# BET Inhibitors: Novel Approaches to Combatting Antimicrobial Resistance

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# BET Inhibitors: Novel Approaches to Combatting Antimicrobial Resistance

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## Abstract

Antimicrobial resistance is an increasing global threat and there is an urgent need for new antimicrobial therapeutics, specifically against multi-drug resistant bacteria. One approach is to use small molecule inhibitors (SMIs) to target specific pathogen virulence traits. BET inhibitors are a type of SMI that target BET proteins, which regulate transcription by reading the acetylation of the lysine residues on histone tails. This study is the first report where the BET inhibitor (+)-JQ1 has been deployed as an antimicrobial agent.

The antimicrobial activity of the (+)-JQ1 SMI was identified from a screening plate containing various SMIs and this showed activity against a multidrug resistant strain of *Staphylococcus aureus* strain USA300 at <456.99 µg mL-1. The Minimum Inhibitory Concentration (MIC) of the biologically active form of (+)-JQ1 against this strain was 128 µg $mL^{-1}$, while the MIC of the stereoisomer derivative (-)-JQ1 was 125 µg $mL^{-1}$. The similarity between these two results, coupled with the lack of BET protein homologues within *S. aureus* led to the hypothesis that the mechanism of antimicrobial action being observed was an off-target effect of the compound. SMI (+)-JQ1 was found to target some of the key virulence factors of *S. aureus* through the inhibition of biofilm formation and haemolytic toxin production. While no cellular morphological changes were observed after bacterial (+)-JQ1 exposure, this SMI was found to depolarise the membrane of *S. aureus* strain USA300 offering an insight into the potential antimicrobial mechanistic activity.

This research demonstrated a novel off-target antimicrobial application of the BET inhibitor (+)-JQ1 and the stereoisomer (-)-JQ1. There is potential for this SMI to be explored further as a topical wound dressing or wound cleaning agent against the multidrug resistant pathogen *S. aureus*.

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# 1. Introduction

## 1.1 Introduction to Antimicrobial Resistance (AMR)

Antimicrobial resistance (AMR) is one of the leading public health issues (Prestinaci, Pezzotti and Pantosti, 2015). Resources to treat bacterial, viral, fungal and parasitic infections are threatened by the development of resistance mechanisms. This issue is particularly concerning in the treatment of bacterial infections with antibiotics. The last few decades have seen the emergence of resistance mechanisms to most classes of antibiotics. Newer antibiotics that are brought to clinical practice are also susceptible to resistance.

There is a major need for alternative antibacterial treatment strategies. This has been recognised by the World Health Organization (WHO) since 2001 when the Global Strategy for Containment of Antimicrobial Resistance was introduced (World Health Organization, 2001). This set out a framework to reduce the spread of resistance in bacterial pathogens. In 2014, the true extent of the problem was recognised for the first time with the publication of the first WHO Global report on surveillance of AMR (World Health Organization, 2014). This report recognised the global spread of antibiotic resistance across various bacterial strains, with 50 % of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* strains showing resistance to common antimicrobials.

A 2022 study compiled data from 204 countries and territories from 2019 to give the most comprehensive analysis of AMR (Antimicrobial Resistance Collaborators, 2022). In 2019, AMR infections directly caused 1.27 million deaths and a further 4.95 million deaths were indirectly associated with AMR. Six leading pathogens accounted for 929,000 of the deaths directly caused

by AMR, including *E. coli, S. aureus*, *K. pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. The leading single pathogen with multiple drug resistances was methicillin resistant *S. aureus* (MRSA), which caused over 100,000 deaths.

By 2050 it is estimated that AMR will cost between $300 billion and $1 trillion annually (Dadgostar, 2019). It is also estimated that annual deaths will rise to 10 million, superseding cancer mortality rates.

## 1.2. Classical Antibiotics: Mechanisms of Activity

Traditionally antibiotics are used to treat bacterial infections and there are five main mechanisms by which this occurs. The first method is inhibition of cell wall synthesis. This mechanism is seen in β-Lactams, including penicillin, and glycopeptides (Reygaert, 2018). β-lactams target penicillin binding proteins which mediate the cross linking in the peptidoglycan structure of the cell wall (Bush and Bradford, 2016). Glycopeptides bind to D-alanyl D-alanine in precursor peptidoglycan, this prevents the peptidoglycan from binding to penicillin binding proteins (Kapoor, Saigal and Elongavan, 2017).

Other antibiotics disrupt the function of the cell membrane, such as polymyxins and lipopeptides. The main mechanism of polymyxins involve the hydrophobic fatty acid chain of the polymyxin interacting with lipid A in the lipopolysaccharides of the outer bacterial membrane, resulting in increased permeability (Mohapatra, Dwibedy and Padhy, 2021). Daptomycin is currently the only approved lipopeptide. The mechanism of action is debated in several studies, but it is widely

agreed that it targets phosphatidylglycerol in the bacterial membrane (Ledger, Sabnis and Edwards, 2022).

Aminoglycosides, tetracyclines, and macrolides are types of protein synthesis inhibitors. Aminoglycosides bind to the A-site of the 30S ribosomal subunit, increasing mistranslation in protein synthesis (Krause *et al.*, 2016). Tetracyclines also target the 30S subunit, they prevent the binding of aminoacyl-tRNA to the acceptor site on the ribosome (Chopra and Roberts, 2001). Macrolides bind to the nascent peptide exit tunnel located on the 50s subunit and block it, preventing proteins from exiting the ribosome (Vázquez-Laslop and Mankin, 2018).

Quinolones and rifampicin inhibit nucleic acid synthesis. Quinolones target the enzymes, gyrase and topoisomerase IV, to create double-stranded breaks in the bacterial chromosome (Aldred, Kerns and Osheroff, 2014). Rifampicin binds to DNA-dependent RNA polymerase, inhibiting its RNA synthesis (Hardie and Fenn, 2022).

The final antibiotic mechanism is action as metabolites, this is seen in antibiotics such as sulphonamides and trimethoprim. Sulphonamides inhibit folic acid synthesis, they act as competitive inhibitors for p-aminobenzoic acid (Ovung and Bhattacharyya, 2021). Trimethoprim targets the dihydrofolate reductase region on tetrahydrofolic acid, which is an active form of folic acid and a co-factor in DNA synthesis (Masters *et al.*, 2003).

## 1.3. Mechanisms of Antibiotic Resistance

As classical antibiotics function against very specific targets within the bacterial cell, there are clear risks of resistance evolution. There are four main methods by which bacteria can develop resistance: alterations of the bacterial target site, alterations to the structure of the antibiotic, the presence of efflux pumps and innate resistance (Munita and Arias, 2016).

One mechanism centring on the bacterial target involves blocking the target site to prevent activity by the antibiotic. The best studied example of this resistance is tetracycline resistance which is mediated by the ribosomal protection proteins Tet(O) and Tet(M) (Connell *et al.*, 2003). These proteins prevent tetracycline from binding to the ribosome by physically blocking the ribosome. Another resistance mechanism focused the bacterial target site involves altering the bacterial target site. This can take place through point mutations, enzymatic alterations or complete replacement of the target site (Munita and Arias, 2016). This mechanism is observed in MRSA. The bacteria acquire the *mecA* gene which encodes for PBP2a, a penicillin binding protein with a low affinity for β-lactams (Vestergaard, Frees and Ingmer, 2019).

Bacteria have the ability to produce enzymes which can induce biochemical changes in the antibiotic (Munita and Arias, 2016). The most common types of biochemical changes are acetylation, phosphorylation and adenylation. This mechanism of resistance is seen widely against aminoglycosides, aminoglycoside modifying enzymes produced by bacteria modify the hydroxyl or amino acid groups of the aminoglycoside, this structural modification reduces the drugs affinity for its target (Ramirez and Tolmasky, 2010). Other enzymes have the ability to degrade the antibiotic as commonly observed in β-lactams through the action of β- lactamases

(Tooke *et al.*, 2019). β- lactamases are a type of enzyme that degrade the amide bond of the beta lactam ring. Alongside the *mecA* gene, β- lactamases are a major factor in the rise of *S. aureus* antibiotic resistance.

Efflux pumps act to move toxic compounds out of the bacterial cell (Munita and Arias, 2016). Some of these pumps act on a specific antibiotic, for example the TetA efflux pump which is specific to tetracycline. While others act on a variety of antibiotics conferring multidrug resistance, such as the MexAB-OprM, NorA and BmrA efflux pumps (Pathania, Sharma and Gupta, 2019).

Gram-negative bacteria are intrinsically resistant to various antibiotic classes due to their permeability barrier (Maher and Hassan, 2023). Some bacteria have developed mechanisms to further alter the permeability barrier to prevent the action of antibiotics.

## 1.4. Development of AMR

The leading issue causing the rapid increase of AMR is overprescription and inappropriate usage of antibiotics. One study found that 30 % of antibiotics prescribed in US ambulatory care visits were prescribed for infections that did not require antibiotics or had a more preferable antibiotic available (Fleming-Dutra, Hersh and Shapiro, 2016). Incomplete treatment is also a major issue, 38 % of patients are unaware of the issues with not completing a full course of antibiotics (McCullough *et al.*, 2016). This can lead some bacteria to survive and develop resistance against the antibiotic (Uddin *et al.*, 2021). Action plans to control AMR are less likely to be implemented in low and middle-income countries due to a lack of funds, control, infrastructure and technology (Otaigbe and Elikwu, 2023). However, the knowledge of inappropriate use of antimicrobials is

similar between doctors from high income countries and doctors in low and middle-income countries (Taylor *et al.*, 2022). Other public health factors such as vaccination rate, migration, tourism, sanitation and population density also play a major role in the spread of AMR (Holmes *et al.*, 2016).

There has also been an increase in the non-therapeutic use of antibiotics in agriculture, with more antimicrobials used in food production than in human healthcare settings (Holmes *et al.*, 2016). They are used in healthy animals to promote growth, increase feed efficiency and prevent disease (Shea, 2004). The types of antibiotics used in livestock are the same or similar to those prescribed to humans for therapeutic use (Manyi-Loh *et al.*, 2018). A particular concern is the use of antibiotics in farming in underdeveloped countries as there is a lack of regulation. Farmers in poorer countries expand their farming practices to a large scale and use high levels of antimicrobials to increase the size of their animals to generate greater profits. However, Swedish agricultural data showed no decreased in production after a ban on antibiotic growth promoters was introduced in 1986 (Cogliani, Goossens and Greko, 2011).

Between 1962 and 2000 no new major antibiotics were introduced, and this time period is referred to as the discovery void (Silver, 2011). This decline can be attributed to a lack of funding available from major pharmaceutical companies in the area of antibiotic discovery. It is estimated to cost £0.5–£1 billion to develop and bring a new antibiotic to market (Sabtu, Enoch and Brown, 2015). Antibiotics have a lower success rate during development than other types of drugs and the profit margins are lower as antibiotics are only prescribed for short periods. This makes investing in antibiotics less desirable than other pharmaceutical opportunities. The use of incentive strategies has been suggested to try and increase the desirability of the antimicrobial

market to pharmaceutical companies (Batista *et al.*, 2019). Strategies such as loans, tax benefits, open-access knowledge sharing, market entry rewards and advances market commitments have been suggested.

In 1960 MRSA resistance was reported simultaneously by two British laboratories (Knox, 1961; Parker and Jevons, 1964). This discovery occurred within one year of the introduction of specific anti-staphylococcal penicillins (Turner *et al.*, 2019). However, recent genetic evidence suggests that methicillin resistance had been present in hedgehogs prior to the clinical use of antibiotics as a co-response to the spread of *S. aureus* (Larsen *et al.*, 2022). *S. aureus* resistance occurs in epidemic waves as a response to concentrated outbreaks rather than resulting from one global strain (Chambers and DeLeo, 2009). Historically these outbreaks were healthcare-associated, but now MRSA outbreaks are frequently observed within the community.

## 1.5. New Approaches to Combatting AMR

Due to the threat of AMR over the past few decades various new antimicrobial treatments have been suggested. One approach is the use of bacteriophages, which are viruses that specifically infect bacteria. Bacteriophages are the most abundant organism and are readily found in various sources including the ocean, soil and the human body (Clokie *et al.*, 2011). The main appeals of phage therapy is their abundance, natural specificity towards bacteria and their adaptability. However, their specificity would cause issues in therapeutic application as each bacterial target would require the discovery of a specific bacteriophage (Wittebole, De Roock and Opal, 2013). The long-term effects of bacteriophage therapy are unknown and there is the possibility that bacteria could develop resistance towards bacteriophages.

Genome editing using CRISPR-Cas is another potential treatment for AMR. It is a precise method to target and disable genes that confer resistance, making the bacteria once again susceptible to antibiotics they were previously susceptible to (Kundar and Gokarn, 2022). The main challenge with this approach is adapting the lab-based technique of CRISPR-Cas for use within humans in a clinical setting. There is also the possibility of bacteria developing resistance to genome editing.

Another method to combat AMR is the implementation of control programmes. The WHO created the Global Action Plan on Antimicrobial resistance in 2015 and encouraged countries to develop their own national action plans (Willemsen, Reid and Assefa, 2022). In 2019 the UK released its five-year national action plan. It included three main sections: reducing the need for antimicrobials, optimising the use of antimicrobials and investing the innovation of new therapeutics, vaccines and diagnostics (HM Government, 2019).

## 1.6. Introduction to Small-Molecule Inhibitors (SMIs)

One approach to new antimicrobial therapeutics is the use of small-molecule inhibitors (SMIs), which are classified as compounds that are smaller than 500 Da that target and inhibit the action of proteins (Megino-Luque *et al.*, 2020). They have emerged as a promising therapeutic across a variety of disciplines with a prevalence in oncology due to their targeted nature (Liu *et al.*, 2022). However, there are some toxicity concerns. While SMIs are celebrated for their targeted approach there is a lack of absolute specificity and the therapy can also target host cells that feature similar structures (Yu *et al.*, 2021). This issue is less relevant in the use of SMIs as antimicrobials due to the fact that the molecules can be designed to target biochemical processes not present in

eukaryotes, such as those involved in building and maintaining the prokaryotic cell wall (Gurevich and Gurevich, 2013).

Another appeal of SMI therapy is their broad spectrum of targets. SMIs have various targets, in particular targets relating to antimicrobial activity include enzyme inhibition, cell wall synthesis inhibition, membrane permeability inhibition, quorum sensing inhibition and efflux pump inhibition. New SMI targets are continually being discovered and notably some of these targets are molecules that had previously been considered as challenging to target, such as sotorasib which targets the KRAS mutation commonly seen in colorectal cancer (Hong *et al.*, 2020).

## 1.7. Types of Small-Molecule Inhibitors (SMIs)

There are various classes of SMIs which each target different protein families. One class of antimicrobial relevant SMIs are enzyme inhibitors such as protease inhibitors, kinase inhibitors and DNA gyrase inhibitors. Protease inhibitors were the first successful antiviral drugs, they act as competitive inhibitors for HIV protease (Wang, Lv and Chu, 2015). Protease inhibitors are used alongside other types of inhibitors as part of highly active antiretroviral therapy. Protease inhibitors have also been used as antivirals against other types of viruses such as hepatitis C and COVID-19 (de Leuw and Stephan, 2017; Sagawa, Inoue and Takano, 2020). One of the most prevalent SMI classes are protein kinase inhibitors, these target kinases preventing the phosphorylation of proteins (Wu, Nielsen and Clausen, 2015). The first kinase inhibitor approved for use was a protein tyrosine kinase inhibitor, imatinib mesylate, which was approved in 2001 as a treatment for leukaemia, increasing interest in the field (Savage and Antman, 2002). Whilst

initially recognised for their applications in oncology, bacterial kinases are vital to bacterial growth, virulence and biofilm formation, making them promising therapeutic targets (King and Blackledge, 2021). The first example of a kinase inhibitor being utilised as an antimicrobial occurred in 1998 when RWJ-49815, a known kinase A inhibitor, inhibited the growth of *S. aureus, Enterococcus faecium*, and *S. pneumoniae* (Barrett *et al.*, 1998). DNA gyrase inhibitors target DNA gyrase which is responsible for the organisation of DNA within the cell (Eakin *et al.*, 2012). Two existing classes of antibiotics target DNA gyrase, these being quinolones and aminocoumarins.

SMIs can inhibit quorum sensing which is beneficial to the prevention and dismantling of the biofilm structure. Furanones were first isolated from marine algae and have been shown to effectively inhibit biofilm formation in several bacterial species (Gómez *et al.*, 2022). Recently novel furanones were synthesised and were shown to be able to inhibit biofilm formation in *S. enterica*, *S. aureus* and *P. aeruginosa*. Acyl homoserine lactone (AHL) analogues are another type of quorum sensing inhibitor (Chbib, 2020). AHL has been shown to be of major importance in quorum sensing in Gram-negative bacteria. AHL is synthesised from S-adenosylmethionine, analogues such as S-adenosylhomocysteine 6 and sinefungin can be used to inhibit the synthesis of AHL.

The action of efflux pumps can be inhibited. Efflux pump inhibitors have been shown to inhibit biofilm formation in vitro (Ikonomidis *et al.*, 2008). Carbonyl cyanide-m-chlorophenylhydrazone (CCCP) is a proton motive force inhibitor, which has been shown to inhibit the MexAB-OprM efflux pump, in turn reducing biofilm formation in *P. aeruginosa*. This is a developing area and

further development and funding is required to assess the practicality of efflux pump inhibitors as therapeutics (Al Matar *et al.*, 2020).

## 1.8. BET Proteins

One promising type of SMI targets the bromodomain and extra terminal domain (BET) protein family, which consists of BRD2, BRD3, BRD4 and BRDT (Cheung, Kim and Zhou, 2021). These proteins are comprised of two bromodomains, BD1 and BD2, at the N-terminal and an extra-terminal domain at the C-terminal (Wang *et al.*, 2023). Bromodomains are a group of proteins first described in 1992 after being observed in the Drosophila genus (Tamkun *et al.*, 1992). BET proteins regulate transcription through the binding of the bromodomains to acetylated lysine residues on the tails of histones (Josling *et al.*, 2012). In response to this, BET proteins regulate gene expression through recruitment of transcription factors or through direct chromatin remodelling (Guo, Zheng and Peng, 2023). They interact with a range of transcription factors including c-Myc, E2F1, EWS/ETS and Twist. Due to this function BET proteins are exclusive to eukaryotes (Sayou and Govin, 2022). BET proteins are expressed in various tissues, with expression levels varying between the different sub-types (Table 1). Dysregulation of BET proteins can lead to various diseases including cancer, inflammatory disorders and neurodegenerative disorders.

BRD2 is expressed ubiquitously in many tissue including the bone marrow, thyroid, testes, ovaries and lymph nodes (Fagerberg *et al.*, 2013). In mice models of obesity BRD2 was found to regulate obesity induced inflammatory responses, such as insulin resistance, protecting against obesity coupled diseases (Wang, Deeney and Denis, 2013). It has been shown to protect cardiomyocytes

against myocardial ischemia-reperfusion injury, by regulating the Nrf2/HO-1 signalling pathway (Liu *et al.*, 2023). BRD2 plays a role in cholesterol homeostasis, regulating the expression of the cholesterol receptor sigma-2 (Shen *et al.*, 2020). BRD2 also promotes spatial mixing and genome compartmentalisation of chromatin through interacting with acetylated targets (Xie *et al.*, 2022).

BRD3 is primarily expressed in the endometrium, brain, prostate, spleen and testes (Fagerberg *et al.*, 2013). BRD3 acts as an epigenetic regulator for nuclear TYRO3, which is an indicator of metastasis in colorectal cancer (Hsu *et al.*, 2023). NUT carcinomas are caused by fusion between the NUT1 and BRD3/ BRD4 genes (Gozalez *et al.*, 2021). Recently it was discovered that fusion between BRD3 and NUTM2B also results in NUT carcinomas. BRD3 also plays a regulatory role in inflammation in rheumatoid arthritis (Seifritz *et al.*, 2023).

BRD4 is primarily expressed in the placenta, testes, spleen, appendix and ovaries (Fagerberg *et al.*, 2013). It is the most widely studied member of the BET protein family due to its significant association with cancer (Yang *et al.*, 2021). It plays a crucial role in the epigenetic regulation of super-enhancers and oncogenes (Donati, Lorenzini and Ciarrocchi, 2018). It plays a role in tumorigenesis of multiple cancers including glioblastoma (Duan, Yu and Chen, 2023). BRD4 also regulates haematopoiesis and inflammation, it is required for hematopoietic stem cell expansion and progenitor development (Ozato *et al.*, 2019). BRD4 in particular regulates transcriptional elongation, a key stage of transcription (Altendorfer, Mochalova and Mayer, 2022). Beyond transcription, BRD4 also regulates DNA damage checkpoints, telomere maintenance and genome folding (Linares-Saldana *et al.*, 2021). There are multiple isoforms of BRD4 and these can have opposing mechanisms even within the same type of tissue (Wu *et al.*, 2020). In breast cancer, BRD4-S is oncogenic and BRD4-L is tumour suppressive.

Unlike the other BET proteins, BRDT is solely expressed in the testes (Fagerberg *et al.*, 2013). Due to its function as an epigenetic regulator in the testes it is thought that inhibition of BRDT could be a form of male contraception (Wisniewski and Georg, 2020). BRDT also plays a role in various types of cancer, overexpression of BRDT was first observed in lung cancer (Bourova-Flin *et al.*, 2017). It is also overexpressed in ovarian cancer tissue and plays a regulatory role in renal cell carcinoma (Chen *et al.*, 2020; Wan *et al.*, 2020).

**Table 1.** The tissues in which each of the BET proteins (BRD2, BRD3, BRD4 and BRDT) are primarily expressed in, information from Fagerberg *et al.* (2013).

| **Protein** | **Tissues Expressed In** |
|---|---|
| BRD2 | Bone marrow, thyroid, testes, ovaries and lymph nodes |
| BRD3 | Endometrium, brain, prostate, spleen and testes |
| BRD4 | Placenta, testes, spleen, appendix and ovaries |
| BRDT | Testes |

## 1.9. BET Inhibitors

Over the past fourteen years several BET inhibitors have been developed as treatment options to combat the dysfunction of BET proteins. In 2010, the first two BET inhibitors were developed these being I-BET762 and (+)-JQ1 (Filippakopoulos *et al.*, 2010; Nicodème *et al.*, 2010). Several other lead candidate BET inhibitors were then developed over the following years, which have shown efficacy against a variety of conditions (Table 2).

One particular area of focus for BET inhibitor treatment is haematologic malignancies such acute myeloid leukaemia, acute lymphoblastic leukaemia and multiple myeloma. BET inhibitors have been shown to downregulate the expression of a range of haematological oncogenes, in particular *c-Myc* is notable due to its prevalence across a variety of oncological malignancies (Munshi, Abedin and Boddy, 2016). The first clinical trial using BET inhibitors began in 2012 using OTX015 against acute myeloid leukaemia and other haematologic malignancies (Berthon *et al.*, 2016). This study resulted in three out of forty-one patients reaching total remission, and another two patients showing partial remission.

BET inhibitors have also been used against solid tumours such as breast cancer and lung cancer. Specific breast cancer types appear promising targets for BET inhibitor therapy, in particular BRD4 is overexpressed in 20.6 % of basal-like breast cancer cases (Sahni and Keri, 2018). (+)-JQ1 has been shown to inhibit the growth of human luminal breast cancer cell lines and mice models, however interestingly BRD4 expression was unaffected (Pérez-Salvia *et al.*, 2017). BET inhibitors also have applications as combination therapeutics. In non-small cell lung cancer, treatment with the chemotherapy drug cisplatin also results in the overexpression of the protein PD-L1 which exerts an oncogenic function. Treatment with the BET inhibitors (+)-JQ1 and ARV-771, alongside cisplatin, inhibit PD-L1 transcription resulting in improved treatment outcomes (Wang *et al.*, 2022).

NUT carcinoma is a rare and aggressive malignancy, which is caused by a translocation mutation in the NUT1 gene and partner genes (Lauer *et al.*, 2022). Most commonly NUT carcinoma is associated with a translocation of BRD4 as the partner gene, but BRD3 can also act as the partner

gene. NUT carcinomas are characterised by a rapid progression, making them particularly challenging to treat. Treatment with OTX015 has shown to reduce the size of NUT carcinoma tumours and result in symptomatic relief (Stathis *et al.*, 2016). A phase 1 clinical trial using OTX015b against NUT carcinoma found a partial response in three out of ten patients (Lewin *et al.*, 2018).

BET proteins also regulate a range of proinflammatory and immunoregulatory genes making BET inhibitors a therapeutic option for several inflammatory disorders (Wang *et al.*, 2021).

Rheumatoid arthritis is characterised by the presence of fibroblast-like synoviocytes. Fibroblast-like synoviocytes from rheumatoid arthritis patients exposed to (+)-JQ1 showed decreased expression of key pro-inflammatory cytokines TNFα, IL-6, IL-1β and IL-8 (Xiao *et al.*, 2015).

In systemic lupus erythematosus (SLE), Fcγ receptors are activated resulting in the over expression of pro-inflammatory cytokines such as IL-1β and TNF. Treatment with I-BET151 regulated the expression of the Fcγ receptors, in turn decreasing the expression of the pro-inflammatory cytokines.

In inflammatory bowel disease (IBD) the key pro-inflammatory cytokines are TNF-α, IL-1, IL-6, IFN-γ, and IL-13 (Ma *et al.*, 2023). Three BET inhibitors ZL0454, ZL0590 and ZL0516 have been identified as being suited for use IBD specifically. They were found to reduce the inflammatory response through inhibiting the activation of NFκB, which stimulates the transcription of the key pro-inflammatory cytokines.

It is thought that BET inhibitors could be effective in treating neurodegenerative disorders (Singh and Sartor, 2020). I-BET858 has been shown to alter the expression of genes controlling neuronal

transmission, neurotransmitter receptor signalling, dendritic and axon development and autism (Sullivan *et al.*, 2015).

Multiple pre-clinical studies have shown the efficacy of BET inhibitors in treating Alzheimer's disease. (+)-JQ1 has been shown to improve cognitive function, improve memory retrieval, improve brain plasticity, reduce inflammation and reduce tau phosphorylation (Badrikoohi, Esmaeili-Bandboni and Babaei, 2022; Benito *et al.*, 2017; Magistri *et al.*, 2016).

In Parkinsons disease the most common pharmaceutical treatment is Levodopa. Long term use of Levodopa can lead to Levodopa-induced dyskinesia (LID) (Figge and Standaert, 2017). The use of (+)-JQ1 in combination therapy with Levodopa prevented LID in rat models, through the downregulation of LID associated genes including *fosB*, *dab1* and *ntrk2*.

**Table 2.** Summary of lead candidate BET inhibitors and their target conditions / sites within previous literature.

| Inhibitor | IUPAC Name | Condition | Target (cell line) | Reference |
|---|---|---|---|---|
| (+)-JQ1 | tert-butyl 2-[(9S)-7-(4-chlorophenyl)-4,5,13-trimethyl-3-thia-1,8,11,12-tetrazatricyclo[8.3.0.02,6]trideca-2(6),4,7,10,12-pentaen-9-yl]acetate | Acute lymphoblastic leukaemia | B-cell acute lymphoblastic leukaemia cell lines (NALM-6, MHH-CALL4, MUTZ-5, CEMO-1, Reh, 697, RS4;11, and SEMK2) | (Ott *et al*., 2012) |
| | | Multiple myeloma | Human MM cell line (KMS11, MM.1S and OPM1) | (Delmore *et al*., 2011). |
| | | Ovarian cancer | Epithelial ovarian cancer cell lines (PEO1) and ID8-Defb29/Vegf-a syngeneic mouse model | (Zhu *et al*., 2016) |
| | | Lung cancer | NSCLC cell lines (A549, H460, H1299, H1975, H292) and immune-competent C57BL/6 mice inoculated with Lewis cells | (Wang *et al*., 2022) |
| | | Lung cancer | Pulmonary sarcomatoid carcinoma | (Yuan *et al*., 2021) |

| | | | | |
|---|---|---|---|---|
| | | | tumour cells (PSCC and PSCN) | |
| | | Colorectal cancer | Colorectal cancer (CRC) cell lines (HCT116, SW480, SW620, HT29, LOVO, and RKO) | (Lei *et al*., 2020) |
| | | Neuroblastoma | Neuroblastoma cell lines (LA-N-6, SK-N-Be , and SMS-KAN) | (Mazar *et al*., 2020) |
| | | Rheumatoid arthritis | Synovial tissue samples from arthritis patients | (Xiao *et al*., 2015) |
| | | Inflammation | RAW264.7 cells | (Meng *et al*., 2014) |
| | | Alzheimer's disease | C57Bl/6j and APP/PS1–21 mice | (Benito *et al*., 2017) |
| | | Alzheimer's disease | 3xTg mice | (Magistri *et al*., 2016) |
| | | Parkinson Disease | 6-Hydroxydopamine (6-OHDA)-Lesioned Parkinson's Model rats | (Wan *et al*., 2022) |
| | | Inflammatory response in *Listeria monocytogenes* | Bone marrow-derived macrophages from C57BL/6 mice infected with *L. monocytogenes* strain LO28 | (Wienerroither *et al*., 2014) |
| | | Murine leukaemia virus (MuLV) | HEK293T cells infected with MLV | (Das Gupta *et al*., 2013) |

| | | | | |
|---|---|---|---|---|
| | | | or HIV-1 vector supernatants | |
| | | Feline leukaemia virus (FeLV) | FeLV-negative feline kidney fibroblast cell line 81C, FeLV-negative lymphoma cell line 3201 and FeLV-infected feline lymphoma cell line FL-74 | (Moll, Swenson and Yuzbasiyan-Gurkan, 2023) |
| | | Epstein-Barr virus | Mutul and Akata-Zta cells | (Keck *et al*., 2017) |
| | | SARS-CoV-2 | Calu-3 cells infected with SARS-Related Coronavirus 2, Isolate hCoV-19/USA/MD-HP05647/2021 | (Vann *et al*., 2022) |
| | | SARS-CoV-2 | Human pluripotent stem cell-derived cardiac cells CW30382A and CW30318C | (Mills *et al*., 2021) |
| | | *T. cruzi* | *Trypanosoma cruzi* epimastigotes (Dm28c strain) | (Alonso *et al*., 2016) |
| I-BET762 | 2-[(4*S*)-6-(4-chlorophenyl)-8-methoxy-1-methyl-4*H*-[1,2,4]triazolo[4,3-a][1,4]benzodiazepin-4-yl]acetic acid | Prostate cancer | Human prostate cancer cells (Human PCa cells (DU145, RRID:CVCL_0105; PC3, RRID:CVCL_0035; 22Rv1, RRID:CVCL_1045; | (Ippolito *et al*., 2022) |

| | | | | |
|---|---|---|---|---|
| | | | and LNCaP, RRID:CVCL_4783) ) | |
| | | T-cell mediated inflammation | CD4+ T cells from 2D2 TCR-transgenic mice | (Bandukwala *et al*., 2012) |
| | | Sepsis/ endotoxic shock | Bone marrow-derived macrophages from C57BL/6 mice | (Nicodème *et al*., 2010) |
| | | Epstein-Barr virus | Mutul and Akata-Zta cells | (Keck *et al*., 2017) |
| OTX015 | 2-[(9*S*)-7-(4-chlorophenyl)-4,5,13-trimethyl-3-thia-1,8,11,12-tetrazatricyclo[8.3.0.$0^{2,6}$]trideca-2(6),4,7,10,12-pentaen-9-yl]-*N*-(4-hydroxyphenyl)acetamide | Acute myeloid leukaemia and other leukaemia | Phase 1 clinical trial | (Berthon *et al*., 2016) |
| | | Lung cancer | Pulmonary sarcomatoid carcinoma tumour cells (PSCC and PSCN) | (Yuan *et al*., 2021) |
| | | NUT carcinoma | Patients with advanced NUT carcinoma | (Stathis *et al*., 2016) |
| | | Neuroblastoma | Neuroblastoma cell lines (The Chp-212, Chp-134, GI-M-EN, IMR-5, IMR-32, NB69, SK-N-AS, SK-N-BE, and SK-N-BE) | (Henssen *et al*., 2015) |
| | | SARS-CoV-2 | Calu-3 cells infected with SARS-Related Coronavirus 2, Isolate hCoV- | (Vann *et al*., 2022). |

| | | | | |
|---|---|---|---|---|
| | | | 19/USA/MD-HP05647/2021 | |
| I-BET151 | 7-(3,5-dimethyl-1,2-oxazol-4-yl)-8-methoxy-1-[(1*R*)-1-pyridin-2-ylethyl]-3*H*-imidazo[4,5-c]quinolin-2-one | Rheumatoid arthritis | Synovial tissue samples from arthritis patients | (Klein *et al*., 2014) |
| | | Systemic lupus erythematosus | Wild-type C57BL/6 mice and Fcγr2b-deficient mice | (Banham *et al*., 2022) |
| | | *T. cruzi* | *Trypanosoma cruzi* epimastigotes (Dm28c strain) | (Alonso *et al*., 2016) |
| | | *T. brucei* | *T. brucei* BF cells (strain Lister 427 antigenic type MITat1.2 clone 221a) | (Schulz *et al*., 2015) |
| | | *Toxoplasma sp.* | Human foreskin fibroblasts infected with *Toxoplasma* type I RH strain parasites | (Jeffers *et al*., 2017a) |
| RVX-208 | 2-[4-(2-hydroxyethoxy)-3,5-dimethylphenyl]-5,7-dimethoxy-3*H*-quinazolin-4-one | Alzheimer's disease | AD patients | (Cummings *et al*., 2021). |
| | | Epstein-Barr virus | Mutul and Akata-Zta cells | (Keck *et al*., 2017) |
| | | HIV | J-Lat A2 cells, J-Lat 10.6 cells, U1 cells and ACH2 cells | (Zhang *et al*., 2018b) |
| ARV-771 | (2*S*,4*R*)-1-[(2*S*)-2-[[2-[3-[2-[[2-[(9*S*)-7-(4-chlorophenyl)-4,5,13-trimethyl-3-thia-1,8,11,12- | Lung cancer | NSCLC cell lines (A549, H460, H1299, H1975, H292) and immune- | (Wang *et al*., 2022) |

| | | | | |
|---|---|---|---|---|
| | tetrazatricyclo[8.3.0.$0^{2,6}$]trideca-2(6),4,7,10,12-pentaen-9-yl]acetyl]amino]ethoxy]propoxy]acetyl]amino]-3,3-dimethylbutanoyl]-4-hydroxy-*N*-[(1*S*)-1-[4-(4-methyl-1,3-thiazol-5-yl)phenyl]ethyl]pyrrolidine-2-carboxamide | | competent C57BL/6 mice inoculated with Lewis cells | |
| AZD5153 | (3*R*)-4-[2-[4-[1-(3-methoxy-[1,2,4]triazolo[4,3-b]pyridazin-6-yl)piperidin-4-yl]phenoxy]ethyl]-1,3-dimethylpiperazin-2-one | Lung cancer | Pulmonary sarcomatoid carcinoma tumour cells (PSCC and PSCN) | (Yuan *et al*., 2021) |
| CPI-0610 | 2-[(4*S*)-6-(4-chlorophenyl)-1-methyl-4*H*-[1,2]oxazolo[5,4-d][2]benzazepin-4-yl]acetamide | Lung cancer | Pulmonary sarcomatoid carcinoma tumour cells (PSCC and PSCN) | (Yuan *et al*., 2021) |
| Mivebresib | *N*-[4-(2,4-difluorophenoxy)-3-(6-methyl-7-oxo-1*H*-pyrrolo[2,3-c]pyridin-4-yl)phenyl]ethanesulfonamide | Systemic lupus erythematosus | C57BL/6J mice | (He *et al*., 2023) |
| PFI-1 | 2-methoxy-N-(3-methyl-2-oxo-1,2,3,4-tetrahydroquinazolin-6-yl)benzenesulfonamide | Systemic lupus erythematosus | Healthy and SLE patients and Brd4flox/floxCD19-cre+ mice | (Zeng *et al*., 2022) |
| ZL0454 | 4-[(2-amino-4-hydroxy-5-methylphenyl)diazenyl]-*N*-cyclopentylbenzenesulfonamide | Inflammatory bowel disease | Human IBD biopsy tissues | (Ma *et al*., 2023) |

| | | | | |
|---|---|---|---|---|
| ZL0590 | 1-[4-[(2*S*)-2-(morpholin-4-ylmethyl)pyrrolidin-1-yl]sulfonylphenyl]-3-[4-(trifluoromethyl)phenyl]urea | Inflammatory bowel disease | Human IBD biopsy tissues | (Ma *et al*., 2023) |
| ZL0516 | 2-[4-[(2*R*)-2-hydroxy-3-(4-methylpiperazin-1-yl)propoxy]-3,5-dimethylphenyl]-5,7-dimethoxychromen-4-one | Inflammatory bowel disease | Human IBD biopsy tissues | (Ma *et al*., 2023) |

## 1.10. Histones

The key target of BET proteins are histones which are the proteins alongside DNA make up chromatin (Shechter *et al.*, 2007). There are four histone proteins: H2A, H2B, H3, H4. These are linked with H1 to form the chromatin unit. All of the histone proteins are vital for maintaining the structure of chromatin but they also have individual functions. H2A and its isoforms control DNA damage response pathways, maintaining the structure and functionality of the genome (Oberdoerffer and Miller, 2023). H2B controls gene expression and maintains the chromatin structure through regulation of chromatin remodelling (Chandrasekharan, Huang and Sun, 2010). H3 plays a role in gene expression, which can be influenced by post-translational modifications influencing H3 to uncover specific genomic loci (Ray-Gallet and Almouzni, 2021). H4 regulates genome stability through the modulation of chromatin remodelling (Kumar, Moirangthem and Kaur, 2020). Alongside H3, H4 makes up the chromatin core.

Histones can undergo post translational modifications, one of these modifications is acetylation (Shvedunova and Akhtar, 2022). This is a reversible process carried out by the enzymes, lysine

acetyltransferases (KATS). These enzymes catalyse the addition of acetyl groups to the lysine residues on the histone tails. This process can be reversed by lysine deacetylases (DKATS), which remove the acetyl group.

Chromatin has two states, euchromatin which has a looser structure and therefore has more gene expression, and heterochromatin which is a tighter structure with a lesser gene expression (Morrison and Thakur, 2021). The addition of acetyl groups by KATS decreases the positive charge of the histone core weakening the attraction between the negatively charged DNA and the positively charged histones (Kumar, Thakur and Prasad, 2021). This allows RNA polymerase and transcription factors to bind more readily to their target genes initiating transcription.

In eukaryotic cells histone acetylation plays a vital role in the regulation of the chromatin structure and gene expression (Shen, Wei and Zhou, 2015). In most cases acetylation results in increased gene expression, while deacetylation results in decreased gene expression (Kumar, Thakur and Prasad, 2021). Other post-translational modifications may occur alongside acetylation and deacetylation. Different combinations of modifications at differing points in the amino acid sequence will result in differing effects on the chromatin structure.

Histone acetylation has been implicated in the progression of various diseases. KAT P300 has been shown to increase the expression of various oncogenes associated with breast cancer (Xiao *et al.*, 2011). Acetyl-CoA is the source of the acetyl group in acetylation, an increase in Acetyl-CoA is observed in cancer cells causing an increase the rate of acetylation (Halbrook, Nelson and Lysstiotis, 2019). Both acetylation and deacetylation have been shown to increase and decrease chronic pain due to alterations in the transcription of nociceptive processing genes (Khangura *et*

*al.*, 2017). Increasing acetylation has been shown to improve the cognitive function in 5×FAD mice models of Alzheimer's disease (Lin *et al.*, 2023). However, to date classical histones, or a role for such proteins, have not been identified in prokaryotic cell types.

## 1.11. Histone-Like Proteins in Bacteria

Most bacteria have a single chromosome, which is located inside the nucleoid and coated with histone-like proteins (HLPs), also referred to as nucleoid-associated proteins (NAPs) (Jha *et al.*, 2012; Levin, 2001, pp.339–350). HLPs regulate gene expression, contribute to the organisation of the nucleoid and maintain chromosomal structure (Dorman and Deighan, 2003; Pettijohn, 1988). There are four main groups of histone like proteins: histone-like protein *E. coli* U93 (HU), histone-like nucleoid structuring proteins (H-NS), integration host factors (IHF), and factors for inversion stimulation (FIS) (Anuchin *et al.*, 2011).

HU protein has various functions involving a range of cellular processes. It causes DNA compaction, initiates DNA replication, regulates gene expression and acts as a crucial transcription factor (Guttula *et al.*, 2018; Mangan *et al.*, 2011; Schramm and Murray, 2022; Stojkova and Spidlova, 2022). The vital role of HU in genetic expression makes it a key regulator of the expression of virulence genes (Stojkova *et al.*, 2018). HU has been shown to interact with the proinflammatory cytokine Interleukin-1β, indicating that it may modulate the inflammatory response during infection through host-pathogen interaction (Paino *et al.*, 2012). HSa is a type of HU protein which is exclusively found in *S. aureus* (Viter, Shaw and Gennaro, 1999). However, the specific function and mechanism of HSa is unknown.

H-NS carries out a variety of functions involved in the maintenance of the nucleoid structure and regulation of gene expression (Winardhi, Yan and Kenney, 2015). Various types of H-NS protein have been identified in a range of bacteria including *E. coli*, *V. cholerae* and Salmonella (Ayala, Silva and Benitez, 2017). H-NS is most widely recognised for its specific role as a silencer of transcription. It disrupts the promotor binding of RNA polymerase, preventing transcription (Landick, Wade and Grainger, 2015). This silencing of genes has been shown to trigger the synthesis of regulatory proteins involved in the expression of bacterial virulence factors (Falconi *et al.*, 1998).

IHF plays a role in DNA packaging, integration of viral DNA and recombination (Lin *et al.*, 2022). IHF binds to DNA at Holliday junctions inducing a bend of 160° resulting in stabilisation of the structure. This stabilisation has been shown to stimulate the mechanism of a range of virulence factors (Chen *et al.*, 2022). In *V. cholerae* and *V. fluvialis*, IHF is one of the key regulators in the expression of the resistance gene *vgrG* (Zhang *et al.*, 2021). IHF also plays a role in the expression of the protein GbdR, which regulates choline metabolism in *P. aeruginosa* (Sánchez *et al.*, 2017).

Similarly to the other HLPs, FIS regulates the expression of a variety of bacterial genes, including those involved in virulence (Chakraborti *et al.*, 2020). FIS is involved in DNA recombination, RNA synthesis and cellular DNA organisation (Duprey, Reverchon and Nasser, 2014). In *E. coli*, FIS increases adherence through the downregulation of curli expression (Saldaña *et al.*, 2009). FIS also been shown to activate *virF* in *E. coli*, which is a key regulatory protein in transcription (Falconi *et al.*, 2001a). In *V. cholerae* FIS regulates the production of HapR, which in turn regulates quorum sensing (Lenz and Bassler, 2007). Identifying SMIs that inhibit the action of HLPs in prokaryotic cells would offer a new antimicrobial therapeutic pathway.

## 1.12. Targeting BET Proteins in Fungi

Whilst not present in bacteria, BET proteins are present in fungi. There are two subtypes of fungal BET proteins, Bdf1 and Bdf2 (Sayou and Govin, 2022). Some fungal species have both types of protein, whilst others only have Bdf1. *Saccharomyces cerevisiae* has both types of fungal BET protein. At least one of the proteins is required for cells to be viable, Bdf1 is suspected to be the dominant protein as the inhibition of Bdf1 resulted in stunted gene expression (Matangkasombut *et al.*, 2000). Bdf1 has been shown to be essential for the expression of gene involved in sporulation and mitosis in *S. cerevisiae* (García-Oliver *et al.*, 2017).

The antifungal properties of Bdf1 inhibition were first explored in *Candida albicans* (Mietton *et al.*, 2017). Mutations in Bdf1 that impacted the bromodomain binding activity caused severe suppression of growth. Inhibition of both bromodomains of Bdf1 showed a decrease in fungal load in murine models. Bdf1 in *C. albicans* has a smaller binding pocket than human BRD4, which prevented Bfs1 from being inhibited by a range of chemically different human BET inhibitors, including (+)-JQ1, PFI-1 and IBET-151. Two compounds were identified which target Bdf1 without targeting any human bromodomains. However unlike human BET inhibitors which target both bromodomains, fungal BET inhibitors only targeted one domain. Further work is needed to develop a fungal BET inhibitor which targets both bromodomains of Bdf1.

*Candida glabrata* is an increasing public health concern and is distantly related to *C. albicans* (Wei *et al.*, 2023). The inhibition of Bdf1 was shown to be lethal, however similarly to the previous work in *C. albicans* inhibition of both bromodomains is required. A compound that selectively inhibits Bdf1 in *C. glabrata*, over human BRD4 was identified. It was also found that the human

BET inhibitor I-BET726, while having a potency for human BET proteins, showed significant inhibition of Candida species. It has been suggested that modification of I-BET726 could cause an inverse of this potency.

## 1.13. Targeting Human BET Proteins During Infection Processes

While bacterial BET protein homologues have not previously been utilised as a therapeutic target, BET inhibitors have been used as a therapeutic to mediate inflammatory responses to bacterial infections. I-BET762 has been used to target human BET proteins resulting in the inhibition of the macrophage mediated inflammatory response (Nicodeme *et al.*, 2010). This inhibition was able to protect against the development of endotoxic shock and sepsis. I-BET151 and (+)-JQ1 have been used to supress the inflammatory response in periodontitis patients (Maksylewicz *et al.*, 2019). (+)-JQ1 treatment has also been shown to reduce the expression of *L. monocytogenes*-induced genes including Nos2, IL1rn and IL-6 (Wienerroither *et al.*, 2014).

BET inhibitors have been used in similar ways in viral infections. BRD4 inhibition by (+)-JQ1, OTX-015 and I-BET151 has been used to enhance the innate immune response and inhibit the attachment of both RNA and DNA viruses through the cGAS-STING pathway (Wang *et al.*, 2020). Treatment with the BET inhibitors PLX51107, I-BET762, ZL0580, and ARV-825 has been shown to reduce transcription of African swine fever virus during infection (Zhao *et al.*, 2022).

## 1.14. Virulence Factors of *S. aureus*

As previously stated, the most prevalent antibiotic resistant bacteria is MRSA. Typically *S. aureus* colonises skin and soft tissue causing uncomplicated infections, however *S. aureus* can enter the bloodstream resulting in bacteraemia which can develop into sepsis (Kwiecinski and Horswill, 2020). Due to the wide scale global spread of *S. aureus* and its typical non-life-threatening presentation, the exact epidemiology is difficult to quantify. However, it is estimated that roughly a third of the population is colonised with *S. aureus* (Salgado, Farr and Calfee, 2003). Between April 2022 to March 2023 in the UK there were 13,912 cases of *S. aureus* bacteraemia, with 5.7% of these being caused by MRSA. Compared to the previous year this was a 7.2% increase in overall *S. aureus* bacteraemia and a 16.6% increase in MRSA bacteraemia (*Annual epidemiological commentary: Gram-negative, MRSA, MSSA bacteraemia and C. difficile infections, up to and including financial year 2022 to 2023*, 2022).

*S. aureus* utilises a wide range of virulence factors in its pathogenesis. One feature is the peptidoglycan present in the cell wall of gram-positive bacteria (Sutton *et al.*, 2021). The cell wall is essential for maintaining the cellular structure. O-acetylation is modification of the individual glycan strands that induces resistance to lytic enzymes (Jones, Anderson and Clarke, 2021). The *oatA* gene encodes for the synthesis of O-acetyltransferase, which catalyses the O-acetylation reaction, this gene was first observed in *S. aureus* (Bera *et al.*, 2004). Thickening of the cell wall can lead to resistance to antibiotics that target the cell wall, such as vancomycin (Cui *et al.*, 2003). This cell wall thickening can be due to a variety of mechanisms including incorporation of N-acetylglucosamine, increased levels of cytoplasmic murein monomer precursor and increased production of production of penicillin-binding proteins 2.

Another virulence factor of *S. aureus* is staphylococcal protein A (SpA), SpA consists of five domains (E, D, A, B, and C) that all bind to the Fc region of immunoglobulin G (IgG) with high affinity (Moks *et al.*, 1986). This binding resulting in decreased opsonisation and phagocytosis (Peterson *et al.*, 1977). SpA also acts as a pro-inflammatory activator, activating TNFR1 and induces TNF-α-like responses leading to pneumonia (Gómez *et al.*, 2004).

*S. aureus* produces a range of toxins with specific functions to enhance virulence. One category is leukocidins, the most widely studied member of this family is Panton-Valentine leukocidin (PVL) (G. Abril *et al.*, 2020). PVL targets the mitochondria and induces apoptosis of neutrophils, monocytes, and macrophages (Genestier *et al.*, 2005). PVL is associated with more severe infections, potentially leading to necrotising pneumonia, osteomyelitis, septic arthritis, sepsis and multiorgan failure (Castellazzi *et al.*, 2021). LukePQ and LukMF' are two further leucoidin secreted by *S. aureus* that have been observed in non-human hosts. LukPQ is has been observed in equine hosts, strains positive for LukPQ show enhanced cytotoxicity towards neutrophils (Koop *et al.*, 2017). LukMF' is observed in bovine hosts and has also been shown to target neutrophils (Vrieling *et al.*, 2016).

Enzymes such as coagulase, catalase and hyaluronidase are another type of toxin secreted by *S. aureus*. Coagulase increases coagulation through the activation of prothrombin which catalyses the conversion of fibrinogen to fibrin (Cheng *et al.*, 2010). Increased coagulation is beneficial for the formation of staphylococcal abscesses and bacteraemia. Catalase degrades hydrogen peroxide produced by macrophages, therefore preventing hydrogen peroxide from killing *S. aureus* (Das and Bishayi, 2009). Hyaluronidase cleaves bonds in hyaluronic acid, which is found in abundance in many of the tissues commonly infected with *S. aureus* (Ibberson *et al.*, 2014).

Haemolysins are another category of toxin. The most widely studied member of this group is α-toxin where the main function is lytic activity against red blood cells, resulting in tissue damage and immunomodulation (Berube and Wardenburg, 2013). γ-Hemolysin is a bi-component toxin that displays haemolytic activity towards neutrophils and monocytes, in particular those expressing chemokine receptors (Spaan *et al.*, 2014). β-Hemolysin, unlike the other haemolysins of *S. aureus* is non-spore forming, it degrades sphingomyelin inducing damage to the cell membrane (Vandenesch, Lina and Henry, 2012). δ-Hemolysin was the first *S. aureus* haemolysin to be discovered, it is hypothesised that it acts as a surfactant towards the cell membrane in a concentration-dependent manner (Dinges, Orwin and Schlievert, 2000; Verdon *et al.*, 2009).

Superantigens are another class of toxin produced by *S. aureus*, one of these superantigens is toxic shock syndrome toxin-1 (TSST-1) which activates the release of cytokines leading to toxic shock syndrome (Peng *et al.*, 2021). There is a group of toxins referred to as the staphylococcal enterotoxins, there are over 20 members of this group and they each have a specific function, the most widely studied members of this group at SEA and SEB (Pinchuk, Beswick and Reyes, 2010). SEA results in DNA damage and oxidative stress in hepatocytes and liver tissue (Chi *et al.*, 2023). SEB increases the release of cytokines, in particular the release of IFN-γ., with the highest levels of activity occurring in the spleen (Bae *et al.*, 2020). SEB has also been studied for its potential as a biological warfare agent (Ler, Lee and Gopalakrishnakone, 2006).

*S. aureus* is one of the most common pathogens observed in biofilm formations (Peng *et al.*, 2023). The bacteria form an extracellular matrix with extracellular polymeric substances, such as polysaccharides, nucleic acids, and proteins. Biofilm formation protects the bacteria from antibiotics and the host immune response (Vestby *et al.*, 2020). Many factors influence the

increased antimicrobial resistance observed in biofilm formation including the biofilm matrix being challenging for antimicrobials to permeate, altered levels of enzymatic and metabolic activity and the presence of efflux pumps (Singh *et al.*, 2017).

The presence of antibiotic resistance genes is a major virulence factor. The resistance to methicillin in MRSA is caused by an altered penicillin binding protein with reduced affinity for β-lactams, known as penicillin binding protein 2a (PBP2a) (Utsui and Yokota, 1985). This alteration is encoded for by a mutation in the *MecA* gene (Ubukata *et al.*, 1989). Staphylococcal cassette chromosome mec (SCCmec) is the genetic component encoding for *mecA* and *mecC* (Lee *et al.*, 2018). SCCmec is acquired by different *S. aureus* strains through horizontal gene transfer.

## 1.15. Research Focus

Preliminary screening by the Butler group using an SMI compound library identified the BET inhibitor (+)-JQ1 as showing antimicrobial action against MRSA. BET inhibitors have not been previously used to directly target bacteria. This study aimed to explore and characterise the therapeutic potential of (+)-JQ1 and other prominent BET inhibitors against MRSA and other multidrug resistant pathogens. The cytotoxicity of these compounds was also examined in relation to human tissues to assess any potential cytotoxicity issues.

## 1.16. Aim of the Study

Determine the antimicrobial therapeutic potential of BET SMIs against MRSA and other multidrug resistant pathogens.

### 1.17. Objectives of the Study

1. Characterise the antimicrobial properties of lead candidate BET protein inhibitors against MRSA and other multidrug resistant pathogens.
2. Determine the anti-virulence properties of the SMIs.
3. Evaluate the potential of cytotoxicity against mammalian tissues.

## 2. Methods

### 2.1. Ethics and Risk Assessment

Full ethical approval was obtained from the Manchester Metropolitan University research ethics council before this study was conducted (approval code: 59284). Full risk assessments were completed, including all relevant COSHH documentation.

### 2.2. Bacterial Strains and BET Inhibitor Preparation

The bacterial strains used in this study were *S. aureus* strain USA300, *L. monocytogenes* strain Scott A, *Enterococcus faecalis* strain 12697, *E. faecium* strain 7171, *K. pneumoniae* strain LEEDS, *A. baumannii* strain LEEDS, *P. aeruginosa* strain PAO1 and *E. coli* MG1655 which were cultured in

Mueller-Hinton broth (MHB) (Oxoid) for 18 h ±2 h at 37 °C, unless otherwise stated. The BET inhibitors and control compounds used in this study were (+)-JQ1 (SML1524), (-)-JQ1 (SML1525), (+/-) JQ1 (SML0974), PFI-1 (SML0352), OTX015 (SML1605) and (+)-JQ-1 carboxylic acid (SML2623) (Merck, UK). These were all solubilised according to the manufacturer's instructions in dimethylsulfoxide (DMSO) (Thermo Scientific). The HepG2 cells were cultured in a media containing 500 mL high glucose Dulbecos modified eagle medium (DMEM) (Corning Incorporated), 50 mL foetal bovine serum (Thermo Fisher Scientific) and 10 mL Penicillin-Streptomycin (Thermo Fisher Scientific).

## 2.3. Bioinformatics

The similarity between the sequences of BET proteins (BRD2, BRD3, BRD4 and BRDt) and *S. aureus* were examined using the Basic Local Alignment Search Tool (BLAST) and HMMER software. Protein sequences were identified using the UniProt database. The NIH BLAST tool and HMMER software were used to compare similarities between the two sequences. E-values ($\leqslant$ 0.01), identity percentages, and alignment scores were examined to determine the similarity between the sequences.

## 2.4. Identification of (+)-JQ1

(+)-JQ1 was initially identified through preliminary screening by the Butler group. It was screened alongside several other lead candidate SMIs on a non-commercially available SMI screening plate. (+)-JQ1 demonstrated antimicrobial activity against MRSA at < 456.99 µg mL-1.

## 2.5. Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) was determined for (+)-JQ1 against all bacterial strains used in this study and all other BET inhibitor compounds were screened against *S. aureus* USA300, in a broth microdilution assay. Column 11 rows B-D of a 96 well microplate plate (Sarstedt) contained 180 µL of MHB and 20 µL of the respective compound. While column 11 rows E-G contained 180 µL of MHB and 20 µL of DMSO. A series of two-fold dilutions were performed so that row 3 contained 0.39 % of the starting concentration. Bacterial cultures were diluted to an $OD_{600}$ of 0.005, which is equivalent to $10^6$ colony forming units per mL (CFU $mL^{-1}$) of viable bacterial cells. Each well had the addition of 100 µL of these bacterial cultures. Each plate had 200 µL of MHB in the outer wells as a negative control. The 96 well microplate was then incubated for 18 h ±2 h at 37 °C, after which the plates were visually examined for growth to determine minimum inhibitory concentration. Three biological replicates were performed, each with three technical replicates.

## 2.6. Minimum Bactericidal Concentration

A sterile 96 pin multi-blot replicator was used to transfer the contents of the 96 well microplate onto Mueller-Hinton agar (MHA) (Oxoid). These plates were then incubated for 18 h ±2 h at 37 °C, before being visually examined to determine minimum bactericidal concentration. Three biological replicates were performed, each with three technical replicates.

## 2.7. Disc Diffusion Assays

Bacterial cultures were grown for 24 h and diluted to an $OD_{600}$ of 0.1. Four sterile filter paper discs were placed evenly on the MHA plate. Twenty microlitres of various (+)-JQ1 concentrations were placed on each disc. The concentrations of (+)-JQ1 used were 256 µg $mL^{-1}$, 128 µg $mL^{-1}$and 64 µg $mL^{-1}$, a 10 % DMSO control was also used to represent a 0 µg $mL^{-1}$ concentration. These plates were incubated for 18 h ±2 h at 37 °C, after which the zones of inhibition were measured using a digital calliper. Three biological replicates were performed, each with three technical replicates.

## 2.8. Antimicrobial Synergy Testing/Checkerboard Assay

(+)-JQ1 was used in combination with penicillin-G (Sigma Aldrich LTD) and vancomycin (Melford) in checkerboard synergy assays. Wells A1-A8 of 96 well microplates were made up of MHB containing a X4 MIC of each antibiotic and a series of two-fold serial dilutions were performed vertically. (+)-JQ1 was added to the wells of column 8 with a starting concentration of 512 µg $mL^{-1}$, and 10 fold serial dilutions were performed horizontally. Bacterial cultures previously grown for 24 h of *S. aureus* USA300 were diluted to an $OD_{600}$ of 0.005, these cultures were then added to

the wells. MHB and bacterial cultures were used as negative and positive controls respectively. These plates were then incubated for 18 h ±2 h at 37 °C. After which the FIC values and synergies were calculated. Three biological replicates were performed, each with three technical replicates.

$$FIC = \frac{\begin{matrix} MIC\ of\ (+)-JQ1\ in\ combination \\ with\ antimicrobial \end{matrix}}{MIC\ of\ (+)-JQ1\ alone} + \frac{\begin{matrix} MIC\ of\ antimicrobial\ in\ combination \\ with\ (+)-JQ1 \end{matrix}}{MIC\ of\ antimicrobial\ alone}$$

## 2.9. Bacterial Growth Curve

Bacterial 24 h cultures were prepared to an $OD_{600}$ of 0.005. The wells of a 96 well microplate were inoculated with bacterial cultures and various concentrations of (+)-JQ1, with 10 % DMSO being used as a solvent control. The 96 well microplate was then placed in a microplate reader (BMG Spectrostar Nano) which took an optical density reading every 30 min over an 18 h period, to determine bacterial growth. Three biological replicates were performed, each with three technical replicates.

## 2.10. Time-Kill Kinetic Assay

Bacterial cultures were grown for 24 h and diluted to an $OD_{600}$ of 0.0025 and were then treated with 256 µg $mL^{-1}$, 128 µg $mL^{-1}$and 64 µg $mL^{-1}$ of (+)-JQ1. Untreated cultures were used as a positive control and cultures treated with 2 % DMSO were used as a solvent control. At each time point, 200 µL was taken from each culture and added into the left most row of a 96 well microplate. A series of ten-fold dilutions were performed up to the $10^{-8}$ dilution. Twenty microlitres from each

dilution well was plated using Miles-Misra techniques onto MHA in triplicate. These plates were then incubated overnight at 37 °C. The liquid test cultures were incubated at 37 °C for 2 h on an orbital shaker, after which the serial dilution and plating steps were repeated. This was also repeated at 4 h, 6 h and 24 h after exposure to (+)-JQ1. Resulting colonies were counted and CFU $mL^{-1}$ was calculated using the below formula as a measure of bacterial viability. Three biological replicates were performed, each with three technical replicates.

$$CFU/mL = \frac{Number\ of\ colonies\ \times dilution\ factor}{Volume\ of\ culture\ on\ plate}$$

## 2.11. Scanning Electron Microscopy Analysis of Bacterial Morphological Changes

At each time point of the previously described time-kill assay, 20 µL of each culture was added onto polycarbonate coupons which had been previously fixed with 4 % glutaraldehyde (Sigma-Aldrich) for 24 h. These coupons then underwent an ethanol (Thermo Fisher) gradient wash using a series of increasing ethanol concentrations as follows: sterile $dH_2O$, 10 % ethanol, 30 % ethanol, 50 % ethanol, 70 % ethanol, 80 % ethanol, 90 % ethanol and 100 % ethanol. Coupons were dried in a desiccator for 72 h, after which they were sputter coated with gold (Polaron, Quorum Technologies, Lewes, UK) for 30 s (parameters: power 5 mA, 30 s, 800 V, vacuum 0·09 mbar, argon gas). Each coupon was then examined using a JEOL JSM 5600LV scanning electron microscope, at 5000x, 10000x and 25000 x magnifications in three different locations to observe any changes in bacterial morphology. Images were taken from three locations from three biological replicates.

## 2.12. Biofilm Inhibition Assay

Bacterial cultures of *S. aureus* USA300 were grown for 24 h and were diluted to an $OD_{600}$ of 0.1. Aliquots of 180 µL of the bacterial suspension was added into the wells of a 96-Well Microplate. 64 µg $mL^{-1}$, 32 µg $mL^{-1}$ and 16 µg $mL^{-1}$ of (+)-JQ1 were added to the wells in 20 µL aliquots, with 10 % DMSO being used as a solvent control. Microplates were incubated for 24 h at 37 °C without shaking. After which the wells were washed with sterile $dH_2O$, then phosphate buffered saline (PBS) (Oxoid). Cells were stained with 0.1 % crystal violet (Sigma-Aldrich) for 1 h. Following this the crystal violet was aspirated and the wells were washed a further three times with sterile $dH_2O$, then the microplates left to air-dry. Glacial acetic acid (33 %) was added at room temperature for 15 min to solubilise the biofilms. The contents of the wells were mixed and then transferred to a new 96 well microplate and the absorbance was measured at 590 nm. Three biological replicates were performed, each with three technical replicates.

## 2.13. Haemolytic Activity Assay

Defibrinated horse blood (TCS Biosciences Ltd) was centrifuged at 1500 g for 5 min. The supernatant was aspirated, with care taken not to disrupt the pellet. PBS was used to wash the cells, then the cells were centrifuged at 1500g for 5 min. Washing and centrifuging steps were repeated a further three times. A 1:10 volume of PBS was added to the final pellet. The erythrocyte suspension was added into the wells of a sterile v-bottom 96-well microplate (Corning Inc) in 90 µL aliquots. (+)-JQ1 final concentrations of 256 µg $mL^{-1}$, 128 µg $mL^{-1}$, 64 µg $mL^{-1}$, 32 µg $mL^{-1}$ and 16 µg $mL^{-1}$ were added into the wells. Triton-X 100 (2 %) (Merck) was used as positive

lysis control and PBS was used as a negative control. The plates were incubated for 1 h at 37 °C without agitation, then centrifuged at 1500 g for 5 min. Aliquots of 10 µL of the supernatant from the previous plate was transferred to a new plate containing 90 µL of PBS. The absorbance was measured at 450 nm and cell haemolysis was quantified using the below calculation. Three biological replicates were performed, each with three technical replicates.

$$\frac{Test\ OD_{450} - PBS\ OD_{450}}{Triton\ X\ OD_{450} - Test\ OD_{450}}$$

## 2.14. Bacterial Haemolytic Activity Assay

*S. aureus* strain USA300 cultures were diluted to an $OD_{600}$ of 0.0025 and were then treated with 64 µg $mL^{-1}$, 32 µg $mL^{-1}$ and 16 µg $mL^{-1}$ of (+)-JQ1 for 18 h ±2h at 37 °C. After incubation, the cultures were centrifuged at 1500 g for 10 min. A 0.22 µM filter (Whatman) was used to filter sterilise the supernatant. Defibrinated horse blood was centrifuged at 1500 g for 5 min. The supernatant was aspirated, with care taken not to disrupt the pellet. PBS was added to wash the cells which were centrifuged at 1500g for 5 min. Washing and centrifuging steps were repeated a further three times. A 1:10 volume of PBS was added to the final pellet. The erythrocyte suspension was added into the wells of a sterile v-bottom 96-well microplate (Corning Inc) in 100 µL aliquots, in a 1:1 ratio of the treated bacterial supernatant. The plates were incubated for 1 h at 37 °C without agitation. The plates were centrifuged at 1500 g for 5 min, then 10 µL of the supernatant from the previous plate was transferred to a new plate containing 90 µL of PBS. The absorbance was

measured at 450 nm and the cell haemolysis was quantified using the previously shown calculation. Three biological replicates were performed, each with three technical replicates.

## 2.15. Mammalian Cytotoxicity Assays

Hep G2 (HEPG2) cells were removed from storage in liquid nitrogen in cryovials and incubated in a 37 °C water bath for 3 min. Media was prepared containing 500 mL Dulbecco's Modified Eagle Medium (DMEM) (Corning Incorporated), 50 mL Fetal Bovine Serum (Thermo Fisher Scientific) and 1 % Penicillin-Streptomycin (Thermo Fisher Scientific). Cells were incubated in this media in a T75 flask (Thermo Fisher Scientific) in a 5 % $CO_2$ humidified incubator for 24 h at 37 °C. After incubation the media was aspirated and the cells were washed with PBS three times. DMEM was added and the cells were incubated under the previous conditions for 48 h. Cells were washed three times with PBS, Trypsin (Thermo Fisher Scientific) was added to dislodge the adherent cells and the cells were incubated at 37 °C for 5 min. The cells were washed with DMEM and centrifuged at 300 x g for 3 min, with supernatant discarded. After which the cells were transferred to a reservoir and then added into the wells of a Nunclon-delta treated 96-well plate (Thermo Fisher Scientific). The plate was incubated under previous conditions for 24 h, after which media was changed. Concentrations of (+)-JQ1 were added and 10 % DMSO was used as a solvent control. The plates were incubated for 18 h±2 h. After incubation, 10 µL of WST-8 assay reagent (Abcam) was added to each well. The cells were incubated for 3 h after which the absorbance was measured at 450 nm to determine cell metabolic activity. Three biological replicates were performed, each with three technical replicates.

## 2.16. Membrane Depolarisation Assay (diSC$_3$(5))

Bacterial cultures of *S. aureus* USA300 were prepared at 37 °C under constant agitation until the growth had reached mid-exponential phase ($OD_{600}$ ~ 0.6). The cultures were washed with a buffer containing 5 mM sodium HEPES (Merck, UK) and 20 mM glucose at a pH of 7.4, then resuspended in the same buffer. The suspension was then diluted to an $OD_{600}$ of 0.05 and incubated with 0.4 µM 3,3′-Dipropylthiadicarbocyanine iodide (diSC$_3$). 0.1 M potassium chloride was subsequently added to the solution until equilibrated. A 2 mL aliquot of the solution was transferred into a quartz cuvette and the fluorescence was measured using a fluorescence spectrometer (Agilent Cary Eclipse). 128 µg mL$^{-1}$ of (+)-JQ1 was added to the cell suspension and 1 % Triton-X-100 was used a positive control. For each condition three replicates were performed (*n*=3).

## 2.17. RNA Extraction

Bacterial cultures of *S. aureus* USA300 were prepared at 37 °C in a shaking incubator until the growth had reached mid-exponential phase ($OD_{600}$ ~ 0.6). Cultures were then treated with 128 µg mL$^{-1}$, 64 µg mL$^{-1}$, 10 % DMSO and an untreated culture was used as a positive control. RNA protect was added to the cultures and they were centrifuged at 5000 x g for 4 min to pellet the cells and supernatant was discarded. RNA was extracted using the ISOLATE RNA Mini Kit (Bioline/ Meridian Bioscience). Pellets were resuspended in a TE Buffer containing 3mg mL$^{-1}$ of lysozyme. Lysis Buffer R was added, and then incubated at room temperature for 3 min. The samples were

then subjected to homogenization with BashingBead Lysis Tubes (Zymo Research) using a MP Biomedicals™ FastPrep-24™ 5G Bead Beating Grinder and Lysis System. Samples were transferred to a Spin Column R1 placed inside a collection tube. The samples were centrifuged at 10000 x g for 2 min and the spin column was discarded. 70 % ethanol was added to the filtrate and this was mixed well by hand. This solution was transferred to a Spin Column R2 in a Collection Tube and this was centrifuged at 10000 x g for 1 min. Wash Buffer AR was added and the samples were centrifuged at 10000 x g for 1 min, with filtrate discarded. Wash Buffer BR was added to the sample and this was centrifuged again at 10000 x g for 1 min, after which filtrate was discarded. This was centrifuged at 10000 x g for 3 min and the spin column was placed in a 1.5 mL Elution Tube. 50 µL of RNase-free water was added the spin column and this was incubated at room temperature for 1 min, after which the samples were centrifuged at 6000 x g for 1 min, to extract RNA from the spin column. RNA was extracted from three biological replicates.

## 2.18. DNA Extraction

DNA was extracted using a Quick-DNA Fungal/Bacterial Microprep Kit (Zymo Research). *S. aureus* USA300 cultures were centrifuged at 4500 x g for 10 min. The supernatant was aspirated and the pellet was resuspended in nuclease-free water. The samples were then subjected to homogenization with BashingBead Lysis Tubes, as previously described in the RNA extraction methodology. The supernatant was then transferred to Zymo-Spin III-F Filters placed in collection tubes. The cells were centrifuged at 8000 x g for 1 min, then the spin filter was discarded. Genomic lysis buffer was added to the filtrate in the collection tubes and then mixed. The filtrate was added

to a Zymo-Spin IC Column in a collection tube and centrifuged at 10000 x g for 1 min, with filtrate discarded. DNA Pre-Wash Buffer was added to the spin column and the samples were centrifuged at 10000 x g for 1 min. g-DNA Wash Buffer was added to the column and the samples were centrifuged at 10000 x g for 1 min. The spin column was then transferred to a collection tube and DNA elution buffer was added. This was incubated at room temperature for 1 min, after which the samples were centrifuged at 10000 x g for 1 min to extract DNA. DNA was extracted from three biological replicates.

## 2.19. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The previously extracted DNA underwent a serial dilution to create 20 ng $\mu L^{-1}$ to 0.0390625 ng $\mu L^{-1}$ concentrations. The treated RNA samples were diluted to form 20 ng $\mu L^{-1}$ stocks. The forward and reverse primers for *gyrA*, *icaB*, *sasG*, *sigB*, *norA, recA* and *clpP* were diluted in nuclease-free water to 20 µM (Table 3). The qRT-PCR preparation was carried out by a Myra Liquid Handling System (Bio Molecular Systems) into a 96-well semi-skirted PCR plate (BIO-RAD Laboratories). The RNA stocks, DNA stocks, primers, 2× Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies), DTT (Agilent Technologies), RNase block (Agilent Technologies) and nuclease-free water were all added to the system. The prepared plate was analysed by a Bio-Rad thermocycler, which performed the following cycles:

- 50 °C for 10 min, 1 cycle
- 95 °C for 3 min, 1 cycle
- 95 °C for 5 s, 60 °C for 12 s, for 39 cycles

- 95 °C for 30 s, 1 cycle
- 60 °C for 1 min, 1 cycle
- 95 °C for 50 s, 1 cycle
- 60 °C for 15 s, 1 cycle

Three biological replicates were performed, each with three technical replicates.

**Table 3.** Sequences of the in house designed primers for the targeted genes of qRT-PCR on (+)-JQ1 treated *S. aureus* USA300 cells.

| Primer | Sequence |
|---|---|
| *gyrA* F | GCGGTAGGTATGGCAACGAA |
| *gyrA* R | CCGCCTCCACGTTCTTCAAT |
| *icaB* F | TCACAGGTCATGTTGGGGAA |
| *icaB* R | ATGCAAATCGTGGGTATGTGT |
| *sasG* F | TGTACCCGTTTTTGGTCCGT |
| *sasG* R | TCGGTGGCGAGAAAATACCG |
| *sigB* F | TGGTCATCTTGTTGCCCCAT |
| *sigB* R | AGCGTTCACCTTCTATCAGTGA |
| *recA* F | GCTTCGGCGATTTCAAGACC |
| *recA* R | TAGCGCTTCACGCTATTGCT |
| *clpP* F | GAAACAACAAACCGCGGTGA |
| *clpP* R | TCTCTGAGTCTTGCGCTTGT |
| *norA* F | TAGGACCAGGGATTGGTGGA |
| *norA* R | GAAGCCGCTTGTCGTAGACT |

## 2.20. Data Analysis

All data analysis was carried out using GraphPad Prism 10.3.1 (GraphPad Software, San Diego, CA, USA). All assays were performed in triplicate with means calculated from this. Standard deviation and standard error of the mean (SEM) were calculated from the means. Paired t-tests, one-way ANOVAs, two-way ANOVAs and Tukey HSD post hoc tests were performed as appropriate to

determine significant difference between groups. GraphPad Prism 10.3.1 was also used to produce all figures.

# 3. Results

## 3.1. Bioinformatics comparison of BET proteins and *S. aureus*

Sequence similarity between the BET proteins BRD2 (P25440), BRD3 (Q15059), BRD4 (O60885) and BRDt (D4A7T3) and *S. aureus* (Taxonomy ID: 1458279) was assessed using BLAST and HMMER software. However, no sequence homology was observed between the sequences when considering the e-value, identity percentage, and alignment.

## 3.2. Minimum inhibitory and bactericidal concentrations of JQ1 and other lead BET inhibitors

BET inhibitor (+)-JQ1 demonstrated a minimum inhibitory concentration of 128 µg $mL^{-1}$ against *S. aureus* strain USA300 (Table 4). Other key BET inhibitors PFI-1 and OTX015, which are both prominent inhibitors in Eukaryotic research were also screened. However, no bacterial growth inhibition was observed at the highest concentrations used in the assays (PFI-1 >500 µg $mL^{-1}$; OTX015 >1000 µg $mL^{-1}$). (+)-JQ1 was then screened against several other prominent ESKAPE bacterial strains but the minimum inhibitory concentration was greater than the highest concentration screened for all strains (Table 5).

**Table 4.** MIC values of (+)-JQ1 and other lead candidate BET inhibitors (PFI-1 and OTX015) against *S. aureus* strain USA300.

| **BET Inhibitor** | **MIC (μg mL$^{-1}$)** |
|---|---|
| (+)-JQ1 | 128 |
| PFI-1 | >500 |
| OTX015 | >1000 |

**Table 5.** MIC values of (+)-JQ1 against ESKAPE bacterial pathogens.

| **Bacteria** | **MIC (μg mL$^{-1}$)** |
|---|---|
| *L. monocytogenes* | >1000 |
| *E. faecalis* | >1000 |
| *E. faecium* | >1000 |
| *K. pneumoniae* | >1000 |
| *A. baumannii* | >1000 |
| *P. aeruginosa* | >1000 |
| *E. coli* | >1000 |

JQ1 chemical synthesis can occur in several derivatives due the structural properties of the compound. During synthesis, it can be subjected to stereoisomerism where enantiomers are formed. These are pairs of compounds with the same connectivity but opposite three-dimensional shapes which are non-superimposable. This can lead to significant changes in biological activity. Two enantiomers of JQ1 termed (+)-JQ1 and (-)-JQ1 were examined for antibacterial activity via MIC assays, with (-)-JQ1 being the stereoisomer which is reported to lack biological activity. Both derivatives had similar antibacterial activity against *S. aureus* strain USA300 with MIC values of 128 μg $mL^{-1}$ for the biologically active (+)-JQ1 and 125 μg $mL^{-1}$ (Table 6) when cells were exposed to the inactive (-)-JQ1 form. This suggests that either off-target effects are being observed or an alternative region of the compound is mediating the observed antibacterial activity. A racemic mixture containing equal concentrations of both stereoisomers ((+/-)-JQ1) was also examined for antibacterial activity against *S. aureus* strain USA300 but this demonstrated MIC values of >100 μg $mL^{-1}$. Finally, to improve compound solubility and potential biological lipophilicity, a (+)-JQ1 derivative modified with a carboxylic acid ligand was also examined for antibacterial activity, with MIC values of >100 μg $mL^{-1}$ being observed against *S. aureus* strain USA300. Both the racemic mixture and carboxylic acid derivatives of JQ1 exhibited low solubility at higher concentrations which limited the final highest MIC assay concentration to 100 μg $mL^{-1}$.

**Table 6.** MIC values of JQ1 derivatives against *S. aureus* strain USA300.

| **JQ1 Derivative** | **MIC (μg $mL^{-1}$)** |
|---|---|

| | |
|---|---|
| (+)-JQ1 | 128 |
| (-)-JQ1 | 125 |
| (+/-)-JQ1 | >100 |
| (+)-JQ-1 carboxylic acid | >100 |

Experiments were conducted to determine the minimum bactericidal concentration (MBC) for each compound with each respective bacterial species. However, due to low solubility of each JQ1 compound at high concentrations, it was not possible to determine accurate MBC values with all being significantly greater than the observed MIC values. Given the (+)-JQ1 enantiomer is the recognised biologically active stereoisomer, this compound was selected for further investigation.

### 3.3. Antimicrobial activity assessed by disc diffusion assays

Disc diffusion assays were performed using 256 µg $mL^{-1}$, 128 µg $mL^{-1}$ and 64 µg $mL^{-1}$ of (+)-JQ1 against *S. aureus* strain USA300, alongside DMSO (10 %) solvent controls. However, no zones of inhibition were observed at these concentrations.

### 3.4. Growth dynamics of *S. aureus* strain USA300 after exposure to (+)-JQ1

After previously determining the MIC value for (+)-JQ1 against bacterial cells of *S. aureus* strain USA300, this study then investigated the growth dynamics of the strain in response to different concentrations of the lead BET inhibitor. *S. aureus* strain USA300 was inoculated to an $OD_{600}$ of approximately 0.1 and incubated with final compound concentrations of 16 µg $mL^{-1}$, 32 µg $mL^{-1}$,

64 μg $mL^{-1}$ and 128 μg $mL^{-1}$. Growth over an 18 h period was assessed in comparison to DMSO (2 %) solvent control and untreated bacterial cells. Untreated bacteria increased in growth to approximately $OD_{600}$ of 1.5 by 9 h (Fig. 1, black circles). There were no significant differences ($p$ >0.05) in growth between the untreated cells, DMSO treated cells and those incubated with 16 μg $mL^{-1}$ and 32 μg $mL^{-1}$ (+)-JQ1 (Fig. 1, compare black circle with black square, clear circle, black diamond). Upon exposure to 64 μg $mL^{-1}$ (+)-JQ1, bacterial growth was significantly affected compared to the controls with an $OD_{600}$ of approximately 0.5 being observed after 18 h incubation (Fig. 1, inverted triangles). Finally, after bacterial exposure to 128 μg $mL^{-1}$, no significant increase in growth was observed at any time point over the 18 h incubation period. Compared to the untreated control, a concentration of 128 μg $mL^{-1}$ (+)-JQ1 significantly inhibited bacterial growth at 18 h ($p$ < 0.0001) (Fig. 1, black triangles).

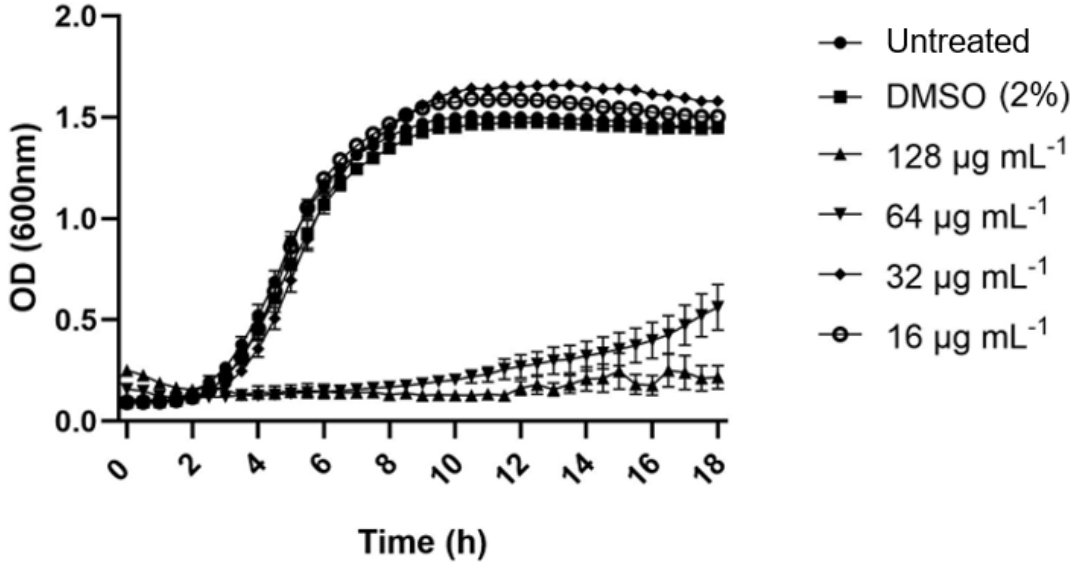

**Figure 1.** Growth curve of *S. aureus* strain USA300 in the presence of (+)-JQ1 at 128 µg $mL^{-1}$, 64 µg $mL^{-1}$, 32 µg $mL^{-1}$ and 16 µg $mL^{-1}$ compared to untreated and solvent treated bacteria. Results show the mean of $n = 3$ and error bars represent standard error of the mean.

### 3.5. Exposure of *S. aureus* strain USA300 to (+)-JQ1 resulted in a significant reduction in bacterial viability over a 24 h period.

To investigate the effects of (+)-JQ1 on bacterial viability, growth kinetics of *S. aureus* strain USA300 were observed over a 24 h period following exposure to the compound. Bacterial cells at approximately $10^6$ colony forming units per mL (CFU $mL^{-1}$) were incubated with 64 µg $mL^{-1}$, 128 µg $mL^{-1}$, 256 µg $mL^{-1}$ of (+)-JQ1 and bacterial viability was determined at time points over 24 h compared to an untreated and DMSO (2 %) solvent treated cells. Untreated bacterial cultures and DMSO treated cells grew to approximately $10^9$ CFU $mL^{-1}$ by 24 h (Fig. 2, squares and triangles respectively). Likewise, bacterial cells treated with 64 µg $mL^{-1}$ showed evidence of growth inhibition at 2 h and 6 h, although by 24 h the culture had recovered to growth levels observed in the untreated cells (Fig. 2, circles). After treatment with 128 µg $mL^{-1}$, an approximately one-log reduction in bacterial growth was observed at 6 h compared to the culture starting viability, with a one-log increase observed by 24 h (Fig. 2, diamond). However, compared to the untreated control there was a significant reduction ($p < 0.01$) in bacterial growth at 24 h (Fig. 2, compare diamond with squares) which equates to approximately three-log reduction in viability. Finally, when bacterial cells were exposed to 256 µg $mL^{-1}$ (+)-JQ1 growth remained static until 24 h when a significant five-log reduction ($p < 0.01$) in viability was observed compared to the untreated control (Fig. 2, inverted triangle compared to square). Overall, these results suggest that the

compound was acting as a bacteriostatic agent as viability did not decrease significantly beyond the initial starting inoculum after 24 h even at one-fold above MIC concentrations.

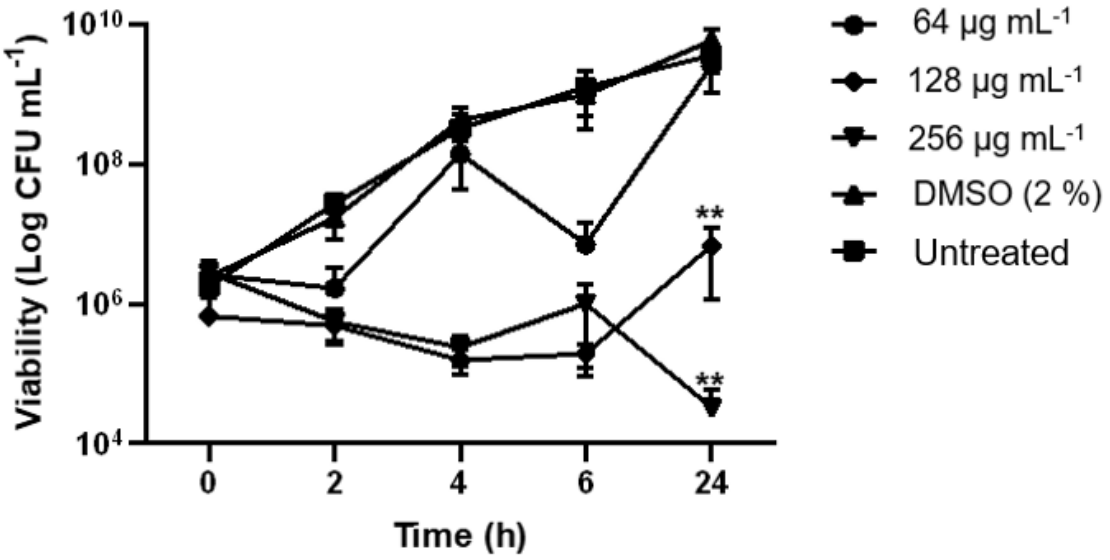


**Figure 2.** Time-kill kinetic assays of *S. aureus* strain USA300 when exposed to 256 µg $mL^{-1}$, 128 µg $mL^{-1}$ and 64 µg $mL^{-1}$ concentrations of (+)-JQ1 compared to untreated and DMSO (2 %) treated cells. Results show the mean of $n$ = 3 and error bars represent standard error of the mean. ** denotes significance of $p$ <0.01.

## 3.6. Antimicrobial synergy of (+)-JQ1 with traditional antibiotics

Potential synergistic activity was evaluated between (+)-JQ1 and two commonly used treatments for *S. aureus* infections (penicillin G and vancomycin) using checkerboard synergy assays. The MIC of penicillin G against *S. aureus* USA300 was 2048 µg $mL^{-1}$ and the MIC of vancomycin against *S.*

*aureus* USA300 was 2 µg $mL^{-1}$. As part of the synergy assay, concentrations up to four times the MIC of (+)-JQ1 and the antimicrobials were used, with two-fold serial dilutions being carried out up to 1/32 of the MIC value. The MIC values were determined for (+)-JQ1 in combination with each antibiotic to calculate the fractional inhibitory concentration (FIC) value. The FIC values for both antibiotics screened were approximately 1.0. The FIC index classifies FIC values between 0.5 – 4 as showing indifference (White *et al*., 1996). This means the combined use of (+)-JQ1 alongside the antibiotics did not enhance or decrease their individual antimicrobial activity profiles (Table 7).

**Table 7.** FIC values and their interpretation as determined through checkerboard assay synergy testing.

| **Antibiotic** | **FIC Value** | **Interpretation** |
|---|---|---|
| Penicillin G | 1.01 | Indifferent |
| Vancomycin | 1.02 | Indifferent |

## 3.7. *S. aureus* strain USA300 biofilm formation reduced after exposure to (+)-JQ1

Biofilm formation is a major virulence factor which contributes to *S. aureus* pathogenicity during infection and disease processes. One approach to reducing bacterial pathogenicity is developing strategies to inhibit or limit biofilm formation using an anti-virulence approach. To determine the anti-virulence potential of (+)-JQ1, the biofilm forming ability of *S. aureus* strain USA300 was determined with exposure to sub-inhibitory concentrations (64 µg $mL^{-1}$, 32 µg $mL^{-1}$, 16 µg $mL^{-1}$)

of the compound. Bacterial cells were cultured with (+)-JQ1 over a 24 h period and biomass accumulation was determined by crystal violet staining. At the 64 µg $mL^{-1}$ concentration (1/2 MIC value) a significant 32.16 % reduction ($p < 0.001$) was seen in biofilm formation (Fig. 3, light grey bar) compared to an untreated control and the DMSO solvent treated cells (Fig. 3, white bar) ($p < 0.01$). After treatment with 32 µg $mL^{-1}$ and 16 µg $mL^{-1}$ concentrations, 15.35 % and 14.10 % inhibition in biofilm formation respectively was observed. This level of inhibition was similar to that observed after treatment with a DMSO solvent control, which had a reduction of 6.23 %.

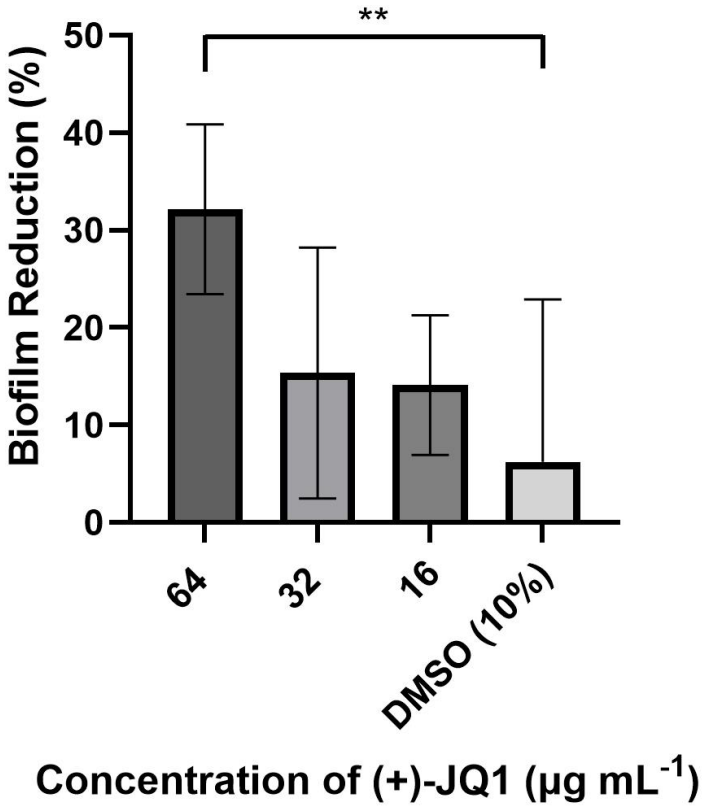

**Figure 3.** Percentage biofilm reduction mediated by 64 µg $mL^{-1}$, 32 µg $mL^{-1}$ and 16 µg $mL^{-1}$ concentrations of (+)-JQ1. Error bars represent the mean ± standard deviation from three biological replicates in triplicate (*n*=3).

## 3.8. Effect of (+)-JQ1 exposure on haemolysis of erythrocytes

To assess the cytotoxicity of (+)-JQ1 against erythrocytes, a range of inhibitory and sub-inhibitory (+)-JQ1 concentrations were applied to haemolytic assays. There was a significant increase in the percentage of haemolysis of the erythrocytes after exposure to 128 µg $mL^{-1}$, 64 µg $mL^{-1}$, 32 µg $mL^{-1}$ and 16 µg $mL^{-1}$ of (+)-JQ1 compared to the DMSO solvent control treated cells (Fig. 4) ($p < 0.0001$). Exposure of cells to (+)-JQ1 resulted in a haemolysis of 11.2 - 24.8 %, suggesting that it caused moderate cytotoxic damage to erythrocytes. Interestingly, the lowest percentage of haemolysis was observed at the highest (+)-JQ1 concentration tested.

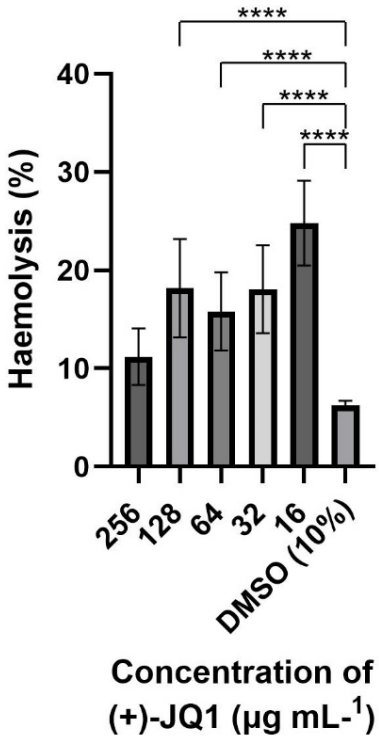


**Figure 4.** Percentage haemolysis observed after erythrocytes were exposed to 256 µg $mL^{-1}$, 128 µg $mL^{-1}$, 64 µg $mL^{-1}$, 32 µg $mL^{-1}$ and 16 µg $mL^{-1}$ (+)-JQ1 compared to Triton X-100 (positive control) and DMSO (10 %) solvent control. Error bars represent standard error of the mean of *n = 3.*

## 3.9. Effects of (+)-JQ1 exposure on bacterial toxin mediated haemolysis

Bacterial toxin production is a major cause of *S. aureus* virulence and pathogenicity. To determine if (+)-JQ1 effects secreted toxin production of *S. aureus* strain USA300, cultures were exposed to sub-inhibitory concentrations of (+)-JQ1 for 24 h and the haemolytic activity of filtrates was determined. After cells were pre-treated with 64 µg $mL^{-1}$ of (+)-JQ1, there was a significant reduction ($p$ < 0.0001) in secreted toxin mediated haemolysis at 0.78 % (Fig. 5, black bar)

compared to untreated samples at 27.04 % (Fig. 5, light grey bar). Cells pre-treated with 32 µg $mL^{-1}$ and 16 µg $mL^{-1}$ produced supernatants which resulted in 11.75 % and 40.37 % haemolysis respectively (Fig. 5). Pre-treatment of cells with a DMSO solvent control resulted in supernatants with a haemolytic rate of 12.73 %. These results indicated that at sub-inhibitory concentrations of (+)-JQ1 (32 µg $mL^{-1}$ and 64 µg $mL^{-1}$), bacterial toxin production was significantly reduced compared to untreated cells. All treated cultures had the same end point bacterial viability.

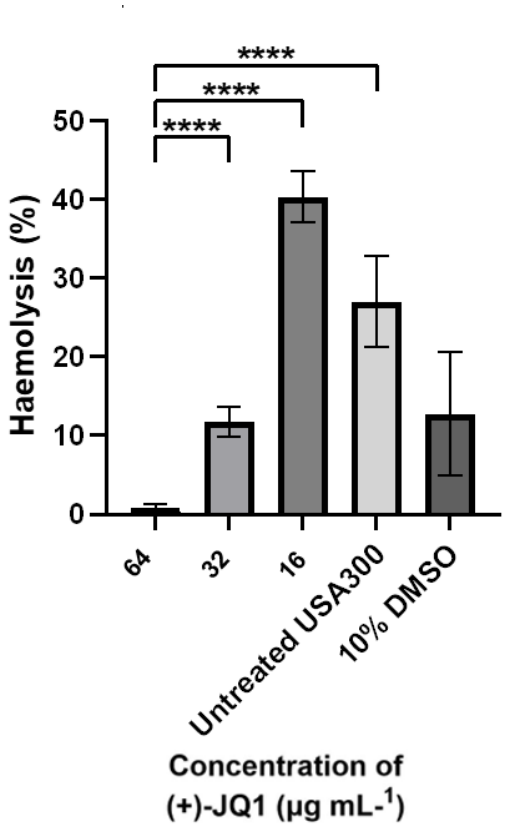


**Figure 5.** Percentage haemolysis observed from filtered extracts of *S. aureus* strain USA300 pre-exposed to 64 µg $mL^{-1}$, 32 µg $mL^{-1}$ and 16 µg $mL^{-1}$ concentrations of (+)-JQ1 compared to Triton X (positive control) and 10 % DMSO solvent control. Error bars represent SEM of *n = 3.* **** denotes $p < 0.0001$.

## 3.10. (+)-JQ1 showed no cytotoxic effects against mammalian cell lines

To assess the cytotoxicity of (+)-JQ1 against human cells, HepG2 cells were exposed to various concentrations of (+)-JQ1. Significant levels of cytotoxicity were observed, with a significant decrease in the survival of HepG2 cells exposed to (+)-JQ1 and the solvent control compared to

exposure to untreated cells ($p < 0.0001$) (Fig. 6). Similar levels of cytotoxicity were observed across all concentrations of (+)-JQ1 and the DMSO solvent control.

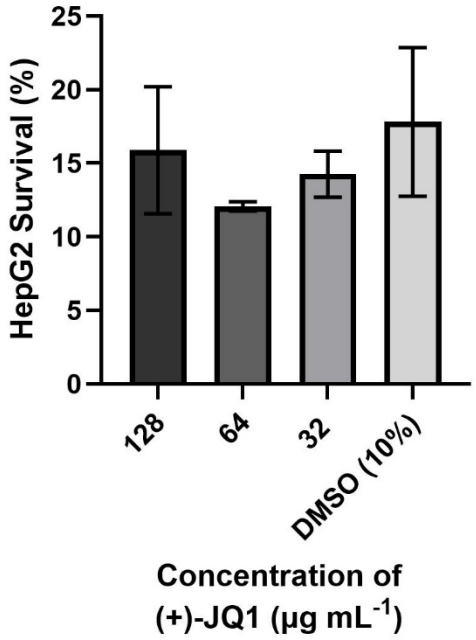


**Figure 6.** Percentage cytotoxicity observed in HepG2 cells after exposure to 128 µg $mL^{-1}$, 64 µg $mL^{-1}$ and 32 µg $mL^{-1}$ (+)-JQ1, and 10 % DMSO solvent. Error bars represent SEM of *n = 3*.

## 3.11. Scanning electron microscopy (SEM) analysis revealed no changes in bacterial cellular morphology after treatment with (+)-JQ1

To determine the potential mechanistic activity for the observed antimicrobial activity of (+)-JQ1, *S. aureus* strain USA300 was exposed to 128 µg $mL^{-1}$ and 64 µg $mL^{-1}$ of compound for 24 h. Bacterial samples were fixed, dehydrated and subjected to coating and analysis by SEM. No visible

changes in cell morphology or ultrastructure were observed in the treated cells (Fig. 7B and C) compared to the untreated cells (Fig. 7A). DMSO (2 %) also had no observed effects on cell morphology (data not shown).

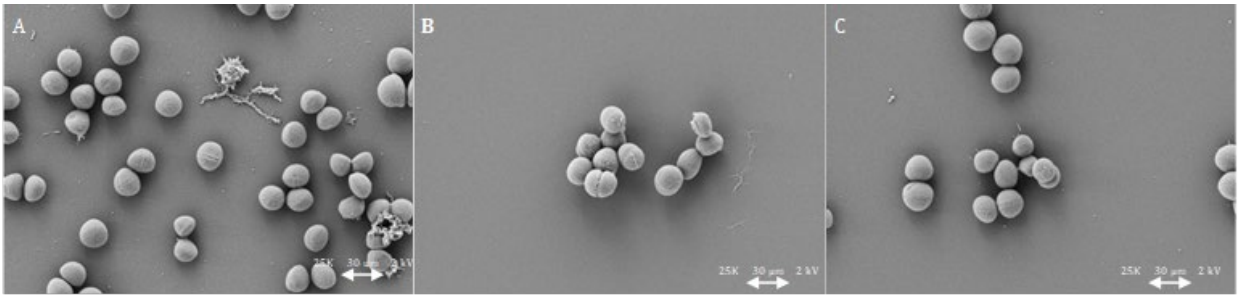


**Figure 7**. SEM micrographs of *S. aureus* strain USA300 after 24 h of exposure to **(B)** 128 µg $mL^{-1}$ and **(C)** 64 µg $mL^{-1}$ (+)-JQ1 compared to **(A)** untreated cells. Scale bars are shown in µm. Images represent examples of *n* = 3 biological replicates.

## 3.12. Exposure to (+)-JQ1 resulted in membrane depolarisation

Some antimicrobial compounds either directly or indirectly target the cell membrane. A fluorescence dye assay was used to determine the role of (+)-JQ1 in bacterial membrane localisation. *S. aureus* strain USA300 was incubated with $diSC_3$ which incorporated into the bacterial cytoplasmic membrane. Upon depolarisation, this fluorescent dye was released from the membrane and the rate of activity was recorded over time. The Triton X-100 (1 %) detergent was used to fully depolarise the bacterial membrane which resulted in a final intensity of 143.13 a.u. after 1 min (Fig. 8, green line). Likewise, bacterial cells exposed to the DMSO solvent control depolarised the membrane to a value of 49.95 a.u. intensity (Fig. 8, blue line). Upon addition of

128 µg mL$^{-1}$ (+)-JQ1 the final fluorescence intensity from *S. aureus* strain USA300 was 103.13 a.u. (Fig. 8, red line).

At the 1 min time point, there was a significant difference in fluorescence between the 10 % DMSO control with both the (+)-JQ1 treated bacteria and the positive Triton X-100 control ($p < 0.0001$). There was also a significant difference between the fluorescence of the Triton X-100 and 128 µg mL$^{-1}$ of (+)-JQ1 ($p < 0.001$). This leads to the conclusion that (+)-JQ1 depolarises the membrane of *S. aureus* USA300 at a significantly higher level than the solvent control, but not at the level of the positive control Triton X-100 as expected.

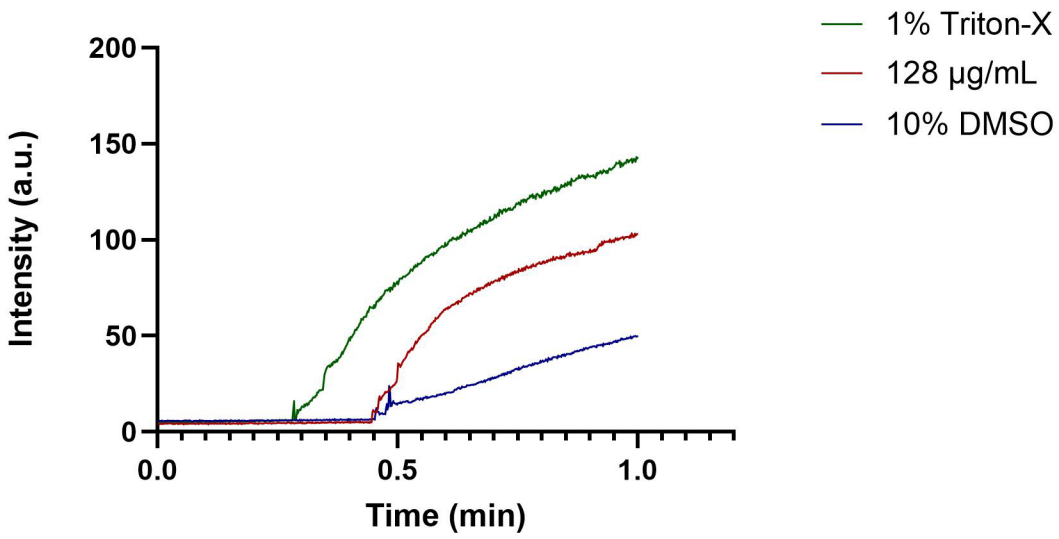


**Figure 8.** Fluorescence intensity observed due to membrane depolarisation of *S. aureus* USA300 after exposure to 128 µg mL$^{-1}$ of (+)-JQ1 compared to Triton X-100 (positive control) and 10 % DMSO solvent control. Image is a representative example of $n = 3$.

## 3.13. Bacterial genetic responses to (+)-JQ1 treatment

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) assays were performed to assess the regulatory changes in gene expression of key *S. aureus* genes after 20 min (one generation) of exposure to 128 µg $mL^{-1}$ and 64 µg $mL^{-1}$ (+)-JQ1. The *gyrA* gene was used as a housekeeping gene to standardise gene expression. For *icaB, sasG, sigB, recA* and *clpP* there were no significant fold changes observed ($p > 0.05$) (Fig. 9A, 9B, 9C, 9D and 9E). For *norA* a significant fold change was observed between 128 µg $mL^{-1}$ of (+)-JQ1 and the positive control, which had a representative fold change of 1.0 ($p < 0.01$) (Fig. 9F).

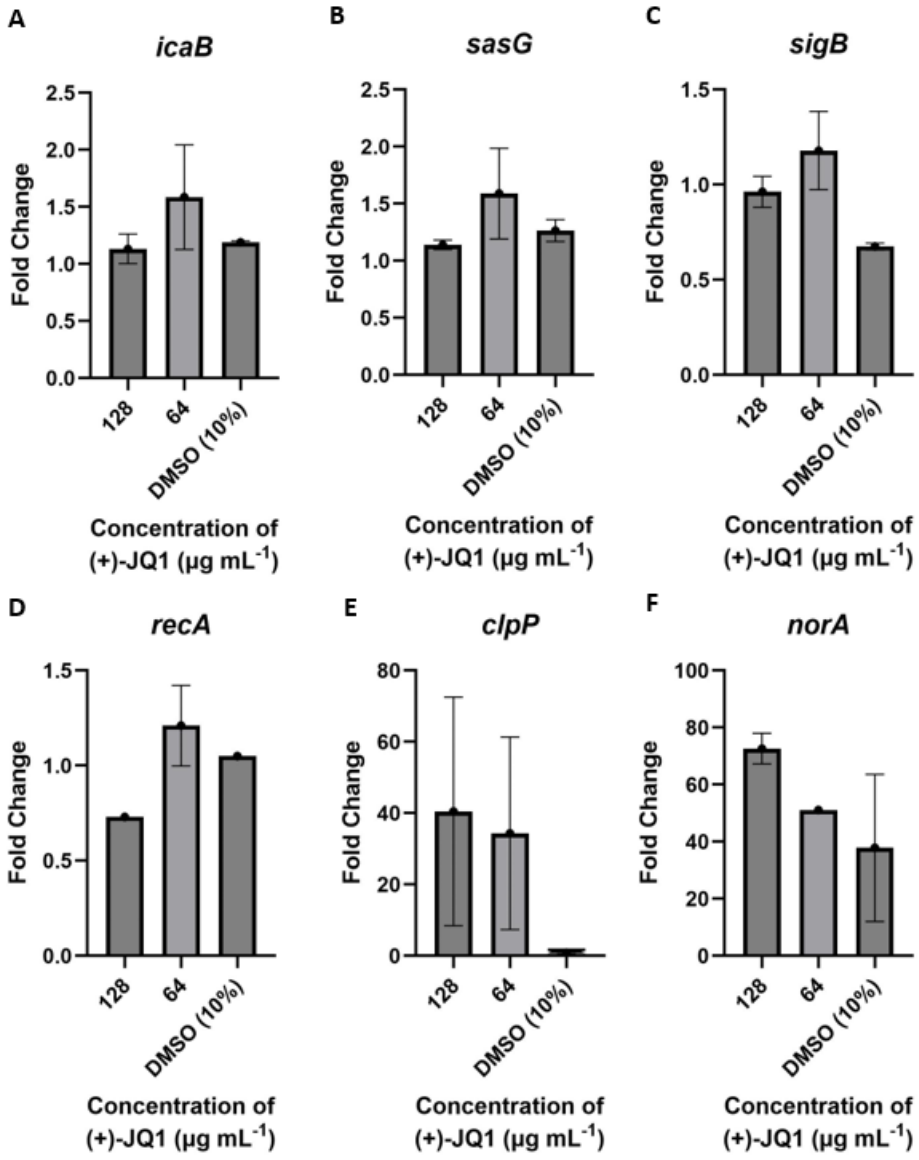


**Figure 9.** Fold change in the expression of genes *icaB* **(A)**, *sasG* **(B)**, *sigB* **(C)**, *recA* **(D)**, *clpP* **(E)** and *norA* **(F)** after exposure to 128 µg $mL^{-1}$ and 64 µg $mL^{-1}$ of (+)-JQ1 alongside a 10 % DMSO solvent control, in relation to *gyrA* housekeeping gene (n=3).

# 4. Discussion

## 4.1. Use of BET Inhibitors as Antimicrobial Therapeutics

This study showed a novel off target effect of the BET inhibitor (+)-JQ1 and its enantiomer (-)-JQ1 as antibacterial agents against the MRSA *S. aureus* strain USA300. This is the first time any BET inhibitor has shown antimicrobial efficacy against prokaryotes. However, there have been previous uses of BET inhibitors within the field of microbiology to target other microorganisms.

As previously described in the introduction, BET inhibitors have been used to target fungal BET proteins. The fungal BET proteins Bdf1 and Bdf2 have a smaller binding site than the human BET proteins and therefore human BET inhibitors such as (+)-JQ1 do not show inhibitory action against fungal BET proteins. Instead, specific fungal BET inhibitors have been developed.

(+)-JQ1 has been previously used in viral infections including infections caused by Epstein-Barr virus (EBV). (+)-JQ1 blocks the EBV lytic cycle at two different stages. The first stage (+)-JQ1 inhibits is the expression of the protein BZLF1 and target the lytic origin of replication (OriLyt) genetic elements to prevent late-stage gene expression (Keck *et al.*, 2017). (+)-JQ1 has also been show to inhibit the pathogenesis of viral infections of both murine leukaemia virus (MuLV) and feline leukaemia virus (FeLV) (Gupta *et al.*, 2013; Moll, Swenson and Yuzbasiyan-Gurkan, 2023). In MuLV and FeLV infection models (+)-JQ1 inhibited proviral integration. In all documented instances of (+)-JQ1 being used as a therapeutic against viral infections, the compounds target human BET proteins to regulate viral replication rather than directly targeting the virus.

(+)-JQ1 has also been used in studies of parasitic disease, it exhibited a lower $IC_{50}$ against *Trypanosoma cruzi* than the current recommended treatment benznidazole (Alonso *et al.*, 2016). *T. cruzi* express a protein called *Trypanosoma cruzi* bromodomain factor 3 (TcBDF3). Parasites

overexpressing TcBDF3 have altered differentiation and exhibit resistance against BET inhibitors including (+)-JQ1. Whilst (+)-JQ1 would not be suitable as a treatment against *T. cruzi* due to relatively high $IC_{50}$ values and its inhibition is the presence of recombinant TcBDF3, there is scope for other BET inhibitors to be developed to specifically target the bromodomain containing proteins of *T. cruzi*.

## 4.2. Previously Described Off Target Effects of (+)-JQ1

The observed MIC of the biologically active enantiomer and corresponding stereoisomer of JQ1 were closely aligned which suggested that the mechanism of action of JQ1 in prokaryotes is likely an off-target effect. The likelihood of this is increased due to the lack of identifiable BET proteins in bacteria as no BET protein homologues were identified from the bioinformatics analysis that was conducted as part of this study. This is further supported by the role of BET proteins in binding to the lysine residues of histones, which are not by current paradigms present in prokaryotes. However, the presence of histone like proteins should be considered in further work to elucidate a potential target site.

Under classical conditions (+)-JQ1 acts as a BET protein inhibitor by binding to BET proteins, primarily BD4, preventing the binding of the acetylated lysine residues. However, off-target effects of (+)-JQ1 have been described in the previous literature.

The study which initially identified (+)-JQ1 as a BET inhibitor also identified two off target effects from a screening plate containing ligand and ion receptors (Filippakopoulos *et al.*, 2010). These were the partial inhibition of binding between [Nleu-10]-NKA to the neurokinin NK2 receptor and

IB-MECA to the adenosine A3 receptor. Binding of the NK2 receptor was inhibited by 56 % and binding of the A3 adenosine receptor was inhibited by 61 %. The NK2 receptor is a type of tachykinin receptor which is involved in the activation of multiple pathways including intestinal motor functions, smooth muscle contraction and inflammation (Wenjing *et al.*, 2022). The A3 adenosine receptor is a type of G protein-coupled receptor found in various tissues throughout the body but expressed at highest levels in the testes (Zhou *et al.*, 1992).

In further studies utilising (+)-JQ1 off target effects were identified and confirmed to be off-target effects through (-)-JQ1 exhibiting similar levels of activity. One of these instances is the use of (+)-JQ1 as an agonist for the pregnane X receptor (PXR) (Huber *et al.*, 2023). JQ1 is metabolised by the enzyme CYP3A4, PXR is the main ligand-dependent receptor of CYP3A4. It was determined that (+)-JQ1 acts as an agonist of PXR and upregulates CYP3A4-luciferase. (-)-JQ1 exhibited similar potency with greater efficacy than (+)-JQ1. The *tert*-butyl group of JQ1 was directly involved in the binding to the PXR rather than the bromodomain group utilised in binding to BET proteins. The binding pockets of BET proteins are smaller than the PXR binding pockets, restricting the rotation of the compound only allowing (+)-JQ1 to bind. Whereas the large binding pockets of PXR allowed (-)-JQ1 to also bind. This suggests that the target site in prokaryotes must also have a large enough binding pocket to allow for rotation of the compound to facilitate (-)-JQ1 binding.

Another off-target effect of (+)-JQ1 was observed in its ability to inhibit aortic contraction (Yan *et al.*, 2023). (+)-JQ1 upregulated levels of endothelial nitric oxide synthase (eNOS) and protein kinase B (AKT) through the PI3K/AKT/eNOS cascade. (-)-JQ1 was used to confirm that this inhibition was indeed an off-target effect and not caused by the inhibition of BET proteins.

## 4.3. Effect of BET Inhibitors as Anti-virulence Agents Against Biofilm Formation and Toxin Production

One of the key virulence factors of *S. aureus* and other multidrug resistant pathogens is the ability to form biofilms. Specifically in the case of *S. aureus* the biofilm formation occurs in five stages, these being attachment, multiplication, exodus, maturation and dispersal (Moormeier and Bayles, 2017). During the attachment phase cell wall-anchored (CWA) proteins attach the bacteria to a surface, the types of CWA proteins involved in this process varies depending on the components of the host's extracellular matrix. Examples of common CWA proteins include fibronectin-binding proteins, serine-aspartate repeat family proteins, clumping factors, collagen adhesin and Protein A. In the multiplication phase the *S. aureus* cells begin to rapidly divide and replicate, this process is supported by proteins which stabilise cell to cell interactions and promote intercellular attachment. Some of these proteins are CWA proteins involved in attachment which serve dual functions, but there are also some specific multiplication proteins including *S. aureus* surface protein G (Speziale *et al*., 2014). Approximately six hours into the biofilm formation process the exodus stage occurs, this is an initial release of some cells that results in restructuring of the biofilm that is triggered by Nuc1 (Schilcher and Horswill, 2020). The exodus stage is mediated by the degradation of eDNA. After this microcolonies begin to form between the *S. aureus* cells in the maturation phase. These microcolonies formation causes encapsulated bacteria to initiate increased production of extracellular polymeric substances, which protect the biofilm and sustain continued development (Luo *et al*., 2022). The final stage of the *S. aureus* biofilm is the dispersal stage, which is primarily mediated by the accessory gene regulator (Agr) quorum sensing system

(Boles and Horswill, 2008). This system triggers modulation of the biofilm matrix and *S. aureus* cell dispersal.

The biofilm production of *S. aureus* is clinically relevant due to the impact biofilm structure has on antibiotic susceptibility. The presence of the biofilm matrix decreases the efficacy of classical antimicrobials as the compounds need to penetrate the matrix to target the individual bacterial cells (Donlan, 2000). This can result in recommended antibiotic dosages becoming sub-lethal as increased dosages would be required to penetrate the biofilm matrix. These increased dosages result in higher concentrations of the antibiotic being required, which also contributes to antimicrobial resistance evolution (Mirghani *et al.*, 2022). It is vital that novel antimicrobials are able to penetrate this matrix or prevent initial biofilm formation as to counteract this crucial virulence factor.

This study found that (+)-JQ1 significantly reduced biofilm formation of *S. aureus* strain USA300. This biofilm reduction could be attributed to a number of transcriptional changes occurring within the bacterial cell, including alterations to the genes involved in quorum sensing, adhesion, charge and biofilm aggregation. Given that no upregulation was observed in the *icaB* or *sasG* genes (responsible for biofilm accumulation and attachment respectively) it can be suggested that (+)-JQ1 does not target matrix formation or biofilm adhesion. The upregulation of *norA* (an efflux transporter) suggests that (+)-JQ1 is acting on an intracellular target. One potential mechanism of (+)-JQ1 is the inhibition of quorum sensing. Quorum sensing is one of the key functions of a biofilm, which allows the biofilm to coordinate gene expression in response to stimuli (Yamazaki *et al.*, 2024). The Agr system is involved in the regulation of quorum sensing, along with several other key bacterial virulence factors (Khan *et al.*, 2015). Due to this the Agr system has been

proposed as a promising target for novel therapeutics. It is possible that (+)-JQ1 is targeting one of the genes involved in the Agr process: *agrA, agrB, agrC* or *agrD*. Another potential target of (+)-JQ1 could be proton motive force (PMF), given that (+)-JQ1 causes depolarisation of the bacterial membrane (Mohiuddin *et al.*, 2022). Previously, inhibitors targeting PMF have been shown to reduce the growth of *S. aureus*. PMF inhibitors have also been shown to reduce biofilm formation by an undefined mechanism, further implicating PMF as a potential target of (+)-JQ1 (Ikonomidis *et al.*, 2008).

Another key virulence factor of *S. aureus* is the production of toxins, particularly haemolysins. There are various types of haemolysins that *S. aureus* produce including α-haemolysin, β-haemolysin, γ-haemolysin, δ-haemolysin and Panton Valentine leukocidin (PVL) (Divyakolu *et al.*, 2019). The production of these toxins is mediated by the Agr quorum sensing system. α-haemolysin forms pores in the cell membrane of haemolytic cells resulting in haemolysis. β-haemolysin is a sphingomyelinase, which breaks down sphingomyelin, a vital lipid in the plasma cell membrane (Vandenesch, Lina and Henry, 2012). γ-haemolysin and Panton-Valentine leukocidin are also types of pore forming toxins, they both specifically target leukocytes (Divyakolu *et al*., 2019). δ-haemolysin has various mechanisms of action against haemolytic cells, including pore forming capabilities, destabilisation of the membrane and action as a detergent to dissolve the membrane. Toxins, including haemolysins, lead to host tissue destruction which can increase the spread of the infection. The lysis of red blood cells releases iron which is an essential nutrient of *S. aureus*, creating a nutrient dense environment which promotes continued spread of the infection (Spaan *et al.*, 2015). Treatment with (+)-JQ1 showed a reduction in the haemolytic activity of *S. aureus,* due to the inhibition of haemolytic toxin production. This suggest that (+)-

JQ1 could be inhibiting the Agr system, which is the key regulator of haemolytic toxin production. As previously mentioned, the Agr also has key functions in the regulation of biofilm formation further supporting this as a potential (+)-JQ1 target.

## 4.4. Cytotoxicity of BET Inhibitors Against Mammalian Cell Lines

This study found that (+)-JQ1 exhibited significant levels of cytotoxicity against mammalian HepG2 cells. The levels of cytotoxicity were similar to the levels observed with exposure of *S. aureus* USA300 to 10 % DMSO solvent control, indicating that the cytotoxic effects were due to the solvent rather than the compound itself.

The previous applications of (+)-JQ1 as a BET inhibitor utilised lower concentrations of the compound. The first use of (+)-JQ1 by Filippakopoulos et al., 2010 used (+)-JQ at an IC50 of 77 nM (35.2 ng $mL^{-1}$) and 33 nM (15.1 ng $mL^{-1}$). (+)-JQ1 has been used in oncology research at various IC50 concentrations, some as high at 500 nM (228.8 ng $mL^{-1}$) (Delmore *et al.*, 2011; Ott *et al.*, 2012). The antimicrobial effects of (+)-JQ1 presented in this study were observed at the higher concentration of 128 µg/mL.

The antimicrobial susceptibility assays were conducted using 2% DMSO to match the concentration used during the preliminary screening. However, low solubility of (+)-JQ1 and (-)-JQ1 and the relatively high MIC values resulted in higher concentrations of DMSO being required. This makes systemic use of the compound unlikely and instead potential applications for the antimicrobial effects of the compounds could include use in topical wound dressings and wound cleaning agents. Indeed, concentration of up to 10 % DMSO are suitable for use in topical

applications and have previously been shown to enhance the rate of early wound healing (Kant, Jangir and Kumar, 2020).

Infections are common complication of cutaneous wounds, potentially resulting in further complications leading to severe outcomes such as sepsis and death (Yousefian *et al.*, 2023). Antimicrobial wound dressings are utilised in situations where bacterial colonisation or local infections are suspected. Wound dressings maintain suitable levels of moisture to promote the growth on new epithelium, whilst acting as a physical barrier between the wound and bacterial colonisation. There are various types of antimicrobial wound dressing available utilising a variety of antimicrobial agents such as chemical antiseptics, silver, iodine, antibiotics, manuka honey and essential oils.

Another application of (+)-JQ1 and (-)-JQ1 is the potential use in wound cleaning agents. Wound cleaning agents are typically antiseptics with a wider spectrum of activity than traditional targeted antibiotic therapies (Atiyeh, Dibo and Hayek, 2009). However, due to the prevalence of *S. aureus* in skin and soft tissue infections a targeted wound cleaning agent could be beneficial, particularly given that (+)-JQ1 and (-)-JQ1 showed antimicrobial activity against a highly resistant strain of *S. aureus*.

*S. aureus* is the most common pathogen isolated from skin-and-soft-tissue infections in the United States, 46 % of which were MRSA strains (Ray, Suaya and Baxter, 2013). The global mortality rate of skin and subcutaneous bacterial infections caused by *S. aureus* was 0.5 in 2019 (GBD 2019 Antimicrobial Resistance Collaborators, 2022). *S. aureus* skin infections can present in

various ways including folliculitis, boils, carbuncles, impetigo, cellulitis, mastitis and folliculitis (Linz *et al.*, 2023).

Typically these infections are treated systemically through the use of antibiotics but this contributes to the increase in antimicrobial resistance. The use of topical treatments such as the wound dressings and wound cleaning agents containing antimicrobials, could lead to slower progression of resistance as the antimicrobial is being directly targeted to the localised area of the wound site rather than in systemic treatments where the antimicrobial is spread throughout the body (Bandyopadhyay, 2021). The direct application of the antimicrobial to the target site also ensures high levels of bioavailability resulting in lower amounts of the antimicrobial being required. There are some disadvantages to topical use of antimicrobials including concerns around the superficial depth limitations, to insure this does not impact patient treatment antimicrobial wound dressings can be used in combination with systemic antibiotics if further infection is suspected.

Further screening work to determine if the antimicrobial properties of (+)-JQ1 and (-)-JQ1 are illustrated against any other common cutaneous infectious agents would be beneficial for furthering the antimicrobial applications of the compounds.

## 4.5. Epigenetic Changes Observed Post BET Inhibitor Treatment

Given that the antimicrobial action of (+)-JQ1 is most likely an off-target effect the exact mechanism of action of the compound is unknown. To elucidate the mechanism of action qRT-PCR was performed post (+)-JQ1 treatment to determine if any transcriptional changes occurred

in key genes. The genes chosen for this were *icaA, sasG, sigB, recA, clpP* and *norA* due to their key functions within various bacterial processes, with a focus on biofilm formation.

The intercellular adhesion (*ica*) operon, is comprised of four genes: *icaA, icaB, icaC* and *icaD*. This operon regulates staphylococcal biofilm formation and antibiotic susceptibility, with each gene carrying out a specific function (Mollaahmadi, Anzabi and Shayegh, 2021). One of the key extracellular polymeric components of staphylococcal biofilms is polysaccharide intercellular adhesin (PIA). PIA is a partially deacetylated form of poly-β-1,6-N-acetyl-d-glucosamine (PNAG), the synthesis of which is mediated by *icaB* (Arciola *et al.*, 2015). A strain of *S. aureus* with modified *icaB* was shown to produce significantly less PNAG, which rendered the strain susceptible to antibody-independent opsonic killing and decreased bacterial survival in a murine bacteraemia model (Cerca *et al.*, 2007). The prevalence of *icaB* genes varies between studies, one study found *icaB* genes to be present in 31 % of MRSA strains (Rawat *et al.*, 2022). While another study found that *icaB* genes were present in 53.3 % of general staphylococcal isolates (Abdel-Shafi *et al.*, 2022). It has been previously shown that biofilm formation can be prevented through the inhibition of the IcaB and IcaA proteins, and these proteins were identified as potential targets of the antibiotic linezolid (Bi, Deng and Liu, 2022). No changes were observed in the expression of *icaB* in *S. aureus* after exposure to sub-lethal concentrations of (+)-JQ1. This suggests that the inhibitory effect on biofilm formation observed after exposure to (+)-JQ1 is a result of targeting a key biofilm pathway that does not involve *icaB*. Since *icaB* is involved in the formation of the biofilm matrix, (+)-JQ1 could be targeting a pathway involved in cell adhesion or quorum-sensing.

One of the key genes mediating *S. aureus* adhesion is *sasG*, which encodes for the *S. aureus* surface protein G (SasG) (Corrigan *et al.*, 2007). Gene *sasG* mediates cell to cell adhesion during

the accumulation phase of biofilm formation (Formosa-Dague *et al.*, 2015). This process is dependent on zinc ions to enhance the rigidity of the cell wall, which activates the adhesive function of *sasG*, resulting in zinc-dependent homophilic bonds forming between cells. Strains of *S. aureus* expressing *sasG* exhibited biofilm formation, whereas strains negative for *sasG* did not form biofilms, with this process occurring independent of the *ica* operon (Corrigan *et al.*, 2007). The formation of biofilms is dependent on the level of *SasG* expression and the number of *SasG* repeats present in the strain. *S. aureus* has ten dominant clonal complexes, 50 % of these have the ability to express the *sasG* gene. Furthermore, 93 % of strains from these clonal complexes were found to express the gene. Following exposure to (+)-JQ1 no significant changes were observed in the expression of *sasG*. Similar to the lack of observed changes in expression of *icaB*, it can be suggested that (+)-JQ1 targets another biofilm pathway, potentially quorum-sensing or biofilm aggregation phase. There is still the potential that (+)-JQ1 does target the initial phase of adhesion as *S. aureus* has several other key proteins involved in the aggregation phase, including BAP (biofilm-associated protein) and fibronectin-binding proteins (Peng *et al.*, 2023).

Sigma factor B (*sigB*) is a stress response regulator which regulates the expression of over 200 genes in *S. aureus* (Guldimann *et al.*, 2016). The *sigB* gene acts alongside three other genes, *rsbU, rsbV* and *rsbW,* to form the *sigB* operon in *S. aureus* (Senn *et al.*, 2005). The activation of the *sigB* gene is dependent on another member of the *sigB* operon, rsbU (Giachino, Engelmann and Bischoff, 2001). The *sigB* gene is responsible for regulating various process including antibiotic resistance, virulence, and biofilm formation (Guldimann *et al.*, 2016). The presence of the *sigB* gene has been shown to confer resistance to oxacillin, vancomycin, methicillin and teicoplanin (Singh *et al.*, 2003). While the exact mechanism of this action is unknown, it is thought that *sigB*

regulates the expression of proteins that confer resistance to antibiotics that target the cell wall. *SigB* is also involved in the regulation of other virulence factors (Schulthess, Bloes and Berger-Bächi, 2012). EsxA is a protein which is secreted by the ESX secretion pathway (Ess) which acts as a transport module. The transcription of *esxA* is regulated by *sigB*. Another key function of *sigB* is its role in biofilm formation, where *S. aureus* strains with a *sigB* deletion mutation are unable to form biofilms (Lauderdale *et al.*, 2009). No significant change was observed in the expression of *sigB* after (+)-JQ1 exposure. This suggests that the mechanism of action of (+)-JQ1 does not activate a broad stress response pathway, adding to the hypothesis that (+)-JQ1 specifically targets another aspect of biofilm formation.

The *recA* gene is involved in the response to bacterial DNA damage and mediating repair mechanisms (Kiran and Patil, 2022). In *S. aureus*, *recA* binds to and invades ssDNA. *recA* exhibits ATPase activity to initiate the synthesis of the nucleoprotein filament, which is essential for the strand chain reaction. The strand chain reaction results in the formation of the displacement loop, a key stage of the homologous recombination DNA repair mechanism. This response mechanism, termed the SOS response, is triggered after exposure to traditional bactericidal antibiotics targeting three of the key antimicrobial targets: DNA replication, protein synthesis and cell wall maintenance (Kohanski *et al.*, 2007). It has been suggested that targeting *recA* in combination therapy with traditional antimicrobials could prevent DNA repair and therefore counteract antimicrobial resistance (Kiran and Patil, 2023). Gallic acid inhibits the formation of the RecA protein DNA complex, in turn preventing the ATPase activity, strand chain reaction and formation of the development loop. No significant change was observed in the expression of *recA* post (+)-JQ1 treatment. This suggests that (+)-JQ1 does not cause significant damage to the DNA of *S.*

*aureus* or activate the SOS response pathway. Once again supporting the conclusion that (+)-JQ1 directly targets a later aspect of biofilm formation.

Another key virulence factor regulator is the Clp protease which identifies and degrades misfolded proteins (Ju *et al.*, 2021). The genes *clpP* and *clpX* mediate the production of *S. aureus* virulence factors through the agr/sar regulatory network. Mutant *clpP* strains of *S. aureus* were shown to reduce abscess formation in a murine model and rendered the cells sensitive to misfolded proteins, showing the importance of the *clpP* gene in bacterial growth and stress tolerance (Frees *et al.*, 2003). Gene *clpP* plays a direct role in AMR as it is also involved in the regulation of toxin–antitoxin (TA) modules, which are crucial to the formation of persister cells. Mutant *clpP* strains of *S. aureus* treated with oxacillin and erythromycin were shown to have lower levels of persister cells compared to wild-type strains (Springer *et al.*, 2016). Due to the role of the *clpP* gene in virulence it makes a promising therapeutic target for antimicrobials. The β-lactones cystargolide A and B act as a ClpP inhibitors preventing their virulence action (Illigmann *et al.*, 2023). The expression of *clpP* was unchanged by (+)-JQ1 treatment, suggesting that (+)-JQ1 does not cause protein misfolding or stress. This supports the suggestion based on the lack of change in *recA* expression that (+)-JQ1 does not evoke a global stress mechanism and instead has a more targeted approach.

The *norA* gene encodes for NorA efflux system which acts against a wide variety of structurally and functionally different drugs (Deng *et al.*, 2012). Expression of *norA* is regulated by the MgrA transcription regulator and the ArlRS two-component system in an iron dependent system. Due to the important role of multi-drug efflux pumps on the development of AMR, the NorA efflux pump is a leading therapeutic target. Vitamin K3 has been shown to inhibit the NorA efflux pump

in a dual action, directly interacting with the NorA and also indirectly inhibiting the *norA* gene (Tintino *et al.*, 2020). A similar dual mechanism is observed with the use of tannic acid, which acted both directly on the NorA protein and also inhibited the *norA* gene through inhibition of the ArlAS pathway (Tintino *et al.*, 2023). A nanocomposite, NiFe2 O4 @Ag, exhibited inhibitory action the *norA* gene and when used in combination with ciprofloxacin resulted in increased levels of *S. aureus* bacterial growth inhibition (Pourmehdi *et al.*, 2020). The expression of *norA* was upregulated after (+)-JQ1 exposure in *S. aureus* which showed that the NorA efflux pump was activated and *S. aureus* recognised (+)-JQ1 as requiring export from the cell. This also suggested that (+)-JQ1 was acting on a target inside the bacterial cell. Given that *norA* was the only gene upregulated after (+)-JQ1 exposure it can be suggested that (+)-JQ1 targets an intracellular aspect of biofilm formation such as quorum sensing or metabolic processes. Whilst the expression of the *norA* gene does not directly influence the antimicrobial activity of (+)-JQ1, this result suggests that (+)-JQ1 acts intracellularly providing a foundation for future work to elucidate the mechanism of action.

## 4.6. Future Work

The future directions of this project would include furthering the cytotoxicity work to ascertain the cytotoxic effects specifically associated with the use (+)-JQ1 as an antimicrobial. Given that the findings of the mammalian cell culture research found significant levels of cytotoxicity, it would be beneficial to also assess other human cell lines for cytotoxicity. Given the potential applications for using (+)-JQ1 as a topical agent, human skin cells would be ideal for continued

work. Further investigations into cytotoxicity could include *in vivo* work using *Galleria mellonella* larvae and wound modelling using cell culture scratch assays.

To elucidate the exact mechanism of antimicrobial activity of (+)-JQ1 against *S. aureus* pull down assays and affinity binding assays could be used to determine the interactions between proteins of interest identified from RNA-sequencing experiments. These assays could be supported by tagging assays, including fluorescent tagging, and cross-linking assays.

The similarity in MIC values between (+)-JQ1 and (-)-JQ1 indicate that the active site utilised during antimicrobial applications of the compound is not the same active site utilised in its action as a BET inhibitor. Future work could involve modification of the compound or the synthesis of novel similar compounds with the BET inhibitor active site inactivated. This could potentially reduce cytotoxicity occurring due to the BET inhibitory action of (+)-JQ1.

Identifying changes in quorum sensing gene expression in response to (+)-JQ1 by RNA-sequencing analysis would be beneficial in determine the anti-virulence role of this compound. Report gene assays could be used to identify the extent of any quorum sensing inhibition.

The gene expression research performed in this study could be expanded to look at lower concentrations of (+)-JQ1 and their impact on specific genes of interest and by expanding the range of genes examined by qRT-PCR.

To expand the findings further other resistant strains of *S. aureus* and similar bacteria commonly found on the skin could also be screened for potential antimicrobial effects of (+)-JQ1.

It would also be of interest to continue the described assays with (-)-JQ1 also to confirm the off-target effect. It would also be beneficial to continue to look at additional potential off-target effects of (+)-JQ1, confirmed by the use of (-)-JQ1. Any potential off-target effects are important considerations as (+)-JQ1 continues to be developed and adapted for clinical use.

Given the rapid development of antimicrobial resistance and the ongoing development of resistance to other antibiotic alternatives, there is the possibility for the development of resistance to SMIs, such as BET inhibitors. To explore the potential development of resistance by *S. aureus* to (+)-JQ1, passage experiments could be utilised to determine changes in *S. aureus* over several replications after exposure to (+)-JQ1.

## 5.0 Conclusion

To conclude this study identified a novel off-target antimicrobial effect of the lead candidate BET inhibitor (+)-JQ1 and the enantiomer (-)-JQ1 against *S. aureus* strain USA300. The mechanism by which (+)-JQ1 exerts antimicrobial activity is yet to be elucidated, but it is likely that this process directly targets an intracellular biofilm mechanism. Further work is necessary to identify the antimicrobial target site and the active site of (+)-JQ1.

BET inhibitors have been shown to exhibit activity against an array of conditions, including cancer, inflammatory disorders and neurodegenerative disorders. Specific BET inhibitors have been synthesised as antifungal agents for use against fungal BET proteins. However, this study is the first instance in which a BET inhibitor has demonstrated activity against prokaryotes.

While off-target effects of (+)-JQ1 have been previously identified, the extent of these effects and their potential impact on the clinical use of (+)-JQ1 is unknown. Ongoing research into the use of (+)-JQ1 should include considerations of potential off-target effects and further work could be undertaken to identify further unknown off-target applications.

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