A study on the antibacterial activity of pyrrolobenzodiazepine dimers

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Abstract

The pyrrolobenzodiazepines (PBDs) are a family of tricyclic antibiotics that interact within the minor groove of DNA to form monovalent adducts. PBD dimers cross-link double-stranded DNA in a sequence selective manner and interfere with DNA transcription and translation. The aim of this study was to determine the antibacterial profile of three PBD dimers with differing sequence selectivity (SJG-136, DRG-16 & ELB-21) against clinical isolates from a range of bacterial infections and to investigate their mechanism of antibacterial action. MICs of the compounds were determined against a total of 123 clinical isolates, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). MIC\(_{90}\) values for the PBD dimers against Gram-positive isolates were \(< 0.5\) mg/l. All Gram-negative isolates were insensitive with MIC\(_{90}\) of \(\geq 16\) mg/l. ELB-21 was the most potent compound against all strains. Time kill kinetics of the PBD dimers against two epidemic strains of MRSA, EMRSA-15 and EMRSA-16, and a VRE isolate were determined; the PBD dimers were bactericidal within 2 hours. Addition of the PBD dimers to genomic EMRSA-16 DNA significantly increased the melting temperature of the DNA, indicating that PBD dimers efficiently cross-link bacterial DNA. ELB-21 was a more potent cross-linking agent than SJG-136. Southern blotting experiments using *mecA* and *16S* rDNA probes provided further evidence for site-selective cross linking of the strands of the DNA duplex by subinhibitory concentrations of ELB-21 and SJG-136; these compounds blocked *EcoR1* cleavage at restriction sites that encompass sites of PBD dimer cross-linking. Two-dimensional gel electrophoresis indicated that the observed cross-linking resulted in up- and down-regulation of a number of EMRSA-16 proteins. An initial evaluation of the efficacy of ELB-21 and SJG-136 in a murine peritonitis model proved inconclusive. This study demonstrates that the PBD dimers exert a bactericidal effect on Gram-positive pathogens by cross-linking strands of duplex DNA at selected sites on the bacterial genome.
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This thesis describes research conducted in the School of Pharmacy, University of London between October 2002 and October 2005 under the supervision of Professor Peter Taylor. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Signature

Date 13/06/2006
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>CA-MRSA</td>
<td>community acquired MRSA</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical Laboratory Standards Institute</td>
</tr>
<tr>
<td>dATP</td>
<td>2'-deoxyadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>2'-deoxycytidine 5'-triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>2'-deoxyguanosine 5'-triphosphate</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>2'-deoxythymidine 5'-triphosphate</td>
</tr>
<tr>
<td>dUTP</td>
<td>deoxyuridine triphosphate</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ds</td>
<td>double stranded</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ESBL</td>
<td>extended spectrum β-lactamases</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HARP</td>
<td>heteroaromatic polycyclic</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>IPG</td>
<td>immobilised pH gradient</td>
</tr>
<tr>
<td>l</td>
<td>liter</td>
</tr>
<tr>
<td>log</td>
<td>logarithmic</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>M</td>
<td>mole</td>
</tr>
<tr>
<td>MALDI TOF</td>
<td>matrix-assisted laser desorption/ionization, time of flight</td>
</tr>
<tr>
<td>MDR</td>
<td>multi-drug resistant</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
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CHAPTER ONE
INTRODUCTION
The development of antibacterial agents in the mid-20th century transformed the pattern of morbidity and mortality associated with bacterial infections. Many previously deadly infectious diseases are now curable and life expectancy has significantly increased as a result. Early indications of the potential for antibacterial chemotherapy appeared in the late 19th century from the work of Louis Pasteur and Paul Ehrlich. The discovery of penicillin in 1928 by Alexander Fleming marked the beginning of a remarkable era in the discovery of antimicrobial agents produced by living organisms. Mass-production of penicillin began in 1944 as a response to victims of World War II with infected wounds and saved the lives of many. Unfortunately, as bacteria adapted to a new, unfavourable environment, the first cases of penicillin resistance were recorded (Barber & Rozwadowska-Dowzenko, 1948). Resistance follows the natural path of Darwinian evolution and the survival of the fittest; by killing susceptible bacteria, the antimicrobial agent selects for pre-existing variants that are carrying resistance genes.

Over the next three decades, many new classes of antibiotics were discovered: these included the macrolides, glycopeptides, chloramphenicol, tetracyclines, aminoglycosides and quinolones, but resistance quickly developed. The increased use of these antibiotics in hospitals, and more recently in the community, has selected for bacteria carrying multiple resistance genes and has gradually led to the emergence of multi-drug resistant pathogens (Levy, 2005). Worryingly, some of the resistant isolates are only susceptible to one or two antibiotics and in the worst cases to none. Resistance was tackled by the introduction of new antibiotics that were chemically modified compounds of existing classes, although cross-resistance was not uncommon. More recently, new generations of antibiotics have been introduced: these include the oxazolidinones, streptogramins and lipopeptides (Shah, 2005). Nevertheless, resistance continues to develop and evolve at a faster rate than new antibiotics can be introduced and limited therapeutic options are available against many pathogens.

Some of the most important pathogens amongst Gram-positive (MRSA, VRE and Streptococcus pneumoniae) and Gram-negative bacteria (Pseudomonas aeruginosa and ESBL-producing Enterobacteriaceae) have been the most successful in evading the consequences of antimicrobial chemotherapy.
1.1. *Staphylococcus aureus*: the evolution of a resistant pathogen

1.1.1. *S. aureus*

*S. aureus* colonises the nasal cavity of some 30-40% of the population and may also survive on drier skin, for example the hands, or other moist mucosal surfaces, such as the armpits (Peacock *et al.*, 2001). These generally do not cause any harm to healthy individuals, although minor infections such as skin boils and abscesses are not uncommon. *S. aureus* is an opportunistic pathogen and can cause more serious infections in the immunocompromised or in patients with open wounds and burns. Blood, heart, lungs, bones and joints are prime tissue targets of infection. Superantigens released into the blood stream lead to toxic shock syndrome and high fever. *S. aureus* also causes food-poisoning by releasing enterotoxins. *S. aureus* secretes a number of other virulence factors such as haemolysin and leukocidin toxins that are damaging to eukaryotic cells (Lowy, 1998). *S. aureus* is a versatile pathogen that has adapted well to environmental pressures over the years and become resistant to most currently used antibiotics (Livermore, 2000). It is not only the most common hospital-acquired pathogen, but is now an increasing cause of community-acquired infections in healthy individuals at low risk of developing infection (Chapman *et al.*, 2005; Enright, 2003).

1.1.2. The β-lactams and peptidoglycan synthesis

The β-lactams, including the penicillins, cephalosporins, carbapenems and monobactams, are potent bactericidal agents. They interfere with the growing bacterial cell by binding to penicillin binding proteins (PBPs), which are involved in peptidoglycan assembly and cross-linking of the cell wall (*Fig. 1.1*). The lack of cross-linking of the glycan chains leads to weakening of the cell wall and cell death (Waxman & Strominger, 1983). The antibiotics are structural analogues to the acyl-D-Ala-D-Ala terminus of the stem peptide in the growing glycan chain, the substrate for PBPs. The β-lactams bind to and inhibit the active domains of the PBPs that catalyse transpeptidation; the function is necessary for cross-linking of the new glycan chain to pre-existing chains (Stapleton & Taylor, 2002; Waxman & Strominger, 1983; Yocum *et al.*, 1979).
Fig. 1.1. Schematic representation of the structure of peptidoglycan. PBPs catalyse the synthesis of peptidoglycan; transglycosylation polymerises the monomers thus extending the growing glycan chain, and transpeptidation cross-links tetrapeptides via pentaglycine bridges. The β-lactams inhibit transpeptidation by binding to and blocking the active site of PBPs that catalyse this reaction. The glycopeptides, vancomycin and teicoplanin, bind to the terminal D-Ala-D-Ala residues of both peptidoglycan monomers and unlinked residues of the growing chain.

*S. aureus* has four penicillin-binding proteins, PBP1, 2, 3 and 4; there are still uncertainties about the role that each performs. It appears that PBP2 is the only bifunctional protein and is essential for cell survival; it performs both transglycosylation and transpeptidation (Georgopapadakou *et al*, 1986; Leski & Tomasz, 2005; Murakami *et al*, 1994). Other studies have concluded that PBP1 (Wada & Watanabe, 1998) but not PBP3 (Pinho *et al*, 2000) or PBP4 (Katayama *et al*, 1999).
are essential for survival of *S. aureus* and it is likely that PBP1 and PBP2 are the main targets for β-lactams.

### 1.1.3. First use of penicillin and penicillin resistance

The first therapeutic trials of penicillin were conducted in the Radcliffe Infirmary in Oxford in 1940 on several patients with staphylococcal or streptococcal infections (Abraham *et al.*, 1941). Penicillin was first given intravenously to a patient with a mouth sore, which yielded *S. aureus* and *S. pyogenes* cultures. Although the patient died, due to insufficient supply of penicillin, his condition did improve for a period of time following treatment. No toxic effects as a result of penicillin administration were observed and penicillin was administered into other patients who responded well to treatment. However, cases of penicillin-resistant bacteria emerged rapidly (Barber & Rozwadowska-Dowzenko, 1948). In 1946 there were relatively few cases of resistance, although by 1948 more than 50% of isolates were penicillin-resistant. The responsible genetic element was an acquired gene, *blaZ*, encoding a β-lactamase enzyme, which hydrolyses and inactivates the β-lactam ring of the antibiotic. This gene is carried by a plasmid in *S. aureus* and can be easily transferred between bacteria; therefore resistance can spread rapidly to previously susceptible bacteria (Rush *et al.*, 1969). As it is a mobile genetic element, it is capable of integration into the bacterial chromosome (Novick *et al.*, 1975).

### 1.1.4. Methicillin and methicillin resistance

Methicillin was introduced in 1961 and was stable to β-lactamases. However, the first cases of methicillin resistance were reported soon after its introduction (Barber 1961, Jevons 1961). Methicillin resistance is invariably due to the gene *mecA*, which encodes a penicillin-binding protein, PBP2a, with a low affinity for penicillins in comparison to the high affinity PBPs 1-4 (de Jonge & Tomasz, 1993; Hartman & Tomasz, 1984). Initially, it was believed that PBP2a substituted for the functions of all other PBPs after they had been inactivated by the β-lactams, but recent evidence suggests that PBP2 is also necessary for maintaining resistance and possibly works together with PBP2a (Pinho *et al.*, 2001a; Pinho *et al.*, 2001b). Each domain of the
PBP2 protein was investigated separately and it was shown that inactivation of the transglycosylase domain drastically reduced the MIC values of methicillin against the strain, while inactivation of the transpeptidase had no effect on resistance levels. The authors concluded that PBP2a performs the transpeptidase function to complement the transglycosylase of PBP2 but an intact PBP2a alone is not sufficient for conferring high level methicillin resistance. It is clear that PBP2a is essential for conferring resistance to methicillin in *S. aureus*, although the exact mechanisms, roles and interactions of the PBPs involved in resistance remain to be elucidated.

### 1.1.4.1. Origin of the *mecA* gene

How the *mecA* gene found its way into methicillin-susceptible *S. aureus* (MSSA) is still uncertain. *Staphylococcus sciuri* has been proposed as the likely source, as 134 strains that were screened with a *mecA* probe gave positive results (Couto *et al*, 1996). Interestingly, the majority of strains were not resistant to methicillin as expected. The *mecA* gene in *S. sciuri* was sequenced and when compared to *mecA* of methicillin-resistant *S. aureus* (MRSA) there was a high degree of homology between the amino acid sequences (Wu *et al*, 1996). There were also two domains, a putative transglycosylase with a 68% identity of the amino acid sequence and a transpeptidase with 91% identity to *S. aureus*. Wu *et al* (2001) exposed a methicillin-sensitive *S. sciuri* strain to increasing concentrations of methicillin and were able to induce resistance to methicillin. After sequencing the *mecA* gene of this new resistant strain, they found a point mutation responsible for a significant increase in transcription rates of the gene. This suggests that the *mecA* gene in *S. sciuri* has the potential to confer resistance to methicillin and is the likely precursor of *mecA* in MRSA. In addition, two strains of *S. sciuri* have been isolated from humans and found to be methicillin-resistant as a result of a point mutation and an IS256 element insertion, again associated with up-regulation of transcription, highlighting the role of bacterial adaptation in different environments (Couto *et al*, 2003).

### 1.1.4.2. SCCmec types I-V

The *mecA* gene in MRSA is located on a unique staphylococcal cassette chromosomal *mec* (SCCmec) element that contains the *ccrA* and *ccrB* genes involved in DNA
recombination with the chromosome (Katayama et al., 2000). The size of SCCmec is similar to other genetic elements such as pathogenicity islands, bacteriophage and transposons. However, SCCmec lacks the structural components of these elements (Ito et al., 1999). Five main types of SCCmec have been identified, each one described on the basis of the ccr gene and mecA complex. Types I-III are large in size (34-67kb) and types II and III carry other resistance genes in addition to mecA (Ito et al., 2001). These types are mainly associated with hospital acquired MRSA and type I SCCmec was determined in a strain isolated in 1961, while types II and III are the typical SCCmec elements isolated from more recent hospital strains that may have accumulated other resistance genes by homologous recombination (Ito et al., 2001). Type IV and a recently described type V SCCmec are smaller in size (21-27kb) than the other three and do not carry other resistance genes other than mecA (Ito et al., 2004; Okuma et al., 2002). Types IV and V are usually associated with community acquired MRSA (CA-MRSA) strains and the elements of the SCCmec are believed to have been obtained from coagulase-negative strains in the community (Ito et al., 2004). The mechanisms of recombination and the process by which mecA and SCCmec found their way into S. aureus are still in question. Enright et al (2002) performed multilocus sequence typing on 912 S. aureus strains from 20 different countries and concluded that it is highly likely that MRSA clones evolved by horizontal transfer of the mecA gene into different MSSA lineages rather than from one initial clone that diversified with time. They found five major lineages from which the current common epidemic MRSA clones have developed. They provided evidence that one clone of MSSA progressed to MRSA on several independent occasions, as the same clone was isolated with different SCCmec types. Robinson and Enright (2003) have further suggested that type IV is the most common SCCmec type found in MRSA isolates and predicted that clones with type IV SCCmec may become more prevalent in MRSA infections than other types. It is likely that the smaller DNA fragment for type IV is advantageous for efficient DNA recombination, and excision and integration occur more readily than with the larger SCCmec types.

1.1.5. Epidemic MRSA strains

During the 1980s a number of commonly occurring isolates of MRSA in UK hospitals were designated as epidemic strains and were designated EMRSA-1 through to
EMRSA-14 (Kerr et al, 1990). These strains persisted in hospitals until they were displaced by two newer epidemic strains, EMRSA-15 and EMRSA-16, which emerged almost simultaneously in the early nineties.

EMRSA-15 emerged in the Midlands and South East England in 1991 (Richardson & Reith, 1993). The strain then spread to most regions of the UK and has been detected in other countries, including Australia, New Zealand, Germany, Spain and Singapore (Hsu et al, 2005; Pearman et al, 2001; Perez-Roth et al, 2004; Smith & Cook, 2005; Witte et al, 2001). EMRSA-15 strains are resistant to ciprofloxacin and variably resistant to erythromycin.

EMRSA-16 was first detected in a hospital in Northamptonshire in 1991 and rapidly spread within the hospital to other wards as well as to other local hospitals (Cox et al, 1995). The strain was highly virulent and resistant to erythromycin and ciprofloxacin; some isolates were also resistant to trimethoprim and gentamicin. EMRSA-16 spread to other hospitals within the UK (CDR, 1993; Murchan et al, 2004) and has also become widespread in other countries. In the US, EMRSA-15 has not been identified but EMRSA-16 is the second most common hospital-associated strain (McDougal et al, 2003). In addition, EMRSA-16 isolates from Spain and Norway have been reported (Perez-Roth et al, 2003; Tveten et al, 2003). EMRSA-15 and EMRSA-16 strains have been very successful epidemic clones; they accounted for 95.6% of all bacteraemia MRSA isolates from UK hospitals participating in the Antibiotic Resistance Monitoring and Reference Laboratory survey conducted between 1998 and 2000 (Johnson et al, 2001). Recently, the genome of EMRSA-16 was sequenced (Holden et al, 2004). It was highly conserved in comparison to the genomes of the hospital strains N315 and Mu50. However, some 6% of the EMRSA-16 genome contained additional genetic elements not previously described. Interestingly, these elements carried neither virulence nor resistance genes, but accessory genes encoding for proteins with metabolic functions. It is possible that these proteins ensure the survival of EMRSA-16 in the hospital setting, and may be partly responsible for its success.

More recently, another epidemic strain, EMRSA-17, has emerged in the South of England (Aucken et al, 2002). EMRSA-17 isolates from the affected hospitals were
multi-drug resistant; the strains were resistant to ciprofloxacin, fusidic acid, rifampicin, aminoglycosides and tetracycline and variably resistant to erythromycin and mupirocin.

MLST and mec gene analysis of over 300 MRSA clones collected between 1961 and 1999 identified EMRSA-15 clones that contained type IV SCCmec; EMRSA-16 contained type II and EMRSA-17 carried type I SCCmec (Enright et al, 2002). As discussed earlier, Robinson and Enright (2003) predicted that more MRSA clones with type IV SCCmec, rather than other SCCmec types, will become widespread, which is so far consistent with the prevalence of EMRSA-15 type IV SCCmec in hospitals (Johnson et al, 2001). In addition to the hospital EMRSA-15 strain, most CA-MRSA strains carry type IV SCCmec (Daum et al, 2002; Ma et al, 2002).

1.1.6. CA-MRSA

CA-MRSA has emerged recently and is affecting healthy individuals with no apparent risk of developing MRSA infection (Herold et al, 1998).

The genome of the CA-MRSA strain MW2 has been sequenced and its genome compared to the hospital strains N315 and Mu50 (Baba et al, 2002). There were 18 unique virulence genes specific to MW2. One of these encodes the Panton-Valentine leukocidin, an important virulence factor that is associated with skin and soft tissue infections and severe necrotising pneumonia (Gillet et al, 2002). The MW2 strain carried the smaller type IV SCCmec, which does not encode other resistance genes, and is smaller in size than the other types of SCCmec (Baba et al, 2002). Therefore, the strain was susceptible to a wider range of antibiotics than typical hospital strains. These findings establish that there are significant genetic differences between community-acquired and hospital-acquired MRSA strains.

Worryingly, there has been an increase in the spread of CA-MRSA; not only are these strains found in the community highly virulent, but they appear to have adapted to the hospital setting (Carleton et al, 2004). A study of 490 MRSA strains collected from San Francisco hospitals between 1996-2002 were characterised and it was established that there was a gradual replacement of the hospital type II SCCmec by the
community-associated type IV SCCmec (Carleton et al., 2004). The authors concluded that a CA-MRSA strain that most likely originated in the community was introduced into the hospital by a patient and spread within the hospital; therefore, a CA-MRSA strain was capable of relocating from the community to the hospital setting. If CA-MRSA and hospital strains circulate in the same environment, a multi-drug resistant (MDR) CA-MRSA strain, which is difficult to treat but spreads with ease in the community, could be selected. Although CA-MRSA strains are less resistant than their hospital counterparts, they are still collecting resistance genes and MDR CA-MRSA isolates resistant to clindamycin, erythromycin, tetracycline, trimethoprim-sulfamethoxazole, ofloxacin and gentamicin have been reported (Lu et al., 2005). The emergence of MDR CA-MRSA poses a great threat to communities and must be closely monitored.

1.1.7. MDR MRSA

In addition to methicillin and penicillin, MRSA is resistant to other β-lactams as well as to non β-lactam antibiotics (Fig. 1.2) (Pfaller et al., 1998).

Fluoroquinolones are effective agents against MRSA but reduced susceptibility to ciprofloxacin emerged in MRSA strains soon after the antibiotic became available (Ena et al., 1993). Fluoroquinolones target two enzymes that are essential for bacterial DNA replication: DNA gyrase, which introduces negative superhelical twists in the DNA, and topoisomerase IV, which is involved in separating the replicated DNA molecules (Blondeau, 2004). In S. aureus, resistance to fluoroquinolones is mediated by mutations in the genes encoding subunit GyrA of DNA gyrase and GrlA subunit of topoisomerase IV, leading to a reduced affinity of the antibiotic for its targets (Ferrero et al., 1995; Yamagishi et al., 1996). In addition, a mutation in the chromosomally located norA gene, resulting in overproduction of an active efflux pump, confers resistance to some hydrophilic fluoroquinolones such as norfloxacin (Noguchi et al., 2004). Several studies have reported fluoroquinolone susceptibility among MRSA isolates to be as low as 10%, while 90% of MSSA were found to be susceptible (Fluit et al., 2000; Fluit et al., 2001b). This difference is indicative of the close relationship between methicillin resistance and resistance to other antibiotics such as fluoroquinolones, macrolides and aminoglycosides (Fluit et al., 2000).
Aminoglycosides bind to the 30S subunit of the ribosome and inhibit protein synthesis. Resistance to those agents in \textit{S. aureus} is a result of modification of the antibiotics by aminoglycoside-modifying enzymes such as nucleotidyltransferases, acetylation transferases and phosphotransferases thus preventing the antibiotic’s binding to the ribosome (Azucena & Mobashery, 2001). One study showed that resistance to aminoglycosides in MRSA was much higher than resistance to MSSA, as 65-95\% of MRSA isolates were resistant to different aminoglycosides; only 4-6\% of the MSSA isolates were resistant (Fluit \textit{et al}, 2000; Schmitz \textit{et al}, 1999a).

Resistance to the macrolides, which inhibit protein synthesis by binding to the 50S subunit of the ribosome, was also higher in MRSA than MSSA isolates; in one study 94\% of MRSA isolates were resistant to erythromycin, while only 20\% of MSSA isolates were resistant (Schmitz \textit{et al}, 1999b).

A study of over 3 000 \textit{S. aureus} strains isolated from hospitals established that more than 80\% of MRSA were MDR; MDR was defined by resistance to at least five different antibiotics (Fluit \textit{et al}, 2001b). In comparison, only 2\% of MSSA strains from the same study were MDR. Similar observations have been reported by others (Bell & Turnidge, 2002; Fluit \textit{et al}, 2001a).

![Fig. 1.2. Use of key antibiotics and the development of resistance by \textit{S. aureus}.](image)

Vancomycin remains an effective antibiotic against MDR MRSA and is currently one of the main drugs used in the treatment of MRSA infections. However, MRSA isolates with reduced susceptibility and high-level resistance to vancomycin have emerged.
1.1.8. Vancomycin and vancomycin resistant MRSA isolates

Vancomycin, a glycopeptide antibiotic, inhibits bacterial growth by interfering with peptidoglycan assembly and cell wall synthesis (Fig. 1.1). In contrast to penicillin, vancomycin does not bind to the PBPs but recognises the peptidoglycan precursor at the terminal D-Ala-D-Ala sequence, as well as the same terminal sequence of the growing peptidoglycan chain. It therefore inhibits peptidoglycan polymerisation and disrupts the mechanical stability of the cell wall (Nieto & Perkins, 1971; Perkins & Nieto, 1974).

It was not until 1997 (Fig. 1.2) that the first cases of MRSA strains with reduced susceptibility to vancomycin were reported; these Japanese isolates were known as hetero-resistant Mu3 (Hiramatsu et al, 1997a) and Mu50 (Hiramatsu, 1998; Hiramatsu et al, 1997b). Hiramatsu et al (1997a) defined the Mu3 strain as hetero-resistant, as a subpopulation of the cells were intermediately resistant to vancomycin even though the minimum inhibitory concentration (MIC) for the strain was within the sensitive range (<8 mg/l). According to the Clinical Laboratory Standards Institute (CLSI, previously known as the National Committee for Clinical Laboratory Standards) guidelines these strains are defined as vancomycin-intermediate *S. aureus* (VISA) and show MIC values between 8-16 mg/l; according to the British Society for Antimicrobial Chemotherapy, MIC values of 8 mg/l define these strain as vancomycin-resistant *S. aureus* (VRSA) (Tenover et al, 2001).

Since the initial report, a number of MRSA strains with a reduced susceptibility to vancomycin have been reported from a number of countries (Oliveira et al, 2001; Ploy et al, 1998; Song et al, 2004; Trakulsomboon et al, 2001).

Kuroda and colleagues (2001) have sequenced the Mu50 strain and the hospital MRSA strain N315, and identified key genes involved in pathogenicity and antibiotic resistance. They reported 96% homology of the two sequences. The major differences between the strains seemed to be due to transposable elements, including bacteriophage genes that have become incorporated into the genome. Mu50 contained an extra bacteriophage and genomic island and several additional insertion sequences. Apart from these differences, the sequences were highly conserved. It appears that
many of the acquired genes have come from other bacterial species, confirming that \textit{S. aureus} can readily obtain genetic elements from distantly related organisms.

The mechanism of resistance in VISA strains has been linked to cell wall thickness, reduced peptidoglycan cross-linking and an increased synthesis of peptidoglycan murein monomers (Cui \textit{et al}, 2000; Cui \textit{et al}, 2003; Hanaki \textit{et al}, 1998b; Hanaki \textit{et al}, 1998a). A thickened wall necessitates that vancomycin must pass through several peptidoglycan sheets in order to reach its murein monomer target at the cellular membrane. An increase in the amount of unlinked peptidoglycan sheets not only retards the passage of vancomycin molecules but also leads to a higher number of false targets as binding sites for vancomycin. As a result, a higher concentration of vancomycin is necessary to achieve a successful therapeutic outcome.

1.1.8.1. VRSA isolates with a high level of vancomycin resistance

Noble \textit{et al} (1992) demonstrated transfer of the \textit{vanA} gene, which confers vancomycin resistance in \textit{Enterococcus faecalis}, to \textit{S. aureus in vitro}, and emphasised the potential threat of the emergence of vancomycin resistance in MRSA in the clinical setting. It was therefore surprising that the isolates with reduced vancomycin susceptibility did not contain this gene, and that the mechanism of resistance was independent of the \textit{van} genes. However, the predicted mechanism of resistance did emerge, when the first clinical isolate of a VRSA containing the \textit{van} gene was reported in Michigan in 2002 with an MIC of 1024 mg/l (Chang \textit{et al}, 2003; MMWR, 2002a). The patient had received multiple courses of antibiotics to treat various conditions such as a chronic foot ulcer, haemodialysis graft infection and MRSA bacteraemia. The patient had received a total of six and a half weeks of vancomycin therapy over a period of six months. A concomitant \textit{E. faecalis} infection was identified and upon comparison of the \textit{vanA} gene in the VRSA and \textit{E. faecalis} isolates the sequences were identical. Close contacts of the patient were investigated and no VRSA isolates were found. These observations raise the strong possibility that horizontal gene transfer between the two strains had occurred under clinical conditions (Weigel \textit{et al}, 2003). Only a couple of months later, another VRSA strain containing the \textit{vanA} gene was isolated from a patient in Pennsylvania (MMWR, 2002b). The MIC for this strain was much lower (16-64 mg/l) than the Michigan strain and, interestingly, there was no
concomitant infection of *Enterococcus* spp., although the *vanA* sequence was similar to a known *E. faecalis* strain (Tenover et al, 2004). A separate, epidemiologically unrelated VRSA isolate was reported in New York two years later, also containing the *vanA* gene and with a vancomycin MIC of >256 mg/l (MMWR, 2004).

Studies were conducted to establish the mechanism of resistance in the new VRSA strains and it was determined that *mecA* and *vanA* genes induce a different set of enzymes and actually have antagonistic effects (Severin et al, 2004a). It was also shown that deletion of the *mecA* gene did not affect vancomycin resistance, indicating that *mecA* is not involved in vancomycin resistance, an observation that is further supported by isolation of a MSSA strain with vancomycin resistance (Bobin-Dubreux et al, 2001). In addition, Severin and colleagues (2004b) have shown that the presence of *vanA* resulted in a modified peptidoglycan layer, with an increase in tetrapeptides and a decrease in pentapeptides, as well as a reduction of the pentaglycine branches. It was later established that PBP2a cannot use the modified substrates, so peptidoglycan synthesis in the *vanA* containing VRSA strain is undertaken by PBP2 (Severin et al, 2004b).

Fortunately, cases with high level vancomycin resistance are not common and control measures have been in force to prevent such occurrences such as pre-operative antibiotic prophylaxis, controlled antibiotic use and close monitoring and isolation of patients infected with MRSA (MMWR, 1997; Tenover et al, 2001). The likely transfer of the *vanA* gene from enterococci to *S. aureus* highlights the importance of VRE in the clinical setting.

### 1.2. *Enterococcus* spp. and evolution of resistance

#### 1.2.1. *Enterococcus* spp.

Enterococci colonise the gastrointestinal tract and are carried by healthy individuals; they may cause serious infections in immunocompromised individuals. There are several members in the *Enterococcus* genus but only two are commonly associated with hospital infections: *E. faecalis*, which accounts for the majority of infections, and
E. faecium (Gordon et al, 1992). The prime loci of infection are the urinary tract, surgical wounds, blood and abdominal sites. In addition, endocarditis is a serious infection resulting as a consequence of VRE bacteraemia. The organisms can also survive in the environment and can be transmitted via the hands of health workers, although how much these factors contribute to infection rates is not clear (Bonten et al, 1996).

It has been difficult to establish exactly how much VRE contributes to mortality rates, as there are usually other concomitant infections present in the affected patients. In many cases, severe underlying diseases in patients are likely to be the sole cause of death, with no link to VRE (Pfaller et al, 1998).

1.2.2. Resistance to β-lactams

Resistance to β-lactam antibiotics by a plasmid-mediated transferable β-lactamase has been reported, although β-lactamase producing enterococci are only infrequently isolated (Murray & Mederski-Samaroj, 1983; Rice & Murray, 1995). The main mechanism of resistance in enterococci is mediated by a constitutively expressed low-affinity PBP, PBP5 (Fontana et al, 1983). The role of PBP5 was shown by: firstly, the direct proportional relationship between the amount of PBP5 and the level of resistance to β-lactams (Fontana et al, 1983), and secondly, by mutants with non-functional PBP5 displaying high susceptibility to β-lactams (Fontana et al, 1985). Fontana and colleagues have shown that PBP5 takes over the functions of the remaining PBPs once these have been saturated with β-lactams (Canepari et al, 1986; Fontana et al, 1983).

1.2.3. Resistance to aminoglycosides

Enterococci are intrinsically resistant to aminoglycosides as a result of the low permeability of these agents into the target bacterial cell. This type of resistance is of a low or moderate level (MIC < 500 mg/l) and can be overcome by a synergistic combination of an aminoglycoside and a penicillin, which enhances cellular uptake of the aminoglycoside (Moellering, Jr. et al, 1971). In addition, enterococci also exhibit
high-level acquired resistance (MIC > 2,000 mg/l), which is either ribosomal or due to the production of an aminoglycoside inactivating enzyme (Mederski-Samoraj & Murray, 1983). In this case, high-level acquired resistance cannot be overcome by the synergistic properties of penicillin. Although resistance to gentamicin and streptomycin are mediated by different enzymes and no single enzyme can inactivate all aminoglycosides, many strains have accumulated multiple genes and are therefore resistant to all known aminoglycosides (DeLisle & Perl, 2003).

1.2.4. Resistance to vancomycin

Vancomycin became the drug of choice for the treatment of enterococcal infections after resistance to β-lactam and aminoglycosides developed and before vancomycin resistance was first reported in 1986 (Uttley et al, 1988; Uttley et al, 1989). Vancomycin was first introduced in the US in 1958 but its use increased in the seventies with the emergence of MRSA. There was a rapid increase in the frequency of VRE isolates in the US and in 1999 approximately 25% of isolated enterococci were vancomycin resistant (NNIS, 2000). Fortunately, in Europe the incidences of hospital VRE cases were significantly less than those in the United States and the strains isolated were not resistant to as many antibiotics.

There are six known phenotypes of vancomycin-resistance, described as VanA, VanB, VanC, VanD, VanE and VanG. Enterococci have developed two pathways with similar mechanisms of resistance by modifying the pentapeptide termini, which are the target for vancomycin. One group of enterococci, which carries the genes vanA, vanB and vanD, confer high-level resistance to vancomycin by modifying the D-Ala-D-Ala terminus to D-Ala-D-Lac. Enterococci with vanC, vanE and vanG genes confer low-level resistance to vancomycin and the D-Ala-D-Ala terminus is replaced by D-Ala-D-Ser.

1.2.4.1. VanA, B and D

The best-characterised glycopeptide resistance mechanism is that for VanA. The predominant VanA phenotype, VRE, has a high-level resistance not only to vancomycin but also to the glycopeptide teicoplanin. The vanA gene cluster is located
on the transposable genetic element Tn1546, which was originally discovered on a plasmid in *E. faecium* (Leclercq *et al.*, 1988). In addition to the transposition genes *orf1* and *orf2*, Tn1546 carries the regulatory genes *vanR* and *vanS*, the genes necessary for vancomycin resistance (*vanA, vanH, vanX*) and the genes *vanY* and *vanZ* that are not essential for resistance but contribute to the levels of resistance to vancomycin and teicoplanin respectively (Arthur *et al.*, 1993). The *vanA* gene encodes a ligase, which has altered specificity and catalyses the formation of D-Ala-D-Lac termini (Bugg *et al.*, 1991). D-Lac is not normally produced in enterococci and is not taken up by the bacteria. It is synthesised from pyruvate, which is catalysed by the *vanH* encoded dehydrogenase (Arthur *et al.*, 1991). VanX is a D,D-dipeptidase, which is responsible for reducing the number of D-Ala-D-Ala substrates produced by the host ligase (Reynolds *et al.*, 1994a). This leads to an increase in the number of D-Ala-D-Lac pentapeptides incorporated in the cell wall.

The D,D-carboxypeptidase encoded by *vanY* cleaves the terminal D-Ala of any pentapeptides that may have been synthesised, again further reducing any potential vancomycin targets (Arthur *et al.*, 1994). VanY is not essential for resistance, as VanX seems to efficiently cleave D-Ala-D-Ala before it is incorporated into a pentapeptide, although it can contribute to levels of resistance (Arthur *et al.*, 1994). These genes are up-regulated in the presence of vancomycin by the two-component system mediated by VanR and VanS (Arthur *et al.*, 1992). VanS is a putative membrane sensor that detects the presence of glycopeptides or some early damage to the peptidoglycan layer caused by the agents, although the exact mechanism is not clear. Phosphorylated VanR has a high affinity for the *vanHAX* promoter, so it appears that the active form of VanR is the phosphorylated form (Wright *et al.*, 1993). Arthur *et al.* (1997) have shown that in the absence of both VanR and VanS there is no activation of the *vanHAX* promoter, while in the absence of VanS there was full activation. This suggests that VanR is negatively controlled by VanS, most likely by dephosphorylation, and activates VanR by phosphorylation.

The VanB cluster is similar to the one described for VanA and is encoded in mobile genetic elements within the chromosome (Quintiliani & Courvalin, 1994), although they have also been described as residing on plasmids in clinical isolates of enterococci (Woodford *et al.*, 1995). VanB type also results in the synthesis of the D-
Ala-D-Lac precursor, although VanB phenotype VRE remain susceptible to teicoplanin (Evers & Courvalin, 1996). VanA and VanB are the main clinical phenotypes responsible for high-level resistance in hospitals, with the majority of the isolates being of the VanA type (Clark et al, 1993).

VanD type enterococci gene operons are organised in a similar fashion to VanA and VanB; however, VanD genes are always chromosomally located and are not transferable (Depardieu et al, 2003). Unlike the inducible VanA and VanB phenotypes resistance, VanD is characterised by a constitutive resistance of moderate level to vancomycin and low level resistance to teicoplanin (Perichon et al, 1997). In enterococci containing the VanD type, the production of D-Ala-D-Lac is constitutive regardless of the presence or absence of glycopeptides. Different strains of enterococci were found to have either a mutation in the \( vanR_D \) gene or an insertion/deletion in \( vanS_D \), which disrupts the normal activity of the two-component regulatory elements and results in a permanently active form of VanR\( _D \) (Depardieu et al, 2004). These structural changes account for the constitutive resistance of the VanD phenotype to vancomycin, although it is still not clear why resistance to teicoplanin is low, considering the constitutive expression of the D-Ala-D-Lac peptides.

1.2.4.2. VanC, E and G

This phenotype has been characterised as constitutive or inducible low-level resistance to vancomycin and susceptibility to teicoplanin in \( E.\ gallinarum \) (VanC-1), \( E.\ casseliflavus \) (VanC-2) and \( E.\ flavescens \) (VanC-3) (Leclercq et al, 1992a; Navarro & Courvalin, 1994). The VanC operon is chromosomally located. \( VanC \) encodes a ligase with some sequence similarity to the VanA ligase; however, VanC catalyses the synthesis of D-Ala-D-Ser peptides rather than D-Ala-D-Lac (Reynolds et al, 1994b). Inactivation of VanC leads to loss of vancomycin resistance and to the production of D-Ala-D-Ala precursors, suggesting that the bacteria have the machinery to produce D-Ala-D-Ala precursors in addition to D-Ala-D-Ser and that VanC is essential for resistance (Dutka-Malen et al, 1992; Reynolds et al, 1994b).
A similar mechanism of resistance to the VanC type was described in *E. faecalis*. The VanE type confers inducible low-level vancomycin resistance but the strains remain susceptible to teicoplanin. The VanE operon is located chromosomally and the functions of the encoded protein are similar to VanC type (Fines *et al*, 1999).

VanG is similar to VanC and VanE in that it also results in the synthesis of D-Ala-D-Ser precursors, but there are distinct differences between VanG and the other types (McKessar *et al*, 2000). VanG confers inducible moderate level resistance to vancomycin in *E. faecalis* but susceptibility to teicoplanin is maintained. The VanG phenotype is unusual, as the strains are vancomycin resistant even in the presence of D-Ala-D-Ala vancomycin targets.

1.2.4.3. Origin of the van genes

The complexity of the resistance mechanisms to vancomycin observed in VRE raises questions as to the origin of the genes. The different mechanisms of resistance employed by intrinsically resistant enterococci, as described above, indicate that the vanA/B genes have been acquired from other sources. Other species that are intrinsically resistant to vancomycin have been identified; these include *Leuconostoc* spp., *Lactobacillus* spp. and *Pediococcus* spp. (Billot-Klein *et al*, 1994). It is unlikely that the high-level resistance phenotypes VanA and VanB emerged from these species, as the homology of the sequenced ligases between *Leuconostoc* spp., *Lactobacillus* spp. and VanA/B ligases was only 26-35% (Elisha & Courvalin, 1995).

There are several bacterial species that are potential sources of the van genes; these are *Paenibacillus popilliae*, *Amycolatopsis orientalis* and *Streptomyces toyocaensis* (Marshall *et al*, 1997; Marshall & Wright, 1998; Patel *et al*, 2000). The latter two organisms are glycopeptide producers and it is feasible that these species carry glycopeptide resistance genes to protect against the antimicrobial actions of the agents. Marshall *et al* (1998) proposed that the van genes originated in these species; they describe an identity of 54-64% in the amino acid sequence of the VanH, VanX and VanA proteins and their relative homologues. Patel and colleagues have reported even higher identities of 74-79% of the amino acid sequences in *Paenibacillus popilliae* Van homologues to VanH, VanX and VanA (Patel, 2000; Rippere *et al*, 1998). It is possible that other organisms such as *Paenibacillus popilliae* initially
acquired the \textit{van} genes from glycopeptide-producing bacteria, before the genes were taken up by enterococci, although the exact route of \textit{van} genes into enterococci remains to be elucidated (Patel, 2000).

It is clear that vancomycin resistance in enterococci is highly complex and continues to evolve. The most worrying aspect of this scenario is that enterococci have become a reservoir for a variety of resistance genes that have the potential to be transferred to other pathogens, such as \textit{S. aureus}. To date, there have been no reports of transfer of the \textit{van} genes to other species, with the exception of \textit{S. aureus}.

\textbf{1.3. Antibiotic resistance of \textit{Streptococcus} spp.}

\textit{S. pneumoniae} is the leading pathogen causing community acquired pneumonia (Hoban \textit{et al}, 2001). The bacteria colonise the nasopharynx in a small proportion of adults and a large proportion of children. Invasive infections are most common in children under the age of two and in the elderly (Burman \textit{et al}, 1985).

The first cases of resistance to penicillin in \textit{S. pneumoniae} were reported more than twenty years after the antibiotic was introduced. However, resistance has since been increasing at an alarming rate and an average of 30-40\% of recently reported isolates are now penicillin resistant (Hansman \textit{et al}, 1971; Hoban \textit{et al}, 2005; Hoban \textit{et al}, 2003). Similarly to \textit{S. aureus} and enterococci, resistance to penicillin and other \(\beta\)-lactams by \textit{S. pneumoniae} is mediated by low-affinity PBPs. In addition, resistance to macrolides, tetracyclines, chloramphenicol, trimethoprim/sulfamethoxazole and, more recently, fluoroquinolones has been described in \textit{S. pneumoniae} (Doern \textit{et al}, 2001; Jones \textit{et al}, 1998b). Resistance to macrolides is widespread and in some parts of the world it is more prevalent than penicillin resistance (Felmingham \textit{et al}, 2002). The most common mechanisms of resistance are due to \textit{mef}-encoded drug efflux pumps and the methylation of the target ribosomal site by an \textit{ermB} encoded enzyme (Farrell \textit{et al}, 2005).

Resistance to other antibiotics is more common in penicillin-resistant \textit{S. pneumoniae} than in penicillin-sensitive \textit{S. pneumoniae} and several MDR clonal strains have been
identified worldwide (McGee et al, 2001). Several studies have found that 15-20% of the isolates are MDR, although linezolid and vancomycin remain effective (Doern et al, 1999; Doern et al, 2001). Fluoroquinolones are generally effective against *S. pneumoniae*, with a small proportion of strains identified as highly resistant (Odland et al, 1999).

Another member of the species, *S. pyogenes*, is also a common respiratory pathogen, especially in children, and although it remains susceptible to penicillin, resistance to macrolides and reduced susceptibility to fluoroquinolones have been reported (Felmingham et al, 2004; Malhotra-Kumar et al, 2005a; Malhotra-Kumar et al, 2005b). *S. agalactiae* is a common cause of infections during pregnancy and in infants; in a similar fashion to *S. pyogenes*, the organism is susceptible to penicillin with reports of macrolide resistance and more recently high-level fluoroquinolone resistance (Marimon et al, 2005; Wehbeh et al, 2005).

### 1.4. MDR *Mycobacterium tuberculosis*

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is the world’s second leading infectious disease after HIV/AIDS, and according to the WHO, approximately one third of the population is infected (Dye et al, 1999). The great majority of TB infections occur in areas of the developing world, such as sub-Saharan Africa, where co-infections with HIV are common, and in Asia: India, China, Bangladesh and Pakistan (Frieden et al, 2003). TB infections have increased following the HIV/AIDS pandemic and as many as 13 million people may be infected with both pathogens (Nunn et al, 2005). Mycobacteria are intrinsically resistant to many broad-spectrum antibiotics, due to their waxy impermeable cell wall and the presence of antibiotic efflux pumps (Morris et al, 2005). Nevertheless, TB infections have been treated and controlled with the antibiotics isoniazid, rifampicin, streptomycin, and ethambutol. Treatment schedules are long-term and involve a combination of drugs (Frieden et al, 2003). Treatment is usually carried out under professional supervision to ensure completion of the medication course. However, MDR-TB strains have emerged that are resistant to isoniazid and rifampicin, the two most effective antibiotics against the disease. In 2000, it was estimated that 3.2% of all new TB cases were MDR (Dye et
Although this is a relatively small proportion of the total number of infections due to *M. tuberculosis*, infections due to MDR *M. tuberculosis* are extremely difficult to treat. Resistance to an antibiotic is mediated by the selection of resistant mutants of *M. tuberculosis* and this is much more likely to occur when a single drug is used, therefore, multi-drug therapy is recommended. Resistant mutants are likely to emerge in patients that have not received complete treatment due to non-compliance or have been treated with a single drug (Sharma & Mohan, 2004). MDR *M. tuberculosis* strains pose a serious threat to health professionals and the community.

1.5. Antibiotic resistant Gram-negative pathogens of clinical importance

1.5.1. *Klebsiella pneumoniae*

*Klebsiella* spp. are opportunistic pathogens associated with nosocomial infections in immunocompromised patients and frequently cause pneumonia and urinary tract infections. They colonise the nasopharynx and the intestinal tract of healthy individuals, and colonisation rates increase with the length of stay in hospital. There appears to be a correlation between colonisation and infection (Selden *et al.*, 1971). *Klebsiella* spp. are problematic because of the multiple resistance observed in some strains, usually mediated by extended spectrum β-lactamases (ESBLs) (Rupp & Fey, 2003).

The first ESBL enzyme was discovered in *K. pneumoniae* in 1983, which was also the first reported case of resistance to third generation cephalosporins (Knothe *et al.*, 1983). The ESBL enzyme is a mutated derivative of other β-lactamases and hydrolyses third and fourth generation cephalosporins, in addition to monobactam. A large number of ESBLs have been described and others are being identified. ESBLs can be transferred to previously susceptible strains by plasmids and can pass between members of the *Enterobacteriaceae* family (Shah *et al.*, 2004). Furthermore, resistance to aminoglycosides and trimethoprim-sulfamethoxazole can be co-transferred on the same plasmid. ESBLs are commonly found in *E. coli, P. mirabilis*...
and *P. aeruginosa*, in addition to *K. pneumoniae*, although *K. pneumoniae* strains more often express ESBL (Hirakata *et al.*, 2005). In some parts of the world, ESBL-producing *K. pneumoniae* isolates can be as high as 60% (Edelstein *et al.*, 2003; Rupp & Fey, 2003). Although carbapenem is the antibiotic of choice for ESBL-producing strains, resistant isolates to carbapenems have been reported, leaving very few therapeutic options against this pathogen (Bradford *et al.*, 1997; Woodford *et al.*, 2004).

### 1.5.2. *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is an opportunistic pathogen; it frequently causes infections in immunocompromised patients, in particular those with cystic fibrosis, and is infrequently found as a component of the commensal flora of healthy individuals. *P. aeruginosa* is commonly isolated from infected skin wounds and burns and also causes meningitis, endocarditis and respiratory infections (Cross, 1985). It is inherently resistant to a number of antibiotics, due to membrane impermeability and to multi-drug efflux pump systems, as well as to a chromosomally-encoded inducible AmpC β-lactamase (Juan *et al.*, 2005; Li *et al.*, 2000).

There are several constitutively expressed efflux pumps responsible for inducing resistance to β-lactams, fluoroquinolones, tetracyclines and chloramphenicol (Li *et al.*, 1994b; Li *et al.*, 1994a; Li *et al.*, 1995; Oh *et al.*, 2003). Although some strains of *P. aeruginosa* are susceptible to fluoroquinolones, aminoglycosides and some β-lactams, resistance to those antibiotics by a number of genetic mutations is common. Mutations in the topoisomerase II and IV genes confer resistance to fluoroquinolones; those resulting in up-regulation of the efflux pumps lead to increased resistance to penicillins, cephalosporins, fluoroquinolones, aminoglycosides and some β-lactams (Oh *et al.*, 2003; Poole, 2001). Mutants with an up-regulated AmpC β-lactamase possess enhanced resistance to aminopenicillins and cephalosporins (Juan *et al.*, 2005). *P. aeruginosa* can also acquire ESBL, facilitating resistance to third and fourth generation cephalosporins, and clinical isolates with resistance to carbapenems have also emerged, making this pathogen multi-drug resistant (Poirel *et al.*, 2000; Poirel *et al.*, 2001; Senda *et al.*, 1996).
1.5.3. *Proteus* spp.

*P. mirabilis* is an opportunistic pathogen usually associated with urinary tract infections, although it has also been isolated from burns and wound infections (Rozalski et al, 1997). The organism colonises the gastrointestinal tract and contributes to the development of infection in patients with underlying disease (Chow et al, 1979). *P. mirabilis* is not highly virulent, although infections can be difficult to treat, especially as many clinical isolates have developed resistance to aminoglycosides, β-lactams and quinolones (De Champs et al, 2000; Gordon & Jones, 2003). Resistance to β-lactams and aminoglycosides is associated with acquisition of a plasmid and the percentage of strains carrying ESBL varies between geographical regions (Bell et al, 2002; Hirakata et al, 2005).

1.5.4. *Citrobacter* spp.

*Citrobacter* spp. are opportunistic pathogens, frequently carried in the intestinal tract, and cause infections in immunocompromised patients and in individuals with underlying diseases (Drelichman & Band, 1985). Although infrequently isolated in hospitals, infections caused by *Citrobacter* spp. may be fatal as a result of multiple resistance (Shih et al, 1996). Resistance to third-generation cephalosporins has been described in a significant number of isolates (Jones et al, 2000). Resistance to third-generation cephalosporins is often mediated by the chromosomally encoded Amp C β-lactamase in *Citrobacter freundii* (Lindberg et al, 1985). Many of the isolated strains were resistant not only to β-lactams but also to trimethoprim-sulfamethoxazole, aminoglycosides and fluoroquinolones; this pattern of resistance is typical of ESBL pathogens (Pepperell et al, 2002).

1.6. Antibiotic development

1.6.1. The industrial development of antibiotics

The complexity of resistance and the rapid emergence of a variety of MDR pathogens emphasise that antibiotics with new mechanisms of action are urgently needed.
Trimethoprim was introduced in the late sixties and was the last discovered antibiotic with a new mechanism of action before the introduction of the oxazolidinones in 2000 (Fig. 1.3).

**Fig. 1.3.** Key time points in the development of antibiotics.

Although there were no discoveries of new generations of antibiotics during these three decades, many agents from existing classes were successfully modified in order to enhance their activity against resistant strains. The cephalosporins are a perfect example of such drugs. Third generation cephalosporins are effective against bacteria that are resistant to the early cephalosporins (Thornsberry, 1985). Also, newer generation quinolones have been developed with greater potency against resistant pathogens (Martin et al, 1998). However, cross-resistance to newer generation antibiotics is common.

Discovering new classes of antibiotics involves the identification of unique bacterial targets. Research and development of new antibiotics is a lengthy process that requires a significant financial input by the pharmaceutical industry. While resistance has been increasing, antibacterial research by pharmaceutical companies has been decreasing for several reasons. Although there is a profitable market for antibacterial agents, the two leading profitable fields are currently cardiovascular and central nervous system disorders (Fox, 2003). Recently, a number of pharmaceutical companies have merged to form large corporations such as Pfizer, GalloSmithKline and Novartis. As a result, many of the smaller companies terminated any ongoing
antibacterial research programmes (Projan & Shlaes, 2004). Finally, regulatory requirements have been tightened over the years and a higher cost is involved in taking a drug through completion of clinical trials. The cost to take a drug to the market may be so high (close to $1 billion) and the time taken for a drug to be approved so long (10-15 years on average), that pharmaceutical companies are having to be very selective in their choices of candidate drugs (DiMasi, 2001).

Despite all those hurdles, there have been several antibiotics with a novel mechanism of action, which have been recently licensed in Europe and the US. Unfortunately, for some, resistance has already been reported, emphasising the urgent need for more research in the field.

1.6.2. Recently introduced antibacterial agents

1.6.2.1. Oxazolidinones

Linezolid is the first member of this new class of antibiotics to be licensed in the UK since 2001. It inhibits protein synthesis in bacteria by binding to the 50S ribosomal subunit and blocks the formation of tRNA^{Met}/mRNA/ribosome protein initiation complexes (Champney & Miller, 2002). There is no cross-resistance between linezolid and other protein synthesis inhibitors such as chloramphenicol, tetracycline, aminoglycosides, macrolides, lincosamides and streptogramins (Fines & Leclercq, 2000). Due to its novel mechanism of action, it is an attractive candidate for the treatment of antibiotic-resistant pathogens. MIC determinations have shown that the antibiotic is equally effective against both resistant and susceptible strains of \textit{S. aureus} and VRE (Gemmell, 2001). \textit{In vitro}, linezolid is bacteriostatic against enterococci and \textit{S. aureus}, and bactericidal against streptococci (Dailey \textit{et al}, 2001; Zurenko \textit{et al}, 1996). Linezolid is licensed for the treatment of infections caused by MSSA and MRSA, vancomycin-resistant \textit{E. faecium} and streptococci; indications for linezolid are nosocomial and community acquired pneumonia and skin and soft tissue infections (Ament \textit{et al}, 2002; Clemett & Markham, 2000). However, there are already reports of some resistant isolates of enterococci and \textit{S. aureus} (Gonzales \textit{et al}, 2001; Pai \textit{et al}, 2002; Tsiodras \textit{et al}, 2001).
1.6.2.2. Streptogramins

Synercid was licensed in 1999 in the UK (Blondeau & Sanche, 2002). The streptogramins were discovered as early as the 1960s but were only used in animal feeds. As a result of increasing resistance by bacteria, they were developed as antibacterial agents in man. Synercid is made up of two components: type A (dalfopristin) and type B (quinupristin) streptogramins, that individually are bacteriostatic but combined in a ratio of 30:70, respectively, have a synergistic bactericidal action against most Gram-positive pathogens (Aumercier et al, 1992). The antibiotic acts by inhibiting protein synthesis through binding to the 50S subunit of the ribosome; dalfopristin inhibits peptidyl transferase and quinupristin inhibits peptide chain elongation (Leclercq et al, 1992b; Neu et al, 1992). Synercid is effective against both resistant and sensitive pathogens and can be used as a last choice drug against multi-resistant organisms. A study of over 28 000 clinical isolates tested against Synercid showed that the antibiotic was effective against the major Gram-positive pathogens, including vancomycin-resistant E. faecium, MSSA, MRSA and S. pneumoniae (Jones et al, 1998a). E. faecalis is intrinsically resistant to the antibiotic and therefore discrimination between E. faecalis and E. faecium is important before treating VRE infections. Indications for this antibiotic are nosocomial pneumonia and skin and soft tissue infections, caused by susceptible Gram-positive pathogens (Lamb et al, 1999). Although infrequent, cases of resistance to Synercid have been reported in E. faecium and S. aureus (Chow et al, 1997; Dowzicky et al, 2000; Johnson & Livermore, 1999; Malbruny et al, 2002).

1.6.2.3. Lipopeptides

Daptomycin is a cyclic lipopeptide antibiotic with an unique mechanism of action and was approved for clinical use in the US in 2003 (Steenbergen et al, 2005; Tally et al, 1999). The lipophilic tail of daptomycin intercalates into the bacterial membrane in a Ca$^{2+}$-dependent manner and causes membrane depolarisation by K$^+$ efflux (Silverman et al, 2003). The membrane depolarisation alone is probably not the cause of cell death but it is likely to affect cellular functions such as DNA and RNA synthesis (Canepari et al, 1990; Jung et al, 2004). An advantage of daptomycin over other antibiotics is that it is bactericidal against both exponential phase and stationary phase
cells (Lamp et al, 1992). Because of its novel mechanism of action, daptomycin is active against susceptible as well as multi-resistant Gram-positive pathogens, including MSSA, MRSA, VRSA, VRE and *Streptococcus* spp. (Critchley et al, 2003; Rybak et al, 2000; Sader et al, 2004; Streit et al, 2004b). The safety and efficacy of the antibiotic was determined in Phase III clinical trials of complicated skin and soft tissue infections (Arbeit et al, 2004). The indications for this drug currently include treatment of complicated skin and skin structure infections caused by MSSA, MRSA, vancomycin-susceptible *E. faecalis, S. pyogenes, S. agalactiae* and *S. dysgalactiae*. Cases of resistance are extremely rare; only two cases in more than 1 000 individuals were reported during clinical trials although recently there have been two reports of daptomycin resistance in patients with osteomyelitis caused by MRSA and a case of daptomycin resistance in a patient with MRSA bacteraemia (Hayden et al, 2005; Mangili et al, 2005). The development of resistance to this antibiotic is unwelcome, as early studies had predicted that resistance would be slow to develop, due to the novel mechanism of action and lack of cross-resistance to other agents (Silverman et al, 2001).

1.6.3. New derivatives of existing classes of antibiotics

1.6.3.1. Ketolides

The ketolides are semisynthetic derivatives of the protein inhibitors, the macrolides. Telithromycin was licensed in 2001 in Europe and in 2004 in the USA. Although the ketolides and the macrolides bind to the same target, the 50S ribosomal subunit, telithromycin has a higher affinity towards the ribosome than the macrolides and is therefore active against macrolide-resistant bacteria (Fogarty et al, 2003; van Rensburg et al, 2005). Telithromycin binds to the 23S rRNA of the 50S subunit and inhibits peptide formation as well as assembly of the 50S subunit (Champney & Pelt, 2002). The indications for telithromycin in Europe include the treatment of respiratory infections such as bronchitis and sinusitis, including those caused by erythromycin- and penicillin-resistant *S. pneumoniae* (Reinert, 2004). Cases of resistance to telithromycin have been rare, with only low-level resistance reported in 0.2% of almost 14 000 isolates monitored over a four-year period after the drug’s initial introduction (Douthwaite et al, 2005; Farrell & Felmingham, 2005). However,
recently there has been a report of a clinical isolate with high-level resistance (Faccone et al, 2005).

1.6.3.2. Glycyclines

Glycyclines are tetracycline derivatives and tigecycline is the first glycycline to be licensed in the US in 2005. This antibiotic seems to overlap or share the same binding sites on the 30S ribosome as the tetracyclines and has a similar mechanism of action (Bauer et al, 2004). Tigecycline evades efflux pumps that confer resistance to tetracyclines and is therefore effective against tetracycline-resistant pathogens (Fluit et al, 2005; Fritsche et al, 2005b). Tigecycline is a broad spectrum antibiotic with activity not only against Gram-positive pathogens, such as MRSA, VRE and penicillin-resistant S. pneumoniae, but also against some Gram-negative pathogens (Fritsche et al, 2004). The agent is less active against the problematic Gram-negative pathogens P. aeruginosa and P. mirabilis (Pankey, 2005; Sader et al, 2005). It is currently licensed for treatment of complicated skin and structure infections and complicated intra-abdominal infections (Fritsche et al, 2005a). Resistance to tigecycline due to a mutation in the efflux pump has been achieved in vitro, although it is anticipated that resistance will not develop easily in the clinic (Tuckman et al, 2000).

1.6.4. Investigational drugs

1.6.4.1. Glycopeptide derivatives

Oritavancin inhibits transglycosylation during peptidoglycan synthesis and is active against vancomycin-susceptible and vancomycin-resistant enterococci, as well as against MRSA (Allen & Nicas, 2003). This agent has completed a phase III clinical trial for treatment of complicated skin and skin structure infections (Shah, 2005). Dalbavancin is a semi-synthetic derivative of a teicoplanin analogue, and in similar fashion to the other glycopeptides inhibits cell wall cross-linking. Like oritavancin, it retains good activity against VRE and has greater potency against MRSA than vancomycin (Streit et al, 2004a). Dalbavancin has shown good efficacy in the treatment of catheter-related bloodstream infections (Raad et al, 2005).
lipoglycopeptide televancin appears to act by two mechanisms: it interferes with peptidoglycan synthesis and also dissipates the cellular membrane potential (Higgins et al, 2005). It is more potent than vancomycin against MRSA and is under clinical investigation for the treatment of complicated skin and skin structure infections (Stryjewski et al, 2005).

1.6.4.2. Lipoglycodepsipeptides

Ramoplanin is the first lipoglycodepsipeptide to reach clinical trials. It inhibits the synthesis of peptidoglycan at an earlier stage than the glycopeptides and does not bind to D-Ala-D-Ala (Somner & Reynolds, 1990). Ramoplanin shows no cross-resistance with vancomycin and teicoplanin and is active against both vancomycin-susceptible and vancomycin-resistant enterococci, as well as other Gram-positive pathogens (Collins et al, 1993). Ramoplanin is undergoing clinical trials for the treatment of diarrhoea caused by Clostridium difficile (Farver et al, 2005).

1.6.4.3. Cephalosporins and new fluoroquinolones

Ceftobiprole is a new broad spectrum cephalosporin with activity against both Gram-negative and Gram-positive pathogens, including VISA and VRSA strains (Bogdanovich et al, 2005). It has a high affinity for PBP2a and its efficacy compares to that of vancomycin against MRSA in an in vivo endocarditis model (Chambers, 2005).

Some new quinolones, including WCK-1153 and WCK-1152, are under development; these have potency against quinolone-resistant Gram-positive bacteria (Al Lahham et al, 2005).

1.6.5. DNA-binding antimicrobials

There has until recently been only limited interest in DNA-binding compounds as potential antimicrobials. The main reason for this is their high toxicity against mammalian cells. Furthermore, antibiotics are selected so that they act on specific bacterial targets that are absent in mammalian cells; as such, DNA is not an obvious target. However, the limited number of new classes of antibiotics being discovered
and the rapid developments in genomics has driven some investigators to consider DNA as a potential antimicrobial target.

1.6.5.1. Pyrrole tetraamides

Dyatkina et al (2002) have examined a series of synthetic pyrrole tetraamides that are related to bis-metropsins and bis-distamycins. These compounds bind non-covalently to the minor groove of the DNA at A:T rich sites and were shown to increase DNA melting temperature by 10-30°C. They reported that good DNA-binding was directly related to good in vitro antibacterial activity with MIC values against MRSA and VRE in the range of 0.7 to 45.5 µM. T-cell toxicity was monitored and it was determined that there was no relationship between toxicity and DNA binding or antibacterial activity. The most potent compounds were selected and their structure modified in order to maintain good antibacterial activity but reduce toxicity to mammalian cells. The authors concluded that human toxicity can be modulated for such compounds and that their antibacterial activity showed promise.

1.6.5.2. Heteroaromatic polycyclic (HARP) compounds

Another closely related group of compounds are based on the structure of distamycin A (Burli et al, 2002; Gross et al, 2003). These compounds bind to A:T rich sequences in the DNA minor groove and interfere with DNA transcription, leading to cell death (Ge et al, 2002). Distamycin A displays a low level of antibacterial activity and these authors modified its chemical structure in order to enhance antibacterial activity. Hundreds of compounds were synthesised and their MICs determined against MRSA, VRE and penicillin-resistant S. pneumoniae (Gross et al, 2003). These authors, in contrast to Dyatkina et al, reported no correlation between DNA binding affinity and MIC values. However, they did find a correlation between in vitro potency and in vivo efficacy (Gross et al, 2003). Selected compounds eradicated potentially lethal MRSA infections in a mouse peritonitis model, suggesting that these DNA-binding agents have potential as antibiotics (Burli et al, 2004a; Burli et al, 2004b; Hu et al, 2004). In addition, the compounds were well tolerated, implicating that reversible DNA binding may cause limited damage to cells (Burli et al, 2004b; Gross et al, 2003).
1.7. Pyrrolobenzodiazepines (PBDs)

1.7.1. PBD monomers

The pyrrolo-[2,1-c][1,4]-benzodiazepines are a family of naturally occurring chemotherapeutic agents produced by various *Streptomyces* spp; some well known members are anthramycin, tomaymycin and DC-81 (Fig. 1.4) (Thurston, 1999).

![Fig. 1.4. Examples of naturally occurring PBD monomers.](image)

These agents bind to DNA; early nuclear magnetic resonance (NMR) studies of anthramycin and DNA indicated that the tricyclic molecule covalently binds the amino group (at N2) of a guanine base in the minor groove of B-form DNA via its N10-C11 imine moiety (Fig. 1.5) (Graves et al, 1984).

![Fig. 1.5. PBD interaction with DNA: nucleophilic attack.](image)

These compounds bind duplex DNA and appear to interfere with DNA transcription and replication (Puvvada et al, 1997). Several PBD monomers were investigated for their effect on inhibition of a bacteriophage T7 RNA polymerase, and all inhibited transcription in a concentration-dependent manner (Puvvada et al, 1997).
The PBDs span three base pairs of DNA in a decreasing order of specificity: Pu-G-Pu, Pu-G-Py and Py-G-Py (Hurley et al, 1988). The monomers have been shown to bind to DNA and inhibit cleavage by the endonuclease BamHI in a concentration-dependent fashion (Puvvada et al, 1993). This enzyme recognises 5'-GGATTC-3', which overlaps with some of the favoured binding sites. An increasing order of inhibition by the monomers was established: anthramycin>tomaymycin>DC-81. This degree of potency is related to the saturation of the C-ring at the C2 position. Both tomaymycin and anthramycin are unsaturated at C2, while DC-81 is saturated at that position. Unsaturation at the C2 is clearly favourable for DNA binding and this may be due to a more planar conformation of the molecule that fits better within the minor groove or to reduced electrophilicity at the N10-C11 position (Morris et al, 1990). Reduced electrophilicity at this position decreases attack by nucleophiles such as thiol-containing compounds, stabilising the compound.

1.7.2. PBD dimers

1.7.2.1. PBD dimer-DNA interactions

In order to increase their DNA specificity and enhance their activity, the PBD monomers have been synthetically linked to form dimers that span longer sequences of DNA. The first synthesised PBD dimer was linked through the C7 position (Fig.1.6) (Farmer et al, 1991). Molecular modelling studies have indicated that these dimers cannot adopt a favourable conformation within the minor groove (Farmer et al, 1991; Gregson et al, 2004).

![Fig.1.6. First synthetic PBD dimer linked through the C7 position.](image)

Thurston and colleagues later synthesised potent cytotoxic PBD dimers that were linked through the C8 position and displayed an excellent fit within the DNA minor groove (Fig.1.7) (Smellie et al, 1994; Thurston et al, 1996).
Fig.1.7. DSB-120, the first C8 linked PBD dimer.

These compounds cross-link DNA, as two opposite strands of the DNA are alkylated simultaneously by each monomer. Guanines of Pu-G-Pu triplets of opposite DNA strands are covalently bound by each monomer spanning 6 or 7 bp of DNA (Fig.1.8). Due to their alkylating and cross-linking functions, these compounds have been referred to as bifunctional alkylating agents. NMR studies have shown that in addition to the covalent amine bond that forms with each guanine, hydrogen bonds with adjacent adenines contribute to the stability of the PBD dimer-DNA adduct (Jenkins et al, 1994). The intercalation within the minor groove of these agents does not distort the structure of the double helix and as a result recognition of such lesions is made difficult for DNA repair enzymes (Clingen et al, 2005b).

Fig.1.8. PBD dimers spanning 6 bp (a) or 7 bp of DNA. A model for PBD dimer binding activity within the minor groove of DNA (c).
1.7.2.2. Synthesis of the C8 linked PBD dimer DSB-120

The first example of a C8 linked PBD dimer, DSB-120 (Bose et al., 1992), was formed by joining two DC-81 units via a flexible C8-C8' linker consisting of three methylene groups (Fig. 1.7.) (Thurston et al., 1996). The resulting agent had greater DNA selectivity than the corresponding monomer and modelling studies predicted that its ability to bind to 6 bp with a preference for 5'-Pu-GATC-Py-3' sequences (Jenkins et al., 1994). The PBD dimer had the potential to cross-link both inter- and intrastrand DNA in an irreversible fashion. DSB-120 was highly cytotoxic against five human ovarian carcinoma cell lines due to efficient DNA cross-linking and was found to be 4- to 80-fold more potent than the major groove cross-linker cisplatin (Smellie et al., 1994). This study also compared DSB-120 and cisplatin in their efficiency at cross-linking DNA in human leukaemic K562 cells. Incubation with DSB-120 for 1 h resulted in a high degree of cross-linking for up to 24 h and cross-links were maintained at 48 h, with little evidence of DNA repair. Cross-links induced by cisplatin, on the other hand, were extensively repaired by 24 h. The reason for limited DNA repair observed with DSB-120 is probably due to poor recognition of the DNA lesions by repair enzymes. NMR studies and molecular modelling of the DSB-120-DNA complex have indicated that the dimer binds symmetrically within the minor groove of DNA, spanning 6 bp and causes very little distortion of the helical structure, thus maintaining DNA integrity (Bose et al., 1992; Jenkins et al., 1994). Major groove binders such as cisplatin induce significant DNA distortion and are therefore more likely to be recognised by DNA repair enzymes than DSB-120.

The effect of DSB-120 on the cell cycle was investigated in the K562 cell line; after 1 h incubation at subinhibitory concentrations of the compound there was accumulation of cells in the G2/M phase, consistent with inhibition of DNA replication (Smellie et al., 1994). Although in vitro cytotoxicity of DSB-120 was excellent against both human and rodent cell lines, the reported in vivo anti-tumour activity was poor (Walton et al., 1996). The lack of in vivo activity was attributed to high plasma protein binding (96.6%), high toxicity to normal cells and reaction with thiol-containing nucleophilic groups such as glutathione released from haemolysed blood cells. DSB-120 was highly electrophilic at N10-C11 moiety due to saturation at the C2 position of both monomers, which explains the instability of the dimer and its susceptibility to
nucleophilic attack. In order to retain the potent cytotoxic activity of DSB-120 but reduce attack by nucleophilic groups, other PBD dimers were synthesised containing less electrophilic monomers.

1.7.2.3. SJG-136

The dimer, SJG-136, was synthesised in a similar fashion to DSB-120 but the monomeric DC-81 unit was replaced by C2 methylene DC-81 (Fig. 1.9) (Gregson et al, 2001b; Gregson et al, 2001a). C2 methylene DC-81 is unsaturated at the C2 position and seems to increase the potency of the monomer compared to saturated equivalents, as discussed earlier. A PBD dimer with unsaturated C2 positions was expected to be not only more biologically stable but also more potent than the C2 saturated equivalent. SJG-136 was more potent than DSB-120, as observed by thermal denaturation studies and gel electrophoresis cross-linking assays (Gregson et al, 2001b). Thermal denaturation studies are based on the principle that covalently cross-linked DNA strands denature at a higher temperature than native DNA strands. Incubation of calf thymus DNA with DSB-120 or SJG-136 (DNA: ligand ratio of 5:1) for 18 h increased the melting temperature of the DNA by 15.1°C and 33.6°C respectively (Gregson et al, 2001b). Furthermore, SJG-136 was more cytotoxic than DSB-120 against five ovarian cell lines. In addition, SJG-136 remained active when exposed to higher levels of thiol-containing groups. While SJG-136 exhibited a greater potency than DSB-120 due to unsaturation at the C2 position, molecular modelling studies concluded that both PBD dimers were well accommodated within the minor groove of the DNA with no distortion of the helical structure (Gregson et al, 2001b). Recent studies have shown that SJG-136 is able to enter both the cellular and nuclear membranes of K562 cells and cross-links genomic DNA in a sequence selective manner identical to that studied in vitro (Martin et al, 2005). As predicted, SJG-136 was more potent and biologically stable than DSB-120 and was considered as the best candidate for further preclinical development.

SJG-136 was investigated in vivo and it displayed potent anti-tumour activity (Alley et al, 2004; Hartley et al, 2004). SJG-136 was well tolerated in vivo with no reported drug-related deaths at doses of 0.2 mg/kg. It exhibited broad-spectrum activity against a range of tumour xenografts and was also effective against the cisplatin-resistant
ovarian CHI1 tumour. Cross-links induced by the dimer were detected in vivo at physiological concentrations, indicating that the mechanism of action of this agent is indeed related to its DNA binding potency as predicted by molecular modelling and in vitro studies (Hartley et al, 2004). When cell lines were exposed to nanomolar concentrations of SJG-136, cytocidal effects against some of the more sensitive cell lines were evident in less than 6 h or 12 h for the more resistant cell lines (Alley et al, 2004). It was determined that in general the in vitro potency of the agent corresponded well to the in vivo efficacy against the tested cell lines (Alley et al, 2004). Pharmacokinetic studies of the compound have indicated that there is reduced binding of plasma proteins (65-75%) in comparison to DSB-120 and that an i.p. dose of 0.2 mg/kg resulted in a $C_{\text{max}} = 336$ nM after 30 min (Wilkinson et al, 2004). SJG-136 was detected in mouse plasma 4 h after dose administration and was found to be stable in whole blood and plasma over 6 h. This pharmacokinetic profile was considered favourable and the compound is now undergoing Phase I clinical trials in the UK as an anticancer agent.

![Fig.1.9. PBD dimers a) SJG-136, b) DRG-16 and c) ELB-21.](image-url)
1.7.2.4. DRG-16/ELB-21

DRG-16 (Fig.1.9), an analogue of SJG-136 with a longer diether C8/C8' linkage, is more potent in vitro in comparison to SJG-136 (Gregson et al, 2004). DRG-16 was more cytotoxic than SJG-136 against the K562 line and was also tenfold more potent in a cross-linking gel electrophoresis assay. Furthermore, DRG-16 was a more effective inhibitor of in BamHI restriction nuclease than SJG-136 (Gregson et al, 2004). The greater potency of DRG-16 is explained by the formation of a more stable and favourable adduct within the minor groove, as it spans a longer DNA region of 7 bp instead of 6 bp as in the case of SJG-136, due to the extended linkage group. This increases the opportunities of contact between the dimer and the walls of the minor groove.

Another PBD dimer, ELB-21, was synthesised with the same diether linkage (n=5) as DRG-16, although the PBD monomer was substituted with tomymycin (Fig.1.9). As discussed previously, tomymycin is less potent than anthramycin but more potent than DC-81. This dimer was expected to exhibit greater potency than all other PBD dimers as it contained two factors that contributed to its potency: a longer linker and more active monomers.

1.7.2.5. Effect of linker length on PBD dimers activity

As is evident from these studies, not only does the type of PBD monomer affect the potency of the synthesised PBD dimer, but the length of the bond linking the two monomers also plays a crucial role in the cytotoxic properties of these agents. It was found that only PBD dimers with diether linkages of n=3, 5 (i.e. odd numbers) are able to cross-link DNA; those containing linkages of n=4, 6 did not induce any cross-links as indicated by a gel electrophoresis cross-linking assay (Smellie et al, 2003). Molecular modelling suggested that this was a result of the spatial orientation of the PBD dimer within the minor groove. PBD dimers with a linkage of n=4, 6 adopted a poor conformation within the minor groove that, energetically, did not favour the cross-linking of opposite guanines within the DNA helix; such compounds probably only function as mono-alkylating agents. On the other hand, dimers with a linkage of
n=3, 5 fit snugly within the minor groove, adopting a favourable conformation and able to cross-link and stabilise the DNA duplex.

1.7.2.6. Sequence recognition by PBD dimers

The PBD dimer DSB-120 cross-linked an oligonucleotide containing a 5'-Pu-GATC-Py-3' sequence, while its equivalent PBD dimer with n=5 linkage cross-linked a sequence with an extra base pair, 5'-Pu-GA(T/A)TC-Py-3' (Smellie et al., 2003). It is interesting to note that the n=5 dimer was also capable of binding to the shorter sequence, 5'-Pu-GATC-Py-3', although less efficiently; DSB-120 was not able to recognise the longer 7 bp sequence. Recent foot-printing studies with SJG-136 have confirmed the preferential sequence binding to 5'-Pu-GATC-Py-3' and have also provided evidence for lower affinity binding sites which incorporate the 5'-Pu-GXXC-Py-3' motif, where X=AG, TA, GC, CT, TT, GG, TC (Martin et al., 2005). The DNAase I foot-printing assay determined that the highest affinity binding site was 5'-GGATCC-3'. This study is consistent with molecular modelling studies that have predicted the preferred binding sequence for SJG-136 and DSB-120 as 5'-Pu-GATC-Py-3'.

1.8. Aim of study

Novel classes of antibiotics with little or no cross-resistance to current clinically useful antibiotics are urgently needed. Bacterial DNA has been considered as a potential target by other investigators, although a DNA-binding antibiotic is yet to be licensed for use in man.

The PBD dimers are unique DNA binding agents that have been extensively studied and their biological functions have been optimised. They have shown promising in vitro and in vivo activity against a broad range of cell lines and tumours and have been considered for further clinical development for the treatment of cancer. It is likely that they will exert a potent antibacterial activity in a similar sequence-dependent fashion. As they interfere with DNA replication and transcription, they are therefore likely to target rapidly dividing cells, such as bacteria.
This study aims to investigate the antibacterial activity and mode of action of three selected PBD dimers of differing structures and potencies, SJG-136, DRG-16 and ELB-21, against a wide range of clinically important bacteria.
CHAPTER TWO

IN VITRO ANTIBACTERIAL ACTIVITY
2.1. Introduction

The purpose of this part of the study was to investigate the susceptibilities of 123 clinical isolates to the three selected PBD dimers, ELB-21, DRG-16 and SJG-136. Broth dilution MICs for these compounds were determined against 94 Gram-positive bacteria, 24 Gram-negative bacteria and 5 atypical mycobacteria. The susceptibilities of the Gram-positive isolates to ten antibiotics of different classes were also determined in order to identify any evidence for antibiotic cross-resistance.

2.2. Materials and Methods

2.2.1. Materials and bacterial strains

Culture media and antibiotic discs (oxacillin, vancomycin, trimethoprim, amikacin, gentamicin, tetracycline, rifampicin, ciprofloxacin, erythromycin and clindamycin) were purchase from Oxoid Ltd., Basingstoke, UK. PBD dimers were supplied by Spirogen Ltd, London, UK in powder form and dissolved in analytical grade methanol. All bacterial strains, with the exception of the mycobacteria, were kindly provided by Professor Jeremy Hamilton-Miller of the Royal Free and University College Medical School, London (RFUCMS). The international collection of 38 MRSA strains was assembled over several years (Table 2.1), whilst the remaining Gram-positive and Gram-negative strains were isolated at the RFUCMS. Out of 20 VRE strains, 11 were E. faecalis and 9 were E. faecium. Other Gram-positive bacteria included 12 isolates of Streptococcus pyogenes, 12 of Streptococcus agalactiae and 12 of Listeria monocytogenes. The Gram-negative bacteria included 6 isolates of Citrobacter spp., 6 of Klebsiella spp., 6 of Proteus spp. and 6 of Pseudomonas spp.

M. aurum A+ was obtained from the Pasteur Institute, Paris, France. M. smegmatis (ATCC 14468) was supplied by Lorraine Jex, Department of Pharmaceutical Sciences, De Montfort University, Leicester, UK. M. fortuitum (ATCC 6841), M. abscessus (ATCC 19977) and M. phlei (ATCC 11758) were purchased from the American Type Culture Collection.
Table 2.1. “International” MRSA strains collected at the RFUCMS.

<table>
<thead>
<tr>
<th>Country</th>
<th>Bacterial Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>AUS K, AUS D, AUS 5, AUS 7</td>
</tr>
<tr>
<td>Brazil</td>
<td>BZ 9, BZ 12, BZ 16, BZ 20, BZ 23, BZ 24</td>
</tr>
<tr>
<td>Chile</td>
<td>CHIL 1</td>
</tr>
<tr>
<td>China</td>
<td>HK 1</td>
</tr>
<tr>
<td>Denmark</td>
<td>DEN 2, DEN 3</td>
</tr>
<tr>
<td>Egypt</td>
<td>EG6</td>
</tr>
<tr>
<td>Finland</td>
<td>FIN 10, FIN 12</td>
</tr>
<tr>
<td>France</td>
<td>F 2, F 4, F 25</td>
</tr>
<tr>
<td>Germany</td>
<td>G 1, G 2, G 3, G 13, G 14</td>
</tr>
<tr>
<td>Greece</td>
<td>AT 4, AT 5</td>
</tr>
<tr>
<td>India</td>
<td>IND 3</td>
</tr>
<tr>
<td>Israel</td>
<td>IS 1</td>
</tr>
<tr>
<td>Kuwait</td>
<td>KW 7</td>
</tr>
<tr>
<td>Poland</td>
<td>POL 2, POL 3</td>
</tr>
<tr>
<td>Portugal</td>
<td>P 1, P 3</td>
</tr>
<tr>
<td>Turkey</td>
<td>T 7</td>
</tr>
<tr>
<td>UK</td>
<td>EMRSA-15, EMSRA-16</td>
</tr>
<tr>
<td>USA</td>
<td>SC 4</td>
</tr>
</tbody>
</table>

2.2.2. MIC determinations

Broth MICs for all strains, with the exception of mycobacteria, were performed according to the NCCLS (now known as the CLSI) guidelines (NCCLS, 1997a). The PBD dimers, initially dissolved in methanol, were diluted in MHB (to <1% v/v) and 100 µl aliquots were dispensed in a sterile 96 well microtitre plate to give the desired concentrations. One row of wells contained 100 µl MHB without compound and one row of wells contained dilutions of the compounds without bacteria as controls. The same amount of methanol was incorporated in the control wells to ensure that there was no inhibition of bacterial growth due to methanol. Bacterial cultures grown
overnight in MHB were diluted before 100 µl were dispensed in the wells to obtain a final bacterial count of ~5x10^5 cfu/ml. The final volume per well was 200 µl. The plates were sealed with a plastic cover and shaken gently to suspend the bacteria before incubation at 37°C. The MIC was determined to be the lowest concentration of PBD dimer at which there was no visible growth of bacteria. Bacterial growth was detected in the form of clumps of bacteria at the bottom of the well or as turbidity. A clear well indicated inhibition of growth. Viable counts from the diluted bacterial suspensions were performed to confirm that the correct inoculum was achieved in each well.

The method of Seidel and Taylor (2004) was used to determine the mycobacterial MICs. The mycobacteria were plated onto blood agar plates and incubated at 37°C for 3-4 days or until individual colonies were visible. Bacterial suspensions were prepared by transferring colonies from the plate directly to a bijou containing 0.9% saline and glass beads. The suspensions were shaken vigorously and left to settle. The supernatants containing bacterial cells were transferred to another bijou containing 0.9% saline and diluted to obtain a matching density to a 0.5 McFarland standard (absorbance of 0.08-0.1 at 625 nm), equivalent to ~5x10^5. The PBD dimers were diluted in cation-adjusted MHB (MHB supplemented with 20-25 mg of Ca^{2+} and 10-12.5 mg of Mg^{2+} per litre) and 100 µl of the appropriate concentrations were dispensed into the wells. One hundred µl of the bacterial inoculum was added to each well to give a final bacterial count of ~2.5x10^5 cfu/ml. The plates were sealed with a plastic cover and shaken gently before incubation at 37°C. The MICs were read after 3 days for *M. smegmatis* and *M. fortuitum*, 4 days for *M. abscessus* and 5 days for *M. phlei* and *M. aurum*. Bacterial growth was detected by the addition of a dye; 20 µl of a methanol solution (5mg/ml) of the yellow tetrazolium redox dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Lancaster, UK) was added to each well. The plates were then incubated at 37°C for a further 20-50 min. Bacterial growth was indicated by change of the yellow dye to purple. All MICs were tested in duplicate and on separate days.
2.2.3. Susceptibility of Gram-positive isolates to conventional antibiotics

Susceptibility tests of the isolates against ten different antibiotics were performed in accordance with the NCCLS guidelines (NCCLS, 1997b). Bacterial colonies were transferred from NA plates to bijoux containing MHB, incubated overnight at 37°C and subsequently diluted in sterile saline to match the density of 0.5 of McFarland standard solution. A sterile cotton swab was dipped in the bacterial inoculum and pressed against the bijou walls to remove excess bacterial suspension. MHA plates were thoroughly streaked with the swab three times, turning the plate 60° each time. No more than six discs, incorporating different antibiotics, were placed onto each inoculated plate within 15 min. The plates were incubated at 35°C for 16-18 h for all organisms, excluding VRE and MRSA, which were incubated for 24 h. The experiment was considered valid if there was confluent bacterial growth rather than isolated colonies. Clear zones around the disc were measured with a ruler and interpreted according to the tables provided by the NCCLS (NCCLS, 1997b). Bacteria were classified as sensitive, intermediate or resistant.

2.3. Results

2.3.1. MICs

The MICs of the PBD dimers against 123 bacterial isolates were determined and the MIC$_{90}$ data are summarised in Table 2.2. Gram-negative isolates were insensitive to the three PBD dimers, with all MIC$_{90}$ values of, or greater than, 16 mg/l. Gram-negative bacteria treated with ELB-21 in the presence of 1 mM ethylenediaminetetraacetic acid (EDTA) had increased sensitivity to this PBD dimer as indicated by MIC$_{90}$ values of 0.5-2 mg/l. All Gram-positive isolates were susceptible to the PBD dimers, with MIC$_{90}$ of ≤0.5 mg/l. MIC$_{90}$ values for Mycobacteria spp. were in the range 0.5-2 mg/l.

Overall, the compound that produced the lowest MIC$_{90}$ for all strains was ELB-21. MIC$_{90}$ values for all Gram-positives against ELB-21 were ≤0.06 mg/l. DRG-16 was more potent than SJG-136; the MIC$_{90}$ values for MRSA, S. pyogenes and L.
*monocytogenes* isolates against SJG-136 were four times higher than those obtained against DRG-16.

Table 2.2. MIC<sub>90</sub> data of PBD dimers against a total of 123 bacterial isolates.

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>No. of strains</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SJG-136</td>
<td>DRG-16</td>
</tr>
<tr>
<td>MRSA</td>
<td>38</td>
<td>0.5</td>
</tr>
<tr>
<td>VRE</td>
<td>20</td>
<td>0.5</td>
</tr>
<tr>
<td><em>S. pyogenes</em></td>
<td>12</td>
<td>0.06</td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>12</td>
<td>0.03</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>12</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Citrobacter</em> spp.</td>
<td>6</td>
<td>&gt;32</td>
</tr>
<tr>
<td><em>Klebsiella</em> spp.</td>
<td>6</td>
<td>&gt;32</td>
</tr>
<tr>
<td><em>Proteus</em> spp.</td>
<td>6</td>
<td>&gt;32</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>6</td>
<td>&gt;32</td>
</tr>
<tr>
<td><em>Mycobacterium</em> spp.</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

*MIC data obtained in the presence of 1mM EDTA*

There was little variation in the MICs obtained between different isolates of the same species. The MIC ranges and MIC<sub>50</sub> and MIC<sub>90</sub> values for all Gram-positive isolates are displayed in Table 2.3. There was no difference between the MIC<sub>50</sub> and MIC<sub>90</sub> values for ELB-21 against VRE (0.06 mg/l), *S. pyogenes* (0.015 mg/l) and *S. agalactiae* (0.015 mg/l); the MIC<sub>90</sub> for this dimer was only two-fold higher than that of the MIC<sub>50</sub> against MRSA (0.03 mg/l) and *L. monocytogenes* (0.06 mg/l). There was a fourfold increase of the MIC<sub>90</sub> (0.125 mg/l) from the MIC<sub>50</sub> (0.03 mg/l) for DRG-16 against MRSA strains; all other MIC<sub>50</sub> and MIC<sub>90</sub> values were either identical or there was a two-fold increase of the MIC<sub>90</sub>.  

56
Table 2.3. MIC ranges and distribution for all Gram-positive isolates.

<table>
<thead>
<tr>
<th>Bacterial isolates (no. of strains) and PBD dimer</th>
<th>MIC (mg/l)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
</tr>
<tr>
<td><strong>MRSA (38)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJG-136</td>
<td>0.125-0.5</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>DRG-16</td>
<td>0.015-0.125</td>
<td>0.03</td>
<td>0.125</td>
</tr>
<tr>
<td>ELB-21</td>
<td>0.008-0.06</td>
<td>0.015</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>VRE (20)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJG-136</td>
<td>0.125-1</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>DRG-16</td>
<td>0.125-0.5</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>ELB-21</td>
<td>0.015-0.06</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>S. pyogenes (12)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJG-136</td>
<td>0.03-0.06</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>DRG-16</td>
<td>&lt;0.008-0.015</td>
<td>0.015</td>
<td>0.015</td>
</tr>
<tr>
<td>ELB-21</td>
<td>&lt;0.008-0.015</td>
<td>0.015</td>
<td>0.015</td>
</tr>
<tr>
<td><strong>S. agalactiae (12)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJG-136</td>
<td>0.015-0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>DRG-16</td>
<td>&lt;0.008</td>
<td>&lt;0.008</td>
<td>&lt;0.008</td>
</tr>
<tr>
<td>ELB-21</td>
<td>&lt;0.008-0.015</td>
<td>0.015</td>
<td>0.015</td>
</tr>
<tr>
<td><strong>L. monocytogenes (12)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJG-136</td>
<td>0.06-0.5</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>DRG-16</td>
<td>0.03-0.125</td>
<td>0.06</td>
<td>0.125</td>
</tr>
<tr>
<td>ELB-21</td>
<td>0.015-0.06</td>
<td>0.03</td>
<td>0.06</td>
</tr>
</tbody>
</table>
2.3.2. Antimicrobial susceptibility of Gram-positive isolates against other antibiotics

Susceptibility of the Gram-positive bacteria to a range of antibiotics was determined by disc diffusion assays. The only antibiotic that was effective against all 38 MRSA strains was vancomycin. Resistance to other agents ranged from 11% for rifampicin, ciprofloxacin and erythromycin to 84% for tetracycline. The susceptibility data for MRSA isolates is summarised in Table 2.4.

**Table 2.4.** Antimicrobial activity of 10 antibacterial agents against 38 MRSA isolates.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>% MRSA isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>100</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>-</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>32</td>
</tr>
<tr>
<td>Amikacin</td>
<td>16</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>70</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>84</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>11</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>11</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>11</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>41</td>
</tr>
</tbody>
</table>

All VRE isolates were resistant to oxacillin (Table 2.5). Resistance to other antibiotics varied between 50% for ciprofloxacin and 90% for erythromycin. Susceptibilities to amikacin and rifampicin were not determined.
Table 2.5. Antimicrobial activity of 10 antibiotics against 20 VRE isolates.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>% VRE isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>100</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>100</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>75</td>
</tr>
<tr>
<td>Amikacin</td>
<td>-</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>80</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>60</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>-</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>50</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>90</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>85</td>
</tr>
</tbody>
</table>

The remaining strains, *S. pyogenes*, *S. agalactiae* and *L. monocytogenes*, were resistant to a maximum of three antibiotics (*Tables 2.6, 2.7 and 2.8* respectively). *S. pyogenes* isolates were resistant only to amikacin and were fully sensitive to the remaining antibiotics. All *S. agalactiae* isolates were resistant to both aminoglycosides and 90% were also resistant to tetracycline. All *L. monocytogenes* isolates were resistant to oxacillin and clindamycin but were completely susceptible to other antibiotics.
Table 2.6. Antimicrobial activity of 10 antibiotics against 12 *S. pyogenes* isolates.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistant</th>
<th>Sensitive</th>
<th>Intermediate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Amikacin</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.7. Antimicrobial activity of 10 antibiotics against 12 *S. agalactiae* isolates.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistant</th>
<th>Sensitive</th>
<th>Intermediate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Amikacin</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>90</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2.8. Antimicrobial activity of 10 antibiotics against 12 *L. monocytogenes* isolates.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>% Resistant</th>
<th>% Sensitive</th>
<th>% Intermediate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Amikacin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2.4. Discussion

2.4.1. Gram-negative isolates

From the MIC data it can be concluded that the Gram-positive bacteria, including mycobacteria, but not Gram-negative strains, are susceptible to the PBD dimers. The reduced susceptibility observed with Gram-negative bacteria is most likely due to the barrier function of the outer membrane.

The outer membrane of Gram-negative bacteria restricts the penetration of hydrophobic molecules, whilst hydrophilic agents are able to enter through membrane-integrated porin channels. The high proportion of lipopolysaccharide (LPS), incorporated into the outer membrane, is responsible for its impermeability to hydrophobic molecules. LPS molecules, located exclusively in the outer leaflet of the outer membrane, are tightly packed and are responsible for the rigidity of this bilayer, reducing the uptake of hydrophobic molecules (Nikaido, 2003). Although the outer
membrane is very effective as a barrier to many substances, it cannot solely account for the high levels of resistance observed with a large number of antibiotics. The presence of active efflux pumps has been described as a synergistic mechanism acting in combination with the impermeability of the outer membrane (Nikaido, 1996). These pumps account for the active efflux of a number of antibiotics by Gram-negative bacteria, including tetracyclines, β-lactams and erythromycin. Since some of the excluded substances are hydrophobic, it is clear that the outer membrane cannot completely inhibit the diffusion of such molecules and they are therefore eliminated by active efflux (Nikaido, 1996). Based on these facts, the barrier function of the outer membrane is the most likely explanation for reduced susceptibility of the Gram-negative isolates to the PBD dimers, as perturbation of the outer membrane with EDTA sensitises the bacteria.

ELB-21 was effective when used in synergy with EDTA against the Gram-negative isolates as indicated by the significantly lowered MICs values. EDTA is one of the agents known to destabilise the outer membrane; others include polymixin B-nonapeptide and other polycationic agents (Vaara, 1992). The stability of the outer membrane is dependent on cations that surround the anionic core region of the LPS and prevent repulsion between individual LPS molecules; divalent cations also effect cross-bridging of LPS molecules (Nikaido, 2003). Polycationic agents or chelators, such as EDTA, disrupt the membrane stability by interfering with and competing with the cations, releasing LPS from the bilayer (Leive, 1965b). It has been proposed that the LPS molecules are then replaced by phospholipids and hydrophobic substances can cross the membrane more readily (Nikaido & Nakae, 1979). The effect of EDTA on increasing the permeability of the Gram-negative outer membrane was shown initially on an \textit{E. coli} strain (Leive, 1965a). The author showed that treatment of \textit{E. coli} with EDTA increased the permeability of the bacteria to actinomycin without affecting bacterial viability.

EDTA was employed in this study to determine if reduced susceptibility to PBD dimers was dependent on an integral outer membrane. ELB-21, as the most potent PBD dimer, was selected for these experiments. The MIC\textsubscript{90} value for ELB-21 against the Gram-negative isolates examined was 16 gm/l; however, in the presence of 1 mM EDTA MIC\textsubscript{90} values were in the range 0.5-2 mg/l (Table 2.2). This indicates that the
outer membrane of the Gram-negative isolates restricts the diffusion of the PBD dimer into the cell. Although MICs for the other two dimers in the presence of EDTA were not performed, similar results would be expected.

2.4.2. Mycobacterial MICs

The MIC$_{90}$ values of the three dimers against the five atypical mycobacterial isolates were in the range 0.5-2 mg/l, with the lowest MIC$_{90}$ for ELB-21 of 0.5 mg/l. Although mycobacteria are classed as Gram-positive bacteria, they have unique cell walls that differ from the cell walls of Gram-positive bacteria. The higher MIC values against mycobacteria, in comparison to Gram-positive bacteria, are likely to be related to the natural intrinsic resistance of mycobacteria to many antibacterial substances.

The mycobacterial cell wall is more complex than that of Gram-positive bacteria. In addition to the peptidoglycan layer, the mycobacterial wall contains an arabinogalactan polymer that is linked to the peptidoglycan layer (Crick et al., 2001). Surrounding this structure, and covalently linked to the arabinogalactan polymer, are mycolic acids. These are branched long fatty acids associated with lipids or glycolipids at the surface of the cell wall (Draper, 1998). In similar fashion to the outer membrane of Gram-negative bacteria, there are porin channels that allow the movement of hydrophilic substances across the cell wall. However, such porins are much more infrequent than those associated with Gram-negative bacteria (Trias et al., 1992). Hydrophobic substances, on the other hand, can diffuse across the lipid layer but diffusion is limited due to the tightly packed layer of mycolic acids (Draper, 1998). In addition to a cell wall barrier, some mycobacteria also produce a capsule, which further enhances resistance to antibiotics (Draper, 1998). As with Gram-negative bacteria, mycobacteria are naturally resistant to a number of antibiotics as a consequence of the structure of their outer wall. It would appear that PBD dimers can pass through the cell wall of mycobacteria more readily than they do in Gram-negative bacteria, as reflected by the MIC$_{90}$ values for these species.
2.4.3. Susceptibility of Gram-positive bacteria to PBD dimers and other antibiotics

All Gram-positive bacteria were highly susceptible to the PBD dimers, with the highest MIC_{90} of 0.5 mg/l (Table 2.2). These MICs values are comparable to ones obtained with the heteroaromatic polycyclic DNA-binding agents, which have been investigated as antibacterial agents (Gross et al, 2003). The compounds GSQ-2287, GSQ-11203 and GSQ-10547 were tested against two MRSA strains, one MSSA, one VRE, one vancomycin susceptible *E. faecalis*, one *S. pneumoniae* and one *Bacillus cereus* strain; the MICs were in the range of 0.06-4 mg/l.

Agents with a novel mechanism of action, such as the PBD dimers and the heteroaromatic polycyclic compounds, are unlikely to have cross-resistance to other antibiotics. Burli and colleagues have reported no cross-resistance between their DNA-binding HARP compounds and other antibiotics following testing of both resistant and susceptible pathogens, including MRSA and VRE (Burli et al, 2002; Ge et al, 2002).

There was little or no variation in PBD dimer activity against isolates from the same bacterial species, including the international collection of MRSA isolates, as indicated by the narrow MIC ranges represented in Table 2.3. There was a significant degree of variability in the susceptibility of the MRSA strains to the ten conventional antibiotics examined (Table 2.4), with the exception of the 100% susceptibility to vancomycin. This indicates that the PBD dimers are effective against all strains, independent of the strain’s resistance to other antibiotics. An MSSA strain, ATCC 29213, was also exposed to the PBD dimers and the MICs were comparable to the MIC_{90} for the remaining MRSA strains; the MICs of ATCC 29213 against SJG-136, DRG-16 and ELB-21 were 0.25, 0.125 and 0.015 mg/l respectively.

A similar pattern was observed against VRE isolates (Table 2.5). All other Gram-positive isolates were 100% sensitive to seven or more antibiotics, with the exception of *S. agalactiae*; these isolates were resistant to tetracycline (Tables 2.6, 2.7 and 2.8). It was therefore not possible to establish whether cross-resistance of these strains to other antibiotics existed, although it would appear unlikely.
2.4.4. Potency of PBD dimers

Based on the MIC\textsubscript{90} values, the order of potency of the PBD dimers was as follows: ELB-21>DRG-16>SJG-136 (Table 2.2). This appears to be consistent with the structural differences between the three dimers. SJG-136 and DRG-16 dimers contain the same PBD monomer, C-2 methylene DC-81. However, the monomers in DRG-16 are linked by a \((n=5)\) C-8/C-8' diether linkage, whilst those in SJG-136 are linked by a \((n=3)\) C-8/C-8' linkage (Gregson \textit{et al}, 2004). It has been established that DRG-16 is more potent than SJG-136 in terms of its cross-linking capacity and its cytotoxicity. This was exemplified in the more efficient inhibition of the DNA restriction enzyme \textit{BamH}1 by DRG-16 in comparison to SJG-136 (Gregson \textit{et al}, 2004). Furthermore, whilst SJG-136 spans six base pairs, DRG-16 spans an extra base pair due to the longer linkage, which may stabilise the DNA-ligand interaction (Gregson \textit{et al}, 2004). It is, therefore, not surprising that DRG-16 produced lower MIC\textsubscript{90} values than SJG-136.

As with DRG-16, the monomers in ELB-21 are linked by the longer \(n=5\) diether linkage. However, ELB-21 is comprised of two tomayamycin monomers rather than the C-2 methylene DC-81. The tomayamycin monomer has an exocyclic unsaturation at the C2-C3 position, which has been previously shown to increase DNA-binding potency. Puvvada \textit{et al} (1993) found that the most potent PBD compounds, in a decreasing order, were sibiromycin, anthramycin, tomaymycin and DC-81 according to a \textit{BamH}1 enzymatic inhibition assay. These compounds were unsaturated at the C2-C3 position, with the most potent, sibiromycin and anthramycin, containing an endocyclic unsaturation. Unsaturation at the C2 position favours a more planar molecular shape, which allows better fit within the minor groove (Puvvada \textit{et al}, 1993). It was therefore expected that ELB-21, comprised of more potent monomers, would be more potent than the corresponding \(n=5\) linked C2-methylene DC-81 dimer DRG-16.

The MIC\textsubscript{90} of ELB-21 against \textit{L. monocytogenes}, MRSA and VRE was two, four and eight-fold lower, respectively, than the corresponding DRG-16 values. \textit{S. agalactiae} was the only organism for which the MIC\textsubscript{90} of DRG-16 was lower than that of ELB-21. The MIC\textsubscript{90} values for the two dimers were equal against \textit{S. pyogenes}. 
In conclusion, the different potencies observed with the three dimers are consistent with their structural differences, with ELB-21 being the most potent compound against all strains.
CHAPTER THREE
TIME KILL KINETICS AND POST-ANTIBIOTIC EFFECT OF PBD DIMERS
3.1. Introduction

In this section, the determination of the bactericidal activity and the post-antibiotic effects (PAEs) of the PBD dimers are reported. To assess the bactericidal potency of the PBD dimers, their capacity to reduce the viability of one VRE (VRE-10 - *E. faecium*) isolate and the two UK epidemic MRSA strains EMRSA-15 and EMRSA-16 was examined. The rate of PBD dimer-mediated killing was determined by measuring the number of viable bacteria at selected time intervals after the bacteria were exposed to different concentrations of compound. The EMRSA strains are associated with the majority of MRSA infections in the UK (Johnson *et al.*, 2001).

The PAEs against EMRSA-16 and VRE-10 were determined in order to provide an indication on the effect of the PBD dimers on bacterial growth following brief exposure to the compounds. PAE is the term used to describe continued suppression of bacterial growth after a brief exposure of bacteria to an antimicrobial agent (Craig & Gudmundsson, 1996). A long PAE would favour a less frequent dosing schedule, as an antibacterial effect would still be observed even when drug concentrations drop below the MIC. Therefore, the PAE is clinically significant and plays a role in determining drug dose concentrations and treatment profiles. The duration of the PAE is dependent on a number of factors, the most important being the type of pathogen and the antimicrobial agent used. PAEs are observed for most antibacterial agents, with the exception of β-lactams and agents effective against *P. aeruginosa* (Bustamante *et al.*, 1984).

3.2. Materials and Methods

3.2.1. Time kill kinetics

The time kill experiments were based on the NCCLS guidelines: Methods for Determining Bactericidal Activity of Antimicrobial Agents (NCCLS, 1999). The experiments were conducted in 250 ml Pyrex flasks containing 50 ml of MHB; these were inoculated with overnight bacterial cultures and are subsequently described as lag-phase cultures. Time kill assays were determined for two different growth phases.
of bacteria: exponential and lag. For exponential phase determinations, the flasks were incubated for 2 h; a final bacterial count of \( \sim 10^5/10^6 \) cfu/ml was obtained for both phases. The bacteria were treated with the PBD dimers at the MIC, 2xMIC and 4xMIC. The same amount of methanol solvent was incorporated into a drug-free control flask. Immediately before addition of the compounds (t=0), 100 µl of each flask were removed and serially diluted ten-fold in PBS; 20 µl of the dilutions were plated onto NA plates and the flasks were incubated at 37°C in an orbital shaker at 200 rpm. Samples were removed from the flasks after 2, 4, 6 and 24 h and dilutions were plated onto NA plates. All plates were incubated at 37°C and counted after 24 h. Experiments were repeated on a separate day.

3.2.2. PAE

The PAE method was based on that described by Craig and Gudmundsson (1996). These authors have suggested a number of drug removal methods, depending on the drug used. For the purposes of this study, three different methods of drug removal have been considered: a hundred-fold dilution of cultures, washing and suspending bacteria in fresh medium and the latter method followed by a dilution step.

Preliminary studies were performed to determine if, following a one hundred-fold dilution of treated bacteria, an antibiotic effect that could affect PAE values would be observed. A one hundred-fold dilution of the highest concentration of PBD dimer (4xMIC) was added to 50 ml of exponential-phase cultures of \( \sim 10^7 \) cfu/ml in MHB; viable counts were taken every hour for 5 h and at 24 h. The growth rates of treated and drug-free control bacteria were compared. For all other experiments, exponential-phase cultures of \( \sim 10^7 \) cfu/ml of EMRSA-16 or VRE-10, in 50 ml of MHB, were treated with PBD dimers at different concentrations: MIC, 2xMIC and 4xMIC for EMRSA-16 and 1/4xMIC, 1/2xMIC and MIC for VRE-10. The same amount of methanol was added to a drug-free control flask. The flasks were incubated for 1 h in an orbital shaker (200 rpm) at 37°C. The cultures were then transferred to Falcon tubes (Fisher Scientific, Bidhop Meadow Road, Loughborough, UK) and centrifuged at 5,100 g for 5 min at room temperature. The supernatant was decanted and the pellet suspended in 20 ml of sterile PBS. This wash step was repeated twice more. In initial
experiments, treated cells suspended in 50 ml of pre-warmed MHB and a fifty-fold dilution of the control, were prepared in new sterile flasks (Method A). In subsequent experiments all samples were diluted ten-fold (Method B). The flasks were incubated in an orbital shaker (200 rpm) at 37°C. Viable counts were taken at each stage of this experiment, prior to compound exposure, following compound exposure and washing, and then every hour for 5 h and at 24 h. Samples (100 μl) were removed from the flasks at specified time points, serially diluted ten-fold in PBS and 20 μl of the dilutions plated in duplicate onto NA plates; the plates were incubated at 37°C and colonies were counted after 24 h.

The PAE was estimated as PAE = T - C, where T is the time taken for the viable count in the test cultures to increase 1 log_{10} following drug removal and C is the time taken for bacterial numbers in the control culture to reach the same value (Fig. 3.1). Experiments were repeated on a separate day.

**Fig. 3.1.** PAE determination; PAE = T - C, where T is the time taken for the test culture to reach 1 log_{10} after the wash step and C is the time taken for the control culture to reach the same value; • (Control), ▲ (MIC), ▼ (2xMIC), ◆ (4xMIC).
3.2.3. Electron microscopy

Exponential-phase EMRSA-16 and VRE-10 were grown in MHB for 1 h in the presence or absence of ELB-21. EMRSA-16 was incubated with 1/2xMIC, MIC and 2xMIC of ELB-21 and VRE-10 was incubated with 1/4xMIC, 1/2xMIC and MIC of this PBD dimer. The cells were recovered by centrifugation and washed twice in PBS. The bacteria were fixed in 1.5% glutaraldehyde for at least 2 h at room temperature, treated with osmium tetroxide and embedded in epoxy resin. Sections were stained with uranyl acetate and Reynolds’ lead citrate and images were viewed using a Philips 201 transmission electron microscope.

3.3. Results

3.3.1. Time kill kinetics

3.3.1.1. EMRSA-15 & EMRSA-16

EMRSA-15 cells in both exponential and lag phases were efficiently killed by ELB-21 within 2 h (Fig. 3.2 and 3.3). The compound remained bactericidal at 4xMIC at 24 h. DRG-16 was bactericidal against cells in both growth phases at 2xMIC and 4xMIC after 2 h, although some re-growth at 24 h was observed. Compared to EMRSA-16, EMRSA-15 was more susceptible to SJG-136. At 4xMIC in the exponential phase and at 2xMIC and 4xMIC in the lag phase, the compound was bactericidal and no recovery was seen after 24 h incubation.

Bacterial counts for EMRSA-16 treated with 2xMIC and 4xMIC of ELB-21 were reduced by >3 log after 4 h incubation using cells in both exponential and lag phases; however, there was some re-growth after 24 h (Fig. 3.4 and 3.5). For lag-phase EMRSA-16 exposed to DRG-16 at concentrations equivalent to 2xMIC and 4xMIC, colony counts were reduced by >3 log after 4 h; viable counts showed no significant change following incubation for a further 20 h; however, in the exponential-phase culture at 4xMIC, there was some re-growth observed at 24 h. In similar fashion to the data obtained with VRE (Fig. 3.6 and 3.7), SJG-136 was less effective in killing EMRSA-16 than ELB-21 and DRG-16. The compound was bactericidal only against
the lag-phase culture at 4xMIC. At all other concentrations in both growth phases, there was initial reduction or inhibition of growth but also some re-growth after 24 h incubation.

3.3.1.2. VRE-10

Exponential- and lag-phase cultures of VRE-10 were both susceptible to ELB-21 and DRG-16. There was rapid killing of cells within 2 h, with a 5-log reduction of the initial inoculum over a range of concentrations. No re-growth was observed at 24 h (Fig. 3.6 and 3.7). Susceptibility to SJG-136 was slightly less than that found with the other two PBD dimers, with a bacteriostatic effect observed at the MIC concentration in both phases and a slight re-growth at 24 h. There was a 3-log reduction of the initial inoculum after 2 h at 4xMIC and no re-growth was observed at 24 h. At 2xMIC, the compound was bactericidal by 24 h in both phases.

3.3.1.3. VRE-10 and EMRSA-16 at high inoculum

Both VRE-10 and EMRSA-16 were rapidly killed within 2 h over the range of ELB-21 concentrations utilised: MIC, 2xMIC and 4xMIC. No re-growth was observed 24 h (Fig. 3.8).
Fig. 3.2. Time-kill of lag-phase EMRSA-15 in presence of a) ELB-21, b) DRG-16 and c) SJG-136; ♦ (Control); ■ (MIC); ▲ (2xMIC); × (4xMIC).
Fig. 3.3. Time kill of exponential-phase EMRSA-15 in presence of a) ELB-21, b) DRG-16 and c) SJG-136; ♦ (Control); ■ (MIC); ▲ (2xMIC); ✗ (4xMIC).
Fig. 3.4. Time kill of lag-phase EMRSA-16 in presence of a) ELB-21, b) DRG-16 and c) SJG-136; ♦ (Control); (MIC); ▲ (2xMIC); × (4xMIC).
Fig. 3.5. Time kill of exponential-phase EMRSA-16 in presence of a) ELB-21, b) DRG-16 and c) SJG-136; ♦ (Control); ■ (MIC); ▲ (2xMIC); × (4xMIC).
Fig. 3.6. Time kill of lag-phase VRE-10 in presence of a) ELB-21, b) DRG-16 and c) SJG-136; ♦ (Control); ■ (MIC); ▲ (2xMIC); × (4xMIC).
Fig. 3.7. Time kill of exponential-phase VRE-10 in presence of a) ELB-21, b) DRG-16 and c) SJG-136; ◆ (Control); ■ (MIC); ▲ (2xMIC); ✗ (4xMIC).
Fig. 3.8. Time kill of high inoculum stationary EMRSA-16 (a) and VRE-10 (b) in presence of ELB-21; *(Control); ■(MIC); ▲(2xMIC); ✗(4xMIC).
3.3.2. PAEs

3.3.2.1. Determination of appropriate PAE method

Experiments were performed to establish if PAEs induced by the PBD dimers could be determined using the dilution method described by Craig and Gudmundsson (1996). In this method, drug removal is accomplished by diluting the treated and control cultures one hundred- to one thousand-fold in fresh media. The authors recommend that, following drug removal, additional control flasks of bacterial cultures treated with appropriate dilutions of the compound used in the experiment should be included, in order to ensure that residual drug in the flasks following dilution will not affect the rate of bacterial growth. Such experiments were undertaken to establish if residual PBD dimer has an effect on the rate of growth and to inform on the suitability of the dilution method of PAE determination. Exponential-phase EMRSA-16 cultures were incubated with a one hundred-fold dilution of 4xMIC, the highest MIC concentration to be used in the PAE determinations. Unexpectedly, with ELB-21-treated cells there was a delay of 7.5 h to reach 1 log_{10} in comparison to the control culture (Table 3.1) and other methods of drug removal were therefore investigated.

Table 3.1. Growth delay in reaching 1 log_{10} for exponential-phase EMRSA-16 following incubation with a one hundred-fold dilution of 4xMIC of PBD dimer in comparison to a control EMRSA-16 culture.

<table>
<thead>
<tr>
<th>Conc. of antibiotic</th>
<th>Growth delay (h) (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJG-136</td>
<td>DRG-16</td>
</tr>
<tr>
<td>1/25xMIC</td>
<td>0.5 (0.3-0.7)</td>
</tr>
</tbody>
</table>

Two other methods of drug removal were performed with EMRSA-16 and ELB-21; these involved removal of drug by washing of the bacterial cultures followed by a dilution step. These procedures are designated method A and method B. In method A, the control
culture was diluted fifty-fold after washing to achieve a bacterial count approximating that of the treated cultures. In method B, all cultures were diluted ten-fold after washing. The PAEs obtained with method A were 0.5-2.8 h longer than those obtained with method B (Table 3.2), possibly due to the presence of residual PBD dimer. Method B was used for the rest of the study for all other PAE determinations. The PAEs induced by ELB-21 for exponential and stationary phase EMRSA-16 cultures were determined and no significant differences in the magnitude of the PAEs were observed; all PAEs were less than 1 h (Table 3.2).

Table 3.2. PAEs for lag-phase (method B) and exponential-phase (methods A and B) bacterial cultures of EMRSA-16 treated with ELB-21.

<table>
<thead>
<tr>
<th>Conc. of antibiotic</th>
<th>Lag phase (method B)</th>
<th>Log phase (method B)</th>
<th>Log phase (method A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1xMIC</td>
<td>0.65 (0.6-0.7)</td>
<td>0.2 (0.3-0.1)</td>
<td>1.45 (1.4-1.5)</td>
</tr>
<tr>
<td>2xMIC</td>
<td>0.15 (0.1-0.2)</td>
<td>0.95 (0.8-1.1)</td>
<td>1.45 (1.3-1.6)</td>
</tr>
<tr>
<td>4xMIC</td>
<td>0.4 (0.3-0.5)</td>
<td>0.65 (0.5-0.8)</td>
<td>3.45 (3.4-3.5)</td>
</tr>
</tbody>
</table>

3.3.2.2. PAEs of the PBD dimers

The PAEs of all three PBD dimers were determined for exponential-phase EMRSA-16 and VRE-10 using method B. Representative PAE graphs for EMRSA-16 and VRE-10 with each PBD dimer are shown in Fig. 3.9 and Fig. 3.10 respectively.
Fig. 3.9. Representative PAE data for exponential-phase EMRSA-16 in presence of a) ELB-21, b) DRG-16 and c) SJG-136; • (Control), ▲ (MIC), ▼ (2xMIC), ◆ (4xMIC).
Fig. 3.10. Representative PAE data for exponential-phase VRE-10 in presence of a) ELB-21, b) DRG-16 and c) SJG-136; • (Control), ▲ (1/4xMIC), ▼ (1/2xMIC), ★ (MIC).
The longest PAE observed against EMRSA-16 (Table 3.3) was 2.05 h at 2xMIC SJG-136. Surprisingly, ELB-21 and DRG-16, which showed greater bactericidal activity against EMRSA-16 than SJG-136, produced less pronounced PAEs with the PBD dimer. In contrast to the concentration-dependent bactericidal effect of PBD dimers observed with EMRSA-16, no evidence was found for a concentration-dependent increase in PAE with any of the compounds using EMRSA-16. The PAEs for vancomycin against EMRSA-16 were determined to ensure that the data obtained with this method are consistent with those in the literature (Boswell et al, 1999; Pfeil & Wiedemann, 2000). Reported PAEs for vancomycin against MRSA are 1-2 h. The vancomycin PAEs obtained in this experiment were in the range of 0.7-1.4 h (Table 3.3).

Table 3.3. PAEs for exponential-phase cultures of EMRSA-16.

<table>
<thead>
<tr>
<th>Conc. of antibiotic</th>
<th>PAE (h) (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJG-136</td>
<td>DRG-16</td>
</tr>
<tr>
<td>1xMIC</td>
<td>0.7 (1.2-0.5)</td>
</tr>
<tr>
<td>2xMIC</td>
<td>2.05 (2-2.1)</td>
</tr>
<tr>
<td>4xMIC</td>
<td>1.8 (1.6-2)</td>
</tr>
</tbody>
</table>

The PAEs for VRE-10 against the compounds could not be determined at concentrations above the MIC as the bactericidal activity at 2xMIC and 4xMIC was too great, with little or no bacterial survival following incubation for 1 h. PAEs were performed at the MIC and at subinhibitory MIC concentrations of 1/4xMIC and 1/2xMIC (Table 3.4). More pronounced PAEs for all compounds were observed at the MIC, with the highest value of 5 h for ELB-21. In contrast to EMRSA-16, the most prolonged PAEs were observed with ELB-21 and the shortest with SJG-136. However, it is only feasible to compare the PAE values obtained at the MIC for the two strains as all other PAEs were obtained at different MIC multiples.
Table 3.4. PAEs for exponential-phase cultures of VRE-10.

<table>
<thead>
<tr>
<th>Concentration of antibiotic</th>
<th>PAE (h) (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SJG-136</td>
</tr>
<tr>
<td>1/4xMIC</td>
<td>0.35 (0.3-0.4)</td>
</tr>
<tr>
<td>1/2xMIC</td>
<td>0.7 (0.6-0.8)</td>
</tr>
<tr>
<td>1xMIC</td>
<td>0.8 (0.5-1.1)</td>
</tr>
</tbody>
</table>

3.3.3. Electron microscopy

Electron micrographs of EMRSA-16 and VRE-10 incubated with ELB-21 at various sub-inhibitory and inhibitory concentrations showed a marked degree of morphological heterogeneity (*Figs. 3.11-3.14*). Cell division appears to be impaired and there is some evidence of drug-induced cell lysis. Furthermore, some drug-treated cells appear much larger than control cells and others have a thicker cell wall. The variety of morphological changes is likely to reflect the multiplicity of PBD dimer targets on the bacterial genome.
Fig. 3.11. Representative electron micrographs of EMRSA-16 cells at different magnifications; a) control and b) treated with 1/2xMIC of ELB-21.
Fig. 3.12. Representative electron micrographs of EMRSA-16 cells at different magnifications; a) treated with ELB-21 at MIC and b) at 2xMIC.
**Fig. 3.13.** Representative electron micrographs of VRE-10 cells at different magnifications; a) control and b) treated with ELB-21 at 1/4xMIC.
Fig. 3.14. Representative electron micrographs of VRE-10 cells at different magnifications; a) treated with ELB-21 at 1/2xMIC and b) at the MIC.
3.4. Discussion

3.4.1. Effect of bacterial growth phase on PBD dimer activity

Time-kill studies are usually performed with exponential-phase cultures. However, it is well established that the growth phase of the bacterial culture can affect the extent of killing by antibiotics. Furthermore, an exponential-phase *in vitro* model is not representative of an *in vivo* infection, where nutrient limitations such as reduced levels of glucose, iron and magnesium give rise to a population of slow-growing bacteria.

There have been a number of studies investigating the effect of bacterial growth phases on the bactericidal activity of an antimicrobial agent. Cozens *et al* (1986) demonstrated that β-lactam antibiotics were bactericidal when added to exponential-phase cultures of *E. coli*. However, they were bacteriostatic against non-growing bacteria. Therefore, β-lactams are highly dependent on the growth phase of the bacterial culture to exert their antimicrobial activity *in vitro*. In addition, Eng *et al* (1991) have shown that slow-growing bacteria are less susceptible to killing by antibiotics such as nafcillin and imipenem than are rapidly growing bacteria. These antibiotics only produced a bactericidal effect against exponential-phase cultures. A separate study on ciprofloxacin demonstrated that the antibiotic was bactericidal to both phases of bacteria (Zeiler, 1985). The bactericidal activity of antibiotics such as vancomycin and daptomycin is also most apparent when exponential-phase cultures are used (Aeschlimann *et al*, 1999; Lamp *et al*, 1992). Bactericidal activity is maintained in the lag phase but the time taken to reduce the colony counts by 3-log is longer than that in the exponential phase. Studies using quinopristin/dalfopristin have also indicated that bactericidal activity is observed with an exponential-phase culture but not with a lag-phase culture (Caron *et al*, 1997).

In general, there were only slight differences in the activity of PBD dimers against staphylococcal strains in different phases of growth. In this study and in contrast to the above observations, bactericidal activity was optimal against lag phase rather than exponential-phase cultures of EMRSA-15 and -16. This did not apply to VRE-10, as both
exponential- and lag-phase cultures were equally susceptible to the PBD dimers. This effect was most evident with DRG-16 against both MRSA strains. There was a greater than \( \log_{10} \) reduction in the lag phase at MIC and 2xMIC concentrations in comparison to the exponential phase, and no re-growth was observed at 24 h with the lag phase. ELB-21 was bactericidal against lag-phase EMRSA-15 and EMRSA-16 at the highest concentrations, although bacterial re-growth was observed against exponential-phases of the cultures.

3.4.2. Potential mechanisms involved in the initial rapid decline in bacterial viability

It is interesting to note that the decrease in colony counts within the first 2 h did not seem to differ between the growth phases, suggesting that, initially, the mechanism of action of the drugs is not affected by the growth phase. This would suggest that the drug binds efficiently to DNA in both non-replicating and replicating cells. One possible explanation is that in the exponential phase, many of the proteins necessary for DNA repair have been synthesised and those cells are much better equipped to eliminate DNA damage and excise cross-links within the genome. Lag-phase cells, which are just beginning to switch on their repair machinery, are slower to respond to any damage caused by the compounds. Since the PBD dimers are expected to bind to multiple DNA sequences within the genome, they may be binding to DNA sequences in lag-phase cells that encode DNA repair proteins, thus inhibiting their transcription and translation. Although this is a reasonable explanation for the two MRSA strains, it does not account for the complete killing observed with VRE-10 in both phases by ELB-21 and DRG-16. This difference could be due to different repair mechanisms found in enterococci and staphylococci or to the greater intrinsic susceptibility of enterococci to PBD dimers.

It is worth noting that an investigation of the effect of SJG-136 on transcription of DNA concluded that SJG-136 had little effect on inhibition of transcription once transcription had been initiated (Martin et al, 2005). It was determined that incubation of DNA with the compound prior to induced initiation of transcription was necessary to block transcription. This was possibly a direct result of the bifunctional mode of action of these
compounds; initially, reversible monoadducts with SJG-136 form at the preferred sequences in the DNA before complete cross-linking of both strands occurred. RNA polymerase is halted when it encounters a cross-link, whilst its encounter of a monoadduct could potentially lead to the displacement of the PBD dimer. This may explain why lag-phase bacteria are more susceptible to PBD dimers, as transcription in cells in the early stages of this growth phase has not been initiated and more cross-links are likely to form. In contrast, exponential-phase cells are dividing rapidly and transcribing DNA continuously and are likely to be less sensitive to the action of PBD dimers. Nevertheless, the molecular basis accounting for the differences between the two growth phases remains to be elucidated.

Rapid bactericidal activity within the first 2 h following exposure to PBD dimers was unexpected and is unlikely to be solely dependent on inhibition of DNA replication and transcription. A stress response may induce other cell pathways, leading to cell death. A rapid bactericidal effect is advantageous in certain infections such as endocarditis, osteomyelitis and meningitis, where an immune response does not play a major role in elimination of the pathogen due to the site of infection (with meningitis) or the high levels of dormant bacteria (endocarditis) (Finberg et al, 2004).

3.4.3. Concentration-dependent bactericidal activity

The bactericidal activity of the PBD dimers is concentration-dependent; other agents with concentration-dependent activity include fluoroquinolones (Klugman & Capper, 1997; Odenholt et al, 2000), daptomycin (Akins & Rybak, 2001) and aminoglycosides (Potel et al, 1991). The rate and the extent of killing are greater at increasing multiples of the MIC with 4xMIC being the most effective concentration of those examined in this study. Vancomycin (Lowdin et al, 1998) and β-lactams (MacGowan & Bowker, 1998) show a time-dependent bactericidal activity, although the extent of bacterial killing is no greater at concentrations higher than the MIC than at the MIC; in addition, in these cases the rates of killing are lower in comparison to those achieved with concentration-dependent antibiotics. Such differences are a useful guide in determining the best dosing schedules.
With time-dependent killing, the most important pharmacodynamic factor is the time that
the antibiotic is maintained above the MIC (t>MIC), whilst with concentration-dependent
killing, the bactericidal effect is most effective at the maximum serum concentration \( C_{\text{max}} \)
(a high \( C_{\text{max}}/\text{MIC} \) ratio is favourable) (Drusano, 2004).

The PBD dimers, like the fluoroquinolones, interfere with nucleic acid synthesis and
replication and, not surprisingly, both groups show a marked concentration-dependent
bactericidal activity. This is yet another similarity between these agents, in addition to
their excellent activity against both lag- and exponential-phase cultures. However, the
killing pattern observed with the PBD dimers is different from another group of DNA-
binding antimicrobial compounds, the HARP antibiotics (Ge et al, 2002). These authors
describe time-dependent killing similar to that obtained with vancomycin and they report
a bactericidal effect against MRSA, but only a bacteriostatic effect against enterococci.
In contrast, the PBD dimers are bactericidal against both VRE and MRSA, with the more
potent activity observed against VRE. In addition, the HARP compounds are reported to
be active only against replicating cells, whereas the PBDs are active against dividing and
non-dividing cells. The HARP agents bind non-covalently to DNA and this could partly
account for the variability of bactericidal activity (Gross et al, 2003).

3.4.4. Effect of high inoculum on PBD dimer activity

The bactericidal activity of the PBD dimers was tested against a high inoculum of
stationary EMRSA-16 (~9x10^9 cfu/ml) and VRE-10 (~8x10^8 cfu/ml) cultures.
Interestingly, a rapidly bactericidal effect was observed with both these high inoculum
cultures. Other studies have demonstrated that, with some antibiotics such as nafcillin
and vancomycin, a large inoculum can lead to a loss or reduction of bactericidal activity
(Laplante & Rybak, 2004). The bactericidal activity of ciprofloxacin was reduced against
a high inoculum \( S. \text{ aureus} \) culture of 10^8 cfu/ml in comparison to a culture of low
inoculum of 10^6 cfu/ml (Mizunaga et al, 2005). This effect has not been observed with
the PBD dimers, implicating that there is a good rapid antibacterial activity against high
inoculum cultures.
3.4.5. Bacterial re-growth after 24 h

As described previously, there was some bacterial re-growth observed at 24 h at the lowest MIC concentrations. Reasons for bacterial re-growth at 24 h are believed to be due to selection and emergence of resistant mutants, inactivation of compound or re-growth of bacterial cells that have escaped the antibacterial action by segregating to the walls of the flasks (NCCLS, 1999). The reason for bacterial re-growth after PBD treatment is unclear but it is not due to mutation. The MICs of colonies picked from the 24-hour plates were determined and found to be identical to those of the inoculum. It is unlikely that cells adhered to the wall of the glass flasks, as the flasks were shaken at 200 rpm and were only removed from the shaker for a short time for sampling. It is possible that within the first few hours a high proportion of PBD molecules enter the bacteria, depleting the broth of compound. If any cells have managed to escape the action of the drug or have repaired damaged DNA, they would be able subsequently to replicate.

3.4.6. Selection of an appropriate PAE method

For some concentration-dependent drugs such as the quinolones, where bactericidal activity is at its maximum at multiples of the MIC, the PAE also increases at higher concentrations. However, one problem that arises with increasing bactericidal activity and higher drug concentrations is that in some cases the PAE cannot be determined due to rapid bacterial killing. Various methods have been established for the determination of the PAE and the method chosen should depend on the type of agent involved (Craig & Gudmundsson, 1996). One of the methods frequently used to remove the antibacterial compound involves a dilution step; the treated culture is diluted one hundred-fold or one thousand-fold in fresh pre-warmed medium. A control culture containing the same dilution of drug as the test culture is included in order to ensure that there is no delay in growth due to the presence of residual compound. Another method of removing the agent relies on washing the treated cultures in PBS several times and then suspending the washed cells in fresh pre-warmed medium. If necessary, the bacterial cultures are diluted following the wash step (Craig & Gudmundsson, 1996).
As a result of the rapid killing observed following incubation of the bacteria for 1 h with the PBD dimers, dilution would result in very low starting counts. Furthermore, it was established that residual compound remaining after a one hundred-fold dilution affected bacterial growth. Upon comparison of control and diluted cultures, the delay in growth to reach 1 \( \log_{10} \) for ELB-21-treated cells was 7.5 h (Table 3.1). As a result a PAE could not be established accurately using this method.

These experiments suggest that a method involving washing the cultures with PBS would constitute a more appropriate method of compound removal. Therefore, an experiment was performed using this procedure with ELB-21 against EMRSA-16; the PAE was determined to be 1.45 h at the MIC and 2xMIC, and 3.45 h at 4xMIC (Table 3.2). The control culture in this PAE experiment was diluted fifty-fold to achieve a count closer to that of the treated cultures. The same experiment was repeated, but this time all cultures, including the control, were diluted ten-fold after the wash step. A ten-fold dilution maintained a good initial bacterial count, without excessive dilution of the cultures, and was introduced to ensure dilution of any residual compound remaining after the wash step. The PAEs obtained with this method using ELB-21 against EMRSA-16 were 0.65 h at MIC, 0.15 h at 2xMIC and 0.4 h at 4xMIC (Table 3.2). These results are not consistent with the results obtained with the fifty-fold dilution of the control culture. It is clear that there are differences in PAE values obtained using different methods.

3.4.7. PAE relative to mechanism of action

In general, compounds that interfere with nucleic acid and protein synthesis produce long PAEs as demonstrated with the aminoglycosides, quinolones and rifampicin (Barmada et al, 1993; den Hollander et al, 1998; Guan et al, 1992; Svensson et al, 1997). However, Ge et al (2002) reported a PAE against MRSA of only 0.1 h with the non-covalent DNA binding ligand GSQ1530. To date, these are the only PAEs reported for DNA-binding antibacterial agents other than the PAEs reported in this study. Furthermore, the oxazolidinones, which are protein synthesis inhibitors, exhibit relatively short PAEs
against MRSA and VRE (Rybak et al, 1998). These results suggest that the length of the PAE is not always related to the drug target.

An idea proposed by Li and colleagues (1997) is that cellular damage is directly related to the bactericidal activity of the drug and the greater the damage that has occurred the longer the PAE. They have demonstrated that there was a linear correlation between bactericidal activity and cellular damage and between bactericidal activity and the value of the PAE. Other groups, however, have reported no correlation between bactericidal activity and the PAE after exposure of bacteria to moxifloxacin (Boswell et al, 1999). These studies demonstrate the complexity of the PAE and to date no clear rationale that would account for the data reported above has been proposed.

Some insight into the mechanisms involved in the determination of the extent of the PAE has emerged from investigations of cellular metabolic parameters. Tobramycin led to inhibition of DNA, RNA and protein synthesis and, following removal of tobramycin, DNA and RNA synthesis returned to normal but this did not result in resumption of normal bacterial growth (Gottfredsson et al, 1995). The length of the PAE was related only to the period of inhibition of protein synthesis. Guan et al (1992) reported that DNA synthesis was inhibited in E. coli during and after treatment with enoxacin and the time necessary for the cells to resume normal DNA synthesis directly corresponded to the length of the PAE. In contrast to this finding, Gottfredsson et al (1995) have reported an increase in DNA synthesis in S. aureus, E. coli and P. aeruginosa after treatment with ciprofloxacin and suggested that this increase may be due to an increase in DNA repair initiated by a quinolone-dependent SOS response (Fuursted, 1997). In addition, the authors reported a reduction of DNA synthesis in S. aureus after rifampicin treatment, with a consequent increase before and during re-growth. Clearly, there are discrepancies in data produced by different authors, most likely as a result of methodological differences. However, all studies have shown that PAEs are associated with various metabolic changes. These findings suggest that the PAE reflects the period of time in which sub-lethal damage in bacteria is repaired.
The relatively short PAEs observed with ELB-21 against EMRSA-16 could be explained as rapid recovery from DNA damage. Alternatively, it is possible that ELB-21 is highly lethal, and only the population of cells that have escaped the action of the compound re-establish growth at a similar rate to the control. It is interesting to note the difference in PAEs for ELB-21 and SJG-136 between the two strains. Whilst ELB-21 gave a prolonged PAE against VRE-10 and not EMRSA-16, the opposite was true for SJG-136. Variations of the DNA repair processes between the strains could play a role here and this could be investigated further with DNA repair mutants.

Clearly, the bacterial strain and the nature of the antimicrobial agent are the main factors involved in determining the PAE; however, other factors such as growth conditions, temperature, length of exposure to the antibiotic and the bacterial growth phase also play a role (Boswell et al., 1999; Fuursted, 1997).

3.4.8. Effect of growth phase on PAE

The effect of the bacterial growth phase on the PAE has been investigated using a variety of antibiotics (Boswell et al., 1999; Pfeil & Wiedemann, 2000). The authors have shown that of all the antibiotics tested against MRSA and MSSA, which included vancomycin, moxifloxacin and genatmicin, only quinupristin/dalfopristin induced a PAE in both phases. They concluded that this effect might be due to the accumulation of the agent in the cytoplasm and to ribosome binding. PAEs of ELB-21 against lag- and exponential-phase EMRSA-16 cultures were similar, indicating that these agents induce a PAE in both phases. These results are consistent with the time-kill kinetics reported for this compound, as bactericidal activity was observed in both growth phases.

3.4.9. In vitro and in vivo PAEs

Although many PAEs are determined in vitro, it must be emphasised that the presence of an in vitro PAE does not necessarily predict the existence of a PAE in vivo. The PAE in vitro measures the re-growth of bacteria after complete removal of antibiotic, whilst a
PAE *in vivo* measures the re-growth of bacteria after the antibiotic level has fallen below the MIC; in the latter situation, significant quantities of the drug are still present in the system under investigation (Craig, 1993). To approximate the *in vivo* situation, Odenholt-Tornqvist *et al* (1992) and Oshida *et al* (1990) established the concept of the postantibiotic sub-MIC effect (PA SME). The PA SME is the effect on bacteria exposed to sub-MIC concentrations after previous exposure to higher multiples of the MIC. This variable reflects the *in vivo* PAE more accurately than the *in vitro* PAE, as, after a single dose, the drug concentration declines gradually to sub-MIC levels. They have compared the time delay of bacterial growth between PA SME and the SME of several antibiotics. SME is the effect of any delayed growth after bacterial exposure to sub-inhibitory concentrations without prior exposure to antibiotic. There were significant differences between the PA SME, the PAE and the SME. Antibiotics such as vancomycin, sparfloxacin and amikacin induced reasonable PAEs and variable SMEs against *S. pneumoniae*. The PA SMEs were significantly longer than the SMEs, indicating that bacterial growth is affected to a greater extent in bacteria that have been exposed to high concentrations of antibiotic. Similar observations have subsequently been reported, with clear differences between the SME and the PA SME (Licata *et al*, 1997; Lowdin *et al*, 1998; Odenholt, 2001). These results suggest that an *in vitro* analysis of the PAE alone may not be sufficient to establish that similar effects occur *in vivo* and may also explain why most PAEs *in vivo* are generally longer than those reported *in vitro*, as sub-inhibitory concentrations in serum are likely to have an effect (den Hollander *et al*, 1996; Lowdin *et al*, 1993).

In conclusion, the PAE is a useful pharmacodynamic parameter aiding the design of dosing regimens. However, it may be difficult to draw reliable conclusions from PAE data, as many factors are involved in the determination of the PAE. Furthermore, PAE values can vary widely under different circumstances.
CHAPTER FOUR

STUDIES ON THE MECHANISM OF ACTION OF PBD DIMERS
4.1. Introduction

Previous studies of the cytotoxic action of PBD dimers have established that these molecules bind to and cross-link DNA (Puvvada et al, 1997). For the purposes of the studies reported herein, it has been assumed that their antibacterial action results from penetration of the molecules into Gram-positive, but not Gram-negative, bacterial cells and binding to chromosomal DNA, resulting in inhibition of DNA function. Although these agents have been shown to bind to both naked and cellular mammalian DNA (Martin et al, 2005), this has not been shown in bacteria. The experiments performed in this chapter were conducted to shed light on the mechanism of antibacterial action of PBD dimers. DNA melting (or DNA denaturation) and interstrand cross-linking capacity were determined for ELB-21 and SJG-136 using agarose gel electrophoresis and Southern blotting; extracted and purified EMRSA-16 DNA (naked) and DNA extracted and purified from EMRSA-16 cells that have been treated with PBD dimer (in situ) were employed. In addition, 2-D electrophoresis was performed to determine if PBD dimers were able to modulate the proteome of EMRSA-16.

4.1.1. DNA melting

The technique of DNA melting has been frequently used to assess the potency of DNA-binding compounds (Wilson et al, 1997). DNA denaturation studies are based on the principle that duplex DNA strands dissociate into separate strands upon heating (Wilson et al, 1997). The T_m is the temperature at which half the double stranded DNA (dsDNA) has separated into single DNA strands (ssDNA). Absorbance (at 260 nm) is measured during a gradual increase of temperature; absorbance values increase as more dsDNA strands are transformed into ssDNA (Fig. 4.1). The increase in absorbance is not linear but is described by a sigmoidal curve that results from cooperative nucleic acid interactions. As the strands separate, there is a relatively sharp absorption transition and this increase is due to the unstacking of bases, known as the hyperchromic shift (Wilson et al, 1997). There are a number of factors that affect the T_m; these include GC content,
Fig. 4.1. Schematic representation of DNA melting: a) dsDNA separates into ssDNA upon heating; b) introducing cross-links in the DNA usually stabilises the helix and a higher temperature is required to separate the strands; c) an example of a melting experiment, showing the T_m of untreated DNA and DNA that has been incubated with a cross-linking agent (black line: native DNA; red line: DNA incubated with a cross-linking agent). The ΔT_m is the difference between the T_m of DNA incubated with compound and the T_m of native DNA.
DNA molecule length and salt concentration (Wilson et al., 1997). DNA rich in GC has a higher melting $T_m$ than DNA with a low GC% content, as more energy is required to break G:C bonds than A:T bonds. GC base pairs are more stable than AT pairs due to the presence of three hydrogen bonds rather than the two associated with AT bases. In addition, longer DNA molecules are held together by more hydrogen bonds and therefore require more energy to separate the strands, resulting in a higher $T_m$. The presence of high salt concentrations stabilises the DNA and reduces the $T_m$. Salt reduces the repulsion between the negatively charged phosphate groups of the DNA, thus generating a less energetically favourable environment for strand separation.

In addition to these factors, intercalating DNA-binding agents have an effect on the $T_m$. Generally, an intercalating agent that cross-links the DNA strands stabilises duplex DNA and leads to an increase in the $T_m$ (Wilson et al., 1997). The difference between the $T_m$ of native DNA and the $T_m$ induced by a DNA cross-linking agent is the $\Delta T_m$ (Fig. 4.1). The PBD dimers bind covalently to DNA and have been shown to significantly increase the $T_m$ of calf thymus DNA (Gregson et al., 2001; Gregson et al., 2004). The $\Delta T_m$ values induced by SJG-136 and DRG-16 with calf thymus DNA at a 1:5 ratio of [PBD dimer]/[DNA] were 25.7°C and 24.9°C respectively (Gregson et al., 2004). These values were recorded immediately following mixing of the agent with DNA and are indicative of the rapid and effective binding of PBD dimers to duplex DNA.

**4.1.2. Detection of interstrand cross-links by gel electrophoresis**

The gel electrophoresis procedure for determination of interstrand cross-links has been described by Futscher et al. (1992). It is based on the principle that cross-linked DNA will resist denaturation with agents such as formamide and manifest as dsDNA when run on a neutral gel (Fig. 4.2). Controls of completely denatured and non-denatured DNA samples are simultaneously run on the gel for comparison. Higher concentrations of the cross-linking agent are likely to result in more cross-linked regions of the DNA and a higher quantity of dsDNA. Analysis of cross-link formation at selected sites on the
bacterial genome can be achieved using agarose gel electrophoresis in combination with gene-specific probes.

**Fig. 4.2.** Schematic representation of the gel electrophoresis method for detection of DNA interstrands. DNA controls: a) denatured ssDNA and b) non-denatured dsDNA; c) cross-linked DNA will appear as dsDNA under denaturing conditions.

### 4.1.3. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis can be used to separate proteins by isoelectric focussing in one dimension followed by electrophoresis on the basis of molecular weight in the other. This allows the separation of more proteins than can be achieved by standard one-dimensional gel electrophoresis.
4.2. Materials and Methods

4.2.1. DNA extraction

All materials for DNA extraction were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. DNA was extracted according to the guanidium thiocyanate method described by Pitcher et al (1989). Exponential phase bacteria (40 ml) were harvested by centrifugation at 5,100 g for 5 min, the pellets washed twice in PBS and suspended in 100 µl of 100 mM phosphate buffer (Na₂HPO₄/NaH₂PO₄ at pH 7) containing 25 mg/ml of lysostaphin. The VRE-10 pellet was suspended in 100 µl of TE buffer (10 mM Tris-HCl/1 mM EDTA pH 8) containing 25 mg/ml of lysozyme. The suspensions were transferred to Eppendorf tubes, mixed and incubated in a 37°C water bath for 30-60 min to lyse bacterial cell walls. Cell lysis was completed by the addition of 0.5 ml of GES reagent (5M guanidium thiocyanate, 100 mM EDTA and 0.5% v/v sarkosyl) and the suspension agitated for several minutes using a vortex mixer. The lysates were placed on ice and 0.25 ml of ice-cold 7.5 M ammonium acetate was added, the tubes were mixed and kept on ice for 10 min. The DNA was extracted by addition of 0.5 ml of chloroform: 2-pentanol (24:1), tubes were mixed and centrifuged at 14,500 g for 15 min. The upper aqueous layer containing DNA was transferred to Eppendorf tubes. Ice-cold isopropanol (0.54 vol) was added to precipitate genomic DNA and the tubes were inverted gently until fibrous DNA became visible. The DNA pellet was recovered by centrifugation at 10,000 g for 10-20 sec. The pellet was washed twice with 70% ethanol and air-dried for up to 10 min. Care was taken not to over-dry the pellet, which was suspended in TE or phosphate buffer as necessary and incubated at 4°C for 24 h. DNA was quantified (A=260 nm) using a Perkin Elmer lambda 25 spectrophotometer (Wellesley, USA).

4.2.2. DNA melting

DNA was suspended in 10 mM phosphate buffer to give an absorbance reading of 0.6 at 260 nm in a 1-cm path length 1.4 ml quartz cuvette containing a magnetic stirrer. PBD
dimers were added to the DNA to achieve final concentrations of 0-10 µM and mixtures incubated for 1 h at 37°C in the spectrophotometer prior to determination of the melting profile. For the remaining experiments, bacteria were incubated with PBD dimers at the same concentrations (0-10 µM), the DNA extracted as described above and the melting profile determined. Cuvettes containing phosphate buffer for reference were placed with each DNA sample in the spectrophotometer. DNA melting was determined in a Varian Cary spectrophotometer (Palo Alto, California, USA). The temperature of the DNA was increased at a rate of 1°C/min over the range 40°C to 98°C; this procedure allows for complete denaturation of the DNA samples.

Absorbance values were imported into the Origin 7 computer package (OriginLab, Northampton, Massachusetts, USA) for analysis and the melting temperature determined at the midpoint of the normalised melting profiles. The increase in T_m (ΔT_m) was determined as: ΔT_m = T_m (DNA+compound) - T_m (control untreated DNA). All experiments were performed in duplicate.

4.2.3. Southern blotting

4.2.3.1. Materials

All materials and kits were purchased from Roche (Lewes, East Sussex, UK) unless otherwise stated. The following PCR primers for 16S and mecA probes were constituted by ThermoHybaid, Ulm, Germany:

387 F [5'-CGA AAG CCT GAC GGA GCA AC-3'] and
914 R [5'-AAC CTT GCG GTC GTA CTC CC-3'] amplify a 528-bp fragment of the 16S ribosomal gene, which incorporates an EcoKX restriction site;
449 F [5'-AAA CTA CGG TAA CAT TGA TCG CAA C-3'] and
761 R [5'-CTT GTA CCC AAT TTT GAT CCA TTT G-3'] amplify a 313-bp fragment of the mecA gene.
4.2.3.2. Probe synthesis

Genomic DNA was extracted from EMRSA-16 as previously described and suspended in TE buffer. Absorbance was measured at 260 nm, DNA diluted to a concentration of 0.2 µg/ml and used as a template in the PCR reactions. Digoxigenin (DIG)-labelled probes were synthesised using a PCR DIG probe synthesis kit according to the manufacturer’s instructions. The reaction mixture consisted of the following: amplification buffer (expand high fidelity buffer with 15 mM MgCl$_2$), PCR DIG probe synthesis mix (200 µM of dNTP mix - dATP, dCTP, dGTP, dTTP and DIG-11-dUTP at pH 7), 10 µM of forward and reverse primers, 2.6 U of enzyme mix (in 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM DDT, 0.1 mM EDTA, 0.5% Tween 20 v/v, Nonidet P40 v/v, 50% glycerol v/v) and 1 µg of DNA template. The mixture was made up to a volume of 50 µl with ultra pure water. A control with non-labelled dUTP was included as well as a control without DNA template. The PCRs were carried out in a Techgene thermocycler (Techne, Stone, Staffordshire, UK). The reaction conditions were programmed as follows:

- Initial denaturation: 95°C for 3 min
- Denaturation: 95°C for 30 sec
- Annealing: 62°C for 30 sec
- Extension: 72°C for 40 sec
- Final extension: 72°C for 7 min

The PCR products were run on a 1% (w/v) agarose gel to establish successful labelling; labelled probes have a higher molecular weight than non-labelled probes due to incorporation of the DIG moiety.

4.2.3.3. Formamide denaturation and electrophoresis

Genomic DNA from bacteria was extracted as previously described and suspended in TE buffer. PBD dimers at 1/2xMIC, MIC, 4xMIC and 10xMIC were added to 10 µg of DNA and incubated for 1 h at 37°C. The DNA was recovered by chloroform/pentanol and
isopropanol as described in the DNA extraction method, washed in ethanol and suspended in 40 µl of TE buffer. For other experiments, bacteria were incubated with PBD dimers at the same concentrations prior to DNA extraction and 10 µg of DNA suspended in 40 µl of TE buffer.

The DNA was restricted with EcoRI (5 units/µg DNA; New England Biolabs Inc., Ipswich, Massachusetts) for 2 h at 37°C. Restricted DNA was recovered by chloroform/pentanol and isopropanol as described in the DNA extraction method, washed with ethanol, and suspended in 40 µl of 10 mM phosphate buffer. To compensate for any DNA loss, DNA was quantified (A=260 nm) at this stage. DNA samples (6/7 µg of restricted DNA) were denatured by addition of two volumes of a solution consisting of 96% deionised formamide/1mM EDTA/0.05 % bromophenol blue. The samples were placed in a water bath at 65°C for 5 min and then kept on ice for 1-2 min before loading on a 0.5% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich) in TBE buffer (90 mM Tris/90 mM boric acid/2 mM EDTA). A non-denatured native DNA sample in 50% glycerol/0.1% bromophenol blue was loaded next to the denatured control sample. Electrophoresis was carried out at 60 V for approximately 2.5-3 h. The gel was scanned using a Molecular Imager FX (Bio-Rad, Hemel Hempstead, Hertfordshire, UK).

4.2.3.4. Membrane blotting

Restricted DNA was transferred to a positively charged nylon membrane using a VacuGene XL vacuum blotting system (Amersham Biosciences, Chalfont St. Giles, Buckinghamshire, UK) according to the manufacturer’s instructions. Once vacuum was established and maintained at 75 mBar, 0.25 M HCl depurinating solution was poured over the gel and left for 20 min. The solution was removed by a pipette and replaced with 1.5 M NaCl/0.5 M NaOH for a further 20 min; the gel was then neutralised in 1 M CH₃COONH₄/0.02 M NaOH for 20 min and the solution removed. Finally, 20 x concentrate SSC buffer (20xSSC - 0.3 M sodium citrate, 3 M NaCl, ~ pH 7) was poured over the gel and left for 1 h. The membrane was washed in 2xSSC before the bound
DNA was fixed to the membrane using ultraviolet light (254 nm) for 3 min in a ultraviolet oven (BDH, Biolab Ltd, Auckland, New Zealand).

Prehybridisation was carried out in a MICRO4 rotating incubator (Thermohybaid) with 20 ml of pre-warmed DIG Easy Hyb per 100 mm$^2$ membrane for 30 min at 42°C according to the manufacturer's instructions. DIG-labelled probe (8 μl of either 16S rDNA probe or mecA probe) was denatured by boiling in a water bath for 5 min and placed on ice for 1-2 min. It was immediately added to 3.5 ml of pre-warmed DIG Easy Hyb (per 100 mm$^2$ membrane). The prehybridisation solution was removed and replaced with the probe/DIG Easy Hyb solution. Hybridisation was carried out at 42°C with agitation for 12-16 h. The membrane was washed twice in low stringency buffer (2xSSC/0.1% SDS) for 5 min at room temperature. High stringency buffer (0.1xSSC/0.1% SDS) was pre-warmed to 68°C and the membrane was washed twice for 15 min at 68°C. The membrane was then rinsed in washing buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% v/v Tween 20 - DIG wash and block buffer set) at room temperature for up to 5 min. Blocking solution (10% blocking reagent in 0.1 M maleic acid, 0.15 M NaCl pH 7.5) was freshly prepared and 100 ml per 100 mm$^2$ membrane was incubated for 30 min at room temperature. Anti-DIG, conjugated to alkaline phosphatase, antibody was diluted in 20 ml of blocking solution to 75μU/ml, added to the membrane and incubated for 30 min at room temperature. The membrane was washed twice for 15 min in washing buffer and then equilibrated for 3 min in 20 ml of detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) at room temperature. The membrane was placed with DNA side facing up in a developing cassette and 1 ml of chemiluminescent alkaline phosphatase substrate, disodium 3-(4-methoxyspiro {1, 2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1$^{3,7}$]decan}-4-yl) phenyl phosphate, was applied in drops on the membrane. A plastic sheet was placed over the membrane and large bubbles were gently removed. The cassette was closed and the membrane incubated for 5 min at room temperature. Excess liquid was removed, the cassette closed completely and incubated for a further 10 min at 37°C. The membrane was exposed for 20-50 min in a fluorescence imager (GeneGnome, Syngene, Cambridge, UK).
4.2.4. Two-dimensional gel electrophoresis

4.2.4.1. Materials

All materials, kits and instruments were purchased from Bio-Rad (Hemel Hempstead, Hertfordshire, UK) unless otherwise stated.

4.2.4.2. Electrophoretic conditions

Exponential phase EMRSA-16 cultures were grown in the presence or absence of compound at 1/2xMIC for 1 h. The cells were recovered by centrifugation at 5 100 g for 5 min, the pellet was washed twice in PBS and suspended in 1 ml of PBS in lysing matrix tubes (Q-BIOgene, Cambridge, UK). The bacteria were placed on ice and lysed using a Fast Prep instrument (Q-BIOgene) for 45 sec. The tubes were placed on ice and, following centrifugation at 14 500 g, the supernatants containing the proteins were collected in fresh Eppendorf tubes.

The protein content was quantified by absorbance measurements at 595 nm (Perkin Elmer spectrophotometer) using a protein assay kit. The concentrations of the extracted proteins were estimated using a protein standard curve, derived from known bovine plasma protein concentrations; usually 500-800 μg of protein were isolated from each extraction. A sample (usually 100 μl, but more if necessary) of each extraction was prepared for isoelectric focusing (IEF) using a clean-up kit in accordance with the manufacturer’s instructions. This procedure eliminates contaminants such as salts, lipids and nucleic acids that can interfere with the IEF step. The protein pellet was suspended in the appropriate amount of rehydration/sample buffer (8 M urea, 2% CHAPS, 50 mM dithiothreitol, 0.2% Bio-Lyte 3/10 ampholyte, bromophenol blue) to give the desired concentration of protein. It is recommended that 2-100 μg of protein in 125 μl of sample buffer are loaded on a 7 cm immobilised pH gradient (IPG) strip. Protein concentrations in the range of 30-35 μg per IPG strip were used. The protein samples were loaded in an equilibration/rehydration tray and the IPG strips were carefully positioned over the
sample, ensuring that there were no trapped air bubbles. The strips were overlaid with 2 ml of mineral oil to prevent evaporation of the samples and the strips rehydrated for 14-16 h.

Electrode paper wicks were moistened with 8 μl of ultra pure water and positioned on the electrodes of the focusing tray. The IPG strips were removed from the equilibration/rehydration tray with a pair of forceps and drained of excess oil before positioning in the focusing tray. Mineral oil (2 ml) was overlaid to prevent evaporation of the sample during focusing. The tray was positioned in the PROTEAN IEF cell and the following conditions were used: conditioning step, linear ramp of 250 V, linear voltage ramp to reach 4 000 V at current limit of 50 μA/strip and final focusing for 10 000 V h. The IPG strips were removed immediately after focusing was completed, the excess oil drained and strips either frozen at -80°C or prepared for gel electrophoresis. Frozen strips were thawed and left for no longer than 15 min before proceeding to the next step. The IPG strips were equilibrated in two different SDS buffers. Lyophilised equilibration buffer 1 (6 M urea, 2% SDS, 0.05 M Tris/HCl pH 8.8, 20% glycerol, 2% dithiothreitol) was reconstituted immediately prior to use and the IPG strips were positioned gel side up in 2.5 ml of buffer contained within a clean equilibration/rehydration tray and gently agitated for 10 min. The buffer was discarded and replaced with equilibration buffer 2 (6 M urea, 2% SDS, 0.05 M Tris/HCl pH 8.8, 20% glycerol, 2.5% of iodoacetamide) for a further 10 min, which was subsequently discarded. The IPG strips were dipped in TGS buffer (25 mM Tris, 192 mM glycine, 0.1% SDS at pH 8.3) and loaded onto a 10% Tris-HCl precast gel. The IPG strips were embedded in molten agarose in the gel well. Once the agarose was set, the gels were run in TGS buffer in the Mini-Protean 3 at 200 V for 40 min. The gels were removed carefully from the cassette, placed in clean plastic containers containing a fixing solution of 10% methanol/7% acetic acid and agitated gently for 30 min. The solutions were discarded, replaced with 50 ml of Sypro Ruby protein gel stain and left on an orbital shaker for 16-18 h. The gels were washed with 10% methanol/7% acetic acid for 40-60 min in order to remove background stain and briefly washed with distilled water prior to recording of the images using the Molecular
Imager FX. Gels used for mass spectrometry analysis were also stained with Coomasie G250 stain (Sigma-Aldrich) for 2-3 h and destained in 25% acetic acid for 16-18 h.

4.2.4.3. Mass Spectrometry

Selected proteins were excised from the gel using the cut end of a sterile pipette tip. The gel pieces were transferred to a 1.5 ml Eppendorf tube and washed twice in distilled water. The pieces were destained by addition of 500 µl of 50 mM ammonium bicarbonate: acetonitrile (50:50) and mixed by a vortex mixer for 10 min. The solutions were discarded and the step repeated with fresh solution. This step was repeated until the stain was removed, which involved at least 2-3 further washes, and the solutions discarded. Acetonitrile (100 µl) was added to the gel pieces, the tubes mixed briefly and the solution discarded. After addition of a further 100 µl acetonitrile, the tubes were mixed thoroughly for 10 min and the solution decanted. This step was repeated at least 2-3 times or until the gel pieces were brittle. The gel pieces were dried in a SpeedVac (Thermo Electron, Waltham, Massachusetts, USA) for 30 min at 30°C. Modified trypsin (Promega, Southampton, UK) was suspended in the supplied acid buffer and then diluted to 40 ng protein/µl in chilled 50 mM ammonium bicarbonate. A small amount of trypsin solution (5-10 µl) was added to the dried gel pieces, which were then placed on ice for 30 min to facilitate penetration of the enzyme into the gel. Subsequently, another 20 µl of 50 mM ammonium bicarbonate were added to the gel pieces, mixed and the tubes incubated at 37°C for 14-16 h to achieve complete digestion.

Digested peptides were extracted from the gel pieces as follows: a solution of 5% formic acid in 50% acetonitrile (25 µl) was added to the gel pieces and mixed by vortex. The contents were placed in a water bath sonicator for 10 min. The gel pieces were separated by centrifugation at 14 500 g for 5 min and the supernatants transferred to fresh siliconised 1.5 ml tubes (Thermo Electron). Acetonitrile (30 µl) was added to the remaining gel pieces, the tubes were mixed for 5 min and centrifuged at 14 500 g for 5 min. The supernatants were pooled; 30 µl of 5% formic acid in 50% acetonitrile were added to the gel pieces, tubes were mixed and the above steps repeated 3-4 times. The
collected supernatants were dried in a SpeedVac (Thermo Electron) for 1 h at 40°C. A small volume of around 10 μl usually remained. If there was complete evaporation, the dried samples were rehydrated with 0.1% formic acid in water and the tubes were placed in a water bath sonicator for 10 min before preparation for mass spectrometry.

The samples were treated with 5 μl of 10% trifluoro-acetic acid and mixed thoroughly. A C_{18} Zip Tip (Fisher Scientific, Loughborough, Leicestershire, UK) was used to concentrate the sample. Wetting solution (0.1% trifluoro-acetic acid in 50/50 acetonitrile:water) was aspirated into the tip and dispensed three times. The same step was repeated with 0.1% trifluoro-acetic acid in water, followed by aspiration of the sample solution 10-15 times. The tip was washed three times with 0.1% trifluoro-acetic acid and the peptides released by filling and emptying the pippette 10-15 times into 4 μl of elution solution (alpha-cyanohydroxycinnamic acid in 0.1% trifluoro-acetic acid in 50/50 acetonitrile:water); 1 μl of this solution, containing the peptides, was spotted onto a matrix-assisted laser desorption/ionisation (MALDI) plate for MALDI time of flight (MALDI-TOF) (Applied Biosystems, Foster City, California, UK) analysis.

4.3. Results

4.3.1. DNA melting

Binding of the PBD dimers to duplex DNA covalently links the strands and as a result increases the melting temperature of the DNA. Representative sets of DNA melting curves obtained for naked and in situ treated DNA with ELB-21 are shown in Fig. 4.3 and Fig. 4.4 respectively, and those for SJG-136 in Fig. 4.5 and Fig. 4.6. The melting data imported in the Origin 7 software package was differentiated and normalised in order to estimate the T_m values. The first derivatives of all curves in each individual experiment are summarised in Fig. 4.7 and Fig. 4.8 for ELB-21 and SJG-136 respectively.
Fig. 4.3. DNA melting curves for naked DNA after incubation with ELB-21 for 1 h.
Fig. 4.4. DNA melting curves for *in situ* DNA, extracted following EMRSA-16 incubation with ELB-21 for 1 h.
Fig. 4.5. DNA melting curves for naked DNA after incubation with SJG-136 for 1h.
Fig. 4.6. DNA melting curves for *in situ* DNA, extracted following EMRSA-16 incubation with SJG-136 for 1 h.
Fig. 4.7. First derivatives of melting data for a) naked DNA and b) in situ DNA, following incubation with ELB-21 at different concentrations. The peaks of the curves represent the $T_m$ values of the DNA and there is some evident shift of $T_m$ values to higher temperatures in the presence of high concentrations of PBD dimer.
Fig. 4.8. First derivatives of melting data for a) naked DNA and b) in situ DNA, following incubation with SJG-136 at different concentrations. The peaks of the curves represent the T_m values of the DNA and there is some evident shift of T_m values to higher temperatures in the presence of high concentrations of PBD dimer.
The melting temperature of the DNA was increased by 23.5°C and 21.4°C following treatment of naked DNA with 10 µM of ELB-21 and SJG-136 respectively. The $\Delta T_m$ of in situ treated DNA with ELB-21 at the same concentration was 14°C, while that for SJG-136 was 10-fold lower. In all cases, there were no significant $\Delta T_m$ increases at the lowest 0.1 µM concentration used. The data in Table 4.1 represents the average of two $\Delta T_m$ determinations. The $\Delta T_m$ of EMRSA-16 DNA alone was 69.3°C (±0.3).

Table 4.1. $\Delta T_m$ for naked and in situ treated EMRSA-16 genomic DNA with the PBD dimers ELB-21 and SJG-136. Ranges of two experiments are shown in brackets.

<table>
<thead>
<tr>
<th>PBD dimer</th>
<th>Conc. of compound (µM)</th>
<th>Average $\Delta T_m$ (°C) after 1 h incubation of EMRSA-16 DNA with a PBD dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Naked</td>
</tr>
<tr>
<td></td>
<td></td>
<td>In situ</td>
</tr>
<tr>
<td>ELB-21</td>
<td>0.1</td>
<td>1.9 (1.8-2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.7 (0.9-2.5)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>7.7 (7.4-8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.1 (2-2.2)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>23.5 (21-26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14 (13.4-14.6)</td>
</tr>
<tr>
<td>SJG-136</td>
<td>0.1</td>
<td>1 (0.6-1.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 (0.6-1.4)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.2 (1.7-4.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 (0.2-0.8)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>21.4 (21.2-21.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4 (0.6-2.2)</td>
</tr>
</tbody>
</table>

4.3.2. Southern blots

4.3.2.1. EMRSA-16 genomic digests, agarose gels

Southern blots were performed, based on the methods described by Futscher et al (1992). *EcoR*1 digested DNA fragments were denatured with formamide. DNA fragments containing a cross-link induced by a PBD dimer will renature in the neutral agarose gel and appear as dsDNA. Native and denatured DNA samples were included as controls. The 0.5% (w/v) agarose gels were scanned using the Bio-Rad Imager FX (*Fig. 4.9* and *Fig. 4.10*). The bands in the denatured control lanes (ssDNA) appear less prominent than
those in the native control lanes (dsDNA). Treated DNA lanes resemble the native control lane, indicating that the majority of fragments appear as dsDNA.

**Fig. 4.9.** Formamide-denatured, EMRSA-16 EcoR1 genomic digests of a) naked and b) *in situ* DNA, following incubation with different ELB-21 concentrations; electrophoresis was undertaken in a 0.5% (w/v) agarose gel. Marker bands are from a λ *Hind* III digest (2-23 kb). Cntrl (control), denat. cntrl (denatured control).
Fig. 4.10. Formamide-denatured, EMRSA-16 genomic digests of a) naked and b) in situ DNA, following incubation with different SJG-136 concentrations; electrophoresis was undertaken in a 0.5% (w/v) agarose gel. Marker bands are from a λ Hind III digest (2-23 kb). Cntrl (control), denat. cntrl (denatured control).
4.3.2.2. Southern blot, meca probe

*Fig. 4.11* shows DNA fragments detected using the meca probe after incubation with naked and *in situ* treated DNA for 1 h with ELB-21. The meca gene was detected on a single 20 kb fragment in the restricted native control DNA. The actual gene is 2 kb in length and does not contain an *EcoR* restriction site; this fragment therefore contains 18 kb of adjacent DNA sequence. Double stranded DNA is evident in both naked and *in situ* samples at a concentration equivalent to 1/2xMIC, indicating that the meca gene or adjacent DNA is cross-linked by sub-inhibitory concentration of ELB-21.

*Fig. 4.11.* Southern blot of EMRSA-16 genomic digests of a) naked and b) *in situ* DNA after incubation with different ELB-21 concentrations and probed with meca. Cntrl (control), denat. cntrl (denatured control).
Double stranded DNA bands were evident when naked DNA was incubated with sub-inhibitory concentrations of SJG-136 (Fig. 4.12). However, dsDNA bands were evident only in samples that had been extracted from bacteria exposed to higher concentrations of SJG-136 (equivalent to 4xMIC and 10xMIC); ssDNA was observed at all concentrations.

Fig. 4.12. Southern blot of EMRSA-16 genomic digests of a) naked and b) in situ DNA, after incubation with different SJG-136 concentrations and probed with mecA. The dsDNA fragment is ~ 20 kb. Cntrl (control), denat. cntrl (denatured control).
4.3.2.3. Southern blot, 16S rDNA probe

A more complex pattern was observed when fragments were probed with the 16S rDNA: there are five copies of this gene in EMRSA-16. This gene also contains an EcoRI restriction site. Searching the EMRSA-16 genome (Holden et al, 2004) database using the Artemis DNA viewer program (www.sanger.ac.uk/software/artemis) indicated an EcoRI DNA restriction profile consisting of fragments containing 16S rDNA of the following sizes: 1.1, 1.2, 2.6, 3.5, 3.8, 4.3 and 17.9 kb. These are comparable to those obtained in this experiment (Fig. 4.13 and 4.14).

There was little variation in the pattern of cross-linking between naked and in situ treated DNA with ELB-21 (Fig. 4.13). In contrast, there was a higher degree of cross-linked sites observed within naked DNA incubated with SJG-136 than in situ treated DNA (Fig. 4.14).
Fig. 4.13. Southern blot of EMRSA-16 genomic digests of a) naked and b) *in situ* DNA after incubation with different ELB-21 concentrations and probed with *16S* rDNA. Marker bands are from a λ *Hind III* digest (2-23 kb). Cntrl (control), denat. cntrl (denatured control).
**Fig. 4.14.** Southern blot of EMRSA-16 genomic digests of a) naked and b) *in situ* DNA after incubation with different SJG-136 concentrations and probed with 16S rDNA. Marker bands are from a λ *Hind* III digest (2-23 kb). Cntrl (control), denat. cntrl (denatured control).
4.3.3. DNA sequences, recognised by the PBD dimers, in the EMRSA-16 genome

The genomes of several MRSA strains have been published and recently the 2.9 Mb genome of EMRSA-16 has been sequenced (Holden et al, 2004). Southern blots indicated that the PBD dimers enter the cell and cross-link DNA; it was therefore of interest to estimate the number of potential targets within the EMRSA-16 genome. The DNA sequences recognised by ELB-21 (and DRG-16) and SJG-136 are Pu-GA(A/T)TC-Py and Pu-GATC-Py respectively (Martin et al, 2005; Smellie et al, 2003). The EMRSA-16 genome was searched for the occurrence of these sequences using the Artemis programme (Table 4.2). The most commonly occurring sequence recognised by SJG-136 was AGATCC, which was located 742 times within the genome. The least common sequence, GGATCC, was also recognised by SJG-136 and occurred 167 times. There were a high number of these sequences appearing in the coding region, although they were also found in non-coding regions.

Table 4.2. Incidence of PBD dimer binding motifs in the EMRSA-16 genome.

<table>
<thead>
<tr>
<th>PBD dimer sequence</th>
<th>Coding regions and pseudo genes</th>
<th>Miscellaneous</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELB-21/DRG-16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGATTCT</td>
<td>313</td>
<td>198</td>
<td>512</td>
</tr>
<tr>
<td>AGATTCC</td>
<td>125</td>
<td>99</td>
<td>224</td>
</tr>
<tr>
<td>GGAATCC</td>
<td>76</td>
<td>106</td>
<td>182</td>
</tr>
<tr>
<td>GGATTCT</td>
<td>172</td>
<td>146</td>
<td>318</td>
</tr>
<tr>
<td>SJG-136</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGATCC</td>
<td>430</td>
<td>312</td>
<td>742</td>
</tr>
<tr>
<td>AGATCTC</td>
<td>162</td>
<td>109</td>
<td>271</td>
</tr>
<tr>
<td>GGATCC</td>
<td>92</td>
<td>75</td>
<td>167</td>
</tr>
</tbody>
</table>
4.3.4. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed with extracted and purified proteins from EMRSA-16 cells that had been exposed to sub-inhibitory concentrations of the PBD dimers (Fig. 4.15). Increases and decreases in the relative concentrations of a number of proteins following exposure to these compounds are apparent. The IPG strips used in this experiment separated proteins with isoelectric points within the pH 4-7 range. Therefore, the proteins in Fig. 4.15 are likely to represent a fraction of the total EMRSA-16 proteome and do not include strongly acidic or alkaline proteins (Hecker et al, 2003).
Fig. 4.15. Representative two-dimensional gels of proteins extracted from a) untreated EMRSA-16 culture and EMRSA-16 incubated with 1/4xMIC of b) ELB-21, c) SJG-136 and d) DRG-16. Proteins that appear to have decreased or increased in concentration following PBD dimer treatment are in blue circles and red circles respectively.
4.4. Discussion

4.4.1. DNA melting of EMRSA-16 genome

The aim of these experiments was to determine the DNA-binding capacity of the PBD dimers following incubation with EMRSA-16 and EMRSA-16 DNA. A rise in $T_m$ of DNA extracted from cells exposed to PBD dimers, expressed as $\Delta T_m$, would provide convincing evidence that these compounds have the capacity to penetrate the cell envelope and interact with the chromosome.

Initial experiments to determine $\Delta T_m$ were undertaken using ELB-21 concentrations equivalent to MIC, 2xMIC and 4xMIC. There were, however, no detectable changes in the $T_m$ at these concentrations. The results reported in Chapter 3 clearly demonstrated that concentrations equivalent to 2xMIC and 4xMIC were antibacterial and, in the majority of cases, bactericidal. This suggests that the inability to determine $\Delta T_m$ at these concentrations was due to an inherent lack of sensitivity of the DNA melting technique. The $T_m$ values were therefore determined at higher, non-physiological concentrations over the range 0.1-10 μM. Incubation of naked EMRSA-16 DNA with the PBD dimers at these concentrations resulted in large increases in the melting temperature, demonstrating their capacity to form interstrand cross-links (Table 4.1). There were small differences in the $\Delta T_m$ of DNA from SJG-136-treated cells. The $\Delta T_m$ from ELB-21-treated cells was significantly higher although comparative analysis between the two agents is not appropriate here due to the high non-physiological concentrations used and the differing MIC values for the two dimers. As treating bacteria at high concentration of PBD dimer is likely to induce major cell damage, and differences in melting temperature could only be demonstrated at concentrations well above the MIC, other more sensitive means of determining interactions with the DNA at concentrations approximating the MIC were explored, as reported below. Nevertheless, these experiments have shown that ELB-21 and SJG-136 bind to naked EMRSA-16 DNA, cross-link the DNA strands and increase the $T_m$. 

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4.4.2. Southern blots

The DNA melting experiments indicated that PBD dimers bind to naked DNA at high concentrations; the technique used was unable to detect DNA cross-links at PBD dimer concentrations approximating the MIC. However, the results obtained using formamide-denatured DNA gel electrophoresis have demonstrated extensive DNA interstrand cross-linking at physiological concentrations of the PBD dimers.

The *mecA* southern blots obtained for EMRSA-16, following incubation of naked DNA with ELB-21 and SJG-136 (*Fig. 4.11a and 4.12a*), demonstrate that both ELB-21 and SJG-136 are capable of binding to naked DNA and cross-link the DNA at concentrations below the MIC. ELB-21 was more potent *in situ* than SJG-136, as dsDNA was evident at all concentrations of ELB-21; with SJG-136, dsDNA bands were only observed at the higher concentrations used (4×MIC and 10×MIC). This indicates that either SJG-136 enters the cell less efficiently than ELB-21, or that the *mecA* gene and the surrounding DNA region encodes less favourable binding sites for SJG-136 than ELB-21. Alternatively, the difference could be due to the greater DNA-binding potency of ELB-21. Nevertheless, these are the first results that clearly demonstrate that PBD dimers are able to penetrate bacterial cells and cross-link regions of intracellular DNA.

It is interesting to note that there are two separate dsDNA bands observed with naked DNA following incubation with both PBD dimers (*Fig. 4.11a and 4.12a*). As the restriction enzyme is introduced into the assay mix after 1 h incubation of the DNA with the PBD dimers, it is possible that the compounds block some *EcoR1* sites, interfering with enzymatic function. *EcoR1* cleaves DNA at G/AATTC sites. ELB-21 and SJG-136 recognise Pu-GA(A/T)TC-Py and Pu-GATC-Py respectively; they can, however, also bind to lower affinity sites (Martin *et al.*, 2005), which may include an *EcoR1* site. This may account for the presence of a faint high molecular weight band in some lanes in *Fig. 4.11a and 4.12a*. Double bands were not observed with DNA extracted from PBD dimer-treated cells, possibly because fewer drug molecules gained access to intracellular DNA and these bound preferentially to high affinity sites.
Southern blots for 16S rDNA were more complex than those for meca due to multiple copies of the gene on the EMRSA-16 genome and to the presence of an EcoR1 site within the gene. The 16S rDNA probe also contained an EcoR1 site, so all restricted fragments were detected. The 16S gene was chosen because it covered five different DNA regions of the genome, and was expected to give a broader representation of DNA-binding within the different regions. Although the DNA patterns are more complex, the results are consistent with those obtained for the meca southern blots. There is clear evidence of dsDNA with both naked and in situ DNA exposed to PBD dimer at most concentrations employed. In a similar fashion to meca Southern blots, ELB-21 induced more cross-links after exposure of the bacteria than SJG-136 (Fig. 4.13b and 4.14b). There was a higher degree of cross-linking with naked compared to in situ DNA after incubation with SJG-136 (Fig. 4.14). As observed in gels probed with meca, there were additional bands of higher molecular weight in many of the lanes, most likely due to EcoR1 site inhibition.

It can be concluded that both compounds investigated bind to intracellular DNA although ELB-21 induces cross-links in a more efficient manner, and at lower concentrations, than SJG-136.

4.4.3. Identification of target DNA sequences using Artemis software

Analysis of the EMRSA-16 genome, using the Artemis program, provided information regarding the frequency of target sequences. There were no major differences between the number of sequences recognised by ELB-21/DRG-16 and SJG-136, even though ELB-21 binds to sequences containing an extra base pair (Table 4.2). This may indicate that the frequency of the target sequences does not determine the potency of the PBD dimers. However, studies on SJG-136 have shown that AGATCT and GGATCC are the preferred sequences for DNA binding (Martin et al, 2005; Smellie et al, 2003) and these are much less common in the EMRSA-16 genome than AGATCC, which was found 742 times (Table 4.2). Binding profiles are further complicated by the fact that a PBD dimer with a n=5 diether linker (such as ELB-21 and DRG-16) can recognise 6 bp sequences in addition to the preferred 7 bp sequences (Smellie et al, 2003). Therefore, ELB-21 and
DRG-16 may also bind to the 6 bp sequences recognised by SJG-136 but with lower affinity and their greater potency may be due to these additional binding sites. As described in Chapter 1, the differences in the potencies of the compounds are believed to be due to the length of the diether linker of the two monomers and the exounsaturation of the monomers. How much the frequency of specific binding sites impacts on the potency of the PBD dimers remains to be elucidated.

A large number of PBD dimer binding motifs were found within coding regions and some of these genes are likely to be essential for bacterial survival. However, non-coding regions may also be potential targets if binding sequences are located in close proximity to coding sequences, as binding will inhibit upstream or downstream transcription. In addition, cross-links in non-coding regions may inhibit the separation of the two DNA strands, block DNA polymerase and thereby terminate DNA replication.

As described above, cross-links were observed in DNA extracted from viable bacterial cells treated at sub-inhibitory concentrations. At such concentrations, the extent of cross-linking is likely to be low and insufficient to cause bacterial death; as the damage caused to the DNA may be limited, cross-links may be readily repaired.

4.4.4. Two-dimensional gel electrophoresis

Several genomes of *S. aureus* have been sequenced; some 2,600 proteins are encoded within the genome (Hecker et al., 2003). Proteomics is a useful tool for comparing the expression of proteins by bacteria exposed under different environmental pressures. Experiments were conducted to determine the differential expression of proteins in EMRSA-16 after incubation with PBD dimers.

As proteins encoded by the genome are differentially expressed over the growth cycle, only a proportion of proteins can be resolved at a given time. Furthermore, using a gradient of pH 4–7 selects for proteins with an isoelectric point approximating neutral and proteins with isoelectric points greater than pH 7 remain undetected. Since all cultures,
treated and untreated, were grown under exactly the same conditions, the only determining factor for changes in concentration of any protein should be the presence of PBD dimers (Fig. 4.15).

The main purpose in attempting to identify proteins modulated by the PBD dimers was to establish if incubation with the PBD dimers at sub-inhibitory concentrations had an effect on the transcription of some proteins. If proteins could be identified by mass spectrometry, their corresponding genes could be examined for the presence of PBD dimer binding motifs using the Artemis software, correlating PBD dimer sequence-selective binding to changes in protein expression following PBD exposure. Furthermore, the identification of stress proteins or proteins associated with DNA damage would confirm that PBD dimers target DNA and help to identify proteins involved in DNA repair.

Several proteins that appeared to differ in concentration, following incubation with the PBD dimers, were excised and analysed by MALDI TOF. Protein sequences could not be obtained and there was a high level of background interference, most likely due to insufficient quantities of protein. As discussed previously, there is a limit to the quantity of protein (2-100 µg) that can be loaded onto 7 cm IPG strips; in the experiments reported here ~ 35 µg protein was routinely employed. Several attempts were made to load higher concentrations of proteins on the strips, but in all cases severe streaking was observed (Fig. 4.16). The optimum protein loading concentration was in the region of 35-40 µg (Fig. 4.15), an amount insufficient for amino acid sequencing. This concentration was low in comparison to that reported in another proteomic study of S. aureus by Cordwell et al (Cordwell et al, 2002); these workers employed 250 µg of protein and longer 18 cm IPG strips were used.

The two-dimensional gels for PBD dimer-treated cells obtained using 7 cm IPG strips (Fig. 4.15) indicated reproducible and significant changes in the concentration of certain proteins and this warrants further investigation. However, due to lack of time and resources, proteins of interest could not be analysed by MALDI TOF. The time and
equipment necessary for employment of 18 cm IPG strips is outside the scope of this project. The identification of such proteins should provide crucial information regarding the nature of the modulation of gene expression in PBD dimer-treated bacterial targets.

Furthermore, two-dimensional gel electrophoresis could be used as a tool to compare the protein patterns induced by the PBD dimers to those of other known antibacterial agents in order to shed more light into the mechanism of action of the PBD dimers. A proteomic study investigating the effect of cell wall-active antibiotics identified proteins associated with oxidative damage and the stress response (Singh et al, 2001). Interestingly, these proteins were unaffected by antibiotics that act by inhibiting RNA-polymerase or protein synthesis. This suggests that drugs that act on different cellular targets induce different protein responses. In addition, changes in growth conditions or growth medium and different environmental stimuli can also induce specific physiological states in bacteria that are characterised by differential protein expression; such distinct protein profiles have been termed “proteomic signatures” by VanBogelen and colleagues (1999). The authors have described a variety of protein signatures in E. coli, including protein signatures of cells subjected to DNA damage. For example, DNA damaged cells invariably expressed the RecA protein, which is involved in DNA repair. Protein signatures could be used to identify the physiological state that new agents, such as the PBD dimers, induce in bacteria, whether through DNA damage or inhibition of protein synthesis.
Fig. 4.16. Severe streaking on the two-dimensional gels observed with a high protein concentration load.
CHAPTER FIVE
EFFICACY OF SJG-136 AND ELB-21 IN THE MURINE MRSA PERITONITIS MODEL
5.1. Introduction

The PBD dimers exhibited potent antibacterial activity in vitro and were subjected to an initial investigation of their in vivo efficacy. Before a compound can progress to clinical trial, its efficacy must be determined in animal models to provide a first indication of a therapeutic effect. There is a large number of established animal models and selecting an appropriate model is dependent on the type of pathogen and the antibacterial agent under investigation (O'Reilly et al., 1996). For initial evaluation of novel antibacterial agents, mouse protection models are most commonly used because they are relatively simple and short in duration and usually give a good indication of potential drug potency in man (O'Reilly et al., 1996). In a mouse protection model, the mice are infected in a single step, followed by an injection of the investigational drug either immediately or 0.5-1 h later. Efficacy is usually evaluated by endpoint survival of treated mice monitored over a specified period of time, cfu count, serum pharmacokinetics and/or the 50% effective dose (ED$_{50}$). The ED$_{50}$ is the dose at which 50% of the mice survive. It is important to establish reproducible infections in mouse models and the type of pathogen being investigated will determine whether or not an additional agent to such an adjuvant is necessary to achieve a successful infection. For organisms such as S. pneumoniae and S. pyogenes, infections can be established naturally without the addition of an adjuvant. However, for pathogens such as S. aureus, gastric mucin is usually co-administered in order to enhance the virulence of the organism and achieve reproducible results (O'Reilley et al., 1996). In addition, mice should be infected with virulent human pathogens whenever possible.

For the purpose of this study, an animal model suitable for assessing the antibacterial activity of the PBD dimers against staphylococcal infections was sought. As discussed previously, MRSA causes both localised tissue infections, such as those in surgical wounds, and life-threatening systemic infections; therefore several potential animal models that mimic human infections would suffice in order to provide an initial indicator of efficacy: the neutropenic murine thigh model, the neutropenic murine pneumonia model and the systemic murine peritonitis model. These models are well established, are
described in the literature and are the most commonly used models for the investigation of new antibacterial agents against MRSA (van Ogtrop et al, 2000; Weiss et al, 2004; Zuluaga et al, 2006).

In the neutropenic thigh model, the immune system of the mouse is compromised and the mice are unable to control subsequently induced infections. This is achieved by injecting the mice with immunosuppressive cytotoxic agents such as 5-fluorouracil, methotrexate or more commonly cyclophosphamide. Cyclophosphamide suppresses T- and B- cell function and reduces the neutrophil count and, when given in the appropriate dose (100-200 mg/kg), induces neutropenia in the mice without severe untoward effects (Zuluaga et al, 2006). The thighs of the mice are subsequently injected with the appropriate inoculum (recommended $10^5$ cfu per thigh). This model establishes a localised infection in the thigh muscles only when the animals are immunosuppressed and the infections are usually sub-clinical or mild. Mice are injected with the test compound intravenously, subcutaneously or intramuscularly. Thighs are harvested and homogenised over a specified time period and the cfu deduced from both control and treated mice. This model allows the analysis of the drug-pathogen interaction \textit{in vivo} without the interference of host defences. In addition, this model has been used for the evaluation of \textit{in vivo} PAEs and their relevance and also for analysis of dosing/pharmacokinetics of antibacterial agents (O'Reilly et al, 1996). Another model that also utilises immunosuppression is the murine pneumonia model (Zuluaga et al, 2006). In similar fashion to the thigh model, mice are immunosuppressed and are inoculated by inhalation of the \textit{S. aureus} inoculum dispersed as small droplets. Mice are treated with the test compound either subcutaneously or intravenously, sacrificed at specified time intervals, the lungs are harvested and homogenised and cfu determined.

The peritonitis model is induced by injecting the mice intraperitoneally with a high inoculum of \textit{S. aureus} ($10^7$ cfu) with the addition of gastric mucin as an adjuvant. The mice are subsequently treated intravenously and survival is the endpoint result monitored over a period of 5-7 days (O'Reilly et al, 1996).
For initial studies of the \textit{in vivo} efficacy of the PBD dimers, the systemic peritonitis model was deployed as this model has been used to investigate the \textit{in vivo} efficacy of other DNA-binding agents such as the HARPs (Burli \textit{et al}, 2004a); it is a less complex model than the alternatives as it does not involve induction of the immunocompromised state.

Previous work on the PBD dimer SJG-136 has indicated that \textit{in vivo} activity was well correlated to \textit{in vitro} activity (Alley \textit{et al}, 2004). SJG-136 is well tolerated \textit{in vivo} and there are no drug-related deaths or severe side effects (Wilkinson \textit{et al}, 2004). Furthermore, there is only moderate binding to plasma proteins and the drug is stable in blood for at least 6 h. In the experiments described herein, the efficacy of SJG-136 and the more potent PBD dimer ELB-21 were investigated using an \textit{in vivo} murine peritonitis MRSA model.

\section*{5.2. Materials and Methods}

A single colony of MRSA 187456 (a wound isolate from St. Thomas’ Hospital, London) or EMRSA-16 was used to inoculate 15 ml of pre-warmed BHI broth (Oxoid). The culture was incubated at 37°C with shaking at 200 rpm to a density equivalent of $A_{600} = 1.2$. The cells were recovered by centrifugation at 5 100 g for 6 min, washed once with PBS and suspended in 10 ml of PBS. To 1 ml of the cell suspension, 4 ml of PBS and 5 ml of 10\% mucin (Sigma-Aldrich or Difco) in PBS were added. This gave a final inoculum of $\sim 1.0 \times 10^7$ cfu/ml. Groups of 5-9 five- to six-week-old female ICR CD1 (Harlan, Loughborough, Leicestershire, UK) mice were injected \textit{via} the intraperitoneal route (i.p) with 0.5 ml of the bacterial suspension. One hour later, 100 µl of various concentrations (0.125-7 mg/kg of body weight) of SJG-136 or ELB-21 were administered by intravenous (i.v) or i.p injection. The same number of infected but untreated mice were used a control. The PBD dimers were initially dissolved in DMSO and then diluted in PBS/0.05\% Tween 80 to give a final DMSO concentration of 1\% v/v. The potential protective effect of a dose of SJG-136 or ELB-21 administered 0.5 h pre-infection was also evaluated. Mice were monitored over a five-day period with survival as the primary
endpoint. Animals were closely monitored at least 3 times a day within the first 24-48 h post-infection (the expected time frame for developing a systemic infection) for signs of systemic infection; symptoms of systemic infection include high respiration rate, elevated temperature (>2°C), depressed behaviour, reduced activity, hunched posture, closed eyes and increased sensitivity to light. If any of these signs persisted for more than a day the animals were killed by euthanasia. All procedures were conducted in accordance with Home Office Project Licence PPL 70/6179: “Treatment of Gram positive bacterial infections”.

5.3. Results

Mice infected with STH MRSA were treated 1 h post-infection with either SJG-136 (i.v) or ELB-21 (i.v. or i.p) at different doses. A summary of all experiments, including infected and untreated control mice, are represented in Fig. 5.1 (SJG-136), Fig. 5.2 (ELB-21 i.v) and Fig. 5.3 (ELB-21 i.p). Unexpectedly, 100% killing of infected but untreated mice was achieved in only three experiments overall and there were various degrees of survival (up to 45%) in the remaining experiments. The maximum doses used in the experiments were 5 mg/kg and 7 mg/kg for SJG-136 and ELB-21 respectively. Even at these higher concentrations, there was no significant increase in the survival of treated mice in comparison to those treated with lower concentrations. The highest percentage of survival of treated mice in any experiment was 55% for mice treated with 3 mg/kg of ELB-21 (i.v) (Fig. 5.2). However, due to the variability of mice survival in the MRSA-infected control groups, these results are inconclusive.
Fig. 5.1. Dose-effect relationship of the PBD dimer, SJG-136 (i.v dose), in an experimental mouse peritonitis infection with STH MRSA; multiple experiments are represented. The number of mice (out of a group of 5-9) that survived following inoculation with STH MRSA and injection with SJG-136 was determined after 5 days and the results are expressed as percent survival. All mice that were injected with SJG-136 alone survived (result not shown).
Fig. 5.2. Dose-effect relationship of the PBD dimer, ELB-21 (i.v dose), in an experimental mouse peritonitis infection with STH MRSA; multiple experiments are represented. The number of mice (out of a group of 5-9) that survived following inoculation with STH MRSA and injection with ELB-21 was determined after 5 days and the results are expressed as percent survival. All mice that were injected with ELB-21 alone survived (result not shown).
Fig. 5.3. Dose-effect relationship of the PBD dimer, ELB-21 (i.p dose), in an experimental mouse peritonitis infection with STH MRSA; multiple experiments are represented. The number of mice (out of a group of 5-9) that survived following inoculation with STH MRSA and injection with ELB-21 was determined after 5 days and the results are expressed as percent survival. All mice that were injected with ELB-21 alone survived (result not shown).
5.4. Discussion

Initial studies with EMRSA-16 were unable to establish a satisfactory endpoint as all mice survived after an i.p injection of a lethal inoculum of the bacteria. Since bacterial virulence is often lost after long-term storage, a recent hospital MRSA isolate was sought and used in all subsequent experiments. Studies with the fresh MRSA isolate gave similar results to the preliminary investigation with EMRSA-16 and all mice survived. Mucin is co-administered with the bacterial organisms as an adjuvant to promote the virulence and proliferation of bacteria and to achieve reproducible infections. The quality of mucin used in those experiments may have affected the results; therefore, the source of mucin was changed and the experiments repeated. Upon changing the mucin source from Sigma-Aldrich to Difco the initial experiment achieved 100% mortality although subsequent experiments did not achieve this level of mortality within the control group.

The inoculum used in these studies was of the same order of magnitude to that that achieved 100% mortality in the murine model used to determine the in vivo antibacterial activity of the HARP agents and mortality approaching 100% was expected in the current study (Burli et al, 2004a). However, control group survival varied between 0 and 45% using a similar experimental protocol. The reason for this variability is not clear. ICR (CD1) mice are outbred and their intrinsic susceptibility and immunity to infections may vary, although this has not been raised as an issue in other studies. These experiments should be repeated using inbred mice. There may be a greater likelihood of obtaining a more reproducible set of results if mice were to be infected with a passaged strain, i.e one selected for enhanced virulence towards the mouse strain.

Nevertheless, increases in survival of treated animals can be compared to survival rates of the controls to determine if the PBD dimers were effective in vivo. Two different routes of PBD dimer administration were used for ELB-21, in order to establish if the route of administration affected survival. Neither of these routes enhanced survival. There was no increase in survival in any of the groups, indicating that the PBD dimers were not effective in vivo at the concentrations employed.
Preliminary pharmacokinetic studies of SJG-136 in mice indicated that SJG-136 concentrations obtained in plasma were higher than the concentrations required to produce an antibacterial effect in vitro (Wilkinson et al, 2004). Furthermore, only moderate protein binding (65-75%) by the dimer has been reported (Wilkinson et al, 2004). The maximum tolerated dose for SJG-136 and ELB-21 were estimated as 0.3 mg/kg and 0.005 mg/kg respectively. Initially, doses at or below these values were used, although higher doses were subsequently used in this study. Uninfected controls administered with appropriate concentrations of PBD dimer were included in each experiment and these animals were closely monitored. Observations on the health characteristics, behaviour and feeding habits suggested that the mice were not affected by the higher doses administered and there was 100% survival for each PBD-dimer treated control group. Unfortunately, even at the highest doses used, there was no significant increase in survival of any treated groups in comparison to the control groups.

In contrast to the studies on HARP DNA-binding agents, the concentrations of PBD dimers used in this study are low. The HARP agents rescued 50% of mice with lethal MRSA peritonitis infections at dosing concentrations of 13 mg/kg per body weight and 100% survival at doses $\geq$ 25 mg/kg was achieved (Burli et al, 2004a; Gross et al, 2003). Further studies involving higher doses of PBD dimer are warranted. In addition, evaluation of the PBD dimers using a neutropenic mouse thigh model of infection may reveal in vivo activity not detected in the peritonitis model. Future generations of PBD dimers that are designed to target specific bacterial genes and possess lower intrinsic host cellular toxicity are likely to enable the administration of higher maximum tolerated doses, allowing the administration of higher doses than those utilised in this short investigation.
CHAPTER SIX
GENERAL DISCUSSION
Antibiotics in current clinical use are in the main derived from natural products elicited by microorganisms that are forced to compete for nutrients in hostile habitats such as soil. The first antibacterial agent of natural origin was penicillin and the collection expanded rapidly in the early days of antimicrobial chemotherapy to include compounds such as erythromycin, gentamicin and vancomycin. Many classes of naturally occurring antibiotics were chemically modified in response to increased resistance, although few antibacterial agents have been chemically synthesised; amongst these are the sulfonamides and more recently the oxazolidinones.

There have been limited discoveries of new classes of antibiotics in the last three decades, as discussed previously. There are several reasons for this; firstly, there was a rapid discovery of new antibiotics in the early decades of antibiotic development and the urgency for new discoveries was reduced. Secondly, due to rapidly emerging resistance in bacteria, pharmaceutical companies were reluctant to invest in projects that may have resulted in poor profits or even losses. Finally, many of the resistant pathogens responded well to modified agents, derived from existing classes of antibiotics.

In the last decade, the approach to investigating novel antibacterial agents has changed somewhat as a result of technological advances. Availability of numerous bacterial genomes has led to the hypothesis that new target genes, encoding proteins essential for bacterial survival, could be identified by high-throughput screening (Miesel et al., 2003). Furthermore, with the completion of the human genome and the availability of computer packages that facilitate multiple genome analyses and comparisons, it is possible to identify bacteria-specific targets that should reduce mammalian cell drug-related toxicity. The development of new antibacterial agents through genomics and target-based discovery starts with the identification of a gene that is preferentially conserved in several bacterial species and is essential for bacterial survival. Evaluation of gene essentiality on a genomic scale has been achieved by gene knockout studies, whereby bacterial cell viability is compromised if an essential gene has been rendered non-functional (Miesel et al., 2003). Once a target gene has been identified, it is expressed, purified and its
molecular structure determined, often by NMR and X-ray crystallography. Computer modelling and biochemical assays are then utilised for screening the protein against a wide range of synthetic inhibitor compounds that may be further optimised before being tested against bacterial cells. The idea that gene target selection would yield a large number of potential drug candidates has been less fruitful in practice than expected. The main problem associated with this discovery process is that successful *in vitro* inhibition of the target protein does not guarantee efficacy against whole cells. This may be due to lack of penetration of the compound into the bacterial cell or existing efflux mechanisms that limit access to the selected target. In addition, obtaining a drug that targets one specific gene product may lead to more rapid emergence of a resistant pathogen than if multiple targets were involved.

The genomic target selection approach has resulted in the identification of at least one novel viable target for antibacterial chemotherapeutic intervention. Peptide deformylase is essential for bacterial protein synthesis and bacterial growth (Chen *et al.*, 2004). This enzyme is not necessary for mammalian cell growth and for this reason represents an attractive target. Libraries of peptide deformylase inhibitors have been developed, screened and active compounds selected for *in vitro* and *in vivo* analysis (Chen *et al.*, 2004). Although promising results have been obtained in these studies, target selection initiatives have not produced the predicted influx of successful novel antibacterial agents necessary to combat increasing resistance amongst pathogens.

The recent advances in genomics and genetics have opened up many doors for exploration and have contributed to a greater understanding of gene evolution and diversity. The availability of hundreds of genomes, including the human genome, is likely to have an impact on future antibiotic development and selective toxicity, although genomics alone may not lead us directly to a solution. Genomics may aid in the understanding of resistance, macromolecular synthesis, stress responses and physiological functions and may also help identify potential drug targets. However, the long-term challenges of bacterial evolution and resistance are unlikely to disappear in the future and
genomics may be viewed as a powerful tool in the fight against increasingly problematic pathogens.

The study described herein has utilised the advances in genomics in a different way to the studies described above. Rather than using the genome as a source of information, this study has examined the potential of the genome as the direct target for a new class of agents. The majority of targets for clinically useful natural and semi-synthetic antibiotics are enzymes or structural proteins involved in essential pathways of macromolecule synthesis and many of these have been exploited for antibiotic development. Naturally occurring DNA-binding antibacterial agents such as anthramycin and distamycin have been known for many years but have been viewed as unattractive candidates for antibacterial chemotherapy due to their inherent toxicity (Dervan, 2001). Historically, DNA-binding agents have most often been investigated against tumour cell lines and there has been only limited investigation of their potential as antibacterial agents, due to the perception that the high toxicity of these putative drugs will severely limit their capacity for the treatment of infections in man. The antimicrobial capacity of distamycin and distamycin derivatives has been determined against Plasmodium falciparum, herpes simplex and HIV and found to be highly potent at low concentrations (Reddy et al, 2001). Due to the relentless rise of antibiotic resistance and the limited discoveries of novel classes of antibiotics, the potential of DNA-binding agents as antibacterial agents has recently been explored by several investigators. The HARP compounds, derived from distamycin, bind to DNA at A:T sequences in the minor groove and have been investigated for their antibacterial activity against a number of pathogens (Burli et al, 2002; Gross et al, 2003). They have been reported to have an excellent potency both in vitro and in vivo against Gram-positive bacteria, including MRSA (Burli et al, 2004a; Kaizerman et al, 2003). Another distamycin-related group of compounds, the pyrrole tetraamides, have also exhibited potent antibacterial activity and selected compounds have been modified in order to reduce toxicity to mammalian cells (Dyatkina et al, 2002).

The PBD dimers, investigated in this study, are semi-synthetic agents, synthesised from various naturally occurring compounds such as tomaymycin and DC-81 and comprise
two identical units tethered through a diether linkage (Thurston, 1999). The PBD dimers, like the distamycin-related agents, bind to the minor groove of DNA. It is interesting to note that many DNA-interactive proteins involved in transcription and replication bind to the major groove of the DNA so it is not surprising that small naturally occurring DNA-binding molecules such as the PBD monomers would target the minor groove. The PBD monomers are synthesised by Streptomyces spp.; these bacteria have evolved over time to protect their environmental niche by competing with other organisms for food sources. The advantage of using naturally occurring molecules as chemotherapeutic agents cannot be underestimated. As already mentioned, there are only a handful of synthetic antibacterial agents that have been successfully developed for clinical use; the majority are of natural origin, which emphasises the importance of natural diversity and evolution. Compounds produced by microorganisms such as Streptomyces spp. would not only be more diverse than synthetically produced libraries but are likely to be highly effective against their targets. Therefore, the investigation of natural compounds with inherent and specific antibacterial and antifungal properties seems to be a valuable and efficient way in searching for new antibiotics. The actions of the PBD monomers have been chemically optimised by the synthesis of dimeric forms.

The novelty of the PBD dimers lies in their sequence selective recognition of DNA. Dimers of differing sequence specificity have been developed, in general binding to 6-7 bp of DNA in the minor groove. Compounds that recognise longer DNA sequences are likely to be more specific biological inhibitors. This is an attractive property of the agents, as specific DNA sequences within known genes have the potential to be targeted. The compounds are therefore promising candidates for antibacterial investigation, especially as many bacterial genomes have now become available, exposing the sequences of essential genes for bacterial survival.

It is important to emphasise that the PBD dimers have been thoroughly investigated as potential anti-cancer chemotherapeutic agents due to their potent DNA-binding properties (Alley et al, 2004; Hartley et al, 2004). The agents have been optimised over time to achieve greater DNA-binding potency and reduced mammalian toxicity. Animal studies
on SJG-136 have indicated that the compound is well tolerated and protein binding is moderate (Wilkinson et al., 2004). SJG-136 has been the most successful candidate for anticancer chemotherapy and is currently being investigated in clinical trials.

The PBD dimers ELB-21, DRG-16 and SJG-136 were selected for this study due to the spectrum of their sequence specificity and DNA-binding potency. The primary *in vitro* determinations of antibacterial activity of potential antimicrobial agents are MIC values.

The PBD dimers were found to be ineffective against Gram-negative bacteria as indicated by the high MIC values in Chapter 2; this was most likely due to the barrier function of the outer membrane and the inherent resistance of the bacteria. Gram-positive bacteria and mycobacteria were, however, highly susceptible to the compounds, with excellent MIC values. Gram-negative bacteria were not evaluated further and all remaining antibacterial investigations were performed on Gram-positive bacteria, mainly using a selection of MRSA and VRE strains. The PBD dimers were evaluated for cross-resistance with a number of other antibiotics and as expected there was no evidence of such an occurrence. To determine if the compounds were bactericidal or bacteriostatic, time kill assays were performed for the three dimers against one VRE strain and two EMRSA strains. As a whole the PBD dimers were bactericidal, although bacterial regrowth at 24 h occurred in some experiments. It is interesting to note that the growth phases of the cultures had some effect on the killing pattern of the dimers, which is possibly related to DNA repair processes associated with PBD dimer-induced damage, although further investigation on this hypothesis is necessary. To establish if brief incubation of the bacteria with the PBD dimers elicits any long-term damage and delays the recovery of bacterial cells, PAEs were performed using a MRSA and a VRE strain. The PAEs reported for the dimers were relatively short in comparison to those in the published literature for nucleic acid inhibitors such as the quinolones (Guan et al., 1992). However, the PAE values reported for the DNA-binding HARP compounds were less than an hour (Ge et al., 2002). In order to substantiate that the antibacterial activity of the PBD dimers was due to their DNA-binding properties, naked DNA and DNA extracted following incubation with the dimers was investigated. The ability of the PBD dimers to form interstrand cross-links in naked DNA was clearly demonstrated by DNA melting experiments. Incubation of purified, naked EMRSA-16 DNA with the PBD dimers
resulted in a large increase in the melting temperature. However, this technique was insensitive and unable to detect cross-links at physiological concentrations approximating the MIC; an alternative method was therefore sought. Extensive DNA cross-linking was observed with both naked DNA and DNA extracted following PBD treatment of intact bacteria using a formamide-denaturing Southern blot analysis. These results indicated that PBD dimers effectively penetrated the bacterial cell membrane and cell wall and were capable of cross-linking DNA in regions of the genome recognised by these agents. The extent of DNA cross-linking was greater with ELB-21-treated DNA than with SJG-136-treated DNA, either as a result of differences in drug potency or in the rate of penetration of the agents into the cell. In order to assess the pattern of motifs recognised by the different PBD dimers within the genome, the EMRSA-16 genome (Holden et al, 2004) was analysed using the Artemis DNA viewer programme. In general, there were no major differences in the frequency of binding sites between the dimers, although the PBD dimers ELB-21 and DRG-16 bind to the 6 bp motifs recognized by SJG-136 in addition to their preferred 7 bp sequence motifs. This may contribute to the greater potency observed with ELB-21 and DRG-16. Although a large number of the PBD dimer binding motifs were located within coding regions of the genome, binding to non-coding regions is also likely to inhibit DNA transcription and replication of upstream or downstream genes. It is possible that many of the genes that contain the PBD dimer binding motifs are essential for bacterial survival and their inhibition contributes to cell death. As observed by electron microscopy, the heterogeneous nature of the cell damage is likely to reflect multiple gene dysregulation as a result of a multiplicity of binding motifs located within the genome. It was therefore interesting to determine if there was a characteristic pattern of alteration in protein concentration in PBD dimer-treated bacteria at sub-inhibitory concentrations. Analysis of two-dimensional gels of proteomes, extracted from untreated bacteria and bacteria incubated with PBD dimers, showed changes in the concentrations of some proteins. Stress proteins, induced following bacterial damage, could account for the increases in concentration of some proteins, whilst proteins that had decreased in concentration were possibly inhibited directly by the PBD dimers. Individual proteins could not be analysed by mass spectrometry due to the limited quantity of protein that could be extracted from the gel. Further analysis,
involving larger two-dimensional gels, will be necessary in order to unambiguously
determine changes of protein concentrations due to exposure to PBD dimers. As initial
\textit{in vitro} results were very promising, the compounds were considered for \textit{in vivo} analysis.
The PBD dimers SJG-136 and ELB-21 were examined in the mouse peritonitis model;
this model was also used in investigations of the efficacy of the HARP compounds, which
were well tolerated and eradicated a potentially lethal MRSA infection (Burli \textit{et al},
2004a; Kaizerman \textit{et al}, 2003). Initial studies involving the treatment of peritonitis
infection with ELB-21 and SJG-136 were inconclusive and showed no efficacy; when
larger quantities of the compounds become available, dosing regimens using higher
concentrations of the compounds should be initiated.

The \textit{long-term} objective of this work is to supplement the existing range of antibacterial
agents. This study has demonstrated that the PBD dimers have excellent potential as
unique DNA-binding antibacterial agents, although further exploration into their toxicity
and efficacy is essential.

An important aspect of future studies is the synthesis of PBD dimers with a greater
intrinsic specificity for bacterial genomes. The PBD dimers are more selective than
DNA-binding agents such as distamycin since they bind longer DNA sequences.
Distamycin-derived compounds do not discriminate between different genes as they bind
to all A:T-rich sites within the minor grooves of DNA. Although the PBD dimers also
appear to have little specificity towards key genes, there is scope for developing gene-
selective PBD dimers. Extension of the diether linker joining the two monomers may
enable the PBD dimer to recognise longer sequences of DNA and therefore increase
sequence specificity. Attempts to increase the specificity of the PBD dimers should be
directed towards two aims that currently restrict their utility: the targeting of bacterial
genes essential for bacterial survival and the reduction of toxicity towards mammalian
cells. Furthermore, genomics has facilitated the identification of many of the essential
genes for bacterial survival that are conserved between bacterial species; for example,
genomes involved in DNA replication, such as \textit{dnaA}, \textit{dnaC}, \textit{lig} and \textit{gyrB}, could be prime
targets for future, strategically designed generations of the PBD dimers.
The re-growth observed at 24 h in the time kill assays may be due to DNA repair in bacterial cells damaged by exposure to PBD dimers. Further investigations could be pursued with DNA repair mutants. DNA repair has been widely studied in *E. coli* cells and there are several known pathways. One involves nucleotide excision at the cross-linked DNA region, followed by homologous recombination with another intact DNA strand (Cole, 1973). This pathway was elucidated by investigating DNA repair of cross-links induced by psoralen (Van Houten et al, 1986). It was determined that incisions are made on both sides of the cross-link on one strand by the UvrABC enzyme; a gap is then opened by DNA polymerase I and the intact strand proceeds with homologous recombination catalysed by the RecA protein. The cross-linked fragment still attached to the original strand is subsequently excised. Another pathway following treatment with nitrogen mustards, which is not dependent on recombination and RecA, also involves nucleotide excision enzyme UvrABS, but the missing oligonucleotide is replaced by DNA polymerase II. Part of the other strand containing the cross-link is then excised by the excision enzyme (Berardini et al, 1999). In other cases, repair does not proceed with either of the above pathways; instead, the replication fork is brought to a halt at the cross-link leading to a double-stranded break. The end of one strand is then cleaved by other excision enzymes and RecA catalyses recombination (Cox, 1998). Since different pathways and different repair elements are deployed following treatment with different alkylating agents, it appears that DNA damage is corrected according to the degree and nature of the helical distortion. The PBD dimer SJG-136 fits snugly in the minor groove of DNA and does not seem to cause significant distortion of the helical DNA structure. It is therefore less likely to be recognised by DNA repair elements in comparison to agents that induce more severe helical distortion upon binding, such as cisplatin and mephlan (Clingen et al, 2005a). Homologous recombination repair occurred in mammalian cells following treatment with SJG-136; however, it was less prominent than with other cross-linking agents such as the major-grove binding nitrogen mustard mephlan (Clingen et al, 2005b). Future experiments with bacterial DNA repair mutants could aid the understanding of the mechanisms involved in the repair of PBD dimer-induced cross-links.
Future generations of PBD dimers could be assessed in a similar fashion to the compounds investigated in this study. To correlate gene-selective binding of new PBD dimers, proteins that have been up- or down-regulated following incubation with sub-inhibitory concentrations of the PBD dimers could be identified using two-dimensional gel electrophoresis and mass spectrometry as previously described. This will facilitate the identification of proteins whose expression is modulated by PBD dimer binding to the corresponding gene or up-/downstream regulatory elements. The identified gene could be investigated for the presence of PBD dimer binding motifs using the Artemis software. In addition to using proteomics for analysis of protein expression, DNA microarrays could be deployed for analysis of gene expression following PBD treatment. DNA microarrays have been widely used for the comparison of genomic DNA, not only between different bacterial strains, but also for different physiological states exhibited by a bacterial strain (Brown & Botstein, 1999). DNA microarray technology involves thousands of known genes incorporated onto a small device (the microarray) by a robot. Messenger RNA is extracted from the bacterial cells under investigation, such as a control culture and a PBD dimer treated culture; extracted mRNA represents the products of actively transcribed genes under the different experimental conditions. The mRNA is used for the preparation of cDNA by reverse transcription and the products of this reaction are labelled with fluorescent dyes that can be differentiated on the basis of colour. Differentially labelled cDNA samples are mixed and added to the microarray containing known gene sequences; hybridisation between complimentary DNA-DNA sequences occurs and the fluorescent signals from the different dyes are measured microscopically. The ratio and the strength of the fluorescent signals determine the relative expression levels of the corresponding genes (Brown & Botstein, 1999). DNA microarrays would facilitate the measurement of transcription of all genes simultaneously and therefore provide an essential tool for comparing gene expression modulated by the PBD dimers to that of untreated cells.

In summary, this study has exposed the potential of a novel class of semi-synthetic DNA binding compounds as antibacterial agents. The PBD dimers were highly potent against resistant pathogens such as MRSA and VRE. There is little variation in MIC values determined against strains within the bacterial species investigated, suggesting that a
similar mechanism of inhibition applies in all strains of a bacterial species. The PBD dimers were not effective against Gram-negative bacteria; this lack of efficacy could be overcome by using the dimers in combination with agents that compromise the integrity of the outer membrane. It has been shown that the PBD dimers effectively penetrate the cell wall and the cytoplasmic membrane of EMRSA-16 before they cross-link genomic DNA. Although the *in vivo* studies conducted in this study were inconclusive, it is evident that these agents warrant further investigation. Such studies could involve the identification of PBD dimers that are bactericidal at low concentrations and have a high degree of selectivity for a limited number of genes essential for bacterial survival; these efficacious agents would be less cytotoxic to mammalian cells and represent attractive options for the development of a novel class of antibacterial chemotherapeutics.
References


Chow, A. W., Taylor, P. R., Yoshikawa, T. T., & Guze, L. B. 1979. A nosocomial outbreak of infections due to multiply resistant \textit{Proteus mirabilis}: role of intestinal colonization as a major reservoir. \textit{J. Infect. Dis.} \textbf{139}: 621-627.


Critchley, I. A., Draghi, D. C., Sahm, D. F., Thornsberry, C., Jones, M. E., & Karlowsky, J. A. 2003. Activity of daptomycin against susceptible and multidrug-resistant Gram-


Draper, P. 1998. The outer parts of the mycobacterial envelope as permeability barriers. Front Biosci. 3: D1253-D1261.


# Appendix 1

MIC data for individual Gram-positive strains

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### Appendix 2

**Resistance patterns**

Trimethoprim (Tri), Amikacin (Ami), Gentamicin (Gen), Tetracycline (Tet), Rifampicin (Rif), Vancomycin (Van), Ciprofloxacin (Cip), Erythromycin (Ery), Oxacillin (Oxa), Clindamycin (Cli);

R – resistant, S – sensitive, I – intermediate

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Publications arising

