Combatting antimicrobial resistance: Small-molecule inhibitors as precision anti-virulence agents.

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Combatting antimicrobial resistance: Small-molecule inhibitors as precision anti-virulence agents.

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Abstract

Antimicrobial resistance (AMR) is an ever-increasing global problem and it is estimated that deaths associated with AMR infections will exceed 10 million by 2050, superseding cancer as the leading cause of global mortality. Traditional antibiotics display antimicrobial activity through direct targeting of key bacterial cellular processes such as cell wall formation, which are essential for viability but are susceptible to resistance generation. In contrast, one approach to combating AMR is the development of novel small-molecule inhibitors (SMIs) as anti-virulence agents, which target pathogen specific virulence-related traits, such as enzymes involved in the generation of post-translational modifications, biofilm formation and toxin production. This study aimed to determine the anti-virulence activity of a novel library of SMIs designed for precision targeting of essential epigenetic bacterial targets. Antimicrobial susceptibility and time-kill kinetic assays identified four lead candidates which demonstrated activity against methicillin-resistant *Staphylococcus aureus* (MRSA). One lead SMI was able to resolve MRSA infections in the *Galleria mellonella in vivo* model, with no observed cytotoxicity being exhibited within the larvae. Research is now focused on determining the downstream effects of these SMIs on MRSA virulence traits, such as biofilm formation and quorum sensing, in addition to confirming the bacterial cellular target site via affinity chromatography coupled with determining binding affinity. This research represented a significant advance in the search for novel antimicrobial agents which target essential bacterial processes beyond those associated with traditional antibiotics.

1 Introduction

Antimicrobial resistance (AMR) in bacteria is a major global issue, which is currently responsible for over 700,000 deaths per year worldwide (NICE impact antimicrobial resistance,2018), and it is predicted to overtake cancer as the leading cause of global mortality by 2050. The World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), and the World Organization for Animal Health (OIE) have stated that AMR is a serious risk to human health and is detrimental to the environment (McCubbin et al., 2021). A variety of factors have contributed towards this threat to public health, such as the over-prescription of antibiotics, the overuse and misuse of antibiotic chemotherapy and the use of antibiotics in farming practices. Bacteria have adapted and evolved to evade antimicrobial treatment options by several key mechanisms, including the production of enzymes that degrade the target antibiotic such as the production of beta-lactamases and aminoglycoside-degrading enzymes; intracellular target modification and activation of drug efflux pumps as reviewed by (Gajdács 2019). It is estimated that failing to tackle AMR will result in a decline in the world population by 2050 of between 11 to 444 million (Taylor et al., 2014). Due to this global threat to public health, there is an increasing need to develop alternative antimicrobial strategies for combating AMR infections. Many of the traditional key antimicrobial target sites within bacteria have been extensively explored and developing or modifying existing chemotherapeutic agents is proving challenging.

By utilising intelligent drug design and virtual screening technology, it is now possible to identify unique novel intracellular target sites for the generation of corresponding small molecules to inhibit cellular processes. Designing bespoke small molecule inhibitors (SMIs) against bacterial target sites reduces the possibility of off-

target effects, whilst ensuring differential toxicity between host and pathogen (Mühlen and Dersch, 2016). Several emerging targets are different families of bacterial epigenetic targets, including kinases which are enzymes that phosphorylate proteins to mediate cellular processes, many of which are involved in mediating bacterial virulence, such as quorum sensing and two-component regulatory systems (Defoirdt, 2018). This thesis introduction provides a comprehensive and subjective analysis of current research into the development of epigenetic inhibitors as anti-virulence agents, with a focus on several key classes of kinase including serine / threonine, tyrosine, and histidine kinase inhibitors, in addition to bacterial Autoinducer-2 kinase inhibitors. Host specificity is considered, alongside the potential for clinical deployment of novel SMI inhibitors and future perspectives in this emerging area of anti-virulence agent development.

1.1 Antimicrobial Resistance

The golden-age of antibiotics that stretched from the 1940s to the 1960s produced a valuable selection of naturally derived bacterial and synthetically made products which are still in use today (Jeśman et al., 2011). After this period there was a significant halt to antimicrobial progress, with current antibiotics that are in production being based on already known classes (mainly from the family Actinomycetaceae) rather than new discoveries (Hutchings et al., 2019). The approval rate of the food and drug administration of new systemic antibiotics has reduced to nearly none during the past 30 years (Prescott, 2014). Due to the constant adaptive nature of bacterial genetics in terms of clonality, resistance is an issue that is an ongoing challenge. This also relates to the use of antibiotics in agriculture, which would impact humans via zoonotic pathogens. Two examples would be *Salmonella enterica* and *Campylobacter jejuni*

that would encounter humans through the food chain. Infectious cases and morbidities in humans due to bloodstream infections caused by third generation cephalosporin-resistant *Escherichia coli* strain G3CREC were studied (Collignon et al., 2013) which found found that 56% of the resistance genes in strain G3CREC from human infections were identical to those genes identified in *E. coli* which was isolated from retail chicken samples. However, whole-genome sequencing has allowed the discovery that strains of multidrug-resistant *Salmonella* Typhimurium strain DT104 in the UK spread mostly independently within animal and human populations (Mather et al., 2013). A major focus when managing antimicrobial resistance in hospitals are the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species*) (De Oliveira et al., 2020). These pathogens have been highlighted as a threat to human health as they significantly decrease the amount of treatment options for bacterial infections. In a hospital setting, medical equipment can serve as a method of infection, for example, use of a urinary tract catheter is an invasive procedure and if contaminated, can introduce resistant bacteria internally. The effects of this are well documented as the World Health Organization has named antimicrobial resistant pathogens a threat to human health, which needs constant monitoring.

In the United States, there was a death toll of 35,900 due to resistant pathogens during 2019 which not only impacts healthcare resources and capacity, it also totals to a health care cost of over $4.7 billion (Kadri, 2020). The CDC report on antibiotic resistance in 2019 also stated that cases are more prevalent in critically ill patients who are affected due to often being immunocompromised already, Extended-spectrum β-lactamase (ESBL) Enterobacteriaceae cases increased by 50% over the past 5 years which is the result of clonal spread which consists of plasmids acting as

a carrier, such as those found in the virulent strain *Escherichia coli* ST131 which contains the resistance gene *bla*$_{\text{CTX-M-15}}$ (Awosile and Agbaje, 2020) During 2015 to 2018, one study monitored the antimicrobial consumption in comparison to rates of resistant ESKAPE pathogens within the surgical, clinical, and intensive care unit (Pérez-Lazo et al., 2021). This study found that the average rate of MRSA and vancomycin-resistant *Enterococcus faecium* (VRE) isolates was over 50% and 60% respectively in all hospital areas, with the highest resistance rate found in the ICU (73.3% and 64.3%). These findings correlate with the antimicrobials that had the highest average consumption in the ICU, which were vancomycin (9.16 /100 bed-days) and meropenem (9.13 /100 bed-days). However, the study did not find a significant linear relationship between an antimicrobial drug and an ESKAPE pathogen which meant there was no causal association. Despite this conclusion, the paper created awareness of the number of antimicrobial drugs being used and provided education and reflection to hospital staff on work protocols. Another study from a Nepalese hospital processed 8,756 clinical samples for the identification of ESKAPE pathogens using standard microbiological procedures (Pandey et al., 2021). Specimens included swabs, urine, bodily fluids, sputum, cerebrospinal fluid, pleural fluid, and semen. Results showed the following bacteria were present, *E. faecium* (5.5%), *S. aureus* (33.4%), *K. pneumoniae* (33.0%), *A. baumannii* (8.6%), *P. aeruginosa* (18.6%), and *Enterobacter aerogenes* (0.9%). MRSA and VRE were found in 57.6%, and 20% of samples respectively. An antimicrobial susceptibility test was carried out using the bacterial samples and 92% were resistant to ciprofloxacin, 52% to gentamicin and 20% against vancomycin. A molecular screen targeting the *vanA* and *vanB* genes was also conducted. VRE strains can transfer *vanA* to *S. aureus* via horizontal gene transfer, creating vancomycin-resistant *S. aureus* (VRSA), which limits

antibiotic treatment of infections (Willems et al., 2005). The *vanA* gene was found in all five VRE isolates, which is a signature of high resistance to both vancomycin and teicoplanin. This study highlighted the need to constantly monitor AMR within healthcare settings.

Current studies have found that there are new clones of *S. aureus* that are able to disseminate within community settings and infect those without predisposing risk factors. A review considered new MRSA and VRSA reservoirs, origins and characteristics, including the varied staphylococcal cassette chromosome (Lakhundi and Zhang, 2018). They found that there is a growing reservoir of MRSA in livestock and pets due to irresponsible use of antibiotics. This is in combination with MRSA's evolutionary nature facilitated by the Staphylococcal cassette chromosome (Shore and Coleman, 2013). *S. aureus* causes soft tissue, respiratory tract, skin and bloodstream infections, making it a major component of hospital and community-acquired infections. It can contaminate medical equipment and cause central-line-associated bloodstream infections. MRSA has a modified penicillin binding protein called PBP2a which is coded for by the *mecA* gene and acts by reducing the rate of β-lactam-mediated enzyme acylation along with decreased affinity for β-lactams. MRSA-infected patients usually have a more extended hospital stay and with *S. aureus* strain USA300 being a common causative agent. This study focuses on MRSA, particularly strain USA300, which is a prominent type of gram-positive community associated pathogens. This strain is mostly a community-associated MRSA strain and has become resistant to other drug types including erythromycin, levofloxacin, mupirocin, and tetracycline over the past 5 years. This is due to the acquisition of *erm*(a), *erm*(C) and *tet*(K) which are mediated by plasmid exchange. Virulence factors

that *S. aureus* strain USA300 uses include arginine catabolic mobile element, PVL toxin, α-toxin and phenol-soluble modulators (Lakhundi and Zhang, 2018).

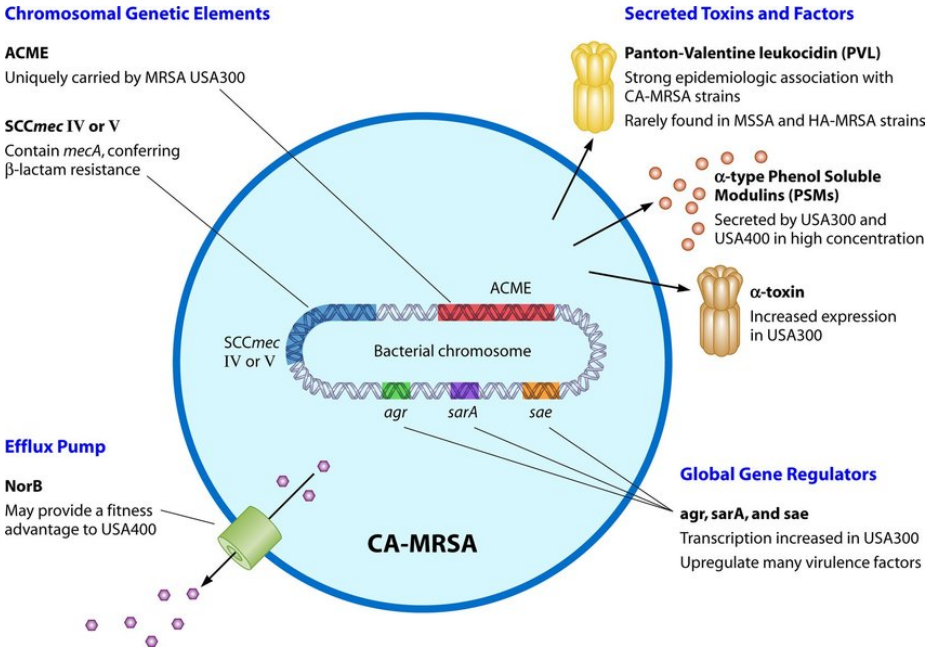


Image 1 (Al-Charrakh and Al-Hassnawi, 2012) depicts the virulence factors that have been identified in community associated MRSA strains including those mentioned above.

Although resistance is a constant threat to public health, there has been progress within recent years to reduce its prevalence. This includes the use of genome sequencing which has been made more widely accessible to investigate secondary metabolites of known antimicrobials. This is due to the price of genome sequencing dropping from thousands to around £100 per isolate as technology advances (Price et al., 2013) This can be further analysed, along with the use of microbial genome mining and synthetic chemistry, to find new antimicrobial drugs. An example of this would be azithromycin which is a second-generation tetracycline and a penicillin derivative. This drug is frequently used to treat skin and respiratory bacterial infections (Katz and Baltz, 2016). Antimicrobial stewardship interventions in hospitals involve using antimicrobials

sparingly and following treatment guidelines to minimise length of hospital stays without impacting quality of care. Within wealthier countries, hospital stewardship consists of the monitoring of antimicrobial use, monitoring resistant patterns, an antimicrobial committee, the evaluation of current intervention strategies and treatment guidelines. However, low to middle income countries have significant limitations due to high patient load to provider ratios, low funding, lack of training on antimicrobial stewardship, which results in high antimicrobial prescription (Kakkar et al., 2020). A study assessed antibiotic usage in Pakistan and their attitudes towards antimicrobial resistance (Waseem et al., 2019). It was found that 55.6% of pharmacists and physicians in a population of 473, had poor knowledge of antimicrobial resistance. A qualified pharmacist did not need to be present during the operation of 90.6% of pharmacies, which resulted in the reduction of specialists.

Recent studies are aiming to identify novel cellular targets and develop strategies to combat antimicrobial resistance. The development of small molecule inhibitors (SMIs) against bacterial virulence factors is an emerging area of research (Kim et al., 2021) (Rice et al., 2021) (Maji et al., 2019). Anti-virulence compounds, such as those which replicate mucus secretions against enteropathogens have been identified. These function by producing mucin glycoproteins which immobilise bacteria (Vaca et al., 2020). The innovation of new strategies and further research utilising existing compounds are both required to progress towards the goal of effective antimicrobial and anti-virulence drug production.

1.2 Identification of potential virulence targets

Bacteria regulate gene expression to control mechanisms used during infection such as the release of toxins. Such mechanisms are essential in bacteria, for *example* *Streptococcus pneumoniae* genes *nan*A and *nan*B encode factors that trigger colonisation (Pettigrew et al., 2006). The gene *rlr*A encodes pilus production and the gene *lux*S encodes the quorum sensing enzyme LuxS which is a regulator of biofilms and nasopharyngeal carriage for *S. pneumoniae* (Sakai et al., 2013). With the new realisation that novel antibiotics should not exert selective pressure on their target bacterial species, alternative mechanisms to disrupt bacterial virulence factor production are now being explored. A recent study focused on preventing *S. aureus* toxin secretion by using anti-virulence agents (Kong et al., 2016). *S. aureus* toxins include leukotoxin, hemolysin, enterotoxin, exfoliative toxin and TSST-1, not including other virulence factors like enzymes or surface proteins. Enzymes can aid in the invasion of a human host cell as well as evading the immune response attack using enzymes such as coagulase, staphylokinase and protease. Monoclonal antibodies have been studied as a potential therapeutic option for neutralising α-hemolysin (Foletti et al., 2013). Here, Mouse animal studies have found that α-hemolysin antibodies impede pneumonia, bacteremia and skin *S. aureus* infections and inhibit necrosis when administered prophylactically. When treatment was administered after the bacterial challenge, survival rates after 72 hours were 100% for mice treated with the LTM14 antibody after 12 hours post infection, which was comparable to mice treated with linezolid antibiotic after 12 hours. A decreased survival rate of 40-50% was observed when treated at 18 hours for both treatment methods. The α-hemolysin:antibody complex showed that the α-hemolysin binds between the cap and the rim domains, so that the antibody neutralises the activity of the toxin by preventing binding to the plasma membrane of susceptible host cells.

Another study has investigated preventing the expression of virulence factors in *Clostridioides difficile* infections using an antisense oligonucleotide (ASO) which would ideally target genes that are non-essential for cell survival (Stewart et al., 2020). This approach would be unique due to being organism specific as ASO would only anneal to mRNA transcripts of the target gene. However, there have been no *in vivo* studies as it has been difficult to produce a significant clinical response using a human host. This study also highlighted a small molecule inhibitor termed Ebselen, which altered proteins through seleno-sulphide conjugation at cysteine residues. Ebselen inactivates toxins TcdA and TcdB produced by *C*. *difficile*, by inhibiting the auto-processing domain and glucosyltransferase domain within the toxin protein structure (Marreddy et al., 2021). Further research found that Ebselen inhibited toxin production, decreased bacterial load, and reduced established biofilms (Thangamani et al., 2015). Ebselen is effective not only on C*. difficile,* but also targets multidrug-resistant strains of staphylococcal infections.

1.3 Kinase Inhibitors as Antimicrobial agents

Protein kinase phosphorylation is an essential process required by bacterial cells for survival in ever-changing environments. It plays a key role in signal transduction by the generation of signalling cascades, which produce secondary messengers to ensure successful transduction (Gao et al., 2019). It is common for proteins to possess an amino acid that acts as a phosphoacceptor to initiate phosphorylation. Phosphate groups are coordinated to hydroxyl residues found in threonine, serine, or tyrosine. Histidine can also be subjected to phosphorylation, but this possesses an imidazole ring which is the

phospho acceptor amino acid side chain (Kennelly, 2002). Phosphate is donated from adenosine triphosphate (ATP) and is coupled to tyrosine, serine, threonine, cysteine, aspartic acid, arginine or histidine, which then initiate protein interactions.

Phosphorylation is required for numerous intracellular processes, such as gene expression to regulate the production of proteins, altering intracellular processes to survive extreme environments and mediating chemotactic responses to environmental stimuli (Grangeasse et al., 2012). This process can be reversed by phosphatases which catalyse the donation of a phosphate group to a substrate from a high-energy molecule, thus allowing the on/off switching of kinase-mediated enzyme activity (Manso et al., 2019).

Eukaryotic protein phosphorylation is a well-characterised phenomenon(Kennelly, 2002) (Manning et al., 1980) . However, protein phosphorylation systems in prokaryotic cells were only discovered in 1979 (Garnak and Reeves, 1979) This discovery occurred whilst researching the catalytic activity of the isocitrate dehydrogenase enzyme in *E. coli*, where it was determined that enzymatic activity was controlled by the phosphorylation of serine residues within the enzyme (Garnak and Reeves, 1979) With the advent of DNA sequencing technology, there are now numerous examples of phosphorylation systems within prokaryotic cells (Adam and Hunter, 2018). Indeed, the global regulation of enzymatic activity mediated by protein kinase signalling in prokaryotic cell is now an emerging area for the development of Anti-virulence compounds.

There are several classes of prokaryotic kinases which are implicated in signal transduction, including histidine kinase two-component regulatory systems, bacterial tyrosine kinase system (termed BY-kinases) and the eukaryotic-like Serine/Threonine kinase system (termed eSTK) (Kyriakis, 2014) .These are essential

pathways for regulating subsequent protein function for example, a subset of eukaryotic-like serine threonine kinase (eSTK) called penicillin binding proteins and serine/threonine kinase associates (PASTA) are commonly found in actinomyces and firmicutes. These kinases bind muropeptides and regulate cell responses to extracellular stresses by constant evaluation of cell wall homeostasis (Pensinger et al., 2018). It was also found that bacterial cells that did not contain sufficient tyrosine kinase protein displayed irregular DNA replication and chromosome distribution, which suggests that it plays a vital role in bacterial physiology (Manuse et al., 2016). The following sections will explore the different classes of protein kinases and detail the role of potential inhibitors which could be employed as anti-virulence agents.

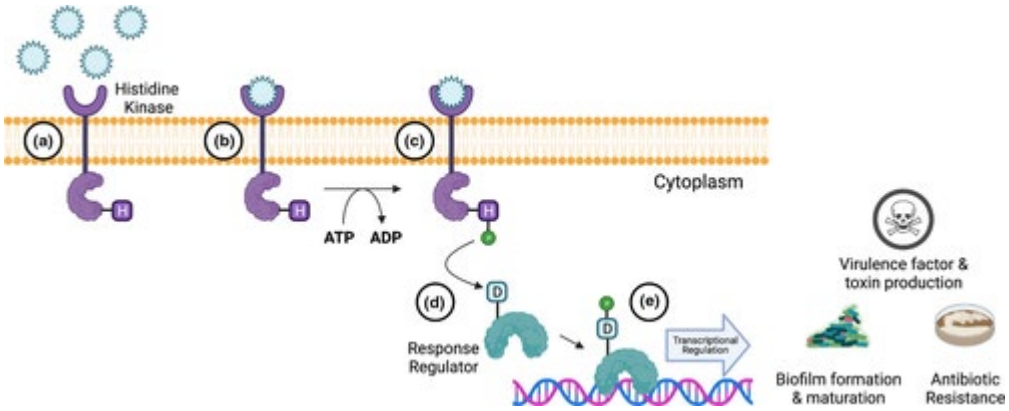


Image 2, (King and Blackledge, 2021) depicts an example of a two-component system cascade. (a) shows a histidine kinase extracellular sensor domain which sits on the cell membrane. (b) shows a signalling chemical compound binding to the histidine kinase's active site. (c) is where a conserved histidine residue undergoes autophosphorylation which is what the green circle represents. (d) is where the phosphoryl group is transferred from histidine to the DNA binding response regulator using histidine kinase. (e) shows The response regulator is phosphorylated which allows it to bind to DNA, leading to transcriptional regulation resulting in toxin or virulence factor production.

1.4 Autoinducer-2 kinase Inhibitors

Quorum-sensing pathways are important targets for the development of novel antimicrobial chemotherapeutics, as these underpin the functional basis for biofilm formation and intercellular communication (Kim et al., 2017). Biofilms are microbial communities which are more resistant to the effects of antimicrobial agents and present some of the most challenging clinical infections, often resulting in chronic disease. In a multitude of bacterial species, including *E. coli* and *S. aureus*, quorum sensing is regulated via the production of small molecules called autoinducers, such as Autoinducer-2 (Ai-2) (Kalia et al. 2019). The LsrK protein belongs to the FGGY kinase family and catalyses phosphate transfer from ATP to multiple sugars such as trioses and heptoses. In enteric bacteria, LsrK phosphorylates a precursor of Ai-2 termed (*S*)-4,5-Dihydroxy-2,3-pentanedione (S-DPD) (Stotani et al., 2019), which is essential for functional quorum sensing cascade activation. Inhibiting the activity of LsrK therefore represents a potential target to reduce the virulence profile of bacterial pathogens.

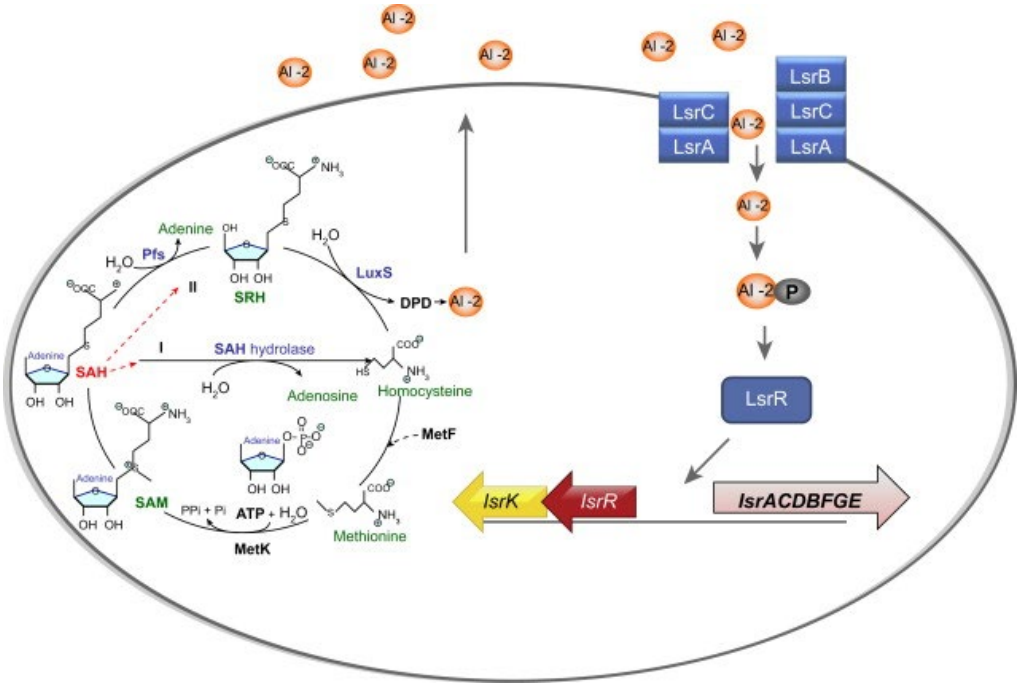


Image 3. This diagram displays the production of autoinducer-2 in a bacterial cell (Sen et al. 2015). This Quorum sensing signalling process is that of *Escherichia coli and Salmonella typhimurium.* LuxS enzymes react with *S-ribosyl homocysteine* (SRH) which results in the production of DPD. DPD undergoes further processing to become active autoinducer-2.

S-DPD is a small signalling molecule that controls quorum sensing in both Gram-negative and Gram-positive bacteria. A study by Stotani et al. (2019) measured the efficacy of a library of small compounds which were derivatives of S-DPD in inhibiting LsrK in *E. coli*. The screening process resulted in 4 compounds out of the 29 screened which showed inhibition of LsrK over 40%: compound 12a (3,5-disubstituted pyrazoles with R^1 = Ph), 12b (3,5-disubstituted pyrazoles with R^1= 2-furanyl), 13a (1,3,5-trisubstitted pyrazoles with R^1 = Ph), 13b (1,3,5-trisubstitted pyrazoles R^1= 2-furanyl). All four compounds produced IC_{50} values between 100 μM and 500 μM, the most successful compound being 13a which demonstrated an IC_{50} of 119 μM (Stotani et al., 2019). Another study screened a 2000 compound library to find compounds which could inhibit LsrK phosphorylation of autoinducer-2 (Gatta et al., 2019).

The remaining compounds were then subjected to dose response assay, selectivity and promiscuity assays which resulted in three compounds: harpagoside, rosolic acid and aurin tricarboxylic acid. An Autoinducer-2 (Ai-2) quorum sensing interference assay was then carried out to assess the compound quorum sensing inhibition ability in a cellular environment. This used *E. coli* strain LW7 pLW11 to monitor activity, which produces beta galactosidase when the quorum sensing cascade is initiated (Gatta et al., 2020). An efflux pump blocker called phe-arg β-naphthylamide dihydrochloride (PAβN) was also used to counteract Gram-negative bacteria using efflux pumps to remove the compounds from the cell. Rosolic acid showed to be the most effective as it inhibited LsrK which in turn, inhibited Ai-2 mediated quorum sensing by 68% at 10 μM, whereas harpagoside had an inhibition percentage of 50% at 50 μM with only a minor inhibitory effect at 10 μM. Aurin tricarboxylic acid had no effect. This was also assayed using *E. coli* strains which had a quorum sensing independent lac promoter but resulted in no effect from both compounds which proved their specificity for inhibiting quorum sensing. However, inhibiting bacterial quorum sensing could result in selective pressures, leading to resistant strains (Fleitas Martínez et al., 2019). Another study identified a new methodology to demonstrate Ai-2 quorum sensing inhibition was controlled by the Lsr promoter (via phosphorylated Ai-2 binding to the Lsr repressor which starts the quorum sensing cascade) (Gatta et al., 2020). This study screened a 91-compound library for those which could target the ATP binding site of LsrK using a kinase inhibition assay with over 70% inhibition rates. Evaluation of different assays facilitates the understanding that the complexity and duration of previously stated quorum sensing interference assays with low sensitivity should be avoided. The pET-Plsrlux plasmid possesses a bacterial luciferase operon,

controlled by *lsr* which is a promoter that acts in response to quorum sensing, which then was constructed into single-gene knock-out *E. coli*. This meant it could act as a bioreporter which could be detected via luminescence due to *lux* gene expression because of Ai-2 quorum sensing, which showed to be more cost effective and reduced assay complexity. 24 compounds displayed quorum sensing inhibition specifically, with an inhibitory rate of over 80%, of which, 6 compounds were found to be LsrK inhibitors. However, the knock-out *E. coli* that was used had a decreased efflux pump activity and when the cell-based assay was performed in *E. coli* cells with normal efflux pump activity, the compounds selected did not exhibit significant inhibition of quorum sensing. This highlights the requirement to study novel cell-based assays within bacterial cells, but also demonstrates that Ai-2 is a viable target for anti-virulence drug discovery.

1.5 Histone Demethylase inhibitors as anti-virulence agents

Multimeric proteins are responsible for posttranslational modifications which provide the ability for cells to adapt to changing surroundings via changes in gene expression. The Jumonji family of proteins act as transcriptional regulators and chromatin modifying enzymes. In eukaryotes, this consists of iron- and 2-oxoglutarate oxygenase with a catalytic Jumonji domain site (also known as JmjC) (Pilka et al., 2015) (Hatch et al., 2017). The Jumonji family is made up of several subfamilies named KDM2-7. These proteins can achieve substrate specificity due to their complex protein structure, but also by conserved helper domains which aid gene targeting. GSK-J4 is a selective inhibitor of H3K27 histone demethylase JMJD3, which is also known as KDM6b, and is a cell-permeable pro-drug of GSK-J1(Heinemann et al.,

2014). KDM6b is found in humans and can promote pro-inflammatory and anti-inflammatory responses by targeting distinct transcription factors in gene promoters in order to aid both adaptive and innate immunity (Salminen et al. 2014). GSK-J1 is a competitive inhibitor of the two cofactors on the JmjC; iron- and 2-oxoglutarate which are required for enzymatic activity. This epigenetic compound has the ability to catalyse the H3K27me3 (trimethylated-histone lysine) to H3K27me2/1 (di/mono-methylated histone lysine) which in turn, regulates gene expression (Wang et al., 2020). There has been extensive research into the activity of GSK-J4 due to the potential for anti-cancer and anti-inflammatory effects (Chu et al., 2020) (Li et al., 2018) (Lochmann et al., 2018).

To date, GSK-J4 has not been used as an anti-virulence or antimicrobial agent against prokaryotic cells. However, recently GSK-J4 was found to demonstrate antiprotozoal activity. The protozoan termed *Plasmodium falciparum* (which is the causative agent of malaria in humans) possess enzymes which have Jumonji domains encoded by three proteins containing JmjC enzymatic domains, designated as PfJmjC1, PfJmjC2 and PfJmj3 (Jiang et al., 2013). When GSK-J4 was used against *P. falciparum* it was found that the drug decreased blood stage asexual development in *P. falciparum* isolates 3D7 (drug sensitive) and Dd2 (multidrug resistant) by 50% at 5 μM and by 100% at 10 μM with an inhibitor EC_{50} of 2.8 ± 0.3 μM (Matthews et al., 2020). Gametocyte development (sexual growth) was also assessed after exposure to Jumonji inhibitors, as well as *N*-acetylglucosamine to kill any asexual parasites, and quantified using flow cytometry after 6 days. GSK-J4 was only effective at higher doses of 6 μM. Parasites were then treated at different specific blood development stages to detect the exact effects of the drugs on the intraerythrocytic developmental cycle (IDC). The parasites were treated with the drug at the EC_{50} concentration and

monitored over a 48-hour period. After 24 hours, there were significantly fewer parasites entering the schizogony phase compared to the untreated parasites. After 48 hours, the parasites exposed to GSK-J4 had a reduced parasitemia percentage of 1.5 to 2%, compared to the 4% increase in the untreated parasites which passed the IDC and were now invading new red blood cells. QRT-PCR then revealed that GSK-J4 reduced the expression of several genes from invasion related gene families such as *clag3.1*, *ron2* and *gap50*. AP2 transcription factor *sip2* and histone trimethylation also displayed reduced gene expression, which collectively, infers that the reduced gene expression of these genes causes a disruption to the IDC and histone trimethylation which results in inhibition of PfJmj3 activity.

Another study also examined GSK-J4 for activity against *Toxoplasma gondii* (Liu et al., 2020). GSK-J4 produced an IC_{50} value of 2.37 μM and a plaque assay resulted in 65% reduction in tachyzoite plaque area when compared to the control data. GSK-J4 was then exposed to intracellular *T. gondii* by synchronising the cell cycle progression to assess the compound effects. Results showed that the compound caused cell cycle arrest at the G1 phase, impeding progressing to S phase, which also disrupted the parasite cell cycle. Another assay looked at the tachyzoites per vacuole, which were 8-16 tachyzoites when treated with DMSO, however when treated with GSK-J4, toxoplasma intracellular growth was inhibited as only 1-2 tachyzoites were counted in vacuoles. GSK-J4 was also examined for anti-toxoplasmosis effects *in vivo* using murine models at 50 mg/kg of body weight. Mice were infected with *T. gondii* strain 100RH then treated with GSK-J4 and continued treatment for the following 4 days. The GSK-J4 treated mice had significantly longer survival rates (15.2 ± 1.69 days, P=0.001) than the mice treated with DMSO (11.9 ± 0.74 days).

The aims of this study were to screen an epigenetic SMI compound library to identify those which possess antimicrobial or anti-virulence activity against a broad range of medically-relevant bacterial pathogens. Lead SMIs were further screened using antimicrobial assays, *in vivo Galleria mellonella* toxicity and infection assays, and mechanistic studies to determine the nature of the observed antimicrobial effects.

2 Methods

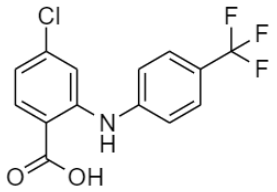
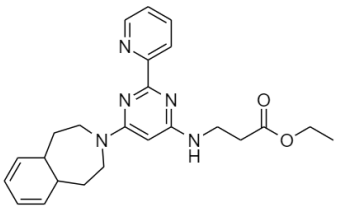
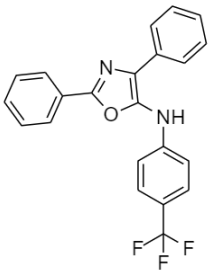
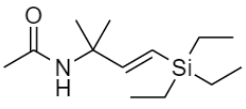
2.1 Bacterial strains and growth conditions

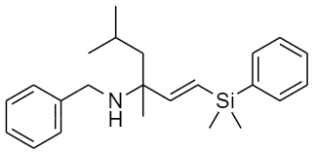
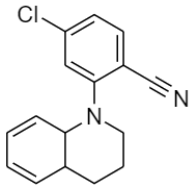
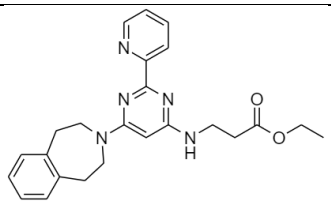
All bacterial strains were stored at -80°C prior to culturing on cationic adjusted Mueller Hinton agar or broth (Oxoid, UK). Broth cultures were incubated at 37°C for 24 h, with agitation at 180 rpm. Fresh isolation plates were prepared every 2 weeks.

Table 1 Bacterial strains

GRAM STATUS	GENUS AND SPECIES	STRAIN	CULTURE MEDIUM
POSITIVE	*Staphylococcus aureus*	SH1000	Mueller-Hinton
	Staphylococcus aureus	USA300	Mueller-Hinton
	Bacillus cereus	9373	Mueller-Hinton
	Enterococcus faecalis	12697	Mueller-Hinton
	Enterococcus faecium	7171	Mueller-Hinton
	Listeria monocytogenes	Scott A	Mueller-Hinton
NEGATIVE	*Pseudomonas aeruginosa*	PAO1	Mueller-Hinton
	Escherichia coli	1655	Mueller-Hinton

Table 2 Lead compounds identified from library screening

Associated compound number	Scientific name	Molecular formula	Molecular weight	Molecular structure	Solvent
89	4-chloro-2-((4-(trifluoromethyl)phenyl)amino) benzoic acid	C15H9ClF3NO3	315.68		DMSO
140	ethyl 3-((6-(1,2,4,5,5a,9a-hexahydro-3H-benzo[d]azepin-3-yl)-2-(pyridin-2-yl)pyrimidin-4-yl)amino)propanoate	-	417.5		DMSO
163	2,4-diphenyl-N-(4-(trifluoromethyl)phenyl)oxazol-5-amine		380.11		DMSO
126	(E)-N-(2-methyl-4-(triethylsilyl) but-3-en-2-yl)acetamide		241.45		DMSO

132	(*E*)-*N*-benzyl-1-(dimethyl(phenyl)silyl)-3,5-dimethylhex-1-en-3-amine		351.61		DMSO
101	4-chloro-2-(3,4,4a,8a-tetrahydroquinolin-1(2H)-yl)benzonitrile		268.74		DMSO
GSK-J4	Ethyl 3-((6-(4,5-dihydro-1H-benzo[d]azepin-3(2H)-yl)-2-(pyridin-2-yl)pyrimidin-4-yl)amino)propanoate	C24H27N5O2	417.5		DMSO

2.2 Antimicrobial compounds and preparation

A set of 139 compounds were synthesized by the McLaughlin laboratory (University of Lancaster, UK). Synthesis was based on known kinase inhibitors of bacterial cells. Compounds were dissolved in dimethyl sulfoxide (DMSO) at a neat concentration of 100 mM or diluted further into 1% DMSO. All compounds were stored at 4°C to prevent degradation. The stability of stock solutions was assessed overtime by confirming antibacterial activity and no evidence of degradation was observed over the course of the experiments.

2.3 Minimum Inhibitory Concentration (MIC) assay

MIC values were determined using Mueller Hinton broth and sterile 96-well plates to perform a micro-dilution assay. Bacterial broth cultures were incubated overnight and adjusted to an OD600 of 0.005, resulting in a final cell concentration of 2.3 x 10^6 Colony-Forming Unit (CFU mL^{-1}). The Compounds were serially diluted to the following dilutions; 1 mM, 500µM, 250µM, 125µM, 62.5µM, 31.2µM, 15.625µM, 7.8125µM, 3.90625µM and 1.95312µM. All plates were incubated at 37°C for 24 h and the MIC results were recorded as the lowest concentration which inhibited visible growth of the bacterial species. Appropriate controls were included using bacteria only, bacteria with neat DMSO solvent at relevant concentrations,neat DMSO solvent only and media only as a negative control. Growth was measured by visual observation of the bacterial growth in wells which would become opaque, wells without growth appearing clear. The dilution with the lowest concentration of compound which resulted in a well without growth, was the dilution read as the MIC.

2.4 Minimum Bactericidal Concentration (MBC) assay

After MIC data was recorded, MBC values were determined using a replica plater for 96 well plates (Sigma-Aldrich, UK) to transfer a sample from the 24 h incubated well plates to Mueller Hinton agar plates. The MBC value was recorded as the lowest concentration of compound to achieve 100% bactericidal effect (no bacterial colonies present). The value would be determined by the lowest concentration where

the sample did not grow colonies. Both MBC and MIC assays were performed in triplicate.

2.5 Time-kill kinetics assay

A bacterial culture was grown overnight and then diluted to an OD_{600} of 0.0025 with Mueller Hinton agar. Aliquots of 5 mL bacterial broth suspension were incubated with compound concentrations at MIC, above MIC, MBC and above MBC levels (concentrations are detailed in table 3). Controls were also used which had no compound and neat DMSO only. Cultures were incubated at 37 °C with agitation at 150 rpm. Viability of time (0 hr, 2 hrs, 4 hrs, 6 hrs and 24 hrs) was determined using the Miles and Misra technique where 20µl droplets were inoculated onto Mueler Hinton agar plates in triplicate and incubated for 24 hrs. The colonies were then counted, which could then be used to calculate the bacterial growth. This time-kill assay was performed with three biological replicates.

2.6 Scanning Electron Microscopy (SEM) Imaging

During the time-kill kinetics assay, at each time point, 10 µL of each sample broth was dried onto 10 cm^2 silicon sterile wafers (Platypus Technologies, USA). The inoculated silicon wafers were submerged into 4% glutaraldehyde fixative for 24 h at 4°C. These were then washed in deionised water and dried before being dehydrated by an ethanol concentration gradient (10%, 30%, 50%, 70%, 90%, 100%), with wafers being submerged for 10 min at each concentration. Wafers were then placed desiccated for 24 h at room temperature. Samples were mounted onto aluminium pin stubs (Agar Scientific, Stansted, UK) and sputter coated with gold to a 10 nm thick

gold layer using a magnetron sputtering system (Polaron, Quorum Technologies, UK). Images were captured on a Supra 40VP scanning electron microscope (Zeiss, Germany) using SmartSEM software (Carl Zeiss Ltd, Germany). Images were used to compare the cellular morphological effect of the antimicrobial compound against cells not exposed to the compound and against positive controls at 5,000x, 10,000x and 100,000x magnification.

2.7 Disc Diffusion Assay

Bacterial cultures were grown overnight and adjusted to an OD_{600} of 0.5 by diluting in Mueller Hinton broth. 100 µL of the inoculated broth was spread over a 25 mL Mueller Hinton agar plate. Aliquots of 20 µL per compound were added to antimicrobial susceptibility discs (Thermo Scientific, UK) and aseptically positioned onto an individual section. The MBC, below MBC and above MBC concentrations were used on three separate agar plates, along with controls of DMSO. The zone of inhibition (ZOI) produced by each compound against the respective bacterial species was determined using digital callipers.

2.8 *Galleria mellonella* Larvae Infection Model

Larvae were stored at room temperature upon arrival, with wood shavings in the dark. Test larvae were selected with a uniform pale colour and larger size, avoiding larvae with melanisation which is a sign of illness or injury. Larvae were then placed into groups of 10. Control conditions included injections with bacteria (OD_{600} of 0.0025), PBS with and without bacteria, 1% DMSO with and without bacteria, no injection and vancomycin (10 mg mL^{-1}). All larvae were swabbed with 70% ethanol around the right

posterior leg prior to injection using a 10 uL Hamilton repeating syringe dispenser. Larvae were injected in the right posterior proleg for bacteria and the left posterior proleg for the assay compounds. Larvae were then incubated at 37°C and monitored over 72 hrs. Larval viability was determined by no movement and complete melanisation. No movement when provoked by gently touching the larvae, was recorded as that larvae not surviving, as well as a visual inspection of complete melanisation. Partial melanisation and movement was recorded as a surviving larvae, as well as no melanisation after visual inspection.

2.9 Biofilm Eradication Assay

2.9.1 Biofilm growth in a CDC Reactor

An culture of MRSA was grown for 24 hrs and diluted in 500 mL of Mueller Hinton broth to produce a concentration of 1 x 10^7 CFU mL^{-1} which was inoculated into a CDC reactor (Biosurface Technologies). Miles and Misra techniques were used to determine bacterial viability. The CDC reactor was incubated at 37°C at 50 rpm for 16 hrs. Coupons were recovered from the reactor and rinsed in deionised water to remove planktonic bacteria. Biofilms were then exposed for 24 hrs to the respective compound. Coupons were submerged in 2 mL Dey-Engley neutraliser for 5 min and bacteria from the coupon biofilms were recovered by bath sonication for 10 min. Miles and Misra techniques were used to quantify the bacterial viability.

2.9.2 Crystal Violet staining of biofilms

An overnight Mueller Hinton broth culture was diluted by 1:100 and split into separate universals to be treated with either a compound concentration or control. 100 µL of each dilution were plated in triplicate on a round-bottomed 96-well plate and incubated at 37°C for 48 h to allow biofilm formation. Unattached planktonic bacteria were

removed from the assay plate and wells were rinsed with deionised water and air dried. All wells were then stained with 0.1% crystal violet (ThermoFisher, UK) for 10 min and rinsed again with deionised water to remove excess staining. The plates were left to dry at room temperature overnight then wells were treated with 30% acetic acid (ThermoFisher, UK) for 10min to solubilize the crystal violet. Aliquots of 125 µL from each well was then transferred to a flat-bottomed 96-well plate which was analysed using a SPECTROstar Omega Absorbance plate reader at OD600. This quantified the crystal violet staining as an indicator of bacterial growth, where increased staining equates to an increase in bacterial cells.

2.10 DNA Demethylase Activity Assay

Demethylase activity was determined using a DNA demethylase (total) Activity Quantification Assay Kit (Abcam, Cambridge UK). The methylated DNA substrate was adhered inside an 8 well strip by applying 80 µL of diluted demethylation substrate into all wells, except for blanks and those wells used to produce a standard curve. Blank and standard curve wells had 80 µL of substrate binding solution added, and for the standard curve a concentration gradient of demethylation standard was included. These were incubated at room temperature for 75 min, when wells were aspirated and washed with a diluted wash buffer. Blank and standard curve wells were treated with 50 µL of demethylase assay buffer. Protein sample control wells were treated with 45 µL of demethylase assay buffer and 10 µL of *S. aureus* USA300 protein extract at 0.571mg/mL, which was prepared by sonicating a culture diluted to OD_{600} of 0.1. Whole cell extract was centrifuged (10k rpm) to remove unbroken cells. Other controls used were protein extracts with saline and DMSO, both in triplicate. Three protein samples were treated with 3 µL of GSK-J4 at 1 µM and 5 µM, with each concentration

was assayed in triplicate. The wells were then incubated at 37°C for 1 hr. Each well was aspirated and washed with an assay buffer prior to the addition of 50 µL diluted capture antibody which was then left to incubate at room temperature for 1 hr on an orbital shaker at 60 rpm. Wells were aspirated and washed with assay buffer, then treated with dilute detection antibody (1:2000 ratio with wash buffer) and incubated at room temperature for 30 min. Further aspiration and well washing was performed prior to the addition of 100 µL of developing solution, The reaction was stopped by adding 50 µL of stopping solution when there was a visible colour change from clear to blue in sample and control wells. The stopping solution changed the blue to a yellow colour, and the absorbance was read using a VANTAstar BMG labtech microplate reader at 450 nm. The yellow colour change was quantified by absorbance. This is due to the detection antibody and capture antibody binding in the presence of demethylation activity which is signified by the release of pigment. The following calculation was used to find the percentage inhibition from the absorbance measured.

$$\text{Inhibition \%} = \left(1 - \frac{[\text{OD (control - blank)} - \text{OD (inhibitor sample - blank)}]}{[\text{OD (control - blank)} - \text{OD (no inhibitor sample - blank)}]}\right) \times$$

2.11 Statistical analysis

GraphPad Prism 9 software was used to analyse the data and to present the data in graph form. A One-way ANOVA was used for all statistical analysis and the P values are reported next to each graph. Each assay was performed in triplicate (n=3) and included control variables appropriate to the assay. A Tukey post hoc test was performed to assess all pairwise differences and showed that the results were significant overall. This data set was suitable for a one-way ANOVA due to there being

only one quantitative variable involved which was bacterial growth with the compound concentrations being the independent variables.

3 Results

3.1 Small molecule screening to identify compounds that inhibit bacterial growth

A selection of compounds that were chemically similar in structure were assessed as possible candidates for the inhibition of bacterial growth or working as an anti-virulence factor. A compound library collection of 103 small molecule kinase inhibitors and 36 epigenetic inhibitors were screened to identify inhibitory effects against Gram-positive (*S. aureus* USA300, *S. aureus* SH1000, *E. faecalis* 12697 and *E. faecium* 7171, *B. cereus* 9373, *L. monocytogenes* Scott A) and Gram-negative (*P. aeruginosa* PAO1. *E. coli* 1165) strains. The preliminary screenings were performed by exposing bacteria to 1mM of each compound and assessing visual growth and viability by culturing on Mueller Hinton agar. This was the first stage of identifying possible kinase inhibitor candidates that possessed antimicrobial abilities. Four compounds were identified (Compounds 89, 140, 132, and GSK-J4) (Table 2) which inhibited 100% of *S.aureus* USA300 and *S. aureus* SH1000 bacterial growth after overnight incubation but did not inhibit growth of the other Gram-positive or Gram-negative bacterial strains. Compounds 163, 126 and 101 (Table 2) also inhibited 100% of *E. faecalis* and *E. faecium* growth, in addition to both *S. aureus* strains after overnight incubation but did not inhibit the growth of the Gram-negative strains of bacteria. All other compounds lacked antimicrobial activity at 1 mM concentrations (data not shown). Compounds 89, 140, 132, 163, 126 and 101 were found to have the most inhibitory activity against bacterial growth, which is why they were then the focus of further testing.

3.2 Antimicrobial activity of lead compounds by determining the Minimum inhibitory and bactericidal concentrations

Seven compounds were further analysed due to being inhibitory at a 1 mM concentration. Compound potency was assessed by calculating the minimum concentrations for inhibition. This allowed the elimination of compounds that could only inhibit bacterial growth at higher concentration. MIC and MBC testing was performed using a concentration gradient of 1 mM to 3.90625 µM of each compound and was replicated in triplicate for each of four bacterial strains. 5 of the kinase inhibitors compounds (89,163,126,132 and 101) had an MBC value ranging from 1 mM to 0.5 mM and an MIC value ranging from 0.125 mM to 0.5 mM (Table 3). Kinase inhibitor compound 140 had slightly lower values with MIC 31.25µM and MBC 62.5µM against S.aureus USA300, which indicates it is a more potent drug candidate. GSK-J4 had the lowest values with MIC 3.906 µM and MBC 15.625µM against *S. aureus* USA300, which implies that it is the most effective antimicrobial agent. This assay revealed that the most potent compounds were GSK-J4 and 140 which display the most inhibition against *S. aureus* USA300.

Table 3 Inhibitory concentrations for focus compounds against a range of bacteria. Minimum Inhibitory Concentration (MIC), above MIC and Minimum Bactericidal Concentration (MBC) against *S. aureus* strains USA300 and SH1000, *E. faecalis* 12697 and *E. faecium* 7171.

compound	*S.aureus USA300*			*S.aureus SH1000*			*E.faecalis 12697*			*E.faecium 7171*		
	Above MIC (mM)	MIC (mM)	MBC (mM)	Above MIC (mM)	MIC (mM)	MBC (mM)	Above MIC (mM)	MIC (mM)	MBC (mM)	Above MIC (mM)	MIC (mM)	MBC (mM)
89	0.25	0.125	0.5	0.5	0.25	1	NA	NA	NA	NA	NA	NA
140	0.046875	0.03125	0.0625	0.5	0.25	1	NA	NA	NA	NA	NA	NA
163	0.5	0.25	1	0.25	0.125	0.5	0.25	0.125	0.5	0.25	0.125	0.5
126	0.5	0.25	1	0.5	0.25	1	0.25	0.125	0.5	0.5	0.25	1
132		0.5	1	0.55	0.5	1	NA	NA	NA	NA	NA	NA
101	0.25	0.125	0.5	0.25	0.125	0.5	0.25	0.125	0.5	0.25	0.125	0.5
GSK-J4	0.007812	0.003906	0.015625	>1	>1	>1	>1	>1	>1	>1	>1	>1

3.3 Antimicrobial activity of lead compounds using the disc diffusion assay

Compounds 140, 89, 101, 132 and GSK-J4 were diluted to their MIC and MBC values and each one was added to filter paper discs on pre-inoculated Mueller Hinton agar containing *S. aureus* USA300. Plates were incubated for 24 hrs and zones of inhibition were measured. However, all compounds did not produce any noticeable inhibitory zones (table 4) which suggests they are unable to diffuse through the agar.

Table 4 Disc diffusion assay zones of inhibition when bacterial strains are exposed to focus compounds at MBC concentration.

compound	Diameter of Inhibition Zones (mm)			
	S. aureus USA300	*S. aureus* SH1000	*E.faecalis* 12697	*E. faecium* 7171
89	0.0	0.0	0.0	0.0
140	0.0	0.0	0.0	0.0
163	0.0	0.0	0.0	0.0
126	0.0	0.0	0.0	0.0
132	0.0	0.0	0.0	0.0
101	0.0	0.0	0.0	0.0
GSK-J4	0.0	0.0	0.0	0.0

3.4 Time-kill kinetics of lead compounds against *S. aureus* USA300

Time-kill assays were performed to determine the rate of antimicrobial activity of compounds 140, 89, 101, 132 and GSK-J4 against *S. aureus* USA300. This assay identifies which compounds had the most fast-acting inhibitory ability against bacterial growth. The assay was performed by exposing bacterial cells to MIC, MBC, and above MIC concentrations of each compound, along with controls of DMSO and no compound. Compounds 140 and GSK-J4 both rendered *S. aureus* USA300 non-viable

by 6 h (Fig. 1A and B), however compounds 89 (Fig. 1C), 101 (Fig. 1D), and 132 (Fig. 1E) required the full 24 h to get to that stage. Control conditions both showed no signs of inhibition over the full 24 hrs.

A, Compound 140 had the most potent inhibitory effect when used at concentrations 0.5mM and 0.25mM as they achieved total inhibition somewhere between 4 and 6 hours of treatment. Compound 140 at 0.125mM did inhibit but did slow bacterial growth. B, Compound 89's highest inhibitory activity at 0.5mM and increasing the concentration to 1 mM did not change the time taken to achieve total inhibition, which occurred between 6 and 24 hrs of treatment. Compound 86 at 0.25mM did inhibit growth from 1.48e+07 cfu/ml to 19400 cfu/ml but did not achieve total inhibition. C, Compound 101 also achieved full inhibition between 6 and 24 hrs, but only at concentrations 0.5 mM and 0.25 mM. The 0.125 mM concentration achieved partial inhibition from 4.5e+06 cfu/ml to 9720 cfu/ml after 24 hrs of treatment. D, Compound 132 treatment resulted in total inhibition after 24 hrs at concentrations 0.5mM and 0.25mM. At 6 hrs, the 0.5 mM treated cultured broth was at 43300 cfu/ml and the 0.25mM broth was at 756000 cfu/ml, which shows the higher concentration producing a stronger inhibitory effect. The 0.125 mM treated broth had 461,000 cfu/ml after 24 hrs, which is around the same value as the highest treatment concentration achieved at 6 hrs. E, Compound GSK-J4 only achieved total inhibition at concentration 3.125 μM between 4 and 6 hrs of treatment. Concentration 1.625 μM treatment after 24 hrs resulted in 211 cfu/ml which is very close to full inhibition. Treatment at concentration 0.78125 μM only slightly changed the starting value of 2.39e+06 at 0 hrs to 4e+06 at 24 hrs. However, this is a large inhibitory effect when compared to the DMSO treated broth cultures at 2.11e+09 after 24 hrs.

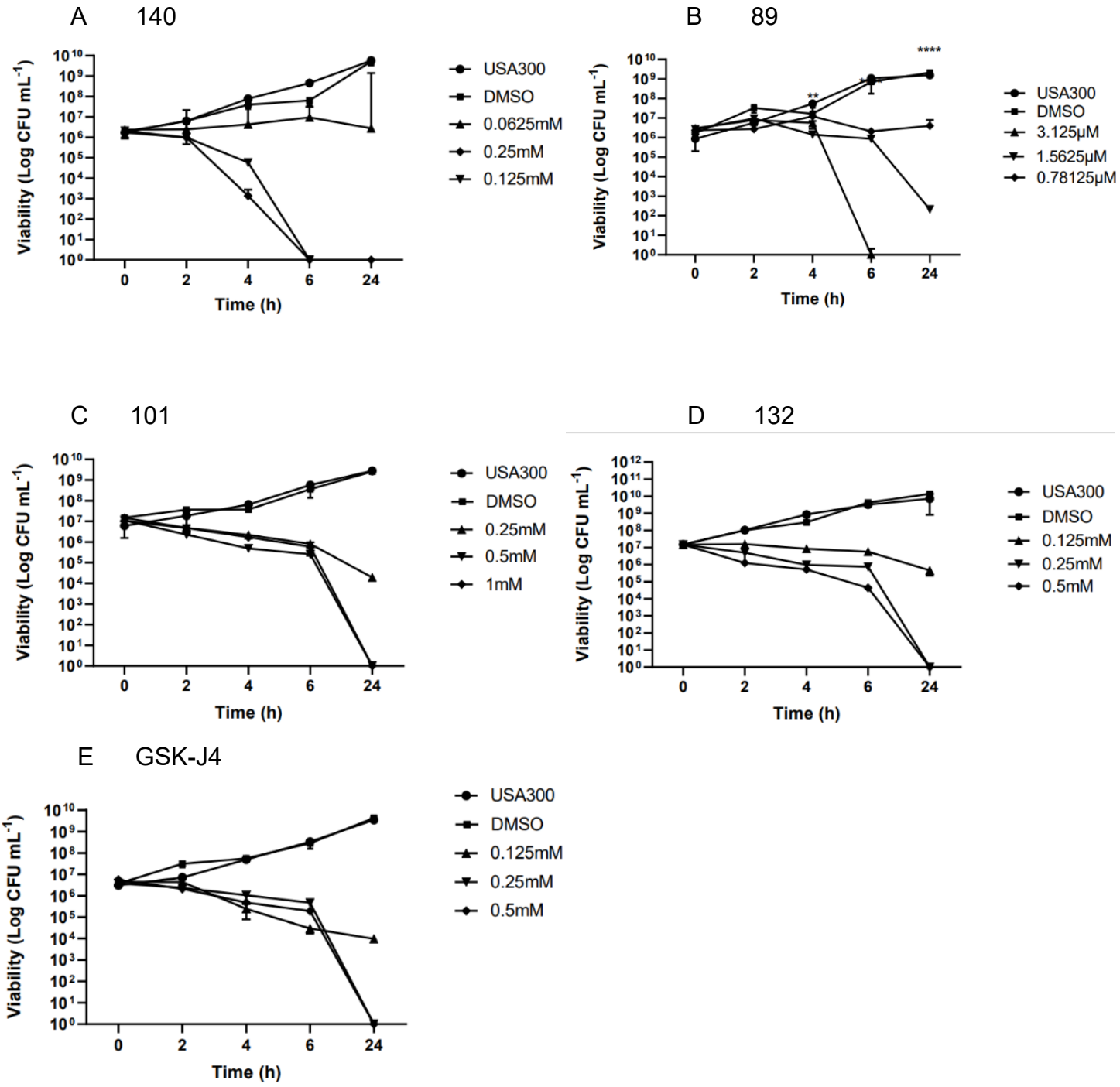
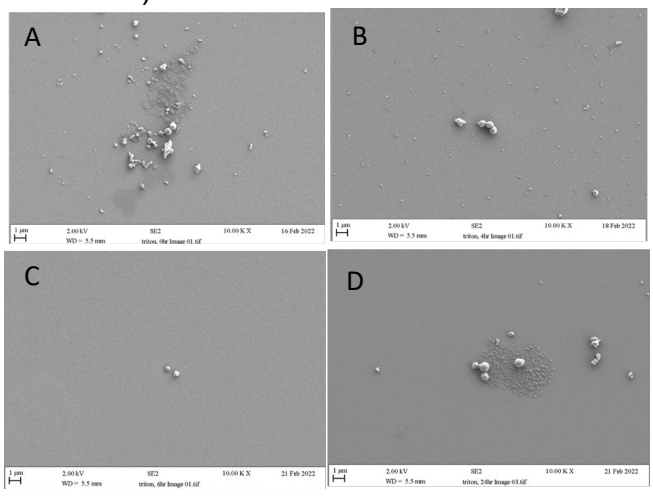


Figure 1, The rate at which antimicrobial activity occurs when *S. aureus* USA300 is exposed to each compound. Time-kill graph that shows the effect of treatment from 0 to 24 hrs quantified using the number of colony forming units produced from samples taken at each time point ($p = 0.001$). The concentrations used were the above MIC, MIC and MBC values previously explained in table 3.

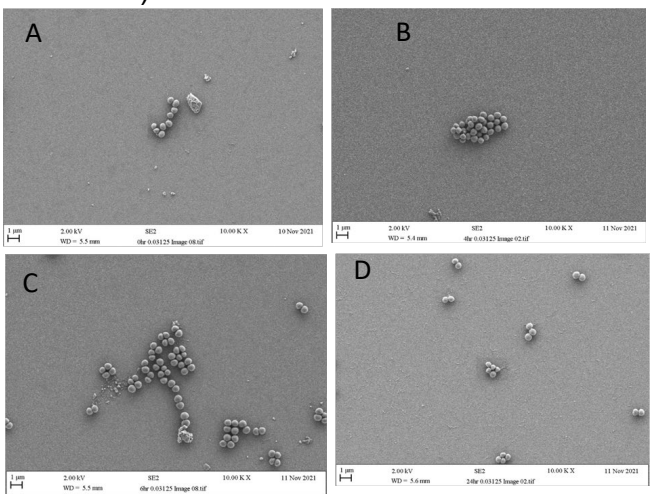
3.5 Evaluation of bacterial cell ultrastructure by Scanning Electron Microscopy (SEM)

A range of images were taken of each sample at 5,000x, 10,000x and 100,000x magnification of areas of interest. This was carried out to observe the method of
C
inhibition of each compound by looking at the bacterial cell structure after exposure to the compounds and would provide further evidence for small-molecule inhibitors affecting cells intracellularly resulting in inhibition of bacterial cell growth. The images do not display any significant cellular morphology differences, however, there is an increase in bacterial cell density on control samples, compared to samples treated with GSK-J4.

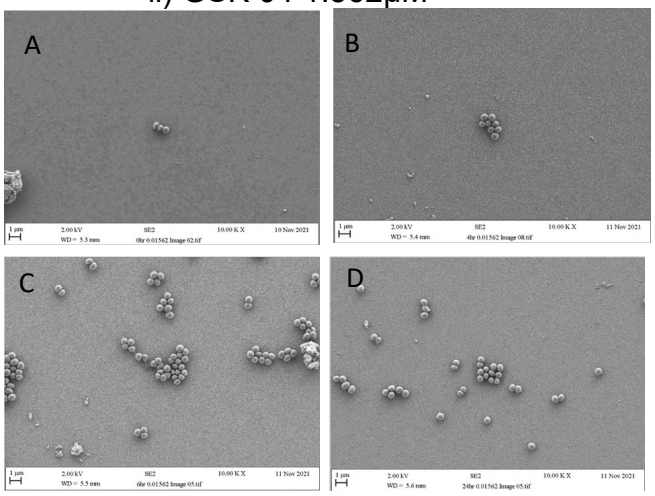
i) Triton



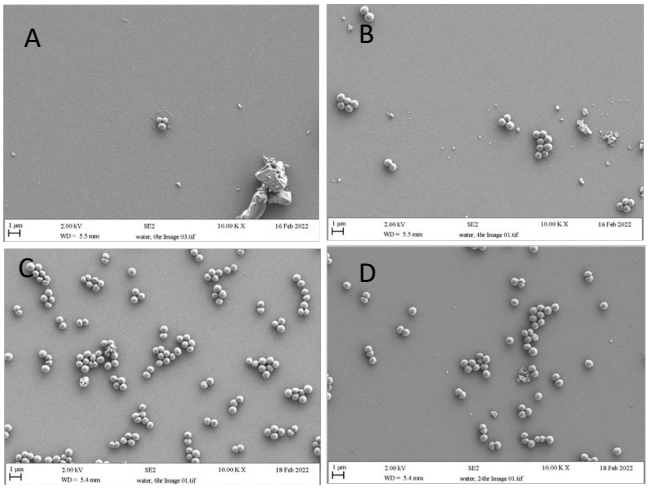
iii) GSK-J4 0.03125mM



ii) GSK-J4 1.562μM



iiii) Water



iiiii) DMSO

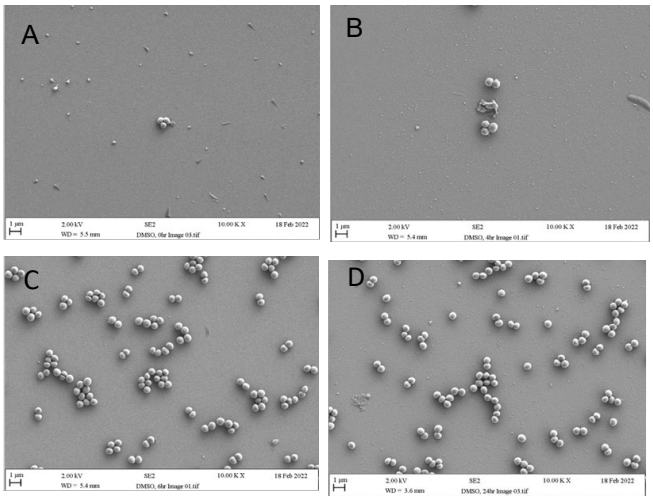


Figure 2. Scanning Electron Microscopy images at 10,000x magnification to observe cell morphology when exposed to GSK-J4.

The effect of GSK-J4 at 1.562μM and 0.03125mM on *S. aureus* USA300 during a time-kill following exposure for (A) 0 hrs, (B) 4 hrs, (C) 6 hrs and (D) 24 hrs at 10,000x magnification. Triton-x100, DMSO and water were used as controls. Triton-x100 eliminates bacterial cell growth, water in order to compare the effect of no compound exposure and neat DMSO was used as the solvent for all compounds. Scale bar is shown and images represent the field of view for three biological replicates.

i) A depicts cells from 0 hrs of triton exposure and shows what looks like damaged cells that are spread across the plane of view. B, after 4 hrs of exposure to triton there are only 4 cells in this image which shows the inhibitors effects of triton which has impeded cell proliferation. C, shows only two cells which confirms the further inhibitory effects of triton. D, although this image shows more cells, each image is from a different area of the silicon wafer, so these cells are most likely damaged by triton but remain visible.

Although no morphology differences were observed as a result of the SEM images, intracellular changes may be occurring which this type of microscopy could not detect.

ii) A shows 0 hrs of GSK-J4 treatment at 1.562μM and has only 3 cells of *S. aureus* USA300 in the field of view. B, at 4 hrs of treatment there is a collection of 8 cells that have proliferated. C, at 6 hrs of treatment there are many congregations of proliferating cells which shows that growth has not been impeded as the cells are thriving. D, at 24 hrs of treatment, there are many cells which do not show signs of damage or impeded growth.

iii) A displays 0 hrs of GSK-J4 treatment at 3.125 μM. There are 9 cells congregated together before the compound has time to take effect. B, at 4 hrs of treatment shows many cells collected in what could be the early stages of a biofilm formation. Judging by the previous time-kill assay, this compound takes effect between 6 to 24 hrs, which could be why there are no inhibitory signs. C, at 6 hrs of treatment, the cells look more spread out however, there are still many cells present. D, at 24 hours cells are seen in smaller groups, with the largest group of cells comprising of 5. These cells are more spread out than in the previous images and more spars, which may be indicative of inhibition.

iiii) A shows at 0 hrs of treatment with water, there are just 3 cells in the field of view. B, at 4 hrs, clusters of cells have become to form with the largest being 8 cells large. C, at 6 hrs shows many cells with a mixture of congregation and smaller groups spread across the area, showing no impediment of cell proliferation. D, at 24 hrs shows a very similar picture to the 6 hr image, with the only difference being it was taken in a different area of the silicon disc.000x

iiiii) A, shows a small collection of 3 cells at 0 hrs of DMSO treatment. B, at 4 hrs of treatment has a pair of two cells and a cluster of 3, showing the start of cell proliferation. C, at 6 hrs the cells have proliferated dramatically as it shows large groups of cells as well as pairs, in a large quantity, spread across the area. D, this image is very similar to image C, indicating no displays of inhibition.

Images that were treated with GSK-J4 did not exhibit any changes in terms of morphology, however there was a visible reduction of bacterial cells in the images treated with the compound.

3.6 *Galleria Mellonella* larvae infection modelling

The compounds focused on in this study have established inhibition of bacterial growth in media, however an *in vivo* test shows how the compounds interact with components of a living organism e.g the immune system. It also shows if the compound would be toxic in a living system, which would prove it to be ineffective as an anti-virulence oral drug. An infection model was also performed where *Galleria mellonella* were inoculated by injection with approximately 1 x 10^6 CFU mL^{-1} *S. aureus* USA300. GSK-J4 at concentrations of 1 μM and 5 μM respectively were subsequently administered to larvae and survival was monitored every 24 hrs over a 72 hr period. After 24 hrs,

90% of larvae survived following the addition of 1 μM and 5 μM GSK-J4, compared to 76.5% after 48 hrs. Following 72 hrs of infection, *G. mellonella* infected with *S. aureus* USA300 had a survival rate of 53.55% (for 1 μM GKS-J4) (Fig. 3, green diamond) and 45.9% (for 5 μM GSK-J4) (Fig. 3, clear triangle), which was significant compared to the bacterial inoculated control group of *G. mellonella* ($P = 0.0062$). The no injection, 1% DMSO and *S. aureus* USA300 plus vancomycin, PBS injected control groups all had a 100% survival rate after 72 hrs. In contrast, larvae infected with *S. aureus* USA300, *S. aureus* USA300 plus PBS or 1% DMSO all had a 0% survival rate after 72 hrs. The larvae were able to tolerate GSK-J4 alone with 85.5% survival following administration of a concentration of 1 μM (Fig. 3, green circle) and 5 μM respectively (Fig. 3, green square with orange outline). This shows that GSK-J4 being a non-toxic drug that is not hindered in terms of inhibition of bacterial growth when in a living system, which is evident by having the highest survival rate.

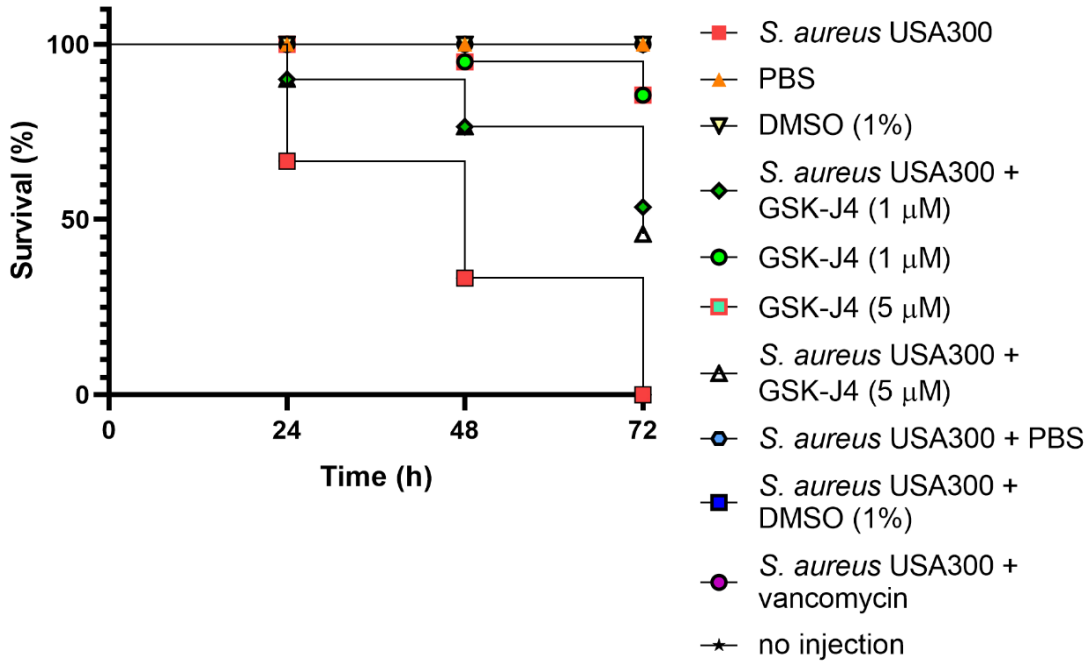


Figure 3. *Galleria mellonella* survival after exposure to *S. aureus* USA300 and following treatment with differing concentrations of GSK-J4 over 72 hrs. Control groups containing bacteria inoculated with *S. aureus* USA300 alone and in combination with PBS, 1% DMSO and vancomycin are shown. Additionally, control groups without bacteria were also used for comparison of survival. Significance was determined by One-way ANOVA (P=0.0062) for larvae inoculated with bacteria alone compared to those following subsequent addition of GSK-J4 at both concentrations. Number of larvae per group = 10.

3.7 Use of GSK-J4 against biofilms of *S. aureus* USA300.

Biofilms were tested against as they are more complex than a normal infection and are more difficult to inhibit. This assay was performed to see if GSK-J4 possessed anti-virulent activity against biofilms which would be indicated by a reduction or an inhibition of bacterial growth. Biofilms of *S. aureus* USA300 were formed within 96 well plates over a 48 h period and treated with GSK-J4. Biofilm recovery was analysed

using the crystal violet biofilm assay, but there was no evidence of biofilm eradication (data not shown). However, biofilms of *S. aureus* USA300 were also grown using a CDC reactor system. Coupons containing 24 hr biofilms were treated with GSK-J4 and bacterial viability was assessed by Miles and Misra techniques. No differences in bacterial recovery were seen following incubation with 1 μM compared to untreated bacterial controls. However, a one-log reduction in viability was observed after biofilms were exposed to 5 μM GSK-J4 (1.52×10^6 CFU mL^{-1}) (Fig. 4, black bar) compared untreated biofilms (1.38×10^7 CFU mL^{-1}) (Fig. 4, dark grey bar), which was deemed a significant reduction ($P < 0.0001$). No evidence of biofilm eradication was observed with 1% DMSO or PBS control groups, whereas biofilm viability was zero after exposure to vancomycin. The results of this assay observed a reduction of viable bacterial cells when exposed to 5μM GSK-J4, however there were no differences in bacterial growth when exposed to 1μM and neither were able to inhibit all viable bacterial cells of the biofilm.

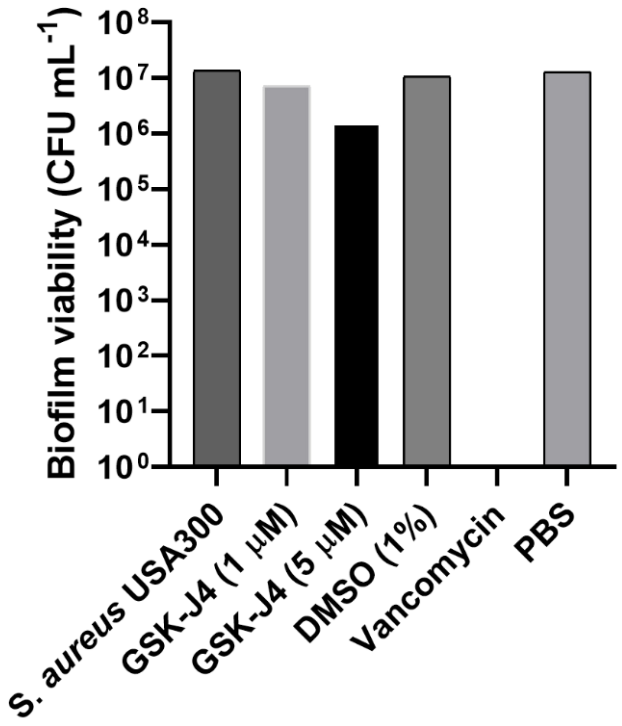


Figure 4. Biofilm eradication assay using *S. aureus* USA300 biofilms treated with concentrations of GSK-J4. Biofilms were grown over 2 days and were treated with 1μM and 5μM of GSK-J4 for 24hrs. A one-way ANOVA was performed to confirm the data is significant with a p value of <0.0001. Control variables included were the *S. aureus* USA300 bacteria alone, 1% DMSO, vancomycin and PBS.

3.8 Determining the demethylase inhibitory activity of GSK-J4.

Demethylase enzyme inhibition was tested as a possible method of action that enables GSK-J4 to inhibit bacterial growth. The inhibitory activity of GSK-J4 against bacterial demethylases was assessed using a colorimetric assay. The demethylase assay produced a colour change, and the absorbance was read from a microplate reader. The mean of each condition repeated in triplicate were entered into the equation below, to calculate percentage inhibition. The 5 mM treated wells exhibited a 23.8833% inhibition of histone demethylases, while 1mM treated wells showed a 9.62667% inhibition. This assay showed that the higher concentration of 5 mM GSK-

J4 exhibits the most inhibition of histone demethylases compared to the lower concentration of 1 mM

10

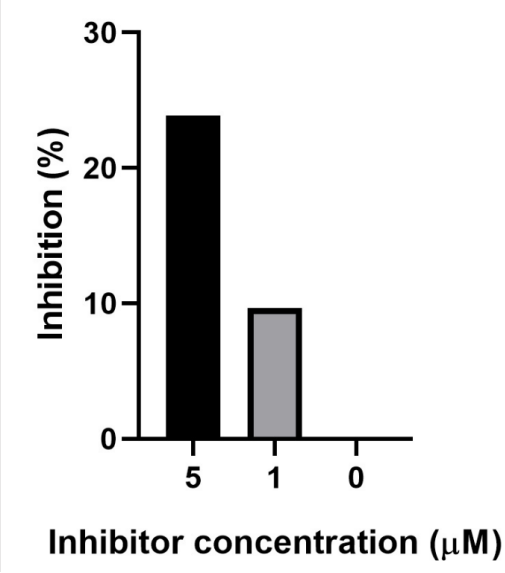


Figure 5. Percentage of demethylase inhibition when *S. aureus* USA300 is treated with GSK-J4. S. aureus USA300 was exposed to 5μM and 1μM of GSK-J4 and the demethylase inhibition percentage was assessed. The control variable used was *S. aureus* USA300 without GSK-J4 treatment to compare the compound's effects. The assay was performed in triplicate and an average value was used. A one-way ANOVA showed that the data is significant (P = 0.0009).

4 Discussion

Epigenetic target inhibitors are a promising novel treatment against antibiotic resistant strains of bacterial pathogens. The results of this study have demonstrated the antimicrobial activity and efficacy of both kinase and demethylase inhibitors. These SMIs all had chemical structures which appear to enable bacterial inhibition without

targeting the cellular ultrastructure. Indeed, kinase inhibitors disrupt the intercellular signalling cascade which decreases virulence and possible biofilm formation communications (King and Blackledge, 2021).

The present study screened a library of 103 putative kinase inhibitors and 36 epigenetic inhibitors of which 6 were identified which inhibited bacterial growth at 1 mM concentrations. Compounds did not achieve complete dissolution when diluted with DMSO, which were removed from the study. Compounds 140, 89, 132 and GSK-J4 all showed inhibitory activity against the Gram-positive *S.aureus* strains USA300 and SH1000. Compound 140 had the lowest MIC against *S.aureus* USA300, and subsequently became the focus kinase inhibitor compound, along with GSK-J4 (table 3). Compound 140 and GSK-J4 have very similar molecular structures and the same molecular weights (table 2). Both GSK-J4 and compound 140 achieved complete USA300 bacterial inhibition by 6 h during the time-kill kinetic assay, whereas the remaining compounds required the full 24 hrs. Higher concentrations of the other compounds would be required to kill the bacteria in a shorter amount of time, meaning they are far less potent than GSK-J4 and compound 140 which already inhibit the bacteria at a lower concentration.

GSK-J4 was highly active in resolving *S.aureus* USA300 infections using the *in vivo* Galleria mellonella model (figure 3). It was discovered that using 100% DMSO as a solvent was toxic towards the *G.mellonella* (data not shown), so a 1% DMSO dilution was used, which improved survival rates considerably and had no detrimental effect on antibacterial activity. Using *G.mellonella* as an in vivo model is an established method to evaluate immune response, tissue burden and replicate mammalian systems (Kay et al., 2019). They have a complex innate and adaptive immune system including phagocytes, encapsulation, clotting, the phenol-oxidase pathway and

opsonins. The results build upon previous studies where GSK-J4 treated mice prevented the onset of *Escherichia coli* infections (Pan et al., 2018). Treatment increased survival rates by 50% after infection, and the bacterial viability from the mice blood samples had significantly decreased. This could be due to a decrease in virulence within the bacterial cell and supports the theory that GSK-J4 is an intracellular inhibitor, which disrupts signalling cascades and communication within the bacterial cell. This would also account for the lack of noticeable changes in cellular ultrastructure as shown by the SEM imaging as internal components are being targeted by SEM imaging rather than physical disruption to the cell. Another study also treated mice with GSK-J4 after exposing them to toxoplasmosis (Liu et al., 2020). Mice were infected with strain 100RH *T. gondii* strain which were then treated with GSK-J4 and continued treatment for the following 4 days. The GSK-J4 treated mice had significantly longer survival rates (15.2±1.69 days, P=0.001) than the mice treated with DMSO (11.9±0.74 days).

GSK-J4 did not have a large inhibitory effect on biofilm formation and did not achieve complete inhibition even at levels above MBC values (figure 4). This may be due to biofilms being notoriously difficult to penetrate and impede growth. Factors working against GSK-J4 include; the extracellular matrix which protects bacterial cells from outer stress and antimicrobial exposure and the secretion of extracellular polysaccharide substance (Roy et al., 2018). This substance traps other bacterial cells in a bio-molecular layer which gives the biofilm structure and stability and channels for waste, nutrients, and water. These assets provide the biofilm with sustenance and defence to survive, which can make it very challenging for compounds to penetrate. However, to advance this study, antibiotics could be used in synergy with GSK-J4.

The histone demethylase assay showed that the highest concentration of GSK-J4 was able to inhibit a higher percentage of demethylation (figure 5). This is evidence to support the hypothesis that GSK-J4 can catalyse the H3K27me3 (trimethylated-histone lysine) to H3K27me2/1 (di/mono-methylated histone lysine) which then is detrimental to virulence gene expression (Wang et al., 2020). Prokaryotic cells do not possess a noticeable homologue for the eukaryotic target of GSK-J4, therefore it is likely there will be low binding affinity for the novel target. Although the percentage inhibition was relatively low, this provided a useful insight to further investigate the binding partner of GSK-J4 within the *S.aureus* bacteria.

Based on the knowledge that the schistosome life cycle needs highly controlled gene-transcription, the effects of GSK-J4 chemotherapy on Schistosoma *mansoni* Schistosomula parasitic infections have been reported (Adeyemi et al., 2018) (Lobo-Silva et al., 2020). Observation of schistosomula exposed to the compound revealed that motility was impaired and adult worm mortality was also induced. These effects became more pronounced when the drug concentration was increased from 5 μM to 10-20 μM. This data demonstrates the potential for using GSK-J4 as a therapeutic application in other areas associated with H3K27 methylation. The study attempted to elucidate the therapeutic target in the inhibition of Smp_034000 protein, however methylation levels of H3K27me3 did not increase during 7.5 μM GSK-J4 treatment and a small decrease at 20 μM. This could be due to a similar observation found in another study where no modulation of H3K27me3 was observed, whereas a decrease in H3K27me1 levels were reported (Morozov et al., 2017). In this study, the inhibition of JMJD3 by GSK-J4 did not cause a global accumulation of H3K27me3, but increased

levels in other promoter regions. This may be the case; however, the target of this drug has not been found so other mechanistic possibilities cannot be discounted.

The histone demethylase inhibitor GSK-J4 is a competitive inhibitor of two cofactors which are required for enzymatic activity and can initiate catalytic reactions to regulate gene expression inside a bacterial cell (Heinemann et al., 2014). The methylation process of lysines by histone methyltransferases can be reversed by lysine demethylases (KDMs), which are known to demethylate histones H3K4, H3K9, H3K27, H3K26 and H4K20 (Maes et al., 2015). GSK-J1 was the first potent KDM6 inhibitor described and inhibits KDM6B in vitro (Kruidenier et al., 2012). However, it is not active in cells due to the highly polar carboxylate group of GSK-J1 which impedes cellular permeability. GSK-J4 is the ethyl ester form of GSK-J1, inhibits H3K27 demethylation and can penetrate the cell. Recent studies have focused on GSK-J4 as treatment for parasitic infections such as Schistosomiasis (Lobo-Silva et al., 2020) (Whatley et al., 2019). It has been found that a potential drug target for eukaryotic pathogens could be eraser enzymes which remove epigenetic markers like demethylases and deacetylases (Arrowsmith et al., 2012).

The major reason for the increased development of antibiotic resistance is the lack of available alternative therapies (Bush et al., 2011). Antimicrobial resistant genes can be acquired by genetic recombination which can include horizontal gene transfer, conjugation, phage transduction, or transformation (Brinkac et al., 2017). Many antibiotics are naturally derived, however synthetic drugs, such as quinolones, can also produce therapeutic inhibition of bacterial growth (Gil-Gil et al., 2019). The essential attributes of these new synthetic drugs are to demonstrate differential toxicity between prokaryotic and eukaryotic cells, demonstrate a broad spectrum of activity and possess a low propensity for resistance generation. For example, the antibacterial

efficacy of several 1,3-bis(aryloxy)propan-2-amines by MIC and MBC assays, exposure to mammalian cells, identifying molecular targets using interaction profiling and homology modelling (Serafim et al. 2019). The results found that the compounds CPD20, CPD21 and CPD22 successfully disrupted the growth of S. aureus, S. pyogenes and E. faecalis. However, the molecular target of the three candidates could not be determined and required further in vitro modelling.

5. Future Work

The research performed has great potential as a basis for further study. A pull-down assay to investigate the protein-SMI interactions occurring in the cell would be integral in finding the drug target within the bacterial cell. This would be the next aim and could enable the screening of more drugs that have a complementary active site to the drug target. GSK-J4 could also be assayed to determine cytotoxicity on human cell-lines such as HeLa, to assess their toxicity on human cells. If successful, an animal model could be used to find the therapeutic concentration in a more complex system beyond the G.mellonella model employed in this study.

Conclusions

Antimicrobial resistance presents a major global issue and there is now an increasing pressure to develop novel methods for targeting bacterial cells beyond the traditional paradigm of antibiotic therapy. Developing SMIs against key bacterial processes which regulate virulence traits represents an emerging area of antimicrobial chemotherapy (Mühlen and Dersch, 2016). The compounds mentioned in this study provide a representation of potential epigenetic targets. GSK-J4 became the focus of this study

due to the ability to inhibit *S.aureus* USA300 bacterial growth at lower concentrations, potentially decreasing the cell virulence abilities in *G.mellonella* with low cytotoxicity. To advance this line of study, further assays should be carried out to discover the GSK-J4 binding site within the bacterial cell. This study has highlighted the demethylase inhibition abilities of GSK-J4 and provides evidence to link the activity to bacterial inhibition. Further research is needed to determine the efficacy of identified kinase inhibitors. Cytotoxicity should also be assessed further, for example by using HeLa and Caco-2 cell lines or mammalian cell lines. If successful, this could create the potential for human trials in later studies to introduce a new antimicrobial drug for use within human healthcare.

Reference List

Adam, K. and Hunter, T. (2018) "Histidine kinases and the missing phosphoproteome from prokaryotes to eukaryotes." *Laboratory investigation; a journal of technical methods and pathology*, 98(2) pp. 233–247.

Adeyemi, O. S., Sugi, T., Han, Y. and Kato, K. (2018) "Screening of chemical compound libraries identified new anti-Toxoplasma gondii agents." *Parasitology research*, 117(2) pp. 355–363.

Al-Charrakh, A. H. and Al-Hassnawi, H. H. (2012) 'Molecular Characterization of Antibiotic Resistance and Virulence Factors of Methicillin Resistance Staphylococcus aureus (MRSA) Isolated from Clinical Cases in Babylon Province.' Figure 1-1

Arrowsmith, C. H., Bountra, C., Fish, P. V., Lee, K. and Schapira, M. (2012) "Epigenetic protein families: a new frontier for drug discovery." *Nature reviews. Drug discovery*, 11(5) pp. 384–400.

Awosile, B. and Agbaje, M. (2020) "Genetic Environments of Plasmid-Mediated blaCTXM-15 beta-lactamase gene in Enterobacteriaceae from Africa." *Preprints*.

Brinkac, L., Voorhies, A., Gomez, A. and Nelson, K. E. (2017) "The Threat of Antimicrobial Resistance on the Human Microbiome." *Microbial ecology*, 74(4) pp. 1001–1008.

Bush, K., Courvalin, P., Dantas, G., Davies, J., Eisenstein, B., Huovinen, P., Jacoby, G. A., Kishony, R., Kreiswirth, B. N., Kutter, E., Lerner, S. A., Levy, S., Lewis, K., Lomovskaya, O., Miller, J. H., Mobashery, S., Piddock, L. J. V., Projan, S., Thomas, C. M., Tomasz, A., Tulkens, P. M., Walsh, T. R., Watson, J. D., Witkowski, J., Witte, W., Wright, G., Yeh, P. and Zgurskaya, H. I. (2011) "Tackling antibiotic resistance." *Nature reviews. Microbiology*, 9(12) pp. 894–896.

Chu, X., Zhong, L., Yu, L., Xiong, L., Li, J., Dan, W., Ye, J., Liu, C., Luo, X. and Liu, B. (2020) "GSK-J4 induces cell cycle arrest and apoptosis via ER stress and the synergism between GSK-J4 and decitabine in acute myeloid leukaemia KG-1a cells." *Cancer cell international*, 20, June, p. 209.

Collignon, P., Aarestrup, F. M., Irwin, R. and McEwen, S. (2013) "Human deaths and third-generation cephalosporin use in poultry, Europe." *Emerging infectious diseases*, 19(8) pp. 1339–1340.

De Oliveira, D. M. P., Forde, B. M., Kidd, T. J., Harris, P. N. A., Schembri, M. A., Beatson, S. A., Paterson, D. L. and Walker, M. J. (2020) "Antimicrobial Resistance in ESKAPE Pathogens." *Clinical microbiology reviews*, 33(3).

Foletti, D., Strop, P., Shaughnessy, L., Hasa-Moreno, A., Casas, M. G., Russell, M., Bee, C., Wu, S., Pham, A., Zeng, Z., Pons, J., Rajpal, A. and Shelton, D. (2013) "Mechanism of action and in vivo efficacy of a human-derived antibody against Staphylococcus aureus α-hemolysin." *Journal of molecular biology*, 425(10) pp. 1641–1654.

Gao, F., Thornley, B. S., Tressler, C. M., Naduthambi, D. and Zondlo, N. J. (2019) "Phosphorylation-dependent protein design: design of a minimal protein kinase-inducible domain." *Organic & biomolecular chemistry*, 17(16) pp. 3984–3995.

Garnak, M. and Reeves, H. C. (1979) "Phosphorylation of Isocitrate dehydrogenase of Escherichia coli." *Science*, 203(4385) pp. 1111–1112.

Gatta, V., Tomašič, T., Ilaš, J., Zidar, N., Peterlin Mašič, L., Barančoková, M., Frlan, R., Anderluh, M., Kikelj, D. and Tammela, P. (2020) "A New Cell-Based AI-2-Mediated Quorum Sensing Interference Assay in Screening of LsrK-Targeted Inhibitors." *Chembiochem: a European journal of chemical biology*, 21(13) pp. 1918–1922.

Gil-Gil, T., Laborda, P., Sanz-García, F., Hernando-Amado, S., Blanco, P. and Martínez, J. L. (2019) "Antimicrobial resistance: A multifaceted problem with multipronged solutions." *MicrobiologyOpen*, 8(11) p. e945.

Grangeasse, C., Nessler, S. and Mijakovic, I. (2012) "Bacterial tyrosine kinases: evolution, biological function and structural insights." *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 367(1602) pp. 2640–2655.

Hatch, S. B., Yapp, C., Montenegro, R. C., Savitsky, P., Gamble, V., Tumber, A., Ruda, G. F., Bavetsias, V., Fedorov, O., Atrash, B., Raynaud, F., Lanigan, R., Carmichael, L., Tomlin, K., Burke, R., Westaway, S. M., Brown, J. A., Prinjha, R. K., Martinez, E. D., Oppermann, U., Schofield, C. J., Bountra, C., Kawamura, A., Blagg, J., Brennan, P. E., Rossanese, O. and Müller, S. (2017) "Assessing histone demethylase inhibitors in cells: lessons learned." *Epigenetics & chromatin*, 10, March, p. 9.

Heinemann, B., Nielsen, J. M., Hudlebusch, H. R., Lees, M. J., Larsen, D. V., Boesen, T., Labelle, M., Gerlach, L.-O., Birk, P. and Helin, K. (2014) “Inhibition of demethylases by GSK-J1/J4.” *Nature*, pp. E1-2.

Hutchings, M. I., Truman, A. W. and Wilkinson, B. (2019) “Antibiotics: past, present and future.” *Current opinion in microbiology*, 51, October, pp. 72–80.

Jeśman, C., Młudzik, A. and Cybulska, M. (2011) “[History of antibiotics and sulphonamides discoveries].” *Polski merkuriusz lekarski: organ Polskiego Towarzystwa Lekarskiego*, 30(179) pp. 320–322.

Jiang, L., Mu, J., Zhang, Q., Ni, T., Srinivasan, P., Rayavara, K., Yang, W., Turner, L., Lavstsen, T., Theander, T. G., Peng, W., Wei, G., Jing, Q., Wakabayashi, Y., Bansal, A., Luo, Y., Ribeiro, J. M. C., Scherf, A., Aravind, L., Zhu, J., Zhao, K. and Miller, L. H. (2013) “PfSETvs methylation of histone H3K36 represses virulence genes in Plasmodium falciparum.” *Nature*, 499(7457) pp. 223–227.

Kadri, S. S. (2020) “Key Takeaways From the U.S. CDC’s 2019 Antibiotic Resistance Threats Report for Frontline Providers.” *Critical care medicine*, 48(7) pp. 939–945.

Kakkar, A. K., Shafiq, N., Singh, G., Ray, P., Gautam, V., Agarwal, R., Muralidharan, J. and Arora, P. (2020) “Antimicrobial Stewardship Programs in Resource Constrained Environments: Understanding and Addressing the Need of the Systems.” *Frontiers in public health*, 8, April, p. 140.

Katz, L. and Baltz, R. H. (2016) “Natural product discovery: past, present, and future.” *Journal of industrial microbiology & biotechnology*, 43(2–3) pp. 155–176.

Kay, S., Edwards, J., Brown, J. and Dixon, R. (2019) “Galleria mellonella Infection Model Identifies Both High and Low Lethality of Clostridium perfringens Toxigenic Strains and Their Response to Antimicrobials.” *Frontiers in microbiology*, 10, July, p. 1281.

Kennelly, P. J. (2002) “Protein kinases and protein phosphatases in prokaryotes: a genomic perspective.” *FEMS microbiology letters*, 206(1) pp. 1–8.

Kim, H., Burkinshaw, B. J., Lam, L. G., Manera, K. and Dong, T. G. (2021) “Identification of Small Molecule Inhibitors of the Pathogen Box against Vibrio cholerae.” *Microbiology spectrum*, 9(3) p. e0073921.

Kim, M. K., Zhao, A., Wang, A., Brown, Z. Z., Muir, T. W., Stone, H. A. and Bassler, B. L. (2017) “Surface-attached molecules control Staphylococcus aureus quorum sensing and biofilm development.” *Nature microbiology*, 2, May, p. 17080.

King, A. and Blackledge, M. S. (2021) “Evaluation of small molecule kinase inhibitors as novel antimicrobial and antibiofilm agents.” *Chemical biology & drug design*, 98(6) pp. 1038–1064.

King, A. and Blackledge, M. S. (2021) ‘Evaluation of small molecule kinase inhibitors as novel antimicrobial and antibiofilm agents.’ *Chemical biology & drug design*, 98(6) figure 1

Kong, C., Neoh, H.-M. and Nathan, S. (2016) “Targeting Staphylococcus aureus Toxins: A Potential form of Anti-Virulence Therapy.” *Toxins*, 8(3).

Kruidenier, L., Chung, C.-W., Cheng, Z., Liddle, J., Che, K., Joberty, G., Bantscheff, M., Bountra, C., Bridges, A., Diallo, H., Eberhard, D., Hutchinson, S., Jones, E., Katso, R., Leveridge, M., Mander, P. K., Mosley, J., Ramirez-Molina, C., Rowland, P., Schofield, C. J., Sheppard, R. J., Smith, J. E., Swales, C., Tanner, R., Thomas, P., Tumber, A., Drewes, G., Oppermann, U., Patel, D. J., Lee, K. and Wilson, D. M. (2012) “A selective jumonji H3K27 demethylase inhibitor modulates the proinflammatory macrophage response.” *Nature*, 488(7411) pp. 404–408.

Kyriakis, J. M. (2014) “In the beginning, there was protein phosphorylation.” *The Journal of biological chemistry*, 289(14) pp. 9460–9462.

Lakhundi, S. and Zhang, K. (2018) “Methicillin-Resistant Staphylococcus aureus: Molecular Characterization, Evolution, and Epidemiology.” *Clinical microbiology reviews*, 31(4).

Li, Y., Zhang, M., Sheng, M., Zhang, P., Chen, Z., Xing, W., Bai, J., Cheng, T., Yang, F.-C. and Zhou, Y. (2018) “Therapeutic potential of GSK-J4, a histone demethylase KDM6B/JMJD3 inhibitor, for acute myeloid leukemia.” *Journal of cancer research and clinical oncology*, 144(6) pp. 1065–1077.

Liu, S., Wu, M., Hua, Q., Lu, D., Tian, Y., Yu, H., Cheng, L., Chen, Y., Cao, J., Hu, X. and Tan, F. (2020) “Two old drugs, NVP-AEW541 and GSK-J4, repurposed against the Toxoplasma gondii RH strain.” *Parasites & vectors*, 13(1) p. 242.

Lobo-Silva, J., Cabral, F. J., Amaral, M. S., Miyasato, P. A., de Freitas, R. P., Pereira, A. S. A., Khouri, M. I., Barbosa, M. M. F., Ramos, P. I. P., Leite, L. C. C., Asojo, O. A., Nakano, E., Verjovski-Almeida, S. and Farias, L. P. (2020) “The antischistosomal potential of GSK-J4, an H3K27 demethylase inhibitor: insights from molecular modeling, transcriptomics and in vitro assays.” *Parasites & vectors*, 13(1) p. 140.

Lochmann, T. L., Powell, K. M., Ham, J., Floros, K. V., Heisey, D. A. R., Kurupi, R. I. J., Calbert, M. L., Ghotra, M. S., Greninger, P., Dozmorov, M., Gowda, M., Souers, A. J., Reynolds, C. P., Benes, C. H. and Faber, A. C. (2018) “Targeted inhibition of histone H3K27 demethylation is effective in high-risk neuroblastoma.” *Science translational medicine*, 10(441).

Maes, T., Carceller, E., Salas, J., Ortega, A. and Buesa, C. (2015) “Advances in the development of histone lysine demethylase inhibitors.” *Current opinion in pharmacology*, 23, August, pp. 52–60.

Maji, B., Gangopadhyay, S. A., Lee, M., Shi, M., Wu, P., Heler, R., Mok, B., Lim, D., Siriwardena, S. U., Paul, B., Dančík, V., Vetere, A., Mesleh, M. F., Marraffini, L. A., Liu, D. R., Clemons, P. A., Wagner, B. K. and Choudhary, A. (2019) “A High-Throughput Platform to Identify Small-Molecule Inhibitors of CRISPR-Cas9.” *Cell*, 177(4) pp. 1067-1079.e19.

Manning, D. R., DiSalvo, J. and Stull, J. T. (1980) “Protein phosphorylation: quantitative analysis in vivo and in intact cell systems.” *Molecular and cellular endocrinology*, 19(1) pp. 1–19.

Manso, J. A., Nunes-Costa, D., Macedo-Ribeiro, S., Empadinhas, N. and Pereira, P. J. B. (2019) “Molecular fingerprints for a novel enzyme family in Actinobacteria with glucosamine kinase activity.” *mBio*, 10(3).

Manuse, S., Fleurie, A., Zucchini, L., Lesterlin, C. and Grangeasse, C. (2016) “Role of eukaryotic-like serine/threonine kinases in bacterial cell division and morphogenesis.” *FEMS microbiology reviews*, 40(1) pp. 41–56.

Marreddy, R. K. R., Olaitan, A. O., May, J. N., Dong, M. and Hurdle, J. G. (2021) “Ebselen Not Only Inhibits Clostridioides difficile Toxins but Displays Redox-Associated Cellular Killing.” *Microbiology spectrum*, 9(2) p. e0044821.

Mather, A. E., Reid, S. W. J., Maskell, D. J., Parkhill, J., Fookes, M. C., Harris, S. R., Brown, D. J., Coia, J. E., Mulvey, M. R., Gilmour, M. W., Petrovska, L., de Pinna, E., Kuroda, M., Akiba, M., Izumiya, H., Connor, T. R., Suchard, M. A., Lemey, P., Mellor, D. J., Haydon, D. T. and Thomson, N. R. (2013) “Distinguishable epidemics of multidrug-resistant Salmonella Typhimurium DT104 in different hosts.” *Science*, 341(6153) pp. 1514–1517.

Matthews, K. A., Senagbe, K. M., Nötzel, C., Gonzales, C. A., Tong, X., Rijo-Ferreira, F., Bhanu, N. V., Miguel-Blanco, C., Lafuente-Monasterio, M. J., Garcia, B. A., Kafsack, B. F. C. and Martinez, E. D. (2020) “Disruption of the Plasmodium falciparum Life Cycle through Transcriptional Reprogramming by Inhibitors of Jumonji Demethylases.” *ACS infectious diseases*, 6(5) pp. 1058–1075.

McCubbin, K. D., Anholt, R. M., de Jong, E., Ida, J. A., Nóbrega, D. B., Kastelic, J. P., Conly, J. M., Götte, M., McAllister, T. A., Orsel, K., Lewis, I., Jackson, L., Plastow, G., Wieden, H.-J., McCoy, K., Leslie, M., Robinson, J. L., Hardcastle, L., Hollis, A., Ashbolt, N. J., Checkley, S., Tyrrell, G. J., Buret, A. G., Rennert-May, E., Goddard, E., Otto, S. J. G. and Barkema, H. W. (2021) “Knowledge Gaps in the Understanding of Antimicrobial Resistance in Canada.” *Frontiers in public health*, 9, October, p. 726484.

Mukherjee, P.K. (2015). “*Chapter 15* - Evaluation of Natural Products against Biofilm-Mediated Bacterial Resistance”. *Evidence-based validation of herbal medicine*. Amsterdam, Netherlands: Elsevier, pp.321–338. figure 15.7.

Morozov, V. M., Li, Y., Clowers, M. M. and Ishov, A. M. (2017) “Inhibitor of H3K27 demethylase JMJD3/UTX GSK-J4 is a potential therapeutic option for castration resistant prostate cancer.” *Oncotarget*, 8(37) pp. 62131–62142.

Mühlen, S. and Dersch, P. (2016) “Anti-virulence Strategies to Target Bacterial Infections.” *In* Stadler, M. and Dersch, P. (eds.) *How to Overcome the Antibiotic Crisis : Facts, Challenges, Technologies and Future Perspectives*. Cham: Springer International Publishing, pp. 147–183.

Pan, Y., Wang, J., Xue, Y., Zhao, J., Li, D., Zhang, S., Li, K., Hou, Y. and Fan, H. (2018) “GSKJ4 Protects Mice Against Early Sepsis via Reducing Proinflammatory Factors and Up-Regulating MiR-146a.” *Frontiers in immunology*, 9, October, p. 2272.

Pandey, R., Mishra, S. K. and Shrestha, A. (2021) “Characterisation of ESKAPE Pathogens with Special Reference to Multidrug Resistance and Biofilm Production in a Nepalese Hospital.” *Infection and drug resistance*, 14, June, pp. 2201–2212.

Pensinger, D. A., Schaenzer, A. J. and Sauer, J.-D. (2018) “Do Shoot the Messenger: PASTA Kinases as Virulence Determinants and Antibiotic Targets.” *Trends in microbiology*, 26(1) pp. 56–69.

Pérez-Lazo, G., Abarca-Salazar, S., Lovón, R., Rojas, R., Ballena-López, J., Morales-Moreno, A., Flores-Paredes, W., Arenas-Ramírez, B. and Illescas, L. R. (2021) “Antibiotic Consumption and Its Relationship with Bacterial Resistance Profiles in ESKAPE Pathogens in a Peruvian Hospital.” *Antibiotics (Basel, Switzerland)*, 10(10).

Pettigrew, M. M., Fennie, K. P., York, M. P., Daniels, J. and Ghaffar, F. (2006) “Variation in the presence of neuraminidase genes among Streptococcus pneumoniae isolates with identical sequence types.” *Infection and immunity*, 74(6) pp. 3360–3365.

Pilka, E. S., James, T. and Lisztwan, J. H. (2015) “Structural definitions of Jumonji family demethylase selectivity.” *Drug discovery today*, 20(6) pp. 743–749.

Prescott, J. F. (2014) “The resistance tsunami, antimicrobial stewardship, and the golden age of microbiology.” *Veterinary microbiology*, 171(3–4) pp. 273–278.

Price, J. R., Didelot, X., Crook, D. W., Llewelyn, M. J. and Paul, J. (2013) “Whole genome sequencing in the prevention and control of Staphylococcus aureus infection.” *The Journal of hospital infection*, 83(1) pp. 14–21.

Rice, A. J., Pesavento, R. P., Ren, J., Youn, I., Kwon, Y., Ellepola, K., Che, C.-T., Johnson, M. E. and Lee, H. (2021) “Identification of Small Molecule Inhibitors against Staphylococcus aureus Dihydroorotase via HTS.” *International journal of molecular sciences*, 22(18).

Roy, R., Tiwari, M., Donelli, G. and Tiwari, V. (2018) “Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action.” *Virulence*, 9(1) pp. 522–554.

Sakai, F., Talekar, S. J., Lanata, C. F., Grijalva, C. G., Klugman, K. P., Vidal, J. E., RESPIRA PERU Group and Investigators Group (2013) “Expression of Streptococcus pneumoniae Virulence-Related Genes in the Nasopharynx of Healthy Children.” *PloS one*, 8(6) p. e67147.

Shore, A. C. and Coleman, D. C. (2013) “Staphylococcal cassette chromosome mec: recent advances and new insights.” *International journal of medical microbiology: IJMM*, 303(6–7) pp. 350–359.

Stewart, D., Anwar, F. and Vedantam, G. (2020) “Anti-virulence strategies for Clostridioides difficile infection: advances and roadblocks.” *Gut microbes*, 12(1) p. 1802865.

Thangamani, S., Younis, W. and Seleem, M. N. (2015) “Repurposing ebselen for treatment of multidrug-resistant staphylococcal infections.” *Scientific reports*, 5, June, p. 11596.

Vaca, D. J., Thibau, A., Schütz, M., Kraiczy, P., Happonen, L., Malmström, J. and Kempf, V. A. J. (2020) “Interaction with the host: the role of fibronectin and extracellular matrix proteins in the adhesion of Gram-negative bacteria.” *Medical microbiology and immunology*, 209(3) pp. 277–299.

Wang, P., Lan, R., Guo, Z., Cai, S., Wang, J., Wang, Quan, Li, Zeyu, Li, Zhenzhen, Wang, Qianqian, Li, J., Wu, Z., Lu, J. and Liu, P. (2020) “Histone Demethylase JMJD3 Mediated Doxorubicin-Induced Cardiomyopathy by Suppressing SESN2 Expression.” *Frontiers in cell and developmental biology*, 8, September, p. 548605.

Waseem, H., Ali, J., Sarwar, F., Khan, A., Rehman, H. S. U., Choudri, M., Arif, N., Subhan, M., Saleem, A. R., Jamal, A. and Ali, M. I. (2019) “Assessment of knowledge and attitude trends towards antimicrobial resistance (AMR) among the community members, pharmacists/pharmacy owners and physicians in district Sialkot, Pakistan.” *Antimicrobial resistance and infection control*, 8, April, p. 67.

Whatley, K. C. L., Padalino, G., Whiteland, H., Geyer, K. K., Hulme, B. J., Chalmers, I. W., Forde-Thomas, J., Ferla, S., Brancale, A. and Hoffmann, K. F. (2019) “The repositioning of epigenetic probes/inhibitors identifies new anti-schistosomal lead compounds and chemotherapeutic targets.” *PLoS neglected tropical diseases*, 13(11) p. e0007693.

Willems, R. J. L., Top, J., van Santen, M., Robinson, D. A., Coque, T. M., Baquero, F., Grundmann, H. and Bonten, M. J. M. (2005) “Global spread of vancomycin-resistant Enterococcus faecium from distinct nosocomial genetic complex.” *Emerging infectious diseases*, 11(6) pp. 821–828.