Novel ruthenium metal-based complexes as antimicrobial agents

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List of Abbreviations

ABC	Adenosine triphosphate binding cassette
AMR	Antimicrobial resistance
AMS	Antimicrobial stewardship
ATP	Adenosine triphosphate
BEN	Benzene
BIP	Biphenyl
CC5	Clonal complex 5
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
CDS	CoDing Sequence
CFU	Colony-Forming Unit
CLSI	Clinical and Laboratory Standards Institute
CT-DNA	Calf thymus deoxyribonucleic acid
CYM	P-cymene
DCFDA	2′,7′-Dichlorofluorescin diacetate
DHA	Dihydroanthracene
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EMEM	Eagle's Minimum Essential Medium
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FBS	Faetal bovine serum
FDA	Food and drug administration
HGT	Horizontal Gene Transfer
IC 50	Inhibitory concentration 50 %
IDSA	Infectious Diseases Society of America
kDa	Kilo dalton
MATE	Multidrug and toxic compound extrusion
MBC	Minimum bactericidal concentration
MDR	Multidrug resistant
MFS	Major facilitator superfamily
mg	Milligram
µg	Microgram
MHRA	Medicines and Healthcare products Regulatory Agency
MIC	Minimal inhibitory concentration
ml	Millilitre
µl	Microlitre
µM	Micromolar
MRSA	Methicillin-resistant Staphylococcus aureus
MSC	Minimum Selective Concentration

NPN	1-N-phenylnapthylamine
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
QAC	Quaternary Amine Compound
QAS	Quaternary Amine Salt
RCF	Relative Centrifugal Force
RNA	Ribonucleic acid
RND	Resistance-nodulation-division
RT-qPCR	Reverse Transcriptase quantitative PCR
SEM	Scanning electron microscope
SNP	Single Nucleotide Polymorphisms
THA	Tetrahydroanthracene
UTI	Urinary Tract Infection
VRSA	Vancomycin-resistant Staphylococcus aureus

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Abstract

Antimicrobial resistance (AMR) is becoming increasingly prevalent amongst clinically significant bacteria. The World Health Organization (WHO) has declared AMR as one of the greatest public health threats facing humanity. There has been a sharp decline in the number of new clinically approved antibiotics, with most new antibiotics being based on pre-existing antibiotic scaffolds. As a result, there is an urgent need for new novel ways to treat infections caused by AMR bacteria. There has been an increased focus on Ruthenium (Ru) complexes acting as antimicrobial agents. This is in part due to their biological compatibility, multiple oxidation states and bonding configuration allowing for specific geometries that are ideally suited for biological applications.

This study evaluated the antimicrobial activity of 12 repurposed Ru complexes. Preliminary screening against a diverse selection of clinically significant bacteria identified Ru complexes 1 ($C_{22}H_{23}Cl_3N_2SRu$) and 7 ($[Ru(NH_3)_6]Cl_3$) as potential lead candidates with the Ru complexes acted as bactericidal agents against *S. aureus* USA300 JE2 and *P. aeruginosa* PAO1 respectively. Eukaryotic cytotoxicity testing against HeLa and HEK 293T cell lines demonstrated Ru complex 7 exhibited no significant cytotoxic effects against both cell lines ($p>0.05$), whilst Ru complex 1 was significantly cytotoxic ($p<0.05$). *S. aureus* USA300 JE2 and *E. coli* EC958 were able to tolerate an 11-fold increase in MIC after long term incrementally increasing concentrations of Ru complex 1. Comparative genome analysis of *S. aureus* USA300 JE2 showed long term exposure to Ru complex 1 increased the rate of mutagenesis and led to 17 de novo mutations being identified within eight genes. Furthermore, significant gene expression changes in *clpP*, *katA* and *norA* were reported in *S. aureus* USA300 JE2 after exposure to Ru complex 1, indicating Ru complex 1 affected a wide array of cellular functions.

The mechanisms of action for antimicrobials Ru complex 1 and 7 were investigated. Ru complex 1 displayed no significant outer membrane or inner membrane permeabilising effects, whilst Ru complex 7 caused no significant outer membrane permeabilising but did stimulate elevated depolarisation of the inner membrane in a number of bacterial species. Scanning electron microscopy confirm that both complexes appeared not to be directly targeting the outer membrane as no cellular morphological changes were observed. Cellular metal uptake studies using Ru complexes 1 and 7 showed elevated intracellular concentrations in *S. aureus* USA300 JE2 and *P. aeruginosa* PAO1 respectively compared to the exposure concentrations. Electrophoretic mobility shift assays (EMSA) and competitive binding assays showed that intracellular concentrations of Ru complexes 1 and 7 had a significant impact on DNA mobility and displacement of SYTO 9 from the SYTO 9/DNA complex. Exposure to Ru complexes 1 and 7 caused elevated but not significant levels of reactive oxygen species generation (ROS) in *S. aureus* USA300 JE2, *P. aeruginosa* PAO1 and *E. coli* EC958

The results of the thesis demonstrate the potential to use mononuclear Ru complexes as antimicrobial agents. Notably, the potent antimicrobial activity of Ru complex 7 against *P. aeruginosa* PAO1, coupled with low levels of cytotoxicity make this an ideal candidate for further *in vivo* investigation.

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Chapter 1: Introduction

Modern antibiotics are ranked amongst the greatest discoveries in history. Antimicrobials have been used throughout human history to varying degrees, but it wasn't until Alexander Fleming discovered penicillin in 1928 the modern era of antibiotics began (Aminov, 2010). Since then, antibiotics have saved the lives of countless millions, enabled challenging operations to become routine, transformed agriculture and livestock farming, altered the way water treatment was undertook and are used routinely during veterinary treatment of domestic animals (Meek *et al*., 2015). Due to the resilience and adaptability of bacteria, the success of antibiotics ironically has become their downfall (Ventola, 2015).

1.1 The Impact of Antimicrobial Resistance in a Clinical Environment

The term antibiotic was first defined in 1947 by Selman A. Waksman, it was defined as natural chemical substance, produced by microorganisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other microorganisms (Waksman, 1947). Since their discovery, antibiotics have been routinely used in clinical environments. Antibiotics have been used in the treatment of a wide range of conditions including urinary tract infections (UTI), streptococci throat, skin infections and pneumonia. In addition, antibiotics are routinely used as a preventative measure against infection (Richman *et al*., 1999). Antibiotics are prescribed in post operation care and during post-chemotherapy care where immunocompromised patients are most vulnerable (Tamma *et al*., 2019). With an ever-increasing demand for antibiotics and the increasing misuse in agriculture, inappropriately prescribed patient treatment, and poor infection control in health care settings, resistance to antibiotics is becoming more prevalent amongst clinically significant bacteria (Ventola, 2015).

Antimicrobial resistance (AMR) can simplistically be defined when a microorganism is no longer sensitive to a drug that it was previously sensitive to (MacGowan., 2008). The issue of

antimicrobial resistance is no longer seen as a regional problem but a global problem. The World Health Organization (WHO) and the Infectious Diseases Society of America (IDSA) have both identified antimicrobial resistance as one of the greatest threats to human health globally (WHO, 2014; Spellberg, 2010). As of 2007, Methicillin-resistant *Staphylococcus aureus* (MRSA) was estimated to kill more people annually in America than Parkinson's disease, HIV/AIDS, emphysema, and murder combined with an estimated total of around 19,000 fatalities (Che *et al*., 2019). In addition, data shows that the number of deaths associated with AMR is increasing annually (Che *c*).

There are wide variety of risk factors associated with acquiring an AMR bacterial infection. Many of these factors are emphasised by the location and in a clinical environment, factors can include; hospitalisation for more than 2 days in the preceding 90 days, residency in a nursing home or extended-care facility, immunosuppression, having an invasive medical device and previous antibiotic treatment (Bennett *et al*., 2018, Song *et al*., 2019). Patients who contract AMR bacterial strains show significantly worse patient outcomes than those who contract non-resistant strains, thus patients' lives and quality of life are being put at risk. In a recent study of patients who contracted MRSA infections, 64% had an increased morality rate over patients who contracted a non-MRSA strain (World Health Organization, 2014). According to a recent report by the WHO, in excess of 700,000 die annually worldwide due to drug resistant strains of bacteria, with 25,000 of those cases within the European Union (Tagliabue, *et al*., 2018). Research by Olchanski *et al.,* (2011) demonstrated that contracting an MRSA infection increased the duration of a hospital stay by 9 days and increases in treatment costs rose by an estimated $27,000 in comparison to a non-methicillin-resistant strain within the US (Olchanski *et al*., 2011).

It is calculated that within the United Kingdom the five-leading drug resistant bacterial species add over £1 billion to hospital treatment and societal costs annually, these species are;

Escherichia coli, *Klebsiella pneumoniae*, *Enterococcus faecium*, *Pseudomonas aeruginosa* and MRSA (Public Health England, 2015). Within Europe the current annual estimated cost of AMR is nine billion euros per year while in the USA, it is estimated to lose around $35 billion in productivity annually (Dadgostar, 2019). When viewing AMR as a global problem, it is estimated by 2050, 10 million deaths annually will be attributed to AMR at an estimated cost of $100 trillion (Neill, 2014). Antimicrobial stewardship (AMS) has proven to be an effective tool in improving patient outcomes as well as reducing the financial burden associated with AMR. In 2016 Naylor *et al*. performed a comprehensive study on the effectiveness of antimicrobial stewardship. Key highlights on two studies showed that hospitals in Germany with AMS saved €2575 per month and AMS could potentially save $2.5 billion for Medicare in America. Four of six studies estimated savings of $415 savings per patient in North America. The average cost per averted death due to AMR was an estimated $19,287.54 (Naylor, *et al*., 2016). In 2017 the WHO created a priority pathogen list. The list highlights pathogens of highest clinical concern due to their AMR the list also considers those that potentially pose a threat in the near future (Table; 1. 1) A monumental effort will be needed to tackle the challenges of AMR using a combination of more frugal use of antibiotics, development of new antimicrobials and increased AMS. The implementation of all these factors could reduce the overall burden of AMR by lowering mortality rates, improving patient outcomes and reducing the economic burden thus allowing funding to be spent in other areas.

Table 1. 1: World health organisation priority pathogen list (2017).

Category	Species	Type of Antibiotic Resistance
Priority 1: CRITICAL	*Pseudomonas aeruginosa*	Carbapenem-resistant
	Acinetobacter baumannii	Carbapenem-resistant
	Enterobacteriaceae spp	Carbapenem-resistant, ESBL-producing
Priority 2: HIGH	*Enterococcus faecium*	Vancomycin-resistant
	Staphylococcus aureus	Methicillin-resistant, Vancomycin-intermediate and resistant
	Helicobacter pylori	Clarithromycin-resistant
	Campylobacter spp	Fluoroquinolone-resistant
	Salmonellae spp	Fluoroquinolone-resistant
	Neisseria gonorrhoeae	Cephalosporin-resistant, Fluoroquinolone-resistant
Priority 3: MEDIUM	*Streptococcus pneumoniae*	Penicillin-non-susceptible
	Haemophilus influenzae	Ampicillin-resistant
	Shigella spp	Fluoroquinolone-resistant

1.2 The Development and Escalation of Antimicrobial Resistance

Antimicrobial resistance amongst clinically significant microorganisms is increasing at an astounding rate, the primary cause of the increase in AMR has been shown to be environmental selection pressure within clinical environments (Vali *et al*., 2004, Fletcher, 2015). There are no restrictions to the development of AMR, it can vary between species, strains and geographical regions. Figure 1.1 demonstrates the rapid evolution of bacteria and the development of AMR and illustrates the year of discovery of a variety of commonly used antibiotics and when the first recorded account of resistance to a particular antibiotic took place.

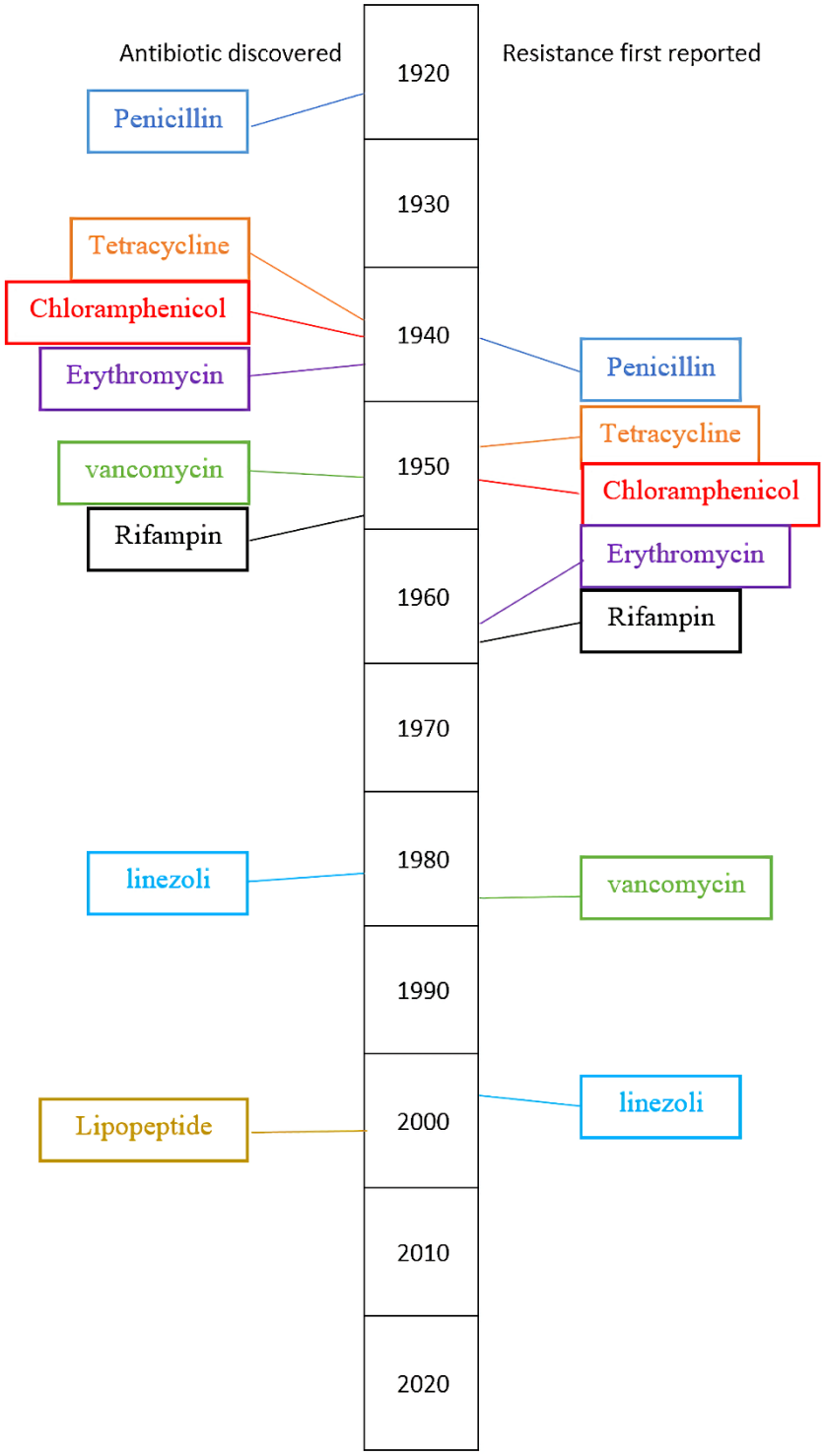


Figure 1. 1: The discovery of new antibiotics and the first reported resistance to those antibiotics.

Many factors can be involved in eliciting environmental pressure on bacteria and these factors can act individually or simultaneously, thus promoting the development of AMR in clinically significant bacteria. The general misuse of antibiotics is one of the largest and leading factors in the development of antibiotic resistance.

The use of antimicrobials in agriculture is extensive, especially as growth promoters. It is widely believed the first use of antibiotics as growth promoters in agriculture was the feeding fermentation offal from the production of chlortetracycline to chickens. This was originally used as a cost cutting measure but produced surprising effects on the growth of the chickens involved (Jukes *et al*., 1953). Since then, other antibiotics such as: streptogramin, bambermycin and efrotomycin have been routinely used in livestock farming to quickly increase mass and reduce infant mortality rates. In 1969, Great Britain took steps by banning the use of antibiotics that may cause cross-resistance with human pathogens (Hao *et al*., 2014). The use of many more antibiotics in agriculture has been banned in Europe since 2006, with a total ban on the use of growth promoters (Castanon, 2007). Other measures within the European union have also been put in place to lower the chance of cross-resistance occurring. The United States of America have only recently introduced these measures on the 1st of January 2017, but the larger problem is within the developing world where little regulation is in place (Wu *et al.*, 2017; Markowiak *et al*., 2018). Plant agriculture is an area that is often overlooked, a by-product of livestock farming is manure, the manure often contains antibiotics and is then used as fertiliser putting low level selection pressure on environmental bacteria (Vidaver, 2002).

The incorrect prescribing and non-completion of antibiotic courses prescribed to patients is another leading factor in the health care environment that has recently been highlighted in the media, and this has become the focus of intensive research, since it is a major concern. In a recent study in the UK, it was shown between 8.8% and 23.1% of all antibiotic prescriptions were incorrectly prescribed (Smieszek *et al*., 2018). These errors in antibiotic prescribing can

be errors related to wrong dose, incorrect frequency in taking the medicine, and duplicate therapy or simply the wrong antibiotic being prescribed (Shrestha *et al*., 2019). This situation is even worse in many other countries where antibiotics can be purchased over the counter, cheaply and without a prescription. Due to poor education around the subject, this often leads to antibiotic misuse (Alhomoud *et al*., 2018). These types of misuse put low level natural selection pressures on the bacteria they are targeting. This is especially noticeable for broad spectrum antibiotics, since low level natural selection pressures allows bacteria to withstand low doses of antibiotics and over time selection pressures allow an individual strain of bacteria to become totally resistance to a particular form of antibiotic (Hughes, 2014). In 2013, the UK set out it's five-year antimicrobial resistance strategy the aim of the strategy was to improve the knowledge and understanding of AMR, conserve and steward the effectiveness of existing treatments and stimulate the development of new antibiotics, diagnostics and novel therapies (Department of Health, 2013). The strategy has shown a reduction of 4.5 percent in defined daily dose of antibiotics from 2013-2017 but mortality rates have continued to increase due to the spread of AMR (NICE, 2018). Modern affordable worldwide travel allows resistant strains to spread globally without time for quarantining. This was demonstrated recently when a new AMR strain of *K. pneumoniae* was discovered in the United States. Upon investigation it was found to be from a patient who had contracted it from a recent visit to India, and the travel of it into the US potentially put other patients lives at risk (Chen *et al*., 2017). In an effort to tackle AMR in 2015, the UK government requested a £1.3 billion commitment from drug manufacturers to development of new antibiotics (Plackett, 2020)

All clinically significant species of bacteria are now showing AMR to at least a one antibiotic while in excess of 70 percent of bacterial strains are showing multidrug resistant (MDR) (Engemann *et al*., 2003). These include certain strains of *S. aureus*, in particular the USA300 strain that has shown MDR rates as high as 46% and more recently *S. aureus* USA 1100 has

also become resistant to vancomycin, as well as methicillin and are known as vancomycin-resistant *S. aureus* (VRSA) (Lee *et al*., 2017). The significance of this is profound due to vancomycin often used as a last resort antibiotic in *S. aureus* infections due to its potent side effects (Sass *et al*., 2012). Originally these VRSA strains carried the vancomycin resistance *vanA* gene. Since the original discovery of the *vanA* gene, five other variants of vancomycin resistance have been characterized *vanB*, *C*, *D*, *E*, and *G* showing that there is an ever-evolving battle against antibiotic resistance (Courvalin, 2006). Rapid detection of AMR strains in patients is vital, advancements in many fields including Biosensor Systems, Lab-on-a-chip systems and MALDI-TOF mass spectrometer all producing rapid viable systems. The rapid detection of AMR strains will help to reduce wrongly prescribed antibiotics (Vasala *et al*., 2020)

1.2.1 Intrinsic, Adaptive and Acquired AMR Mechanisms

Firstly, it is important to highlight the differences between resistance and persistence against an antimicrobial agent. Persistence is generally considered to be the ability of bacteria to enter a dormant phase by which a cell is not actively replicating or metabolising, thus the antimicrobial agent has little or no effect on the cell. However, true resistance occurs when cells undergo binary fission when in contact with the antimicrobial (Brauner *et al*., 2016).

AMR can be divided into three main categories: intrinsic, adaptive and acquired resistance. All three types of AMR employ fundamental mechanisms including regulation at the molecular level, and recent research demonstrated that the development of resistance is often associated with multiple mechanisms (Olivares *et al*., 2013). Bacteria can use a plethora of defence mechanisms to resist or inactivate antibiotics which may include genetic modifications, changes in gene regulation or cell structural changes (Table 1. 2).

Whilst many Ru complexes are known to target the same basic cellular components that traditional antibiotics and antimicrobials target (Munteanu *et al*., 2021). The effects of intrinsic, adaptive and acquired mechanisms on Ru complexes are largely unknown due to the limited work conducted in the field. It is speculated that the (3D) shapes of Ru complexes are more compatible with organic structures and allow for targeting of multiple sites. This could allow a lower rate of resistance development and evasion of innate and adaptive mechanisms compared to tradition antibiotics that are composed of organic fragments have linear (1D) or planar (2D) shapes (Munteanu *et al*., 2021).

Table 1. 2: Mechanisms of horizontal gene transfer and direct changes to cellular DNA (Sultan *et al*., 2018).

Mechanism	Description
Conjugation	The direct transfer of genetic material from one bacterium to another via cell-to-cell contact or using a pilus.
Transduction	The passing of genetic material via a virus or vector.
Transformation	The uptake and incorporation of genetic material form the surrounding environment
Mutation	A permanent alteration of genetic material as a result of factors including environmental factors, repair and copy errors.

The genetic alterations and changes to gene regulation can cause advantageous or deleterious modifications to the cell (Figure 1. 2) including: structural alterations to the cell (thicker cell membrane, reduced LPS), increase expression of efflux pumps, overproduction of the target enzyme, modification of the antibiotic or proteins**,** Expression of alternative proteins and bypassing of pathways inhibited by drugs (Woodford *et al*., 2007)**.** Advantageous adaptations are more likely to be passed to subsequent generations causing proliferation of resistance (Depardieu *et al*., 2007; Munita *et al*., 2016). Acquired resistance is the greatest concern in a clinical environment, due to the ability of mobile genetic elements to propagate to other pathogens (Larsson *et al*., 2021).

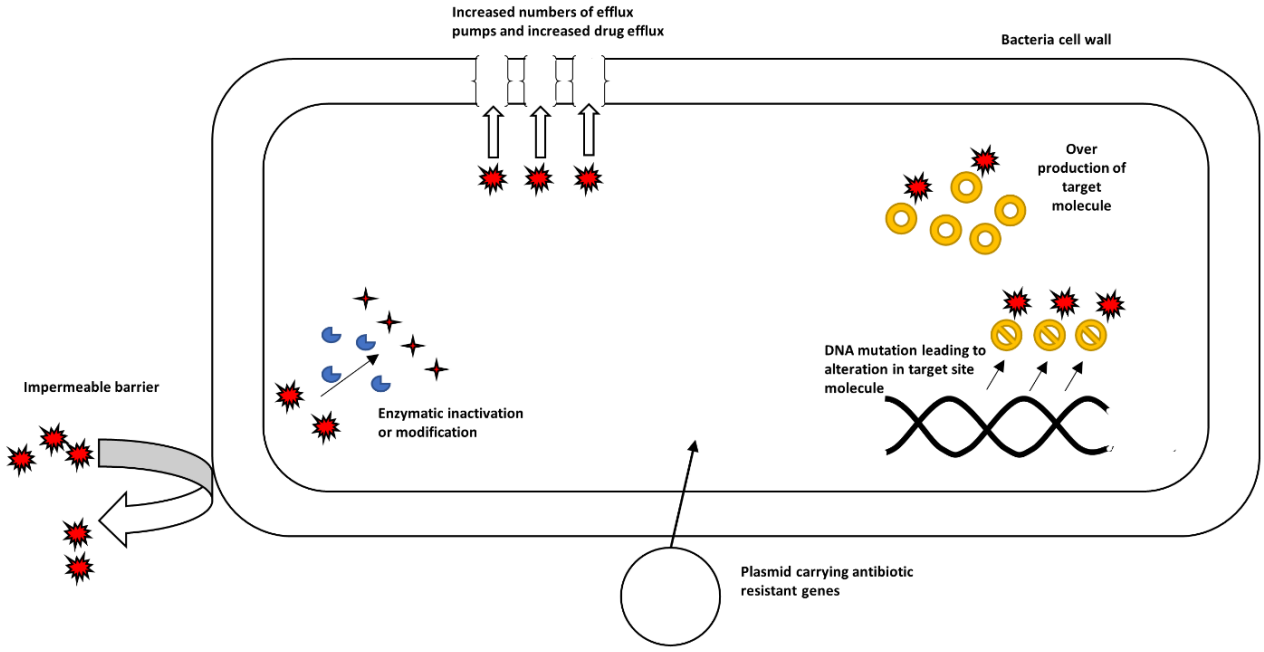


Figure 1. 2: Strategies used by bacteria to resist the effects of antibiotics.

1.2.1.1 Bacterial cell ultrastructure

1.2.1.1.1 Gram-positive Bacterial Cell Envelope

The cell envelope of Gram-positive bacteria is composed of unique components not found elsewhere in nature, which makes them ideal targets for antibiotic therapy (Rajagopal *et al*., 2017). The Gram-positive cell envelope has two distinct layers, the outer peptidoglycan layer known as the cell wall and the inner cytoplasmic membrane. The peptidoglycan layer is composed of repeating interconnecting layers of sugars in the form of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) and amino acids in the form of L-alanine, D-glutamine, L-lysine or *meso*-diaminopimelic acid (DPA), and D-alanine (Lovering *et al*., 2012). Chains of 4 to 5 amino acids combine to form tetrapeptides which bind to the NAM unit allowing the cross linking of NAG and NAM. Interlaced between the peptidoglycan layers are

teichoic acids and lipoteichoic acids, which assist with providing structural integrity in addition to other roles. Lipoteichoic acids also binds the peptidoglycan layer to the cytoplasmic membrane and acts as an adhesion molecule when binding to cells (Ginsburg, 2002). Teichoic acids bind to phosphate groups within the membrane causing an increase in the overall negative charge of the membrane, which helps mediate resistance to antimicrobial peptides and enhances adhesion to surfaces (Drayton *et al*., 2021). In Gram-positive bacteria, the peptidoglycan layer is much thicker than Gram-negative bacteria and accounts for between 60 to 90% of the cell envelope structure. Thicker cell walls and highly cross-linked peptidoglycan plays an important role in mediating resistance to cell lysis (Rice, 2008). The Gram-positive wall is highly permeable, wildtype Gram positive bacteria normally does not restrict penetration of antimicrobial compounds. The upper molecular weight for compound penetration is estimated to be around 50000 Da although this can be structurally dependent (Lambert, 2002).

1.2.1.1.2 Gram-negative Bacterial Cell Envelope

Gram-negative bacteria have intrinsic resistance to many antibiotics due to the structure and composition of the outer membrane (Lee, 2007; Miller, 2016; Cillóniz, 2019). The Gram-negative bacterial envelopes consist of three distinct layers, each with different physical properties (Silhavy *et al*., 2010). Firstly, the inner layer known as the plasma or cytoplasmic membrane, is composed of a phospholipid bilayer that contains outer membrane proteins (OMP). The outer membrane proteins perform a diverse range of functions including; nutrient uptake, cell adhesion, cell signalling and waste export (Rollauer *et al*., 2015). The second layer is a central area known as the periplasm that contains the cross-linked cell wall composed of peptidoglycan and stress sensors that are responsible for maintaining cell integrity under

osmotic stress (Benz, 1988). Thirdly, the outer membrane is an asymmetric bilayer that contains β-barrel proteins known as porins, phospholipids in the form of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin on the inner leaflet and protrusions of lipopolysaccharides (LPS) or lipooligosaccharides (LOS) and porins in the outer leaflet. The LPS is composed of three domains, the outer O-antigen region, central region and lipid A. The O-antigen is derived from repeating hydrophilic O-antigenic oligosaccharide side chain. LPS attaches long oligosaccharides to glycolipids, whereas LOS attaches shorter oligosaccharides (Nikaido, 2003). The central region contains core oligosaccharide which is broken down into an inner and outer core. Thirdly, the lipid A section anchors the entire LPS structure to the outer membrane via non-covalent association between lipid A and phosphate groups creating a negatively charged hydrophobic environment that repulses hydrophobic molecules and Polar molecules (Auer *et al*., 2017). Divalent cations Mg^{2+} and Ca^{2+} helps to electrostatically stabilise the outer membrane by intermolecular bridging interactions linking adjacent negatively charged phosphate groups on the LPS molecules creating a tightly packed LPS outer leaflet (Li *et al*., 2017).

The hydrophobic outer membrane in combination with porins allows selectivity of molecules entering the cell. The porins in the outer membrane resists penetration of hydrophilic molecules greater than 600 Da preventing larger antimicrobials including vancomycin (1448 Da), chlorobiphenyl vancomycin (1649.9 Da), and erythromycin (734.94 Da) entering the cell (Choi, 2019). The outer membrane is also able to exclude toxic molecules from the cell, including macrolides, bile salts, lysozyme and detergents (Ibrahim *et al*., 1991; Ofek *et al*., 1994; Masschalck *et al*., 2001). Small hydrophilic and amphiphilic antibiotics use porins to enter the periplasm. Aminoglycosides such as gentamicin and kanamycin are able permeate the outer membrane thus remaining effective against Gram-negative bacteria (Krause *et al*., 2016).

1.2.1.2 Intrinsic Resistance

Intrinsic resistance is the innate structure or functional characteristics of a bacterial cell that confers resistance to antimicrobial agents and is derived from environmental factors that have contributed to a complex network of genetic loci that form the intrinsic resistance (Cox *et al*., 2013). Intrinsic resistance is not acquired via horizontal gene transfer (HGT) and is formed independently of the antimicrobial that is of clinical concern (Cox *et al*., 2013). Although it has been demonstrated that genes associated with intrinsic resistance could be the origin of some acquired resistance (Zhang *et al*., 2016). Intrinsic resistance can be presented in many forms including: membrane structure, the efflux of toxic substances and the lack of target site for the antibiotic (Peterson *et al*., 2018).

Bacteria can be intrinsically resistant not only a single antibiotic class but multiple antibiotic classes (Reygaert., 2018). The cell wall of *Enterococcus. faecalis* reduces penetration of aminoglycoside antibiotics into the cell, allowing *E. faecalis* to be intrinsically resistant to clinically safe levels of aminoglycosides. *E. faecalis* has also displayed high levels of resistance to streptogramin A, due to efflux pump activity (Singh *et al*., 2002; Hollenbeck *et al*., 2012). The genus *Mycoplasma* lack a cell wall thus are intrinsically resistant to antimicrobials that target the cell wall such as *β*-lactams and glycopeptides (Chernova *et al*., 2016). *Providencia* spp. have an intrinsic resistance to many antibiotics including carbapenem, ampicillin, cefuroxime, tetracycline and penicillin G (McHale *et al*., 1981). These intrinsic resistances are of major concern in a clinical environment due to the possibility of resistance genes passing to clinically significant species via transformation and conjugation (Abdallah *et al*., 2018). Smitten *et al* (2019) demonstrated that dinuclear ruthenium(II) complexes exhibit greater antimicrobial activity against Gram negative bacteria and postulated the dinuclear

ruthenium(II) complexes were binding to the teichoic acids residues of the cell wall, reducing internalization (Smitten *et al.,* 2019)

Collectively Gram-negative bacteria have been shown to possess a higher level of intrinsic resistance in comparison to Gram-positive bacteria (Impey *et al*., 2020). This higher level of resistance is primarily due their outer membrane permeability barrier and the increased number and variety of efflux pumps, varying on a species-by-species basis. Sections 1.2.1.1.1 and 1.2.1.1.2 describes some of the differences between the Gram-positive and Gram-negative membranes and how the differences contribute to intrinsic resistance.

All living cells contain proteinaceous transporters known as efflux pumps on their outer membranes and efflux pumps have proven to be an invaluable survival mechanism for bacteria (Sharma *et al*., 2019). Efflux pumps remove toxic substances such as antibiotics and heavy metals from within the cells via active transport, and this action has been shown to contribute to a rise in antibiotic resistance (Ebbensgaard *et al*., 2020). Efflux pumps also play an important role in intrinsic, adaptive and acquired resistance (Blanco *et al*., 2016). There are five major families of efflux transporters; adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS), and the multidrug and toxic compound extrusion (MATE) family. The majority of the RND efflux pumps are found exclusively in Gram-negative bacteria (Blanco *et al*., 2016). These possess another major mechanism of resistance which involves their two-membrane cell envelopes that provides a low permeability barrier thus preventing many antibiotics entering the cell (Zgurskaya *et al*., 2015). The effluxion of toxic substances can work synergistically with innate structures such as the outer membrane on Gram-negative bacteria, which correlates with resistance to ß-lactam antibiotics. *P. aeruginosa* is one of the most resistant and adaptable pathogens and efflux pumps have been key to adaptability. This is demonstrated with their high intrinsic resistance to β-lactam and

fluoroquinolone antibiotics which is in part due to MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY/OprM efflux pumps and the outer membrane (Dreier *et al*., 2015). These efflux pumps combined with the low permeability of the outer membrane and the production of ß-lactamases render β-lactam antibiotics ineffective. *P. aeruginosa* also demonstrates natural resistance to imipenem due to the lack of D2 porin in the outer membrane (Ochs *et al*., 1999, Okamoto *et al*., 2001). The AcrAB-TolC efflux pumps expressed by *E. coli* contribute towards intrinsic resistance to tetracyclines, fluoroquinolones, ß-lactams and macrolide antibiotics (Chollet *et al*., 2004; Chetri *et al*., 2019). Efflux pumps can further contribute to antibiotic resistance due to their inability to efflux the antibiotic entirely. This leaves a diminished active concentration of antibiotics within the bacterial cell and can allow for natural resistance to occur via DNA mutations (Ebbensgaard *et al*., 2020).

The use of β-lactam antibiotics is extensive, however, this also correlates with resistance generation (Essack., 2001). β-lactamase enzymes are naturally produced by many Gram-positive and Gram-negative species and can be separated into two distinct groups depending on their method of hydrolysis of β-lactam antibiotics. One chemical process forms an acyl enzyme with an active-site serine and the other is facilitated by one or two essential zinc ions in the active site of metallo-β-lactamases rendering the β-lactam antibiotic inactivated ((Bush *et al*., 2009). Species such as *Mycobacterium tuberculosis* have shown an intrinsic resistance to β-lactam antibiotics due to their highly active β-lactamase combined with membrane permeability (Wang *et al*., 2006).

Recently the rapidly evolving extended-spectrum β-lactamases (ESBLs) have posed a more substantial threat due to their ability to inactivate a wider array of antibiotics, thus further driving the need for new novel antibiotics (Shaikh *et al*., 2014).

Table 1. 3: The intrinsic resistance of microorganisms to specific antimicrobials and the method of resistance. Adapted from (Reygaert, 2018).

Microorganism	Intrinsic resistance target	Mechanism
Stenotrophomonas maltophilia	β-lactams	Production of enzymes (β lactamases) that inactivate imipenem before the drug can reach the PBP targets.
Pseudomonas aeruginosa	tetracycline	The MexAB/MexXY efflux systems are able to efflux the antibiotic
Acinetobacter spp.	glycopeptides	Unable to penetrate cell wall due to molecular weight
All gram positives	aztreonam	Lack of penicillin binding proteins (PBPs) that bind and are inhibited by this β lactam antibiotic
Enterococci	aminoglycosides	Lack of sufficient oxidative metabolism to drive uptake of aminoglycosides
Listeria monocytogenes	cephalosporins	Expresses a modified antibiotic target (PBP) that binds to cephalosporins poorly
Anaerobic bacteria	aminoglycosides	Lack of oxidative metabolism to drive uptake of aminoglycosides
Aerobic bacteria	metronidazole	Inability to anaerobically reduce drug to its active form
Klebsiella spp.	ampicillin	Production of enzymes (β-lactamases) that inactivate ampicillin before the drug can reach the PBP targets
Lactobacilli	vancomycin	Lack of appropriate cell wall precursor target to allow vancomycin to bind and inhibit cell wall synthesis
Escherichia coli	vancomycin	Unable to penetrate cell membrane due to high molecular weight
Providencia spp. and *Serratia* spp.	colistin	Modification of the LPS membrane

1.2.1.3 Adaptive Resistance

The widely accepted definition for adaptive resistance is the temporary increase in resistance to toxic environmental substances as a result of gene and/or protein expression (Fernández *et al*., 2012; Sandoval-Motta *et al*., 2016). This emphasises that bacteria are extremely mutable and can adapt to many types of environmental stress (Rossnerova *et al*., 2020, Tan *et al*., 2022). Adaptive resistance has been attributed to many cellular alterations and behaviours including biofilm formation, increased numbers of efflux pumps, reduction in porins, increased mutation rates, increased efflux rate and changes in gene expression (Motta Tan *et al*., 2015, Tan *et al*., 2021). Adaptive resistance acts as an intermediary between intrinsic resistance and acquired resistance (Rizi *et al*., 2018). It is often temporary in nature, normally reverting when environmental pressure is alleviated. This reversion is due to the mechanism of resistance often being energy intensive (Coleman *et al*., 2020). The effects of adaptive resistance on AMR, can be quantified by the increased recommended MIC break points on clinically significant bacteria over recent decades (Jahn *et al*., 2017). Primarily due to the continual use and misuse of antibiotics, recommended patient dosages have substantially increased. In the case of vancomycin against certain strains of *S. aureus*, the recommended breakpoints in 1986 were between 0.25–0.5 μg/mL, those same breakpoints in 2002 are reported at >32 μg/mL (Fernández *et al*., 2011). Similar effects have also been observed in Gram-negative species, where the recommended breakpoints for ciprofloxacin against strains of *P. aeruginosa* in 1990 were reported at <0.5 g/mL but by 2008 the breakpoints had increased to 32–64 g/mL for the same strain (Fernández *et al*., 2011).

Bacteria have shown the ability to adapt and harness a multitude of different structural modifications in an effort to increase resistance to antibiotics, these can include: increased membrane thickness, different profiles of plasma membrane fatty acid composition, changes

to the LPS structure and changes to the overall cell size (Crompton *et al*., 2014). Many Gram-negative species have also demonstrated the ability to resist outer membrane permeabilizing agents (Gogry *et al*., 2021). Colistin (polymyxin E) is a cationic antibiotic belonging to the polymyxin family and acts as dual functioning antibiotic (Cai *et al*., 2015). The polymyxin family are the only antibiotics in current clinical use that targets outer membrane assembly allowing self-promoted uptake. Once the outer membrane is permeabilized colistin then penetrates deeper into the cell targeting the cytoplasmic membrane (Sabnis *et al.,* 2018). Initially, colistin binds to the outer membrane LPS layer displacing divalent cations via competitive binding. This prevents bridging interactions between LPS molecules destabilizing the LPS leaflet (Band *et al.,* 2015). Colistin is used as a last resort in clinical practice due to nephrotoxicity and neurotoxicity (Ustundag *et al.,* 2022). Resistance to permeabilizing agents is often determined by the length of the LPS molecule on the outer membrane, for example, *Acinetobacter baumannii* has the ability to reduce the length of the LPS molecule. By undergoing this reduction, the overall negative charge of the outer membrane is reduced and therefore the effectiveness of colistin is also reduced allowing for temporary resistance in *A. baumannii* (Kamoshida *et al*., 2020).

The over production of cellular target molecules is another adaptation that bacteria utilise to resist antibiotics. This response to antibiotics was initially thought to be clinically insignificant due to the phenomena being only reproducible in a laboratory environment (Palmer *et al.,* 2014). Subsequent research has demonstrated this is not the case and an increasing number of clinical examples are disproving this initial observation. An example of such a process includes the production of prolipoprotein signal peptidase by *E. coli* in response to globomycin (Sakka, *et al*., 1987). Bacteria are also able to utilise overexpression of structural components of the cell and this is demonstrated by modifications to the cell wall of certain *S. aureus* strains. The upregulation of the two-component regulatory system *vraSR* in *S. aureus* strains B6D and D7

results in the overexpression of the cell wall, resulting in a thicker cell wall preventing the diffusion of vancomycin leading to an intermediate resistance phenotype (Howden *et al*., 2008).

Increased expression of efflux pumps plays a key role in many instances of adaptive resistance. The increase in frequency and activity of efflux pumps permits lower intracellular concentrations of toxic substances in comparison to the surrounding environment (Fernández *et al.,* 2012). Hocquet *et al* (2003) demonstrated the importance of increased adaptive expression of the transporter MexY when *P. aeruginosa* is exposed to gentamicin. The results showed the expression of MexY peaked after 2 h and started to recess 4 h after drug removal (Hocquet *et al.,* 2003). The presence of oxidative compounds and the subsequent generation of reactive oxygen species (ROS) influences the expression of efflux pumps. McBee *et al.,* (2017) observed that $O_2^{\bullet-}$ production was responsible for the up regulation of rifampicin efflux pump, BCG_1316c (McBee *et al.,* 2017*)*. Fraud *et al*., (2011) demonstrated ROS generation activated the PA5471 gene in *P. aeruginosa* allowing the expression of the MexXY efflux pump in contributing to resistance against aminoglycosides (Fraud *et al.,* 2011). These observations indicate that the generation of ROS plays an integral role in the expression of efflux systems and contributes towards antibiotic resistance. Recently Varney *et al.,* (2020) was able to induce resistance in *E. coli* strain 958 using a synthesised $[\{Ru(TMP)_2\}_2(tpphz)]^{4+}$ since an 8-fold increase in MIC over a five-week period was observed. This resistance proved to be temporary reverting to wild type levels over an unspecified period. This adaptation was attributed to gene expression levels (Varney *et al.,*2020).

Biofilm formation is also an adaptive response mechanism allowing bacteria to resist exposure to toxic substances (Uruén *et al.,*2021). Bacteria begin to form biofilms by utilising a form of communication signalling known as quorum sensing, Bacteria produce specific molecules which elicit a response by other bacteria (Pena *et al.,*2019). The signals can have profound

alterations to transcription, in response to population density and environmental stresses leading to switching from a planktonic state to biofilm (Miller *et al.,*2001). When bacteria are clustered in biofilms, bacteria within the biofilm can be up to 1000x more resistant to antibiotics to which they are normally sensitive (Potera., 2001). This is in part due to the restricted penetration through the biofilm matrix of antibiotics allowing the bacteria to adapt at lower concentrations. Bacterial exposure to low concentrations of antibiotic has also been implicated in the generation of genetic mutations which is a form of adaptive resistance (Martinez *et al*., 2000).

There are three main classes of mutagens: physical, chemical mutagens and biological (Kodym *et al*., 2009). These mutagen classes can be further broken down into subclasses including; base analogs, ROS, deaminating agents, alkylating agents and viruses (Chatterjee *et al*., 2017). These agents can cause direct mutations or trigger cell response pathways as a result of DNA damage. The activation of the cell response pathways can subsequently increase the rate of DNA mutation. The spontaneous rate of mutations under favourable growth conditions is species and stain dependent and this has been shown to vary from 1 in 10^5 to 10^{10} base pairs and these predominantly occur due to copy and repair errors (Hoek *et al*., 2011; Schroeder *et al*., 2018). Under certain types of environmental pressure, mutation rates can dramatically increase. Environmental pressure and mutagens activate cell response pathways which can subsequently induce mutations in DNA or increase the rate of DNA mutation via DNA repair mechanisms (Galhardo *et al*., 2007). Exposure of bacterial cells to sub-lethal concentrations of antibiotics have been shown to expedite AMR by triggering the SOS response (López *et al*., 2009). These increased mutation rates are also shown to be present following exposure to sub lethal concentrations of Ru complexes. Monti-Bragadin *et al*., (1987) used $RuCl_2$ $(dimethylsulfoxide)_4$ to produce reverse mutations in *Salmonella Typhimurium* and *E. coli* (Monti-Bragadin *et al*., 1987).

The SOS response is a low-fidelity DNA repair mechanism used by bacteria and is a negatively regulated system that uses the LexA repressor protein with the SOS response being initiated when RecA binds to damaged ssDNA (Sánchez-Osuna *et al*., 2021). Any molecule that can damage DNA has the potential to trigger the SOS response. Under significant levels of DNA damage, lower fidelity DNA repair processes are co-initiated including the use of DNA polymerases UmuDC and DinB, which are used in conjunction with low-fidelity DNA repair mechanisms thereby increasing mutation rates, causing single nucleotide polymorphisms (SNP), recombination and enhancing gene transfer (Guerin *et al*., 2009, Úbeda, *et al*., 2005). Both individual SNP and the accumulation of non-related low level resistance mutations can lead to high levels of resistance within the cell (Oethinger *et al*., 1998, Baquero, 2001). Mutations in the *gyrA* gene are primarily associated with Gram-negative spp. with reports of this occurring in *Enterobacteriaceae* spp., which has led to the resistance to fluoroquinolones. Such a mutation alters the target site of fluoroquinolones rendering them ineffective due to being unable to interact with the DNA–topoisomerase complex thus preventing DNA fragmentation leading to cell death (Weigel *et al*., 1998; Jacoby *et al*., 2015).

Antibiotics from the fluoroquinolone class such as ciprofloxacin and norfloxacin, in addition to sulfamethoxazole and trimethoprim, can directly activate the SOS stress response pathway due to the role in mediating DNA changes (Qin *et al*., 2015). Norfloxacin is also known to directly generate ROS within the cell, which has an active role in causing death of the bacterial cell (Liu *et al*., 2017). The SOS response can also be triggered indirectly by antibiotics due to activation of alternative activation pathways for example, in the presence of β-lactams, a two-component signal transduction system that induces the SOS response has been demonstrated in *E. coli* (Miller *et al*., 2004). The generation of ROS has also been shown to trigger the SOS response (Zeng *et al*., 2013).

1.2.1.4 Acquired Resistance

The ability of bacteria to genetically acquire antibiotic resistance was first discovered in Japan during the 1950's. Akiba *et al.* (1960) and Ochiai *et al.* (1959) both published papers on the ability of antibiotic resistance in *Enterobacteriaceae* spp to mediate resistance transfer to *Shigella dysenteriae* strains in liquid culture medium (Saeed *et al*., 2009; Akiba *et al*., 1960). Conjugation was confirmed as the mechanism in 1960 when research showed direct cell to cell contact being involved through plasmid transfer (Davies, 1995). This newly discovered ability of bacteria to transfer AMR was designated acquired resistance and is defined as the ability of a bacterium to acquire resistance to antibiotics from an external genetic source via mobile genetic elements including plasmids, transposons, gene cassettes, integrative, conjugative elements and other mobile elements conferring resistance (Schwarz *et al*., 2017). The transfer of external genetic material is conducted in one of three ways: conjugation, transduction and transformation. Through extensive research conjugative transfer has proven to be the most prevalent and problematic mechanism for the transfer of AMR genes between non-pathogenic organisms to pathogenic organisms (Davies *et al*., 1996; Munita *et al*., 2016).

The contribution of HGT is well established in the development of not only AMR but that of MDR within both laboratory and clinical setting (Evans *et al*., 2020). Once AMR genes have undergone HGT, the alteration to the recipient cell is permanent but once selective pressure from an antimicrobial is alleviated the gene is often downregulated (Rajer *et al*., 2022). This allows the AMR pathogenic bacteria to proliferate, accelerating the spread of AMR genes in clinical settings thus impacting the most susceptible to infection. Many AMR genes from commensal, pathogenic and environmental bacteria are homologous and are readily transferable between genus (Hoffman, 2001). Due to the increased energy requirements of many acquired resistance mechanisms, bacterial cells that have acquired resistance often see a

decline in proliferation in comparison to wild type variants (Reygaert, 2009). The acquisition of AMR genes can lead to a plethora of cell changes that are described in section 1.2.1.

The Gram-positive *Enterococcus* genus is synonymous with AMR. The genus represents a major cause for concern within clinical environments for many reasons, most notably due to intrinsic resistance against a broad range of antibiotics and has been shown to act as a repository for the transfer of AMR genes to non-resistant species (Schjørring *et al*., 2011: Gardete *et al*., 2014). In a recent study, 96.6 % of *Enterococcus faecalis* samples collected from faeces tested positive for antibiotic resistance to gentamicin and erythromycin, while the same study also showed 10.4 % were also resistant to vancomycin. *Enterococcus faecium* species also showed similar levels of resistance (Adesida *et al*., 2017). The *Enterococcus* genus is also highly resistant to ampicillin and encodes at least five genes associated with ampicillin resistance with examples including *pbp5, pbpA* and *pbpB* (Grayson *et al*., 1991: Miller *et al*., 2014). There are many examples of conjugal transfer of AMR genes and one of the most widely published is the vancomycin resistance gene *vanA* containing the transposon Tn*1546* from vancomycin-resistant *E. faecalis* to *S. aureus*. This conjugal transfer occurs in both a laboratory environment but also crucially, in clinical cases with the earliest example being a patient in Michigan USA in 2002 (Sievert *et al*., 2008). This not only shows the *Enterococcus* genus can act as a reservoir to AMR but also pass AMR genes onto species which previously showed no resistance. Furthermore, other resistance genes in the *Enterococcus* genus could be of interest in helping to understand the spread of AMR (Noble *et al*., 1992; Sarathy *et al*.,2020).

Cellular responses to antimicrobial agents also play a key role in the development of AMR. As well as the SOS response contributing to adaptive resistance, it is well established that the SOS response can contribute significantly to the development of acquired AMR. The SOS response does this incidentally by increasing the rate of HGT. Crane *et al.* (2018) demonstrated that by triggering the SOS response in *E. coli* EC43, which under normal circumstances lacks natural

competence, it was able to acquire ß-lactam resistance from *Enterobacter cloacae* strain E_clo_Niagara via conjugation (Crane *et al.,* 2018). The SOS response can also be responsible for the increased incorporation of ssDNA from a donor to a recipient via conjugation. When ssDNA enters the recipient cell, the SOS response is triggered facilitating the integration of the transferred ssDNA into the recipient genome (Baharoglu *et al*., 2010). Not only have AMR genes been transferred, equally important virulence factors have also been integrated into the recipient genome. Maiques *et al.* (2006) demonstrated that β-lactam antibiotics can trigger horizontal transfer of virulence factors by initiating the SOS response in *S. aureus* strains (Maiques *et al*., 2006). As well as contributing to resistance, the SOS response is able to regress resistance to antibiotics in laboratory conditions. Recacha *et al.* (2017) demonstrated the ability of the SOS response to reverse Quinolone resistance in *E. coli* (Recacha *et al*., 2017).

The response of bacteria to toxic environmental substances and the subsequent formation of biofilms have previously been shown to contribute to the formation of acquired resistance. Biofilm interfaces have proven to be conducive to an increased frequency of HGT via conjugation and transformation in comparison to a planktonic state with the transfer of AMR genes leading to acquired resistance (Sørensen *et al*., 2005; Madsen *et al*., 2012). Savage *et al.* (2013) demonstrated the multidrug resistant plasmid pGO1 showed increased transfer rates via conjugation in *S. aureus* strains in a biofilm (Savage *et al.,* 2013). As well as the increased rate of conjugation in a biofilm especially during the initial formation stages, the rate of transformation of mobile elements such as small DNA fragments and plasmids was also shown to increase. This can be critical as the outer interface of the biofilm is inducive to increased DNA mutation rates potentially propagating resistance (Madsen *et al*., 2012). The environment within the biofilm promotes plasmid stability, which has a key role in mediating AMR as plasmids often contain genes that increase host fitness (Lili *et al*., 2007).

1.3 **Antibiotics and Mechanisms of Activity, the need to find Alternatives**

Antimicrobial agents can be categorised into organic and inorganic compounds. Organic antimicrobials are molecules that are derived with carbon moieties while inorganic compounds are defined as molecules that are not usually found or incorporated into a living organism (Saidin *et al*., 2021). Antibiotics are currently classified based on their mechanisms of action and there are over 20 classes of antibiotics which fall within five functional groups: 1) inhibitors of cell wall synthesis, 2) inhibitors of protein synthesis, 3) inhibitors of cell membrane function, 4) inhibitors of nucleic acid synthesis and 5) inhibitors of other metabolic processes (Neu, 1992) (Table 1.4).

Table 1. 4: Antibiotic classes with named examples and mechanisms of antimicrobial activity.

Antibiotic Class	Antibiotic Types	Cellular Target
β-lactam	Penicillin, Amoxicillin, Ampicillin, Ceftazidime, Cephalosporins	Cell wall synthesis
Quinolone	Nalidixic acid, Ciprofloxacin, fluoroquinolone	DNA gyrase and topoisomerase IV
Aminoglycoside	Streptomycin, Gentamicin, Amikacin	30S ribosomal subunit / cell membrane
Macrolide	Erythromycin, Azithromycin	Peptide exit tunnel in 50S ribosomal subunit
Tetracycline	Tetracyclin, Tigecycline	tRNA binding in 30S ribosomal subunit
Oxazolidinones	Linezolid	Peptidyl transferase centre in 50S ribosomal subunit
Phenicol	Chloramphenicol	Peptidyl transferase centre in 50S ribosomal subunit
Glycopeptide	Vancomycin	Cell wall synthesis
Sulfonamides	Sulfamethoxazole	Tetrahydrofolate synthesis
Tetracyclines	Tetracyclin, Tigecycline	tRNA binding in 30S ribosomal subunit
Rifamycins	Rifampicin	RNA polymerase
Polypeptides	Polymyxin B, Polymyxin E	Permeabilization of outer and cytoplasmic membranes
Oxazolidinones	Linezolid	Peptidyl transferase centre in 50S ribosomal subunit
Licosamide	Clindamycin, Lincomycin	Peptide exit tunnel in 50S ribosomal subunit

The majority of antibiotics currently in clinical use are organic molecules or are derivatives of these molecules. In the environment, bacteria and fungi use these molecules as part of offensive and defensive mechanisms against other microorganisms (Peterson *et al*., 2018). There are many fundamental problems with the discovery of new organic antimicrobial agents. The Waksman-platform has been at the forefront in the discovery of antibiotics for nearly half a decade but it is estimated that only 2 % of total microorganisms are culturable under laboratory environmental conditions, therefore research has dwindled or been abandoned completely (Wade *et al*., 2002, Gajdács, 2019). Subsequently, this limits the ability to discover new classes of organic antibiotics. As a result, only two new classes, Oxazolidinones and Lipopeptides, have gained Food and Drug Administration (FDA) approval since 1968 (Powers, 2004). There has only been a single new antibiotic that works via a new mechanism of action discovered within the last 30-years called Teixobactin, which is currently undergoing clinical trials. In contrast between 1930 and 1962 there were over 20 new novel classes of antibiotics discovered. There has been a significant decline in the discovery of new antimicrobials, with 16 being discovered between 1983 and 1987 whilst only 2 discovered between 2008 and 2012 (Figure 1. 3).

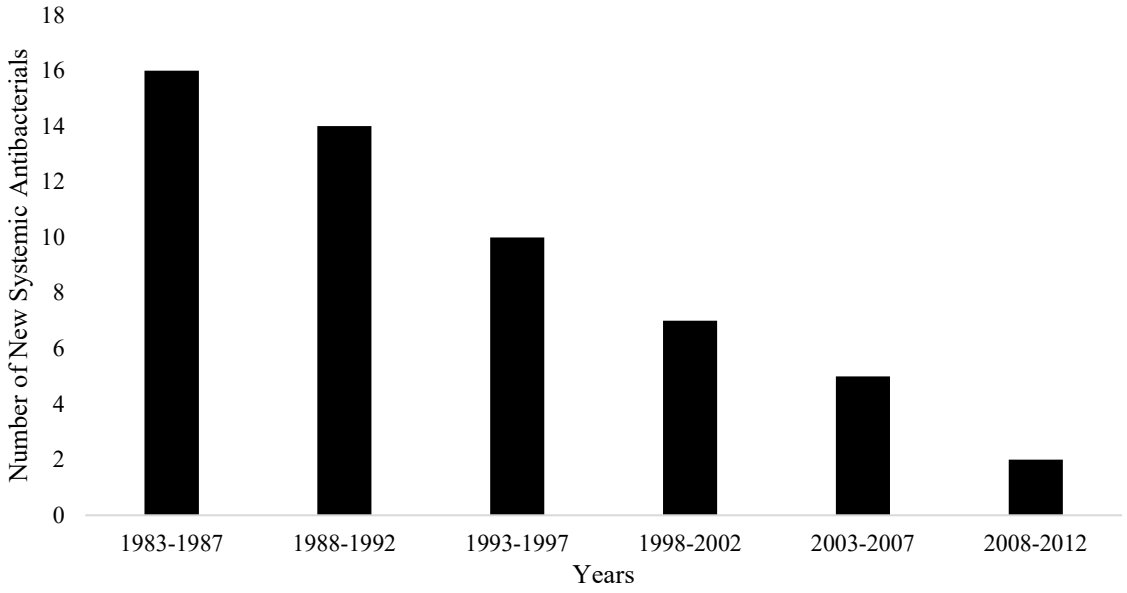


Figure 1. 3: Antibiotic classes with named examples and mechanisms of antimicrobial activity.

Another fundamental issue with the discovery of new organic antibiotics is resistance generation, for each organic antibiotic discovered there will be at least one mechanism of resistance in the wider domain. It is suggested that over time, pathogenic bacteria would be able to acquire such resistance via HGT (D'Costa *et al*., 2011). The development of novel inorganic antimicrobials that have novel mechanisms of antimicrobial activity would reduce the rate of AMR (Udaondo *et al*., 2020).

There are currently three main strategies in the development of new antimicrobials, first being the development of auxiliary agents (Breijyeh *et al*., 2020). Auxiliary agents work symbiotically with existing antibiotics producing a potent effect on bacteria without increasing toxicity to eukaryotic cells. The second strategy is the production of different analogues of existing antibiotics. This involves modifying the molecular structure of existing antibiotics to bypass existing mechanisms of resistance or to further increase the efficacy of the antibiotic. Between 1980 to 1999, 51 new antibiotics based on existing analogues were approved by the

FDA (Mott *et al*., 2016). The final strategy is to discover new novel molecules either synthetic or natural-based that possess new mechanisms of action or are resistant to current bacterial adaptations. The use of metallodrugs can be adapted to fit into all three current strategies (Annunziato., 2019). When Abrusán *et al*. (2019) conducted a detailed study on current clinically used antibiotics, it showed that the most successful antibiotics bind several protein targets such as β-lactams and fluoroquinolones. This also reduces the rate of reported resistance in comparison to single target antibiotics such as, sulphonamides and trimethoprim which often have to be used in combination therapy (Abrusán *et al*., 2019)

This shows there is an urgent need for the development of new antimicrobial agents either in an organic or inorganic form for therapeutic use. Resistance against antimicrobials can develop at a rapid rate often making companies reluctant to invest in the development of new antimicrobials due to the inability to make long term gains on research expenditure (Simpkin *et al*., 2017).

1.4 Inorganic Molecules and Metals as Antimicrobials

The modern era of organic-based antibiotics began in 1928 when Alexander Fleming made observations showing fungal contamination on Petri dishes inoculated with *Staphylococcus* species reduced bacterial growth. The organic molecule was later named penicillin. Only in the 1940s with the advent of batch fermentation did penicillin become more widely available. Organic antibiotic manufacturing had lower production cost, capacity for mass production and demonstrated relatively few side effects, which resulted in a decline in inorganic antimicrobial research. With the ever-increasing rise in AMR, other types of antibacterial development are once again being pursued.

The use of inorganic antimicrobials in the form of metallic-based compounds being used predates the discovery of modern organic antibiotics by millennia. Metallic-based compounds in therapeutic use can also be referred to as metallodrugs. The Egyptians used copper jars to sterilise water whilst Gabor reported the use of silver nitrate between the years 702–705 AD for use in the treatment of wounds (Alexander, 2009.). Silver sulfadiazine is still used in modern medicine in the treatment of burns (Hussain *et al*., 2006).

Many of the earliest modern metallodrugs used in therapy date from the early 1900's were arsenic based. These ware used to treat parasitic infections, such as atoxyl arsanilic acid which was designed for the treatment of trypanosomiasis (sleeping sickness) (Mjos *et al*., 2014). Sulphonamide, while not metallic based, was the first true synthetic antibiotic to be used before organic antibiotics. Sulphonamides were first developed during the early 1930s and are still used in modern medicine. Sulphonamides are a broad-spectrum antibiotic that mimic p-aminobenzoic acid preventing the production of tetrahydrofolic acid (Hitchings, 1973). Humans cannot synthesise tetrahydrofolic acid thus is only consumed in diet, thus these sulphonamides have no effect on humans. Later modifications to heterocyclic sulphonamides introducing copper complexes in the form of $[Ni(L_4)(H_2O)_2]$ have shown improved antibacterial properties (Chohan, 2008). In the 1960's a new class of synthetic antibiotics were first introduced known as fluoroquinolones (Appelbaum *et al*., 2000). These are produced during the synthesis of chloroquine and act as a broad-spectrum antibiotic used to treat a variety of conditions including UTI's. There are currently four generations of fluoroquinolone antibiotics with modern research looking to increase the functionality of the molecules by adding functional groups creating dual action molecules or reducing toxicity (Fedorowicz *et al*., 2018). As of 2019 in the UK and 2018 in Europe usage has been restricted due to their potential side effects (Naeem *et al*., 2016)

1.4.1 Modern Era of Metallodrugs

The synthesis of $[Pt(NH_3)_2Cl_2]$ (cisplatin) in 1845 and its later adaptation as an antitumour drug in 1978 began the era of modern medicinal inorganic chemistry, with substantial amounts of research into antitumor drugs. Many such drugs have been tested for other purposes including antimicrobial activity with some showing promising results (Shah *et al*., 2013; Soo *et al*., 2017).

Currently there are many different metallodrugs which have gained FDA and Medicines and Healthcare products Regulatory Agency (MHRA) approval. These cover a broad range of metals and conditions including lithium carbonate for depression, auranofin, a gold compound used in the treatment of rheumatoid arthritis and cisplatin that uses platinum as a scaffold for the treatment of cancer. Currently there are only two ruthenium (III) complexes that have undergone clinical trials, NAMI-A (imidazolium trans-[tetrachloride (imidazole)(dimethylsulfoxide) ruthenate(III) and KP1019 (indazolium trans-[tetrachloridobis (1H-indazole) ruthenate (III)] both of which are antitumour related yet currently no ruthenium complexes have been approved or entered clinical trials by the FDA or MHRA in relation to antimicrobial activity (Gransbury *et al*., 2016). Currently there is a resurgence in studying inorganic compounds to combat the threat posed by AMR. This is demonstrated by the development of ruthenium-based antimicrobials, with only 669 papers being published over a 15-year period between 1995 and 2010 while over a seven-year period between 2011 and 2018 there were 1066 papers published according to Science Direct using the terms “Ruthenium and Antimicrobial”. Other non-ruthenium based metallodrugs have shown similar increases in research output. Many of these papers have shown promising results (Bolhuis *et al*., 2011; Li *et al*., 2012).

1.5 Ruthenium Coordination Chemistry in Antimicrobial Compounds

Ruthenium (Ru) as element 44 was first isolated by Jedrzej Sniadecki in 1808 and was initially named vestium (Sioda, 2011). In 1844, Karl Ernst Claus reisolated vestium and subsequently renamed this as ruthenium (Ru). Ruthenium has an atomic mass of 101.07 and is in group 8, period 5 of the periodic table and is part of the platinum group of transitional metals. The electron configuration of Ru is $1s^2$ $2s^2$ $2p^6$ $3s^2$ $3p^6$ $4s^2$ $3d^{10}$ $4p^6$ $5s^1$ $4d^7$. Ru possesses many properties that make it an ideal candidate for use as a coordination centre in drug development. The biological compatibility, oxidation states and the bonding configuration of Ru allows for specific geometries which are ideally suited for biological applications. Due to such properties clinical research conducted using Ru complexes is on the increase. Ru complexes have been routinely applied in anticancer therapy trials as well as being recently involved as antimicrobial coatings that are used on the international space station (Van Loi *et al*., 2018).

The use of a central metal atom and the subsequent formation of a metal coordination complex can provide unique modes of action including ligand exchange or release, ROS generation, redox activation and catalytic generation of toxic species or depletion of essential substrates. Many of these mechanisms are not reproducible with organic compounds (Frei *et al*., 2020). The antimicrobial activity of Ru complexes could also be tailored to target specific bacterial species. This could be achieved by small alterations to the coordinated ligands structure while keeping the original scaffold intact. Such specific antimicrobial usage will be limited to intended targets which has the advantage of potentially increasing their longevity in clinical use before resistance generation occurs, however, a downside is the increased overall cost per application. Whilst no species-specific antimicrobial compounds have been developed, compounds that target specific proteins or inhibit kinases have been developed (Nišavić *et al*., 2018; Dömötör *et al*., 2018) Work conducted by Wong *et al.,* (2006) demonstrated the ability

of Ru-oxo-oxalato clusters to inhibit HIV reverse transcriptase (Wong *et al*., 2006). Research conducted by Adhireksan *et al*., (1994) demonstrated that a single ligand substitution on Ru complexes can differentiate between targeting DNA of chromatin and forming of adducts on the histone proteins (Adhireksan *et al*., 1994).

1.5.1 Biological Compatibility

Ruthenium demonstrates similar properties to iron in biological organisms (Sahu *et al*., 2018). One example is the ability of Ru to bind to biological molecules such as human serum albumin and the iron-transporter transferrin (Kratz *et al*., 1994; Śpiewak *et al*., 2015). It is thought this occurs because both Fe and Ru are in the 8^{th} group of the periodic table and share similar orbital sizes. When a Ru complex binds to the target site within the cell, Ru complexes have shown reduced cell efflux levels in comparison to other metal complexes, even after the Ru complex has been displaced. If there is a destabilisation of the Ru complex and elemental toxicity occurs then a similar physiological response is produced in the human body to iron poisoning (Luedtke *et al*., 2003). To be an effective antibiotic, a Ru coordination complex needs to either possess a greater toxicity to prokaryotic cells than eukaryotic cells or have a higher uptake rate than the eukaryotic cell.

Research into the mechanisms of action of Ru complexes has been limited with most being concentrated on the effectiveness of individual compounds as opposed to identifying the mechanisms of action (Cetinkaya *et al*.,1999; Kumar *et al*., 2016; Andrade *et al*., 2020). This is due to each complex having different ligands resulting in different mechanism of action and even potentially being effective against multiple target sites (Chen, 2003; Adhireksan *et al*., 2014). Transition metal complexes which bind randomly to DNA also have been shown to have a greater resistance against bacterial adaptation (Frei *et al*., 2020). This is because bacteria have

no means to counteract the compounds other than efflux pumps, which are never fully effective at removing the compound from the cell, thus making metallodrugs an ideal candidate for further research. (Dwyer, 1964).

Currently there has been limited work conducted on mechanisms of action and resistance against ruthenium-based antimicrobials agents, this is in part due to no successful *in vivo* trials. The limited resources available are invested in the development of new ruthenium-based compounds combined with basic antimicrobial testing oppose to in-depth investigation into the mechanism of action.

1.5.2 Oxidation States of Ru

Ruthenium possesses a total of ten oxidation states with the interconversion energy barrier allowing access to three biologically accessible oxidation states (+2, +3 and +4). In the development of new pharmaceuticals, the Ru +2, +3 oxidation states are predominantly used because these oxidation states are relatively inert with no oxidation reaction occurring (Cotton, 1997; Sahu *et al*., 2018). Ruthenium's three biologically accessible oxidation states allow for slow ligand exchange rates and Reedijk, (2008) recorded the ligand exchange rate range in Ru (II) complexes from 10^{-2} to 10^{-3} s^{-1} (Reedijk, 2008). It has been demonstrated that the slow ligand exchange rate accounts for the good affinity of Ru coordination complexes to biomolecules (Matshwele *et al*., 2020). The lower biologically accessible oxidation states of +2 and +3 are also able to form aqueous cations aiding in the solubility of Ru complexes, which can allow for a more diverse range of pharmaceutical applications (Seddon, 1984).

1.5.3 Ligands Coordinated to Ru for use in Antimicrobial Applications

The choice of ligand selection and the subsequent attachment to the central metal ion is one of the main challenges when developing metal-based antimicrobials (André *al*., 2021). The ligands attached to the central metal atom play a pivotal role when selecting complexes for potential therapeutic applications (Abrusán *et al*., 2019). A ligand is defined as an ion or molecule that binds to a central metal atom to form a coordination complex. The central metal atom acts as an electron acceptor thus is referred as a Lewis acid and the ligands act as an electron pair donor and are referred to as Lewis bases (Braunschweig *et al*., 2011).

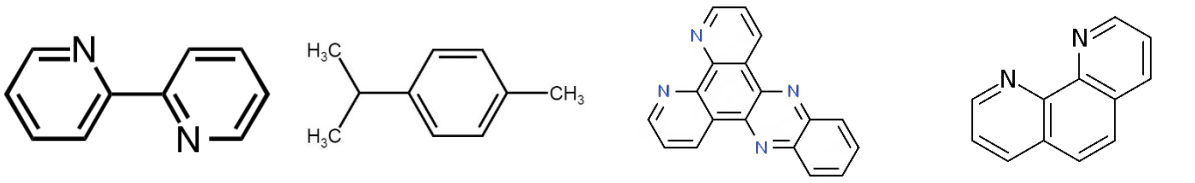
One of the main contributing factors in establishing the antimicrobial properties of an Ru coordination complex is the ligand selection. When designing metal-based antimicrobials, it is important to consider the molecular properties of the molecule. The choice of ligand can alter the charge, lipophilicity, lability, shape and redox potential of the coordination complex (Martin., 2018). It is also important to understand the total number of ligands that can be attached to form a coordination complex and subsequent ligand interactions.

A Ru complex contains atoms, ions, or molecules that bind to a central metal atom or ion and the sum of these is referred to as coordination number. The coordination number determines the geometry of the complex and thus plays an important role in determining antimicrobial activity (Claudel *et al*., 2020). The ligands of coordination complexes can either attach directly or indirectly to the central metal atom. This results in ligands being grouped into inner coordination and outer coordination spheres. The inner coordination sphere that encompasses all ligands that directly attach to the central metal atom and the outer coordination that encompasses ligands that indirectly attach to the central metal atom. The outer coordination sphere is used to balance out the charge of the complex. Recent work conducted by Therrien (2018) demonstrated the importance of considering the second coordination sphere showing that the outer coordination sphere plays a major role in most biological and biomedical applications (Therrien., 2018). Each ligand that is part of the coordination complex contains

donor atoms that either bind to the central metal atom or act as ions to balance the oxidation state and this is referred to as denticity and can be categorised as monodentate, bidentate and polydentate ligands. Ligands that attach to a central metal atom from multiple donor atoms on a single ligand are referred to as chelating ligands. The denticity of the ligands determines the oxidation state of the Ru coordination complex. The natural properties of the central metal atom can either be subdued, enhanced or remain unchanged by the addition of ligands (Matshwele *et al*., 2020).

1.5.3.1 Biologically active ligands utilised in Ru complexes

John Dwyer, Francis Patrick and David Paver Mellor were the first to conduct research into the antimicrobial properties of Ru coordination complexes during the 1950s. They demonstrated that the compound $[Ru(phen)_3]^{2+}$ possessed no antimicrobial activity, until the addition of a methyl group to the phenanthroline ligands (Dwyer, 1964). Since the initial work conducted by Dwyer *et al.*, 1952, many more Ru coordination complexes have been developed which possess antimicrobial properties with new Ru coordination complexes being synthesised daily (Sun *et al*., 2015; Liu *et al*., 2018; Bu *et al*., 2020;). There are many examples of ligands that are commonly used in the development of Ru coordination complexes that possess antimicrobial properties, examples include; 1,10-Phenanthroline (phen), dipyrido[3,2-a:2',3'-c]phenazine (dppz), Chloride (chlorido), 1-Methyl-4-(propan-2-yl) benzene (p-Cymene), 2,2-bipyridyl (bpy) (Figure 1. 4) (Pandrala *et al*., 2013; Abebe *et al*., 2016; Gichumbi *et al*., 2018; Namiecińska *et al*., 2019; Bu *et al*., 2020).



2,2-bipyridyl (bpy)	dipyrido[3,2-a:2',3'-c]phenazine (p-Cymene)	dipyrido[3,2-a:2',3'-c]phenazine (dppz)	1,10-Phenanthroline (phen)

Figure 1. 4: Examples of ligands commonly used in ruthenium complexes.

There has been extensive use of chelating ligands in the synthesis of antimicrobial complexes, and this is thought to be primarily due to the biological activity and use in anticancer treatments (Yan *et al*., 2005; Wang *et al*., 2005). Many chelating ligands focus on nitrogen acting as the donator. Each chelating ligand can confer different properties to the coordination complex. One such ligand is phen and the antimicrobial effects of this are primarily associated with the sequestration of metal ions in biological systems. When acting as a ligand in a coordination complex, the hydrophobic chelating ligand phen facilitates the uptake of cationic metal ions through transporter proteins (Viganor *et al*., 2017). The alkyl-substituted aromatic hydrocarbon 1-Methyl-4-(propan-2-yl) benzene (p-Cymene) is an organic molecule that can be isolated from plant species and is known to possess antimicrobial activity due to the presence of a substituted benzene ring. Usman *et al*. (2021) synthesised a series of Ru complexes with p-Cymene ligands. The complex [RuCl(η^6-p-cymene)(R)] demonstrated antimicrobial activity against *P. aeruginosa*, MRSA, and *Chromobacterium violaceum* with MIC of 8 μg mL^{-1}, 8 μg mL^{-1} and 4 μg mL^{-1} respectively. The complex also inhibited biofilm formation across all three species (Usman *et al*., 2021). Work conducted by Ude *et al*. (2016) synthesised the compound [Ru(η^6-p-cym)($CipA_{-H}$)Cl] utilising the p-Cymene ligand and this compound demonstrated a 91.7 % reduction in colony forming units (CFU mL^{-1}) producing a bactericidal effect against *E. coli*

CL2 at 200 μM concentrations after a 1 h incubation (Ude *et al* 2016). Attached organic ligands on Ru coordination complexes have been shown to increase antimicrobial activity over non-coordinated organic ligands (Santos *et al*., 2014). The ligand Dipyrido[3,2-*a*:2‘,3‘-*c*]phenazine (dppz) is a heterocyclic aromatic ligand that has been utilised in Ru complexes and tested extensively in both anti-tumour and antimicrobial applications. Liu *et al*. (2018) conducted antimicrobial testing on the compound $[Ru(bb_7)(dppz)]^{2+}$ where the compound demonstrated an MIC of 4 μg mL^{-1} against *S. aureus* SH 1000 and 8 μg mL^{-1} against *E. coli* ST 131. The compound showed both DNA intercalating and membrane interacting properties. The increased antimicrobial activity of $[Ru(bb_7)(dppz)]^{2+}$ over other compounds tested was speculated to be a result of the nucleic acid binding properties of the dppz ligand (Liu *et al*., 2018).

Labile ligands have proven to be an effective tool in biological application. Coordination complexes can be designed in such a way that the metal-ligand bond is able to break and this is referred to as ligand liability. Labile ligands have been implemented on a myriad of Ru coordination complexes. Ruthenium compounds containing labile ligands have proven to be effective in the development of anticancer drugs most notably Imidazolium-trans-tetrachloro(dimethylsulfoxide)imidazoleruthenium(III) (NAMI-A) that successfully entered clinical trials (Rademaker *et al.,* 2004). The use of labile ligands in antimicrobial applications is less prolific, nevertheless many compounds have displayed promising results. Pandrala *et al*. (2013) synthesised a series of Ru complexes including $[\{Ru(tpy)Cl\}_2(\mu\text{-}bb_n)]^{2+}$ (Cl-Rubb$_n$). It was thought that the lower cationic charge of the molecule would assist with increased penetration across the bacterial membrane. The compound was able to achieve an MIC and MBC against *S. aureus* ATCC 25923 of 1 and 2 μg mL^{-1} respectively (Pandrala *et al*., 2013).

A Schiff base ligand is formed when a primary amine reacts with an aldehyde or a ketone (Da Silva *et al*., 2011). The attachment of Schiff base ligands to a central metal atom has been heavily utilised in the development of anti-cancer drugs and antimicrobials. This is in part due to cellular enzymes heavy utilisation of Schiff based chemistry (Gao *et al*., 1990; Patel *et al*., 1996; Bulatov *et al*., 2018). The first Ru based anticancer drug to enter clinical trials NAMI-A contained Schiff bases. The chemical compound cinnamaldehyde is often used in the synthesis of antimicrobial Schiff bases, primarily due to its ability to inhibit the growth of microorganisms. Paul *et al.* (2017) used cinnamaldehyde to synthesise $Ru(L)(bpy)_2](ClO_4)_2$ and the resulting compound produced zone of inhibition (ZOI) of 20 mm, 18 mm and 22 mm against *E. coli*, *Vibrio cholerae* and *Shigella* sp. respectively. It was concluded that the complex was able to cross the cell membrane and bind to the cellular DNA (Paul *et al.,* 2017). Lapasam *et al*. (2019) synthesised a series of Ru coordination complexes with a nitrogen donor Schiff-base ligand attached to a central Ru core. The results showed increased antimicrobial activity against Gram-negative bacteria in comparison to Gram-positive bacteria with a 1 μg mL^{-1} MIC against *S. aureus* and a 0.5 μg mL^{-1} against *E. coli.* (Lapasam *et al*., 2019)

Arene or aromatic ligands are hydrocarbons with sigma bonds and delocalized π electrons between carbon atoms forming rings and are amongst the most utilised molecules in metal complexes. Arene and other small low molecular weight molecules can act independently as ligands, or they can be incorporated into larger more complex ligands. This can be demonstrated by the formation of a p-Cymene ligand where an arene benzene molecule forms part of the new p-Cymene ligand. Ligands with increased complexity containing arene structures have been heavily utilised in the development of anti-tumour drugs, such as L-type ligands which act as either actors or spectators (Biersack., 2016; Palmucci *et al*., 2016). The arene ligand acts as a stabiliser for the Ru(II) state under physiological conditions.

1.5.4 Geometry

The geometry of coordination complexes has been shown to affect biological function. Alfred Werner proposed the naming of structures of coordination compounds containing complex ions and later received a Nobel prize in 1913 for his work proposing the octahedral configuration of transition metal complexes (Constable *et al*., 2013). Types of geometry include octahedral, linear tetrahedral and square planar. These different geometries are primarily determined by the coordination number and ligands attached to the central metal atom. Cardoso *et al*., (2017) demonstrated the effect by producing and examining a series of camphorimine complexes for antimicrobial activity against the Candida genus. It was concluded that the geometry of the complexes coupled with the distinct differences between the proteins and lipids of prokaryotic and eukaryotic organisms could allow for binding specificity between cell types (Cardoso *et al*., 2017).

The three-dimensional bonding configuration offered by Ru, allows for the creation of modular complexes that can be tailored to target specific enzymes. Tailored antimicrobials with a Ru coordination centre can achieve modes of action unobtainable to organic compounds, making Ru an ideal candidate when designing and creating novel pharmaceuticals (Adeniyi *et al*., 2016). The three-dimensional structures created by the bonding configuration produce a higher rate of success in clinical trials in comparison to flat molecules (Sundaraneedi *et al*., 2017; Lovering *et al*., 2009). Most research conducted using Ru coordination complexes are hexacoordinate and octahedral geometries.

Recent developments in anticancer and antimicrobial research has shown an increased number of polypyridyl Ru (II) complexes which possess octahedral geometry (Shum *et al*., 2019; Liao *et al.,* 2017). This is in part due to the octahedral geometry allowing for the attachment of multiple active ligands. Ligands can include = active anticancer/antimicrobial ligand but also

allowing other ligands that allow desirable physicochemical properties including water solubility and lipid solubility (Liu *et al.,* 2008). Octahedral geometry Ru complexes have a greater DNA binding affinity that other Ru complex geometries, Due to most anticancer treatments targeting DNA, this in an advantage in anti-cancer treatments where most research have been undertaken (Khanna., 2015; Guk *et al*., 2021). Li *et al.,* 2020 produced a series of polypyridyl Ru (II) complexes intended for anticancer treatments, where each complex underwent minor ligand modifications. The results showed each complex had preferences for different cellular targets such as DNA, both as intercalating and grove binding agents, in addition to cellular protein interactions (Li *et al*., 2020). These minor ligand alterations are relativly simple to undertake allowing for inexpensive and radpid Ru complex develpoment (García-Ortegón *et al*., 2022). Due to prokaryotics cella and eukaryotic cells sharing many similar cellular stuctures including DNA, proteins and membranes, these minor ligand alterations would also be applicable Ru complexes actiong as antimicrobials.

Arene ligands are used extensively in Ru complexes and can form many different structures depending on the molecular makeup of the molecule (Hanif *et al*., 2013; Brissos *et al*., 2018; Popp *et al*., 2019). The number of atoms in the aromatic ring that are bonded to the central metal atom determines the η*-mode, which subsequently determines the geometry of the arene (Soto *et al*., 2012). When all six atoms of a benzene ring are bound to the metal (η^6-mode), the ring is flat and C–C bond lengths are slightly longer than those in the free arene (Goodman *et al*., 2019). When an arene is in η^4-mode the ring is bent and anti-aromatic (Boronski *et al*., 2020). The four-atoms bound to the central metal atom are coplanar and the other π bond is out of the plane. Molecules containing η^6-arenes act as sandwich or piano stool complexes due to their geometries (Kumar *et al*., 2013). Piano-stool complexes allow for the creation of diverse Ru coordination complexes which can use a variety of donor ligands including S-, N-, O- and P- (Namiecińska *et al*., 2019). Arene η^6have a general [(η^6-arene) Ru(X](Y)(Z)] structure. The

variable arene ring can be formed by common arene rings including; biphenyl (Bip), tetrahydroanthracene (THA), dihydroanthracene (DHA), cymene (Cym), and benzene (Ben). The (X) (Y) can represent a single bidentate ligand or monodentate ligands while the (Z) normally represents a leaving group.

1.5.4.1 Multi Nuclear Coordination Complexes

Multi nuclear coordination complexes are a relatively recent advancement in the field of antimicrobials. Multi nuclear coordination complexes are defined as, the use of two or more central Ru atoms with one or more bridging ligands (McCleverty *et al*., 1998). Many research groups have shown promising results with custom synthesised Ru multi nuclear coordination complexes. Unique modes of action have been attributed to dinuclear Ru complexes. Li *et al.,* 2012 demonstrated the compound $[Ru_2(phen)_4(\mu\text{-}bb_n)_2]^{4+}$ (bb_n = bis [4(4′-methyl-2,2′-bipyridyl)]-1,*n*-alkane was able to bind to RNA within a cell and accumulate within the ribosomes which condensed upon the formation of polysomes preventing protein synthesis (Li *et al*., 2012). Oligonuclear polypyridylruthenium (II) complexes have shown selectivity between prokaryotic and eukaryotic cells. Gorle *et al.,* (2016) synthesised a series of Chlorido-containing oligonuclear polypyridylruthenium (II) complexes that possessed potent antimicrobial activity with MIC of 1.4 μM against *E. coli* while IC50 values against HEK-293 cell lines were 10.4±0.5 μM (Gorle *et al.,* 2016).

1.5.5 Binding Targets

The ligands that form Ru coordination complexes have been shown to possess a wide range of binding targets both extracellular and intracellular (Li *et al*., 2015). Ru coordination complexes

have shown a larger array of cellular targets and are able to interact with DNA, RNA, cellular membranes and proteins in a reversable manner (Jabłońska-Wawrzycka *et al*., 2020). As a result of the primary research into Ru complexes being conducted in the field of anticancer chemotherapy, a large number of Ru complexes are designed to target DNA and RNA (Brabec *et al*., 2006; Li *et al*., 2014; Brabec *et al*., 2018). As a ligand is designed to be structurally and molecularly unique, a different antimicrobial mechanism can be associated with individual ligands of Ru coordination complexes. This can be exemplified by research conducted by Chen *et al*. (2003). The group produced a series of Ru coordination complexes including [(η^6-arene)Ru(en)(H_2O)]2+, [(η6-arene)Ru(II)(en)X] where the arene ligand was defined as Bip, THA, DHA, Cym, and Ben. All the Ru coordination complexes showed 100 % affinity to the N7 position on guanine within the DNA molecule. Despite all the Ru coordination complexes having affinity to the same DNA binding position, each compound showed differing modes of action. This was thought to be due to the numbers of coordination sites on the Ru scaffold (Chen *et al*., 2003). Thus, not only is the ligand selection important, the position of each ligand within the complex plays a pivotal role. The combined total mass of the antimicrobial that was discussed in chapter 1. 2 contributes to the antimicrobial properties of a Ru coordination complex.

Ligands on Ru coordination complexes are aided in interactions with cellular targets via electrostatic interactions between the positive charged-metal complex and the negative charged phosphate group nucleic acids (Lipfert *et al*., 2014). Other factors influencing DNA binding include charge of the molecule, the ligand hydrophobicity and the total size of the ions (Claudel *et al*., 2020). Once a Ru coordination complex is attached to its cellular target it can be displaced by molecules with greater affinity for the specific target site (Watson, 2015). In contrast, comparatively little research has been conducted on Ru complexes in relation to other metallodrugs and their antimicrobial activity. Ruthenium coordination complexes can bind to

DNA in three main ways; intercalation, groove binding and electrostatic interaction (Pages *et al*., 2015). DNA intercalators work by reversibly binding via non-covalent interaction between base pairs on double stranded DNA. Groove binding also works via non-covalent interactions, normally via hydrogen bonds and can attach via the major and minor grooves on double stranded DNA. Finally, electrostatic interaction is when a positively or negatively charged molecule is attracted to the opposite charge causing interaction. Each one can provide different outcomes. Some of these interactions are permanent whilst others are temporary interactions and this is dependent on the specific metallic complex being studied (Almaqwashi *et al*., 2016). Sun *et al.,* (2015) synthesised the antimicrobial compound $Ru(phen)_2(p\text{-}BPIP)^{2+}$ (RuBP) and the antimicrobial activity was attributed to the ability to increase the permeability of cell membranes (Sun *et al*., 2015).

The targeting of proteins within the cell is an established mode of action for antimicrobial compounds. Ciprofloxacin and Rifampicin are able to target and inhibit DNA gyrase and RNA polymerase respectively. Sun *et al.,* (2019) synthesised a tetranuclear Ru complex containing the bis[4(4′-methyl-2,2′-bipyridyl)]-1,7-heptane ligand and demonstrated its ability to bind to the protein human serum albumin (Sun *et al*., 2019). Piano-stool complexes have also been primarily associated with DNA intercalation (Colina-Vegas *et al*., 2015; Harringer *et al*., 2020).

Arene ligands have been shown to have a reversible binding affinity to DNA on the N7 position of guanineas demonstrated by Luedtke *et al*., (2003) with the synthesis of the intercalating agent D-$[Ru(bpy)_2$eilatin$]^{2+}$. Upon binding to regions of DNA, the Ru coordination complex was able to prevent the replication of DNA. A series of competitive binding assays were conducted using ethidium bromide proving the Ru coordination complex was binding in a reversable manner (Luedtke *et al*., 2003).

1.6. Repurposing of Ru Complexes as Antimicrobial Agents

A vast array of once potent antibiotics previously capable of treating a multitude of infections are now being rendered ineffective. Coupled with a lack of new antibiotic discoveries (Section 1. 3), therefore new approaches are needed for the discovery of antimicrobial compounds. The repurposing of existing pharmacological indications in medicine is increasing in prevalence and can be referred to as drug repurposing, drug repositioning, reprofiling or re-tasking. Drug repurposing can be defined as the process of identifying new therapeutic uses of existing, old, failed, investigational, already marketed, FDA approved drugs and pro-drugs (Rudrapal *et al*., 2020). Drug repurposing has many advantages over *de novo* drug development: it is cost effective, reduces development times and the pharmacological indications are often as effective as *de novo* drugs. It is estimated that 2.01 % of newly developed *de novo* drugs are successful, and this process takes between 10 to 17 years, at an estimated to cost of 2-3 billion USD (Tobinick., 2009; Li *et al*., 2018). In contrast, it is estimated to cost 300 million USD and take 3 to 12 years to bring a repurposed drug to market.

In the USA, the development of new *de novo* drugs utilises five key stages: discovery and development, preclinical research, clinical research, FDA review, and FDA post-market safety monitoring. Repurposing can often eliminate key stages used in de novo drug development. Drug repurposing has utilised many different approaches including experimental approaches, clinical and computational (Figure. 1. 4).

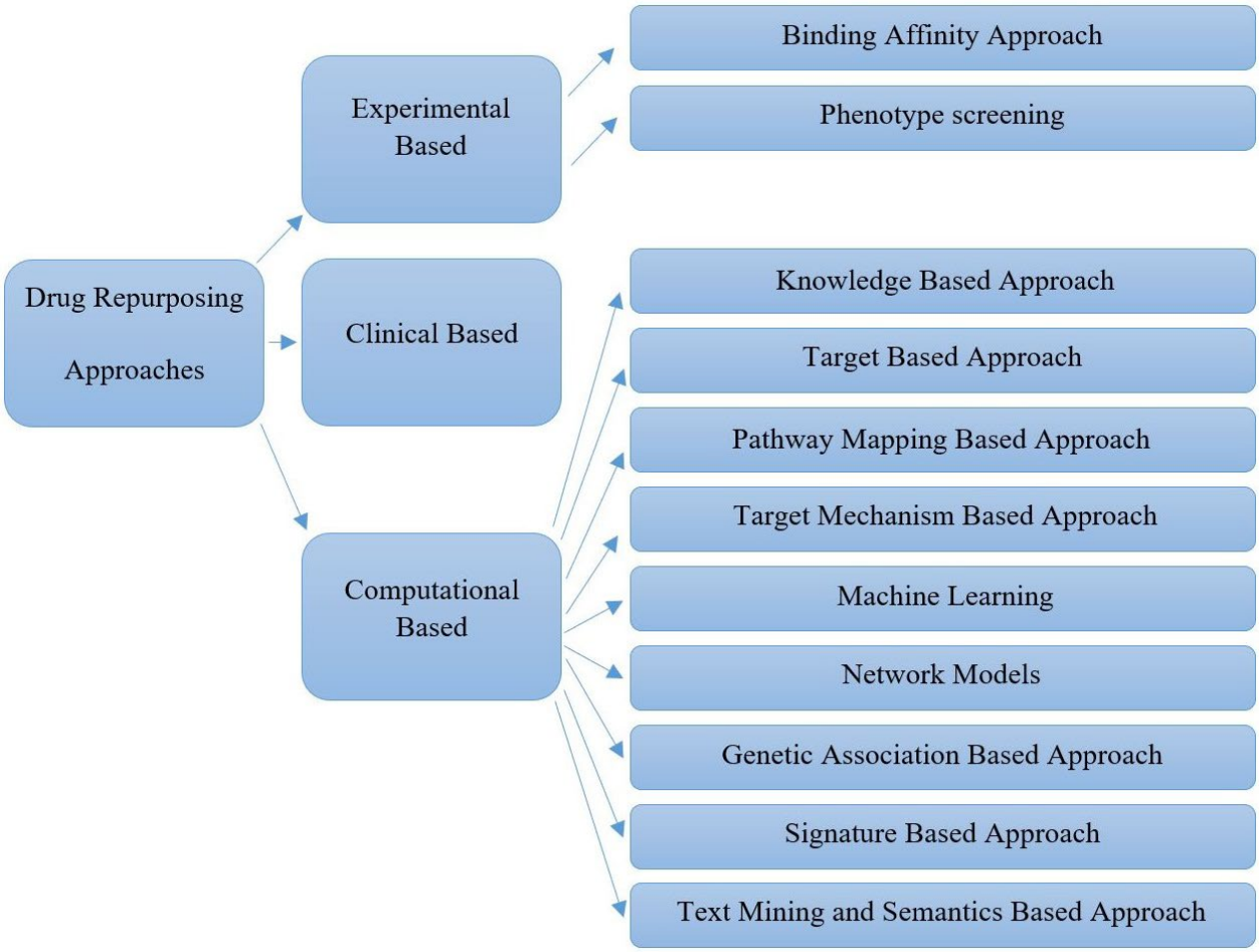


Figure 1. 5: Current and past approaches used for drug repurposing adapted from Dhir et al (2020).

There are currently in excess of 46 approved drugs which have been repurposed for new therapeutic uses (Farha *et al*., 2019). Examples include Acetylsalicylic acid which is one of the oldest examples in modern medicine, originally purposed and marketed as an analgesic in 1899. Later, acetylsalicylic acid was associated with many clinical treatments including the reduction of inflammation, antiplatelet aggregation and decreasing the risk of developing various cancers (Jourdan *et al*., 2020). Sildenafil originally gained FDA approval for use as an antianginal drug and was later repurposed as an erectile dysfunction treatment (Dhir *et al*., 2020).

Two techniques that have been heavily utilised in the pursuit of new antimicrobials are high throughput screening (HTS) and gene expression profiling. HTS of chemical libraries of failed

drugs is a technique that is commonly used in the pharmaceutical industry. This technique is often utilised due to the rapid growth of bacteria and the inexpensive nature of the technique. It is not uncommon for 100,000 compounds to be rapidly screened for antimicrobial activity leading to a hit to lead approach, so that compounds that produce antimicrobial effects can be further studied and refined (Zhu *et al*., 2013). HTS has led to promising discoveries including a novel antimicrobial mechanism against *E. coli* produced by 2-Pyrazol-1-yl-thiazole derivatives (Ivanenkov *et al*., 2019). Gene expression profiling has come to the forefront of repurposing in recent years, and the lower cost of RNA sequencing has given rise to gene expression databases combined with an increase in computational processing; gene expression profiling has become one of the most cost-effective and productive strategies. The use of gene expression profiling has led to discoveries of topiramate in the treatment of inflammatory bowel disease and the use of sirolimus for glucocorticoid-resistant acute lymphocytic leukaemia (Corsello *et al*., 2017).

The repurposing of drugs into antimicrobials has proven to be a successful endeavour, particularly for parasitic, protozoal, viral and fungal diseases. Examples include chlorcyclizine that was originally developed as an allergy medication but repurposed as an antiviral agent. Sertraline which was originally developed as an antidepressant was later repurposed as an antifungal treatment. More recent discoveries have undergone initial testing including the anticancer drug Mitomycin C which demonstrated potent antimicrobial activity against MDR Gram-negative bacteria (Domalaon *et al*., 2019). Weber *et al*. (2020) tested the cationic dye used as a stain for electron microscopy know as Ruthenium red as an antimicrobial. Ruthenium red produced an MIC of 0.004 μg/mL against *K. pneumoniae* indicating its potential as an antimicrobial agent (Weber *et al*., 2020).

1.7 Aims and Objectives

With AMR increasing at an ever increasing rate, alternatives to current antimicrobials are urgently required. There has been extensive development and testing of Ru complexes in the field of oncology, with many Ru complexes showing promising results. Ru complexes possess many physical and chemical properties that make ideal candidates for the development of inorganic antimicrobials. The aim of this PhD project was to identify a selection of Ru complexes with biologically active ligands and assess their antimicrobial potential against a selection of clinically significant bacteria. The Ru complexes with the most potent antimicrobial activity were further assessed to determine potential cellular targets. Resistance to key Ru complexes was induced in bacterial strains and genome comparisons were undertaken to determine along with gene regulation studies.

1.7.1 Objectives

1) To quantify the antimicrobial effects of 12 selected compounds against 11 bacterial species and strains.
2) To determine the cellular target of Ru complexes with the most effective antimicrobial Ru complexes.
3) Induce resistance to an Ru complex to a species that was previously susceptible.
4) To investigate the genetic response to the Ru complexes focusing on gene regulation and genomic mutations.

Chapter 2: Materials and Methods

2.1 Bacterial Strains and Growth Conditions

The bacteria species and strains were selected for the study to give a broad overview of the antimicrobial activity of Ru complexes. Clinical significance, antibiotic resistance profiles and laboratory availability were all taken into account. All Bacterial strains were stored at -80°C prior to culturing on cationic adjusted Mueller Hinton-2 (MH), Tryptic soy (TS) or Brain heart infusion (BHI) agar or broth (Oxoid, Basingstoke UK) (Table 2. 1). Cultures were incubated at 37°C for 24 h, with agitation at 180 rpm for broth cultures only. Fresh isolation plates were prepared every 2 weeks. All OD_{600} reading were conducted using Jenway 6305 Spectrophotometers (Jenway, Staffordshire UK).

Table 2. 1: Bacterial strains and culture media.

Gram status	Genus and species	Strain	Culture Medium
Positive	*Staphylococcus aureus*	USA300:JE2	Mueller-Hinton
	Staphylococcus aureus	USA300	Mueller-Hinton
	Micrococcus luteus	15307	Mueller-Hinton
	Micrococcus luteus	2665	Mueller-Hinton
	Lactobacillus lactis	6681	Tryptic soy
	Enterococcus faecalis	12697	Brain heart infusion
	Enterococcus faecium	7171	Brain heart infusion
	Listeria monocytogenes		Tryptic soy
Negative	*Pseudomonas aeruginosa*	PAO1	Mueller-Hinton
	Escherichia coli	MG1655	Mueller-Hinton
	Escherichia coli	EC958	Mueller-Hinton

2.2 Antimicrobial Compounds and Preparation

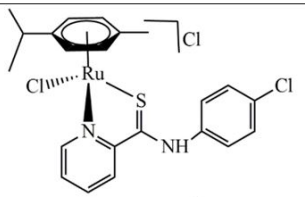
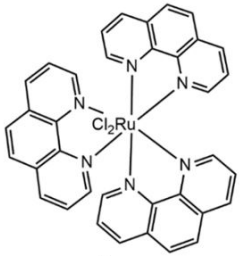
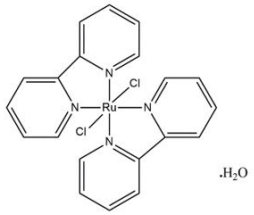
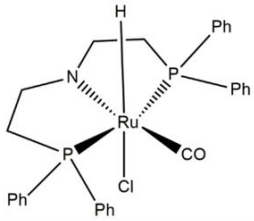
Ru complex 1, [Chlorido(η6-p-cymene)(N-(4-chlorophenyl)pyridine-2-carbothioamide)ruthenium(II)] chloride was synthesized and kindly provided by Professor C. Hartinger (University of Auckland, NZ).

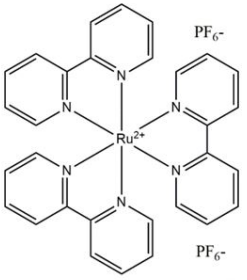
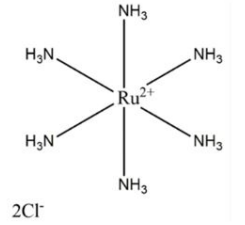
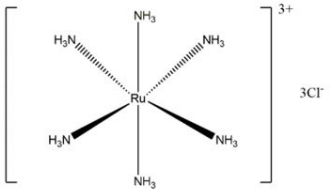
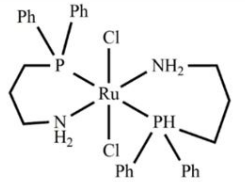
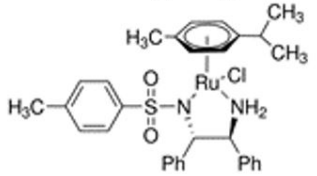
Briefly, Ru complex 1 synthesis was conducted according to Arshad *et al*., (2017). Synthesis utilised a general procedure with substitutions *N*-(4-clorophenyl)pyridine-2-carbothioamide (100 mg, 0.40 mmol) and $[Ru(cym)Cl_2]_2$ (122 mg, 0.20 mmol). A solution of $[Ru(cym)Cl_2]_2$ in dry dichloromethane (DCM) was added to a stirred solution of carbothioamide ligand in dry Tetrahydrofuran (THF). The reaction mixture was stirred for 4 h at 40°C under a nitrogen atmosphere. A change in colour from brown to deep red was observed immediately after the addition of dimer. The solvent was evaporated and the residue was dissolved in a minimal volume of DCM, followed by addition of *n*-hexane that resulted in immediate precipitation. After stored overnight at 4°C, the precipitate was filtered, and dried under reduced pressure. Yield: 77% (171 mg, red solid). Elemental analysis found: C, 48.63; H, 4.24, N, 4.97, calculated for $C_{22}H_{23}Cl_3N_2RuS \cdot 0.15C_6H_{14}$: C, 48.44; H, 4.46; N, 4.93. ^{1}H NMR (400.13 MHz, d_4-MeOD, 25 °C): δ = 9.63 (d, ^3J = 6 Hz, 1H, H-1), 8.40 (d, ^3J = 8 Hz, 1H, H-4), 8.25 (t, ^3J = 8 Hz, 1H, H-3), 7.81 (t, ^3J = 7 Hz, 1H, H-2), 7.56 (m, 4H, H-9/H-11/H8/12), 6.02 (d, ^3J = 6 Hz, 1H, H-15), 5.92 (d, ^3J = 6 Hz, 1H, H-17), 5.87 (d, ^3J = 6 Hz, 1H, H-18), 5.61 (d, ^3J = 6 Hz, 1H, H-14), 2.73 (sept, ^3J = 6 Hz, 1H, H-21), 2.20 (s, 3H, H-19), 1.20 (d, ^3J = 6 Hz, 3H, H-20), 1.13 (d, ^3J = 7 Hz, 3H, H-22) ppm. ^{13}C{^{1}H} NMR (100.61 MHz, d_4-MeOD, 25 °C): δ = 159.9 (C-1), 155.5 (C-5), 140.9 (C-3), 139.8 (C-7), 134.9 (C-10), 130.8 (C-9/C-11), 130.5 (C-2), 127.6 (C-8/C-12), 125.1 (C-4), 107.1 (C-16), 105.2 (C-13), 89.2 (C-15), 89.1 (C-17), 86.5 (C-18), 84.8 (C-14), 32.4 (C-21), 22.9 (C-20), 21.9 (C-22), 18.8 (C-19) ppm. MS (ESI$^+$): *m/z* 483.0236 $[M - 2Cl - H]^+$ (m_{ex} = 483.0231) (Arshad *et al*., 2017). All other

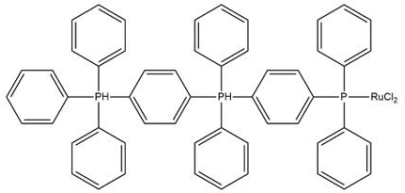
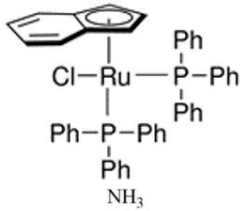
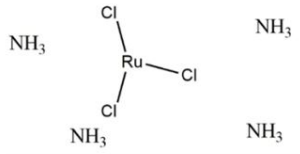
compounds were obtained from Sigma Aldrich (Poole, UK) unless otherwise specified. Compounds were dissolved in either Dimethylsulfoxide (DMSO) ethanol Sigma Aldrich (Poole, UK), or distilled water (Table 3). All compounds were stored in ultra-violet resistant, glass vials (Ossila, UK) and wrapped in tinfoil, then stored at 4°C to prevent degradation. The stability of stock solutions was assessed overtime by confirming antibacterial activity using MIC and MBC protocols (2.5-2.6), and no evidence of degradation was observed over a two-week period.

Prior to initial testing each Ru complex was screened utilising Nephelometric assay with a range of solvents including; Dimethylformamide (DMF), methanol, ethanol, DMSO and water, with the most compatible solvent selected. The Ru complexes were also tested with 10% PEG 400 co solvent in an attempt to increase solubility where necessary.

Table 2. 2: Ru complexes with scientific name, molecular formula, weight and structure, along with respective solvents.

Associated Ru complex number	Scientific name	Molecular formula	Molecular weight	Molecular structure	Solvent
1	[Chlorido(η6-p-cymene)(N-(4-chlorophenyl)pyridine-2-carbothioamide)ruthenium(II)] chloride	$C_{22}H_{23}Cl_3N_2SRu$	554.92		DMSO
2	Dichlorotris(1,10-phenanthroline)ruthenium(II) chloride	$C_{36}H_{24}Cl_2N_6Ru$	712.59		DMSO
3	cis-Bis(2,2′-bipyridine) dichlororuthenium(II)	$C_{20}H_{16}Cl_2N_4Ru \cdot xH_2O$	484.34		DMSO
4	Carbonylchlorohydrido{bis[2-(diphenylphosphinomethyl)ethyl]amino}ethyl]amino}ruthenium(II)	$C_{29}H_{30}ClNOP_2Ru$	607.03		Ethanol

5	Tris(2,2′-bipyridine)ruthenium(II) hexafluorophosphate	$C_{30}H_{24}F_{12}N_6P_2Ru$	859.55		DMSO
6	Hexaammineruthenium(II) chloride	[Ru(NH3)6]Cl2	273.15		Water
7	Hexaammineruthenium(III) chloride	$[Ru(NH3)6]Cl_3$	309.61		Water
8	Dichlorobis(3-(diphenylphosphino)propylamine)ruthenium(II)	$C_{30}H_{36}Cl_2N_2P_2Ru$	658.54		DMSO
9	[*N*-[(1*S*,2*S*)-2-(Amino-κ*N*)-1,2-diphenylethyl]-4-methylbenzenesulfonamidato-κ*N*]chloro[(1,2,3,4,5,6-η)-1-methyl-4-(1-methylethyl)benzene]-ruthenium	$C_{31}H_{35}ClN_2O_2RuS$	636.21		DMSO

10	tris(triphenylphosphine)ruthenium(ii) dichloride	$[(C_6H_5)_3P]3RuCl_2$	958.83		DMSO
11	Chloro(indenyl)bis(triphenylphosphine)ruthenium(II)	$C_9H_7 \cdot RuCl \cdot 2(C_{18}H_{15}P)$	776.25		DMSO
12	Pentaamminechlororuthenium(III) chloride	$[Ru(NH_3)_5Cl]Cl_2$	292.58		Water

2.3 Bacterial Resistance Generation to Ru Compounds

Bacterial cultures were grown overnight in MH broth and adjusted to an Optical Density of 0.005 at 600nm (OD_{600}) using sterile broth. Multiple wells of a sterile 96-well flat-bottom plate (Sarstedt, Germany) were inoculated with 200 µL of adjusted bacterial culture. A concentration of 10 µg/mL added to 3 wells and 3 wells were used to establish controls without an Ru complex the plate was incubated overnight at 37°C. Subsequently, each well was individually sub-cultured using an isolation streak plate method and incubated overnight at 37°C. Single colonies were then added to a further 96-well flat-bottom plate containing 10 x 200 µL MH broth supplemented with incrementally increasing concentration of the target Ru complex. This was repeated until no growth was recorded or the compound had visibly reached its solubility threshold.

2.4 Disc Diffusion Assay

Protocols were followed in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST., 2021). Bacterial cultures were grown overnight and adjusted to an OD_{600} of 0.5. Aliquots of 100 µL were applied to 25 mL of Mueller Hinton Agar. Petri dishes containing agar were divided into 8 equal sections. Aliquots of 20 µL per complex were added to antimicrobial susceptibility discs **(**Thermo Scientific, UK**)** and aseptically positioned onto an individual section. The zone of inhibition (ZoI) produced by each compound against the respective bacterial species was determined using digital callipers. results. Statistical significance was determined using two-way ANOVA with Tukey's post hoc analysis. A value of $p<0.05$ was considered to indicate statistical significance.

2.5 Minimum Inhibitory Concentration (MIC) assay

MIC values were determined using a broth micro-dilution assay which were performed in triplicate using sterile 96-well flat-bottom plates (Sarstedt, Germany). Bacterial cultures were grown overnight and adjusted to an OD_{600} of 0.005 using appropriate broth media, giving a final cell concentration of 2.3 x 10^6 Colony-Forming Unit (CFU mL^{-1}). Viability was confirmed using the Miles and Misra technique. Complexes solubilised in Dimethylsulphoxide (DMSO) had a final DMSO well concentration of 5%. The starting concentration for all compounds was 1024 µg mL^{-1} (in well 11), which was serially diluted to 2 µg mL^{-1} (in well 3). All plates were incubated at 37°C for 24 h and the MIC results were recorded as the lowest concentration which inhibited visible growth of the bacterial species (Andrews *et al*., 2001). Appropriate controls were included using bacteria only, bacteria with solvent only at relevant concentrations (as shown in Table 3), solvent only and media only as a negative control.

2.6 Minimum Bactericidal Concentration (MBC) assay

Following MIC analysis, MBC values were determined using a 96 well multi-replicator (Sigma-Aldrich, UK) by transferring inoculated samples from the wells to the appropriate agar medium. These were incubated for 24 h at 37˚C and the lowest concentration of the compound that recorded a 100% bactericidal effect was recorded as the MBC. Each assay was performed in triplicate.

2.7 Growth Dynamics

Bacterial growth curves were conducted using a microplate growth assay technique, which utilised 96-well flat-bottom plates. All growth curves were performed in triplicate. Bacterial cultures were grown overnight and adjusted to an OD_{600} of 0.005 using appropriate broth media. Complexes were added at a scale ranging from lower than the MIC to above MBC concentrations (Table 4-5), resulting in final well volumes of 200 µL. Plates were incubated at 37 °C in a BMG SPECTROstar Nano (BMG LABTECH Ltd, UK) with plates agitated for 20 s prior to measurements at OD_{600} being taken at 10 min intervals over a 24 h period. To determine the end point viability, Miles and Misra techniques were conducted. Statistical significance was determined using two-way ANOVA with Tukey's post hoc analysis and multiple comparisons at the 24 h time point.

2.8 Time-kill kinetics Assay

Bacterial cultures were grown overnight and adjusted to an OD_{600} of 0.005 using appropriate broth media (Table 2. 1). Ru complexes were added at one concentration below MBC, MBC and one concentration above MBC for relevant bacterial cultures with appropriate controls (no Ru complex and solvent only) (2.6). Cultures were incubated at 37°C with agitation at 150 rpm. Bacterial viability was determined at intervals of 0 h, 2 h, 4 h, 6 h and 24 h using serial dilution and Miles and Misra techniques. Each time-kill kinetics assay was performed in triplicate. Statistical significance was determined using two-way ANOVA with Tukey's post hoc analysis and multiple comparisons at the 24 h time point. A value of $p<0.05$ was considered to indicate statistical significance.

2.9 Haemolytic Assay

Ten mL of defibrinated horse blood (TCS Biosciences Ltd, UK) was aliquoted into a sterile universal and centrifuged at 1000 RCF for 10 min. The supernatant was removed and 5 mL of PBS was added to the solution until fully re-suspended. Erythrocytes were further washed three times at 300 RCF. 96-well V-Bottom plates (Sarstedt, Germany) were prepared with 150 µL of PBS for each assay, supplemented with 50 µL of washed horse erythrocytes. Subsequently, 1 µL was removed and replaced with 1 µL of target compound to produce final concentrations equivalent to MIC and MBC protocols (2.6). A positive control of 0.1% Triton X-100, a negative control and solvent only controls were included. Plates were incubated at 37°C for 1 h, followed by centrifugation at 1000 RCF for 10 min at 4°C. Supernatants were removed and the absorbance was determined at 540 nm using a Multiskan Go microplate reader (Thermo scientific, UK). Each assay was performed in triplicate and Statistical significance was determined using one-way ANOVA with Tukey's post hoc analysis and multiple comparisons with Brown-Forsythe and Welch Tests.

2.10 Resuscitation of Eukaryotic Cells and Cell Culture

HeLa (passage 14) (ATCC® CCL-2) were rapidly defrosted in a water bath at 37°C and inoculated into 10 mL of Eagle's Minimum Essential Medium (EMEM) ATCC® 30-2003, (ATCC, UK) supplemented with 10% Fetal bovine serum (FBS) (Lonza, UK) and 2% penicillin-streptomycin (Lonza, UK). The cells were centrifuged at 300 relative centrifugal force (RCF) for 3 min. The supernatant was removed, and the cell pellet was resuspended in 10 mL of Supplemented EMEM in T75 flasks (Sarstedt, USA). Cells were incubated at 37°C in 5% CO_2. HEK 293T cells (passage 8) (ATCC® CRL-3216) were resuscitated in the same manner but using 10 mL of Dulbecco's Modified Eagle Medium (DMEM) ATCC® 30-2002,

(ATCC, UK) supplemented with 10% FBS and 2% penicillin-streptomycin and cultured at 37°C in 5% CO_2.

Culture passages were performed using trypsin (Lonza, Belgium) by a process of trypsinization. When confluence reached 90%, cell viability was tested using by mixing 50 µL of trypan blue (Fisher Scientific, UK) with 50 µL of culture media containing the appropriate cells then measuring using a haemocytometer. A 90% cell viability was used to conduct experimentation. Culture media was removed from flasks, and cells were washed three times with phosphate-buffered saline (PBS) (Oxoid, UK). The cells were incubated with 2.5 mL of 1% trypsin for 3 min, and the reaction was stopped by adding 10 mL of either DMEM or EMEM cell dependent medium. The solution was transferred to 15 mL falcon tubes and centrifuged at 300 RCF for 3 min. The supernatant was discarded, and the cells were resuspended in 10 mL of appropriate supplemented medium. All microscopy was conducted on a GXM-XDS-5 Phase Contrast microscope (Gtvision, UK), and images were captured on a GXCAM-U3-5 (Gtvision, UK).

2.11 Cell Viability (MTS) Cytotoxicity Assay

HEK 293T and HeLa cells were grown to 70% confluence and over 90% viability. Cells were seeded into 96-well Tc plates (Sarstedt, Germany) at 5×10^4 cells per well in 100 µL of appropriate supplemented media. HeLa and HEK 293T cells were left to adhere for 24 h and 48 h respectively. Prior to experimentation, penicillin-streptomycin free media supplemented with 2% FBS was applied to the cells. Target compounds were added at a scaled concentration. Established controls were 50% ethanol as a positive control for cell viability inhibition, untreated cells and cells treated with 1% solvent. Cells were incubated at 37°C in 5% CO_2 for 2 h, 4 h, 8 h and 24 h. Cells were washed with FBS and penicillin-streptomycin free medium

twice and then re-suspended in fresh medium. Then 10 µL of CellTiter 96® AQueous One Solution Cell Proliferation Assay kit [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) (Promega Corporation, USA) was added to each well and left to incubate at 37°C in 5% CO_2 for 4 h. Colourimetric reactions were measured at 490 nm using a Multiskan Go spectrophotometer. The cytotoxicity tests and half maximal inhibitory concentration (IC50) was calculated in Prism GraphPad version 9.00 (GraphPad Software, USA) by removal of background absorbance then log transforming the concentrations and normalizing the absorbance values to 100% for negative controls and 0% for positive control for HeLa and HEK 293T. A non-liner regression of dose response inhibition was selected. Statistical significance was determined using two-way ANOVA with Tukey's post hoc analysis and multiple comparisons.

2.12 *In Vivo* Ru Complex *Galleria mellonella* Tolerance Assay

The *Galleria mellonella* were purchased from (Urban jungle, UK) freshly on the day of delivery. An average larval mass of 250 mg was obtained. Each experiment used 10 larvae and was repeated in triplicate with different biological batches. Ru complex 1 was solubilised in 10% DMSO with 90% sterile water to produce a compound concentration of 10 mg/Kg while Ru complex 7 was solubilised in water at 50 mg/Kg. Each larva was swabbed with 70% ethanol on the site of injection. Injections were conducted on the last rear proleg on the left side of the larvae in 10 µL volumes. The larvae were then incubated at 37°C and results of live dead counts were recorded at 24 h, 48 h and 72 h intervals. Date was plotted using a Kaplan–Meier survival curve.

2.13 Outer Membrane Permeabilization Assay

Bacterial cultures were grown overnight and adjusted to an OD_{600} of 0.1 and incubated for 4 h at 37°C with agitation at 180 rpm until reaching mid-log phase to an OD_{600} of 0.5. Cells were washed twice by centrifugation at 3000 RCF using a cell suspension buffer consisting of 5 mM sodium (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), 5 µm Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), 5 mM glucose adjusted to pH 7.2. A 0.5 mM stock solution of 1-N-phenylnapthylamine (NPN) was prepared in 10% acetone and 90% cell suspension buffer. All stock solutions were stored away from light to avoid degradation and used as freshly prepared.

Each assay consisted of a 3 mL total sample volume containing bacterial cells with a final NPN concentration of 10 mM in a quartz cuvette (Hellma analytics, UK). Target antimicrobial compounds at MBC concentrations were added at a 70 s interval whilst incubating at 22°C with stirring. Changes in fluorescence were recorded using an Agilent Cary eclipse (Agilent, USA) with λ_{ex} of 350 nm and λ_{em} recorded at 420 nm. Negative controls containing bacteria only, NPN only, and a positive control using 1% Triton X-100 were included. Statistical significance was determined using two-way ANOVA with Tukey's post hoc analysis.

2.14 Membrane Depolarisation

The membrane intercalating fluorescent dye, 3,3′-Diethylthiadicarbocyanine iodide ($DiSC_3(5)$ was used under low light conditions and prepared prior to use.

2.14.1 Cytoplasmic Membrane Depolarisation

For Gram-positive bacteria, cultures were grown overnight and cells were washed three times in DiSC3(5) buffer consisting of 5 mM $MgSO_4$ (Sigma-Aldrich, UK), 250 mM sucrose (Sigma-Aldrich, UK), and 10 mM potassium phosphate (Sigma-Aldrich, UK) (pH 7.0). The final pellet was resuspended in the DiSC3(5) buffer at an OD_{600} of 0.085 and a 1 μM final concentration of $DiSC_3(5)$ was added. Cells were incubated at 20°C for 10 min followed by a further 10 min at 37°C to establish a baseline. For Gram-negative bacteria, cultures were grown overnight and cells were washed three times in PBS. The final pellet was resuspended in buffer containing 20 mM glucose and 5 mM HEPES (pH 7.3) to an OD_{600} of 0.085 and $DiSC_3(5)$ was added at a final concentration of 1 μM. This assay was incubated for 60 min at 37°C to establish a baseline. For both Gram-positive and Gram-negative bacteria, antimicrobial complexes were added at 70 s at MBC concentrations for the respective bacterial strains. Changes in fluorescence were recorded using an Agilent Cary eclipse with an λ_{ex} of 620 nm and an λ_{em} at 670 nm. Experiments were performed at 37°C with stirring set to medium with fluorescence measurements taken at 10 s intervals over a 5 min period. The uncoupling agent, Carbonyl cyanide m-chlorophenyl hydrazine (CCCP) (Sigma-Aldrich, UK) was used as positive control for membrane depolarisation, along with bacteria alone as a negative control. Statistical significance was determined using two-way ANOVA with Tukey's post hoc analysis.

2.15 Reactive Oxygen Species H_2DCFDA Assay

Stock solutions containing 20 mM of the fluorogenic dye, 2′,7′-Dichlorofluorescin diacetate (H_2DCFDA) (Sigma-Aldrich, UK), were prepared in DMSO under low light conditions and stored at -18°C. Bacterial cultures were grown overnight and adjusted to an OD_{600} of 0.2 OD then incubated for 5 h at 37°C until reaching mid log phase to an OD_{600} of 0.5. Aliquots containing 200 µL of cells were added to black Nunc™ 96 microwell plate (ThermoFisher scientific, UK). H_2DCFDA was added from the stock solution to make a final well concentration of 20 µM. Antimicrobial compounds were added at MBC concentrations for the respective bacterial strain and incubated at 37°C for 30 min. Cells were washed in PBS (Oxoid, UK) three times at 3000 RCF for 5 min. Fluorescence was detected using an λ_{ex} of 485 nm and an λ_{em} of 535 nm using an Agilent Cary Eclipse 96 well plate attachment. Statistical significance was determined using two-way ANOVA with Tukey's post hoc analysis.

2.16 Cellular Uptake Study

Bacterial cultures were grown overnight and adjusted to 1 x 10^7 CFU mL^{-1} in appropriate media. Ru-based compounds were added at concentrations below MIC, at MIC and above MIC, along with DMSO and non-treated cells alone. Cells were incubated with agitation at 150 rpm at 37°C for 1 h. Cells were washed three times in 1 mL of PBS and all supernatants were retained for analysis. The final cell pellet was resuspended in 1 mL of 70% of HNO_3 (Sigma-Aldrich, UK) and incubated at 100°C for 1 h. The solution was left at room temperature for 24 h. The sample was diluted to 7% HNO_3 using sterile filtered water. All other supernatant samples were then adjusted to final concentration of 7% HNO_3. Samples were analysed for Ru metal content using an Agilent 7900 ICP-MS system (Agilent, USA). Statistical significance was determined using two-way ANOVA with Tukey's post hoc analysis and multiple comparisons. Statistical significance was defined as (p<0.05).

2.17 Scanning Electron Microscopy (SEM) Imaging

Overnight cultures were washed and resuspended into 5 mL Mueller Hinton broth to an OD_{600} of 0.5. The corresponding antimicrobial complex was added at MIC and MBC concentrations respectively, along with samples without antimicrobial compound as a negative control and a relevant antibiotic acting as a positive control. Cultures were then incubated at 37°C for 2 h with agitation at 180 rpm. Sterile 1 cm^2 silicon wafers (Platypus Technologies, USA) were prewashed in 99.998% ethanol and allowed to aseptically air-dry. Aliquots containing 25 µL of each culture was added to the washed sterile silicon wafers, aseptically air dried and fixed overnight at 4°C in 4% glutaraldehyde (diluted in 10 mM PBS) (Sigma-Aldrich, UK). Samples were dehydrated by exposure to increasing ethanol concentrations (10%, 30%, 50%, 70%, 90% and 100%) for 10 min at each concentration, followed by desiccation overnight at room temperature. Samples were mounted onto aluminium pin stubs (Agar Scientific, Stansted, UK) and sputter coated with gold to a 10 nm thick gold layer using a magnetron sputtering system (Polaron, Quorum Technologies, UK). Images were captured on a Supra 40VP scanning electron microscope (Zeiss, Germany) using SmartSEM software (Carl Zeiss Ltd, Germany). Each sample was repeated in triplicate and was captured at 25,000, 50,000 and 100,000 times magnification at Manchester metropolitan universities central facility. Images were used to compare the cellular morphological effect of each antimicrobial complex compared to untreated cells and against positive controls.

2.18 Extraction of pGEM®-3Zf(+) Plasmid

Plasmid extractions were carried out in accordance with the Qiagen spin miniprep kit protocol. Briefly, *E. coli* strain DH5α containing the pGEM®-3Zf(+) (Promega, UK) plasmid insert were grown in nutrient broth supplemented with ampicillin at a final concentration of 55 µg/mL.

Cultures were adjusted to 1 x 10^8 CFU/mL then centrifuged at 3000 RCF for 6 min. Pellets were resuspended in 250 µL of P1 buffer and transferred to a microcentrifuge tube. A volume of 250 µL of P2 buffer was added then inverted five times allowing thorough mixing. A volume of 350 µL of N3 was immediately added then inverted five times allowing thorough mixing, followed by centrifugation at 17500 RCF for 10 min. The supernatant was added to a QIAprep spin column and centrifuged at 17500 RCF for 60 s. A volume of 500 µL of buffer PB was added to the QIAprep spin column which was centrifuged at 17500 RCF for 60 s. A volume of 750 µL of buffer PE was added to the QIAprep spin column then centrifuged at 17500 RCF for 60 s. The QIAprep spin column was centrifuged for a further 1 min at 20000 RCF. A volume of 50 µL of buffer EB was added, then centrifuged at 17500 RCF for 60 s. The quantity and quality of the DNA were measured using NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, USA).

The restriction digest enzyme FastDigest *Eco*R1 (ThermoFisher scientific, UK) was used to cut the plasmid at base pairs 5-10. The extracted plasmid was stored at -80°C until use.

2.19 Electrophoretic Mobility Shift Assay

The effect of concentration on plasmid DNA was assessed over a 5 h period. Ru complexes at concentrations of 2 mM, 1 mM, 0.5 mM, 0.25 mM, 0.125 mM, 0.0625 mM were incubated with 10 ng/µL of the restriction digested pGEM®-3Zf(+) plasmid and incubated at room temperature for 5 h. Solvent only controls and plasmid DNA without compound were included in parallel for comparison.

Following exposure, DNA samples were subjected to electrophoresis using 0.7% agarose (Cleaver Scientific, UK) in Tris-acetic acid-EDTA buffer, at 80 V for 70 min. Agarose gels

was stained with 1 µL Midori Green (Nippongenetics, Germany) and visualised using a UV transilluminator.

2.20 RNA Extraction

RNA extraction protocol was adapted from Qiagen RNeasy Mini Kit (Qiagen, Germany). Bacterial cultures grown overnight were adjusted to an OD_{600} of 0.1 and incubated at 37°C with agitation at 180 rpm for 4 h until reaching an OD_{600} of 1.0. Cultures were then exposed to a target complex at MBC concentration or without treatment as a control and incubated at 37°C with agitation at 180 rpm for 1 h. Cultures were centrifuged at 3000 RCF for 5 min at 4ºC and the pellet was resuspended in 300 µL of DNA/RNA Shield (Zymo, USA). Samples were incubated at room temperature for 10 min, followed by centrifugation at 6000 RCF for 5 min. Pellets were re-suspended in 350 µL RLT Buffer with 1% β-mercaptoethanol (Sigma-Aldrich, UK), along with 350 µL of TE buffer with 20 µg/mL Lysozyme (Sigma-Aldrich, UK). The solution was added to a 2 mL Safe-Lock tube with 50 mg acid-washed glass beads (150–600 µm diameter) and homogenised using a FastPrep-24 5G for 2 min. A volume of 700 µL of 70% ethanol was added and the beads left to resettle. Supernatants were transferred to a RNeasy spin column with 2 mL collection tube and centrifuged for 20 s at 9000 RCF, repeating if required. The flow-through was discarded and 700 µL Buffer RW1 was added to the RNeasy spin column, which was centrifuged for 20 s at 9000 RCF. The flow-through was discarded and 500 µL Buffer RPE was added to the RNeasy spin column, which was centrifuged for 20 s at 9000 RCF. Again, the flow through was discarded and a further volume of 500 µL Buffer RPE was added to the RNeasy spin column, with centrifugation for 2 min at 9000 RCF. The RNeasy spin column was placed into a fresh 2 mL collection tube and centrifuged at 21000 RCF for 1 min to remove residual buffer. Finally, RNA was eluted from the RNeasy spin

column into a new 2 mL collection tube using 30 µL RNase-free water with centrifugation for 1 min at 10000 RCF. To maximise elution efficiency, the eluate was reapplied to the RNeasy spin column, which was centrifuged for 1 min at 10000 RCF. The quantity and quality of the RNA was measured using a NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, USA). The final eluate was stored at -80°C until use.

2.21 DNA Extraction

DNA extraction was carried out in accordance with the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Germany) protocol with some modifications. Briefly, bacterial cultures were grown overnight, adjusted to an OD_{600} of 0.8 and centrifuged at 3000 RCF for 5 min. The bacterial pellet was resuspended in 180 µL lysis buffer containing acid-washed glass beads (150–600 µm diameter) (Sigma-Aldrich, UK), 10 mM Tris-HCl (Fisher Scientific, UK), 100 mM NaCl, 1 mM Ethylenediaminetetraacetic acid (EDTA) (Fisher Scientific, UK), 5% [v/v] Triton X-100, adjusted to pH 8.0. Bacteria were incubated for 10 min at 37°C followed by vortex mixing for 5 min. A volume of 25 µL proteinase K and 200 µL Buffer AL was added prior to vortex mixing for 20 s. A volume of 200 µL 100% ethanol (Fisher Scientific, UK) was added and vortexed for 20 s. The supernatant was transferred to a DNeasy Mini spin column and placed in a collection tube then centrifuged at 7000 RCF for 1 min. The flow-through was discarded and the DNeasy Mini spin column was placed in a new collection tube. A volume of 500 µL Buffer AW1 was added and centrifuged at 7000 RCF for 1 min. The flow-through was discarded and the DNeasy Mini spin column was placed in a new collection tube. A volume of 500 µL Buffer AW2 was added and centrifuged at 20000 RCF for 3 min. The flow-through was discarded and the DNeasy Mini spin column was placed in a new microcentrifuge tube (Fisher Scientific, UK). A volume of 200 µL Buffer AE was added and incubated at room

temperature for 1 min followed by centrifugation at 7000 RCF for 1 min. DNA samples were eluted from the DNeasy Mini spin column using 50 µL Buffer EB. Spin columns were incubated at room temperature for 1 min then centrifuged at 7000 RCF for 1 min. The quantity and quality of the DNA was measured using a NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, USA). The final elute was stored at -80°C until use.

2.22 DNA Sequencing

The genomes were sequenced by MicrobesNG (Birmingham, UK) using the enhanced genome service to reduce gaps in sequencing. The service used Illumina HiSeq for short reads and Oxford Nanopore for long read sequencing data and created a hybrid assembly which was contig free compete assembly. Genomes were annotated with Prokka 1.14.3 (Seemann, 2014) using default settings and the genome comparison visualisation was constructed in CGView (http://cgview.ca/). Comparative genomes between *S. aureus* USA300 JE2 and *S. aureus* USA300 JE2 (IR) were assessed in Geneious prime 2012.2.2 using default setting with base quality score below 20 filtered to reduce false positive SNPs (Olson *et al*., 2015).

2.23 Competitive Binding Assay

Competitive binding assays were used to determine if the Ru complexes possessed DNA intercalating activity based on methods adapted from Cain *et al*. (1978). Black Nunc™ 96 microwell plates (ThermoFisher scientific, UK) were inoculated with 200 µg of UltraPure™ Calf Thymus DNA Solution (2000 bp) (ctDNA) (Thermo Scientific, USA), 1.2 µM of SYTO 9 (ThermoFisher scientific, UK) and nuclease free water, giving a final well volume of 190 µL. This was incubated in low light conditions at room temperature for 10 min to permit SYTO 9/DNA complex formation. Throughout the assay, the scanning speed was set to 600 nm/min

with the slit set to 5 nm and pmw set to high. All readings were recorded at 22°C with λ_{ex} of 480 nm and λ_{em} at 485-700 nm using an Agilent Cary eclipse (Agilent, USA). Immediately after reading, concentrations of 2 mM, 1 mM, 0.5 mM, 0.25 mM and 0.125 mM of Ru complex was added bringing each final well volume to 200 µL using a maximum of 7.5% total volume DMSO (Sigma-Aldrich, UK). Readings were measured at λ_{ex} of 480 nm and λ_{em} at 485-700 nm every 5 s for 10 min allowing time for Ru/DNA complex formation. Statistical significance was determined using two-way ANOVA with Tukey's post hoc analysis.

2.24 Reverse Transcriptase quantitative PCR (RT-qPCR)

All thermocycling was conducted on a Bio-Rad CFX96 Touch RT-PCR unit (Bio-Rad, USA). RNA extraction was conducted with *S. aureus* USA300 JE2 and *S. aureus* USA300 JE2 induced resistance. DNA was extracted using the DNA extraction protocol in Section 2.21. All assays were conducted using 20 µL reaction volumes and each experiment was conducted with three biological replicates.

Primers were designed using Primer-BLAST (National Centre for Biotechnology Information, USA). Each primer was designed to produce amplicon sizes between 80 to 200 bp in length with primer melting temperatures (Tm) between 57°C to 63°C (optimal 60°C). Primers were between 18 to 30 bp (optimal 20 bp) in size with primer GC content from 20 to 80% (Table 2. 3) and were synthesised by Invitrogen (ThermoFisher scientific, UK).

RT-qPCR was performed using a custom protocol utilising Brilliant III Ultra-Fast SYBR® Green RT-qPCR Master Mix (Agilent, USA) in a one-step reaction. Briefly, Agilent qPCR Semi-Skirted 96-Well Plates (Agilent, USA) were used throughout. All RNA stocks were diluted to 20 ng/µL DNA stocks were diluted into standards of 20 ng/µL, 10 ng/µL, 5 ng/µL, 2.5 ng/µL, 1.25 ng/µL, 0.625 ng/µL, 0.3125 ng/µL, 0.156 ng/µL, 0.078 ng/µL, 0.039 ng/µL,

0.0195 ng/µL. All Primers were adjusted to 5 pM/µL. For constituent makeup of each reaction see (Table 2. 4). Each reaction was conducted with 3 biological replicates.

All RNA samples were assessed on a bio analyser 2100 (Agilent) to verify the level RNA degradation and RNA purity prior to use. Each primer was subjected to an annealing temperature gradient (55-65°C) with a subsequent melt curve to produce optimal conditions and melt curves were used to confirm the samples free from gDNA and primer dimer contamination. A standard curve was constructed for each primer set and primer efficiencies were calculated using the equation $E = (10^{(1/slope)}-1) \times 100$, all primers were recorded above the minimum required 95 % efficiency. An assessment of potential reference genes was undertaken using *gyrA* and *recN*, the gene *gyrA* proved to be more stable across all experimental conditions and was therefore selected as the reference gene. Data analysis of relative gene expression was conducted using the 2–ΔΔCT (Livak) Method (Livak *et al.*, 2001). Statistical significance was determined using two-way ANOVA with Tukey's post hoc analysis and multiple comparisons. All amplicons sizes were validated on an electrophoresis gel (data not shown).

Table 2. 3: Primers used for RT-qPCR reactions

Gene	Direction	Sequence	Melting temperature (Tm)	Product size (bp)
gyrA	Forward	GCGGTAGGTATGGCAACGAA	59.5	190
	Reverse	CCGCCTCCACGTTCTTCAAT	60	
recA	Forward	GCTTCGGCGATTTCAAGACC	59.7	165
	Reverse	TAGCGCTTCACGCTATTGCT	60.2	
mprF	Forward	TTATTGGTGGTGGGGCATCG	60.2	173
	Reverse	CATTGCTCTAACGCCTGCAC	60.1	173
norA	Forward	TAGGACCAGGGATTGGTGGA	59.58	140
	Reverse	GAAGCCGCTTGTCGTAGACT	60.11	
fieF	Forward	GGATGATTTCGATCGGCAGG	58.8	104
	Reverse	GCTGTAAGAGCCGATGCATT	58.7	
katA	Forward	CTGGGATTTCTGGACGGGTC	60.1	150
	Reverse	ACCCAAACACGTTCACCAGA	59.7	
recN	Forward	GCAGCTTGTCCAGATACACC	58.63	122
	Reverse	ATTGCGTCAGGTGGAGAACTT	59.93	
umuC	Forward	TGTACTGTGGGCATTGGTTCT	59.6	148
	Reverse	CCCAAAAATCTCGCAAGGGC	60.1	
icaB	Forward	TCACAGGTCATGTTGGGGAA	58.9	119
	Reverse	ATGCAAATCGTGGGTATGTGT	58.3	
perR	Forward	TTGGAAAATCAGGTGAAAGTGCT	58	118
	Reverse	CGACAAGCAGGCGTAAGAA	58.2	
sasG	Forward	TGTACCCGTTTTTGGTCCGT	59.82	170
	Reverse	TCGGTGGCGAGAAAATACCG	60.46	
ccpA	Forward	ATAAGCGCATCCCTACTGCA	59.2	181
	Reverse	TGTATCAGCGACGAAGAAGCA	59.8	
sstD (yclQ)	Forward	ACCTAAAGGGGAAGGCGGTA	60.3	122
	Reverse	TTACTTCGGGTTTCGTCGCA	60	
sigB	Forward	TGGTCATCTTGTTGCCCCAT	59.6	178
	Reverse	AGCGTTCACCTTCTATCAGTGA	59.2	
clpP	Forward	GAAACAACAAACCGCGGTGA	59.9	148
	Reverse	TCTCTGAGTCTTGCGCTTGT	59.3	
dinB	Forward	GCAGGAAGGTCTGGTCTCAC	60	122
	Reverse	TGCGCAGATGTTTCTCGGTA	59.8	
msrA	Forward	CGGCTTTGTAAAATTGTGACGC	59.6	158
	Reverse	ATCAAGACCGTGGTCCTCAA	58.7	

Table 2. 4: Reagent volumes per 20 uL RT-qPCR reaction.

Component	Volume uL
Sybr green master mix	10
Forward primer	1.25
Reverse primer	1.25
DTT	0.2
RT / RNase block	1
Nuclease free H_20	1.3
RNA/DNA	5

Table 2. 5: PCR cycling program for all RT-qPCR experiments

Cycle	Duration of cycle (Min)	Temperature (°C)	Number of cycles
Reverse transcribe	10	50	1
PCR initial heat activation	3	95	1
Denaturing	0.05	95	39
Combined annealing/extension	0.05	60	
Denaturing	0.10	95	1
Melt curve	0.05	65	1

2.25 Software and Data Analysis

This thesis was constructed using Microsoft word 365. Data sets were stored in Microsoft excel 365. Graphs were created in Prism GraphPad version 9.00. The differences between control and treatments in these experiments were tested for statistical significance by one-way or two-way ANOVA with tukey's post hoc test in Prism GraphPad version 9.00. Absorbance reading were taken using a BMG SPECTROstar Nano utilising the BMG Labtech Reader Control and Data Analysis Software, MARS. Throughout experimentation excitation and emission were recorded in Agilent Cary WinFLR fluorescence software package and exported to excel.

Chapter 3: Evaluating the Antibacterial Activity of Ruthenium Complexes

3.1 Introduction

An estimated 700,000 global deaths are attributed to AMR annually (Blair *et al.*, 2015) and increasing at an astounding rate (Dadgostar *et al.*, 2019). By 2050, it is predicted that 10 million deaths annually will be attributed to AMR (O'Neill, 2014). With only a single new mechanism of activity for an organic antibiotic discovered in the past three decades, coupled with a decline in the discovery of new organic antimicrobials, there has been a renewed urgency for the development of new novel antimicrobials to treat AMR bacteria. (Ling *et al.*, 2015).

Metallodrugs developed over the last century have been deployed in an array of clinical applications, from salvarsan used as an antimicrobial to cisplatin routinely used in anticancer chemotherapy (Dasari *et al.*, 2014; Kolmer *et al.*, 1912). Modern metallodrugs that are developed for chemotherapeutic applications are now simultaneously tested for antimicrobial activity. Failed metallodrugs developed in the past were rarely tested for antimicrobial activity due to cost, time and little need for new antimicrobial compounds (Soo *et al.*, 2016). Recently, there has been renewed focus on the development of metallodrugs, with Ruthenium (Ru)-based complexes being pushed to the forefront of research. Ruthenium acting as a central metal atom offers many advantages over other elements, including lower biotoxicity compared to other central metal atoms, three biologically accessible oxidation states and three-dimensional bonding configuration (Roymahapatra *et al.*, 2015., Southam *et al.*, 2017).

It is fundamental to understand if any of the Ru complexes under investigation (Table 2.2) possess antimicrobial activity. Antimicrobial susceptibility can be determined using a range of techniques, including the MIC, MBC and disc diffusion assays as an initial *in vitro* high-throughput screen (Khan *et al.*, 2019). Ru complexes demonstrating potent antimicrobial activity are further examined for effects on bacterial growth kinetics and time-dependent bactericidal activity using time-kill kinetic assays. In clinical use, the MIC value is often

considered the primary pharmacodynamic endpoint, with the actual patient dosage being much higher to allow for absorption, distribution, metabolism, and excretion (Daley *et al*., 2018). There can be significant variations in MIC values not only between different antibiotics but also with different bacterial species and strains (Vali *et al*., 2004; Fair *et al*., 2014). The European Committee on Antimicrobial Susceptibility Testing (EUCAST) developed guidelines for defining a bacterial species as resistant, intermediate or susceptible to antimicrobials depending on the results of the MIC, and these are defined as the breakpoints (Macgowan *et al*., 2005). The ratio between the MIC and MBC can be used to determine if an antibacterial Ru complex is considered bacteriostatic or bactericidal. An antimicrobial is considered bactericidal if the ratio of the MBC value is no more than four times the MIC value in a 24 h period. When considering this as a percentage reduction, the generally accepted definition of a bactericidal agent is a ≥99.9% reduction in viable bacterial density in an 18–24-h period (French, 2006), while a bacteriostatic antimicrobial can be defined as a 90 to 99% reduction in a 24 h period (Pankey *et al*., 2004). In combination with the MIC and MBC assays, microbial growth and time kill kinetic assays provide a better understanding regarding the antimicrobial efficacy of the Ru complex.

The categorisation of the Ru complexes into broad-spectrum or narrow-spectrum band antimicrobials is also important, as the difference may determine the possible future applications for the antimicrobial. A broad-spectrum antibiotic is defined as an antibiotic that acts against a wide range of pathogenic bacteria or on the two major bacterial groups, such as Gram-positive and Gram-negative bacteria. A narrow spectrum antibiotic is active against a select group of bacterial types (Palmer *et al*., 1995). By testing a variety of bacterial species, including Gram-positive and Gram-negative bacteria, each Ru complex can be categorised. Sun *et al.* (2015) recently demonstrated their synthesised Ru complex $[Ru(phen)_2(tip)]$ $(ClO_4)_2$

acted as a broad spectrum antibacterial by being effective against the Gram-positive species *S. aureus* and the Gram-negative species *E. coli* (Sun *et al*., 2015).

Prior to animal-based modelling (preclinical studies) or patient clinical trials, it is important to study the possible physiological impact of antimicrobial Ru complexes. To do this, a series of *in vitro* techniques can be undertaken, including haemolytic and cytotoxicity assays that are able to mimic *in vivo* Ru complex interactions in an ethical and controlled environment which conforms to the 3R (replacement, reduction and refinement) principles. The use of haemolytic assays assesses *in vitro* drug-induced haemolysis by quantifying phospholipid bilayers rupturing of erythrocytes and the subsequent release of haemoglobin. This assay determines the haemolytic effect of a Ru complex, however, must be taken with caution as exceedingly rare drug-induced haemolysis can be fatal (Chen *et al.,* 2014). Ru complexes capable of permeabilization would show an increased lysis compared to Ru complexes that focus on other types of mechanistic binding. Malik *et al.* (2019) synthesised two Ru complexes to test for anti-tumour and antimicrobial properties: Ru(Cl)2(SB)(Phen] and [Ru(Cl)2(SB)(Bipy). These compounds, whilst lacking the amine group, both showed HD50 value of >100μg/mL; half of the recommended clinically used amount of the antitumour drug cisplatin, thus demonstrating the potential lower toxicity of Ru complexes (Malik *et al.* 2019). Determining the cytotoxic effects on eukaryotic cell lines allows for the quantification of Ru complex concentration that may cause adverse physiological reactions. If cytotoxic levels against eukaryotic cells are substantially higher than those of the prokaryotic cells then such compounds are unsuitable for progression to use within animals or humans (Dzoyem *et al.*, 2017, Pacor *et al.,* 2002). With AMR becoming more prevalent in clinical settings, antibiotics such as colistin and vancomycin that were previously considered too cytotoxic by producing adverse physiological reactions, are now routinely prescribed (Godoy *et al.,* 2016; Filippone *et al.,* 2017). Haemolytic and cytotoxicity assays are linked to a range of physicochemical properties, including membrane

composition. Research into the antimicrobial activity of Ru complexes has shown that Gram-positive bacteria are more susceptible than Gram-negative bacteria (Li *et al.,* 2012; de Sousa *et al.,* 2020; Matshwele *et al.,* 2020; Liao *et al.,* 2017). The susceptibility of Gram-positive bacteria is in part due to the peptidoglycan that antibiotics can bind to, while Gram-negative possess an outer membrane and subsequently have lower permeability. Furthermore, utilisation of efflux pumps is also an important factor in the ability of Gram-negative bacteria to resist antimicrobial compounds.

In this chapter, 12 Ru complexes were selected for repurposing and evaluation for use as antimicrobial agents. These complexes were previously used for a range of applications from electron acceptors in electrochemistry to failed chemotherapy drugs. The 12 Ru complexes selected contain ligands or structural characteristics which contribute towards proposed biological applications. This chapter evaluated and quantified the antibacterial efficacy of 12 Ru complexes as described in (Table 2. 2) against a range of bacterial pathogens (Chapter 2.1) using a series of *in vitro* methods including, micro-dilution MIC and MBC assays, disc diffusion assays, growth dynamics, time-kill kinetics, haemolytic and cytotoxicity assays. Out of the complexes selected, 11 have not previously been examined for antimicrobial activity, with only Hexaammineruthenium (III) chloride previously demonstrating antimicrobial activity in a single limited historical study against *B. subtilis* (Yasbin *et al.,* 1980).

3.2 Aims and Objectives

The aim of this chapter was to determine the antimicrobial efficacy of a series of Ru complexes and to identify the potential for progressing these as antimicrobial agents for use against clinically relevant bacterial pathogens. Lead compounds were selected for further characterisation to determine the effects on microbial growth, in addition to cytotoxicity profiling using representative mammalian cell line models.

3.3 Results

3.3.1 Disc Diffusion Assays

Disc diffusion assays were used as a preliminary method to determine antibiotic susceptibility. All species and strains were selected due to their clinical significance or as a reference standard to evaluate the differences between the AMR strains and non-AMR strains. It was fundamental that the compounds were tested against a wide variety of Gram-positive and Gram-negative bacteria. All concentrations displayed in section 3.4.1 were conducted at a concentration of 256 μg/mL. All assays were conducted in accordance with the guidelines of the EUCAST where applicable. The disc diffusion assay results were segregated into Gram-positive (Table 3.1) and Gram-negative (Table 3.2) results.

The results demonstrated that the Gram-positive bacteria were principally more susceptible to the Ru complexes in comparison to the Gram-negative bacteria. All Gram-positive species and strains showed susceptibility to at least one Ru complex. Gram-negative species were generally resistant to the majority of Ru complexes, with only Ru complexes 6, 7 and 12 being active.

The largest ZOI was produced by Ru complex 7 against *L. lactis* 6681 where a ZOI of 14.18±0.189 mm was observed, demonstrating a high level of susceptibility. This ZOI diameter was a mean 1.65 mm larger than the second largest ZOI of 12.53±1.73 mm which was also caused by exposure of Ru complex 7 against *M. luteus* 2665, no significance was shown (p>0.999). Ru complex 7 produced the largest ZOI against Gram-negative bacteria with 11.12±0.17 mm mean ZOI against *P. aeruginosa* PAO1. This was statistically significant compared to the second largest zone was produced by Ru complex 12 against *E. coli* 958 untreated with a mean ZOI of 8.08±0.78 mm (p<0.001). Ru complex 12 was the only Ru complex that exhibited antimicrobial activity against all three species of Gram-negative bacteria.

Ru complex 1 was the only sample to inhibit growth of all Gram-positive bacteria, with the largest ZOI observed against *L. lactis* 6681 with a diameter of 12.52±0.63 mm. an producing the largest ZOI against *S. aureus* USA300 JE2 at 11.4±0.57 mm.

When viewing ZOI results as aggregates to assess overall antimicrobial performance, Ru complex 1 produced the largest aggregate ZOI for Gram-positive species, totalling 82.24 mm over the eight Gram-positive bacteria tested, averaging a 10.28 mm ZOI. Ru complex 12 possessed a broad range of activity against Gram-negative bacteria producing a ZOI aggregate of 22.27 mm, averaging 7.423 mm across the three species tested. When comparing the aggregate antimicrobial activity of each Ru complex with Gram-positive species grouped, both Ru complex 3 and 10 showed no significance (p=0.717). Ru complexes 5 and 12 showed no significant deference in aggregate ZOI (p>0.999). All other compounds showed significance.

Ru complexes 5 and 11 were the only compounds to produce no antimicrobial activity against any of the 11 species and strains tested.

Table 3. 1: Zone of inhibition data for 12 Ru complexes applied at 256 µg/mL per disc on Mueller Hinton-2 agar against a selection of clinically significant Gram-positive bacteria after 24 h incubation. Mean ZOI values of three biological replicates were recorded in mm.

	Gram-positive bacteria species and strain															
	S. aureus USA300:JE2		*S. aureus* SH1000		*M. luteus* 15307		*M. luteus* 2665		*L. lactis* 6681		*E. faecalis* 12697		*E. faecium* 7171		*L. monocytogenes*	
Ru complex	Zone of Inhibition (mm)	Std Dev	Zone of Inhibition (mm)	Std Dev	Zone of Inhibition (mm)	Std Dev	Zone of Inhibition (mm)	Std Dev	Zone of Inhibition (mm)	Std Dev	Zone of Inhibition (mm)	Std Dev	Zone of Inhibition (mm)	Std Dev	Zone of Inhibition (mm)	Std Dev
1	11.4	0.57	10.8	0.38	10.06	0.29	12.04	2.34	12.52	0.63	8.58	0.14	7.12	0.81	9.18	0.62
2	8.14	0.24	7.21	0.81	6.92	0.14	6..28	0.24	9.62	0.11	0	0	0	0	6.31	0.19
3	0	0	0	0	0	0	0	0	9.42	0.26	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	7.61	1.82	6.76	0.3	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	11.62	0.81	9.38	0.28	0	0	0	0
7	0	0	0	0	11.13	1.36	12.53	1.73	14.18	0.189	0	0	0	0	0	0
8	0	0	0	0	6.77	0.14	0	0	8.86	0.32	0	0	0	0	0	0
9	8	0.75	6.12	0.61	7.51	0.35	6.83	0.93	9.88	0.15	6.44	0.33	0	0	0	0
10	0	0	0	0	7.12	1.11	8.14	0.52	7.01	0.31	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	7.66	0.39	0	0	0	0

Table 3. 2:Zone of inhibition data for 12 Ru complexes applied at 256 µg/mL per disc on Mueller Hinton-2 agar against a selection of clinically significant Gram-negative bacteria after 24 h incubation. Mean ZOI values of three biological replicates were recorded in mm.

	Gram-negative bacteria species and strain					
	P. aeruginosa PAO1		*E. coli 1165*		*E. coli* 958	
Ru complex	Zone of Inhibition (mm)	Std Dev	Zone of Inhibition (mm)	Std Dev	Zone of Inhibition (mm)	Std Dev
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	7.46	1.16	0	0	0	0
7	11.12	0.17	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0
11	0	0	0	0	0	0
12	6.83	1.41	7.36	1.25	8.08	0.78

3.3.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC assays were used as part of the initial screening of each antimicrobial Ru complex, prior to further data collection. The antimicrobial activity of 12 Ru complexes was examined against 11 bacterial species and strains. The MIC was defined as the lowest concentration of a compound that visually inhibited bacterial growth. MBC was defined as the lowest concentration of an antibacterial agent required for a total kill of the bacteria (Andrews, 2001). Ru complexes were considered bactericidal if MBC values were ≤ 4× MIC values and bacteriostatic if MBC values were > 4×MIC values (Levison, 2012).

The MIC and MBC results are summarised in Tables 3.3 and 3.4. All solvents controls resulted in no loss in cell viability at the 5% experimental concentrations (data not shown), indicating the selected Ru complex was responsible for the observed effects. Initial starting concentrations were determined via preliminary ZOI antimicrobial activity screening coupled with Ru complex solubility testing.

Table 3. 3: MIC / MBC values of 12 Ru complexes (µg/mL) in respective broths against a selection of clinically significant Gram-positive bacteria after 24 h incubation.

	Gram-positive bacteria species and strain															
	S. aureus USA300:JE2		*S. aureus* SH1000		*M. luteus* 15307		*M. luteus* 2665		*L lactis* 6681		*E. faecalis* 12697		*E. faecium* 7171		*L. monocytogenes*	
Ru complex	MIC µg/mL	MBC µg/mL	MIC µg/mL	MBC µg/mL	MIC µg/mL	MBC µg/mL	MIC µg/mL	MBC µg/mL	MIC µg/mL	MBC µg/mL	MIC µg/mL	MBC µg/mL	MIC µg/mL	MBC µg/mL	MIC µg/mL	MBC µg/mL
1	8	16	8	8	<1	8	<1	8	16	128	128	128	128	256	32	256
2	8	32	8	16	4	16	4	8	64	128	256	>256	128	>256	32	64
3	64	>1024	32	>1024	64	>1024	64	>1024	64	>1024	64	>1024	64	>1024	64	>1024
4	16	>1024	4	>1024	64	>1024	64	>1024	32	>1024	64	>1024	32	>1024	32	>1024
5	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
6	1024	1024	1024	1024	512	512	512	512	512	512	128	1024	128	1024	256	512
7	>1024	>1024	512	1024	2	2	4	4	512	512	>1024	>1024	>1024	>1024	8	16
8	>256	>256	>256	>256	4	16	4	16	32	64	64	64	64	>256	64	64
9	8	32	8	16	4	8	4	8	256	>256	128	>256	256	>256	32	64
10	>1024	>1024	>1024	>1024	16	32	16	32	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
11	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	64	64
12	1024	>1024	>1024	>1024	256	256	256	256	256	512	256	>1024	256	>1024	1024	>1024

Table 3. 4: MIC / MBC values of 12 Ru complexes (µg/mL) in respective broths against a selection of clinically significant Gram-negative bacteria after 24 h incubation.

	Gram-negative bacteria species and strain					
	P. aeruginosa PAO1		*E. coli* *1165*		*E. coli* 958 Untreated	
Ru complex	MIC µg/mL	MBC µg/mL	MIC µg/mL	MBC µg/mL	MIC µg/mL	MBC µg/mL
1	256	>256	128	>256	128	>256
2	>1024	>1024	>1024	>1024	>1024	>1024
3	>1024	>1024	>1024	>1024	>1024	>1024
4	>1024	>1024	>1024	>1024	>1024	>1024
5	>1024	>1024	>1024	>1024	>1024	>1024
6	32	1024	128	>1024	128	>1024
7	32	128	>1024	>1024	>1024	>1024
8	>256	>256	>256	>256	>256	>256
9	>1024	>1024	>1024	>1024	>1024	>1024
10	>1024	>1024	>1024	>1024	>1024	>1024
11	>256	>256	>256	>256	>256	>256
12	128	256	256	>1024	128	256

The MIC and MBC results were segregated into Gram-positive (Table 3. 3) and Gram-negative (Table 3. 4) bacteria respectively. Overall, Gram-positive bacteria showed a higher susceptibility to the Ru complexes at lower concentrations when compared to the Gram-negative species.

The results showed that Ru complex 1 possessed the most species diverse antimicrobial activity of the 12 Ru complexes examined (Table 3. 3 and 3. 4). Ru complex 1 was the only Ru complex that exhibited antimicrobial activity against all 11 bacterial species and strains tested. The most notable antibacterial activity of Ru complex 1 was against both strains of *M. luteus* and *S. aureus* (Table 3. 3), these produced MIC values of <1 μg/mL and 8 μg/mL with MBC values of 8 μg/mL 16 μg/mL respectively. The MIC to MBC concentration ratio of ≤ 4× MIC indicated that Ru complex 1 was primarily functioning as a bactericidal agent at these concentrations. The bacteria *M. luteus* 15307, *M. luteus* 2665 and *L. monocytogenes* showed the highest susceptibility to the Ru complexes collectively. Ru complex 7 was highly active against these species and strains, with MIC and MBC results of 2-2, 4-4 and 8-16 μg/mL respectively.

There were only 4 of the 12 Ru complexes that exhibited antimicrobial activity against Gram-negative species and strains. Ru complex 7 produced the highest antimicrobial activity against *P. aeruginosa* PAO1 with MIC and MBC values of 32 and 128 μg/mL respectively (Table 3. 4) indicating bactericidal activity. Ru complex 6 produced the same 32 μg/mL MIC against *P. aeruginosa* PAO1 but had a substantially higher MBC concentration of 1024 μg/mL indicating a bacteriostatic effect. Ru complexes 5 produced no antimicrobial activity against all 11 bacterial species and strains.

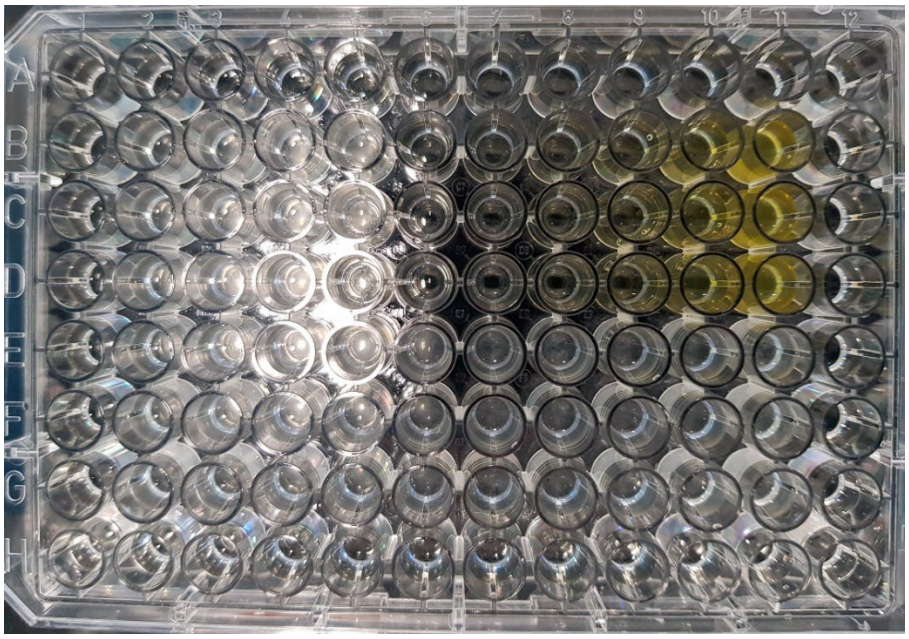


Figure 3. 1: Representative MIC for Ru complex 1 against *S. aureus* USA300 JE2 using broth micro-dilution method displaying concentrations 0.5 µg/mL (B2-D2) to 256 µg/mL (B11-D11). MIC (8 µg/mL) shown in wells B6 to D6. DMSO controls shown 5% (E11-G11) serial diluted to 0.0097% (E2-G2).

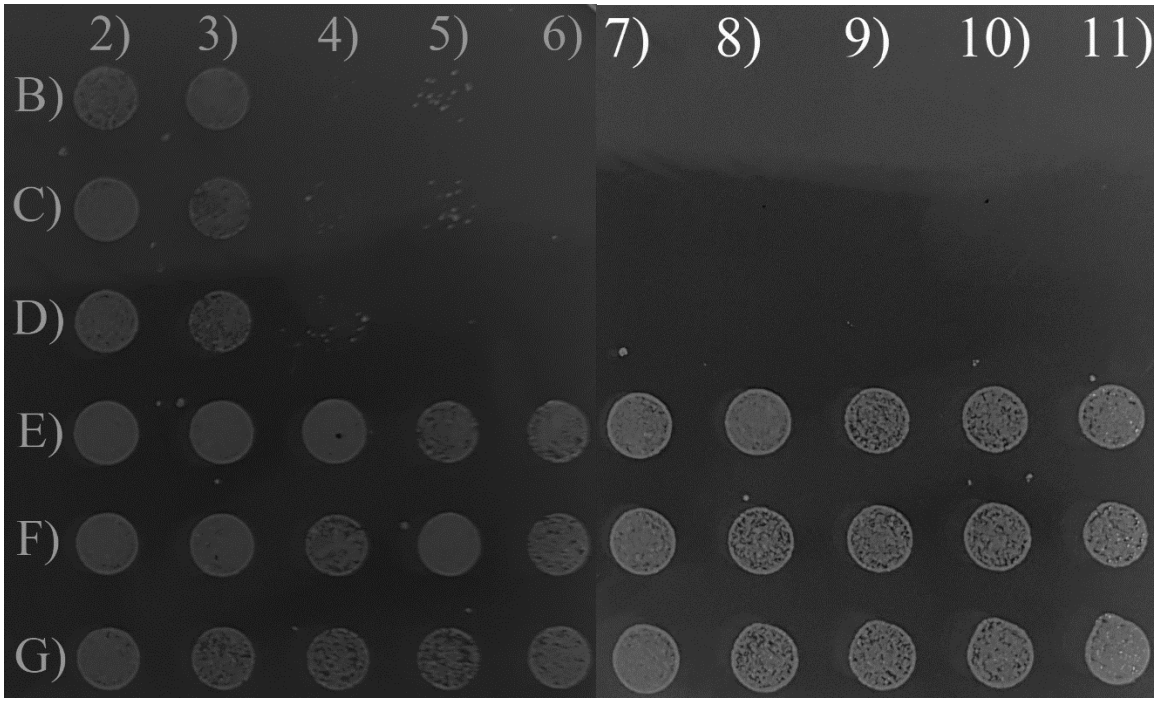


Figure 3. 2: MBC Ru complex 1 against *S. aureus* USA300 JE2 using a multireplicator displaying concentrations 0.5 µg/mL (column 2 row B, C, D) to 256 µg/mL (column 11 row B, C, D). No growth can be seen above 8 µg/mL (column 6 row B, C, D).

3.3.3 Growth Dynamics

Bacterial growth dynamics were used to assess the effect of varying concentrations of Ru complexes 1 and 7 on the growth kinetics of *S. aureus* USA300:JE2 and *P. aeruginosa* PAO1 over a 24 h period. The specific Ru complexes and bacteria species were selected due to the bactericidal effects of the respective Ru complex during the MIC and MBC experimentation (Table 3. 3, Table 3. 4). Viability at 0 h and 24 h was determined using Miles and Misra techniques.

The growth curve for Ru complex 1 against *S. aureus* USA300:JE2 (Figure 3. 3) was seeded at OD_{600} of 0.09 ($8.16x10^6$ CFU/mL). For all concentrations, there was no significant change in OD_{600} values between 0 h and 3 h indicating a bacteria growth lag phase ($P>0.05$). The solvent control showed no inhibition of growth compared to the negative control, with final bacterial viability values of $7.00x10^8$ and $6.81x10^8$ CFU/mL respectively. Zero viability of *S. aureus* USA300:JE2 was recorded at concentrations of 32 and 16 µg/mL, with no change in the OD_{600} 0.09 reading after 24 h was observed and a final 0 CFU/mL after 24 h was recorded. Concentrations of 2 µg/mL and 4 µg/mL showed little inhibition of *S. aureus* USA300:JE2 with final viability of $4x10^8$ and $1.83x10^8$ CFU/mL respectively. Concentrations of 8 µg/mL showed significant inhibition of growth compared to the negative control, with a final OD_{600} reading of 0.161 translating to a $1.10x10^7$ CFU/mL after 24 h ($p<0.0001$).

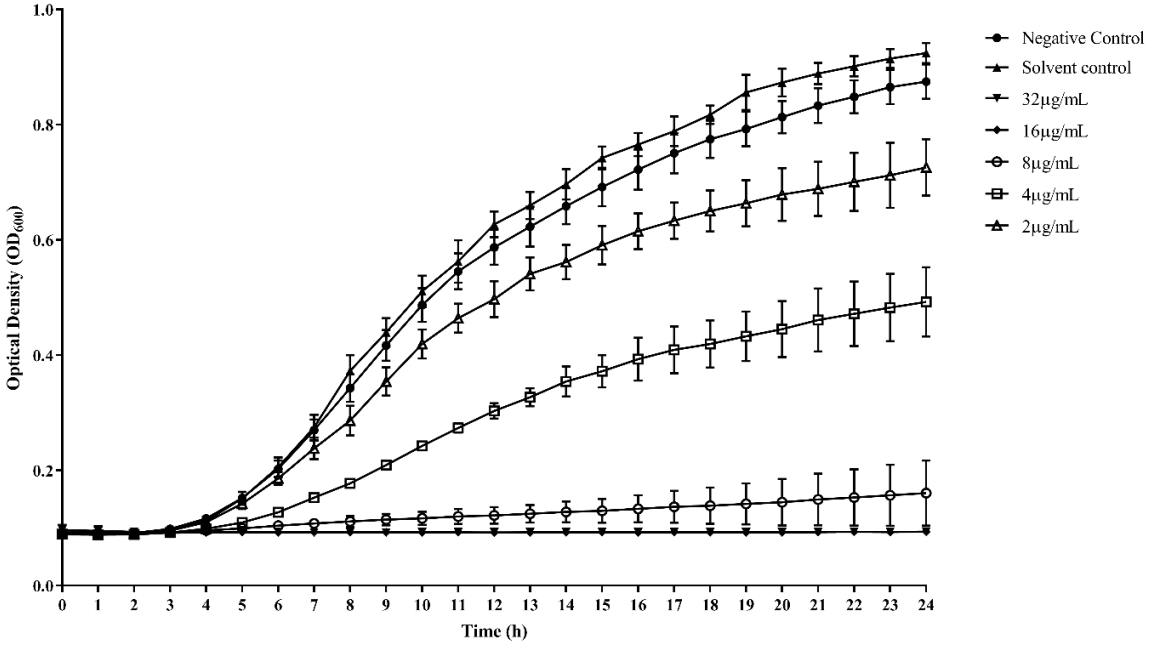


Figure 3. 4: Growth Curve Assay for Ru complex 1 against *S. aureus* USA300:JE2 at concentrations of 2, 4, 8, 16 and 32 µg/mL, solvent and negative control over a 24 h period. Error bars represent standard error of the mean (n=3).

The growth kinetics of Ru complex 7 against *P. aeruginosa* PAO1 was seeded at an OD_{600} of 0.091 or ($6.83x10^7$ CFU/mL). The solvent control had no effect on growth rate or viability after 24 h, when compared to the negative control with OD_{600} readings of 1.799 and 1.949 translating to $1.23x10^{10}$ and $1.18x10^{10}$ CFU/mL respectively. Each of the concentrations tested of Ru complex 7 inhibited growth of *P. aeruginosa* PAO1. MBC data was confirmed, with 128 µg/mL and 256 µg/mL concentrations achieving a total kill of *P. aeruginosa* PAO1. A concentration of 16 µg/mL inhibited growth until 18 h, with an OD_{600} 0.27 reading. At which point exponential growth began, producing an endpoint OD_{600} 0.96 equating to $5.22x10^8$ CFU/mL by 24 h. OD_{600} values for 32 µg/mL and 64 µg/mL showed growth inhibition for the duration of the growth kinetics with final viability of $9.22x10^7$ and $1.05\ x10^5$ CFU/mL respectively.

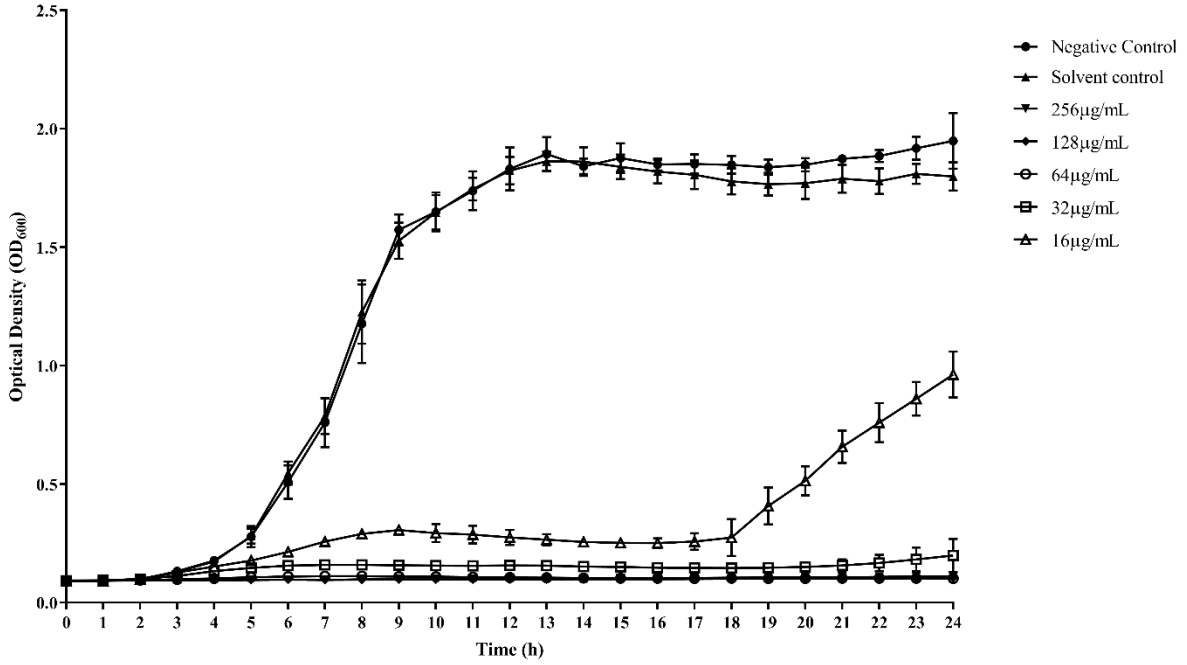


Figure 3. 5: Growth Curve Assay for Ru complex 7 against *P. aeruginosa* PAO1 at concentrations of 16, 32, 64, 128 and 256 µg/mL, solvent and negative control over a 24 h period. Error bars represent standard error of the mean (n=3).

3.3.3 Time-Kill Kinetics Assay

Time-kill kinetics were conducted in accordance with the Clinical and Laboratory Standards Institute (CLSI). A decrease of ≥ 3-log10 CFU/mL on the time-kill curves indicates a 99.9% killing rate and is considered bactericidal. A decrease of ≤ 3-log10 CFU/mL indicates a bacteriostatic concentration effect.

Ru complex 1 was selected for time kill kinetics due to its antimicrobial activity during the MIC and MBC experimentation (Section 3.3.2) against *E. faecium* 7171, *E. faecalis* 12697, *S. aureus* USA300 JE2 while Ru complex 7 was selected due to its activity against *P. aeruginosa* PAO1. All controls showed no significant difference over the negative controls (p>0.05) (Figure 3. 6). All time-kill kinetics data supported the micro well dilution MIC and MBC

values. For all species tested, exponential growth was recorded between 0 h and 4 h for negative and solvent controls, before entering the stationary phase between the 4 h and 24 h.

The time kill kinetics assay of Ru complex 1 against *S. aureus* USA300 JE2 (Figure 3. 5C) was seeded at $1.18x10^6$ CFU/mL and a total kill was achieved at 16 µg/mL and 32 µg/mL concentrations at 4 h and 2 h respectively with ≥ 3-log10 CFU/ml decreases between 0-2 h indicating a bactericidal effect. At the MIC concentration of 8 µg/mL, a ≤ 3-$\log_{10}$ decrease was observed indicating a bacteriostatic effect. At a concentration of 4 µg/mL, no significance was observed after 24 h compared to the negative control ($p>0.05$).

Ru complex 1 achieved a total kill of *E. faecium* at concentration of 256 µg/mL in a 6 h timeframe, while Ru complex 1 achieved at total kill of *E. faecalis* at a 128 µg/mL concentration over a 24 h time period. Ru complex 1 produced a bacteriostatic effect at all concentrations below MBC against *E. faecalis* and *E. faecium* with a < 3-$\log_{10}$ decrease in CFU/mL. At 32 µg/mL concentrations both *E. faecium* and *E. faecalis* increased in viability between the 4 h and 6 h time, with *E. faecium* showing a mean increase from $6.61x10^6$ CFU/mL to $2.52x10^7$ CFU/mL and *E. faecalis* showed a mean increase from $2.82x10^6$ CFU/mL to $1.32x10^7$ CFU/mL. The final viability at 32 µg/mL concentration had dropped from $2.17x10^7$ CFU/mL to $1.17x10^6$ CFU/mL and $4.29x10^6$ CFU/mL for *E. faecium* and *E. faecalis* respectively.

Ru complex 7 achieved a total kill of *P. aeruginosa* at concentrations of 128 µg/mL and 256 µg/mL after 24 h and 8 h respectively showing bactericidal effects. All concentrations below 128 µg/mL showed a bacteriostatic effect with ≤ 3-$\log_{10}$ decrease in viability. At 32 µg/mL concentration an increase in viability was observed between 6 h and 24 h with CFU/mL increasing from $5.00x10^6$ to $1.60x10^8$ CFU/ml.

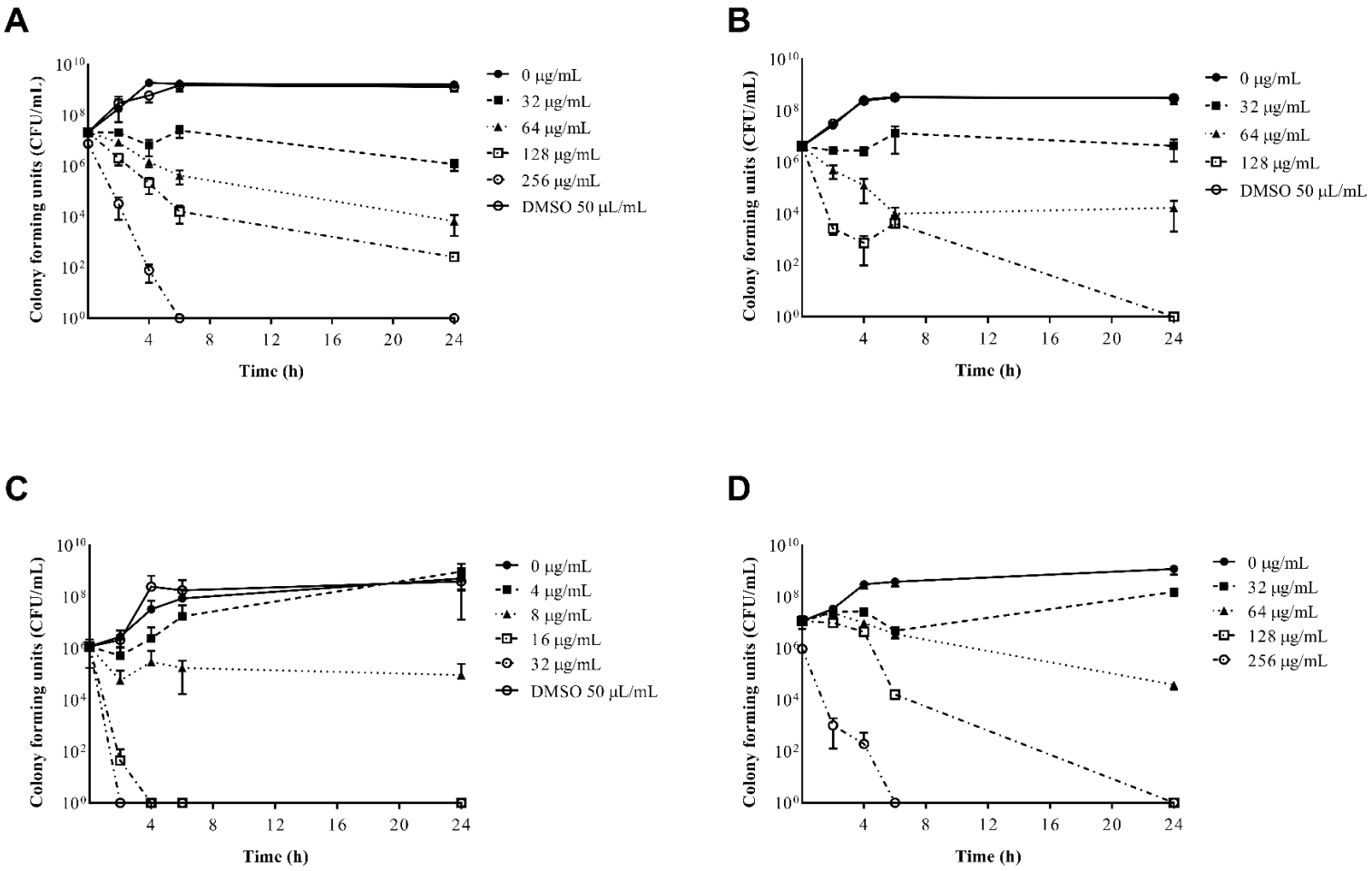


Figure 3. 7: Time-Kill Kinetics Assay over a 24 h period using Ru complexes 1 and 7 at varying concentrations against a selection of bacterial species. Samples were collected at 0,2,4,6 and 24 h. Error bars represent standard error of the mean (n=3). A) Ru complex 1 against *E. faecium* 7171 B) Ru complex 1 against *E. faecalis* 12697 C) Ru complex 1 against *S. aureus* USA300 JE2 D) Ru complex 7 against *P. aeruginosa* PAO1.

3.3.4 Haemolytic Assay

The haemolytic assay was used to assess the potential of the selected Ru complexes to lysis of erythrocytes/red blood cells (RBC). The RBC are also used as an analogue to more sophisticated mammalian cell membranes, allowing for a rapid initial toxicity assessment of each Ru complex. All Ru complexes were tested at concentrations ranging from 8 µg/mL to 1024 µg/mL, with the highest concentration used at MIC level for any single compound (Table 3. 3, Table 3. 4). Applied at lower concentrations, each Ru complex resulted in a reduced percentage of haemolysis in comparison to the data represented in (Figure 3. 6). Triton X-100 positive control was assumed to be 100% RBC lysis. Percent haemolysis was calculated using the equation:

$$\text{haemolysis } \% = \frac{\text{A540 nm of sample} - \text{A540 nm of buffer}}{\text{A540 nm of positive control} - \text{A540 nm of buffer}} \text{x100}$$

The DMSO and water solvent controls showed 1.01±1.21 SD and 0.22±0.04 SD percent lysis respectively. Ru complexes 1 to 12 showed significantly lower haemolytic activity in comparison to the 0.1% Triton X-100 positive control ($p<0.0001$).

Ru complex 8 showed the highest percentage haemolytic activity of all Ru complexes tested, 8.58±0.472% RBC lysis was observed at a concentration of 256 µg/mL, significance was observed over the solvent control was shown ($p=0.0291$). Ru complex 6 showed the lowest percentage of haemolysis of the 12 Ru complexes tested, with a 1.86±0.463% RBC lysis at a concentration of 1024 µg/mL being observed. This was significant compared to the positive control and not significant compared to the colistin control ($p<0.001$ and $p=0.9999$) respectively. The known permeabilising agent colistin was used as a comparable control, which produced a 5.82±0.48% haemolytic effect ($p<0.001$) in comparison to Triton X-100 positive control. Both Ru complex 1 and 7 showed no Significance when compared to colistin

(p=0.9396) and (p=0.3629) respectively. Only compounds 7, 8 and 12 showed significance RBC lysis over their respective controls with (p=0.031), (p=0.029) and (p=0.0208 respectively.

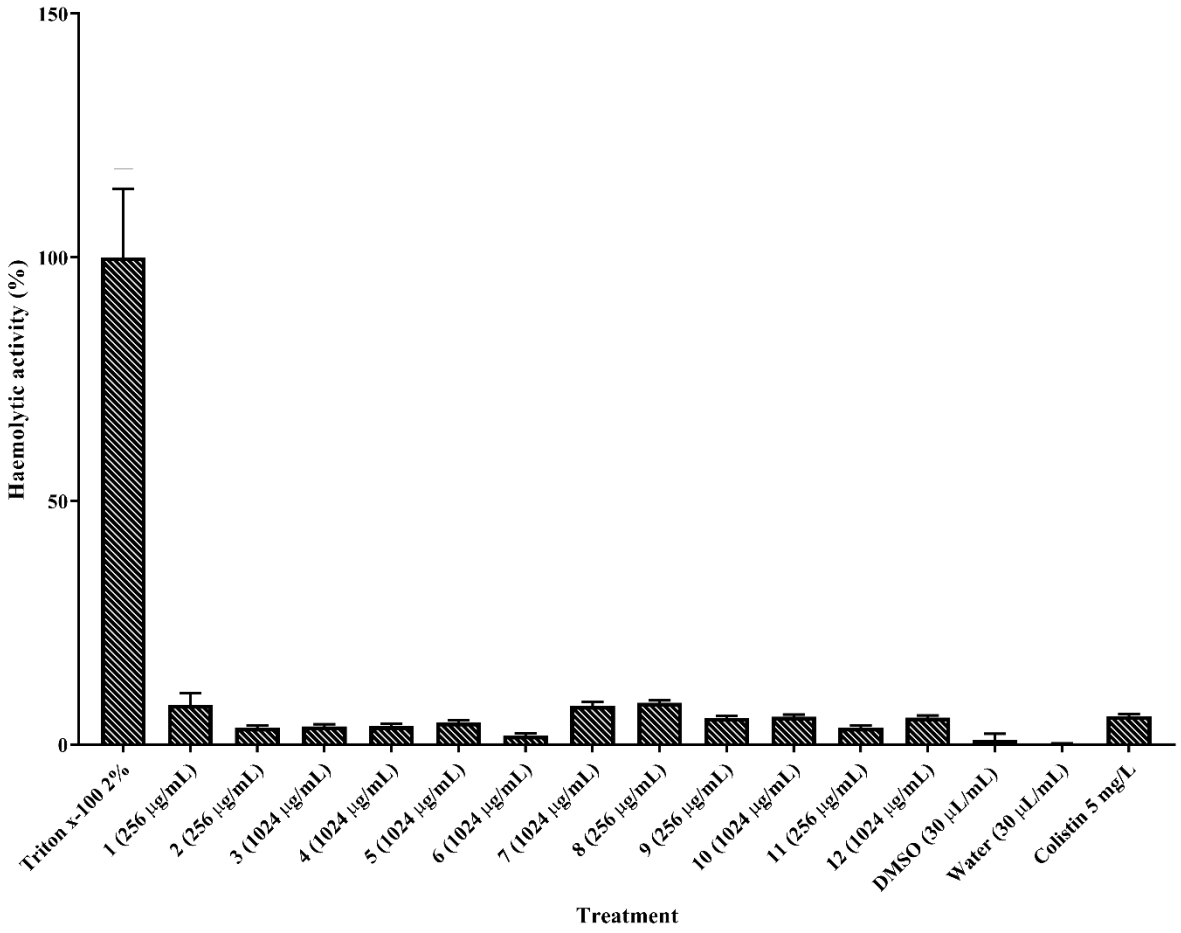


Figure 3. 6: Haemolysis of horse erythrocytes following 1 h exposure to Ru complexes 1-12 (Table 2. 2), solvent controls and colistin compared to a Triton X-100. Absorbance was measured at 540 nm and error bars represent standard error of n = 3.

3.3.5 Cytotoxicity Assay

Cytotoxicity assays were conducted on two distinct cell lines; HeLa and HEK 293T utilising Ru complexes 1 and 7. The Ru complexes were selected based on their potent antimicrobial activity against the key pathogens *S. aureus* USA300 JE2 and *P. aeruginosa* PAO1. Ru complex 1 was conducted over five time points at 0 h, 2 h, 4 h, 8 h and 24 h and Ru complex 7 was conducted over four time points at 0 h, 2 h, 4 h and 24 h (Figure 3. 7: Figure 3. 9). Dose response curves were plotted using the results of MTS cell proliferation assay.

3.3.5.1 Ru complex 1

Ru complex 1 showed varied, yet high levels of cytotoxicity on both cell lines and across all concentrations. Both HeLa and HEK 293T cells that were subjected to treatment with Ru complex 1 showed an extreme reduction in metabolic activity between the 0 h and 2 h, with the exception of HEK 293T cells treated at 1.44 µM concentration (Figure 3. 7 A). Using microscopy, both HeLa and HEK 293T cells also showed distinct morphological changes between the 0 h and 2 h time points (Figure 3. 8). The HEK 293T maintained 97.13±6.53% viability after 2 h, then decreased to 94.94±13.16% after 4 h, before rapidly decreasing in cell viability between the 4 h and 8 h. However, after 2 h exposure to the highest concentration of 46.13 mM, both HeLa and HEK 293T reduced cell viability by 90.20% and 79.56% (mean) respectively.

The HeLa cell line was more sensitive to the cytotoxic effects of Ru complex 1, with all cell viability counts falling below 25% for concentrations above 5.82 µM after 4 h and cell viability falling below 35% across all concentrations after 24 h exposure. Both cell lines were < 50% viable at all concentrations of Ru complex 1 after 24 h. The overall toxicity level was an order of magnitude higher for eukaryotic cells than prokaryotic cells, with exposure to 5.76 µM

resulting in only 4.69±1.55% cell viability after 24 h compared to the higher MBC for *E. faecalis* of 230.66 µM. Inhibitory concentration 50% *(*IC 50) values of 2.73 µM and 2.61 µM were determined after 8 h for HEK 293T and HeLa respectively. The DMSO solvent control showed no significant decline in cell viability for HEK 293T and HeLa (p=0.9999).

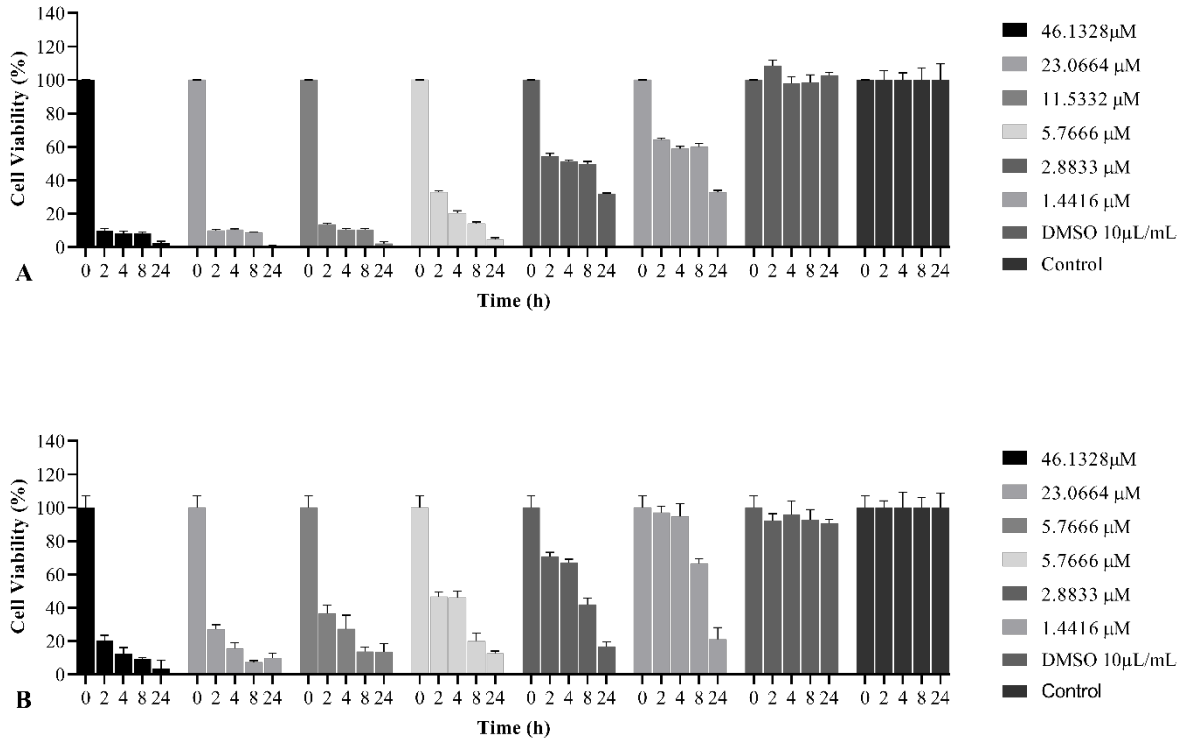


Figure 3. 7: Relative cell viability (%) of (A) HeLa and (b) HEK 293T cells after 2 h, 4 h, 8 h and 24 h exposure to Ru complex 1 at a concentration range of 0 µM to 46.13 µM. Results represent the mean of n =3, with error bars demonstrating SD.

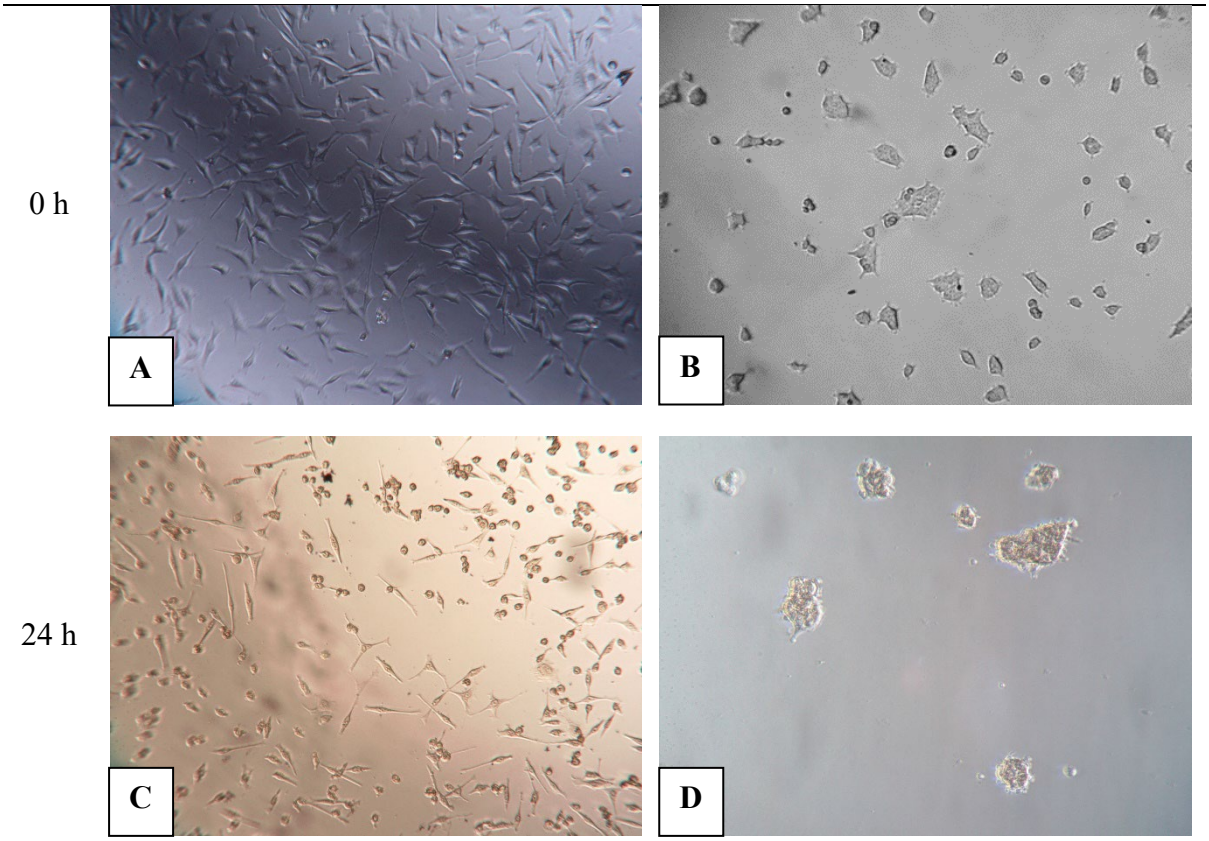


Figure 3. 8: Morphological changes in HeLa and HEK 293T in response to Ru complex 1 exposure at 46.13 µM. A) HeLa cells after 0 h, B) HEK293T cells after 0 h, C) HeLa cells after 24 h incubation D) HEK293T cells after 24 h incubation.

3.3.5.2 Ru complex 7

Ru complex 7 showed low levels of cytotoxicity across both HeLa and HEK 293T cell lines and at all concentrations. No significance difference in viability was observed after 2 h incubation of HeLa cells with Ru complex 7 at concentrations of 826.85 µM, 413.42 µM, 206.711 µM, 103.36 µM and 51.68 µM when compared to the respective controls (p>0.999). Furthermore, increases in cell viability were observed at the concentrations of 826.85 µM (110.02%), 206.711 µM (100.50%), 103.36 µM (107.39%) and 51.68 µM (114.93%). After 24 h of incubation with Ru complex 7 at 826.85 µM, HeLa cell viability decreased to 92.1±5.1%, but this not significant compared to the DMSO solvent (p>0.999) and negative controls (p=0.901) (Figure 3. 9 A).

No significant difference in viability was observed after 2 h incubation of HEK 293T with all concentrations of Ru complex 7 and the solvent controls (p>0.999). At the highest concentration of 826.85 µM, HEK 293T cell viability reduced to 89.49±9.55% after 24 h, with no significance seen between the solvent control (p>0.999) and negative control (p>0.999) (Figure 3. 9 B). No morphological changes to either the HeLa or HEK 293T cell lines were observed over a 24 h period using compound 7 (Figure 3.10).

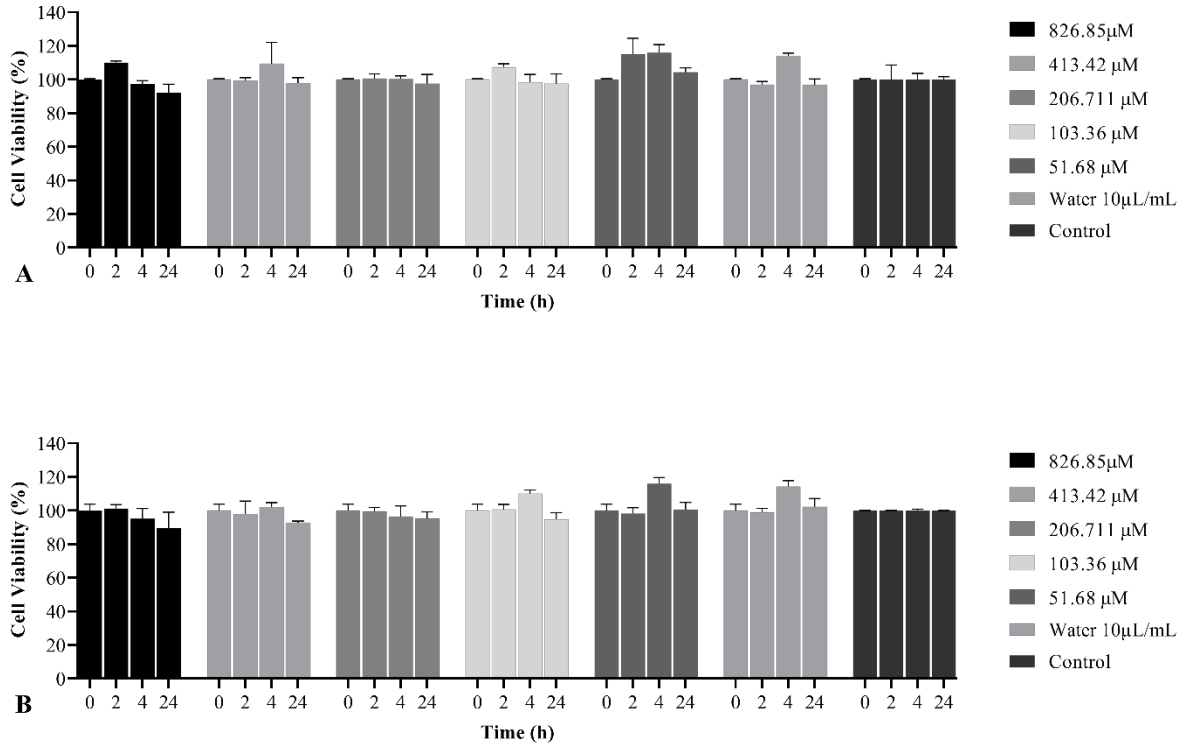


Figure 3. 9: Relative cell viability (%) of (A) HeLa and (B) HEK 293T cells after 2 h, 4 h 8 h and 24 h exposure to Ru complex 7 at a concentration range of 0 µM to 826.85 µM. Results represent the mean of n =3, with error bars demonstrating S

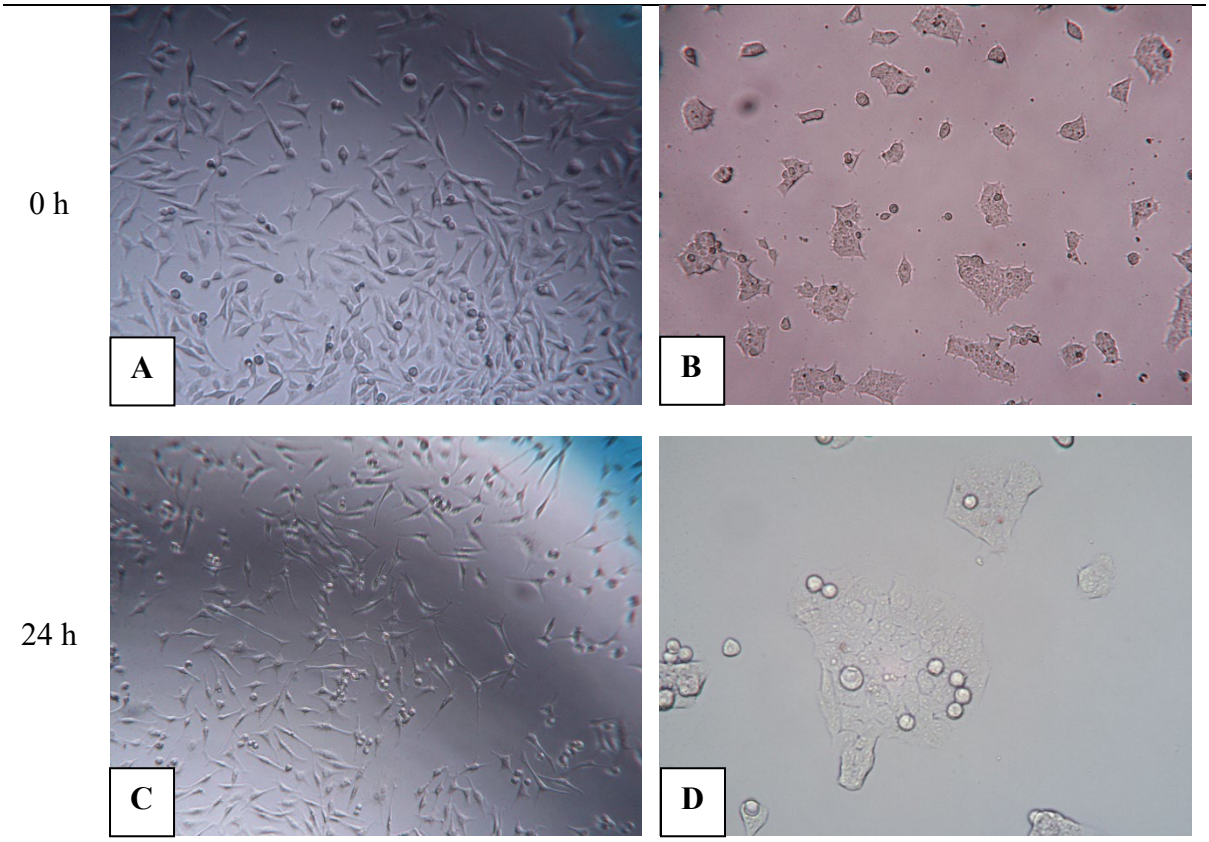


Figure 3. 8: Morphological changes in HeLa and HEK 293T in response to Ru complex 7 exposure at 826.85 µM. A) HeLa cells after 0 h, B) HEK293T cells after 0 h, C) HeLa cells after 24 h incubation D) HEK293T cells after 24 h incubation.

3.3.6 *In Vivo* Ru Complex *Galleria mellonella* Tolerance Assay

Galleria mellonella were used as an *in vivo* model to evaluate the toxicity of The Ru complexes in a live model organism. There was a 100% survival rate of *Galleria mellonella* for Ru complex 1, 7 and control at the 72 h time point. No significance was reported between any treatments.

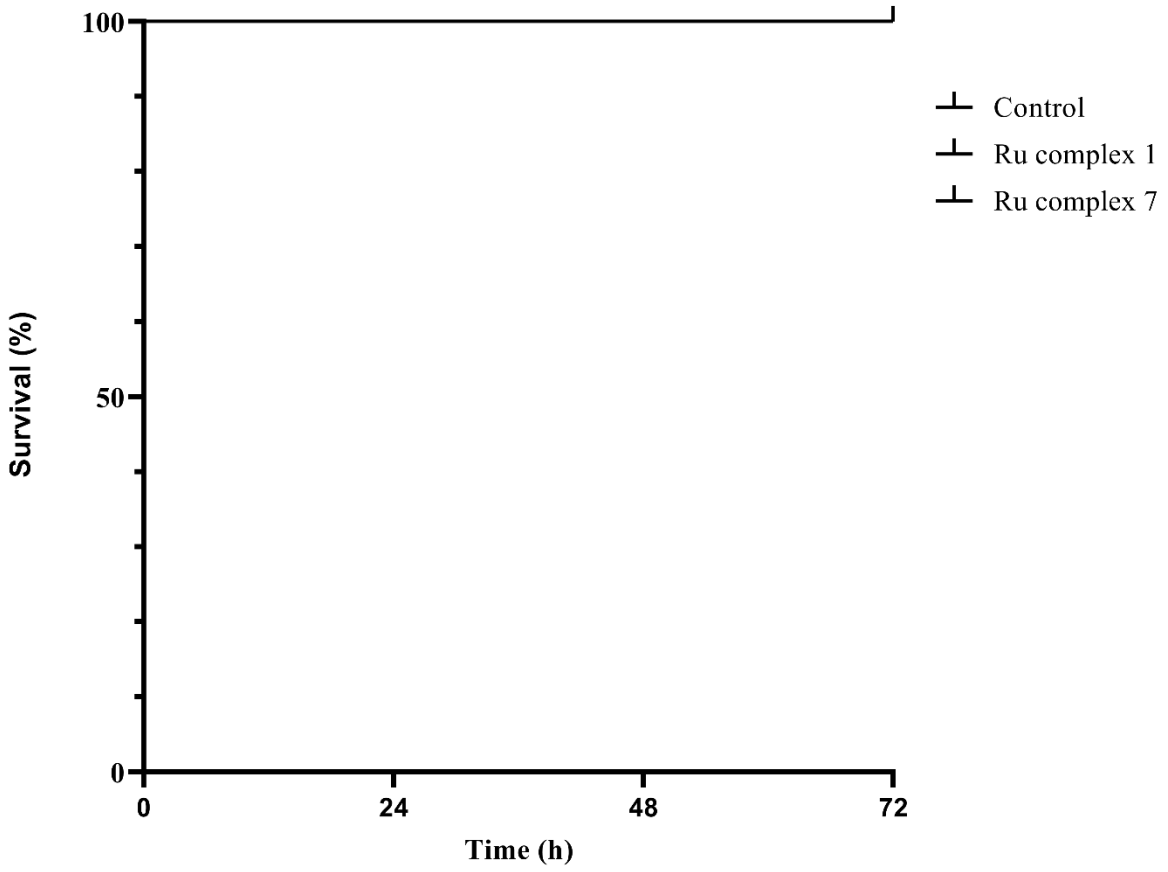


Figure 3. 9: Kaplan–Meier survival curves determining the survival of *Galleria mellonella* Larvae after injecting with Ru complex 1 at 10 mg/Kg, Ru complex 7 at 50 mg/Kg and DMSO (control), The larvae were incubated at 37 °C and live/dead scores were conducted at 24, 48 and 72 hours.

3.4 Discussion

With the world seemingly advancing towards a post-antibiotic era, development of new antimicrobial agents is urgently needed. Over recent years, there has been a dramatic rise in research and development of metallodrugs for use as antimicrobial agents. Many new metallodrugs are based around a Ru core, with many having demonstrated lower levels of cytotoxicity in eukaryotic cells compared to clinically used central metal atoms such as platinum (Motswainyana *et al*., 2015). All 12 Ru complexes selected for repurposing herein contain bioactive ligands designed to maximise potential antimicrobial activity. The objective of this chapter was to quantify and evaluate the antimicrobial activity of the 12 repurposed Ru complexes, utilising a series of *in vitro* experiments against a wide range of bacterial pathogens. The bacterial pathogens were selected due to their clinical significance, representing both Gram-positive and Gram-negative bacteria. Many of the selected strains possess intrinsic or acquired antibiotic resistance and cause a wide range of infections, such as the highly virulent *E. coli* 958 that is synonymous with urinary tract infections and the community acquired methicillin-resistant *S. aureus* USA300 JE2. It was important to understand if any active Ru complexes were bactericidal or bacteriostatic in nature, as this may influence endpoint usage. Bacteriostatic antibiotics, such as tetracycline, function by inhibiting bacterial protein synthesis pathways and require patients to have functional immune systems to be effective. Bactericidal complexes can act as pre-emptive treatments or for use where conditions such as toxic shock are of little concern. The categorisation of Ru complexes into broad or narrow spectrum antimicrobials is also important, whilst the incorrect prescribing is not associated with treatment failure, it is associated with higher rates of adverse events (Gerber *et al*., 2017), which can influence future applications.

The initial results from the disc diffusion assay prompted an increase in the Ru complex concentration. Ru complexes 3, 4, 5, 6, 7, 10 and 12 showed low antimicrobial activity when conducting the disc diffusion assay; thus, the concentrations of these Ru complexes were increased from 256 µg/mL to 1024 µg/mL for conducting the MIC and MBC protocols. At these concentrations, it was unclear if the Ru complexes possessed low antimicrobial activity or low agar diffusion rates. The molecular weight and molecule size contribute to diffusion potential of the Ru complex; larger and higher molecular weight molecules will diffuse at a slower rate than smaller lower molecular weight compounds (Hudzicki., 2009). The solubility of a Ru complex in an aqueous media is also a contributing factor to the size of the ZOI, with low solubility leading to lower diffusion rates resulting in smaller ZOIs. This can potentially lead to misinterpretation by indicating antimicrobial leaching potential instead of actual antimicrobial activity. The increase in concentration caused some Ru complexes to be equal to or above their solubility limit. Ru complexes 4, 10 and 11 all showed low solubility when preparing the 20.48 mg/mL stock concentration. Some of the Ru complexes were solubilised in DMSO, which is known to increase cell membrane permeability and can allow toxic substances and heavy metals to penetrate the membrane (Notman *et al*., 2006).

The MIC and MBC results (Table 3. 4, Table 3. 4) showed that Ru complex 5 was the only Ru complex to possess no antimicrobial activity at experimental concentrations. The antimicrobial activity produced by the 11 other Ru complexes differed depending on the Ru complex and bacterial genus and species. Overall, Gram-positive bacteria displayed greater susceptibility to the 12 Ru complexes compared to Gram-negative bacteria. Eleven of the 12 Ru complexes showed antimicrobial activity against at least one Gram-positive species, whilst antimicrobial activity against Gram-negative bacteria was only achieved by Ru complexes 1, 6, 7 and 12. Against *P. aeruginosa,* Ru complex 6 was bacteriostatic whilst Ru complexes 7 and 12 were

shown to act in a bactericidal manner. These findings are in agreement with other studies which showed Gram-positive bacteria are more sensitive to the antimicrobial activity of Ru complexes when compared to Gram-negative bacteria (Cetinkaya *et al*., 1999; Smitten *et al*., 2019; Matshwele *et al*., 2021).

The intrisic resistance mechanisms of Gram-negative bacteria that confer resistance to many conventional antibiotics may be also applicable to Ru complexes (Reygaert., 2005; Munteanu *et al*., 2021). The intrisic mechanisms of Gram-negative bacteria can infuluence the antimicrobial activity of Ru complexes in a variety of ways. The use of efflux pumps by both Gram-positive and Gram-negative bacteria is one of the primary mechanisms of defence against toxic substances. Gram-negative bacteria exclusively utilise RND efflux pumps, which may aid in the ability to tolerate higher concentrations of Ru complexes (Godoy *et al*., 2010). The RND efflux pumps are able to extrude a wide range of toxic substance from the cell, Ru complexes could potentially be categorised into one of the four efflux grouping catagories, group 1 extrudes a wide range of antibiotics while group 2 extrudes oxidative stress causing agents, group 3 are involved in the extrusion of heavy metals and group 4 extrudes organic and inorganic compounds (Nikolouli *et al*., 2015). It is worth noting that not all Gram-negative species carry genes to produce each type of efflux system. The MexEF-OprN efflux systems are found in the *P. aeruginosa* strain PAO1, but are absent in *E. coli* spp. These efflux systems are known to target antibiotics, dyes, detergents, inhibitors, disinfectants and organic solvents (Poole, 2005), and it has been demonstrated that these systems are directly responsible for AMR in *P. aeruginosa* (Piddock, 2006; Soto, 2013). Bacterial gene expression can be affected by Ru complexes, where genes encoding multidrug efflux pumps, including *mdtABC*, *marAB* and *acrD* were shown to be upregulated in response to CORM-2 (Bang *et al*., 2017). This

indicates that these efflux systems contribute to the ability of Gram-negative bacteria to resist the antimicrobial effects of Ru complexes.

Another key component thought to influence the ability of Gram-negative bacteria to resist Ru complexes is the lower drug permeability of the outer membrane, which is composed of an outer asymmetric bilayer of lipopolysaccharides with glycerophospholipids on the inner leaflet. The outer asymmetric bilayer of Gram-negative bacteria has a lower fluidity, thus reducing spontaneous permutation of the membrane. The low permeability barrier of Gram-negative bacteria likely plays an important role in mediating resistance to Ru complexes (Liu *et al*., 2018; Breijyeh *et al*., 2020). Research has demonstrated that the low permeability barrier reduces uptake of clinically used antibiotics, as well as antimicrobials that have failed clinical trials (Krishnamoorthy *et al*., 2017; Delcour, 2009). In an effort to counteract the low permeability outer membrane, many Ru complexes are designed with a cationic component, which has been shown to affect the lipophilicity of the Ru complex, therefore increasing the penetration of Ru complexes in both Gram-positive and Gram-negative bacteria (Uivarosi *et al*., 2019; Smitten *et al*., 2020).

Ru complexes 6,7 and 12 demonstrated antimicrobial activity against Gram-negative bacteria, these Ru complexes were all water-soluble and of relatively low molecular mass. The quaternary ammonium groups in combination with the chloride counterions allow for solubility in aqueous solvents such as water (Kawai *et al*., 2019). An Ru complex that is soluble in aqueous solvents minimises potential toxicity and allows for increased drug distribution in a clinical setting (Savjani *et al*., 2012). It is unclear if the Ru complexes were actively or passively taken up into the bacteria, or if the cationic charge of the Ru complexes caused attachment to the LPS structures on the outer membrane. Water soluble complexes can utilise

porins within the outer membrane to diffuse into the cell and these are species dependent pore diameter size can influence the diffusion molecules, for example, those in *E. coli* generally allow molecules of 550-800 Da to enter whereas porins in the surface of *P. aeruginosa* permit molecules of up to 5000 Da (Berg *et al*., 2015; Benz *et al*., 1988).

Secondly, Ru complexes can be actively targeted by the iron transport systems pyoverdine and pyochelin siderophores, or the FeoB transporter. Yang *et al*. (2014) successfully synthesised a Ru (II) complex termed R-825 which was able to compete with ferrichrome for use of the ferrichrome-transport pathway in *S. pneumoniae*. As a result, Ru (II) complex R-825 competitive binding it reduced the uptake of iron into *S. pneumoniae* and a decrease in cell growth was achieved (Yang *et al*., 2014).

Thirdly, quaternary amines have demonstrated the ability to permeabilize the other membrane. The cationic complex can absorb onto the cell membrane and then penetrate, leading to membrane depolarization and loss of low-molecular internal components from the cell (Kwaśniewska *et al*., 2020). The protocols undertaken in this chapter were not designed to isolate a mechanism of action, as it is unknown if the Ru complexes acted as permeabilizing agents or if the Ru complexes were targeting a cellular component. Ru complex 7 has previously shown DNA binding and DNA mutagenic effects along with the ability to elicit the bacterial SOS response (Yasbin *et al*., 1980). It is unknown how Ru complex 7 enters the bacterial cell; membrane depolarisation studies would need to be conducted to determine the mechanism of Ru complex 7 when traversing the cell membrane.

Due to the high level of antibiotic resistance and clinical significance of *P. aeruginosa* PAO1, any antibacterial activity was concidered important. Ru complex 7 was of particular interest

with concentrations of 128 µg/mL and 256 µg/mL producing bactericidal effects after 24 h and 6 h respectively. The growth curve kinetics of Ru complex 7 against *P. aeruginosa* revealed that at sub-MBC concentrations of 16 µg/mL and 32 µg/mL, the 18 h extended lag phase showed similar growth dynamics compared to bacteria when exposed to DNA gyrase and topoisomerase IV-targeting antibiotics, such as fluoroquinolones (Theophel *et al*., 2014). Likewise, membrane targeting antibiotics generally cause a reduction in bacterial growth during the lag phase (Kim *et al*., 2019).

Antimicrobial activity against Gram-positive bacteria was higher than against Gram-negative bacteria. All species and strains showed suseptability to at least one Ru complex, with Ru complexes 1, 2 and 9 showing potent antimicrobial activity against a wide range of Gram-positive bacteria, indicating broad spectrum antimicrobial activity. All three Ru complexes showed similar levels of antimicrobial activity against *S. aureus* with MIC results of 8 µg/mL, with Ru complex 1 showing more potent MBC activity. Gram-positive bacteria showed greater susceptibility to Ru complex 1 when compared to Gram-negative bacteria. Gram-positive species such as *S. aureus* and both stains of *M. luteus* were susceptible to 8 µg/mL and <1 µg/mL respectively as demonstrated by the MIC results. Ru complex 1 was the only water insoluable Ru complex to show antimicrobial activity against any Gram-negative bacteria.

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susceptibility to Ru complex 1 when compared to Gram-negative bacteria. Gram-positive species such as *S. aureus* and both stains of *M. luteus* were susceptible to 8 μg/mL and <1 μg/mL respectively as demonstrated by the MIC results. Ru complex 1 was the only water insoluable Ru complex to show antimicrobial activity against any Gram-negative bacteria.

For Ru complexes which target cellular membrane, it is important to determine selectivity and specificity against prokaryotic membranes over eukaryotic membranes in order to prevent off-target effects. This is a long-established principal in conventional antibiotic therapy, for example, β-lactam antibiotics specifically target the penicillin binding proteins in the bacterial membrane. Antibiotics such as penicillin and monobactams have no direct effect on eukaryotic cells (Dewdney, 1986). Here, concentration-dependent Ru complex permeabilization on mammalian cells was measured via a haemolytic assay. The haemolytic assay utilizes RBCs and is an excellent model for examining compound interactions with cellular membranes and has been used across a broad range of research (Evans *et al*., 2013; Pham *et al*., 2014; Malagoli, 2007). The RBC membrane is structurally very similar to other eukaryotic cell membranes, with the exception that it does not contain tubulin (Smith, 1987). All Ru complexes were tested across a wide range of concentrations, with the highest concentrations selected to represent the percentage lysis alluding to the highest possible haemolytic activity. All Ru complexes showed low levels of RBC lysis; Ru complex 8 recoded the highest RBC lysis at 8.58 ± 0.472%. This result was only 2.25% above that of a clinically used antibiotic colistin, and a significance of (p=0.021). However, colistin is known to be more toxic than many other antibiotics due to its cationic charge which helps bind and permeabilise bacterial membranes (Landman *et al*., 2008). Colistin has FDA approval as a last-resort antibiotic against infections from Gram-negative bacteria such as *K. pneumoniae* and *P. aeruginosa*. Some antibiotics such as streptomycin and gentamicin have been shown to reduce haemolysis under certain conditions,

although it is currently unknown if any of the tested Ru complexes act this way (Lijana *et al*., 1986). Other antibiotics such as vancomycin and methyldopa can initiate the condition called drug-induced immune haemolytic anaemia within patients (Gniadek *et al*., 2018). The significant increased lysis of RBCs for Ru complexes 7 and 8 over their respective controls could be indicative of the amino group and quaternary ammonium salts that are contained within the Ru complexes (Bielawski *et al*., 2017).

Compared to eukaryotic cells, the prokaryotic cell membrane contains a greater number of negatively charged structures, including teichoic acids (Gram-positive), phosphatidyl-glycerol and lipopolysaccharides (Dathe *et al*., 1999). These structures can cause greater attraction of the positively charged compounds to the bacterial membrane when compared to eukaryotic cells, and this could aid in *in vivo* testing. The bacteria could potentially attract more of the compound thus lower the overall concentrations within the surrounding tissue in eukaryotic cells.

After initial MIC, MBC, and haemolysis experimentation, Ru complexes 1 and 7 demonstrated the highest antimicrobial efficacy and were selected for further study. Ru complex 1 possessed potent broad-spectrum antimicrobial activity against Gram-positive bacteria, whilst Ru complex 7 possessed potent antimicrobial activity against *P. aeruginosa*.

Time-kill kinetic assays were conducted using Ru complexes 1 and 7 against target bacterial species, where viability corresponded to the MIC and MBC values for each bacterium. The Ru complex 1 time-kill kinetics against *E. faecium* indicated a concentration between 128 µg/mL and 256 µg/mL may produce a complete kill of *E. faecium* due to the 256 µg/mL concentration being fully effective after only 6 h of incubation. Even at the lowest concentration of 32 µg/mL, Ru complex 1 was still efficient at reducing viability of *E. faecium* but at a diminished rate

compared to a concentration of 256 µg/mL. Ru complex 7 was to be bacteriostatic against *P. aeruginosa* at lower concentrations, with an increase in viability between 6 h and 24 h at 32 µg/mL. This could indicate that large amounts of Ru complex 7 were being absorbed by the bacteria which reduced viability, thus also reducing the levels of available compound and preventing any further activity. Another possible explanation is a subpopulation of bacteria within the kinetics assay had a higher tolerance for the Ru complex 7, therefore allowing them to survive and replication through binary fission.

Research into *in vitro* efficacy has demonstrated that many Ru complexes have potent antimicrobial activity, whilst also displaying low levels of eukaryotic cytotoxicity (Laurent *et al*., 2018: Li *et al*., 2012; Ude *et al*., 2016). Using haemolytic assays in conjunction with MTS assays can assist with understanding the nature of potential cytotoxic effects on mammalian cell viability. If there is a high cytotoxic effect during the MTS experiment coupled with low haemolytic activity, then the compound is targeting internal cellular processes. Whereas, a high cytotoxic effect as seen via the MTS assay along with high haemolytic activity suggests that the compound is predominantly targeting the cell membrane (Greco *et al*., 2020).

The choice of MTS for the cytotoxicity assays was due to faster and more efficient rates of reactivity when compared to the older generation MTT assay. MTS is an adenosine triphosphate (ATP) based assay and provides true representation of cell metabolism, and not necessarily if a cell is alive or dead. ATP is known to degrade rapidly in dead and damaged cells, therefore the quantity of ATP present in the cell is thought to provide an accurate representation of the number of viable cells. Many antimicrobial compounds are known to suppress or increase metabolic activity (Wang *et al*., 2010; Zhu *et al*., 2019). Ru complex 1 was originally developed as an antitumour drug but failed in early testing (Arshad *et al*., 2021).

The cytotoxicity levels of the Ru complex 1 were much higher than anticipated; the effective MBC concentration for *S. aureus* USA300:JE2 was 28.83 µM, while cytotoxicity against eukaryotic cell lines was seen at a much lower dose. The IC50 levels after 8 h exposure to Ru complex 1 against HEK 293T and HeLa cell lines were almost an order of magnitude lower in molarity when compared to the MBC results. Research from Arshad *et al.* (2021) confirmed the toxicity levels of Ru complex 1 with IC50 values of 4.3±1.2 µM against the human colon cell line SW480 (Arshad *et al*., 2021). It is unclear whether Ru complex 1 failed to progress further due to high cytotoxicity or other technical reasons.

The phase contrast microscopy images (Figure 3.8; Figure 3.10) showed significant changes in cell morphology across all cell lines, which supports the metabolic cytotoxicity data from MTS assay. The three cell lines often detached from the plate wells, taking a spherical form after treatment with Ru complex 1. Detachment of adherent cells also gives a strong indication of cell death or the cells being in a distressed state. Furthermore, when cytotoxic effects occur, adhered cells often become abnormally shaped with noticeable amounts of compound attached to the outer surface of the cell (Kroemer *et al*., 2009). When HEK 293T and HeLa cells were exposed to Ru complex 1, cell viability decreased rapidly within 2 h, indicating efficient uptake into the cells. Both HeLa and HEK 293T cells are immortalised and require a large quantity of Fe for proliferation. However, Ru can mimic Fe in biological systems which may provide an insight into the observed rapid cytotoxic activity of Ru complex 1 within the first 2 h of incubation (Kratz *et al*., 1994).

Ru complex 7 showed lower cytotoxic effects against HeLa and HEK 293T cell lines, with the highest concentration tested (826.85 µM) only reducing HeLa and HEK 293T cell viability to 92.1±5.1% and 89.49±9.55% after 24 h respectively. This was significant because the concentration was twice the MBC value, indicating very low cytotoxicity to eukaryotic cells at biologically relevant concentrations. Overall, this demonstrates the potential for use in clinical

applications, for example, in the treatment of chronic diseases such as cystic fibrosis where *P. aeruginosa* infections are common (Silva *et al*., 2013). It also has potential commercial applications such as biocidal applications in paints, as *P. aeruginosa* is known to degrade aromatic hydrocarbons such as methylbenzenes which are used in the production of paint (Szoboszlay et al., 2002).

Galleria mellonella were used as an *in vivo* method to test the toxicity of Ru complexes 1 and 7 on living organisms. *Galleria mellonella* were selected due to their ease of access and studies show data obtained from *Galleria mellonella* studies correlates with those of similar mammalian studies (Ramarao *et al*., 2012; Brochado *et al*., 2018; Rochford *et al*., 2018). Results were assessed using Kaplan–Meier survival curves over a 72 h period, both Ru complex 1 and 7 showed no toxicity to *Galleria mellonella* with a 100 percent survival rate and no significance shown between the negative control ($p>005$). This low toxicity has also been reported by other research where *Galleria mellonella* have been able to tolerate concentrations substantially above MIC/MBC concentrations without causing deleterious effects (Smitten *et al*., 2018).

In summary, many of the Ru complexes tested showed potent antimicrobial activity, with Gram-positive bacteria being more susceptible than Gram-negative bacteria to the Ru complexes. Ru complex 1 was highly active as an antimicrobial agent against the *S. aureus* strains and was further investigated. Whilst the cytotoxicity of Ru complex 1 was high against Gram-positive bacteria, the mechanism of action remains to be evaluated. Additionally, the bactericidal effect of Ru complex 7 against *P. aeruginosa* was potent, yet this complex demonstrated low haemolytic activity and no significant mammalian cell cytotoxicity, thus further studies into the mechanism of antimicrobial activity were essential.

Chapter 4: Resistance Development and Gene Regulation in Response to Long Term Exposure to Ru Complexes

4.1 Introduction

Antimicrobial resistance (AMR) is one of the leading threats to global health. The rise of AMR has compromised the ability to treat minor routine infections, leading to increased mortality rates in clinical settings (Kraker *et al.*, 2016). Antibiotics that were once effective at treating bacterial infections are now redundant with HGT and cellular mutations proven to be the primary mechanisms responsible (Martens *et al.*, 2017). Whilst AMR occurs naturally due to environmental pressures, the misuse of antibiotics coupled with a lack of new antibiotics has applied severe selective pressure onto clinically significant bacteria. This, in turn, has accelerated the evolutionary process leading to an increasing rate of AMR amongst clinically significant bacteria (Harada *et al.*, 2010; Von Wintersdorff *et al.*, 2016; Merker *et al.*, 2020). This process can be simulated in an *in vitro* environment utilising long-term selection pressure. This allows bacteria to tolerate increasing concentrations of antimicrobials leading to increased antimicrobial breakpoints against bacterial strains and even permanent resistance (Andersson *et al.*, 2011; Hong *et al.*, 2016). To achieve this, an antimicrobial is added at minimum selective concentration (MSC) at regular intervals to growth media inoculated with a bacterium of interest. This MSC is the lowest concentration that can enrich for resistant mutants and induce mutations. The MSC varies depending on the bacterial species and the antimicrobial in question (Khan *et al.*, 2017). Under high concentrations of antibiotics, bacteria remain dormant, and these cells are referred to as persister cell. These persister cells do not undergo binary fission, so are easily distinguishable from resistant cells (Wood *et al.*, 2013). Due to the inorganic nature and three-dimensional bonding structure of Ru complexes, they often target multiple sites within the bacterial cell leading to several antimicrobial mechanisms of action. Due to this, it has been speculated that both adaptive and permanent resistance would occur at a lower rate (Frei, 2020; Pierce *et al.*, 2020). The development of AMR is normally associated with a

loss of fitness and a reduction in growth which can lead to reversion when the selection pressure is alleviated (Sandegren., 2014).

The mechanisms that determine pre-existing, development, and spread of AMR can be categorised into three areas: Intrinsic, Adaptive and Acquired resistance. Intrinsic resistance to Ru complexes has proven to be relatively common amongst both Gram-positive and Gram-negative bacteria. Efflux pumps and cellular membranes act as the primary mechanisms of intrinsic resistance to Ru complexes, assisting to maintain low levels of Ru complexes within the cell (de Sousa *et al.*, 2020; Matshwele *et al.*, 2020). The membrane acting as an intrinsic resistance mechanism can be exemplified by the low membrane permeability of *P. aeruginosa* to polypyridyl ruthenium (II) complexes (Gorle *et al.*, 2014). Experimentation using long-term selection pressure in a pure isolated culture forces bacteria to utilise adaptive mechanisms to develop AMR to a specific antimicrobial. The adaptive resistance mechanisms utilised by bacteria include the reduction in the cell membrane and LPS layer permeability, reduced porin channel activity, biofilm formation, overexpression of efflux pumps, and DNA mutations (Fernández *et al.*, 2012; Impey *et al.*, 2020).

Mutations within a bacterial genome have been responsible for the development of AMR in both *in vivo* and *in vitro* settings (Martinez *et al.*, 2000; Ocejo *et al.*, 2021). There are five main mechanisms of the genic mutation: Substitution, Deletion, Insertion, Inversion, and Duplication (Griffiths *et al*., 2000). Depending on the location of the mutation within the DNA it can lead to nonsense mutation, missense mutation, silent mutations, and frameshift mutations. All such mutations can have a profound impact on transcription (Zhang *et al.*, 2012; Gorlov *et al.*, 2018). Random mutation rates amongst bacteria species varies. For example, the *S. aureus* genome is comprised of around 2.8 million nucleotide base pairs and under normal conditions, spontaneous mutation rates are estimated to be 0.8×10^{-10} mutations per nucleotide per generation (Windels *et al.*, 2017; Szafrańska *et al.*, 2019). The presence of antimicrobial

compounds within the cell have been shown to increase mutation rates in bacteria promoting the evolution of antimicrobial resistant bacteria (Long *et al.*, 2016; Windels *et al.*, 2017). Mutations within genes can directly confer resistance to an antimicrobial whilst mutations on regulatory areas of DNA within genes can potentially alter gene expression (Wang *et al.*, 2001; Szafrańska *et al.*, 2019). Changes to genomic DNA can be assessed by full genome comparisons pre and post long-term selection pressure, highlighting points of molecular change.

The regulation of molecular mechanisms in response to a stressor is an attempt to maintain cellular homeostasis and results in cellular repair, damage tolerance, and potentially the removal of the stressor. Mutations within global regulatory areas have been a source for increased resistance to antimicrobials. The global regulator MgrA regulates the MDR efflux pumps; NorA, NorB, and NorC, as well as the modulation of cell clumping and virulence in *S. aureus*. The alteration of gene expression can lead to specific genes being up or down regulated in response to a stimulus or mutations in gene regulatory areas. MgrA acts as a positive regulator for *norA* while direct repressor for *norB*. Mutations within *mgrA* have been shown to affect expression of *norA,* directly conferring resistance to the hydrophilic antibiotic fluoroquinolone (Kaatz *et al.*, 2005). In addition to mutation in the regulatory areas of DNA causing changes to gene regulation, bacteria can alter gene regulation naturally as a result of external pressures, in an effort to maintain cell homeostasis (Liao *et al.*, 2020).

Alterations to individual gene expression leading to an increased AMR are plentiful. For example, Gram-positive bacteria are known to upregulate the *mprF* gene to counter the positive charge of cationic molecules. The *mprF* gene encodes for a bifunctional membrane protein that synthase and translocases the positively charged lysyl-phosphatidylglycerol within the cell membrane. The accumulation of the translocated positively charged lysyl-phosphatidylglycerol in the cell membrane can repel cationic molecules (Mishra *et al.*, 2009).

Antimicrobials acting as stressors have shown patterns in gene regulation, there are several techniques that can be utilised to study the molecular mechanisms of resistance in bacteria including RNA-seq, qRT-PCR, microarray analysis, two-dimensional protein gel electrophoresis, gene knockout, and overexpression studies (Hendriksen *et al.*, 2019). Each technique is unique and serves a specific purpose. With recent advancements in bioinformatics and a more in-depth knowledge of genome-wide gene regulation, it is possible to make predictive models of AMR from gene expression profiles (Suzuki *et al.*, 2014).

4.2 Aims and Objectives

As a result of the antimicrobial activity produced by Ru complex 1 against a range of key pathogens within the study, most notably *S. aureus* USA300:JE2. The aim of this chapter was to investigate the potential for bacterial resistance generation towards Ru complex 1 and to elucidate the mechanisms underpinning AMR development.

The original concept for this chapter was to conduct all experimentation on both Ru complex 1 and 7. Due to budgeting and time restraints, full work on Ru complex 7 within this section was not successfully completed and the data was subsequently omitted from the final thesis.

4.2.1 Objectives

- Induce resistance in two clinically significance bacteria towards Ru complex 1 via long-term selection pressure.
- Evaluate morphological changes and growth kinetic changes between the resistant and non-resistant bacteria.
- Determine the genetic basis for the observed bacterial resistance.
- Assess gene expression changes in the induced resistant bacteria.

4.3 Results

4.3.1 Induced Resistance

4.3.1.1 Development of Induced Resistance via Long-term Selection Pressure

To assess the development of resistance to Ru complex 1, *S. aureus* USA300:JE2 and *E. coli* EC958 were subjected to incrementally increasing exposure concentration of Ru complex 1 over a period of 100 days. The 100-day period equated to approximately 4800 generation for *S. aureus* USA300:JE2 and approximately 7200 generations for *E. coli* EC958. Both *S. aureus* USA300:JE2 and *E. coli* EC958 became highly resistant to Ru complex 1, with complex concentrations reaching levels higher than maximum compound solubility levels for *E. coli* EC958. After exposure to incremental daily increases in concentration of Ru complex 1, *S. aureus* USA300:JE2 and *E. coli* EC958 were able to tolerate concentrations of 189.441 µg/mL equating to an 1084.01% increase and 346.3695 µg/mL equating to an 35.301% increase respectively. The now resistant strains were labelled *S. aureus* USA300:JE2 (IR) and *E. coli* EC958 (IR). The cells were deemed not to be persister cells due to the daily growth exposed to Ru complex 1. The resistance gained from the daily exposure was able to revert after 7 days daily sub culturing without exposure to Ru complex 1, indicating a transcriptome response.

4.4.1.2 Morphological Changes in Induced Resistant Cultures

Morphological changes were assessed by visually comparing before and after long term exposure to Ru complex 1. To confirm morphological changes all plates were streaked in triplicate on Mueller-Hinton agar. *S. aureus* USA300:JE2 exhibited distinct changes with a substantial reduction or elimination of the triterpenoid carotenoid pigment Staphyloxanthin (Figure 4.1, compare A with B). There was no observable change to colony size, form or elevation and no observed increase in extracellular mucus production.

A) *S. aureus* USA300:JE2

B) *S. aureus* USA300:JE2 Induced resistance

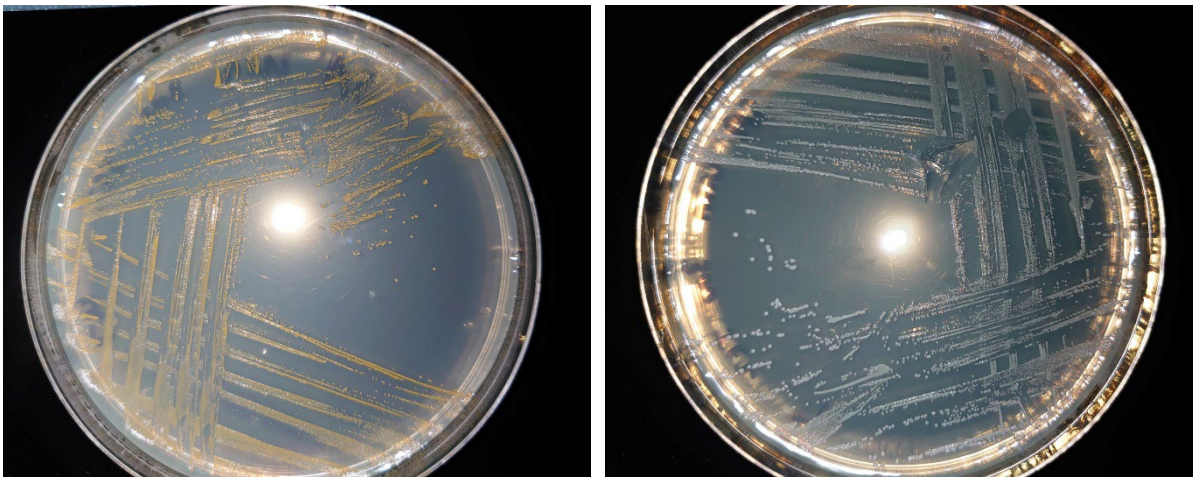

Figure 4. 1: A visual comparison of (A) *S. aureus* USA300:JE2 without induced resistance and (B) *S. aureus* USA300:JE2 with induced resistance after 100 days of incremental increases of Ru complex 1 exposure up to a concentration of 189.441 µg/mL on Mueller Hinton agar.

The *E. coli* EC958 exhibited morphological changes with increased colony sizes, no change in colony form or elevation was observed (Figure 4.2). The most notable morphological change was to the glycocalyx, it was observed that the glycocalyx produced an increased slime layer surrounding the colonies.

A) *E. coli* EC958 B) *E. coli* EC958 (IR)

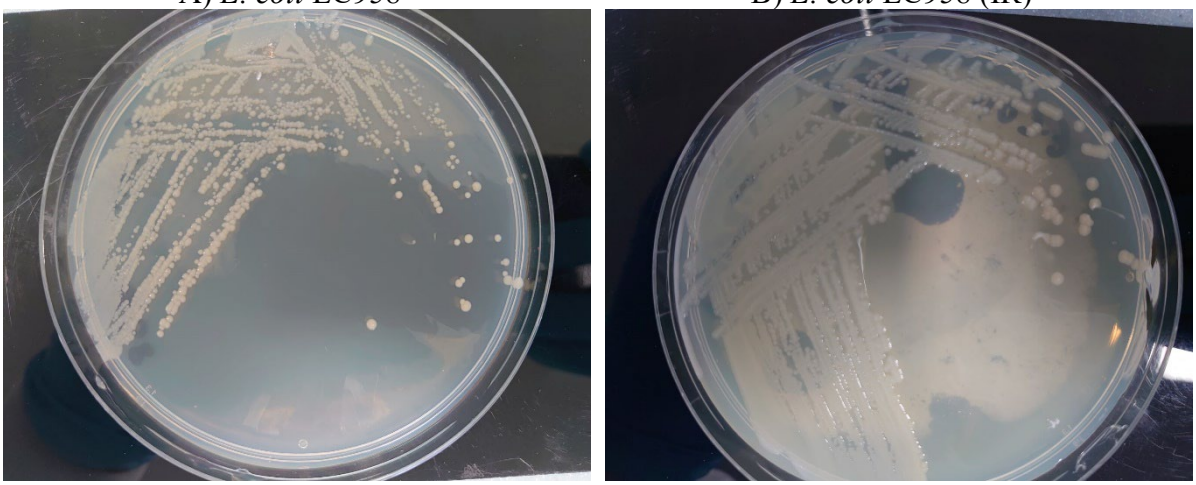

Figure 4. 2: A visual comparison of *E. coli* EC958 without induced resistance and *E. coli* EC958 Induced resistance with induced resistance after 100 days of incremental increase of Ru complex 1 concentration exposure up to a concentration of 346.3695 µg/mL on Mueller Hinton agar. A) *E. coli* EC958 with no compound exposure B) *E. coli* EC958 after 100 days of incremental compound 1 exposure to a concentration of 346.3695 µg/mL.

4.3.2 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration Assays

The 11 remaining Ru complexes were used to assess changes to the MIC and MBC of the induced resistance bacteria *S. aureus* USA300:JE2 and *E. coli* EC985 compared to their non induced resistance counterparts.

The MIC results for *S. aureus* USA300:JE2 (IR) showed a significant increase in resistance to Ru complexes 1, 2, 3 and 4 in comparison to the *S. aureus* USA300:JE2 ($p<0.05$). *S. aureus* USA300:JE2 (IR) showed an increased susceptibility to Ru complexes 7 and 8 with MIC values reducing from >1024 µg/mL to 512 µg/mL and >256 µg/mL to 64 µg/mL respectively. The MBC results for *S. aureus* USA300:JE2 Induced Resistance showed increases for Ru complexes 1 and 2 with increases from 16 µg/mL to >256 µg/mL and 32 µg/mL to 256 µg/mL respectively. Ru complexes 4, 8, 9 showed a decrease in MBC with concentrations reducing from >1024 µg/mL to 512 µg/mL, >256 µg/mL to 64 µg/mL and 32 µg/mL to 16 µg/mL respectively.

Table 4. 1: MIC / MBC values of 12 ruthenium compounds (µg/mL) in respective broths against *S. aureus* USA300:JE2 with and without induced resistance to Ru complex 1 after 24 h incubation.

	S. aureus USA300:JE2 Untreated		*S. aureus* USA300:JE2 (IR)	
Ru complex	MIC µg/mL	MBC µg/mL	MIC µg/mL	MBC µg/mL
1	8	16	>256	>256
2	8	32	128	256
3	64	>1024	>1024	>1024
4	16	>1024	64	512
5	>1024	>1024	>1024	>1024
6	1024	1024	1024	1024
7	>1024	>1024	512	>1024
8	>256	>256	64	64
9	8	32	8	16
10	>1024	>1024	>1024	>1024
11	>256	>256	>256	>256
12	1024	>1024	>1024	>1024

When comparing the effects of Ru complex 1-12 on strain *E. coli* EC958 (IR), it was observed that the MIC value for Ru complex 1, 6 and 12 had increased from 128 µg/mL to >256 µg/mL, 128 µg/mL to >1024 µg/mL and 128 µg/mL to 256 µg/mL respectively. Only Ru complex 12 showed an increase in MBC concentration, increasing from 256 µg/mL to >1024 µg/mL. For all other Ru complexes, the MIC and MBC values against the bacteria were above the maximum concentration tested.

Table 4. 2: MIC / MBC values of 12 ruthenium compounds (µg/mL) in respective broths *E. coli* EC958 with and without induced resistance to Ru complex 1 after 24 h incubation.

	E. coli EC958 Untreated		*E. coli* EC958 (IR)	
Ru complex	MIC µg/mL	MBC µg/mL	MIC µg/mL	MBC µg/mL
1	128	>256	>256	>256
2	>1024	>1024	>1024	>1024
3	>1024	>1024	>1024	>1024
4	>1024	>1024	>1024	>1024
5	>1024	>1024	>1024	>1024
6	128	>1024	>1024	>1024
7	>1024	>1024	>1024	>1024
8	>256	>256	>256	>256
9	>1024	>1024	>1024	>1024
10	>1024	>1024	>1024	>1024
11	>256	>256	>256	>256
12	128	256	256	>1024

4.3.3 Assessment of Growth Dynamics

Growth curves were undertaken using *S. aureus* USA300:JE2 and *S. aureus* USA300:JE2 (IR) (Fig. 4.3A and B) with a starting OD_{600} of 0.09 (8.16 x 10^6 CFU/mL) using the same concentrations of Ru complex 1 and a temperature of 37°C.

At all concentrations following exposure to Ru complex 1 no reduction in growth was recorded for *S. aureus* USA300:JE2 (IR) against the positive control. The lag phase of *S. aureus* USA300:JE2 lasted from 0 h to 3 h while for *S. aureus* USA300:JE2 (IR), the lag phase lasted from 0 h to 1 h. The rate of exponential growth was also increased for *S. aureus* USA300:JE2 (IR) even at higher concentrations of Ru complex 1. After 6 h at a concentration of 32 µg/mL, viability of *S. aureus* USA300:JE2 (IR) was 3.22 x 10^8 CFU/mL, compared to *S. aureus* USA300:JE2 at the 6 h time point for the negative control was 2.67x10^7 CFU/mL.

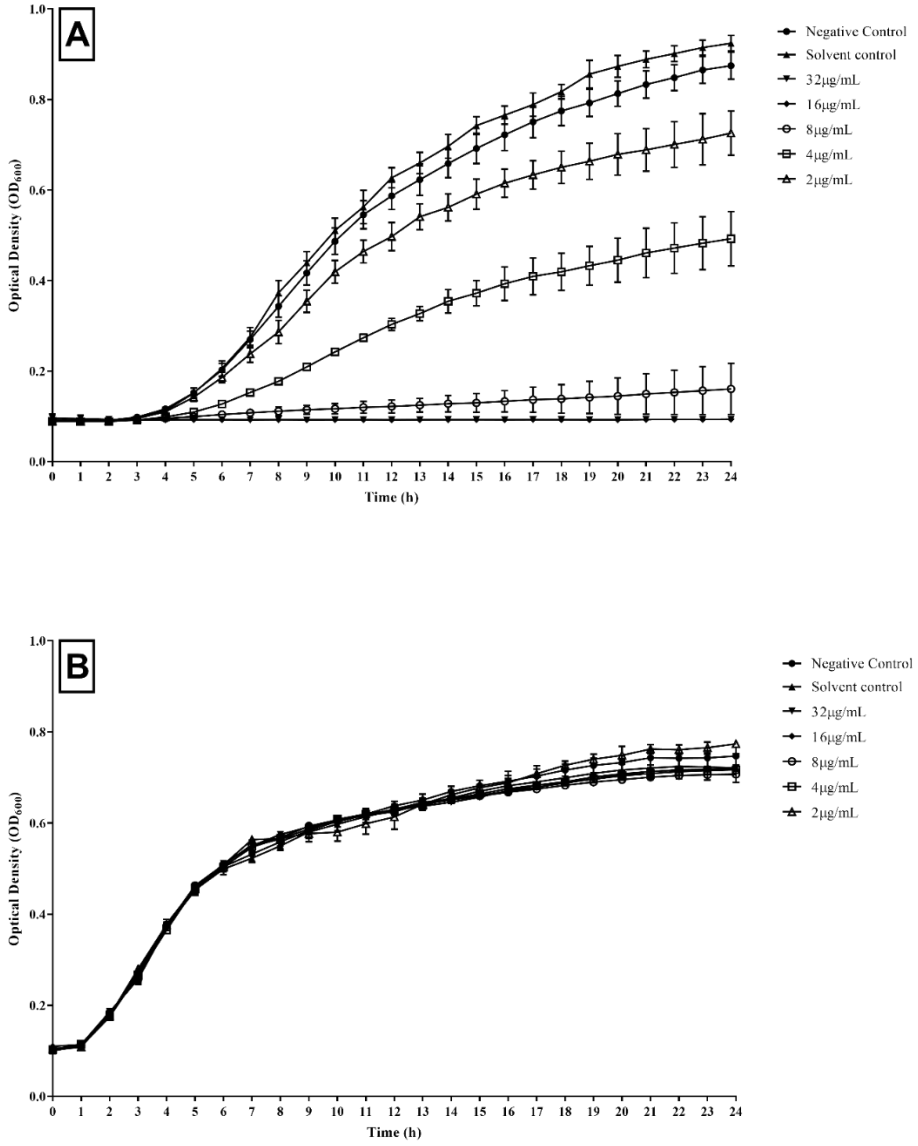

Figure 4. 3: Growth curve assay for (A) *S. aureus* USA300:JE2 *and (B) S. aureus* USA300:JE2 (IR) following exposure to Ru complex 1 at concentrations of 2, 4, 8, 16 and 32 μg/mL, DMSO solvent and negative control over a 24 h period.

4.3.4 Whole Genome Sequencing of *S. aureus* USA300 JE2 and *S. aureus* USA300 JE2 (IR).

The assembled genome of *S. aureus* USA300 JE2 contained one single circular chromosome of 2,697,311 bp in size and a guanine–cytosine (GC) content of 32.8% with a predicted 2427 protein CoDing Sequences (CDS) and 62 tRNA genes. The *S. aureus* USA300 JE2 (IR) contained one single circular chromosome of 2,697,263 bp in size and a guanine–cytosine (GC) content of 32.8% with proteins (CDS) 2429 and tRNA 62 genes. The nucleotide sequence pairwise identity between the *S. aureus* USA300:JE2 and the *S. aureus* USA300:JE2 (IR) entire genomes was 99.997% with a total of 17 separate *de novo* mutations being detected across 8 different genes (Table 4.3). Based on average binary fission times under optimal conditions, it was estimated that *de novo* mutation rate between *S. aureus* USA300 JE2 and *S. aureus* USA300 JE2 (IR) occurred at a rate of 1.313^{-9} per base pair per generation.

Thirteen of the 17 genome mutations were reported as substitution mutations. Two C > T substitution mutations were identified in regions deemed as non-coding of the *S. aureus* USA300 JE2 (IR) genome. A total of five silent mutations observed with four occuring in the *sasG* gene each producing a base pair substitution from G > A resulting in a codon sequence change GAC > GAT, no change to the translated aspartate amino acid occurred. The other silent mutation occurred on a CDS encoding hypothetical protein at the base pair 425,612 with an A > G change leading to a codon change of GAA > GAG with no change in the resulting transcribed glutamate amino acid. Base pair substitutions in the genes; Oleate hydratase protein (GGT > AGT), *tarF* (GAA > GCA), *purK*2 (CTT > ATT) and a hypothetical protein (ACT > ATT) resulted in nonsynonymous mutations.

Nonsense mutations were detected in the *fieF* and *ydfJ* genes, these mutations initiated a stop codon at bp 2,361,738 and 2,515,688 respectively. The substitution mutation in the *fieF* gene resulted in a bp change of C > A resulting in a codon change (GGA > TGA) leading to a stop

codon in place of glycine amino acid. Whilst the gene *ydfJ* also underwent a substitution G > T at bp 2,515,688 this resulted in an amino acid change to TCA >TAA leading to a stop codon in place of a serine amino. Deletion mutations were detected in the genes *metI* and *purR.* The *metI* deletion occurred at bp 359,147 and resulting in a frame shift mutation while the *purR* deletion occurred between bp 472,512-472,571.

Insertion mutations were reported in the *purR* and *sigB* genes. The *purR* had an insertion (tandem repeat) of TTT between 472,571-472,575 bp. The insertion of a thymine at bp 2,008,621 in the *sigB* gene resulted in a frame shift.

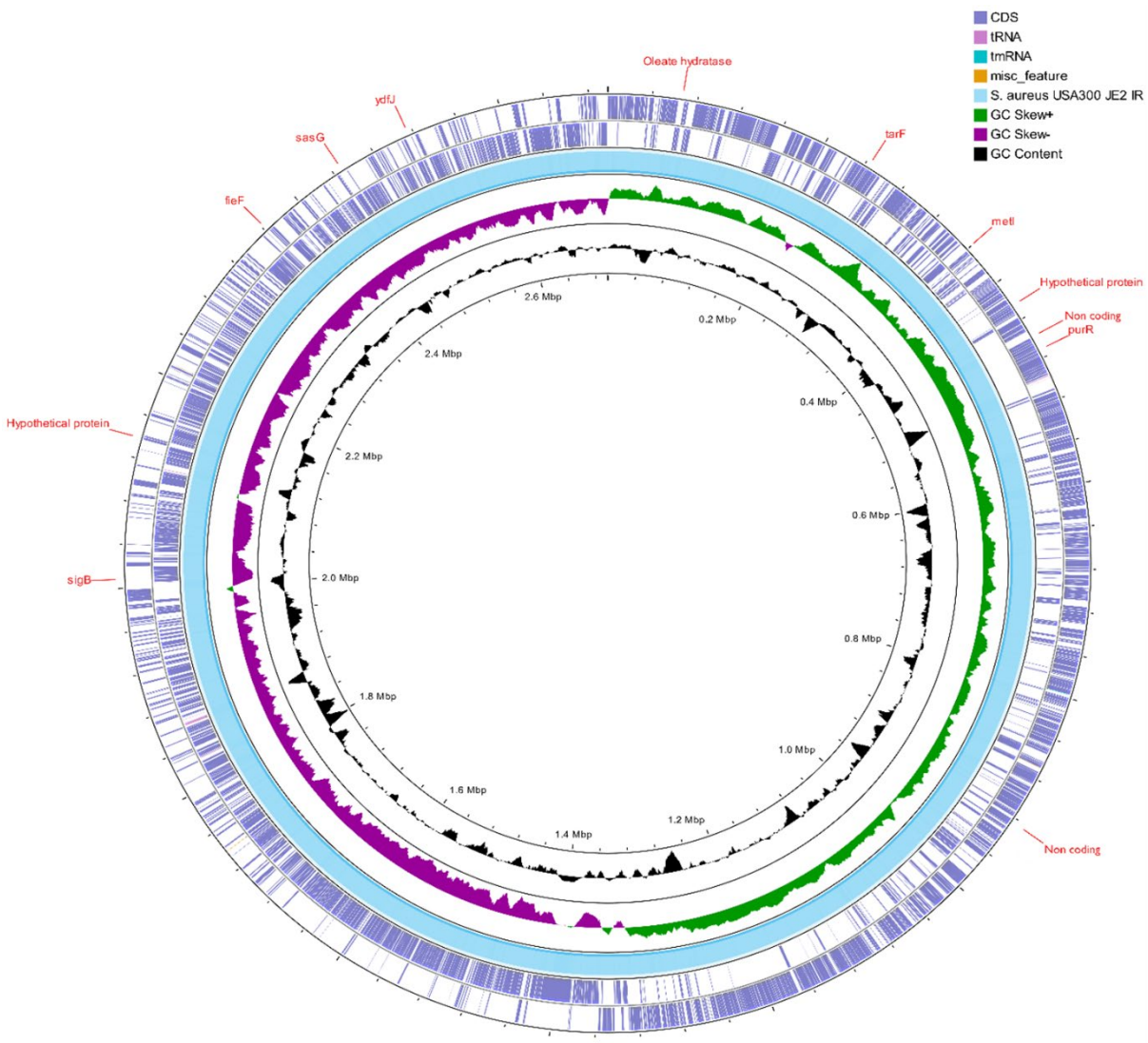

Figure 4. 4: Comparative circular genome (CGView) visualization of the 2.6 Mb bacterial chromosome of *S. aureus* USA300 JE2 against *S. aureus* USA300 JE2 (IR). The genome map highlights the genes affected by base pair changes and their position on the genome map, when resistance was induced in *S. aureus* USA300 JE2. From the outer most circle, *S. aureus* USA300 JE2 genes on the + (circle 1) and – (circle 2) strands, CGView DNA to DNA search of *S. aureus* USA300 JE2 against *S. aureus* USA300 JE2 (IR) (circle 3), GC skew (circle 4), GC content (circle 5).

Table 4. 3: Mutation analysis of *S. aureus* USA300:JE2 and *S. aureus* USA300:JE2 (IR) after increasing incremental exposure to Ru complex 1. Amino acids are shown as Glycine (G), Serine (S), Glutamic Acid (E), Alanine (A), Leucine (L), Isoleucine (I), Threonine (T), Aspartic acid (D), Stop (*).

Gene	Mutation position	Protein	Mutation	Codon	Amino acid	Protein effect	Effect on bp
?	66,217	Oleate hydratase	C > T	GGT>AGT	G>S	Substitution	Transition
tarF	244,246	Teichoic acid glycerol-phosphate transferase	A > C	GAA>GCA	E>A	Substitution	Transversion
metI	359,147	Cystathionine gamma-synthase/O-acetylhomoserine (thiol)-lyase	G > *			Frame shift	Deletion
?	425,612	Hypothetical protein	A > G	GAA>GAG	E>E	None	Transition
Non coding	457,866	Noncoding	C > T			Substitution	Transition
Non coding	920,858	Noncoding	C > T			Substitution	Transition
purR	472,512-472-571	Pur operon repressor				Deletion	Deletion
purR	472,571-472,575	Pur operon repressor	*** > TTT			Insertion (tandem repeat)	Insertion
*purK*2	1,038,649	N5-carboxyaminoimidazole ribonucleotide synthase	C > A	CTT>ATT	L>I	Substitution	Transversion

sigB	2,008,621	RNA polymerase sigma-B factor	* > T			Insertion	Frame shift
?	2,138,146	Hypothetical protein	C > T	ACT>ATT	T >I	Substitution	Transition
fieF	2,361,738	Ferrous-iron efflux pump	C > A	GGA>TGA	G>*	Truncation	Transversion
sasG	2,447,192	surface protein G	G > A	GAC>GAT	D>D	None	Transition
sasG	2,447,576	surface protein G	G > A	GAC>GAT	D>D	None	Transition
sasG	2,447,960	surface protein G	G > A	GAC>GAT	D>D	None	Transition
sasG	2,448,334	surface protein G	G > A	GAC>GAT	D>D	None	Transition
ydfJ	2,515,688	Membrane protein	G > T	TCA>TAA	S>*	Truncation	Transversion

4.3.5 Evaluation of Gene Expression using Reverse Transcription Quantitative PCR (RT-qPCR)

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used to assess regulation of target genes relative to the standardised reference gene *gyrA* in the control strain *S. aureus* USA300 JE2. The control was used as a reference organism to determine relative gene regulations changes of *S. aureus* USA300 JE2 (IR) and *S. aureus* USA300 JE2, before and after 1 h exposure to Ru complex 1.

There were no significant gene regulation changes exhibited between the control *S. aureus* USA300 JE2 and *S. aureus* USA300 JE2 (IR) without exposure to Ru complex 1. The genes *norA*, *clpP* and *katA* exhibited significant gene regulation change compared to the control *S. aureus* USA300 JE2 after exposure to 16 µg/mL of Ru complex 1 (Figure. 4.5). The gene *norA* showed the highest fold increase in gene up-regulation after *S. aureus* USA300 JE2 had been exposed to Ru complex 1 for 1 h, showing an observed 8.815±1.149 fold increase compared to the untreated *S. aureus* USA300 JE2 ($p<0.001$) The gene *clpP* demonstrated the second highest change in gene up-regulation with 4.5685±1.207 fold increase in *S. aureus* USA300 JE2 that had been exposed to Ru complex 1. Also, *clpP* was the only gene to show a significant fold increase in gene expression between *S. aureus* USA300 JE2 and *S. aureus* USA300 JE2 (IR) post exposure to Ru complex 1 ($p=0.008$).

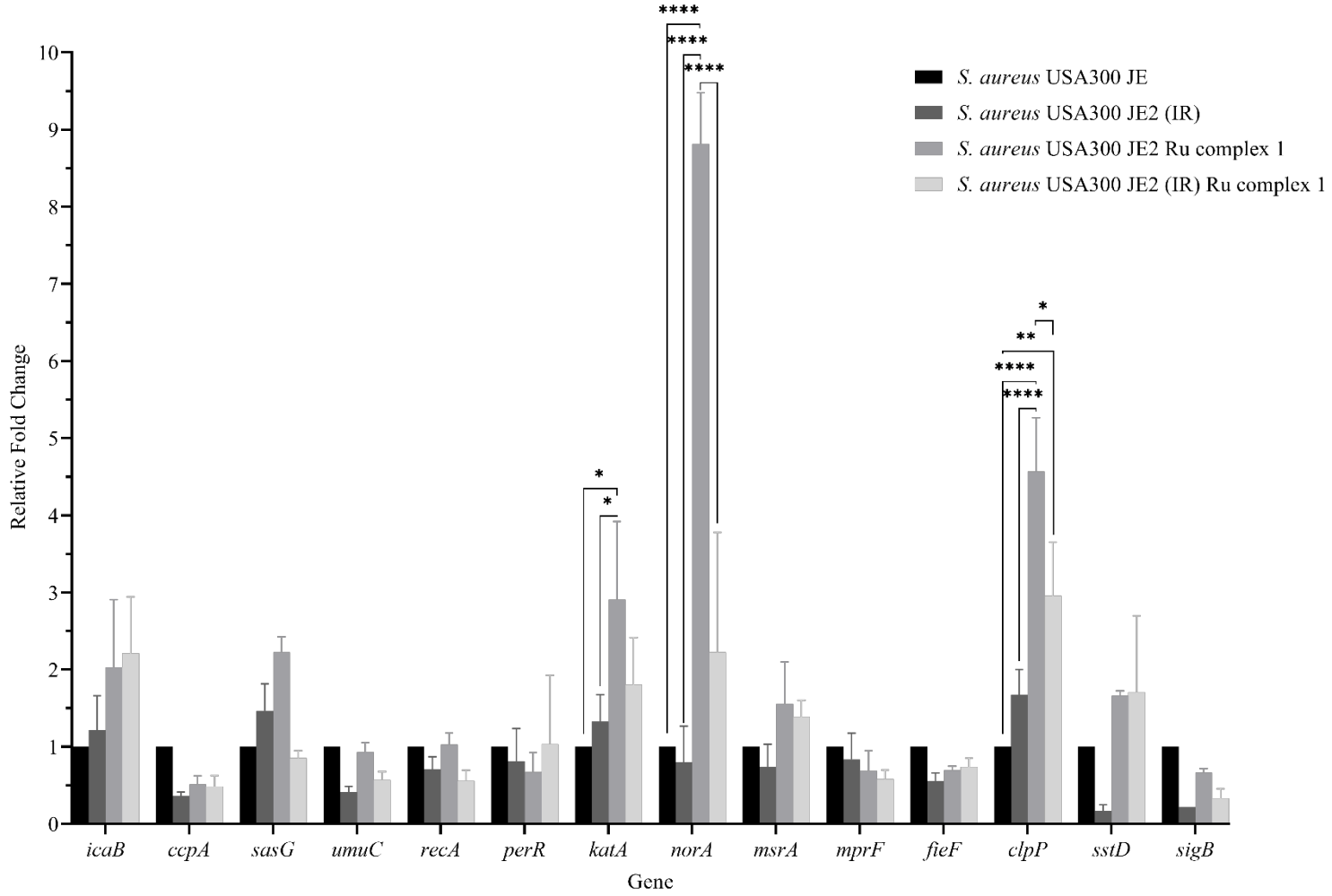

Figure 4. 5: Relative gene expression of 14 target genes under 4 different experimental conditions. The expression levels were calculated using the 2-ΔΔCq method and data are expressed as the mean ± SD. Significance equals (∗∗∗∗p < 0.0001, ∗∗p < 0.01, ∗p < 0.05).

4.4 Discussion

Bacteria in clinical settings now exhibit resistance to nearly all antibiotics (Khalifa *et al.*, 2019; Marston *et al.*, 2016). The primary cause of AMR development in the clinical setting is environmental selective pressure leading to rapid bacterial genome evolution and changes to the transcriptome (Kolář *et al.*, 2001; Ventola, 2015). Once bacteria have become genetically resistant to conventional antibiotics, they thrive in normally hostile environments while further propagating resistance in other species (Hoek *et al.*, 2011). It has been speculated that the use of Ru complexes as antimicrobial agents can reduce the rate of resistance development compared to conventional antibiotics (Claudel *et al*., 2020). This is thought to be caused by the inorganic non-biological nature of Ru complexes coupled with an ability to target multiple cellular components (Pokrovskaya *et al.*, 2010). Previously, it was shown that resistance to metal complexes is often reverted upon the removal of the selective pressure (Dunai *et al.*, 2019).

To assess the rate of resistance development of *S. aureus* USA300:JE2 and *E. coli* EC958 to Ru complex 1, the selected strains were exposed to incrementally increasing concentrations of the Ru complex over a 100-day period. This method has been shown to induce resistance to a wide variety of antibiotics in clinical species (Jahn *et al*., 2017; Wistrand-Yuen *et al*., 2018). Post exposure to Ru complex 1, *S. aureus* USA300:JE2 and *E. coli* EC958 were able to tolerate an 11-fold increase in Ru complex 1 concentration over the pre-exposure MBCs, thus showing a high tolerance or total resistance to this specific Ru complex. Daily growth of *S. aureus* USA300:JE2 and *E. coli* EC958 in the presence of Ru complex 1 confirmed the cells were resistant to the Ru complex and not purely persister cells. Persister cells arise during the stationary phase by reducing metabolic activity and not undergoing binary fission; they contribute up to one percent of any culture (Wood *et al.*, 2013; Grassi *et al.*, 2017). Other

research corroborates the possibility of resistance development to Ru complexes via selective pressure. Dwyer *et al* (1964) demonstrated that a highly virulent strain of *Staphylococcus pyogenes* known as var. Phillips was only able to increase the MBC two-fold after 25 subcultures over a 48-hour period with the synthesised Ru complex $[Ru(Me_4phen)_2(acac)]^+$. In the same period, *S. pyogenes* var. Phillips was able to withstand a 10,000-fold increase to penicillin (Dwyer, 1964). Varney *et al* (2021) exposed *E. coli* EC956 to incrementally increasing concentrations of their synthesised Ru complex 1^{4+}, and *E. coli* EC956 was able to tolerate an 8-fold increase to this Ru complex with concentrations (MIC) increasing from 1.5µM to 12.5µM. The resistance reverted directly after streak plating without exposure to the Ru complex, and the authors assumed that efflux pump regulation was the contributing factor (Varney *et al.* 2021). The resistance generated to Ru complex 1 by *S. aureus* USA300:JE2 and *E. coli* EC958 also proved to be temporary. After five days of subculturing without exposure to Ru complex 1, the resistance reverted to the original MIC and MBC. This reverted resistance indicated that transcriptome changes were likely the cause of the temporary resistance.

The comparative analysis of *S. aureus* USA300 JE2 and *S. aureus* USA300 JE2 (IR) genomes showed that *S. aureus* USA300 JE2 (IR) underwent a total of 17 separate *de novo* mutations across eight different genes. The estimated mutation rate between genomes of *S. aureus* USA300 JE2 (IR) and *S. aureus* USA300 JE2 was 1.313^{-9} per base pair per generation. The mutations within the *S. aureus* USA300 JE2 (IR) genome appeared to have no significant impact on the binary fission rate when compared to *S. aureus* USA300 JE2. Many factors can influence mutation rates within a cell; and under stress-free conditions, spontaneous mutations rates in *S. aureus* occur between 1.6×10^{-10} and 3.4×10^{-10} mutations per nucleotide per generation (Szafrańska *et al*., 2019). The introduction of selective pressure has been shown to accelerate the rate of spontaneous mutation (Engelhardt *et al*., 2019), which varies dramatically between species and strains but is estimated to occur at rates between 10^{-7} to 10^{-10} per nucleotide

per generation (Wang *et al*., 2001; Björkholm *et al*., 2001; Nyinoh *et al*., 2018; Sánchez *et al*., 2014). The exposure concentration of an antimicrobial also contributes to the rate of spontaneous mutation and therefore the rate of resistance development (Oz *et al*., 2014; Andersson *et al*., 2014). The results demonstrated an increased rate of mutation for the *S. aureus* USA300 JE2 (IR) when compared to Szafrańska *et al*. (2019) and indicated that selective pressure caused Ru complex 1 to accelerate the mutational rate within the *S. aureus* USA300 JE2 (IR) genome. A more accurate mutation rate could have been achieved by fully sequencing of the *S. aureus* USA300 JE2 control genome that was passaged in parallel with the *S. aureus* USA300 JE2 (IR). The exact mutation rate could have been calculating for the control under the same physiological conditions and using the identical strain. Due to budget limitations sequencing of the control genome was not undertaken.

The bacteria that were subjected to long-term incremental exposure of Ru complex 1 exhibited distinct morphological changes when compared to their control counterparts. The most notably morphological difference between *E. coli* EC958 and *E. coli* EC958 (IR) was the enhanced slime layer produced by *E. coli* EC958 (IR). The enhanced slime layer subsidised within two generations after no exposure to Ru complex 1, indicating a transcriptome response, directly linking exposure to Ru complex 1 with the enhanced production of extracellular matrix in *E. coli* EC958. The increased slim layer that is produced by *E. coli* EC958 (IR) was most like a defensive mechanism and has been shown to aid in antimicrobial resistance and was mostly likely a contributing factor in the increased resistance to Ru complex 1 (Elsayed *et al*., 2019)

The most notable difference was that *S. aureus* USA300 JE2 (IR) exhibited a substantially reduced staphyloxanthin expression when compared to *S. aureus* USA300 JE2, which resulted in an elimination of the golden pigmentation. This reduction in staphyloxanthin expression persisted even after the resistance to Ru complex 1 had reverted, indicating possible mutations within the genome. The extended use of sub culturing under stress free conditions has

previously been associated with reduced expression of virulence factors including staphyloxanthin (Ansari *et al*., 2011; Mohammad *et al*., 2019). Mohammad *et al* (2019) subcultured four different strains of *S. aureus* demonstrating pH, culture medium and temperature all contributed to staphyloxanthin expression during daily repeated sub culturing (Mohammad *et al*., 2019). A *S. aureus* USA300 JE2 control was passaged in parallel with *S. aureus* USA300 JE2 (IR), there was no visual reduction in staphyloxanthin expression compared to the *S. aureus* USA300 JE2 that had not been sub cultured.

This is potentially significant, as staphyloxanthin has the ability to act as a virulence factor and helps protect *S. aureus* from host defences by impairing neutrophil killing and promoting virulence through its antioxidant activity (Lang *et al.*, 2000; Liu *et al*., 2005). Xue *et al*., (2019) speculated that if a method to control production of staphyloxanthin could be found, it would reduce viability of *S. aureus* in a host organism (Xue *et al*., 2019). This would make the staphyloxanthin biosynthesis pathway a potential target in the treatment of *S. aureus* infection. Because growth media has been shown to alter the expression of staphyloxanthin, the same batch of Mueller-Hinton growth media was used for both cultures to eliminate the possibility of nutrients being a contributing factor (Mashburn *et al*., 2004).

The complete synthesis of staphyloxanthin is carried out by the *crtOPQMN* operon and is directly regulated by *sigB* (Antonic *et al*., 2013). Other genes have been shown to influence the expression of staphyloxanthin, in particular the purine biosynthesis family genes *purN*, *purH,* and *purD* (*Lan et al*., 2019; Xue *et al*., 2019). Three genes underwent mutational change in the *S. aureus* USA300 JE2 (IR) genome, potentially explaining the altered staphyloxanthin expression. The most notable mutation was an insertion (tandem repeat) mutation, which lead to a frameshift in the *crtOPQMN* operon regulator *sigB.* Understanding the role of this frameshift mutation on regulating expression levels of staphyloxanthin remains to be determined, but RT-qPCR analysis confirmed decreased expression levels of SigB across all

conditions when compared to the *S. aureus* USA300 JE2 control. Mutational changes to the *sigB* gene have also been shown to reduce tolerance to antibiotics such as oxacillin and vancomycin, although other factors were involved (Singh *et al*., 2003). Spontaneous mutations also occurred within two gene members of the purine biosynthesis family. A substitution mutation occurred in the *purK*2 gene, leading to an amino acid change L>I while a 51 bp deletion mutation occurred in the *purR* gene. While existing literature shows no direct link of *purK*2 or *purR* genes to staphyloxanthin regulation, this research establishes a potential link. If the mutations in the purine biosynthesis family of *S. aureus* USA300 JE2 (IR) were linked to decreases in staphyloxanthin expression, this would be unusual, as Xue *et al*. (2019) showed mutations in the genes *purN, purH, purD, and purA* were all linked to increases in staphyloxanthin expression (Xue *et al*., 2019).

Four other genes that underwent mutagenesis in the *S. aureus* USA300 JE2 (IR) genome are of potential interest—namely, the *fieF*, *ydfJ, tarF*, and *metI* genes. The *ydfJ* and *fieF* genes underwent nonsense mutation, leading to early termination of the genes. The *fieF* gene is part of the divalent metal cation transporter family and aids in ferrous ion efflux; Grass *et al*. (2005) showed that the transcription rate of the *fieF* gene directly correlates with the intracellular iron concentration (Grass *et al*., 2005). If the nonsense mutation in the *fieF* gene affected iron efflux from the cell, it is possible iron accumulation could occur. This would increase the number of hydroxyl radicals within the cell via the Fenton reaction and would have a detrimental effect on the growth rate of *S. aureus* USA300 JE2 (IR) (Pi *et al*., 2017). Yet, the growth curves showed no reduction in growth for *S. aureus* USA300 JE2 (IR), indicating there was either no excess of iron or the nonsense mutation and downregulation of the *fieF* gene had no effect on iron efflux. The *ydfJ* gene acts as an inner membrane metabolite transport protein, and members of the YdfJ family have been associated with heavy-metal ion export, solvent tolerance, multidrug resistance, and transport of oligosaccharides (Serizawa *et al*., 2005). While the *ydfJ*

gene was unlikely to be involved in the efflux of antibiotics, it was likely to be involved in the efflux of noxious compounds including heavy-metal ions and dyes (Serizawa *et al*., 2005). The nonsense mutation also appeared to have no negative impact on the growth rate of *S. aureus* USA300 JE2 (IR) across all concentrations tested. The gene *metI* is responsible for methionine-biosynthesis enzyme cysteine-γ-synthase and is a known virulence factor that assists in the nasal colonization of humans. Mutants with *metI* deficiencies have been shown to have a severe reduction in colonising capability (Ramsey *et al*., 2016). The effects of the frameshift mutation on the colonising capability of *S. aureus* USA300 JE2 (IR) remain unknown, and further study of *metI* would be required to identify any changes in colonisation. The *tarF* gene is involved in glycerol phosphate oligomerization during the synthesis of teichoic acid and is essential to cell survival. Past efforts to remove the gene have resulted in deleterious effects on the cell (D'Elia *et al*., 2006; Qian *et al*., 2016). The incomplete production of teichoic acid is known to cause cell death by accumulation of toxic substances (Qian *et al*., 2016). This would indicate the substitution mutation appeared to have no detrimental effect on cellular function; thus, the full synthesis of teichoic acid must have occurred.

The 14 target genes selected for RT-qPCR performed a wide variety of key cellular functions, primarily relating to antimicrobial activity. The aim was to determine the effects on expression profiles for selected target genes within *S. aureus* USA300 JE2 and *S. aureus* USA300 JE2 (IR) in response to Ru complex 1 exposure. The target genes included the DNA damage repair mechanism enzyme *recA*; *katA* which neutralises the effects of hydrogen peroxide within the cell and *mprF*, which reduces the negative charge on the membrane potentially repulsing cationic molecules (Barrière *et al*., 2002; Giliberti *et al*., 2006; Andrä *et al*., 2011). Of the 14 genes tested, only *norA*, *clpP*, and *katA* genes produced significant changes in gene regulation in response to exposure to Ru complex 1. Due to the vastly different cellular functions

performed by these genes, exposure to Ru complex 1 affects a wide variety of cellular functions.

The *norA* gene is part of the major facilitator superfamily and codes for the NorA efflux pump. The NorA efflux pump enables resistance to fluoroquinolone antibiotics, biocides, dyes, quaternary ammonium compounds, and antiseptics (Costa *et al*., 2019). The upregulation of the *norA* gene in *S. aureus* USA300 JE2 (IR) and *S. aureus* USA300 JE2 indicated a potential cellular response that would lead to a higher extracellular tolerance of Ru complex 1. By altering the regulation of the *norA* gene it may be possible to reduce the resistance to Ru complex 1. Zimmermann *et al*. (2019) recently showed the tyrosine kinase inhibitor nilotinib was able to inhibit the NorA efflux system; thus, the combination of nilotinib and Ru complex 1 could potentially lead to lower MIC and MBC concentrations for Ru complex 1 (Zimmermann *et al*., 2019). A second efflux system was also assessed for gene regulation changes. The *msrA* gene that codes for an ATP transporter known to efflux erythromycin and streptogramin B, so was a potential target for antimicrobials (Ojo *et al*., 2006). The *msrA* showed no significant upregulation across any condition but was not significantly upregulated for *S. aureus* USA300 JE2 (IR) with and without exposure to Ru complex 1, indicating there could be a general trend in the upregulation of efflux systems from a global regulator.

The upregulation of the *clpP* gene could indicate a significant increase in abnormally folded proteins. The *clpP* gene produces the ClpP subunit of ClpXP protease. Under stressful conditions, ClpXP protease breaks down abnormally folded proteins and acts as a rapid adaptive response, contributing to growth and survival of bacteria (Gaillot *et al*., 2000; Moreno-Cinos *et al*., 2019). This is of potential significance due to the cationic nature of Ru complex 1 and its ability to bind to anionic structures such as DNA and proteins, causing irreversible damage. The upregulated *clpP* gene could indicate a protein damage as a potential mechanism of action for Ru complex 1.

Hydrogen peroxide is a cell damaging agent and is generated within the cell as a by-product of aerobic respiration. The *katA* gene neutralises the effects of hydrogen peroxide by producing the enzyme catalase, which neutralises the harmful effects of hydrogen peroxide by decomposing H_2O_2 into H_2O and O_2 (Heck *et al*., 2010). The upregulation of the *katA* gene indicates an increase in ROS within the cell. Clinically used antibiotics such as ampicillin, kanamycin, and norfloxacin are known to cause a dramatic increase in oxidative stress within a cell, leading to deleterious effects on DNA and proteins within the cell (Dwyer *et al*., 2014). Due to the upregulation of the *katA* gene and as 6 of the 17 mutations occurred at cytosines, this corroborates work by Degtyareva *et al*. (2010) which stated that mutations induced by oxidative stress primarily occur at cytosines (Degtyareva *et al*., 2010). The *perR* gene was also assessed for expression changes; this gene has a primary role as a ferric uptake regulator and is known to control transcription of the *katA* gene and contribute to virulence in *S. aureus* (Horsburgh *et al*., 2001). While there was no significant change in expression of the *perR* gene, there was a trend of downregulation across all conditions. The non-significant decrease in expression of PerR was likely enough to trigger a significantly increase KatA expression. Thus, it could be hypothesized that exposure to Ru complex 1 leads to increased levels of intracellular ROS further promoting oxidative stress and subsequent mutagenesis.

Clinically used antibiotics such as ciprofloxacin are known to cause DNA damage, increasing transcription and translation of the DNA associated repair genes *recA and umuC*, thus highlighting two potential target genes (Qin *et al*., 2015). Liu *et al*. (2018) hypothesised that arene ligands, similar to the one present in Ru complex 1, would bind to the genomic DNA, leading to DNA damage and subsequently causing genes involved in DNA repair to be upregulated when exposed to Ru complex 1 (Liu *et al*., 2018). The RT-qPCR showed both *recA and umuC* were downregulated across all experimental conditions. The expression of the *recA* gene is greatly stimulated by agents that block normal chromosomal DNA replication and

is activated in the presence of single-stranded DNA (Casaregola Liu *et al*., 1982). If exposure to Ru complex 1 did not increase the quantity of single-stranded DNA, this may explain why the rate of transcription of the *recA* gene may not have increased. The regulation of *recA* also plays a major role in the regulation of error-prone polymerase *umuC*. The UmuC protein is often involved in mutagenesis and it was speculated there may have been regulatory changes to the *umuC* gene allowing increased tolerances of Ru complex 1 in *S. aureus* USA300 JE2 (IR) (Reuven *et al*., 1998). As there were no significant gene expression changes to *recA*, there would also be no regulation changes to UmuC prevalence due to the *umuC* gene being reliant on the *recA* gene (Qin *et al*., 2015).

Whilst no quantification of biofilm formation was undertaken, it was important to assess genes relating to biofilm formation due to the ability to contribute to resistance generation and pathogenicity (Bowler *et al*., 2020; Vestby *et al*., 2020). The following three genes relating to biofilm formation were evaluated: *icaB, sasG, and ccpA*. Genes *icaB and sasG* encode for protein products which promote adhesion of bacterial cells, whilst *ccpA* encodes for catabolite control protein A and influences biofilm formation (Seidl *et al*., 2008). Both *icaB and sasG* showed a non-significant increase in transcription across all experimental conditions after exposure to Ru complex 1 for one hour. These two genes are directly responsible for the adhesion of bacteria to surfaces. Any increase in gene expression would indicate that Ru complex 1 was initiating biofilm formation in *S. aureus* USA300 JE2. Conversely, some Ru complexes containing chlorine have been shown to inhibit the formation of biofilms in *E. coli*, *S. aureus*, and *P. aeruginosa* PAO1 (Jabłońska *et al*., 2020).; however, this was not the case when *S. aureus* USA300 JE2 was exposed to Ru complex 1. The *icaB* gene has previously been implicated in studies relating to Ru-based antimicrobials (Vaishampayan *et al*., 2018*).* Vaishampayan *et al*. (2018) showed that the regulation of the *icaB* gene is time dependent where the Ru-based antimicrobial coating AGXX showed a -104.2-fold regulation after 80

minutes, yet after 120 minutes the regulation increased to a 7.8-fold increase (Vaishampayan *et al*., 2018). The downregulation of *ccpA* was reported across all conditions. This was unexpected due to the ability of *ccpA* to reduce resistance to selected antimicrobials such as oxacillin and teicoplanin in *S. aureus* strains. (Seidl *et al*., 2006). The ability of the *ccpA* gene to influence biofilm formation is generally dependent on nutrient availability, the same batch was used throughout discarding the slight gene regulation changes that can occur (Zheng *et al*., 2012). There was a non-significant downregulation of *mprF* across all experimental conditions, indicating a possible increase in the negative charge of the membrane when compared to the *S. aureus* USA300 JE2 control. Smitten *et al.* (2019) showed that increased transcription of *mprF* played a significant role in repelling cationic Ru complexes, significantly increasing survival of *S. aureus* SH1000 mutant strains (Smitten *et al.*, 2019). The downregulation of *mprF* shows that it did not contribute to the ability of *S. aureus* USA300 JE2 (IR) to tolerate higher concentrations of Ru complex 1. The *sstD* gene is involved in metal cation acquisition and is upregulated in iron scarce environments to allow survival and proliferation (Morrissey *et al*., 2000). The results of the RT-qPCR showed that there was a non-significant but distinct downregulation of *sstD* when *S. aureus* USA300 JE2 was exposed to Ru complex 1. Conversely, *sstD* showed a distinct but not significant upregulation for *S. aureus* USA300 JE2 (IR) with or without exposure to Ru complex 1. This distinct downregulation when *S. aureus* USA300 JE2 was exposed to Ru complex 1 could be due to lower tolerances of Ru complex 1 and an increased bactericidal effect compared to *S. aureus* USA300 JE2 (IR) exposure to Ru complex 1. The *S. aureus* USA300 JE2 would focus on conservation of life genes opposed to genes focusing on proliferation.

This chapter demonstrated that both *E. coli* EC958 and *S. aureus* USA300 JE2 were able to tolerate over an 11-fold increase in exposure concentration of Ru complex 1 after 100 days of

increasing incremental exposure. It could be hypothesised that Ru complex 1 was entering *S. aureus* USA300 JE2 in sufficient quantities to trigger a significantly increased efflux response. Upon penetrating the bacterial membranes Ru complex 1 was likely binding to anionic structures such as proteins and DNA causing deleterious effects. The DNA/protein damage caused by Ru complex 1 subsequently generated an increased level of ROS within the cell leading to increased mutation rates. Further experimentation of individual mechanisms of action would need to be conducted to validate the hypothesis. By better understanding resistance development to Ru complexes, it may also give a greater insight when developing new Ru complexes for antimicrobial applications (Chellat *et al.*, 2016).

Chapter 5: Mechanistic Actions of Ruthenium Complex

5.1 Introduction

Identifying and understanding the cellular target of an antimicrobial agent is a key stage in the development of novel antimicrobial therapies. Conventional organic antibiotics target one of five cellular processes; cell wall synthesis, protein synthesis, RNA synthesis, DNA synthesis, and intermediary metabolism (Kohanski *et al.*, 2010). *In-vitro* data demonstrates that synthetic antimicrobial agents exhibit a broader range of mechanistic activity than purely organic antimicrobials (Munteanu *et al*., 2021).

The use of ruthenium (Ru) as a central atom in antimicrobial complexes provides several advantages over organic antimicrobials. The three-dimensional bonding configuration can be tailored to specific cellular targets, and the biologically accessible oxidation states make Ru complexes ideal candidates for binding negatively charged biological structures such as cell membranes, nucleic acids and proteins (Adeniyi *et al*., 2016). While research into antimicrobial Ru complexes is less comprehensive than that of their antitumour counterparts, Ru complexes have still been implicated in targeting various cellular processes. Ru complexes have been shown to permeabilize Gram-positive and Gram-negative membranes, induce SOS repair response, cause mutagenic effects, inhibit RNA transcription, and induce DNA degradation (Pereira *et al*., 2008; Maeda *et al*., 2019; Munteanu *et al*., 2021). Several of these antimicrobial mechanisms are not known to occur in bacteria when challenged with organic compounds (Frei *et al*., 2020). Some *in-vitro* experiments have revealed single or multiple mechanisms of action of organic and synthetic antimicrobial agents (Munita *et al.*, 2016). The structural and molecular properties of each ligand affect the ability of the Ru complex to reach and interact with its intended subcellular target. Among several factors, the molecular mass, lipophilicity, wettability, and charge primarily contribute to the potency and cellular target of the antimicrobial compound (Claudel *et al*., 2020).

An ideal antimicrobial target site must be exclusively present in prokaryotic cells, therefore selectively reducing the potential side effects in clinical settings. Antimicrobials can produce non-anti-infective and off target effects, with profound consequences in clinical settings (Meier *et al.*, 2017, Sullivan *et al.*, 2018). The use of vancomycin exemplifies the non-anti-infective effects of antimicrobial agents. Vancomycin targets the prokaryotic specific d-Ala-d-Ala terminus of peptidoglycan**.** This binding site is not present in eukaryotic cells, yet vancomycin causes unrelated nephrotoxicity by inducing oxidative stress via compromised mitochondrial function, which subsequently lead to dose-dependent proliferation of proximal tubular cells (Filippone *et al.*, 2017). Most modern antibiotics produce some degree of off-target cytotoxic effect in eukaryotic cells. However, if the therapeutic efficacy is considerably higher against the prokaryotic counterparts, then the antimicrobial properties remain viable.

The cellular membranes are the initial contact site of any antimicrobial agent with a prokaryotic organism. These provide structural stability and a protective barrier which confers homeostasis within the cell by selectively controlling the movement of substances across the membrane via porin and efflux pumps (Silhavy *et al.*, 2010). Permeabilization of the cellular membranes by antimicrobial agents disrupts the cellular homeostasis, exposes the cellular content to the outer environment, culminating in cell death. The differences in structure, composition, and charge between prokaryotic and eukaryotic membranes make them an ideal target for antimicrobial agents. Eukaryotic membranes possess a neutral charge, while prokaryotic membranes possess an anionic charge due to the presents of teichoic acids. This anionic charge electrostatically attracts and binds counter-charged molecules. Next, the ligands within the complex can interact and destabilizes the membrane (Omardien *et al.*, 2016). Although the differences between Gram-positive and Gram-negative membranes are less profound, they still offer attractive molecular candidate targets for antimicrobial agents to exploit. The metal-chelating agents EDTA, sequesters the outer membrane-stabilizing divalent cations Mg^{2+} and Ca^{2+}, which

consequently reduces the outer membrane stability and cell viability in Gram-negative bacteria (Alakomi *et al.*, 2006). Furthermore, quaternary ammonium compounds (QACs) can target the cytoplasmic membrane in Gram-positive and Gram-negative bacteria. These cations produce an antimicrobial effect by electrostatically binding to the membrane, which at a high concentration disrupts the membrane, and discharges the cytoplasmic contents to the exterior (Jennings *et al.*, 2015).

To target intracellular components, an antimicrobial agent is required to penetrate the cellular membranes. Gram-negative bacteria possess a greater intrinsic ability to resist antimicrobial molecules than Gram-positive bacteria (section 1.2.1.2). The molecules enter the cytoplasm in one of four ways: facilitated diffusion, through ion channels, passive, and active transport. Three major factors can influence the ability of molecules to enter the bacterial cytoplasm: mainly the lipophilicity, molecular weight, and hydrogen bond counts (Yang *et al.*, 2015). An antimicrobial agent must possess adequate lipophilicity to disseminate through the biological membranes, yet it should be hydrophilic enough to penetrate the cytoplasm (Stoica *et al.*, 2015). The utilisation of methyl groups increases the lipophilicity of molecules and leads to higher uptake of antimicrobial molecules (Harrold *et al.*, 2013). The lipophilicity of a molecule also contributes to the amount of absorption, distribution, metabolism, and elimination (ADME) of therapeutic drugs (Stoica *et al.*, 2015). The molecular weight of an antimicrobial agent partly dictates the ability to enter the cell, with lower molecular weight molecules transiting more easily into Gram-negative and Gram-positive bacteria. The upper molecular weight limit of 500 kDa and 6 kDa is suggested for Gram-positive and Gram-negative bacteria respectively (Lambert, 2002; Zgurskaya *et al.*, 2015). Small hydrophilic molecules such as bile salts, quaternary ammonium salts, and other cations can permeate through the outer membrane of Gram-negative bacteria via self-promoted uptake (Davis *et al.*, 2014)

Upon penetration of the cellular membranes, intracellular targets become accessible to the antimicrobial agent; DNA, RNA, and proteins are common targets of Ru complexes (Sun et al., 2018; Chakrabortty *et al*., 2017; Li *et al*., 2015). The interaction of Ru complexes on intracellular targets have been shown to result in deleterious or mutagenic effects (Yasbin *et al*., 1980). A significant proportion of the research conducted on antimicrobial Ru complexes has focused on nucleic acid binding (Turel *et al*., 2010; Liu *et al*., 2018; Yang *et al*., 2018). This is partly due to the repurposing of failed antitumour Ru complexes that often focus on nucleic acid binding as a mechanim of action. These Ru complexes often utilise aromatic ligands and possess specific physicochemical properties such as cationic charges, with an affinity for nucleic acid binding (Lombardo *et al*., 2015). Nucleic acid interactions can result in increased mutation rates, complete degradation of DNA, and errors in transcription. Alternatively, RNA interactions can lead to reduced protein translation and thereby inhibiting binary fission (Hong *et al*., 2014). The resulting nucleic acid damage increases intracellular ROS production (Kang *et al*., 2012; Rowe *et al*., 2008; Liao *et al.*, 2017) which leads to higher mutation rates and impaired cellular physiological function (Zhao *et al*., 2014; McBee *et al*., 2017).

5.2 Aims and Objectives

The aim of this chapter was to determine the cellular targets of Ru complex 1 and 7 and evaluate the effects of these mechanisms of activity on key bacterial species.

5.2.1 Objectives

- Determine the nature of interactions between Ru complexes and cellular membranes and visualise the effects.
- Quantify the uptake of the Ru complexes into the bacterial cell and determine potential nucleic acid interactions.
- Evaluate and quantify ROS generation within the cell after exposure to the Ru complexes.

5.3 Results

5.3.1 Outer Membrane Permeabilization Assay

The outer membrane permeabilization assay was conducted using fluorescent compound NPN. In a hydrophilic environment the ability of NPN to fluoresce is quenched, but in the presence of a permeabilizing agent the outer membrane of bacteria becomes depolarized and NPN is able to enter the phospholipid membrane. Once inside the hydrophobic environment of the phospholipid membrane, NPN increases in fluorescent intensity. The two pathogens *P. aeruginosa* PAO1 (Figure. 5.1) and *E. coli* EC958 (Figure. 5.2) were selected for the outer permeabilization study against Ru complex 7. Ru complex 1 was omitted from experimentation due to low antimicrobial activity against these Gram-negative bacterial pathogens. The addition of water as a solvent control resulted in no significant changes in depolarisation when compared to the negative controls (data not shown).

The Triton X-100 positive control and Ru complex 7 were added after 20 s and permeabilization of the *P. aeruginosa* PAO1 outer membrane began instantly when exposed to Triton X-100, with fluorescence rising from 2.05 a.u to a peak of 42.34 a.u. The fluorescence signal plateaued at 39.48 a.u after 140 s showing significant membrane permeability compared to the negative control ($p<0.001$). No permeabilizing effects were observed when Ru complex 7 was added at the MBC concentration of 128 µg/mL after 300 s and no significance was recorded when compared to the negative control ($p>0.999$). Peak fluorescence of Ru complex 7 was again significantly lower than the peak fluorescence of Triton X-100 ($p<0.001$).

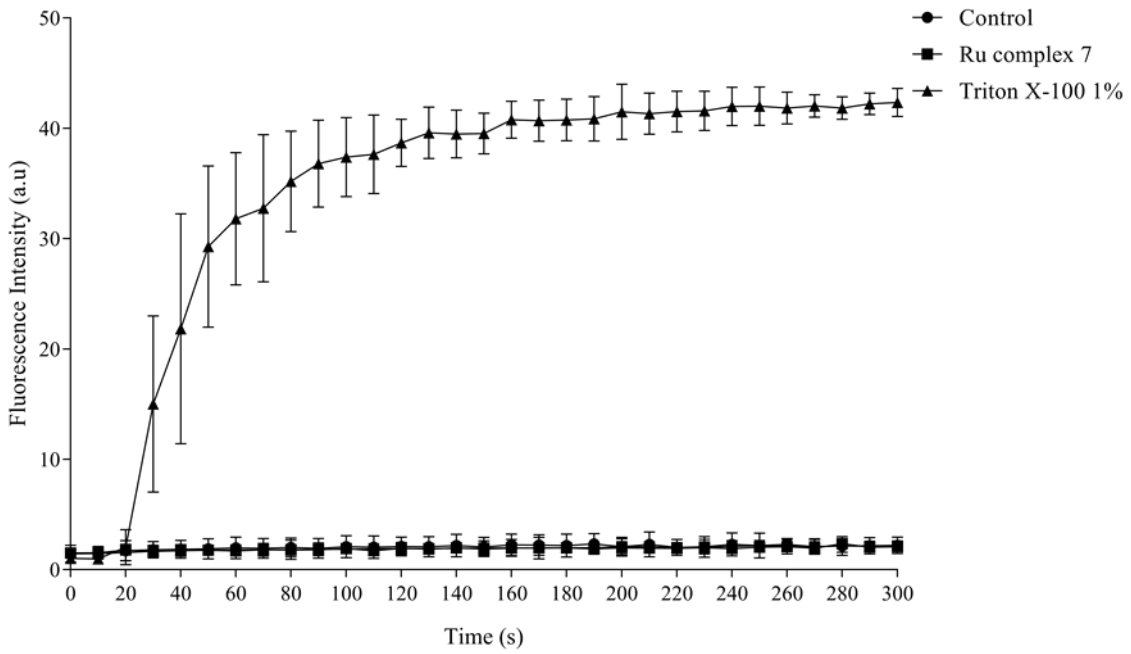

Figure 5. 1: The outer membrane permeability of *P. aeruginosa* PAO1 induced by Triton X-100 (▲), Ru complex 7 (■) and negative control (●). The NPN uptake was monitored at λ_{ex} 350 nm and λ_{em} 420 nm. Values are the mean ± SD of three biological replicates.

The outer membrane permeabilization assay was also performed using Ru complex 7 against *E. coli* EC958 (Figure 5.2). Exposure of cells to Triton X-100 resulted in immediate permeabilization of the outer membrane with fluorescence rising from 4.37 a.u to a peak of 150.77 a.u. The fluorescence plateaued after 140 s indicating either the membranes were fully permeabilized, the membranes were saturated with NPN or the NPN concentration was exhausted. Treatment with Triton X-100 showed a statistical significance in terms of permeability of the outer membrane when compared to the negative control (p<0.001). When Ru complex 7 was applied to the bacterial cells, fluorescence remained relatively static reaching a peak of 2.88 a.u, which was statistically comparable to the fluorescence values observed in the negative control (p=0.9716). Peak fluorescence after exposure of bacterial cells

to Ru complex 7 was significantly lower than the peak fluorescence resulting from incubation with Triton X-100 (p<0.001).

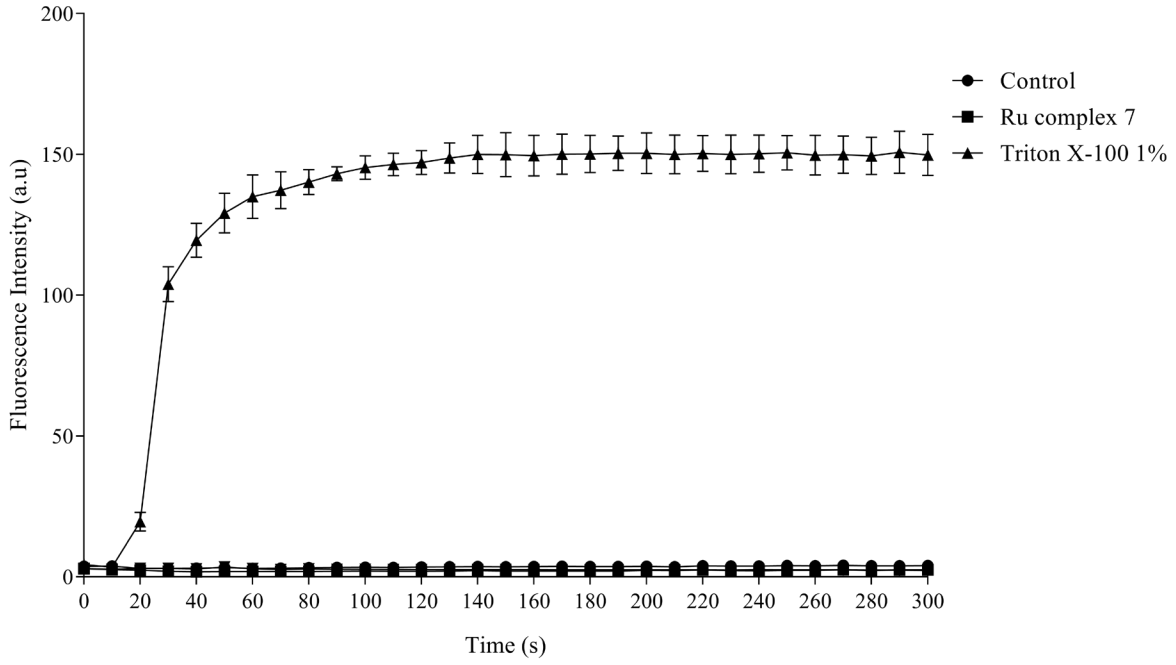

Figure 5. 2: The outer membrane permeability of *E. coli* EC958 induced by Triton X-100 (▲), Ru complex 7 (■) and negative control (●). The NPN uptake was monitored at λ_{ex} 350 nm and λ_{em} 420 nm. Values are the mean ± SD of three biological replicates.

5.3.2 Cytoplasmic Membrane Depolarisation

The cytoplasmic membrane depolarization assay was used to assess the ability of Ru complexes to depolarize the cytoplasmic membrane. The assay utilises $DiSC_3(5)$ which is a cationic membrane potential sensitive dye, where $DiSC_3(5)$ possesses sufficient hydrophobicity to penetrate lipid bilayers and partition itself in the bacterial cell membrane. Once partitioned within the bacterial cell membrane the fluorescence is quenched, if cell membrane depolarization occurs $DiSC_3(5)$ is dequenched as it leaks into the surrounding medium (Winkel *et al*., 2016). Treatment of bacterial cells with solvent controls demonstrated no significant depolarizing effect on the cytoplasmic membrane (data not shown). The cytoplasmic membrane depolarization assay was conducted over a 60 min period, the bacterial cells and $DiSC_3(5)$ solution was allowed to stabilise for 14 min within the cuvette before adding Ru complex 1, Ru complex 7 and Triton X-100.

After 60 min of exposure to Ru complexes 1 and 7, *S. aureus* USA300 JE2 and *S. aureus* USA300 JE2 (IR) exhibited no significant cytoplasmic membrane depolarisation compared to the negative control (p=0.9740) (p=0.8924) and (p=0.0763) (p=0.0869) respectively (Figure. 5.3A and B). Exposure of Ru complex 1 and 7 to *S. aureus* USA300 JE2 resulted in a 1.601% and 5.736% normalised fluorescence increase after 60 min respectively. While Ru complexes 1 and 7 against *S. aureus* USA300 JE2 (IR) produced 1.406% and 1.505% normalised fluorescence increase after 60 min respectively.

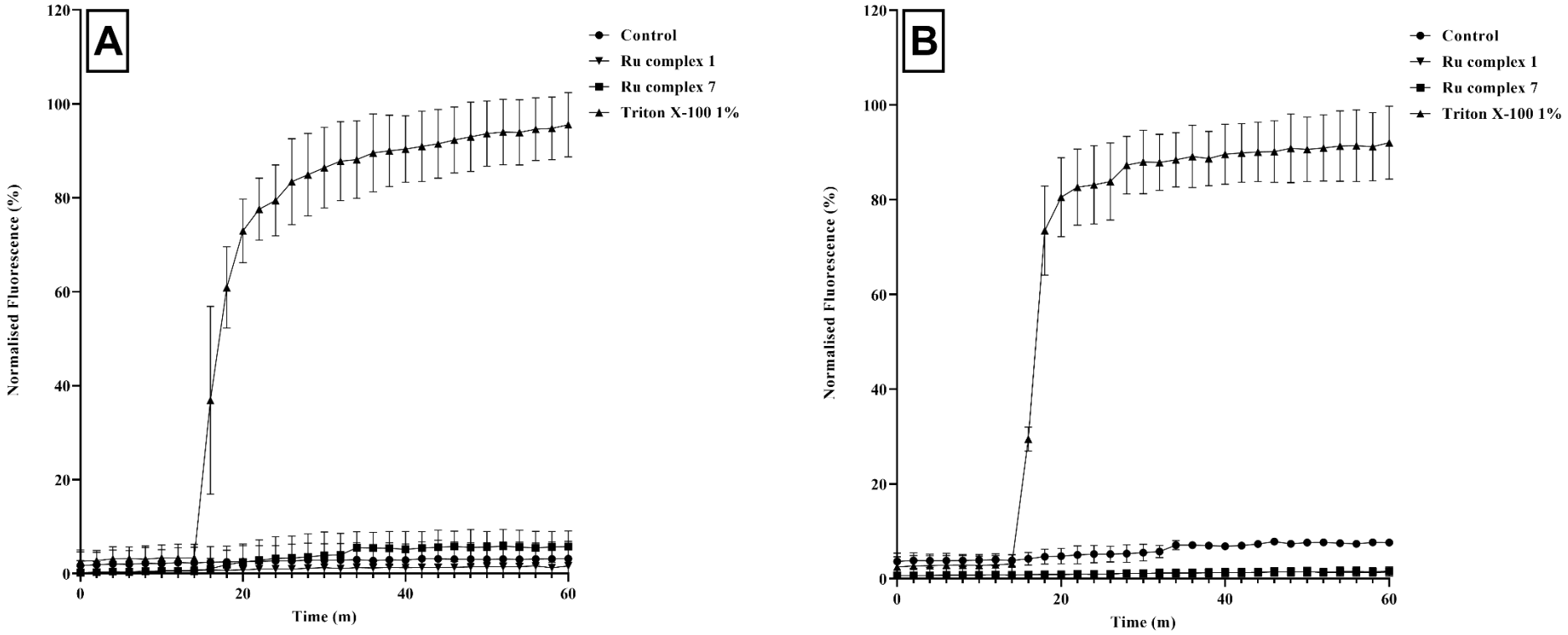

Figure 5. 3: Cytoplasmic membrane depolarisation of A) *S. aureus* USA300 JE2 and B) *S. aureus* USA300 JE2 (IR) (●), by Ru complex 1 at 16 µg/mL(▼) Ru complex 7 at 256 µg/mL (■) and Triton X-100 at 1 % (▲) using the membrane potential-sensitive dye $DiSC_3(5)$. Fluorescence was measured at λ_{ex} of 620 nm and an λ_{em} at 670 nm. Values are the mean ± SD. All experiments were performed in triplicate.

No significance changes in cytoplasmic membrane depolarization of *P. aeruginosa* PAO1 was observed when exposed to Ru complex 1 and 7 (p>0.999) and (p=0.4792) respectively (Figure. 5.4) compared to cells exposed to Triton X-100 with normalised fluorescence increase of 13.394% and 6.880% respectively.

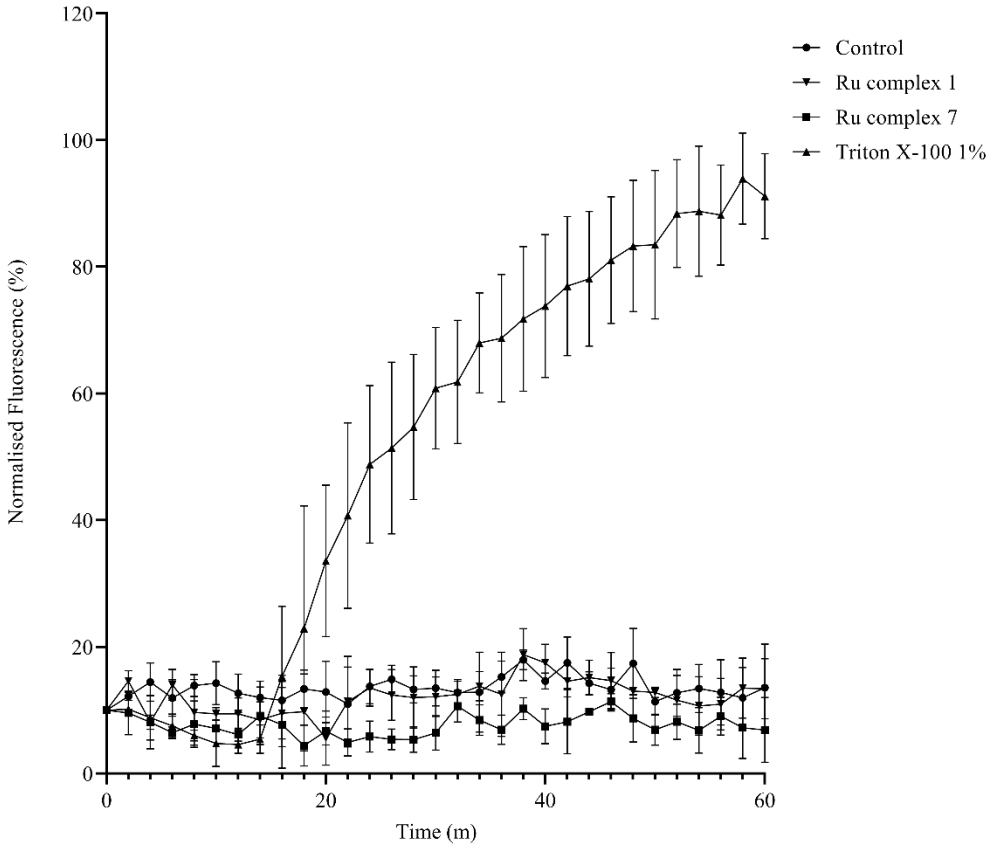

Figure 5. 4: Cytoplasmic membrane depolarisation of *P. aeruginosa* PAO1 (●) by Ru complex 1 at 16 µg/mL (▼), Ru complex 7 at 256 µg/mL (■) and Triton X-100 at 1 % (▲) using the membrane potential-sensitive dye $DiSC_3(5)$. Fluorescence was measured at λ_{ex} of 620 nm and an λ_{em} at 670 nm. Values are the mean ± SD. All experiments were performed in triplicate.

Ru complex 7 caused significant cytoplasmic membrane depolarisation against *E. coli* EC958 (Figure. 5.5A) and *E. coli* EC958 (IR) (Figure. 5.5B) after 60 min of exposure compared to the untreated control (p<0.001) and (p<0.005) respectively. Ru complex 1 caused no significant changes in bacterial membrane depolarisation against *E. coli* EC958 (Figure. 5.5A) and *E. coli* EC958 (IR) (Figure. 5.5B) (P=0.9987) and (p=0.9917) respectively. The percent normalised fluorescence for Ru complex 7 was significantly lower than that of the positive control Triton X-100 for *E. coli* EC958 and *E. coli* EC958 (IR) (p<0.001) and (p<0.001) respectively.

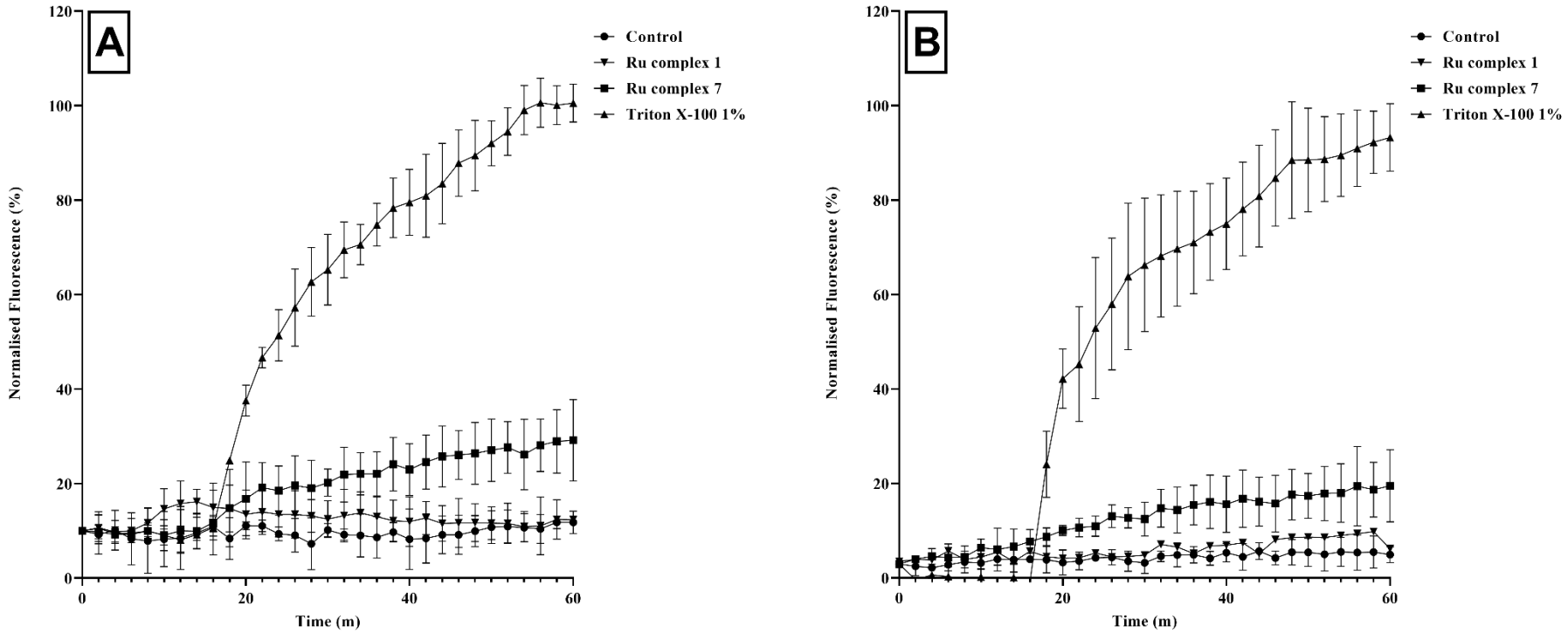

Figure 5. 5: Cytoplasmic membrane depolarisation of A) *E. coli* EC958 and B) *E. coli* EC958 induced resistance (●) by Ru complex 1 at 16 µg/mL (▼), Ru complex 7 at 256 µg/mL (■) and Triton X-100 at 1% (▲) using the membrane potential-sensitive dye $DiSC_3(5)$. Fluorescence was measured at λ_{ex} of 620 nm and an λ_{em} at 670 nm. Values are the mean ± SD. All experiments were performed in triplicate.

5.3.3 Analysis of Bacterial Cellular Morphology.

Scanning Electron Microscopy (SEM) was used to further characterise the effect of Ru complexes 1 and 7 on cellular morphology, cellular clustering, changes to membrane integrity and increased extra cellular matrixes in comparison to an untreated negative control after a 2 h exposure period.

S. aureus USA300 JE2 and *S. aureus* USA300 JE2 (IR) untreated negative controls showed coccoid morphology with minimal extra cellular matrix, the cell surface appeared to be smooth with an intact membrane (Figure. 5.6A and B respectively). When exposed to Ru complex 1 for a 2 h period, the *S. aureus* USA300 JE2 (Figure. 5.8C) showed no morphological changes, but there was an observed increase in extracellular matrix and cell clustering which suggested a propensity for increased biofilm formation. The cell surface appeared to show heterogeneity when compared to the negative control but no decrease in membrane integrity was observed. After exposure to Ru complex 1, *S. aureus* USA300 JE2 (IR) (Figure. 5.6D) showed no visible change when compared to the untreated negative control. The exposure of *S. aureus* USA300 JE2 and *S. aureus* USA300 JE2 (IR) to Ru complex 7 (Figure. 5.6E and F respectively) showed no visible changes in cell ultrastructure when compared to the respective negative controls.

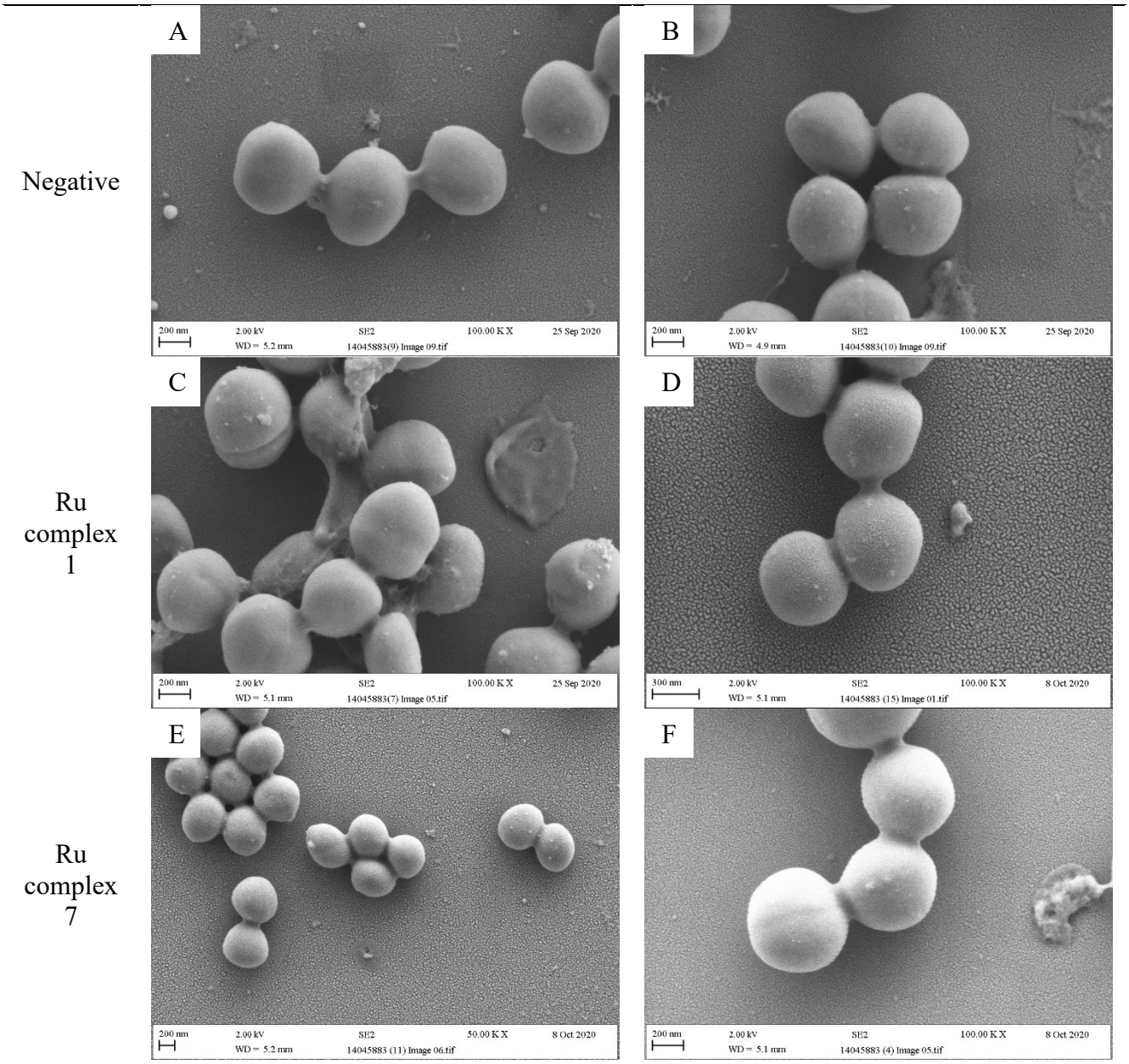

Figure 5. 6: SEM micrographs of *S. aureus* USA300 JE2 and *S. aureus* USA300 JE2 (IR) bacterial cells displaying the effects of different antimicrobial compounds on cell and membrane morphology after 2 h exposure with respective compound at MBC concentration (Table 4) and negative controls. A) *S. aureus* USA300 JE2 negative control. B) *S. aureus* USA300 JE2 (IR) negative control. C) *S. aureus* USA300 JE2 incubated with Ru complex 1 for 2 h at 16 µg/mL. D) *S. aureus* USA300 JE2 (IR) incubated with Ru complex 1 for 2 h at 16 µg/mL. E) *S. aureus* USA300 JE2 incubated with Ru complex 7 for 2 h at 256 µg/mL. F) *S. aureus* USA300 JE2 (IR) incubated with Ru complex 7 for 2 h at 256 µg/mL. Images are representative examples of the field of view for 3 biological replicates. Images were captured at the Manchester metropolitan university S.E.M central facility at 25,000, 50,000 and 100,000 times magnification.

E. coli EC958, *E. coli* EC958 (IR) and *P. aeruginosa* PAO1 untreated negative controls had rod shaped morphologies with minimal extra cellular matrix, relatively smooth cell surfaces and intact membranes. After 2 h exposure to Ru complex 1, *E. coli* EC958, *E. coli* EC958 (IR) and *P. aeruginosa* PAO1 showed little change in morphology compared to the respective negative controls. *P. aeruginosa* PAO1 showed the greatest change in cell ultrastructure after exposure to Ru complex 1 (Figure. 5.7F), where the membrane had increased heterogeneity compared to the negative control (Figure. 5.7C). When compared to the colistin 2 μg/mL positive control (Figure 5.7J, K, and L), exposure to Ru complexes 1 and 7 resulted in reduced extracellular matrix, intact membrane and a smooth membrane surface. (Figure. 5.7 D, E, F, G, H and I).

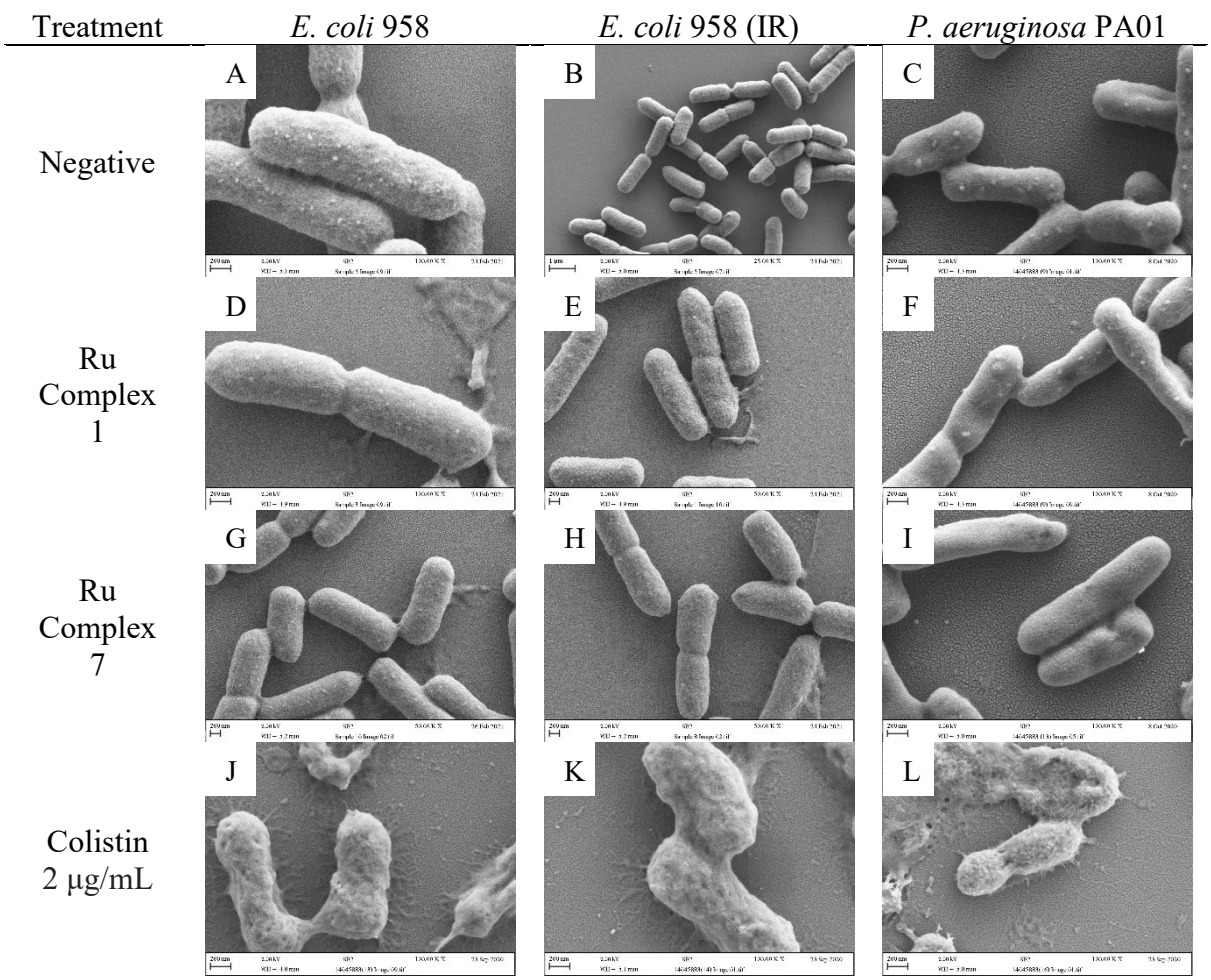

Figure 5. 7: SEM micrographs of *E. coli* EC958 (A, D, G and J), *E. coli* EC958 (IR) (B, E, H and K) and *P. aeruginosa* PAO1 (C, F, I and L) treated with Ru complex 1 and 7, colistin 2 µg/mL positive control and an untreated negative control. (A-C) untreated negative controls of *E. coli* EC958, *E. coli* EC958 (IR) and *P. aeruginosa* PAO1 respectively, (D) *E. coli* EC958 exposed to Ru complex 1 at 128 µg/mL, (E) *E. coli* EC958 (IR) exposed to Ru complex 1 at 128 µg/mL (F) *P. aeruginosa* PAO1 exposed to Ru complex 1 at 128 µg/mL, (G) *E. coli* EC958 exposed to Ru complex 7 at 256 µg/mL, (H) *E. coli* EC958 (IR) exposed to Ru complex 7 at 256 µg/mL, (I) *P. aeruginosa* PAO1 exposed to Ru complex 7 at 128 µg/mL, (J-L) *E. coli* EC958, *E. coli* EC958 (IR) and *P. aeruginosa* PAO1 treated with colistin at 2 µg/mL respectively. Images are representative examples of the field of view for 3 biological replicates. Images were captured at the Manchester metropolitan university S.E.M central facility at 25,000, 50,000 and 100,000 times magnification.

5.3.4 Bacterial Intracellular Ru Uptake Study

To quantify the amount of the Ru complexes that had penetrated the bacterial membranes and accumulated intracellularly, a cellular uptake study was conducted using ICP-MS. An increased amount of Ru complexes within the cell indicated the Ru complex was able to penetrate the membranes of the bacterial cell leading to possible interactions with intracellular targets. To evaluate the uptake of Ru complex 1, *E. coli* EC958, *E. coli* EC958 (IR), *S. aureus* USA300 JE2 and *S. aureus* USA300 JE2 (IR) were used. To evaluate Ru complex 7, *P. aeruginosa* PAO1 was used. The exposure time for each treatment was 1 h.

ICP-MS measurements of uptake showed, between equal exposure concentrations statistically significance uptake was only observed between *E. coli* EC958 and *E. coli* EC958 (IR) at exposure concentrations of 0.360 mM of Ru complex 1 (Figure. 5.8) (p=0.0001). The quantity of Ru complex 1 within *E. coli* EC958 was 6.75^{-3}±2.37^{-4} pg/cell whilst *E. coli* EC958 (IR) uptake was recorded at 1.22^{-2}±1.58^{-3} pg/cell. No significant difference in uptake of Ru complex 1 was observed between any bacteria at set concentrations of 0.180, 0.090, 0.0288, 0.01442 and 0.009 mM (p>0.05). *S. aureus* USA300 JE2 and *S. aureus* USA300 JE2 (IR) exhibited a higher uptake of Ru complex 1 at a lower exposure concentration of 0.0288 mM compared to *E. coli* EC958 and *E. coli* EC958 (IR) at an exposure concentration of 0.180 mM. The *S. aureus* USA300 JE2 and (IR) derivative strains recorded Ru complex 1 uptakes of 4.18^{-4}±9.69^{-6} pg/cell and 3.80^{-4}±1.05^{-6} pg/cell respectively at a concentration of 16 µg/mL, whilst the *E. coli* EC958 and (IR) derivative strains recorded cellular uptakes of 4.06^{-4} ±1.55^{-5} pg/cell and 3.83^{-4} ±9.39^{-6} pg/cell respectively at exposure concentrations of 0.090 mM.

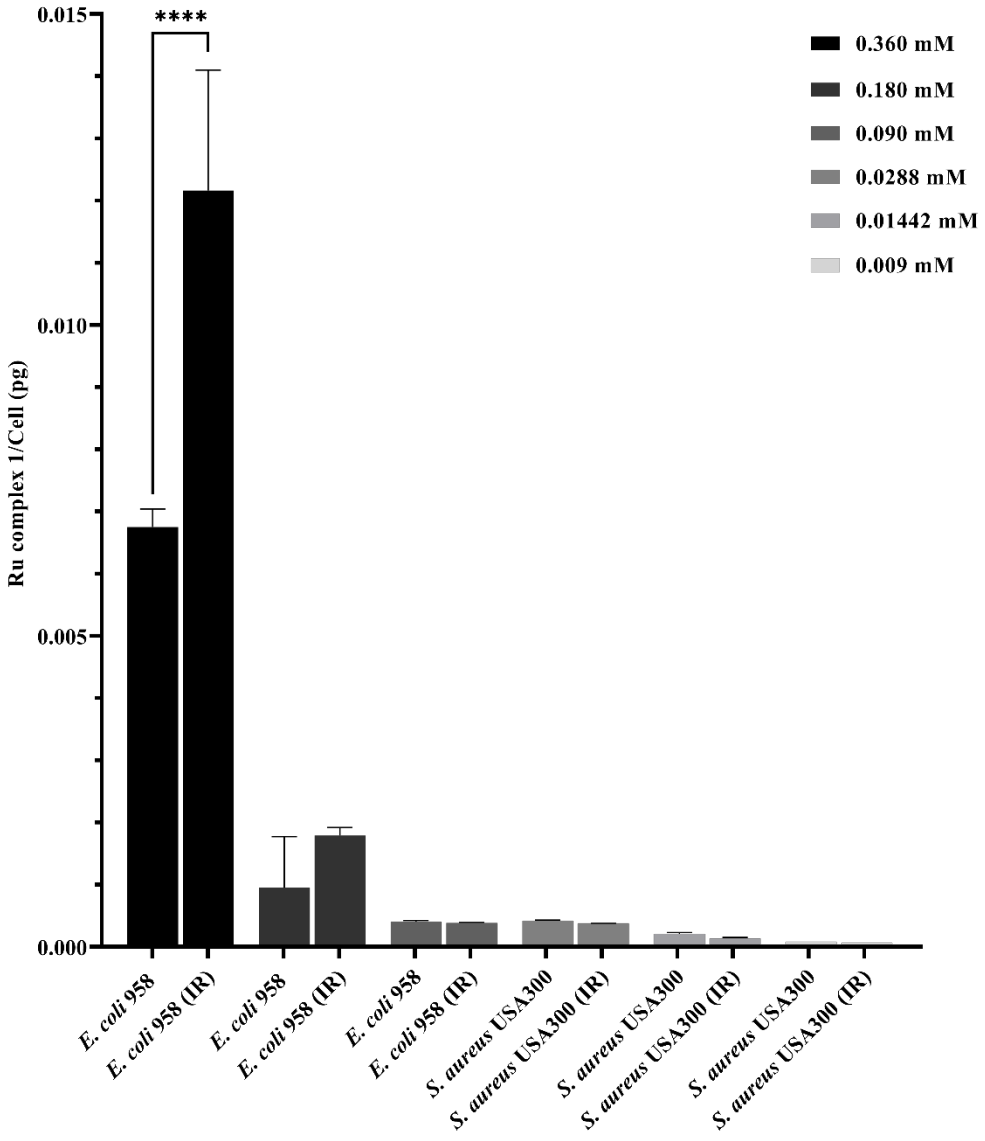

Figure 5. 8: Cellular uptake of Ru complex 1 at concentrations of 0.360, 0.180, 0.090, 0.0288, 0.01442 and 0.009 mM against a variety of bacterial species after 1 h exposure. Bars represent the mean of 3 biological replicates, with error bars representing SD. The absence of error bars on any particular point indicates that the size of the calculated SD is smaller than the symbol used for that point. Significance equals **** = ($p \leq 0.0001$)

The uptake study was also conducted using *P. aeruginosa* PAO1 with exposure to Ru complex 7 for 1 h. Statistically significant uptake was observed between exposure concentrations of 0.82685 mM and 0.2067 mM (p=0.0123) (Figure. 5.9). The cellular uptake for 0.82685 mM,

0.4134 mM and 0.2067 mM were recorded as $1.73^{-3}\pm2.12^{-4}$ pg/cell, $1.09^{-3}\pm1.49^{-4}$ pg/cell and $7.48^{-4}\pm2.77^{-5}$ pg/cell respectively.

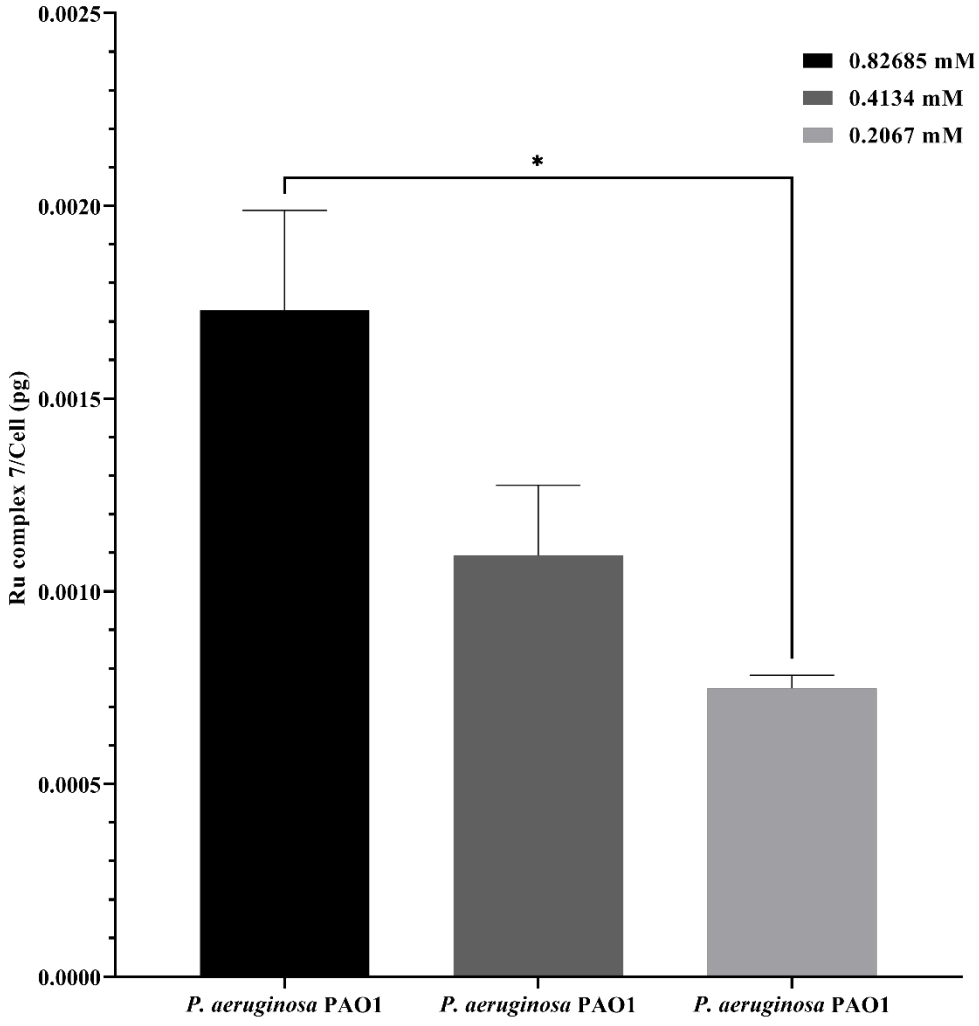

Figure 5. 9: Cellular uptake of Ru complex 1 at concentrations of 0.82685, 0.4134 and 0.2067 mM against a *P. aeruginosa* PAO1 after 1 h exposure. Bars represent the mean of 3 biological replicates, with error bars representing SD. Significance equals * = ($p \leq 0.05$)

To calculate the intracellular concentrations the average cellular volumes of each species were *E. coli* 1.0-15 μm^3, *S. aureus* 0.23 μm^3 and *P. aeruginosa* 1.33 μm^3 were used (Kubitschek *et al*., 1986; Maass *et al*., 2011; Ghanbari *et al*., 2016). The μm^3 volumes were converted to µL. and the pg/cell was converted to mass concentration. Finally, the formula mass / (volume * molar mass) = molarity was used to calculate the Intracellular Ru complex concentration (mM). After 1 h exposure to the respective Ru complex, intracellular concentrations were greater than the original extracellular exposure concentrations across all concentrations and conditions. After exposure to Ru complex 1 at a concentration of 0.360 mM *E. coli* EC958 (IR) contained the highest intracellular concentration of 21.912 mM. The lowest intracellular concentration was recorded by *S. aureus* USA300 JE2 (IR) after being exposed to Ru complex 1 at 0.009 mM the intracellular concentration was recorded at 0.5486959 mM. All *S. aureus* USA300 JE2 internal cellular concentrations were higher than counterparts *S. aureus* USA300 JE2 (IR) counterparts (Table 5.1).

Table 5. 1: Cellular uptake of Ru complexes, results shown as a percentage of original exposure concentration and the estimated intracellular Ru complex concentration per cell single cell.

Bacteria	Ru complex exposure concentration (mM)	Uptake of original exposure per Cell (%)	Intracellular Ru complex concentration (mM)
E. coli EC958	0.360	6.7075^{-10}	12.1614
E. coli EC958	0.180	2.95426^{-10}	1.7116
E. coli EC958	0.090	1.66916^{-10}	0.7321734
E. coli EC958 (IR)	0.360	1.01746^{-09}	21.9119
E. coli EC958 (IR)	0.180	3.73109^{-10}	3.2272
E. coli EC958 (IR)	0.090	1.55085^{-10}	0.6902078
S. aureus USA300 JE2	0.0288	5.13714^{-10}	3.2736
S. aureus USA300 JE2	0.01442	4.75947^{-10}	1.6087
S. aureus USA300 JE2	0.009	4.07332^{-10}	0.6392079
S. aureus USA300 JE2 (IR)	0.0288	4.75701^{-10}	2.9759
S. aureus USA300 JE2 (IR)	0.01442	3.67864^{-10}	1.0957
S. aureus USA300 JE2 (IR)	0.009	3.51561^{-10}	0.5486959
P. aeruginosa PAO1	0.82685	1.35^{-10}	4.1998
P. aeruginosa PAO1	0.4134	1.65915^{-10}	2.6557
P. aeruginosa PAO1	0.2067	2.43718^{-10}	1.8171

5.3.5 ROS DCFDA Assay

The quantification of general ROS was undertaken using the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). ROS is generated in many forms including $O^{\bullet-}_2$, $OH^{\bullet}$ $^1O_2$ and H_2O_2 and these are detectable through a series of reactions by the non-ROS specific H_2DCFDA. The non-fluorescent H_2DCFDA molecule diffuses through the cell membrane, if ROS are present within the cell they are able to cleavage the acetate groups converting H_2DCFDA to the highly fluorescent 2',7'-dichlorofluorescein (DCF).

All bacteria exposed to Ru complex 1 at their relevant concentration exhibited an increased level of ROS compared to the respective untreated negative controls, but no statistical significance was observed (Figure. 5.10, compare black with dark grey bars). There was a significant increase in fluorescence intensity between following exposure of *E. coli* EC958 to Ru complex 1 and H_2O_2 400 μM positive control (p=0.014), all other species and strains exhibited no significance between the positive control 400 μM H_2O_2 and Ru complex 1 after 30 min. *E. coli* EC958 (IR) was the only bacterial strain to show no statistical significance between all three conditions.

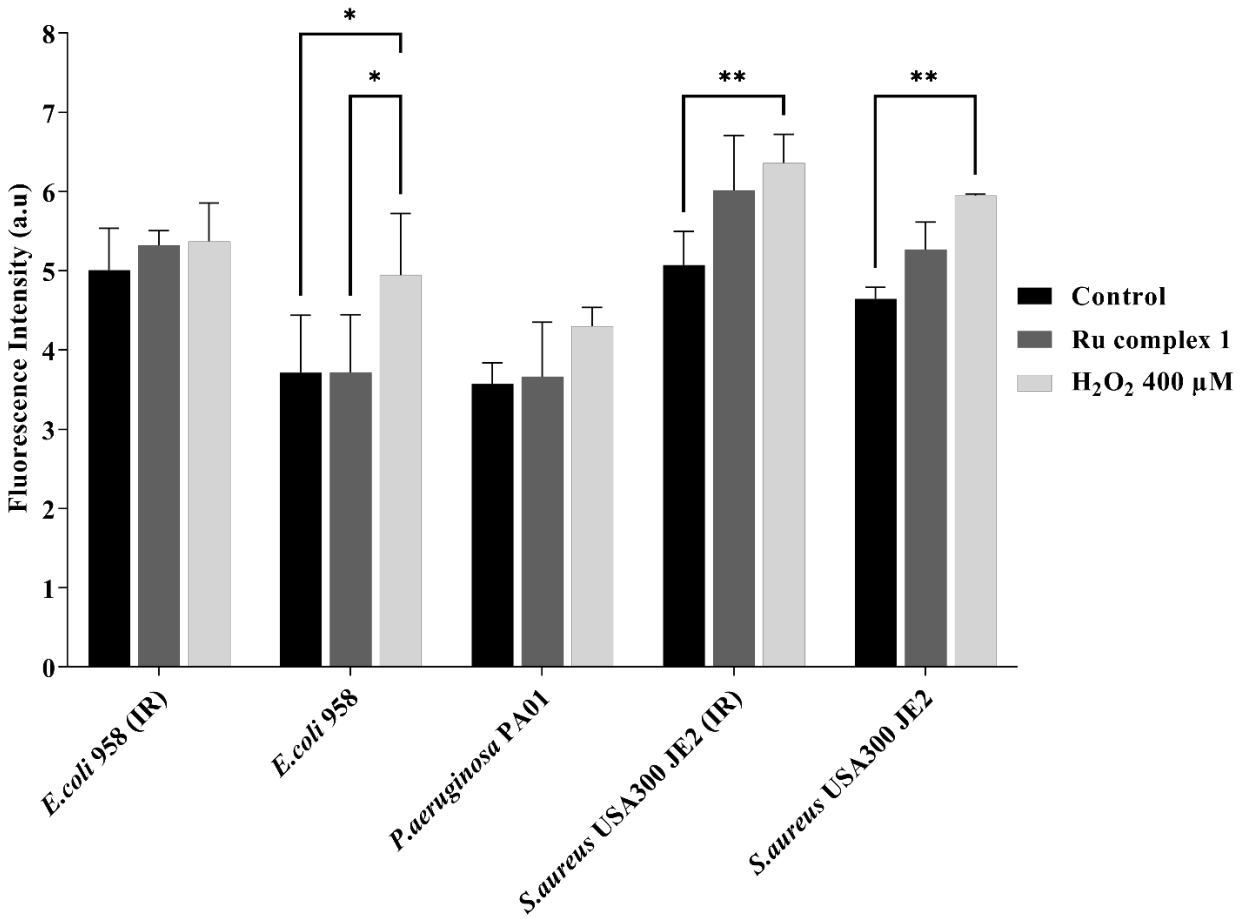

Figure 5. 10: ROS generation in a series of clinically significant bacteria after exposure to Ru complex 1 at *E. coli* EC958 256 µg/mL, *E. coli* EC958 (IR) 256 µg/mL, *P. aeruginosa* PAO1 256 µg/mL, *S. aureus* USA300 JE2 16 µg/mL and *S. aureus* USA300 JE2 (IR) 16 µg/mL. Significance equals * = (p<0.05) and ** = (p<0.01).

All bacteria exposed to Ru complex 7 exhibited an increased level of ROS compared to the respective negative control, but no statistical significance was observed (Figure. 5.11, compare black with dark grey bars). The generation of ROS in *S. aureus* USA300 JE2 was significant when comparing untreated cells or those exposed to Ru complex 7 with those exposed to 400 µM H_2O_2 positive control (p=0.0002) and (p=0.0031) respectively). *S. aureus* USA300 JE2 (IR) showed significance between negative and H_2O_2 400 µM positive control (p<0.0001). A significant increase in ROS production was also observed when comparing bacterial exposure

to Ru complex 7 and the H_2O_2 400 µM positive control (p=0.0031). All other bacteria showed no significance in terms of increases in ROS generation across all three conditions.

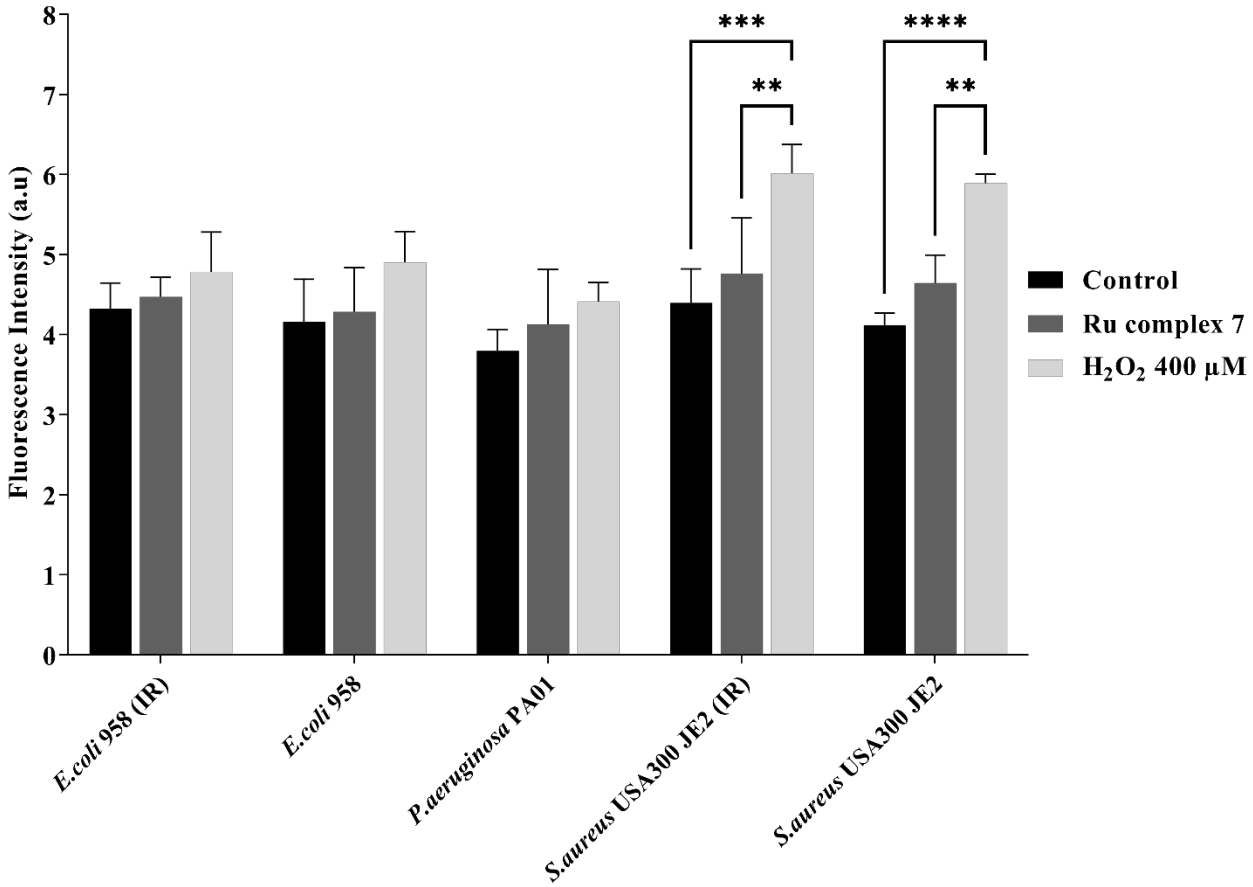

Figure 5. 11: ROS generation in a series of clinically significant bacteria after exposure to Ru complex 7 at 128 µg/mL. Significance equals ** = (p<0.01), *** = (p<0.001) and **** = (p<0.0001).

5.3.6 Electrophoretic Mobility Shift Assay

Initial assessment of interactions between DNA and individual Ru complexes were determined using gel electrophoretic mobility shift assays. The electrophoretic mobility shift assay is a rapid and sensitive method that is primarily used to detect protein-nucleic acid interactions but can also be used to detect interactions of other molecules with DNA (Kothinti *et al*., 2011).

The effect of Ru complexes 1 and 7 on an EcoR1 restriction digested linearised *E. coli* pGEM®-3Zf (+) plasmid was determined using DNA agarose gel electrophoresis and subsequent staining. A standard of 10 ng μL^{-1} pGEM®-3Zf (+) plasmid DNA was used per assay. Ru complex 1 was applied to linearised plasmid DNA at variable concentrations between 0.125 mM and 2 mM at a set 5 h timepoint. Concentrations of 2 mM and 1 mM respectively (Figure 5.12, lane 4 and 5) totally degraded the DNA with no visible traces remaining of the gel. Concentrations 0.5 mM and 0.25 mM (Figure. 5.12, lane 6 and 7) respectively caused severe degradation and shift to the plasmid DNA with a minor reduction in migration. Exposure of plasmid DNA to 0.125 mM Ru complex 1 (Figure. 5.12, lane 8) remained unchanged when compared to the solvent and negative controls.

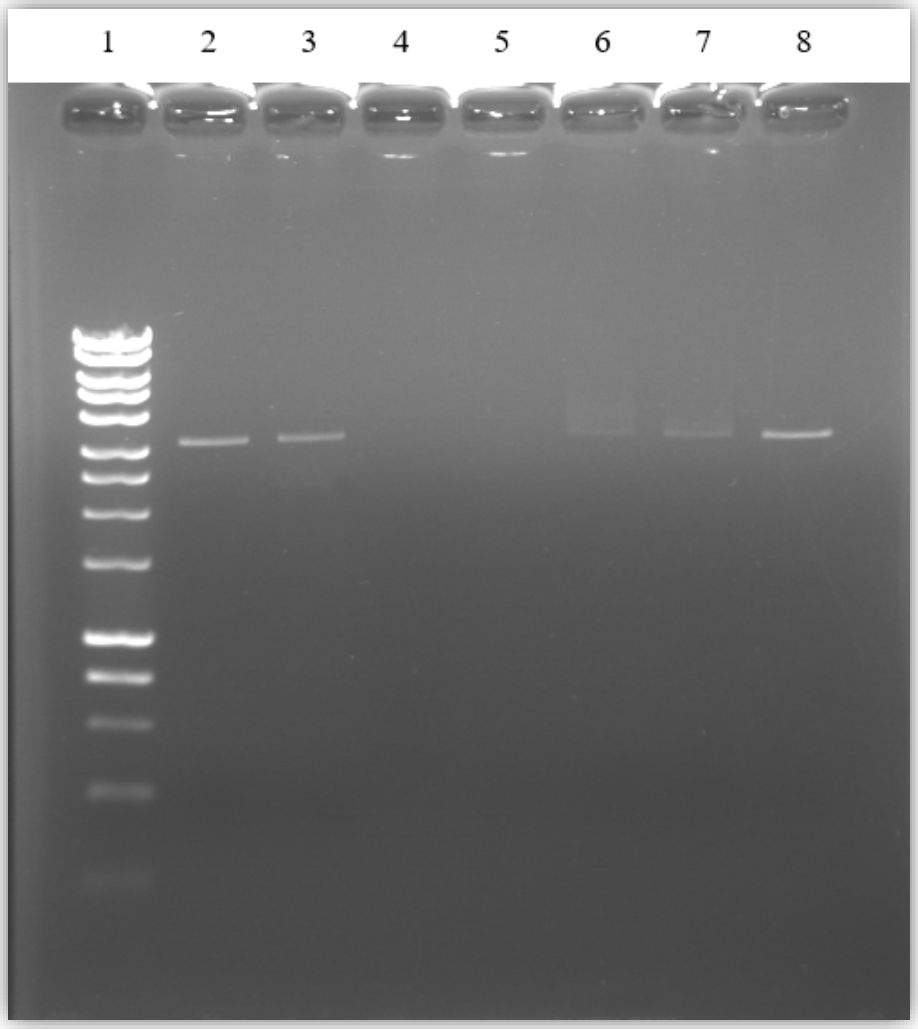

Figure 5. 12: Effect of compound 1 at varying concentrations over a 5 h incubation period on ecor1 restriction digested pGEM®-3Zf (+) 3197 bp Plasmid. Lane 1: 1kb Hyper ladder; lane 2: Plasmid only control; lane 3: DMSO control 5 h incubation; lane 4: 2 mM concentration; lane 5: 1 mM concentration; lane 6: 0.5 mM concentration; lane 7: 0.25 mM concentration; lane 8: 0.125 mM concentration.

The effect of Ru complex 7 on pGEM®-3Zf (+) 3197 bp plasmid over a set 5 h period was also determined (Figure. 5.13). Concentrations of Ru complex 7 between 10 and 0.625 mM were incubated with the linearised plasmid DNA. All tested concentrations resulted in varying levels of DNA mobility changes and evidence of degradation. The three highest concentrations 10 mM, 5 mM and 2.5 mM (Figure. 5.13, Lanes 4, 5 and 6) resulted in reduced levels of plasmid DNA electrophoretic mobility and noticeable degradation on the gel. Concentrations of 1.25

mM and 0.625 mM (Figure 5.13, Lane 7 and 8) showed a decrease in luminescence, but there was no evidence of degradation.

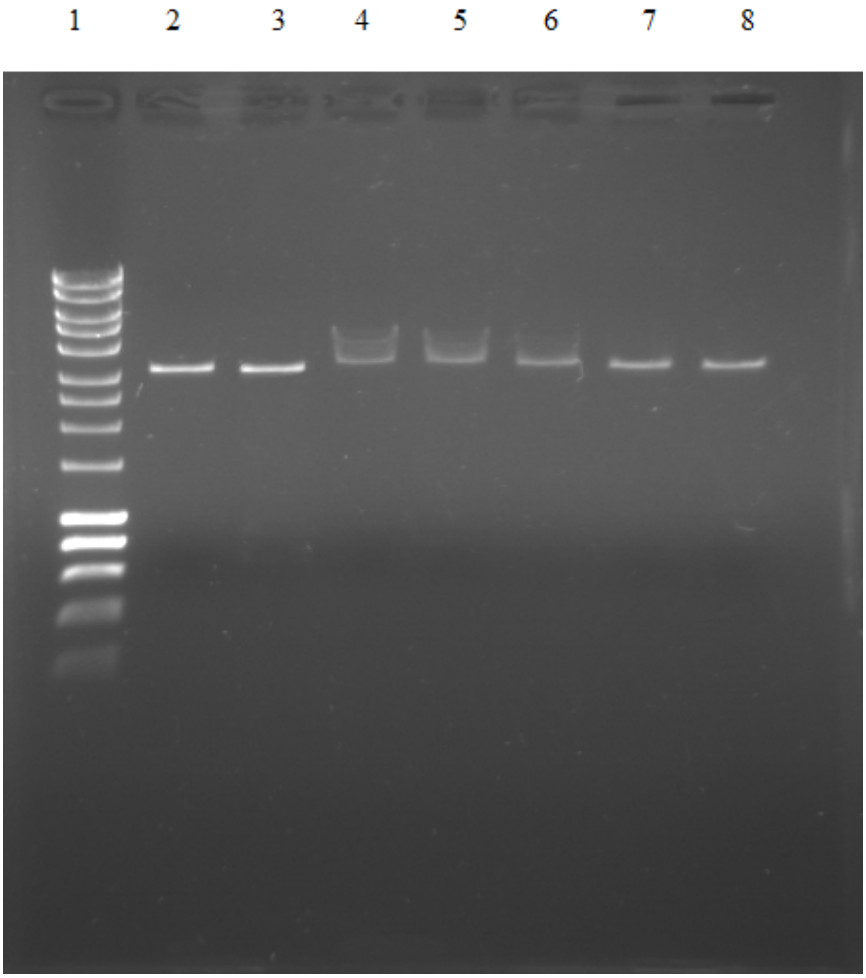

Figure 5. 13: Effect of compound 7 at varying concentrations over a 5 h incubation period on ecor1 restriction digested pGEM®-3Zf (+) 3197 bp Plasmid. Lane 1: 1kb Hyper ladder; lane 2: Plasmid only control; lane 3: Solvent control 5 h incubation; lane 4: 10 mM concentration; lane 5: 5 mM concentration; lane 6: 2.5 mM concentration; lane 7: 1.25 mM concentration; lane 8: 0.625 mM concentration.

5.3.7 DNA Competitive Binding Assay

The competitive DNA binding assay was used to assess the DNA binding potential of the Ru complexes to dsDNA and further expand the results from the electrophoretic mobility shift assay. The fluorescent probe SYTO 9 produces low amounts of fluorescence in the absence of DNA. By adding SYTO 9 to dsDNA, the SYTO 9 can bind to the dsDNA resulting in a greatly increased fluorescence. An excitation emission matrix was conducted to find the optimum excitation and emission for SYTO 9 bound to dsDNA. The Ru complexes were then added to the solution resulting in a potential drop in fluorescence emissions if the Ru complexes showed a greater affinity to the dsDNA. UV absorption studies were conducted on the Ru complex 1 and 7 to demonstrate no absorption interference occurred at examined wavelengths.

To demonstrate that fluorescence intensity decreases at a linear rate with the subsequent addition of Ru complex 1, a standard curve was constructed producing an R^2 value of 0.9133 (Figure. 5.14). From the standard curve a 50% reduction in fluorescence intensity occurred at 0.911762 mM. Statistical significance was shown between the post treatment negative control and Ru complex 1 at concentrations 2 mM, 1 mM and 0.5 mM ($p<0.001$, $p<0.001$, $p=0.00018$ and $p<0.0001$ respectively). Concentrations of Ru complex 1 at 2 mM, 1 mM, 0.5 mM, 0.25 mM and 0.125 mM resulted in percentage reductions in fluorescence output of 85.64%, 63.98%, 29.22%, 31.86% and 14.21% respectively.

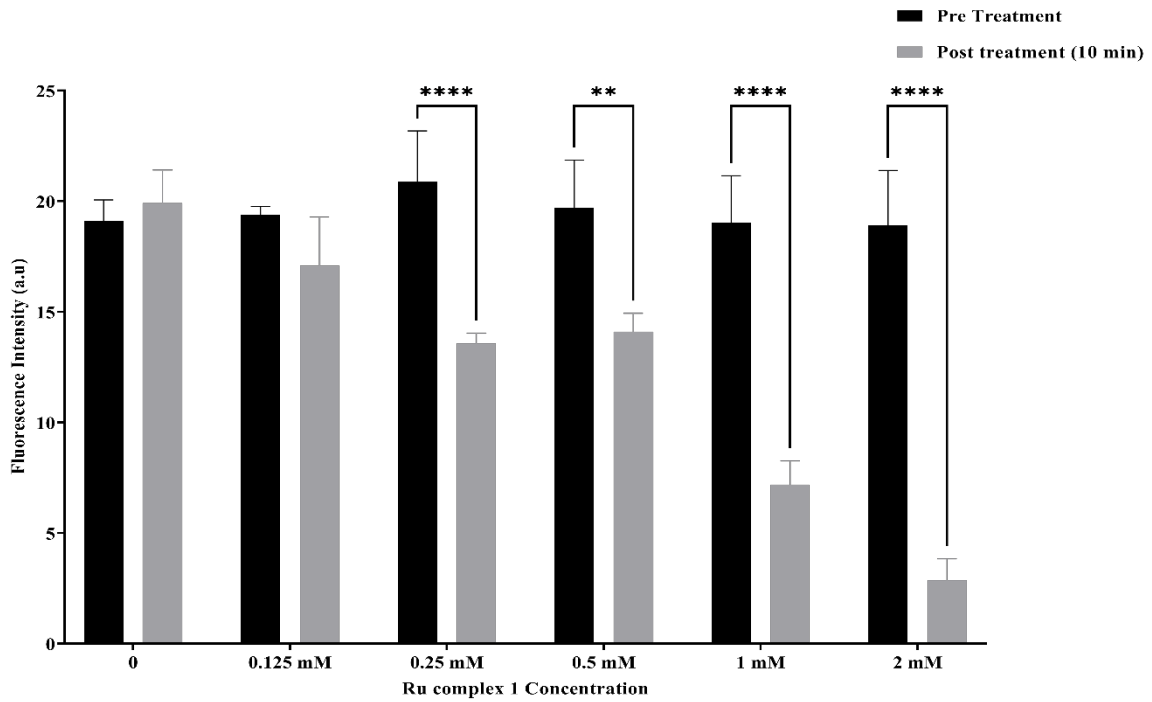

Figure 5. 14: Competitive displacement assays of CT-DNA and SYTO 9 complex with Ru complex 1. SYTO 9-DNA complex was λ_{ex} 480 nm and emission spectra were recorded from 485–700 nm. Peak recording was measured at 520nm with fluorescence intensity decreasing with increasing concentration of Ru complex 1. Bars represent the SD of 3 biological replicates. Significance equals * = ($p \leq 0.05$), ** = ($p \leq 0.01$), *** = ($p \leq 0.001$) and **** = ($p \leq 0.0001$)

A standard curve was constructed plotting concentration against fluorescence output and an R^2 value of 0.624 (Figure. 5.15) was shown with a 50% reduction in fluorescence intensity occurred at 1.203023 mM. Statistical significance was shown between the post treatment negative control and post treatment with Ru complex 7 at 2 mM, 1 mM, 0.5 mM, 0.25 mM and 0.125 mM concentration (p<0.001, p<0.001, p<0.001, p=0.0147 and p=0.0388 respectively). Exposure of CT-DNA to Ru complex 7 resulted in a 57.38%, 61.38%, 48.98%, 27.78% and 21.95% reduction in fluorescence intensity for concentrations of 2 mM, 1 mM, 0.5 mM, 0.25 mM and 0.125 mM respectively.

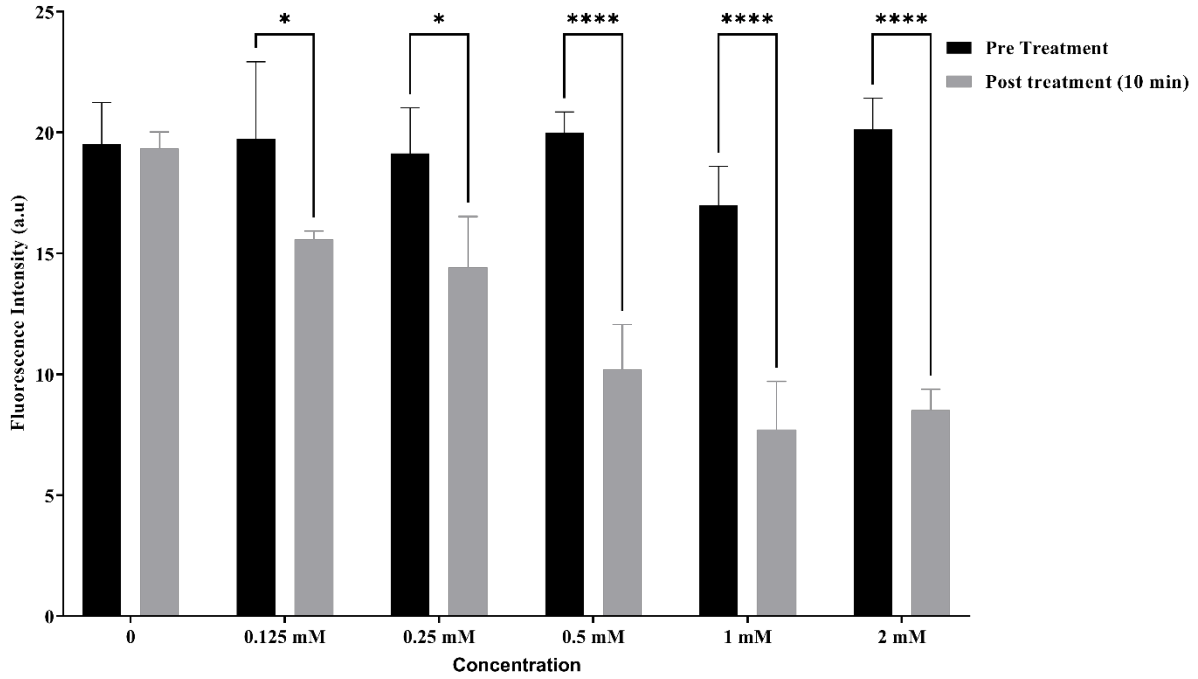

Figure 5. 15: Competitive displacement assays of CT-DNA and SYTO 9 complex with Ru complex 7. SYTO 9-DNA complex was λ_{ex} 480 nm and emission spectra were recorded from 485–700 nm. Peak recording was measured at 520nm with fluorescence intensity decreasing with increasing concentration of Ru complex 7. Bars represent the SD of 3 biological replicates. Significance equals * = ($p \leq 0.05$), ** = ($p \leq 0.01$), *** = ($p \leq 0.001$) and **** = ($p \leq 0.0001$)

5.4 Discussion

To develop and utilise new antimicrobials in a clinical setting, it is first essential to understand the cellular target of the proposed antimicrobial agent. This assists with both determining the most appropriate clinical application and also highlights any potential toxicity risks (Pankey *et al*., 2004). All current clinically prescribed antimicrobials directly target one of five cellular targets: bacterial cell wall, the cell membrane, protein synthesis, DNA and RNA synthesis, and folic acid synthesis (Kohanski *et al*., 2010). Many antimicrobials also possess the ability to indirectly trigger other antimicrobial processes within the cell (Dwyer *et al*., 2014). Antibiotics within the quinolones family, such as ciprofloxacin, directly target DNA but are also able to trigger ROS production within the cell. The production of ROS can damage lipids, DNA, RNA, and proteins (Masadeh *et al*., 2017). Ru complexes have demonstrated the ability to target the same cellular targets as clinically prescribed antimicrobials. Upon reaching their cellular target, Ru complexes binding interactions result in a variety of deleterious effects including: permeabilization of membranes, induction of SOS repair responses, mutagenic effects, inhibition of RNA transcription and induction of DNA degradation (Yasbin *et al*., 1980; Pauly *et al*., 2002; Li *et al*., 2013). Ru complexes have also been associated with multiple mechanistic actions and the subsequent triggering of antimicrobial cellular effects (Kenny *et al*., 2019; Burmeister *et al*., 2021). This targeting of multiple cellular components has been shown to reduce the development of AMR (Peschel *et al*., 2006). The ligands that form Ru complexes 1 and 7 have been associated with a wide variety of cellular targets including cell membrane activity and nucleic acid binding (Alsaeedi *et al*., 2020; Matshwele *et al*., 2020). This chapter assessed the interactions of the main bacterial cellular targets and investigated possible subsequent interactions of Ru complexes 1 and 7.

The initial contact of any antimicrobial is with the cell membrane, and this can have a profound impact on the antmicrobial activity due to the compositional differences between Gram-negative and Gram-positive bacteria. In Gram-negative bacteria, the function of the outer

membrane is primarily to sustain mechanical forces (Rojas *et al*., 2018); however, due to the molecular makeup and the structures embedded within the membrane, it is able to act as a barrier to help maintain homeostatis within the cell (Koebnik *et al*., 2000). In particular, the anionic lipid polysaccharides of the Gram-negative bacteria have been shown to severely limit the permeation of hydrophobic drugs (Nikaido, 1994). Due to the lack of antimicrobial activity against Gram-negative species and the potent antimicrobial activity against Gram-positive species, it was reasoned the Ru complex 1 was not targeting the outer membrane of Gram-negative bacteria, and the outer membrane permeabilization assay was not conducted. The ultrastructure of Gram-negative and Gram-positive bacteria was analysed by SEM to provide a visual representation of possible membrane interactions with Ru complex 1. In accordance with the time kill kinetics (Figure. 3.12), if membrane damage was occurring, the 2 h timepoint should have shown visual changes to the membranes. No visual alteration to any membranes occurred, confirming the lack of interaction between the outer membrane and Ru complex 1 for Gram-negative *E. coli* and *P. aeruginosa*.

There are structural similarities between Ru complex 7 and quaternary ammonium salts (QAS). This is significant due to QAS acting as cationic detergents and disinfectants (Devinsky *et al*, 1985). A QAS possesses a cationic charge on the central nitrogen atom, and this cationic charge shifts to the central ruthenium core on Ru complex 7. This movement of cationic change would still allow for electrostatic interactions with the cytoplasmic, outer membranes and other negatively charged cellular structures. Thus, it was hypothesised that the primary mode of action of Ru complex 7 would be via destabilising effects on bacterial membranes (Alkhalifa *et al*, 2020; Daood *et al*, 2020). While there is no definitive antimicrobial mechanism known for QAS, one of the leading theories is the perturbation of cytoplasmic and outer membrane lipid bilayers via the electrostatic interactions of the positively charged QAS with the polar head groups of acidic phospholipids (Jiao *et al*., 2017). Several molecular properties of Ru

complex 7 indicated similar theoretical physical effects on membranes. First, the cationic charge of the Ru complex 7 may also contribute to the efficiency of antimicrobial activity by electrostatically binding to anionic charged membranes (Wenzel *et al*, 2014). Second, the hydrophilic properties of Ru complex 7 would allow for easier permeation into the cell membrane. Upon permeation of the membrane, the charged groups would alter the alignment of the polar ends of the lipid bilayer, causing disruption to the membrane (Butler, 2001; Vance, 2008; Loew, 2017).

The outer membrane permeabilization assay demonstrated that Ru complex 7 produced no permeabilising effects on the outer membrane of *E. coli* EC958 or *P. aeruginosa* PAO1 at 128 µg/mL concentrations (Figure. 5.1, Figure. 5.2). Preliminary tests were also conducted up to concentrations of 619.24 µg/mL; these also exhibited no outer membrane permeabilization. This demonstrated NPN remained in a hydrophilic environment and no interaction with phospholipids occurred, thus indicating the bactericidal effect on Gram-negative bacteria produced by Ru complex 7 was not outer membrane related. The 128 µg/mL concentration of Ru complex 7 was at MBC level; it has been demonstrated with other compounds that concentrations at MBC are able to rapidly permeabilize the outer membrane, producing increased fluorescence intensity in the assay (Lv, 2014). The increased fluorescence output of *E. coli* EC958 over *P. aeruginosa* PAO1 may in part be down to the thickness of the peptidoglycan layer. *E. coli* peptidoglycan layer 3.0 nm this is twice the thickness of *P. aeruginosa* 1.5 nm thus allowing more interaction of NPN with peptidoglycan (Yao, 1999). *P. aeruginosa* has also shown a greater resistance to detergents than *E. coli,* which could reflect in the increased fluorescence intensity produced by the Triton X-100 permeabilization (Ayres *et al*., 1999). To substantiate the outer membrane permeabilization studies, SEM was conducted (Figure. 5.6; Figure. 5.7) There were no visual effects observed for Ru complex 7

on the outer membrane of *E. coli* EC958, *E. coli* EC958 (IR) and *P. aeruginosa* PAO1 in comparison to the negative control.

The protonophore CCCP was used throughout the outer membrane permeabilization assay to reduce false positive fluorescence results by preventing cells from switching energy states and inhibiting energy-dependent efflux systems (Sekyere, 2017; Hohl, 2019). CCCP at concentrations of 20 µg/mL has the ability to increase permeability of the outer membrane to many different molecules, including terpinen-4-ol, c-terpin-ene and tea tree oil, whilst not affecting cell viability (Cox, 2007). Due to these studies, concentrations of 5 µm were used throughout the experiments.

There have been many examples of Ru complexes permeabilizing the membranes of Gram-negative bacteria. For example, Kumar *et al*. (2016) demonstrated that a synthesized $[Ru(octpytri)3]^{2+}$ was able to cause cell wall/membrane disruption by allowing the normally membrane-impermeable dye propidium iodide to access the bacterial DNA. Kumar *et al*. (2016) also synthesised Ruthenium (II) R-pytri complexes that are structurally similar to cationic detergents and could potentially target outer membranes in Gram-negative bacteria, causing permeabilizing effects (Kumar *et al*., 2016).

The inner membrane differs in location and composition to the outer membrane of Gram-negative bacteria, thus providing an alternate cellular target for Ru complexes. The inner membrane is present in all prokaryotic organisms and acts as a mediator between the internal cellular environment and the external environment, helping to maintain homeostasis within the cell (Miller *et al*., 2018). The inner membrane contributes to many different cellular functions, including cellular signalling, respiration, transport of nutrients and maintaining energy gradients; disruption to the inner membrane can have effects on essential inner membrane functions (Mykytczuk *et al*., 2007). For an antimicrobial to target and depolarise the inner

membrane, it needs to bind to the surface of the inner membrane in sufficient quantities to reach a critical antimicrobial/lipid ratio (Hollmann *et al*., 2018). Depolarisation of the inner membrane can occur without directly contributing to bacterial cell death, although the depolarisation would likely be a contributing factor (Winkel *et al*., 2016; Yasir *et al*., 2019). If depolarisation and subsequent degradation of the inner membrane occurs, initial peaks of fluorescence can potentially be quenched by the release of genomic material and other contents of the cytoplasm (Wang *et al.*, 2011). The data showed there was no peak followed by a dip in fluorescent intensity, indicating that there was unlikely a release of genomic material into the surrounding media. The one-hour incubation period for the inner membrane depolarisation assay was selected due to research showing that biomolecules such as Hen Egg-White Lysozyme requires a 2-hour incubation period before deviation above baseline occurs, while non-biomolecules, such as detergents, possess rapid depolarising effects (Derde *et al*, 2012). Ru complexes are known to cause depolarising effects on the inner membrane. Li *et al*. (2013) demonstrated that dinuclear polypyridylruthenium(II) complexes were able to depolarise the cell membrane in *S. aureus,* showing that uptake was not dependent on the membrane potential (Li *et al.*, 2013).

Both *E. rcoli* EC958 and *E. coli* EC958 (IR) exhibited significant inner membrane depolarisation after 1 hour of incubation with Ru complex 7. While there was significant inner membrane depolarisation compared to the negative control, the inner membrane depolarisation caused by Ru complex 7 was significantly lower when compared to the Triton X-100 positive control. Both *S. aureus* USA300 JE2 and *S. aureus* USA300 JE2 (IR) also showed increases in membrane depolarisation when exposed to Ru complex 7 compared to the negative controls, but this was not statistically significant. These results further indicated that membrane interaction was contributing to the antimicrobial activity of Ru complex 7. *P. aeruginosa* PAO1 was the only bacteria that did not exhibit any inner membrane depolarisation compared to the

negative control, contradicting the MIC/MBC results showing that Ru complex 7 potent antimicrobial activity against *P. aeruginosa* PAO1.

P. aeruginosa has been shown to be one of the more adaptable and resilient species when exposed to quaternary ammonium compounds (Méchin *et al*., 1999; Guerin *et al.*, 2000; Loughlin *et al.*, 2002; Tabata *et al.*, 2003). The fatty acid composition and expression of membrane protein OprR is thought to contribute to resistance against quaternary ammonium compounds in *P. aeruginosa* (Méchin *et al*., 1999; Loughlin *et al*., 2002). Ru complex 7 exhibited low level inner membrane depolarisation across a range of different bacterial species, but for all conditions, the inner membrane depolarisation was significantly below the positive control, indicating a secondary mode of action for bacterial cell death.

There was no significant inner membrane depolarisation after exposure to Ru complex 1 when compared to the negative control for any of the experimental conditions, indicating inner membrane interactions had no contribution to the antimicrobial activity produced by Ru complex 1. While no previous membrane studies had been conducted on Ru complex 1, the inner membrane studies (Section 5.3.2) and haemolytic assays (Section 3.4.4) showed that Ru complex 1 did not exhibit membrane interactions. Ru complex 1 was originally synthesized as a nucleic acid targeting antitumour drug; the finding confirmed by Arshad *et al*. (2017) indicated that Ru complex 1 contained lipophilic ligands, allowing Ru complex 1 to pass through membranes (Arshad *et al.*, 2017).

Ru complexes with potent antimicrobial activity but with have no interactions or permeabilising effects on bacterial membranes, must therefore interact with intracellular components. The uptake or absorption of molecules can be classified into three distinct areas: attachment to the cell surface, accumulation inside the cell, and the accumulation outside the cell (Kahraman., 2020). The cationic charge of the Ru complexes would bind to negatively

charged cellular components such as proteins, DNA, cellular membrane and RNA, increasing the intracellular concentration of the Ru complex (Pandrala *et al*., 2013; Lei *et al*., 2019). Due to the positive charge of the Ru complexes, a cellular uptake assay would record all Ru complex molecules attached to the outer exterior components of the cell, as well as the intracellular accumulation. Ru complex that are bound to the membrane would cause no internal effects but would artificially increase the concentration of the uptake study. To minimize this, 0.5% HNO_3 washing stages were used.

It has been demonstrated that Ru complexes can enter cells via passive diffusion, facilitated diffusion or active transport (Li *et al*., 2012; Li *et al*., 2014; Li *et al*., 2016). The uptake method can have a profound impact on the rate of uptake and intracellular concentration (Chopra,. 1988). Active transport of Ru complexes into a bacterial cell allows intracellular concentrations to exceed extracellular concentrations, and the rate has been shown to be dependent on concentration, temperature, pH, surface and the amount of energy available to the cell (Alagga *et al*., 2021). The 1 hour incubation time used in these studies was selected in part due to work conducted by Li *et al*., (2012) who demonstrated that *S. aureus*, MRSA, *E. coli* and *P. aeruginosa* exposed to their synthesised Ru complexes showed no significant differences in uptake between 30 min and 2 hour (Li *et al*., 2012). This work was also confirmed by Smitten *et al*. (2020), who also demonstrated the rapid uptake of Ru complexes between the 0 and 5 min time points, with only a minor increase between the 5 and 60 min timepoints, idicating that Ru complexes have a rapid initial uptake in bacterial cells (Smitten *et al*., 2020). Molecular structure and physicochemical parameters play a greater role in influencing cellular uptake of molecules, and the attached ligands play a primary role in the structural and physicochemical parameters of a molecule (Davis *et al*., 2014). Ligands on the Ru complex can also contribute to the rate of uptake into the cell; the use of chloride ligands have been shown to increase

cellular uptake but reduce antimicrobial activity (Pandrala *et al*., 2013). Further, Richter (2017) showed that non-sterically encumbered ionizable nitrogen in the form of primary amines aided in the accumulation with a lower efflux rate within Gram-negative bacteria (Richter, 2017).

Ru complexes with increasingly complex ligands often have a higher molecular weight and occupy an increased molecular space, which can have detrimental effects on uptake and accumulation of the Ru complexes. Bacterial membranes can exclude larger molecules while selectively screening small molecules, and combined with efflux pumps, can extrude toxic substances from the cell, limiting accumulation within the cell (Zhou *et al*., 2015). Bacteria are known to efflux at different rates depending on the molecular weight of the compound. Lower molecular weight molecules efflux at reduced rates compared to higher molecular weight molecules. A recent study by Manchester *et al*., (2012) demonstrated that compounds with a molecular weight between 475 kDa and 600 kDa show an 80% efflux rate, while compounds with a molecular weight between 725 kDa and 850 kDa show a 100% efflux rate (Manchester *et al*., 2012). The molecular weights of the 12 Ru complexes tested in this study ranged from 273.15 to 958.83 kDa, and these differences in molecular weight could contribute to bacterial resistance against a specific Ru complex. The molecular weights of Ru complexes 1 and 7 were 554.92 kDa and 309.62 kDa, respectively, indicating that both compounds would potentially have a lower level of efflux from bacterial cells compared to larger molecules, thus potentially allowing for greater accumulation compared to higher molecular weight antibacterial agents.

Ru complex 1 has previously been shown to bind to cellular targets in other eukaryotic studies (Arshad *et al*., 2017). *S. aureus* USA300 JE2 showed a higher uptake of Ru complex 1 compared to *E. coli* EC958 at the same exposure concentrations. This indicated a level of intrinsic resistance by the Gram-negative *E. coli* EC958. The uptake of Ru complex 1 positively

correlated with the extracellular concentrations of Ru complex 1, with higher extracellular concentrations having increased intracellular concentrations. Exposure to higher concentrations of antimicrobials has previously been linked to a rate of linear cellular uptake at lower concentrations (Zhou *et al*., 2015).

The accumulation of Ru complex 7 in *P. aeruginosa* PAO1 was lower compared to similar exposure concentrations of Ru complex 1 in the Gram-negative species *E. coli* EC958. This in part maybe due to the presence of the OmpF porin in *E. coli*, which permits the transportation of Ru complexes through the cell membrane (Ho *et al*., 2011). It is unknown if Ru complexes 1 and 7 were taken into the cell via active transport or passive diffusion into the cell; performing the uptake study in the absence and presence of glucose may provide a greater insight into the transport mechanisms allowing to differentiate if the Ru complexes were up taken into the cell via active transport or passive diffusion (Smitten *et al*., 2020). Due to the intracellular concentration being higher than the extracellular concentration, it is likely the Ru complexes were actively transported into the cell.

The intracellular concentration of Ru complex 7 were higher than the original exposure concentration across all concentrations tested. Ru complex 7 was theorised to function similarly to quaternary ammonium compounds and *P. aeruginosa* are known to possess a high-level intrinsic resistance to quaternary ammonium compounds; this could be a contributing factor in the lower uptake of Ru complex 7 (Langsrud *et al*., 2003). Another factor that may contribute to the lower accumlation in *P. aeruginosa* is the highly active RND efflux pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY. *P. aeruginosa* is known to simultaneously overexpress multiple efflux systems, potentially contributing to the lower accumilation of Ru complex 7 (Llanes *et al*., 2004). With this low permability and high efflux rate, *P. aeruginosa* has been shown to be able to maintain a constant concentration of Ru complexes within the cell over extended periods of time, where work conducted by Sun *et al*., (2018) demonstrated

P. aeruginosa was able to maintain a constant concentration of Ru complexes within the cell after a 2 h time period (Sun *et al*., 2018).

The ROS H_2DCFDA assay was used to determine possible increases in ROS within the bacterial cells after incubation with the Ru complexes. ROS is generated in many forms, including $O^{\bullet-}_2$, $OH^{\bullet}$ $^1O_2$ and H_2O_2; each of these ROS molecules can interact and inactivate biomolecules, including DNA, lipids, carbohydrates, and proteins (Therond, 2006). The quantity, type and tolerance of ROS within a cell is species and strain dependent (Fang., 2011; Feng., *et al*., 2020). Whilst ROS is primarily generated by metabolic activity in the mitochondria, it can also be caused by stress from environmental factors and as a result of DNA damage (Magerand *et al*., 2021). Under stress free conditions, *E. coli* generates around 14 μM hydrogen peroxide (H_2O_2) per second and maintains an intracellular concentration of 50 nM of H_2O_2 (Seaver *et al*., 2001; Imlay., 2015). In *S. aureus,* 400 μM concentrations of H_2O_2 have been shown to extend the lag phase (Painter *et al*., 2015), while accumulation of ROS at concentrations of H_2O_2 between 1 and 2 mM has been shown to extend the lag phase of growth and produce MIC_{90} in many species (Brudzynski *et al*., 2011; Painter *et al*., 2015). Bacteria utilise both nonenzymatic and enzymatic antioxidants to control ROS levels within the cell to prevent cellular damage. The ability to neutralise ROS is vital to their survival in a clinical environment, just as the production of H_2O_2 by neutrophils is a critical host defence mechanism (Painter *et al*., 2015).

The ability of Ru complexes to generate ROS within a cell has been demonstrated on several occasions; for example, the utilisation of polypyridyl ligands such as bipyridine have been associated with the increased production of ROS within cells (Le Gilles *et al*., 2018; Shum *et al*., 2019; Hua *et al*., 2020). Further, Ru complexes tricarbonyldichlororuthenium (II) dimer (CORM-2), tricarbonylchloro(glycinato)ruthenium (CORM-3) and $[Ru(CO)_2Cl_2]n$ have all

been directly attributed to stimulating increased levels of cellular ROS (Abreu *et al*., 2016; Ghosh *et al*., 2021).

All ROS experiments were performed in the exponential growth phase and the H_2O_2 positive control was conducted at 500 μM, just above the 400 μM threshold that has shown to inhibit *S. aureus* growth (Painter *et al*., 2015). H_2O_2 does not directly damage DNA; instead, it as able to interact with Fe^{2+} and generate a hydroxyl radical ($HO^{\bullet}$) via the Fenton reaction. These hydroxyl radicals are then able to damage DNA (Mendoza-Chamizo *et al*., 2018). The specific type of ROS generated within the cell in these experiments was unknown, as the H_2DCFDA dye acts as a general ROS indicator; other ROS indicators can identify specific ROS species. For initial probing experiments, it was deemed that H_2DCFDA would be sufficient to detect if any type of ROS generation was occurring.

The H_2DCFDA assay showed that exposure to Ru compexes 1 and 7 increased ROS generation within all cell types tested and across all conditions compared to their respective negative control, although the levels of ROS generation were not statistically significant after 30 min of exposure. ROS production in *E. coli* EC958 when exposed to Ru complex 1 was significantly lower than the positive control, similar to ROS production in *S. aureus* USA 300 JE2 and *S. aureus* USA 300 JE2 (IR) exposed to Ru complex 7. Using a variety of exposure times over a 24 h period would have provided a better insight into the role of ROS on cell death. Theoretically, low level ROS-mediated cellular damage over a short time period could lead to a perpetual cycle where ROS-mediated cellular damage leads to the production of increasing levels of ROS within the cell, eventually leading to cellular death (Hong *et al*., 2019). Due to the low levels of ROS produced during the assay, damage to biomolecules within the cell as a result of exposure to the Ru complexes is a likely cause of the increase of ROS within the cells (Rowe *et al*., 2008).

Damage to the genomic DNA in a cell can compromise the ability to form cellular structures, biomolecules and potentially increase DNA mutation rates (Volkova *et al*., 2020). Many of the Ru complexes tested were failed anti-tumour drugs repurposed for antimicrobial applications, and one of the primary targets for anti-tumour drugs is DNA (Shahabadi *et al*., 2019). Ru complex-DNA interactions were assessed using an electrophoretic mobility shift assay and DNA competitive binding assays. While these assays only directly examined DNA-Ru complex interactions, it is likely that RNA within a cell would also be a target due to the molecular similarites to DNA (Philips *et al*., 2012). Ru complex 1 contains arene ligands that strongly indicated potential DNA-Ru complex interactions (Gkionis *et al*., 2008; Arshad *et al*., 2017). Work conducted by Yasbin *et al.,* (1980) previously demonstrated Ru complex 7-DNA interactions and was able to elicit revertant mutations in *Salmonella* strains TA98 and TA100 (Yasbin *et al.,* 1980). It was theorised, if Ru complex 7 was able to penetrate the cell membrane, that Ru complex 7 would then be able to bind to the negatively charged phosphate backbone of DNA via electrostatic forces (Sato, 2012, Andrews, 2019)

The results from the EMSA demonstrated DNA-Ru complex interactions from both Ru complexes 1 and 7. The EMSA results for Ru complex 1 showed visible DNA-Ru complex interaction down to a concentration of 0.25 mM, with total degradation of DNA at 1 mM and 2 mM after a 5 h incubation period. This indicated that Ru complex 1 possessed a high affinity for DNA and produced a DNA cleavage effect. At the MBC concentration of 16 µg/mL (28.833µM) against *S. aureus* USA300 JE2, the pGEM®-3Zf (+) plasmid showed little visual degradation when exposed to Ru complex 1 for five hours; this lack of visual degradation at MBC levels could be a result of the resolution of the assay or intracellular concentrations being substantially higher than exposure concentrations (Pizarro *et al*., 2009). The uptake study (Table 5.1) showed that at the 16 µg/mL (28.833µM) exposure concentration for *S. aureus* USA300 JE2, the actual intracellular concentration was 1.6514 mg/mL (2.9759 mM). At this

concentration there was total degradation of the pGEM®-3Zf (+) plasmid after five hours. This result further signified the DNA-Ru complex interactions.

The EMSA demonstrated that Ru complex 7 also exhibited DNA-Ru complex interactions, although the interactions were less profound than when the pGEM®-3Zf (+) plasmid was exposed to Ru complex 1. Ru complex 7 still exhibited minor visual changes on the EMSA, indicating DNA-Ru complex interactions even at the lowest concentation tested (0.625 mM). These findings also confirm the cellular uptake study, where intracellular concentrations were above those of extracellular concentrations. While testing the antimicrobial activity of Ru complex 7 against *P. aeruginosa* PAO1, the extracellular concentration at MBC was 413.41 μM and the intracellular concentration was shown to be 2.6557 mM (Table 5.1). This is significant due to the effects on DNA as demonstrated by the EMSA as discussed earlier, where at concentrations of 0.625mM there were minor visual effects on the pGEM®-3Zf (+) plasmid while concentrations of 2 mM produced a significant effect on the DNA (Figure. 5. 13).

To confirm the results of the EMSA, a competitive binding assay was conducted for Ru complexes 1 and 7. The competitive binding assay modified the traditionally used ethidium bromide competitive binding assay, replacing ethidium bromide with the fluorescent molecule SYTO 9. Due to the general withdrawal of ethidium bromide from research use, the fluorescent molecule SYTO 9 was used as a suitable substitute. This molecule is utilised in live / dead staining in conjunction with propidium iodide, where the lower affinity DNA binding SYTO 9 is able to penetrate live cells and bind to DNA. When a bacterial cell loses viability, the higher DNA affinity molecule propidium iodide is able to permeate and displace SYTO 9 from the DNA (Robertson *et al*., 2019). Fangfei *et al*. (2013) has previously described Ru complexes which competitively bind to RNA-SYTO 9 complexes, therefore a substitution of SYTO 9 was made to the ethidium bromide competitive binding assay (Fangfei *et al* 2013). Careful consideration of the absorption spectra of each compound was undertaken. Full spectrum UV-

VIS scans were undertaken to assess potential fluorescent molecules with little or no absorbance interference from Ru complexes 1 and 7. An excitation emission matrix for the binding of SYTO 9 to the pGEM®-3Zf (+) plasmid was also constructed. The results from the competitive binding assay corroborated the EMSA results confirming Ru complexes 1 and 7 DNA interactions. These results also support that the arene ligands of Ru complex 1 are likely binding to the DNA, confirming other research conducted on arene ligands and DNA binding. Previous research has shown the likely target would be combined binding to the N7 of guanine, non-covalent arene intercalation and minor groove binding (Liu *et al*., 2018).

The chloro ligands on both Ru complexes 1 and 7 may also contribute to the DNA-Ru complex interactions. Schmitt *et al.* (2018) demonstrated that Ru complexes with subsitituted chloro ligands that interacted with DNA resulted in decreased DNA mobility, indicating that chloro ligands play an important role in DNA interactions with Ru complexes. The study also noted that chloro ligands do not correlate with an increased eukaryotic cytotoxicity compared to other ligands (Schmitt *et al*., 2018). Metal ammine complexes similar to Ru complex 7 have been well-studied in regard to DNA interaction. Work conducted by Katner *et al*., 2018 on ammine-DNA interaction utilised $[Ru(NH_3)6]^{3+}$ and $[RuCl(NH_3)5]^{2+}$ in their DNA ammine binding study. $[RuCl(NH_3)5]^{2+}$ produced an EC_{50} of >1000 μM and $[Ru(NH_3)6]^{3+}$ produced an EC_{50} of 853 ±186 μM, where EC_{50} equals a concentration that it takes to displace 50% of the competing ethidium bromide from DNA (Katner *et al*., 2018). The use of ammonium salt has also demonstrated the ability to interact with phosphate anions present in DNA chains (Meisel *et al*., 2017).

This chapter identified the mechanistic actions of Ru complexes 1 and 7. The work demonstrated Ru complex 1 had no significant membrane interactions while Ru complex 7 produces a significant depolarizing effect on the cytoplasmic membrane of *E. coli* EC958 and produced an elevated but not significant increase in fluorescent intensity in *S. aureus* USA300

JE2. The intracellular concentrations of Ru complex 1 and 7 were elevated above the original exposure concentrations indicating both Ru complexes were accumulating within the cells. The elevated intracellular concentrations were of particular interest for the Ru complex/DNA studies where the EPMSA showed Ru complexes/DNA interactions from Ru complexes 1 and 7 at intracellular concentrations and the DNA interactions were confirmed via a DNA competitive binding assay. The Ru complex/DNA interactions potentially lead to elevated but not significant increases in intracellular ROS. It can be concluded that Ru complex 7 direct interactions with cell membrane and nucleic acids were the likely cause of Ru complex 7s antimicrobial activity while Ru complex 1s antimicrobial activity appeared to solely as a result of nucleic acid interactions.

Chapter 6: Discussion and Future Work

6.1 General Discussion

With the increase in prevalence of MDR bacteria in clinical settings coupled with the decline in novel antibiotics being approved for clinical use, bacterial infections have once again come

to the forefront of public health issues (Jernigan *et al*., 2020; Vivas *et al*., 2019; Klemm *et al*., 2018). As a result, urgent research is required to identify and develop new antimicrobials. The use of metallodrugs has proven to be successful in many clinical applications including: diagnostics, cancer treatments, antimicrobials and antiarthritics (Mjos *et al*., 2014). The concept of ruthenium acting as the central metal atom in antimicrobials was first envisaged by Dwyer *et al*. during the 1950s. However, research in the field did not progress further due to the abundance of effective antimicrobials available during the period (Dwyer, 1964). Ru acting as a central metal atom in a complex makes it an ideal candidate for the development of antimicrobials due to its biological compatibility, multiple oxidation states and accessible bonding configuration (Cotton, 1997; Sahu *et al*., 2018; Kratz *et al*., 1994; Śpiewak *et al*., 2015; Adeniyi *et al*., 2016).

6.1.1 Preliminary Antimicrobial Evaluation of Ru complexes

The initial stages of the study selected 12 Ru complexes from failed custom anticancer treatments and commercially available Ru complexes incorporating bioactive ligands (Table 2.2). The selected Ru complexes were repurposed for antimicrobial evaluation against a range of clinically significant bacteria. Many of the Ru complexes exhibited potent antimicrobial activity, with Gram-positive bacteria generally showing greater susceptibility. This substantiates other research showing Gram-negative bacteria having greater resistance towards antimicrobials due to the presence of an outer membrane (Li *et al*., 2015; Munteanu *et al*., 2021). The outer membrane of Gram-negative bacteria has been shown to provide intrinsic resistance to some Ru complexes. Multiple studies have demonstrated Ru complexes containing the DNA intercalating ligand (dppz) show little or no antimicrobial activity against Gram-negative bacteria. This is thought to be due to complex lipophobicity, thus these are

unable to penetrate the two membranes of Gram-negative bacteria (Liu *et al*., 2018; Bolhuis *et al*., 2011). Li *et al*. (2012) showed lower uptake of their synthesised dinuclear ruthenium(II) complexes in Gram-negative bacteria, which was attributed to lower permeability of the outer membrane of Gram-negative species (Liu *et al*., 2012). After the preliminary antimicrobial evaluation, two antimicrobials were selected for further in-depth study. Ru complexes 1 and 7 were chosen due to potent antimicrobial activity against *S. aureus* USA300 JE2 and the Gram-negative *P. aeruginosa* PAO1 respectively (Chapter 3.4.2).

The results of the eukaryotic cytotoxicity studies showed Ru complex 7 exhibited no significant cytotoxicity compared to the negative controls. This result was further confirmed by using the *in vivo Galleria mellonella* model where 100% survival rate was achieved at 50 mg/Kg. The *in vitro* cytotoxic testing of Ru complex 1 showed it was highly toxic to eukaryotic cells, substantiating work from Arshad *et al*., (2017) where cytotoxicity studies of Ru complex 1 had IC_{50} values of 5.5 μM and 6.2 μM against HCT116 and NCI-H460 cell lines respectively (Arshad *et al*., 2017). Conversely, the *in vivo G. mellonella* studies showed a 100% survival rate at 10 mg/Kg. It is not understood why Ru complex 1 showed no toxicity to *G. mellonella,* however, the use of *G. mellonella* for synthetic antimicrobial evaluation is somewhat controversial, primarily due to variations in antimicrobial activity that synthetic antimicrobials have produced between *in vitro* and *in vivo* experimentation (Huggins *et al*., 2017; Vellé *et al*., 2017; Tran *et al*., 2018). Further research would need to be undertaken to determine the nature of the observed *in vitro* cytotoxic effects, compared to the low toxicity recorded during *in vivo* studies.

6.1.2 Resistance Development

It is widely believed that AMR to synthetic antimicrobials develops at a slower rate compared to organic antimicrobials, and recent research alludes that AMR development towards Ru

complexes is slower compared to conventional antibiotics (Konaklieva *et al*., 2019; Frei, 2020). In order to develop resistance in a pure culture, a bacterial cell must undergo transcriptome changes and/or *de novo* genomic mutations (Reygaert., 2018). Exposure to sublethal concentrations of antibiotics causes selective pressure and mutagenesis, which has been shown to induce *de novo* antibiotic resistance (Kohanski *et al*., 2010; Davies *et al*., 2010; Gullberg *et al*., 2011). A current literature review suggested there is no published research into using whole genome sequencing to assess the effect of Ru complexes on mutagenesis. The limited mutation research that has been undertaken, has primarily focused on Ames testing (Benkli *et al*., 2009; Alanyali *et al*., 2010; Pavan *et al*., 2013; Inami *et al*., 2013).

After 100 days of incremental exposure to Ru complex 1, *E. coli* EC958 and *S. aureus* USA300 JE2 were able to tolerate an 11-fold increase in MBC. The prolonged exposure elevated the rate of mutagenesis and resulted in 17 *de novo* mutations being observed across 8 different genes in *S. aureus* USA300 JE2. Sadly, due to funding, mutagenesis in the *E. coli* EC958 genomes were not assessed. The mutations in the *S. aureus* USA300 JE2 (IR) and *E. coli* EC958 genome did not appear to confer resistance to Ru complex 1, the resistance reverting to pre-incremental exposure levels after a week of subculturing in *S. aureus* USA300 JE2 and after a single day of subculturing in *E. coli* EC958, this indicated changes to the transcriptome were responsible.

The reverting of resistance substantiates work done by done by Varney *et al*., (2020) where *E. coli* EC958 was only able to tolerate a 4- to 8-fold increase in MIC and the resistance rapidly reverting upon the removal of the selective pressure (Varney *et al*., 2020). Bu *et al*., (2020) also experimented on resistance development in *S. aureus* with $[Ru(bpy)_2(BTPIP)](ClO_4)_2$. The results showed only a 2-fold increase in resistance after 20 passages, whilst in the same period a 1000-fold increase in resistance to ampicillin was observed (Bu *et al*., 2020). These results demonstrate that microorganisms are able to temporarily adapt to Ru complexes, but

they appear to be unable gain a permanent resistance. It is widely believed that this is due to Ru complexes often utilising multiple or novel antimicrobial mechanistic actions (Frei., 2020; Bu *et al*., 2020; Munteanu *et al*., 2021). To confer full resistance to an antimicrobial, the microorganism would often need to undergo *de novo* mutations across multiple loci in set locations (Porse *et al*., 2020). As a result, it was unsurprising that changes to the transcriptome were responsible.

The changes to gene regulation were varied and some unexpected results were observed. As a result of the DNA binding studies it was expected that there would be up regulation in the *recA* and possibly the *umuC* genes, but this was not the case as both genes showed no significant alteration in expression. Indeed, both showed a general trend of down regulation. The cytotoxic effects of Ru complex 1 were rapid producing a bactericidal effect in under 2 hours, which is sufficient time to generate a regulation response from the *recA* gene which has near instantaneous response times upon detection of DNA strand breaks (Patel *et al*., 2010). The rationale underpinning a lack of expression changes in *recA* are unknown, but it could be hypothesised the cationic nature of Ru complex 1 preferentially targets anionic proteins. This would potentially explain the significant upregulation of *clpP*, but further studies are required to confirm this hypothesis.

The genes *norA* and *katA* were both significantly upregulated. The multidrug resistant transporter NorA is considered the first line of defence to antimicrobials, and upregulation indicated the presence of Ru complex 1 was treated in the same manner as biocides, dyes, quaternary ammonium compounds, and antiseptics (Costa *et al*., 2019). The upregulation of *katA* was expected as the gene is pivital to cellular oxidative stress response. The oxidative stress response occurs as a result of respiration, autooxidation reactions, intracellular redox reactions, and antibiotics (Gaupp *et al*., 2012). The three genes examined which relate to biofilm formation, *icaB, sasG and ccpA*, were all non-significantly upregulated. This trend

indicates a generic survival response, as bacteria within biofilms are able to tolerance higher concentrations of disinfectants, antibiotics, and phagocytosis (Slany *et al*., 2017). Likewise, due to the cultures being in constant motion during gene expression studies, biofilm formation was unlikly to occur.

6.1.3 Mechanistic Actions

A series of experiments were undertaken to elucidate the mechanisms of antimicrobial action of Ru complexes 1 and 7. The study showed that the Ru complex 1 had no significant membrane interaction and the antimicrobial activity was likely as a result of DNA/Ru complex interactions. The results of the EMSA and DNA competitive binding assay correlated with the accumulation of intracellular concentrations of Ru complex 1. The DNA binding of Ru complex 1 is likely due to the complexes arene ligands as these are known to bind to N7 of guanine often causing irreparable DNA damage (Meier *et al*., 2012). DNA damage and elevated cellular metabolism in response to antimicrobials are known to elevate intracellular ROS levels (Zhao *et al*., 2014; Imlay, 2015), which could explain the significant increase in the *katA* upregulation, coupled with observed elevated intracellular ROS levels. It is probable that the intracellular ROS generation was higher during exposure to Ru complex 1 but this was counteracted by significant upregulation of ROS decomposing genes to maintain non-lethal intracellular ROS concentrations. An elevated level of ROS production can lead to a positive feedback loop resulting in cell death. However, this is unlikely to be the case due to such low elevation (Hong *et al*., 2019). The upregulation of the *clpP* gene in *S. aureus* USA300 JE2 and *S. aureus* USA300 JE2 (IR) also indicated possible RNA/protein interactions. The *clpP* gene is critical to protein hydrolysis of damaged and modified proteins (Moreno-Cinos *et al*., 2019). The anionic charge of proteins makes them a potential target of cationic Ru complexes and Ru

complexes/protein interactions have been demonstrated multiple times (Williams *et al*., 2012; Wang *et al*., 2012). Further experimentation of Ru complex/protein interactions would need to be undertaken to confirm this potential site of activity.

The potent antimicrobial activity of Ru complex 7 against *P. aeruginosa* PAO1 made it an ideal candidate for mechanistic action study. Due to its similarities to QAS, it was originally speculated that outer membrane interactions would be the primary mode of antimicrobial activity for Ru complex 7 (Ohta *et al*., 2008; Kwaśniewska *et al*., 2020). This proved not to be the case, Ru complex 7 showed no significant outer membrane activity over the H_2O_2 control. The cytoplasmic membrane depolarisation assay showed Ru complex 7 had no activity against Gram-positive species but did show significant depolarisation against *E. coli* EC958 and *E. coli* EC958 (IR). The depolarisation was not as rapid or as profound as the Triton X-100 positive control but did indicate a possible mode of action or contributing factor. The increased depolarisation of the cytoplasmic membrane of Gram-negative bacteria over Gram-positive bacteria was unusual. While QAS produce antimicrobial activity against both Gram-positive and Gram-negative bacteria, the antimicrobial action tends to be more effective against Gram-positive bacteria (Knauf *et al*., 2018; Kwaśniewska *et al*., 2020). Ru complex 7 lacked activity against the cytoplasmic membrane in P. aeruginosa PAO1 which might be in part due to the high level of resistance P. aeruginosa possess against QAC (Adair *et al*., 1969). Whilst other QAS compounds have shown a much more rapid depolarisation of the cytoplasmic membrane, the 60-minute end point of the cytoplasmic membrane depolarisation assay may have been a limitation of the assay (Xiao *et al*., 2008; Murakami *et al*., 2016). Due to the nature of the assay, all cultures were grown on the day to maintain the highest number of viable cells and the lowest membrane deterioration. The time kill kinetics for P. aeruginosa PAO1 indicate this 60-minute time point should have been extended but this was not feasible. The results of the EMSA and competitive DNA binding assay showed Ru complex 7/DNA interactions, where

the DNA showed reduced mobility indicating Ru complex 7/DNA interactions. This was confirmed in the DNA competitive binding assay where significant fluorescence reduction was shown down to 0.125 mM concentrations. There were also elevated levels of intracellular ROS, which could be either due to DNA interactions or as a result of the stress response increasing metabolic activity (McBee *et al*., 2017). Unfortunately, no gene regulation studies were conducted on Ru complex 7, leaving the possibility of undertaking them in future work.

6.2 Conclusion

The purpose of this study was to identify and repurpose a series of Ru complexes and evaluate their potential as antimicrobial agents. Ru complexes with the potent antimicrobial activity were assessed for resistance development via long term selective pressure and subsequently assessed for changes to gene expression and mutagenesis. Finally, further investigation elucidated to the Ru complexes mechanisms of action.

The initial antimicrobial screening demonstrated the potential of the selected Ru complexes acting as antimicrobial agents with many of the Ru complexes possessed potent antimicrobial activity. The initial antimicrobial highlighted that Gram-positive bacteria showed greater susceptibility to the selected Ru complexes. Ru complex 7 was of particular interest due to its antimicrobial activity against *P. aeruginosa* and low eukaryotic cytotoxicity. While Ru complex 1 exhibited potent antimicrobial activity against a broad spectrum of bacteria. The antimicrobial actions were varied, Ru complexes 7 targets included; cytoplasmic membrane depolarisation, elevated ROS levels and DNA damage. Ru complex 1 produced elevated ROS and severe DNA damage.

After long term exposure to Ru complex 1, *E. coli* EC958 and *S. aureus* USA300 JE2 were able to tolerance a temporary 11-fold increase in MBC, this temporary resistance indicated

gene regulation changes were responsible for the increased tolerance to Ru complex 1. Gene regulation analysis showed a significant upregulation of genes across a wide range of cellular functions, these included; efflux systems, ROS decomposers and protein hydrolysis. Based on these findings, Ru complexes acting as antimicrobial agents have a promising future but require further investment. The work showed that Ru complexes are able to target a range of cellular components and potentially having a lower resistance development compared to conventional antibiotics. The antimicrobial activity produced by Ru complexes 1 and 7 warrant further in-depth study and *in vivo* experimentation.

Finally, the modification to the competitive binding assay proved to be successful and the Ru complexes were able to displace SYTO 9 from the ctDNA. This assay modification could potentially have benefits to other research.

6.3 Future work

The work conducted for this thesis has demonstrated the potential of Ru complexes acting as antimicrobial agents. The work highlighted key areas where further research and experimental changes would contribute to furthering knowledge in the field of antimicrobial Ru complexes. The long-term selective pressure induced a number of mutations withing the *S. aureus* USA300:JE2 (IR) genome, some of these mutations were classified as non-sense mutations. The use of western blots would confirm the changes to the proteins produced non-sense mutations of the *S. aureus* USA300:JE2 (IR) genome. Full sequencing of the *E. coli* EC958 and the *E. coli* EC958 (IR) genomes could be undertaken to compare possible mutations, this would be followed by RT-qPCR on selected genes to indicate possible reasons for the increased tolerance of Ru complex 1.

To quantify and better understand which the cellular structures Ru complex were accumulating within. A subcellular fractionation study followed by ICP-MS analysis of the constituent parts would prove beneficial (Prochnow *et al*., 2018). A dissection followed by ICP-MS analysis of *Galleria Mellonella* injected with differing Ru complexes would give a better understanding of the distribution of Ru complex within the organism. The low cytotoxicity of Ru complex 7 to eukaryotic cell lines combined with the low toxicity during the *in vivo Galleria mellonella* assay makes Ru complex 7 an ideal candidate for Murine skin infection model. This would also give a better understanding of the pharmacodynamics of the complex within a higher organism.

The project originally proposed the use of RNA-SEQ, compared to RT-qPCR it would have given a better understanding of transcriptome wide response to Ru complexes. Cost and time restraints led to project alterations, subsequently a more focused use of RT-qPCR was selected for the gene expression study. By using long-term selective pressure to induce resistance to an array of Ru complexes in a variety of bacteria species simultaneously, then analysing the transcriptomes via RNA-SEQ a better understanding of Ru complex resistance development could be achieved. The data could potentially identify novel responses or locate patterns in gene expression that could further help in the development of new Ru complexes via custom synthesis.

Chapter 7: References

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Chapter 8: Appendix

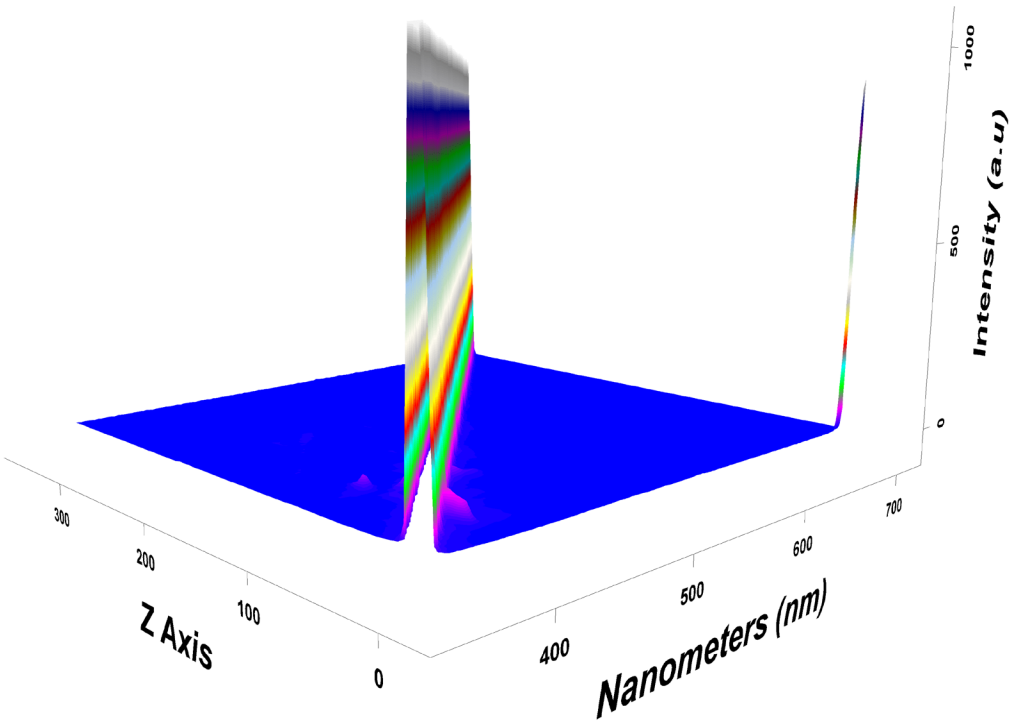

Figure 8. 1: The excitation and emission spectrum of Ru complex 1 excitation from 350 to 700 nm and emission scan from 360 to 700 nm

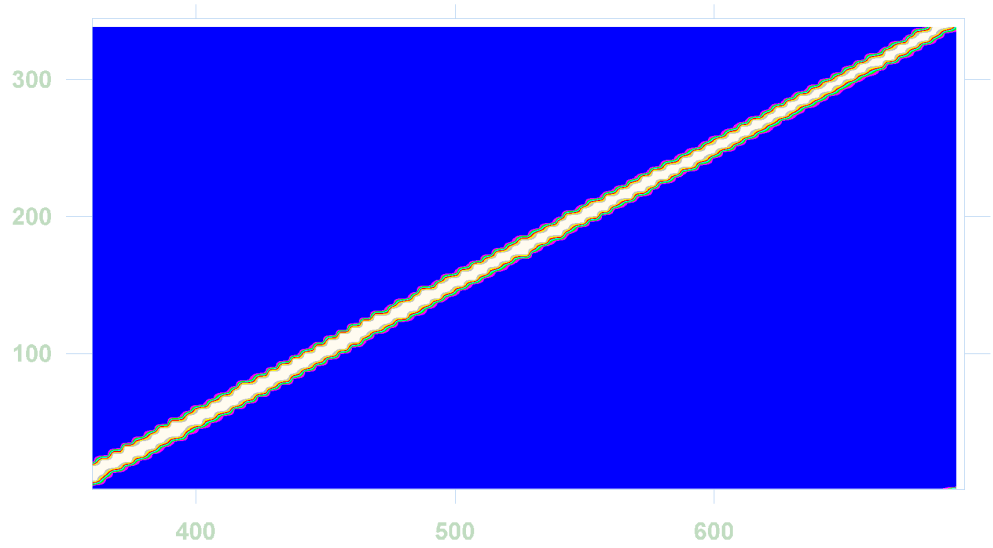

Figure 8. 2: A contour graph of the excitation and emission spectrum of Ru complex 1 excitation from 350 to 700 nm and emission scan from 360 to 700 nm

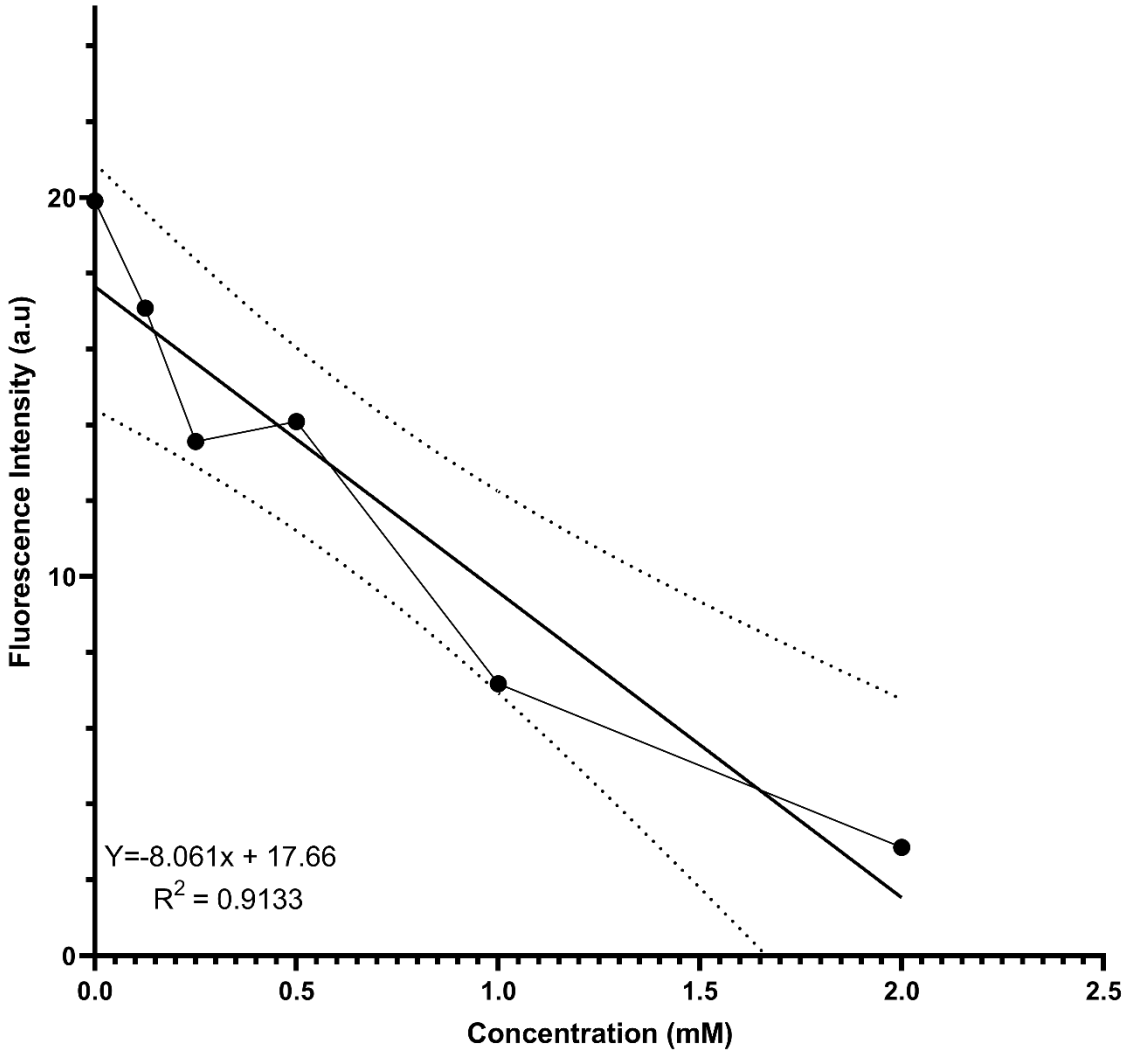

Figure 8. 3: A Standard curve constructed for the competitive binding assay of Ru complex 1 and CT-DNA showing the R^2 value.

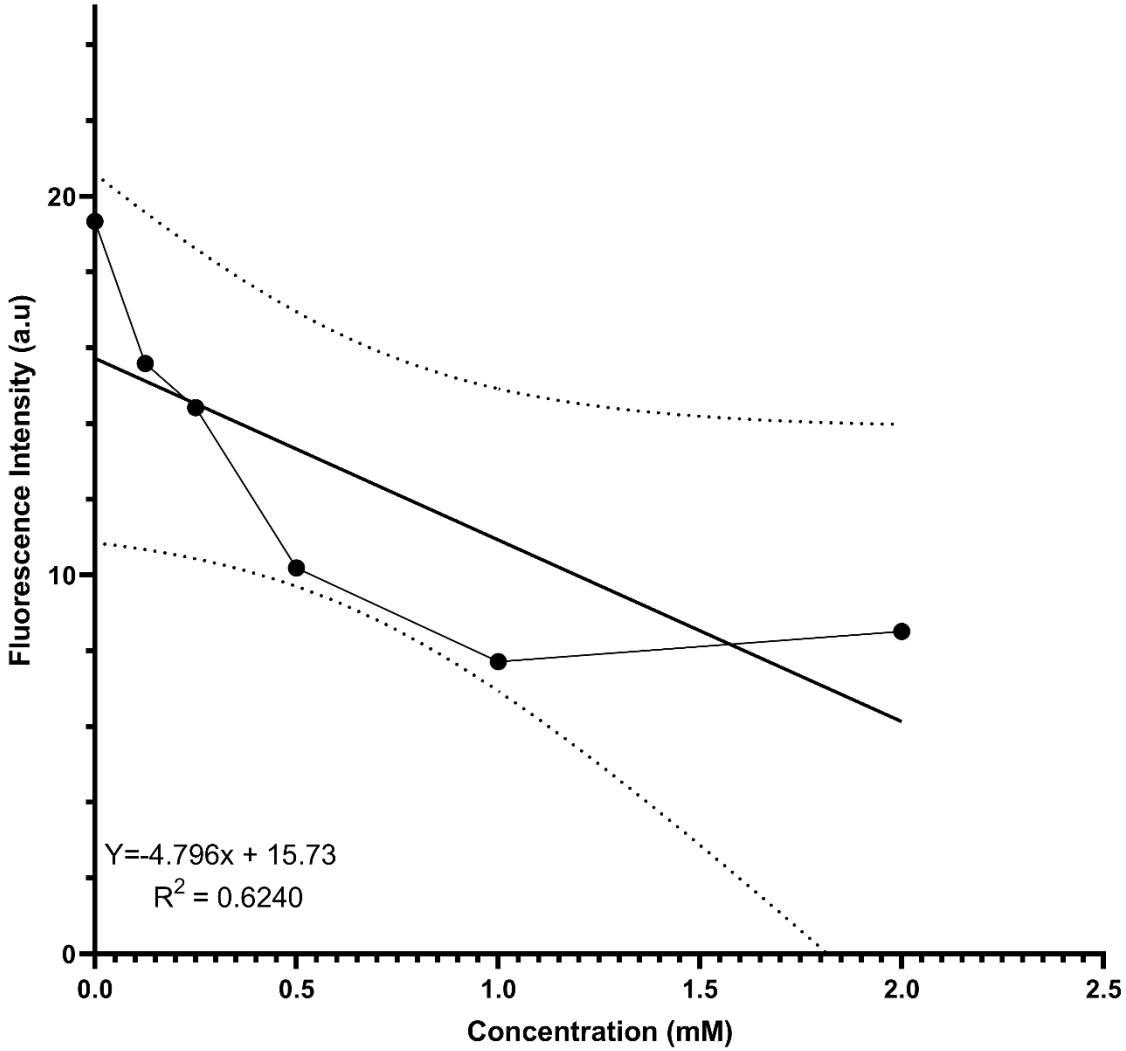

Figure 8. 4: A Standard curve constructed for the competitive binding assay of Ru complex 7 and CT-DNA showing the R^2 value.

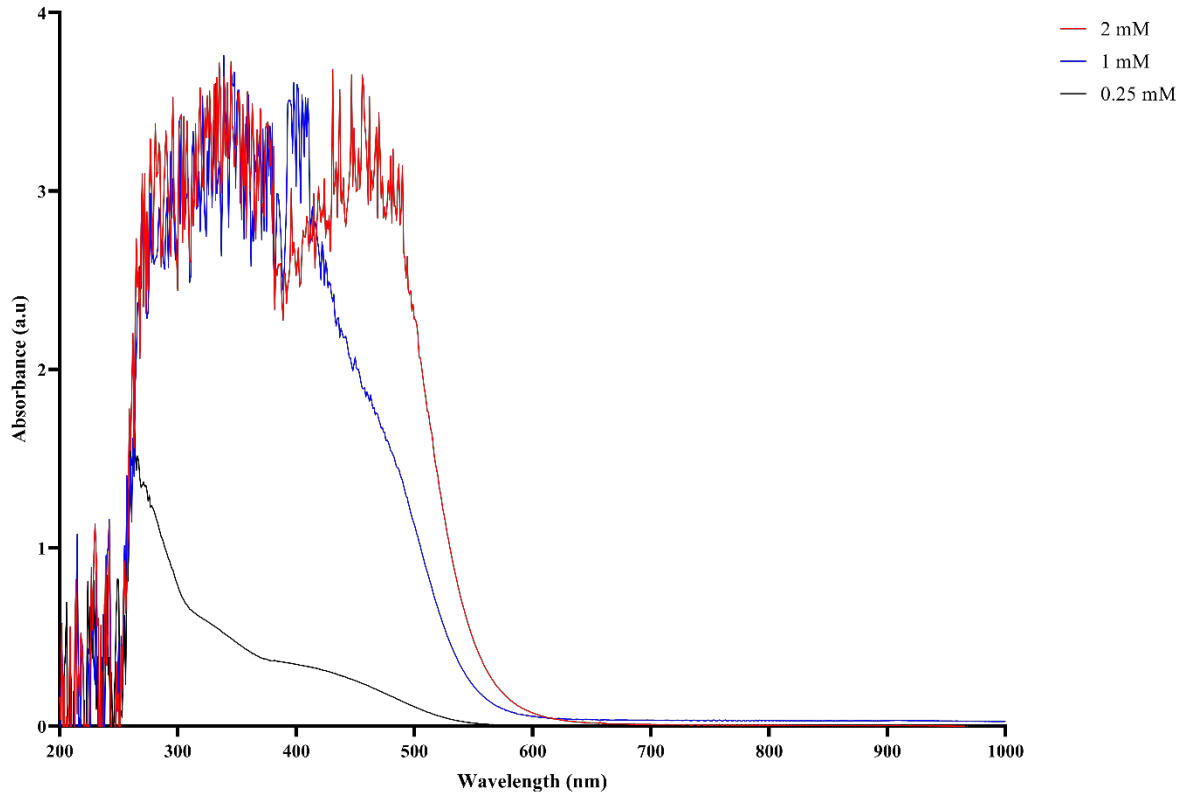

Figure 8. 5: Absorption spectra between 200 and 1000 nm for Ru complex 1 at 2mM, 1mM and 0.25mM concentrations.

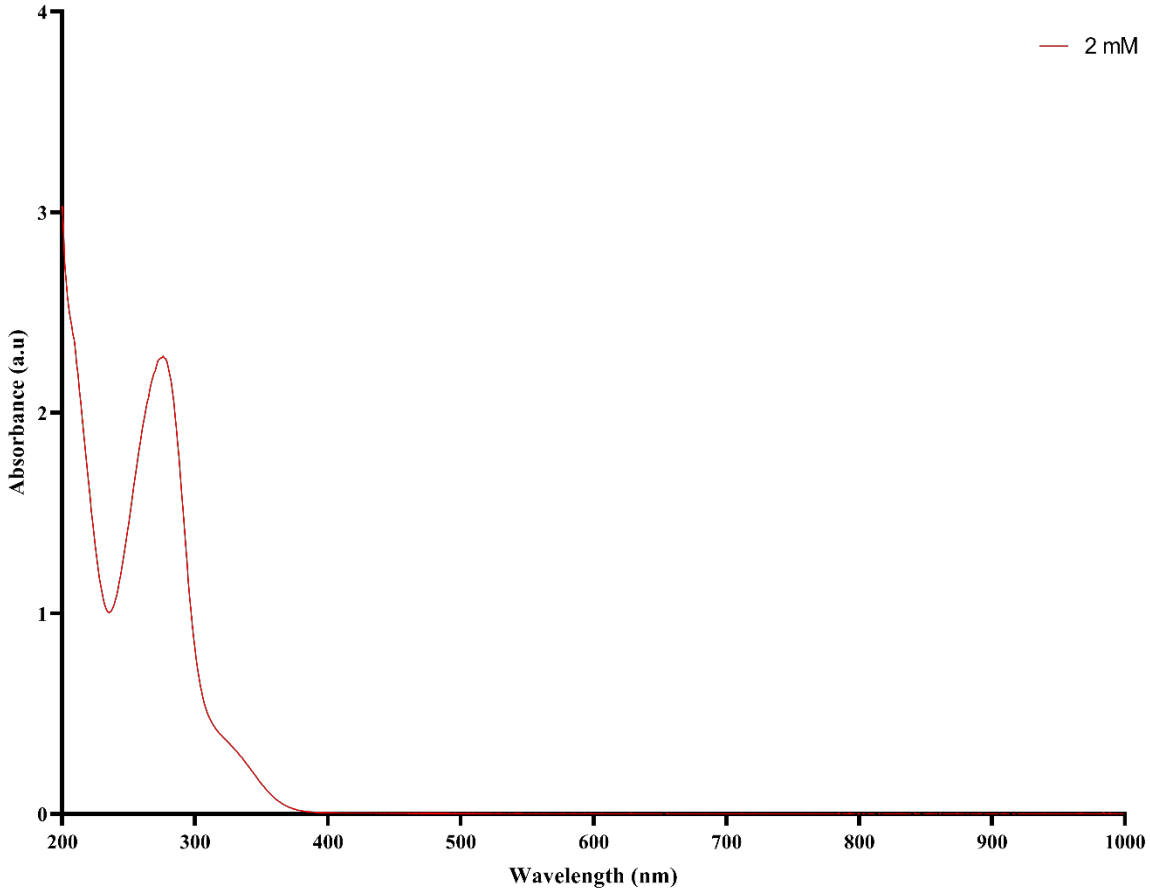

Figure 8. 6: Absorption spectra between 200 and 1000 nm for Ru complex 7 at 2mM concentration.

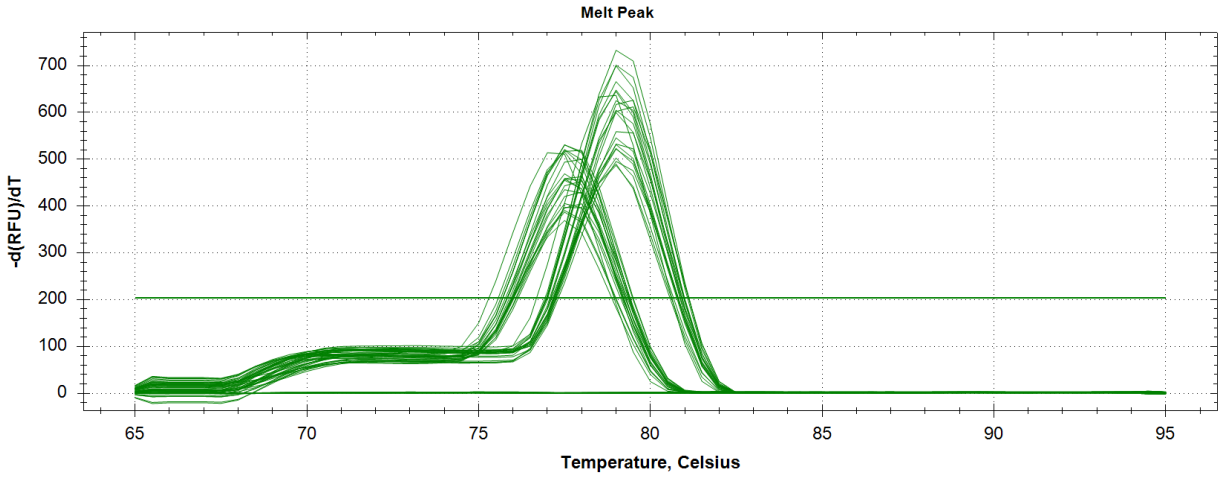

Figure 8. 7: Representative melt peak for RT-qPCR. Melt peak for norA and sasG.

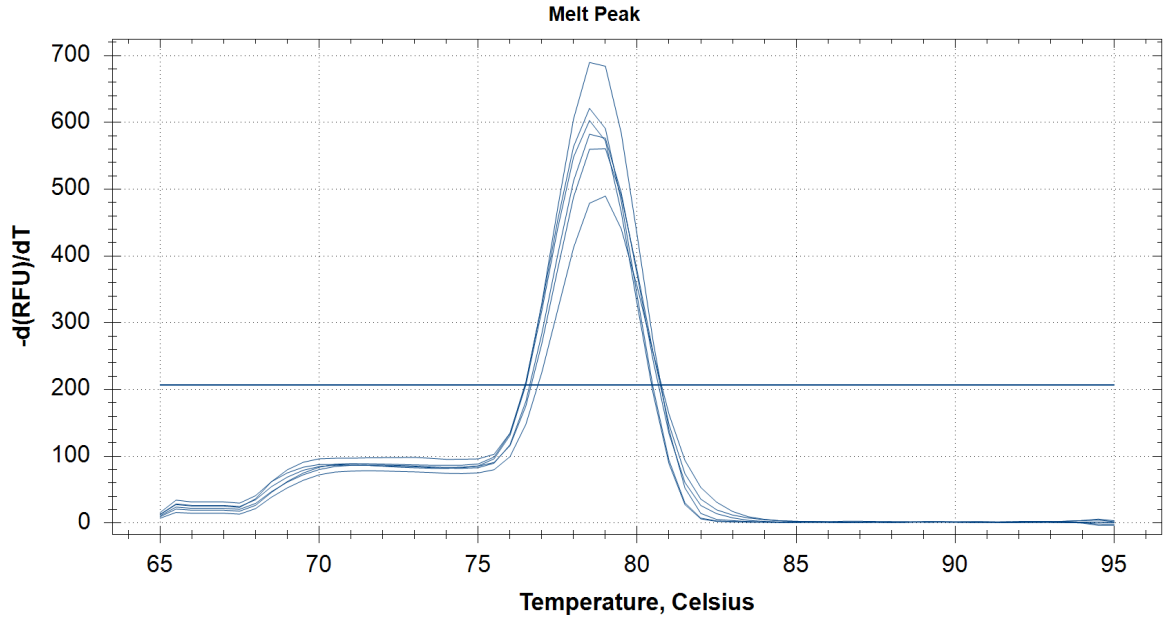

Figure 8. 8: Representative melt curve analysis for primer specificity at different temperatures. Graph showing recA.