2h without any residual adverse effects, and are not expected to suffer pain following the non-surgical IT procedure. Infected mice in the control groups (antibiotics alone) are expected to have a high rate of morbidity and mortality within 24-72h following infection due to severe pulmonary compromise or disseminated infection. They will be monitored three times per day for clinical symptoms for 72h.
5. Euthanasia as per SOP 301 at 72h or earlier if clinical endpoints have been reached as per SOP 410 Human interventions points, including weight loss >20%, respiratory distress, a BSC level 1+ or 0 as described below.

Experiment 3. Subcutaneous P. aeruginosa abscess in immunocompetent mice

1. Skin preparation: The fur on the backs of the mice will be removed by shaving and application of chemical depilatories.
2. Subcutaneous infection of bacteria (10^6 to 10^8 CFU per mice) in combination with HAQ compounds or carrier control (PBS

+/- 1% DMSO) in a total volume 50uL per animal.
3. Antibiotics +/- HAQ (50 l) will be directly injected subcutaneously into the infected area [intra-abscess (IA) injection] at 1 h post infection and daily when indicated without anesthesia.
4. Daily care and evaluation: Animals are not expected to suffer pain or distress following the SC injections and abscess infection. In most infection, the SC abscess is well tolerated without signs of distress or morbidity. All mice will be monitored for the appearance of the abscess wound. Abscess lesion sizes will be measured daily using a caliper. In some infection, infected mice may show morbidity within 24-72h following infection due disseminated infection. They will be monitored twice daily for clinical symptoms for 72h. All mice will be euthanized after day 7 for enumeration of bacterial load and assessment of tissue histopathology.
5. Euthanasia as per SOP 301 at 72h or earlier if clinical endpoints have been reached as per SOP 410 Human interventions points, including weight loss >20%, a BSC level 1+ or 0, or if the abscess appearance is of level 3 or 4 as the score below.

Abscess appearance:
0 Abscess is healing (usual dry scabbing) or becoming smaller (<1.0cm)
1 Site is red and swollen but not ulcerated (<1.0cm)
2 Site is red and swollen and has a small but dry ulcer (<1.5 cm)
3 Site is ulcerated and moist but no visible pus, animal is scratching or chewing damaging the normal skin around the abscess (<1.5cm)
4 Skin is ulcerated (visible pus), moist wound, animal is self mutilating (scratching or chewing) damaging the normal skin around the abscess (>1.5cm)

Experiment 4. Severe S. aureus pneumonia in immunocompromised mice

1. To induce an immunocompromised state prior to S. aureus infection, mice will be treated with 200 mg/kg cyclophosphomide, injected IP at 1 and 2 days prior to infction.
2. Anesthesia: as per SOP 110_03 using ketamine/xylazine/acepromazine cocktail injected IP (SOP 404-01).
3. Non-surgical intra-tracheal (IT) instillation of bacteria (S. aureus 10^7 to 5x10^8 CFU per mice) in combination with HAQ compounds or carrier control (PBS +/- DMSO) in a total volume 50uL per animal. The procedure is performed as described above in Experiment 1.
4. Antibiotics will be given IP within 4h after bacterial inoculation.
5. Daily care and evaluation: Animals will be monitored starting after cyclophosphomide IP, administration. Animals typically recover completely from anesthesia and IT instillation within 1-2h without any residual adverse effects, and are not expected to suffer pain following the non-surgical IT procedure. Infected mice in the control groups (antibiotics alone) are expected to have a high rate of morbidity and mortality within 24-72h following infection due to severe pulmonary compromise or disseminated infection. They will be monitored three times per day for clinical symptoms twice daily for 72h. Possible adverse effects include pulmonary or systemic toxicity.
6. Euthanasia as per SOP 301 at 72h or earlier if clinical endpoints have been reached as per SOP 410 Human interventions points, including weight loss >20%, respiratory distress, a BSC level 1+ or 0 as described below.

Experiment 5. Subcutaneous S. aureus abscess in immunocompetent mice
1. Skin preparation: The fur on the backs of the mice will be removed by shaving and application of chemical depilatories.
2. Subcutaneous infection of bacteria (10^6 to 10^8 CFU per mice) in combination with HAQ compounds or carrier control (PBS +/- 1% DMSO or MeOH) in a total volume 50uL per animal.
3. Antibiotics +/- HAQ (50 l) will be directly injected subcutaneously into the infected area [intra-abscess (IA) injection] at 1 h post infection and daily when indicated without anesthesia.
4. Daily care and evaluation: Animals are not expected to suffer pain or distress following the SC injections and abscess infection. In most infection, the SC abscess is well tolerated without signs of distress or morbidity. All mice will be monitored for the appearance of the abcess wound. Abscess lesion sizes will be measured daily using a caliper. In some infection, infected mice may show morbidity within 24-72h following infection due disseminated infection. They will be monitored twice daily for clinical symptoms for 72h. All mice will be euthanized after day 7 for enumeration of bacterial load and assessment of tissue histopathology.
6. Euthanasia as per SOP 301 at 72h or earlier if clinical endpoints have been reached as per SOP 410 Human interventions points, including weight loss >20%, a BSC level 1+ or 0, or if the abscess appearance is of level 3 or 4 as per Exp 3.

Experiment 6: Pilot experiment for P. aeruginosa or S. aureus abscess in immunocompetent mice
A pilot experiment of procedures described in Exp 3 and 5 will be performed and a report will be submitted to the FACC prior to carrying out Exp 3 and 5.

Optional procedure:

As an optional procedure for Experiments 2, 3, 4, 5 and 6, we will add in vivo imaging of infected mice with the SAIL imaging suite. Animals will be infected with P. aeruginosa or S. aureus (bacteria carrying a bioluminescent genetic reporter) as described in the respective experiments. On the day prior to Xtreme imaging, mice will be anesthetized with Ketamine + Xylazine. Once there are no pedal or ocular reflex, we will shave the mice abdomen with an electric razor, followed by cream hair remover (Nair). The mice are then after washed under warm water and dried with clean paper towel. After the shaving procedure, the mice will be moved to the CL2 holding area, be placed in a heat source and observed until their complete awakening.

At different time points (as specified in each experiment's procedure) infected mice will be brought from CL2 holding room into the SAIL imaging suite as per SAIL-SOP-03. Mice will be anesthetized as per SAIL-Xtreme-SOP-02 using Ketamine + Xylazine + acepromazine (100/10/3 Mg/kg) with a volume of 0.1ml/10g of Body weight inject intraperitonealy, and an additional half dose

may be administered, as per SOP 110, if anesthesia needs to be extended for more than 30 minutes. Alternatively, mice will be anesthetized using isoflurane as per SOP 110 in order to allow for safe repeated anesthesia and imaging over short periods of time (e.g. once or twice daily).

While anesthetized, the animal will be moved to the Bruker In-Vivo Xtreme optical imager and placed in the supine position in an imaging tray. The animals will be maintained at ~37oC using an air-warming system, and are expected to remain under anesthesia for a total time of approximately 30 minutes. Biohazard considerations regarding the handling of infected are specifically discussed in the Biohazard section. Following completion of imaging, animals will be given approximately 0.5 mL of sterile, warmed saline subcutaneously, returned to the procedure suite, and monitored during recovery from anesthesia under a warming lamp. The imaging tray and surrounding surfaces will be cleaned with disinfectant between each animal scan and after imaging, as described in the Biohazard section.

Attachments List

File Spec	Description	Created
2016-7801_2_MICE_0001_110-mouse_anesthesia.pdf	SOP 110	06/27/2017
2016-7801_2_MICE_0001_SAIL-SOP-03-v01 - CL2 Animals.doc	SAIL SOP CL2	06/27/2017

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33- SOP Deviations

Need help?

Species Name	Mice
Will there be any SOP deviations on the animals of this species?	YES
If yes, please specify which procedure(s) deviates from the Standard Operating Procedure, supply detailed information on the procedure(s) and justify why the deviation is being requested.	SOP# 705 will not be followed as a microscope is needed for the administration of the CL2 agent. However, we will: 1- Perform all handling over plastic-backed absorbent paper (Ex: blue pad). 2- When withdrawing a needle from a stoppered bottle, wrap the needle and bottle cap in a disinfectant-soaked absorbent in order to minimize the generation of aerosols. SOP-SAIL-02 will not be followed as we will also use isoflurane anesthesia during imaging. Please see Procedures section for details.

You may attach supporting documentation in the "Attach File" section below.

Attachments List

File Spec	Description	Created

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34- Environmental Enrichment

Environment enrichment is covered by species specific Standard Operating Procedures (SOPs)

Need help?

Species Name	Mice
Will standard environment enrichment be provided according to standard operating procedure (SOP)by the animal facility staff?	YES
If not using SOP, what will be used (if any), by whom and what is the justification? If not applicable (such as for wild animals in the field), state N/A.	

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35- Personnel List

List the names of all individuals who will be in contact with animals in this study and their employment classification (co-investigator, technician, research assistant, undergraduate / graduate student, teaching assistants and fellow). Do not add a person if he/she will not be starting to work soon.

Note that the **Principal Investigator's name** is automatically included BUT his/her name needs to be clicked in order to answer the questions and select the species and Activities performed by the PI.

If this is a renewal, an amendment or a copy from an existing protocol, you must **click each of the names to ensure all questions for this person are answered and Activities assigned.**

Training is mandatory for all personnel listed here. Refer to Training Requirements for details.

For new people added to the list, it may take a few days to receive access to Darwin. If access is urgently needed, please request it via the Web form."

Everyone is covered under an **Occupational Health Program (OHP)**. For all students as well as McGill staff and faculty, see the Environmental Health and Safety Web site to apply. For staff in hospitals with a Health Office, please contact them; if no Health Office, you may apply to McGill's OHP. To find out more, visit the OHP section of the Theory course.

If you have removed procedures, drugs, euthanasia methods, hazards and surgeries, please uncheck the box for the corresponding Activity for each person who had been assigned to perform or use it.

NOTE: Sometimes the red dot does not change to green for this section (intermittent issue), continue with the next sections and submit the protocol or amendment as usual; it will not prevent you from submitting it to the FACC. If you are unable, please contact Darwin Support, darwin@mcgill.ca.

Need help?

Name	Phone + Ext	Email	Cell Phone	Emergency Contact	Monitor?
Nguyen, Dao	934-1934 42534	dao.nguyen@mcgill.ca		Yes	
Houle, Daniel	934-1934 X76396	daniel.houle555@gmail.com		Yes	Yes
Casgrain, Pierre-Andre	44857	pierre-andre.casgrain@mail.mcgill.ca		Yes	Yes

Note that if a person is removed from all the protocols under this Principal Investigator (PI), then Darwin login access will be revoked entirely.

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Personnel Information

If this person is designated as an emergency contact, a minimum of 2 phone numbers are required.

Only an institutional email address is permitted such as first.last@mcgill.ca and not first.last@gmail.com.

Need help?

Name	Nguyen, Dao
Classification	Principal Investigator
If an undergraduate student, specify who will be supervising him/her:	
Phone + Ext	934-1934 42534
Email	dao.nguyen@mcgill.ca
Fax	514-933-3962
Home Phone	
Cell Phone	
Card Access ID	
This is an Emergency Contact on this Protocol	Yes
This person will be monitoring the animals:	

You MUST answer "Yes" or "No" to ALL the questions below. This will prompt the list of Activities (procedures, drugs, euthanasia...) specified earlier in the protocol on the next page. You will then need to choose which Activities the person will be performing.

Need help?

Will person be handling live animal?	NO
Will person be performing animal procedures?	NO
Will person be handling drugs?	NO
Will person be handling controlled substances?	NO
Will person be administering hazardous agents?	NO
Will person euthanize animals?	NO

Must select the species handled by this person.

Species Name

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Personnel Protocol Related Activities

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Personnel Information

If this person is designated as an emergency contact, a minimum of 2 phone numbers are required.

Only an institutional email address is permitted such as first.last@mcgill.ca and not first.last@gmail.com.

Need help?

Name	Houle, Daniel
Classification	Research Assistant
If an undergraduate student, specify who will be supervising him/her:	
Phone + Ext	934-1934 X76396
Email	d
Fax	
Home Phone	
Cell Phone	
Card Access ID	000005
This is an Emergency Contact on this Protocol	Yes
This person will be monitoring the animals:	Yes

You MUST answer "Yes" or "No" to ALL the questions below. This will prompt the list of Activities (procedures, drugs, euthanasia...) specified earlier in the protocol on the next page. You will then need to choose which Activities the person will be performing.

Need help?

Will person be handling live animal?	YES
Will person be performing animal procedures?	YES
Will person be handling drugs?	YES
Will person be handling controlled substances?	YES
Will person be administering hazardous agents?	YES
Will person euthanize animals?	YES

Must select the species handled by this person.

Species Name
Mice

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Personnel Protocol Related Activities

Please check the boxes for all agents and procedures that this person will be using/performing. Animal related training verification depends on the information supplied here.

Need help?

Person Activities > Drugs

Species Name	Agent
Mice	quinolone (e.g. ofloxacin, ciprofloxacin)
Mice	hydroxy-alkyl-quinolones
Mice	Ketamine-xylazine-acepromazine
Mice	Ketamine-xylazine
Mice	Carbapenem (e.g. imipenem, meropenem)
Mice	Ampicillin
Mice	Aminoglycosides (e.g. tobramycin ot gentamicin)

Person Activities > Euthanasia

Species Name	Euthanasia Method
Mice	Isoflurane and CO2 followed by cervical dislocation

Person Activities > Hazardous Agents

Species Name	Agent	Type
Mice	hydroxy-alkyl-quinolones	Toxic Chemical
Mice	Staphylococcus aureus	Infectious
Mice	Pseudomonas aeroginosa	Infectious
Mice	Cyclophosphamide	Toxic Chemical

Person Activities > Procedures

Species Name	Procedure Name
Mice	Intratracheal Instillation
Mice	Intratracheal Injection
Mice	Injection - Subcutaneous
Mice	Injection - Intraperitoneal
Mice	Injection - Intramuscular
Mice	Blood Collection - Cardiac Puncture - terminal
Mice	Anesthesia - Injection
Mice	Anesthesia - Inhalant
Mice	Analgesia/sedation

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Personnel Information

If this person is designated as an emergency contact, a minimum of 2 phone numbers are required.

Only an institutional email address is permitted such as first.last@mcgill.ca and not first.last@gmail.com.

Need help?

Name	Casgrain, Pierre-Andre
Classification	Research Assistant
If an undergraduate student, specify who will be supervising him/her:	
Phone + Ext	44857
Email	pierre-andre.casgrain@mail.mcgill.ca
Fax	
Home Phone	
Cell Phone	
Card Access ID	
This is an Emergency Contact on this Protocol	Yes
This person will be monitoring the animals:	Yes

You MUST answer "Yes" or "No" to ALL the questions below. This will prompt the list of Activities (procedures, drugs, euthanasia...) specified earlier in the protocol on the next page. You will then need to choose which Activities the person will be performing.

Need help?

Will person be handling live animal?	YES
Will person be performing animal procedures?	YES
Will person be handling drugs?	YES
Will person be handling controlled substances?	YES
Will person be administering hazardous agents?	YES
Will person euthanize animals?	YES

Must select the species handled by this person.

Species Name
Mice

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Personnel Protocol Related Activities

Please check the boxes for all agents and procedures that this person will be using/performing. Animal related training verification depends on the information supplied here.

Need help?

Person Activities > Drugs

Species Name	Agent
Mice	quinolone (e.g. ofloxacin, ciprofloxacin)
Mice	Ketamine-xylazine-acepromazine
Mice	Carbapenem (e.g. imipenem, meropenem)
Mice	Ampicillin
Mice	Aminoglycosides (e.g. tobramycin ot gentamicin)

Person Activities > Euthanasia

Species Name	Euthanasia Method
Mice	Isoflurane and CO2 followed by cervical dislocation

Person Activities > Hazardous Agents

Species Name	Agent	Type
Mice	Staphylococcus aureus	Infectious
Mice	Pseudomonas aeroginosa	Infectious
Mice	hydroxy-alkyl-quinolones	Toxic Chemical
Mice	Cyclophosphamide	Toxic Chemical

Person Activities > Procedures

Species Name	Procedure Name
Mice	Intratracheal Instillation
Mice	Injection - Subcutaneous
Mice	Injection - Intraperitoneal
Mice	Injection - Intramuscular
Mice	Blood Collection - Cardiac Puncture - terminal
Mice	Anesthesia - Injection
Mice	Anesthesia - Inhalant
Mice	Analgesia/sedation

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36- The 3Rs: Replacement, Reduction and Refinement

Need help?

Address the alternatives you have examined in relation to the 3 R's (Replacement, Reduction, Refinement), refer to the CCAC 3 Rs Microsite.

Replacement: We are interested in testing the tolerability and antibacterial activity of novel compounds (called HAQ) in the treatment of several bacterial infections. As part of this project, we will perform in vitro experiments using bacteria and cell lines to obtain preliminary data on the toxicity and antibacterial activity of the novel compounds. The murine experiments will be done to establish the in vivo activity of these drugs, as there are many parameters critical to the pre-clinical assessment of potential drugs, which cannot not be assessed solely through in vitro studies.

Reduction: The minimum number of mice is always used, while ensuring that we obtain the sufficient number of animals to reach statistical power. Our procedures are associated with potential complications, and there is always inherent variability in how mice respond to infections and treatment. In order to minimize the number of mice, we have added in vivo luminescence imaging which will provide an alternative assessment of the bacterial burden in the infected mice. This may allow us to reduced the number of time points where mice will be euthanized. We have included all of the procedures known to maximize the success of the procedure and minimize the variability. Further reduction in the numbers may jeopardize our ability to obtain sufficient numbers for proper interpretation and publication of results.

Refinement: The intracheal instillation technique has been refined from a traditional surgical intratracheal tehcnique to a non-surgical technique where injection is performed under direct visualization of the vocal cords. The subcutaneous abscess infection model is expected to cause minimal morbidity and is less likely to cause disseminated infections than the severe pneumonia model Through these refinements, we minimize the pain and discomfort of the mice, and reduce the mortality associated with the infections.

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Electronic Signature

Principal Investigator	Nguyen, Dao
Protocol Application Number	2016-7801
Document Type	

The information in this application is exact and complete. I declare that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be renewed on an annual basis. Approval from the committee does not guarantee availability of space, equipment and services; contact the animal facility supervisor for requirement. Everyone listed in the protocol must have read the Animal Use Protocol's sections pertinent to what they are mandated to perform.

Important note to Apple OS X Safari users:
A recent update of the Apple OS X Safari browser causes an Autofill compatibility issue with several web forms, including Darwin web forms. Safari Autofill overwrites the last text field above your password on web forms. Click here for a solution.

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