Characterizing the Antimicrobial Activity of N^2,N^4-Disubstituted Quinazoline-2,4-Diamines Towards Multidrug Resistant *Acinetobacter baumannii*

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Abstract

We previously reported a series of N^2,N^4-disubstituted quinazoline-2,4-diamine as dihydrofolate reductase inhibitors, with potent in vitro and in vivo antibacterial activity against MRSA strains. In this work we extend our previous study to the Gram-negative pathogen *Acinetobacter baumannii*. We determined that optimized N^2,N^4-disubstituted quinazoline-2,4-diamines are strongly antibacterial against multi-drug resistant *A. baumannii* strains when the 6-position is substituted with a halide or an alkyl substituent. Such agents display potent antibacterial activity, with MICs as low as 0.5 μM, whilst proving to be strongly bactericidal. Interestingly, these compounds also possess the potential for anti-biofilm activity, eradicating 90% of cells within a biofilm at or near MIC concentrations. Using serial passage assays we observed a limited capacity for the development of resistance towards these molecules (4-fold increase in MIC) compared to existing folic acid synthesis inhibitors, such as trimethoprim (64-fold increase) and sulfamethoxazole (128-fold increase). We also identified limited toxicity towards human cells, with LD_{50} values of ≤ 23 μM for lead agents **4** and **5.** Finally we demonstrate that our lead agents have excellent in vivo efficacy, with lead agent **5** proving more efficacious than tigecycline in a murine model of *A. baumannii* infection (90 % survival vs 66%), despite being used at a lower dose (2 mg kg^{-1} vs 30 mg kg^{-1}). As such, we demonstrate that N^2,N^4-disubstituted quinazoline-2,4-diamines have strong antimicrobial and anti-biofilm activity, against both Gram-positive organisms and Gram-negative pathogens, suggesting strong potential for their development as antibacterial agents.

Introduction

Acinetobacter baumannii is one of the most successful nosocomial pathogens, causing infections that have over the past few decades become increasingly difficult to treat. The prolonged ability of *A. baumannii* to survive on abiotic surfaces, alongside broad antimicrobial resistance, allows it not only to survive, but thrive in hospital settings.(1) Consequently, there has been an alarming increase in mortality associated with infections caused by this difficult to treat organism.(2) In addition to eliciting fatal nosocomial infections, this pathogen is a primary agent of infections in military personnel, often resulting from combat trauma or burns.(3, 4) These often result in chronic wound infections and biofilm-mediated disease, with the latter resulting from surgery and implanted devices.(4) Such chronic *A. baumannii* infections lead to complications, extended rehabilitation, increased use of hospital resources, and considerably increased mortality.(4)

Drug resistance in *A. baumannii* has resulted in few antibiotics left to eradicate the infections it causes, with clinicians often turning to last resort, toxic treatment options.(1),(5, 6) The worldwide incidence of pan drug resistant (PDR) *A. baumannii* has spread quickly, at least in part due to its naturally transformable nature, leading to an increased capacity to acquire new determinants of resistance.(1), (6) The occurrence of PDR isolates, with no effective treatment options, seemingly marks the beginning of a post-antibiotic era for *A. baumannii*; thus, measures must be taken to identify effective therapeutic options.(7)

Quinazolines are an emerging class of compounds that have a broad range of biological activities ranging from anti-cancer, anti-inflammatory, anti-psychotic, anti-diabetic, anti-leishmanial,(8, 9) and anti-bacterial.(10-15) Kung et al. discovered a series of 2-substituted quinazolines with broad spectrum antibacterial activity, inhibiting RNA synthesis and translation in a number of bacterial species.(16) More relevant to this study, Harris et al. revealed 5-substituted 2,4-diaminoquinazolines that inhibited the dihydrofolate reductase (DHFR) enzyme of *Escherichia coli* and *S. aureus*.(17) In so doing, they

67 determined that the 5-substituted position of the 2,4-diaminoquinazolines was not as important for
68 enzyme binding affinity as the general structural type of the group. Unfortunately, these molecules were
69 not specific towards the bacterial DHFR enzyme, but also inhibited the bovine liver DHFR enzyme.(17)
70 Further analysis revealed that smaller substituents created greater activity in bacterial cells while larger
71 substituents were more active towards the bovine enzyme. However, unlike the quinazolines identified in
72 this study, the 5-substituted 2,4-diaminoquinazolines proved ineffective in animal models of
73 infection.(17)

74 Our group has recently shown the utility of N^2,N^4-disubstituted quinazoline-2,4-diamines for the treatment
75 of *S. aureus* infections.(18) Specifically, we have shown them to be active against a library of MRSA
76 isolates, displaying strong bactericidal activities, with limited cytotoxic and hemolytic capacities towards
77 human cells. Mechanism of action profiling reveals that, much like other quinazoline compounds, they
78 appear to function by targeting bacterial dihydrofolate reductase.(18-21) We have also shown their
79 potential for anti-biofilm activity, low frequencies of mutation, and in vivo efficacy using murine models
80 of infection.(18)

81 In this study, we have further explored the impact of N^2,N^4-disubstituted quinazoline-2,4-diamines as
82 antibacterial agents, focusing specifically on the Gram negative species *A. baumannii*. Using a library of
83 multi-drug resistant isolates, we reveal that these compounds are broadly bactericidal, dihydrofolate
84 reductase inhibitors. In addition, we observed that these compounds have low incidences of resistance and
85 possess the potential for anti-biofilm activity. Finally, we show the compounds are efficacious in vivo
86 using a murine model of *A. baumannii* infection. As such, we demonstrate for the first time the very real
87 potential of quinazoline derived compounds as antibacterial agents against the important human pathogen,
88 *A. baumannii*.

89

Materials and Methods

General. All strains used in this study are listed in **Table S1**.

Synthetic Protocols and Compound Characterization: Full details of compounds synthesis and characterization can be found in the supplemental materials.

Antibacterial activity assessment. Minimal inhibitory concentration **(**MIC) and minimal bactericidal concentration (MBC) assays were performed in this study as documented by us previously.(18, 22-24) Briefly, *A. baumannii* strains were grown in tryptic soy broth overnight cultures at 37 °C with shaking. MIC determination was performed in a 96-well plate by diluting overnight cultures 1:1000 in Mueller Hinton broth (MHB) and adding 195 µL to each well. Subsequently, 5 µL of quinazoline (or control compounds) were added before incubation for 24 hours at 37 °C. Following, this MICs were determined as the lowest concentration to produce a complete absence of growth. All compounds were diluted prior to testing in DMSO to assess multiple concentrations with the addition of the same volume of solvent. MBC assays were performed in an identical manner to MIC experiments, however after 24 hours incubation, bacterial cells were serially diluted in phosphate buffered saline (PBS) and recovered on antibiotic free tryptic soy agar (TSA) for 24 hours at 37 °C. MBC_{90} values were calculated using linear regression of the percent recovery compared to no treatment controls.

Biofilm eradication determination assay. These assays were performed as described by us previously,(24, 25) as follows. Each of the *A. baumannii* strains were grown overnight in MHB. The next day these were used to seed fresh MHB to an OD_{600} of 0.5, with 150 µL then added to the wells of a 96 well plate and grown for 24 hours at 37 °C. After 24 hours, the planktonic bacteria were carefully removed and fresh MHB was added with increasing concentrations of lead quinazolines. After incubation at 37 °C for 24 hours, planktonic cells were removed and biofilms were washed three times with PBS. Biofilms were then resuspended in PBS and plated for cell viability on TSA. Biofilm recovery was assessed compared to no drug controls, and determined as percent eradication. This was used to determine

$MBEC_{90}$ values (Minimal Biofilm Eradication Concentration), where the viability of cells within the biofilm was reduced by 90%.

Investigating the Mechanism of Action of Quinazoline-Based Compounds. To evaluate the effect quinazolines have on DHFR reduction of dihydrofolic acid, a tetrahydrofolic acid rescue assay was performed as described by us previously.(18) *A. baumannii* strain 1403 was grown overnight in LB, and then diluted 1:1000 into fresh media. These cultures were then seeded into a sterile 96-well plate with tetrahydrofolic acid added at concentrations ranging from 0 to 225 μM. Lead quinazoline **25** was then added at 1X, 2X, and 5X the MIC and cultures were incubated at 37 °C for 18 hours. MICs were determined and used to assess whether the addition of tetrahydrofolic acid rescued *A. baumannii* growth from quinazoline inhibition. Assays were repeated in triplicate, alongside trimethoprim and sulfamethoxazole controls.

Serial Passage assay. In order to test potential resistance towards the quinazolines, a serial passage assay was performed alongside control compounds (sulfamethoxazole and trimethoprim), as described by us previously.(24) *A. baumannii* strain 1403 was grown overnight in LB media at 37 °C. The next day cultures were diluted 1:100 in fresh media and seeded into a 96-well plate. Lead quinazolines or control agents were added to respective wells at half MIC concentrations. Plates were then incubated for 24 hours at 37 °C, followed by the removal of aliquots from these cultures to inoculate fresh media (1:100 dilution) containing compounds at a 2-fold higher concentrations. These were then grown overnight, and the procedure repeated for a total of eight days. The cultures were observed for a lack of growth, indicating strains were no longer able to resist the action of a given compound. Each experiment was performed in triplicate, yielding identical results.

HepG2 cytotoxicity. Cytotoxicity assays were performed using human HepG2 cells (human liver epithelial with hepatocellular carcinoma), as described by us previously.(18, 24) Cells were cultured in Dulbecco's modified eagle medium (DMEM), supplemented with 10% fetal bovine serum and 1%

penicillin-streptomycin for 3 days at 37 °C and 5% CO_2. Cells were then diluted to 1 x 10^5 ml^{-1} using fresh DMEM and added to 96 well tissue culture plates at a volume of 100 µL. Plates were incubated for 24 hours at 37 °C and 5% CO_2, allowing the cells to adhere to the plastic. After this time, media was carefully removed and 200 µL fresh DMEM added with test compounds at concentrations: 0, 1, 2, 5, 10, 15, 30, and 50 µM. Plates were then incubated for 48 hours at 37 °C and 5% CO_2. After 48 hours the DMEM was removed and 100 µL of new media was added containing MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), followed by incubation for four hours at 37 °C and 5% CO_2. After 4h, 75 µL of media was removed, replaced with 50 µL of 16% w/v SDS and DMSO followed by incubation for ten minutes at 37 °C to solubilize any formazan produced. A Biotek plate reader was used to measure the absorbance of formazan production at 540 nM. Lead compounds were solvated in 100% DMSO for these studies, which served as the negative control. LD_{50} values were determined for each compound by comparison to vehicle only controls.

Hemolysis assay. A hemolysis assay was performed using whole human blood (Bioreclamation), as described previously.(24) Briefly, human red blood cells (hRBCs) were resuspended in 20% v/v 1X HA buffer (4.25 mL 10% NaCl; 1mL $CaCl_2$ in 50 mL sterile water), before lead compounds were added at 2 µM, 10 µM and 20 µM, in a final volume of 100 µL. Cells were incubated for 15 minutes at 37 °C before being centrifuged at 5,500 g for 1 minute to pellet non-lysed hRBCs. The supernatant was removed, added to a 96-well Microtiter plate and the OD_{543} read using a BioTek Synergy2 plate reader. The negative control was vehicle only (DMSO), and the positive control was 1% triton X-100. Assays were performed in triplicate, with data displayed as percent hemolysis compared to controls, defined as: Percent Hemolysis = (OD_{543} test sample – OD_{543} no drug control) / (OD_{543} triton X-100 – OD_{543} no drug control) x 100.

In vivo Efficacy Testing Using a Murine Model of Lethal Peritonitis. A murine model of lethal peritonitis was used to demonstrate the effectiveness of the lead quinazolines to clear bacterial infections, as described by us previously.(24) Six mice per group were infected via I.P. injection (right side) with 7.5

x 10^8 CFU mL^{-1} of *A. baumannii* 1646 in PBS containing 5% mucin. After 1h, mice were inoculated by I.P. injection to the left side of the abdomen with either 2 mg/kg of lead agent **25** (test group); 30 mg/kg tigecycline (positive control); or vehicle alone (45% w/v (2-hydroxypropyl)-β-cyclodextrin in water (negative control). Mice were monitored twice daily for five days to assess mortality. All animal studies received written approval after review by the Institutional Animal Care & Use Committee in the Division of Comparative Medicine & Division of Research Integrity & Compliance at the University of South Florida. The clinical endpoint was reached for this study when the mice reached a pre-moribund state. The number of mice surviving between control and treatment groups was compared and analyzed for statistical significance using a log-rank (Mantel-Cox) test.

Results and Discussion

N^2,N^4-disubstituted quinazoline-2,4-diamines are active against multi-drug resistant *A. baumannii* isolates. We have previously reported the activity of N^2,N^4-disubstituted quinazoline-2,4-diamines against MRSA strains (18). To determine if our compounds have activity against any other bacterial species, we screened them against the other ESKAPE pathogens. In so doing, we identified a number of analogues that were effective against *A. baumannii*, but lacked activity towards other members of the ESKAPE pathogen set. To explore these findings more broadly, we expanded our studies to include a clonally diverse collection of *A. baumannii* isolates **(Table 1)**. Strong activity was found against a number of strains, with single digit micromolar MICs noted for three benzenoid substituted N^2-benzyl-N^4-methylquinazolin-2,4-diamines **1**, **2**, and **3** against the 1646 strain.

Following the identification of active quinazolines **1**, **2**, and **3**, additional N^2-benzyl-N^4-methylquinazolin-2,4-diamines were made with either 6- or 7- substitutions **(Table 2)**. Substitution at the 6-position with a bromo or a methyl group was found to be more beneficial for activity than substitution at the 7-position when comparing 6-bromoquinazolin-2,4-diamine **4** with its 7-substituted counterpart **6** or the 6-methyl-substituted quinazoline **5** with its 7-substituted analogue **7**. Importantly, N^2-benzyl-N^4-methylquinazolin-2,4-diamine analogue **8**, which lacks any substitution at the benzenoid ring, was inactive with an MIC of ≥50 μM and therefore demonstrated the importance of a 6- or 7-substuent on the benzenoid ring.

A similar trend was observed with N^4-benzyl-N^2-methylquinazolin-2,4-diamine analogues when comparing 6-substituted compounds **10** and **11** with the 7-substituted analogues **14** and **15 (Table 3).** Furthermore, substitution in 6- or 7-position with an electron withdrawing chloro or a bromo moiety yielded quinazolines **9**, **10**, **13**, or **14** which were more potent than corresponding methyl- or methoxy-substituted analogues **11**, **12**, or **15**. Of all the quinazolines of the first two subseries tested, only compound **5** was active against the clinically important 1403 strain with an MIC of 10 μM, leading us to believe that continued work on the benzenoid ring would be highly beneficial.

Extending the N^2-benzyl chain to a N^2-phenethyl was investigated to see if an increase in activity would be found **(Table 4)**. Compound **16** with no benzenoid substitution was 4-fold more active than the benzyl analogue **8 (Table 1).** Compounds **17** – **20** were also found to be slightly more potent than the benzyl analogues **9** – **12 (Table 3)** with MICs of 2 or 4 μM.

With the importance of substitution at the 6-position identified, new analogues were evaluated with vinyl, alkyl or aryl substitutions **(Table 5) (Figure S1)**. While the MIC barrier of 2 μM was not broken against the most susceptible strain (1646), major advances were seen in activity against the most resistant isolate (1403). In particular, *n*-pentyl-, cyclohexenyl- and cyclohexyl-substituted quinazolines **27**, **29** and **30** had MICs of 2 μM against most isolates besides the 1652 strain for which they had MICs of 10 μM and 30 μM. These three compounds revealed that large, bulky and lipophilic groups at the 6-position are not only tolerated but beneficial for inhibiting the growth of *A. baumannii*. Phenyl- and furanyl-substituted quinazolines **31** and **32** were less active, as were the vinyl and ethyl analogues **22** and **23**, the isopropenyl and isopropyl analogues **24** and **25**, and the cyclopentenyl-quinazoline **28**.

Lead Quinazolines are Bactericidal in Activity. Lead quinazolines **4**, **5**, **26**, **29**, **27**, and **30** were selected to be further evaluated for antimicrobial effects. The first assay utilized was a minimal bactericidal concentration (MBC) assay, to assess whether leads compounds were bacteriostatic or bactericidal. The six lead agents were screened to identify their MBC_{90} towards each of the six *A. baumannii* isolates used in the SAR studies **(Table S2)**, with data for isolate 1646 detailed in **Table 6**. Lead agents were all found to be broadly bactericidal, with MBC_{90} values ranging from 0.77 μM to 1.8 μM. Compounds **4** and **5** were found to be the most efficacious at eliminating bacterial growth, with MBC_{90} values of 0.81 μM and 0.77 μM respectively. Further to this, we were able to obtain complete eradication of bacterial growth for these two compounds at 1 μM for **24** and 5 μM for **25**. Although marginally less effective, compounds **26**, **29**, **30**, and **27**, all still efficiently reduced bacterial viability, with MBC_{90} values of 1.8 μM, 1.5 μM, 1.1 μM, and 1.1 μM respectively. Moreover, compound **26** resulted in complete bacterial eradication at 5 μM, which is only 5x its MIC.

Front Runner Agents Impact the Viability of Cells Within a Biofilm. *A. baumannii*, like many nosocomial pathogens, utilizes biofilm formation to increase persistence and decrease sensitivity to the action of antibiotics. Accordingly, the ability to impact cell viability within a biofilm is an important attribute for novel antimicrobial compounds. As such, we next tested our isolates for this activity, again using our library of multi-drug resistant strains **(Table S2)**, with data from strain 1646 shown in **Table 6**. As with our bactericidal profiling, lead quinazolines **4** and **5** again had the most promising activity, with 90% biofilm eradication ($MBEC_{90}$) seen at 3.3 µM and 2.8 µM, respectively **(Table 6)**. Further to this, analogue **5** was the most effective lead agent with a 3-log reduction in biofilm viability observed at 10 µM. Compound **4** reduced biofilm viability by 3.6-log, but not until a concentration of 50 µM. Lead quinazoline **30** had biofilm eradication potential similar to **4**, reducing viability by 4.2-log at 50 µM, although its $MBEC_{90}$ (1 log reduction) was found to be close to this value at 41 µM. Compounds **26** and **29** also had promising activity with both displaying $MBEC_{90}$ at a concentration of 8.9 µM. Extended testing with these two quinazolines revealed that compound **29** reduced biofilm viability by 1.6-log at 50 µM, while **26** resulted in a 1.4-log reduction in biofilm viability at the same concentration.

Lead Quinazoline Derivatives Appear to Function by Targeting Dihydrofolate Reductase. To determine if lead quinazolines inhibit the *A. baumannii* dihydrofolate reductase (DHFR) enzyme, similar to that seen for sister compounds in our work with MRSA, an in vitro rescue assay was performed.(18) Accordingly, the viability of *A. baumannii* cells was tested using lead quinazoline **5** in the presence or absence of tetrahydrofolic acid (THF, 0 µM-225µM), the end product produced by DHFR. After 24 hours incubation, we determined that only 10 µM of THF was sufficient to rescue bacterial growth from the inhibitory effects of lead agent **25**. This data suggests the potential mechanism of action for our compounds is perhaps via inhibition of tetrahydrofolic acid production. Whilst this finding supports data generated by ourselves and others regarding the impact of quinazoline molecules on bacterial cells,(18-21) we cannot discount the possibility of other potential targets within for these compounds within *A. baumannii*.

Quinazolines Derived Compounds Induce Limited Capacity for Resistance. An important attribute of novel antibiotics is the ability to fend off the development of resistance towards their effects. To assess this capacity, *A. baumannii* strains were incubated overnight with 0.5x MIC of each of the frontrunners. The next day cells were washed and used to inoculate fresh media that contained a two-fold increase in drug. This was repeated for a total of 8 days, alongside sulfamethoxazole (SMX) and trimethoprim (TMP) controls, both of which target the same pathway as our lead agents (**Figure 1**), as well as an unrelated agents, tetracycline (TET).(26) Upon analysis, we determined that all of our frontrunner compounds out performed SMX, TMP and TET, generating much lower incidences of resistance. Specifically, lead agents **4** and **5** had the most striking effects with MICs increasing over the 8-day test period by only 4-fold, compared to 64-fold (TMP) and 128-fold (SMX and TET) for the control agents. Each of the other 4 agents were similarly impressive in their ability to limited resistance, resulting in an increased MIC of only 16-fold, which, whilst not as promising as **4** and **5**, is still profoundly reduced compared to our controls.

Front Runner Quinazolines Have Limited Toxicity Towards Human Cells. In order to gain a sense of the toxicity of lead quinazolines towards eukaryotic cells, we determined LD_{50} values for human HepG2 cells **(Table 6, Figure 2A)**. Importantly, we observed >50% cell viability for all compounds at concentrations up to 6 µM. Furthermore, 4 of our 6 leads returned >50% viability at 12 µM, whilst **26** and **29** were only marginally less promising, returning HepG2 cell viabilities of 49% and 42%, respectively, at this concentration. When treated with 25 µM of each lead quinazoline or control antibiotics we observed only fractionally less than 50% recovery. Importantly, lead agents **4** and **5** at 25 µM performed the best, with 43% and 44% viability observed, respectively. Similarly, lead agents **26** and **30** allowed for 41% and 39% respective viability at this concentration, while treatment with **27** and **29** resulted in 31% and 32% viability, respectively. The control compounds sulfamethoxazole and trimethoprim returned 46% and 45% viability at the highest concentration tested, which is in line with data generated from our front runners. To place lead compound data in context, **4** and **5** have the greatest therapeutic window for

infection treatment. Specifically, lead agent **4** possesses a 46-fold preference in specificity towards bacteria, with an MIC (0.5 μM) much lower than the LD_{50} (23 μM) towards human liver cells. Similarly, lead agent **5** displayed a 22-fold activity index (AI =LD_{50} / MIC), which is a measure of specificity towards bacterial cells **(Table 6)**. As an additional measure of toxicity, we next tested the hemolytic capacity of the front runners using whole human blood (**Figure 2B**). Importantly, we observed negligible capacity of our lead quinazolines to lyse human red blood cells when incubated for 1 hour at a concentration of 10 μM. Specifically, we observed average hemolysis well below 1% (range = 0.24% – 0.47%), whilst the positive control (1% triton X-100) produced 100% lysis during a similar time frame.

***N*^{2},*N*^{4}-disubstituted quinazoline-2,4-diamines are efficacious in vivo.** As a final assessment, we used a murine model of lethal *A. baumannii* infection to determine the efficacy of quinazolines in vivo. This was performed using frontrunner **5**, which had the most promising properties from all of our biological testing. Accordingly, mice were inoculated with a lethal dose of *A. baumannii* via intraperitoneal injection on the right side of the abdomen. One hour post challenge, mice were treated with an intraperitoneal injection of 2 mg kg^{-1} of frontrunner **5** on the left side of the abdomen. As a control, we also performed similar testing using 30 mg kg^{-1} of tigecycline, which we already know our test strain to be susceptible to in vitro. In so doing, we determined that quinazoline **5** resulted in a statistically significant survival rate of 83% of infected animals, compared to only 17% for vehicle only controls **(Figure 3)**. We also saw significant survival of animals injected with tigecycline, although this was at a rate of 66%, which is inferior to that of our frontrunner agent. As such, this would suggest that our class of *N*^{2},*N*^{4}-disubstitutedquinazoline-2,4-diamines have excellent potential for development as antibacterial agents targeting multi-drug resistant *A. baumannii* infections.

Concluding Remarks: A library of *N*^{2},*N*^{4}-disubstituted quinazoline-2,4-diamines, which was previously shown to have antibacterial activity against MRSA,(18) was also found to have potent effects towards the multi-drug resistant Gram negative species *A. baumannii*. We assessed seventy-three *N*^{2},*N*^{4}-disubstituted quinazoline-2,4-diamines and found that 6- or 7-substituted *N*^{2}-benzyl-*N*^{4}-methylquinazoline-2,4-

297 diamines displayed promising activity, with MICs ranging from 0.5 to 30 μM against the six strains of *A.*
298 *baumannii* tested. Over thirty molecules were designed and synthesized to conduct a structure-activity
299 relationship study to systematically probe the substituents in the N^2-, N^4-, 6-, and 7-positions. The most
300 potent in vitro activities were obtained with quinazoline-2,4-diamines bearing a N^2-benzyl moiety and a
301 N^4-methyl group. Furthermore, quinazolines substituted in 6-position with a halide or alkyl group were
302 more potent compared to analogs substituted at the 7-position. 6-*n*-Pentyl- and 6-cyclohexyl-substituted
303 quinazolines **27** and **30** were among the most effective agents since they were equipotent with single-digit
304 μM MICs against the six tested *A. baumannii strains*. Following, frontrunner compounds **4**, **5**, **26**, **29**, **27**,
305 and **30** were tested for bactericidal activities and biofilm eradication. We found the lead quinazolines **4**
306 and **5** displayed the strongest bactericidal and biofilm activity towards *A. baumannii*, with MBC_{90} values <
307 1 μM and $MBEC_{90}$ values < 4 μM. These compounds also allowed for limited resistance development,
308 displaying only a 4-fold increase in MIC against *A. baumannii* over an 8-day period, which was only a
309 fraction of that observed for control compounds. Using a murine model of infection, we determined that
310 lead agent **5** was more effective, and at lower concentrations, in rescuing mice from a lethal dose of *A.*
311 *baumannii* than our control agent tigecycline. Our results reveal the potent antibacterial activities of N^2-
312 benzyl-N^4-methylquinazoline-2,4-diamines against *A. baumannii*, and show their potential for
313 development to treat both Gram-positive and Gram-negative multidrug resistant infections.

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398 **Figure 1. Lead quinazolines generate limited resistance by *A. baumannii* isolates.** The six lead
399 quinazolines were tested in a stepwise serial passage assay, alongside sulfamethoxazole (SMX),
400 trimethoprim (TMP) and tetracycline (TET) controls. The first passage began with 0.5 X MIC and with
401 each passage the concentration of all compounds was increased by 2-fold. The graph displays the fold
402 increase in the MIC over the course of 8 days. Representative data generated using strain 1403 is shown.

403

404 **Figure 2. Cytotoxicity towards human cells. A:** Lead quinazolines were tested at 25 µM, 12 µM, 6 µM,
405 and 3 µM, against human HepG2 cells compared to solvent only controls. The known antibiotics
406 sulfamethoxazole (SMX) and trimethoprim (TMP), which target the same pathway as our quinazolines,
407 were also tested in parallel. **B:** The six lead quinazolines were tested at 10 µM against whole human
408 blood for the ability to lyse erythrocytes. Shown is the percent lysis of human red blood cells compared to
409 a triton X-100 (T) control at a concentration of 1%.

410

411 **Figure 3. Lead quinazolines are efficacious in vivo.** Mice were administered with a lethal dose of *A.*
412 *baumannii* 1646. They were then give lead quinazoline **5** at 2 mg kg^{-1}, tigecycline at 30 mg kg^{-1}, or
413 vehicle. Survival was monitored over a 5 day period. Statistical significance was determined using a log-
414 rank (Mantel-Cox) test; *=$p<0.05$.

415

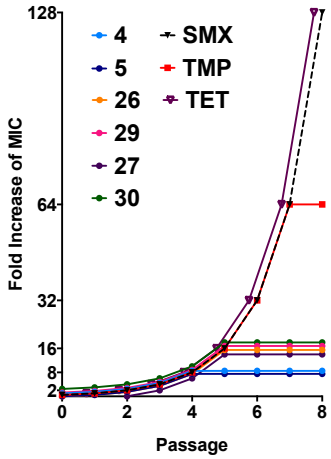


Figure 1. Lead quinazolines generate limited resistance by *A. baumannii* isolates. The six lead quinazolines were tested in a stepwise serial passage assay, alongside sulfamethoxazole (SMX), Trimethoprim (TMP) and Tetracycline (TET) controls. The first passage began with 0.5 X MIC and with each passage the concentration of all compounds was increased by 2-fold. The graph displays the fold increase in the MIC over the course of 8 days. Representative data generated using strain 1403 is shown.

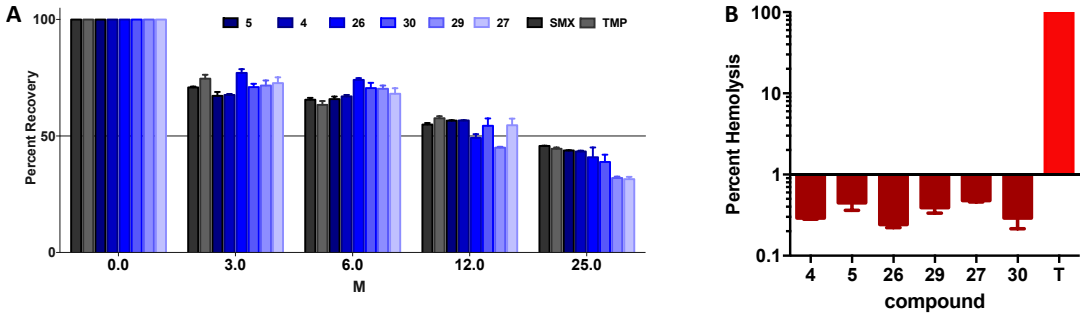


Figure 2. Cytotoxicity towards human cells. A: Lead quinazolines were tested at 25 µM, 12 µM, 6 µM, and 3 µM, against human HepG2 cells compared to solvent only controls. The known antibiotics sulfamethoxazole (SMX) and trimethoprim (TMP), which target the same pathway as our quinazolines, were also tested in parallel. **B:** The six lead quinazolines were tested at 10 µM against whole human blood for the ability to lyse erythrocytes. Shown is the percent lysis of human red blood cells compared to a Triton X-100 (T) control at a concentration of 1%.

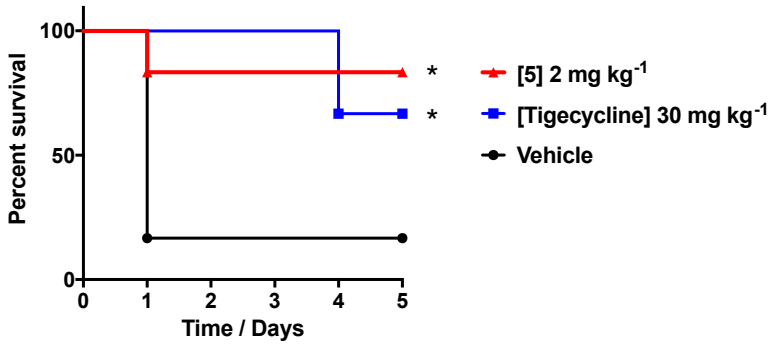
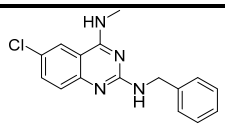
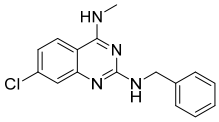
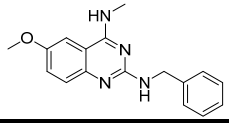


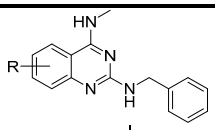
Figure 3. Lead quinazolines are efficacious in vivo. Mice were administered with a lethal dose of *A. baumannii* 1646. They were then give lead quinazoline **5** at 2 mg kg^{-1}, tigecycline at 30 mg kg^{-1}, or vehicle. Survival was monitored over a 5 day period. Statistical significance was determined using a log-rank (Mantel-Cox) test; *=$p<0.05$.

Table 1. SAR focusing on benzenoid ring substitution of various quinazoline-2,4-diamines[a]

Compound	R	1403 MIC (μM)	1646 MIC (μM)	1649 MIC (μM)	1650 MIC (μM)	1651 MIC (μM	1652 MIC (μM)
1		50	2	25	15	6	20
2		>50	6	30	>50	10	50
3		>50	2	25	50	10	30

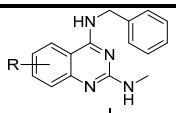
[a] Sulfamethoxazole (SMX) and trimethoprim (TMP) are internal controls for each in vitro MIC assay: SMX, 138 μM 1403, 118 μM 1646, 118 μM 1649, 118 μM 1650, 118 μM 1651, and 118 μM 1652; TMP, 103 μM 1403, 34 μM 1646, 517 μM 1649, 120 μM 1650, 103 μM 1651, and 103μM 1652.

Table 2. Probing benzenoid substitution of N^2-benzyl-N^4-methylquinazolin-2,4-diamines[a]

 Compound	R	1403 MIC (μM)	1646 MIC (μM)	1649 MIC (μM)	1650 MIC (μM)	1651 MIC (μM)	1652 MIC (μM)
4	6-Br	50	2	12	15	8	20
5	6-Me	10	2	25	50	10	20
6	7-Br	>50	6	25	>50	10	>50
7	7-Me	50	12	25	50	12	35
8	6-H, 7-H	>50	50	>50	>50	>50	>50

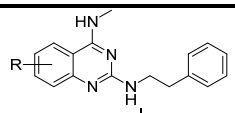
[a] Sulfamethoxazole (SMX) and trimethoprim (TMP) are internal controls for each in vitro MIC assay: SMX, 138 μM 1403, 118 μM 1646, 118 μM 1649, 118 μM 1650, 118 μM 1651, and 118 μM 1652; TMP, 103 μM 1403, 34 μM 1646, 517 μM 1649, 120 μM 1650, 103 μM 1651, and 103μM 1652.

Table 3. Probing benzenoid ring substitution of N^4-benzyl-N^2-methylquinazolin-2,4-diamines[a]

 Compound	R	1403 MIC (μM)	1646 MIC (μM)	1649 MIC (μM)	1650 MIC (μM)	1651 MIC (μM)	1652 MIC (μM)
9	6-Cl	>50	4	50	50	6	>50
10	6-Br	>50	2	50	>50	>50	>50
11	6-Me	>50	6	>50	>50	12	>50
12	6-OMe	>50	6	>50	>50	8	>50
13	7-Cl	>50	4	>50	>50	>50	>50
14	7-Br	>50	4	>50	>50	>50	>50
15	7-Me	>50	12	>50	>50	>50	>50

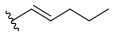
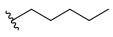
[a] Sulfamethoxazole (SMX) and trimethoprim (TMP) are internal controls for each in vitro MIC assay: SMX, 138 μM 1403, 118 μM 1646, 118 μM 1649, 118 μM 1650, 118 μM 1651, and 118 μM 1652; TMP, 103 μM 1403, 34 μM 1646, 517 μM 1649, 120 μM 1650, 103 μM 1651, and 103μM 1652.

Table 4. Benzenoid ring substitutions of N^4-methyl-N^2-phenethylquinazolin-2,4-diamines[a]

 Compound	R	1403 MIC (μM)	1646 MIC (μM)	1649 MIC (μM)	1650 MIC (μM)	1651 MIC (μM)	1652 MIC (μM)
16	6-H	>50	12	>50	>50	>50	>50
17	6-Cl	>50	2	>50	>50	>50	>50
18	6-OMe	>50	4	>50	>50	>50	>50
19	6-Br	>50	2	>50	>50	10	>50
20	6-Me	>50	2	>50	>50	10	>50

[a] Sulfamethoxazole (SMX) and trimethoprim (TMP) are internal controls for each in vitro MIC assay: SMX, 138 μM 1403, 118 μM 1646, 118 μM 1649, 118 μM 1650, 118 μM 1651, and 118 μM 1652; TMP, 103 μM 1403, 34 μM 1646, 517 μM 1649, 120 μM 1650, 103 μM 1651, and 103μM 1652.

Table 5. Extension of the 6-position of N^2-benzyl-N^4-methylquinazolin-2,4-diamines[a]

Compound	R	1403 MIC (μM)	1646 MIC (μM)	1649 MIC (μM)	1650 MIC (μM)	1651 MIC (μM)	1652 MIC (μM)
21	6-Me	10	2	25	50	10	20
22		25	2	10	20	25	25
23		30	2	10	30	2	30
24		>50	2	25	>50	10	>50
25		>50	25	35	>50	35	>50
26		10	2	10	25	2	30
27		2	2	2	2	2	10
28		>50	2	50	>50	30	>50
29		2	2	10	10	2	30
30		2	2	2	2	2	10
31		30	10	20	20	2	50
32		40	25	>50	>50	15	>50

[a] Sulfamethoxazole (SMX) and trimethoprim (TMP) are internal controls for each in vitro MIC assay: SMX, 138 μM 1403, 118 μM 1646, 118 μM 1649, 118 μM 1650, 118 μM 1651, and 118 μM 1652; TMP, 103 μM 1403, 34 μM 1646, 517 μM 1649, 120 μM 1650, 103 μM 1651, and 103μM 1652.

Table 6. In vitro antibacterial assessment of front runner quinazoline compounds against multi-drug resistant *A. baumannii*. Activity Index = LD50 / MIC

Compound	**4**	**5**	**26**	**29**	**27**	**30**
MIC [μM]	0.5	1	1	2	1	2
MBC_{90}[μM]	0.8	0.8	1.8	1.5	1.1	1.1
$MBEC_{90}$ [μM]	3.3	2.8	8.9	8.9	11.5	41.2
LD_{50} (HepG2) [μM]	23	22	16	23	12	11
Activity Index	46	22	22	12	12	6