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Potential strategies for the eradication of multi-drug resistant Gram-negative bacterial infections

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1   Potential strategies for the selective eradication of multi-drug resistant Gram-negative

2   bacterial infections

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Abstract

Antimicrobial resistance is one of the leading threats to society. The increasing burden of multidrug-resistant Gram-negative infection is particularly concerning as such bacteria are demonstrating resistance to nearly all currently licensed therapies. Various strategies have been hypothesised to treat multidrug-resistant Gram-negative infections including: targeting the Gram-negative outer membrane; neutralization of lipopolysaccharide; inhibition of bacterial efflux pumps and prevention of protein folding. Silver and silver nanoparticles, fusogenic liposomes and nanotubes are potential strategies for extending the activity of licensed, Gram-positive selective, antibiotics to Gram-negatives. This may serve as a strategy to fill the current void in pharmaceutical development in the short-term. This review outlines the most promising strategies that could be implemented to solve the threat of multidrug-resistant Gram-negative infections.

Introduction

There is a drastic need for innovative therapeutic solutions that selectively target multi-drug resistant Gram-negative infections. This can be attributed to resistance to nearly all conventional antibiotics used clinically, and a lack of effective antibiotics in reserve. Gram-negative bacteria, particularly: *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*, are an ever-increasing threat to health and

44 particularly that of hospitalized patients who commonly are immunocompromised, have co-

45 morbidities and are less able to fight infection [1]. Recently, emphasis has been placed on the

46 rapid detection of specific, causative antimicrobial resistant strains. This has catalysed the

47 drive to develop pathogen-specific, narrow spectrum antimicrobials. This change in focus

48 from broad-spectrum microbial annihilation to more targeted therapy, acknowledges the

49 major contribution empirical prescribing has on increasing drug resistance, and its impact on

50 beneficial human microbiota [2].

51

52 Nosocomial infections are a major contributor to healthcare associated infections and

53 antimicrobial resistance. Approximately 20-40% are attributed to transfer of commensal

54 microorganisms from the skin of healthcare workers to patients or even the patients' own

55 commensal flora [3]. Healthcare associated infections affect approximately 4.1 million

56 patients annually within the European Union. They are a major contributor to morbidity

57 causing 37, 000 deaths annually and a further 100,000 deaths in those with co-morbidities

58 [4]. In terms of antimicrobial resistant infections, recent UK government reports estimate that

59 these contribute to around 25,000 deaths annually in Europe alone [5].

60

61 Gram-negative bacteria are a particular problem due to multiple inherent resistance

62 mechanisms, most notably the presence of a lipopolysaccharide (LPS) outer membrane and

63 efflux pumps [6]. As a result of improper and overuse of antimicrobials, the resistance rates

64 to current therapeutic agents have increased to serious levels. This dilemma has attracted the

65 attention of scientists, the general public, health authorities and politicians. It is now

66 recognized as a considerable global health problem [3]. As mentioned, the significant lack of

67 newly licensed antimicrobial pharmaceuticals translating from the laboratory to patients is

68 concerning. In the past 25 years, only two new cephalosporin-beta-lactamase inhibitor

69 combinations- ceftolozane/tazobactam in 2014 and ceftazidime/avibactam in 2015 have been

70 approved to treat systemic bacterial infections caused by multi-drug resistant Gram-negative

71 bacteria [7]. There are a multitude of reasons for the decline in antimicrobial drug

72 development, most notably the high financial commitment and time required for developing

73 and registering a new drug. On average it costs approximately $800 million to introduce a

74 new drug to market with development times normally in excess of 10 years. Parallel to this,

75 the pharmaceutical industry has focused over the past 30 years on the more financially

76 rewarding novel therapies for chronic diseases such as diabetes and cardiovascular disorders.

77 These products are likely to be required as lifetime treatments in contrast to antibiotics that

78 are most commonly short-term acute treatments (typically 5- 14 days) [8]. Other contributing

79 factors include clinical trial requirements, particularly the challenge of proving novel

80 therapies produce greater clinical outcomes compared to existing products, and that they are

81 sufficiently safe for use. Pharmaceutical companies also express reservations about future

82 resistance development that may reduce drug longevity [9][10]. In order to increase the

83 approval and registration of new antimicrobials, the US Food and Drug Administration have

84 indicated that it may be ready to alter its strict clinical-trial requirements and reassess the

85 antimicrobial approval regulations in order to increase the potential availability of novel

86 treatments [11]. The primary barriers to overcome, as will be discussed further in this review,

87 include the specific targeting of Gram-negative bacteria in order to produce selective

88 antibiotics that are suitable candidates for clinical trials and transition from the lab bench to

89 the clinic.

90

91 **The Gram-negative outer membrane as a barrier to therapy**

I. Bacterial cell wall structure

Understanding the mechanisms that govern Gram-negative bacterial resistance requires a fundamental appreciation of their cell morphology. The unique structure of the outer membrane of Gram-negative bacteria plays an important role, providing an additional layer of mechanical protection, without affecting the selectivity or exchange of material needed for bacterial survival [12]. The Gram-negative cell wall is composed of an outer LPS membrane and an inner cytoplasmic membrane. A thin layer of peptidoglycan and lipoproteins exist within the periplasmic space. The inner cell membrane is composed of a phospholipid bilayer, whilst the outer membrane consists of phospholipids on its interior leaflet and of LPS on its outer leaflet [13]. Porins and specialized transporters are also present within the outer membrane channels and mediate the influx of a variety of compounds including nutrients and minerals such as sugars, amino acids, phosphates and ions. Porins play an important role in bacterial metabolism and growth, and are therefore a valuable target for antimicrobial drug development [14]. Gram-negative bacteria continuously alter the expression and function of outer membrane porins hence this may affect the sensitivity of antimicrobial agents. Loss of or changes in porin amino acids could influence the ability or rate of entry of antibiotics and contribute to resistance. In contrast to Gram-negative bacteria, Gram-positive bacteria lack an outer membrane and are composed of a single lipid membrane surrounded by numerous interconnecting layers of peptidoglycan and lipoteichoic acid (Figure 1) [15]. Although Gram-positive bacteria possess a cell membrane, the lack of a protective outer membrane makes them more susceptible to antibiotics.

II. Antimicrobial resistance mechanisms of Gram-negative bacterial cell wall

116   The outer membrane of Gram-negative bacteria acts as a selective barrier by adding a

117   hydrophobic lipid bilayer to the specific size-exclusion properties of porins. The outer

118   membrane has the ability to block the entry of numerous toxic compounds and prevent the

119   uptake of molecules with a molecular mass greater than 600 Daltons [16]. The influx of

120   metabolites such as sugars, phosphates and hydrophilic molecules is mainly directed by

121   porins. The continuous alteration in lipid or protein composition of the outer membrane leads

122   to drug-resistance. This involves the increasing of outer membrane hydrophobicity, changing

123   porin specificity or increasing the number and efficacy of efflux pumps [17].

124

125   Reducing the negative charge of LPS within the bacterial outer membrane is one of the key

126   strategies employed by Gram-negative bacteria to negate the action of membrane active

127   cationic antimicrobials, such as chlorhexidine and cationic antimicrobial peptides. This is

128   achieved via the addition of positively charged residues such as aminoarabinose and

129   galactosamine sugars to LPS or by the removal of negative charged moieties. This

130   modification leads to increased bacterial survival as demonstrated by both *Pseudomonas*

131   *aeruginosa* and *Francisella novicida* after exposure to the cyclic cationic lipopeptide

132   polymyxin B [18]. Amines are also harnessed by Gram-negatives to increase LPS membrane

133   cationicity as demonstrated by *Salmonella typhimurium* which increases tolerance to

134   polymyxin B by conjugating phosphoethanolamine to one of the phosphate groups present

135   within outer membrane lipid A [19]. Bacteria are also able to remove anionic phosphate

136   groups to reduce the overall anionic surface charge of LPS, proven by the removal of the 4′-

137   phosphate group from lipid A in *Helicobacter pylori*. This results in increased resistance to

138   membrane active cationic antimicrobial peptides [20]. Phospholipids present in the Gram-

139   negative outer membrane are also susceptible to modification. *Salmonella typhimurium* has

140   the ability to increase the levels of outer membrane glycerophospholipids resulting in

141   increased membrane hydrophobicity and reducing the permeability of charged, water soluble

142   molecules [21].

143

144   Alteration of outer membrane porins prevent intracellular diffusion of small hydrophilic

145   antibiotics such as beta-lactams, tetracycline, chloramphenicol and fluoroquinolones.

146   Research has revealed that functional changes in porins are directed by specific mutations in

147   a variety of pathogens, including *Escherichia coli, Pseudomonas aeruginosa* and *Neisseria*

148   *gonorrhoeae* [14][22]. A relatively minor change in porin structure can have a significant

149   effect on functionality. For example in *Enterobacter aerogenes*, substitution of glycine with

150   aspartate within the peptide structure of its porin, results in a narrower lumen, affecting

151   intracellular cephalosporin transport and lowering susceptibility to antimicrobials [14].

152

153   Efflux pumps are membrane bound proteins that regulate the intracellular environment active

154   transport mechanisms to extrude toxic compounds such as bile salts, fatty acids and heavy

155   metals outside of bacterial cells [23]. They are important cellular machinery in increasing

156   Gram-negative bacteria's ability to resist diverse classes of antibiotics including beta lactams,

157   aminoglycosides and fluoroquinolones via expulsion out of the cell. These antibiotics often

158   target intracellularly hence their expulsion restricts activity. Efflux pumps also contribute to

159   bacterial virulence and the formation of biofilms [24]. The resistance-nodulation-division

160   family (RND), one of five families of bacterial efflux pumps, is the only one that is

161   specifically implicated in Gram-negative bacteria. Other families of efflux systems are

162   extensively spread across both Gram-positive and Gram-negatives [25]. RND efflux pumps

163   are able to expel a wide range of antibiotics with a high degree of specificity. Both RND-

164   based efflux pumps in *Pseudomonas aeruginosa*, MexAB-OprM and MexXY-OprM, can

165 expel tetracycline, fluoroquinolones, and chloramphenicol, whilst for beta-lactams and

166 novobiocin, expulsion occurs via the MexAB-OprM system [24].

167

168 **Strategies for extending therapeutic activity against Gram-negatives**

169 **I. Antimicrobial peptides**

170 Antimicrobial peptides were first isolated by Dubos in 1939 from *Bacillus* bacteria derived

171 from soil [26]. The amphipathic nature of most antimicrobial peptides proves advantageous

172 for antimicrobial activity. The presence of hydrophilic and hydrophobic domains allows

173 interaction with both lipid and phospholipid groups present in the bacterial cytoplasmic

174 membrane [27]. The majority of antimicrobial peptides are cationic in character. These

175 naturally occurring molecules mediate an innate immune response in a multitude of

176 organisms [28]. They possess several optimal properties for therapeutic applications. Cationic

177 antimicrobial peptides have the ability to bind to LPS and therefore negate the production of

178 host pro-inflammatory cytokines [29]. Most cationic antimicrobial peptides exert their

179 bactericidal action via targeting of bacterial membranes, resulting in membrane

180 disintegration, cell lysis and death [28]. A variety of antimicrobial peptides demonstrate an

181 ability to permeate bacterial cell membranes at low concentrations, inhibiting DNA

182 replication and protein synthesis without altering membrane integrity [27]. For example,

183 buforin-II binds to DNA and RNA without disrupting the bacterial cell membrane

184 architecture [30]. Cationic antimicrobial peptides have great potential to fill the current void

185 in antimicrobial drug development because of their selectivity for negatively charged

186 microbial membranes compared to neutral sterol-rich mammalian forms. Antimicrobial

187 peptides tend to demonstrate rapid bactericidal activity utilising multiple modes of extra- and

188 intra-cellular action. They therefore have a reduced tendency to promote bacterial resistance

189   compared to many currently licensed antimicrobials which tend to target only a single

190   biomolecular mechanism. Antimicrobial peptides are already in clinical use and such

191   examples include lysostaphin, polymyxin B and gramicidin S, demonstrating their potential

192   for clinical translation and ability to fill the void in current antimicrobial drug development

193   [31].

194

195   Polymyxins are a class of cationic cyclic lipopeptides, first discovered in 1947, isolated from

196   the spore-forming bacteria *Paenibacillus polymyxa* present in soil. Polymyxin E (colistin) and

197   polymyxin B are classified as narrow spectrum Gram-negative selective antibiotics. Their

198   clinical use decreased in the 1970s due to concerns regarding nephro- and neuro-toxicity.

199   Most recently there has been a revival in their potential clinical use and research has focused

200   on the design of novel polymyxin derivatives with markedly lower mammalian toxicity and

201   higher bactericidal activity [32]. The exact bactericidal mechanism of polymyxins has

202   remained a topic for debate amongst researchers. It has been hypothesised that the protonated

203   amino acids within the cyclic peptide structure of polymyxins, bind directly to the lipid A

204   part of LPS present in the outer membrane of Gram-negative bacteria, facilitating insertion of

205   hydrophobic motifs into the outer membrane. This enables the formation of pore-like

206   aggregates thus increasing outer membrane permeability [33]. Polymyxin B, for example, has

207   the ability to attach to the anionic surface of LPS in the outer membrane resulting in self-

208   promoted uptake into the periplasmic space and cytoplasmic membrane. It is more difficult

209   for bacteria to generate resistance against such physical interactions as it would require

210   reorganisation of vast areas of the membrane architecture. However, plasmid-borne resistance

211   has been reported recently against colistin and this is concerning as colistin is typically

212   considered a drug of last resort for Gram-negative infections [34]. The *mcr*-1 plasmid,

213   identified in an *Escherichia coli* isolate present in a pig in China, encodes an enzyme that

214 directs the addition of phosphoethanolamine to lipid A decreasing the anionic charge of the

215 outer membrane. Whilst this addition has been elucidated previously, the fact that the process

216 is mediated via a plasmid is crucially significant, as it will allow resistance to readily spread

217 to other species. This discovery highlights the urgent need for investment to elucidate

218 antimicrobial resistance mechanisms and for tailored therapies to combat these.

219

220 Research into polymyxin-like molecules has been on-going, especially with regard to

221 producing less toxic derivatives (nephro- and neuro-toxic) and compromising the integrity of

222 the Gram-negative outer membrane barrier to increase the activity of existing antibiotics [35].

223 Structurally similar cyclic antimicrobial peptides are also of interest as future synthetic

224 therapies as they possess increased serum stability relative to linear forms. They may also

225 provide a basis for designing cost-effective, low molecular mass, anti-LPS compounds [36].

226 Cyclic peptide variants are synthesised by directly conjugating the two terminals of the

227 primary amino acid sequence to form an amide bond, or via another form of linkage such as

228 lactone or disulfide bonds. Generally, cyclic peptides are more effective than their linear

229 analogues because of the structural rigidity that enables cyclic peptides to bind selectively to

230 bacterial targets. They can also adapt an ordered amphipathic structure that allows them to

231 insert deeper within the bacterial membrane, with extended action *in vivo* due to their

232 increased stability to proteases [37]. Almost all known natural cyclic peptides display high

233 antibacterial activity. For example, polymyxin B, colistimethate and gramicidin S show high

234 bactericidal activity against *Pseudomonas aeruginosa* with minimum bactericidal

235 concentrations of 0.125, 4 and 8 μg/ml respectively [38]. Despite their significant bacterial

236 activity *in vitro*, many cyclic peptides are highly haemolytic and currently lack the bacterial

237 selectivity required for clinical translation [39].

238

II. Combinational antibiotic treatment for Gram-negative bacteria

Synergistic therapy, a combination of two or more antibiotics, is a commonly employed

strategy to resolve Gram-negative infections. In comparison to monotherapy, combination

therapy takes advantage of the additive effects of multiple antimicrobial mechanisms for each

drug therapy to lower the risk of resistance developing. Combination therapy has also been

demonstrated to lower mortality and improve clinical outcomes. It is recommended for

patients whose infection is suspected or confirmed to be multidrug-resistant Gram-negative

bacteria [40]. Synergy between two or more antimicrobial agents means that the combined

effect will be greater than their individual effects. Combination therapy allows lower

prescribed doses of individual antimicrobials and shortens the duration of treatment reducing

the risk of adverse side effects to the patient [41]. Generally each individual antibiotic

employed varies with respect to their mode of action [42]. However, the use of multiple

therapies does not come without risk. Combination therapy has been associated with an

increase in nephrotoxicity, especially when prescribed in long term chronic infections.

Another disadvantage is the increased complications associated with multiple treatment

schedules [43]. A model combination therapy includes a broad-spectrum beta-lactam with an

aminoglycoside, macrolide or fluoroquinolone for treatment of *Pseudomonas* infections [40].

A novel combination between cephalosporins and a beta-lactamase inhibitor has been

recently approved [7]. A synergistic approach is a beneficial strategy that is available

currently to reduce the burden of antimicrobial resistance, whilst efforts intensify to identify,

design and test new antimicrobial therapies.

260

III. The activity of silver against Gram-negative bacterial infection

262   Silver has been known to protect against infection for over 2,000 years and continues to be

263   used widely in many antimicrobial applications, especially within the biomaterial industry.

264   Morones-Ramez and colleagues demonstrated that silver ions (Ag^+) have a synergistic effect

265   with beta-lactam, aminoglycoside and quinolone antibiotics against a variety of Gram-

266   negative bacteria. Silver has been shown to increase the production of reactive oxygen

267   species, including hydroxyl radicals (OH•), increasing the permeability of the outer

268   membrane to commonly employed antibiotics [44]. Silver also acts intracellularly to

269   inactivate bacterial protein synthesis and enzymes responsible for a range of biochemical

270   processes, including deoxyribonuclease and ribonuclease. Silver has also been implicated in

271   DNA degradation and activation of cysteine proteases, namely the cysteine-dependent

272   aspartate-directed proteases, which play an important role in bacterial cell apoptosis. Silver

273   ion's cationic properties bestow affinity for anionic minerals present in the host, such as

274   chloride or phosphate, or proteins such as albumin. The complexes that form are inactivated

275   by precipitation or deposit in tissue debris with the potential to cause toxicity. Problems such

276   as these have led to questions regarding the safety and widespread use of silver for

277   antibacterial applications. More recently studies have focused on improving silver's ability to

278   selectively target bacterial metabolic pathways via a silver nanoparticle system [45]. Silver

279   nanoparticles have attracted interest in the development of new pharmaceutical products.

280   They have been introduced into wound dressings, medical device coatings, and are

281   increasingly utilized as drug delivery nanomaterials. Silver nanoparticle dressings, when

282   compared to silver sulfadiazine cream, have been found to decrease wound-healing time and

283   improve the clearance of bacteria from the infection site. Within medical devices, silver

284   nanoparticles have been tested as novel coatings for catheters, which are typically liable to

285   bacterial infections leading to complications such as device failure and sepsis. Furthermore,

286 they have the potential to be administered as drug delivery platforms, acting as carriers for

287 licensed antibiotics and enabling penetration of the Gram-negative outer membrane [46].

288

289 **Specific methods to target Gram-negative pathogens**

290 As highlighted, the development of bacterial resistance towards existing antimicrobial agents

291 has led to an urgent need for effective, alternative strategies. There is a necessity to develop

292 novel classes of antibiotics and different methods to bypass current resistance mechanisms of

293 Gram-negative bacteria [6]. There are multiple hypothesised mechanisms by which this can

294 be achieved including: targeting membrane integrity by binding to LPS; interacting with the

295 DsbA-DsbB enzyme system; or blocking the intracellular expulsion of antibiotics via

296 inhibition of efflux pumps. Innovative drug delivery platforms are also considered to be

297 "smart" approaches to enhance the efficacy of existing and future antibiotics. Genetic

298 engineering of phage lytic enzymes is also a promising strategy with the potential to kill

299 specific Gram-negative bacterial strains. Whilst all these approaches hold great promise, their

300 potential for pharmaceutical scale-up and related regulatory barriers have to be considered

301 early in the drug development process. Additionally, the high cost and the requirement to

302 prove quality, efficacy and safety considerations are the main reason behind clinical trial

303 failure and cessation of antimicrobial drug development [47]. Despite this, we will look

304 further at the most promising approaches to resolving the clinical and resistance barriers that

305 govern Gram-negative bacterial infection.

306

307 **I. Negating the biological effects of Gram-negative lipopolysaccharide**

308 As well as being the major constituent of the outer membrane, LPS signals bacterial invasion

309 and triggers an aggressive host immune response resulting in the release of pro-inflammatory

310   mediators, cytokines, chemokines, and lipoproteins [48]. Lipid A is the hydrophobic portion

311   of LPS that is chiefly responsible for biological toxicity. Within the outer membrane it

312   protects Gram-negative bacteria from host immune defences by forming a gel-like layer of

313   low fluidity. This layer limits the influx of hydrophobic solutes into the cell including many

314   antibiotics [49]. Excessive host response to LPS causes organ dysfunction, septic shock and

315   can even result in death. Antibiotics currently used to treat Gram-negative infections

316   exacerbate the immune crisis by causing bacterial cell lysis, resulting in the release of

317   significant amounts of LPS into the systemic circulation and creating an infection that is

318   difficult to treat effectively [50]. The risk of these events requires consideration prior to

319   initiation of empirical therapy as demonstrated in 2011, when the European Union witnessed

320   a haemolytic uremic syndrome outbreak caused by Shiga toxin-producing *Escherichia coli*

321   O104:H4. Treatment with antibiotics such as quinolones enhanced the release of its virulence

322   factors, including LPS, resulting in multiple deaths [51].

323

324   The severity of the host response is mediated by plasma lipoproteins and the LPS-binding

325   receptor CD14 that appears on the surface of host macrophages and neutrophils [52].

326   Examples of plasma lipoproteins include lipopolysaccharide-binding protein (LBP),

327   bactericidal/permeability-increasing protein (BPI), phospholipid transfer protein and

328   antimicrobial proteins secreted by neutrophils. Their binding to LPS causes a variety of

329   cellular effects [53]. Both soluble LBP and CD14 are present in the blood and are known to

330   enhance the effects of bacterial LPS. When LPS binds to LBP, the complex is recognized by

331   host CD14 receptors that in turn activate the production of pro-inflammatory cytokines and

332   type-I interferon, leading to local and systemic inflammatory reactions [52]. In contrast, BPI

333   binding to LPS is thought to be inhibitory and therefore beneficial in preventing an

334   exaggerated immune response. Recombinant and modified forms of BPI have been assessed

335 in clinical trials in patients with severe sepsis or septic shock. For example, recombinant BPI

336 (rBPI$_{21}$) is composed of the amino-terminal half of naturally occurring BPI and possesses

337 antibacterial and anti-LPS effects. When one amino acid cysteine residue of BPI is replaced

338 with alanine biological stability is significantly improved without affecting the neutralizing

339 properties of BPI [54]. This highlights how naturally occurring biomolecules can be altered

340 synthetically to improve pharmacological and pharmaceutical properties. If harnessed

341 correctly it will enable a wealth of potential therapies to be explored.

342

343 Throughout history nature has been the most significant source of antimicrobial therapies and

344 there has been an increased focus on identifying novel molecules of interest from natural

345 sources. Limulus anti-LPS factor (LALF) is an example of a small cyclic basic peptide found

346 in haemocytes of marine chelicerates, demonstrating a strong affinity to LPS. It shows the

347 ability to neutralize LPS by inhibiting the inflammatory cytokine tumour necrosis factor-

348 alpha produced as a result of LPS stimulation of the immune response. The amino acid

349 sequence that is responsible for LALF activity is found between amino acids 31 and 52

350 within the primary peptide sequence. The synthetic peptides derived from LALF 31-52 bind

351 to LPS with high affinity and inhibits binding of LPS to LBP in a dose-dependent manner.

352 The protective effect of LALF has been shown *in vivo* via *Escherichia coli* and *Pseudomonas*

353 *aeruginosa* sepsis models in mice, with administration of LALF resulting in extended life

354 span and decreased mortality [55].

355

356 **II. Targeting disulfide bond formation by the bacterial DsbA-DsbB enzyme system of**

357 **Gram-negative bacteria**

358   The folding, stability and activity of a multitude of proteins in prokaryotic and eukaryotic

359   cells are attributed to disulfide bonds formed between pairs of cysteines within peptide

360   monomer units. Formation of a covalent disulfide bridge, via oxidation of sulfhydryl groups

361   (-SH) on corresponding cysteines, is important for the stabilization of the protein tertiary

362   structure. In bacteria disulfide bond formation is mediated by the DsbA-DsbB enzyme

363   system. The Gram-negative bacterial genotype encodes for a diversity of cysteine-based

364   disulfide bound proteins that are responsible for many bacterial virulence factors including

365   toxins, adhesins, flagella, fimbriae, and secretion systems [56]. For example, *Escherichia coli*

366   has around 300 proteins consisting of even numbers of cysteine residues that require DsbA

367   for folding [57]. It is hypothesized therefore that inactivation of enzymes that mediate the

368   creation of disulphide bonds in such proteins will disturb the stability and activity of related

369   virulence factors.

370

371   In Gram-negative bacteria the periplasmic enzyme DsbA is a member of the thioredoxin

372   family and oxidizes complementary pairs of cysteines to form disulfide bonds during their

373   movement through the cytoplasmic membrane into the cell envelope (Figure 2) [56][58]. The

374   resulting reduced active site cysteine of DsbA is re-oxidised by the inner membrane partner

375   protein DsbB, restoring DsbA's activity. The subsequent reduced DsbB is reoxidized and

376   restored using the oxidizing power of membrane-embedded quinones [59]. A number of

377   molecules have been found that disrupt this enzymatic pathway. Landeta and colleagues

378   discovered during screening of compounds that 4,5-dichloro-2-(2-chlorobenzyl)-3(2H)-

379   pyridazinone inhibits disulfide bond formation in *Escherichia coli* by blockage of the DsbB

380   enzyme *in vitro*. This compound was shown to bind covalently to the DsbB-DsbA system and

381   inhibit *Escherichia coli* growth. 4,5-dichloro-2-(2-chlorobenzyl)-3(2H)-pyridazinone was

382   also shown to inhibit DsbB enzymes in eight of nine tested Gram-negative pathogenic

383   bacteria [56]. Since the DsbA-DsbB system is responsible for disulfide bond formation in

384   Gram-negatives, it is an essential process for the correct folding and assembly of multiple

385   virulence factors and the bacterial cell envelope. This makes it a key target for the

386   development of new drugs to tackle Gram-negative infection. These compounds also exhibit

387   synergistic effects with a variety of antibiotics including beta-lactams, kanamycin,

388   erythromycin, novobiocin, and ofloxacin [60].

389

390   **III. Inactivating Gram-negative efflux pumps**

391   RND efflux pumps in Gram-negative pathogens play an important role in bacterial resistance

392   to a wide range of antibiotics, and so they are considered as a valuable field for development

393   of efflux pump inhibitors (EPI) for use in combination therapy. EPIs are envisaged to

394   increase the intracellular retention time and therefore efficacy of co-administered antibiotics

395   [61]. As outlined previously, RND pumps in Gram-negative bacteria are responsible for

396   exporting drugs and other toxic cations out of the cell. Their expression is upregulated in

397   response to external stress factors, including reactive oxygen species, cell membrane injury or

398   ribosome blocking agents [62]. The main RND efflux pumps expressed in Gram-negatives

399   are AcrAB-TolC in *Escherichia coli* and MexAB-OprM in *Pseudomonas aeruginosa*.

400   Located within the inner cell membrane their efflux action is mediated by bacterial

401   periplasmic adaptor proteins and an outer membrane channel (Figure 3). If an antimicrobial

402   agent successfully transverses the outer membrane via diffusion or porin channels, it enters

403   the periplasmic space. Once the antibiotic is in the periplasmic space, it binds to the

404   substrate-binding pocket of periplasmic adaptor proteins. The drug is actively transported to

405   the outer membrane channel and into the extracellular environment. *Pseudomonas*

406   *aeruginosa* PAO1 alone has 12 different RND efflux systems demonstrating the varying

407   complexity of bacterial efflux systems and the significant contribution they have in Gram-

408   negative resistance [61].

409

410   The physicochemical properties of the antibiotic molecule also determines its extrusion rate

411   by efflux pumps. RND pumps are mainly composed of an amino acid sequence with

412   lipophilic side chains. Small hydrophilic molecules, which move rapidly through porins,

413   possess a low efflux rate limiting their expulsion from the periplasmic space. However in

414   *Pseudomonas aeruginosa,* porins only allow a much slower entry of small molecules and so

415   efflux pumps can rapidly export them out of the cell. RND pumps also effectively efflux

416   more lipophilic and larger molecules, as they diffuse slowly through the hydrophobic layer of

417   the outer membrane. The rate of influx and active efflux of a drug can influence the minimum

418   inhibitory concentration (MIC) of an antibiotic *in vitro* [63].

419

420   Researchers have attempted to inhibit RND efflux pumps to restore the activity of antibiotics

421   previously deemed unusable due to the development of resistance [62]. Peptidomimetic

422   molecules were the first synthesized EPIs. Phenylalanyl-arginyl-β-naphthylamide is a

423   peptidomimetic compound that inhibits the levofloxacin efflux in *Pseudomonas aeruginosa*

424   overexpressed with MexAB-OprM efflux pumps. It achieves this by directly competing with

425   the antibiotic sites on MexAB-OprM [21]. Another novel EPI is the pyranopyridine

426   derivative, MBX2319, which increases *Escherichia coli* sensitivity to ciprofloxacin,

427   levofloxacin and piperacillin by inhibiting AcrAB-TolC efflux pumps. Peptidomimetic EPIs

428   often possess cidal antibacterial activity alone but are more likely to form an important role

429   within future clinical strategies as part of combination therapy [63].

430

Methods to extend the spectrum of activity of existing narrow spectrum Gram-positive antibiotics to Gram-negatives

The majority of antimicrobial agents, especially within the field of antimicrobial peptides, characterized in the laboratory setting are commonly more active against Gram-positive than Gram-negative bacteria [28]. A similar scenario exists clinically with a worrying lack of effective treatment options in reserve. Of greatest significance is the increase in resistance attributed to the Gram-negative pathogens *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*, due in part to a lack of available, effective narrow spectrum Gram-negative selective antibiotics. The majority of antibiotics reserved for resistant Gram-positive infection have no activity against Gram-negatives as they are incapable of crossing the Gram-negative LPS outer membrane barrier. The critical need for urgent action in the licensing and availability of effective antimicrobials to treat Gram-negative infections clinically has led researchers to concentrate their efforts on uncovering new and effective drug delivery systems to expand and target the spectrum of activity of currently licensed antibiotics. Various platforms, including fusogenic liposomes and nanotubes are in development. They represent a novel approach to tackle the current deficit in Gram-negative antibiotics and hope to rapidly extend the currently available antibiotic library using regulatory approved Gram-positive drugs.

I. Fusogenic liposomes

Liposomes are small vesicular systems composed of an amphipathic phospholipid bilayer with an aqueous interior core. They are attractive from a drug delivery perspective due to their varying hydrophobic (membrane) and hydrophilic (core) architecture that allows the incorporation of both hydrophobic and hydrophilic drugs, including a vast range of

455   antibiotics. Liposomal vesicles vary widely in diameter from 0.025 to 2.5 μm [64] and

456   demonstrate high biocompatibility and biostability resulting in prolonged circulation life [65].

457   Liposomes are promising molecules for antimicrobial drug delivery as the amphipathic

458   properties of the phospholipids enable strong interactions with the bacterial membranes and

459   enhance the release of the encapsulated drugs across them [66]. Interactions between

460   liposomal vesicles and bacterial membranes occur via multiple mechanisms, including

461   physical adsorption, lipid exchange and fusion. Liposomal-cell interactions are influenced by

462   the composition of the bacterial cell membrane, the exterior structure of liposomal carrier and

463   the biological environment [67].

464

465   Fusogenic liposomes are a variation on standard liposomal formulations consisting of

466   inactivated Sendai virus envelope components (mainly for targeting of eukaryotic cells) or

467   nonviral vectors involving the inclusion of specific lipids, for example amphiphilic

468   derivatives of cholesterol including cholesterol hemisuccinate, that increase fluidity of

469   liposomal vesicles to promote weakening of biological membranes. They demonstrate an

470   enhanced ability to fuse with cell membranes, mixing with their lipids, resulting in delivery

471   of vesicular contents into the cytoplasm [64]. They are promising as potential molecules to

472   transverse the Gram-negative outer membrane, enabling delivery of antibiotics such as

473   vancomycin to the periplasmic space. Vancomycin is a glycopeptide antibiotic with a

474   complex chemical structure and a high molecular weight (approximately 1450 daltons). It is

475   used clinically in the treatment of severe, multi-drug resistant Gram-positive infections. It

476   exerts a bactericidal effect by inhibiting the synthesis of peptidoglycan, the major component

477   of the bacterial cell wall [66]. The Gram-negative outer membrane is impermeable to

478   vancomycin macromolecules, therefore they are intrinsically resistant. Encapsulation of

479   vancomycin within fusogenic liposomes composed of dioleoylphosphatidylethanolamine,

480   dipalmitoylphosphatidylcholine and cholesterol hemisuccinate enables delivery to the

481   periplasmic space therefore allowing activity against Gram-negative bacteria. In a study by

482   Nicolosi and collagues, non-encapsulated vancomycin demonstrated high MIC values,

483   greater than 512 µg/ml for *Escherichia coli* and *Acinetobacter baumannii*, which reduced

484   significantly to 6 µg/ml upon inclusion within this liposomal platform [68]. This

485   demonstrates the potential of liposomal drug delivery platforms to extend the therapeutic

486   efficacy of narrow spectrum Gram-positive therapies.

487

II. Carbon and peptide nanotubes

489   Nanotechnologies, for example nanotubes, are at the forefront of research to tackle the most

490   difficult diseases in human and animal health. Nanotubes are materials consisting of hollow

491   cylindrical tubes with nanoscale morphology [69]. Organic-based nanotubes are attracting

492   increased attention as therapeutic applications, with researchers attempt to synthetically

493   replicate the nanoscale architectures of biomolecules such as DNA. Two of the most

494   promising nanomaterial formats are carbon and peptide-based systems [70]. Due mostly to

495   their increased structural strength and biological stability, carbon nanotubes have attracted the

496   attention for a range of applications throughout nanomedicine [71]. They can be formed by

497   coiling a single layer of graphene sheet to form single-walled carbon nanotubes, or by rolling

498   several layers to form multi-walled carbon nanotubes. The diameter of single-walled carbon

499   nanotubes varies from 0.4 to 3.0 nm with their length ranging from 20 to 1000 nm. Their

500   formation is driven by van der Waals intermolecular interactions increasing their structural

501   flexibility. Multi-walled carbon nanotubes are easier to manufacture than single-walled

502   variants, possessing an outer diameter ranging from 2 to 100 nm and inner diameter of 1 to

503   3 nm respectively. However, their length of 1 to several micrometres limits their structural

504   flexibility compared to single wall forms. Non-functionalized carbon nanotubes are insoluble

505   in aqueous physiological media making formulation difficult and some concerns do exist

506   regarding their safety in humans. For example, some studies have demonstrated toxicity to

507   mammalian cells including mediators of the immune response such as macrophages due

508   mainly to their high hydrophobic character [72].

509

510   Carbon nanotubes also lack homogeneity in terms of their size (diameters, length) this makes

511   it difficult to effectively link the type of formulation (e.g. suspension) and concentrations to

512   biological activity [73]. For future antimicrobial drug delivery purposes, carbon

513   nanostructures will likely require functionalization before attachment of a drug and this can

514   prove difficult due to the lack of chemical versatility provided by the rigid carbon-carbon

515   covalent bond. Covalent and noncovalent surface functionalization can be performed on the

516   synthesized carbon nanotubes facilitating the conjugation of antimicrobial agents such as the

517   antifungal amphotericin B [69]. Specific antibacterial activity has also been demonstrated for

518   carbon nanotubes against Gram-negative pathogens including *Escherichia coli*. Single walled

519   nanotubes are particularly effective due to their smaller diameter and therefore increased

520   ability to penetrate the cell wall. Carbon nanotubes display inherent antibacterial activity via

521   physical disruption of *Escherichia coli*'s bacterial cell membrane and oxidation of bacterial

522   glutathione resulting in oxidative stress and cell death [74]. The addition of hydroxyl (-OH)

523   and carboxylic acid (-COOH) groups to the surface of single-walled carbon nanotubes has

524   also been shown to enhance antimicrobial activity against Gram-positive and Gram-negative

525   bacteria. This is due to the formation of cell-nanotube aggregates and subsequent cell wall

526   lysis and DNA release [73]. Interestingly multi-walled carbon nanotubes do not display

527   similar efficacy due to increased length and a reduced ability to aggregate with bacterial cells

528   [74]. To date the majority of antibacterial carbon nanotube strategies are broad spectrum in

529 focus including coating with copper to eradicate *Escherichia coli* and *Staphylococcus aureus*

530 [75]. As the Yang group confirmed, neither the difference in cell wall structures between

531 Gram-negative and Gram-positive isolates nor the bacterial cell shape (cocci or rods), alter

532 the effectiveness of the single-walled carbon nanotubes [73]. Carbon nanotube research has

533 therefore been unable to selectively target Gram-negatives but the platform holds great

534 promise in the delivery of current and future drug molecules across the outer membrane

535 barrier.

536

537 Peptide-based nanomaterials have also received attention from researchers in the past decade

538 due to their chemical and functional versatility. Peptide nanomaterials have many advantages

539 over current synthetic-based materials utilised throughout healthcare. Peptides possess vast

540 chemical flexibility attributable to variation of the amino acid R-group. As a result they can

541 be utilised to create nanomaterials with very specific functionalities and have the potential to

542 conjugate to a variety of molecules including antimicrobial drugs. Amino acids are the

543 building blocks of peptides, proteins and tissues, existing throughout the body. The primary

544 amino acid sequence of peptides can be modified in order to drive self-assembly to

545 nanomaterials structures (nanofibers, nanotubes) in response to a range of physiochemical

546 stimuli (pH, temperature, ionic strength, presence of specific enzymes). Self-assembling

547 peptide platforms are gaining increasing interest as potential future antimicrobial

548 nanotherapies. The properties required for peptide assembly to occur are similar to those that

549 confer antimicrobial activity to the peptide, namely hydrophobic and electrostatic interactions

550 [76].

551

552 Some of the most successful approaches to target Gram-negative bacteria have focused on

553 utilising self-assembling linear and cyclic peptides. This is due to their ability to target

554 bacterial cell membranes and their structural similarities to naturally occurring polymyxins

555 [77]. Cyclic peptide nanotubes are primarily hexamers or octamers, composed of alternating

556 amphiphilic D- and L-amino acid residues, for example L-tryptophan and D-leucine. They

557 self-assemble into flat ring-shaped structures, with different channel diameters ranging from

558 0.2 to 1.3 nm [78]. Cyclic peptides can arrange into tubular open-ended structures via

559 intermolecular interactions including hydrogen bonding. When adsorbed onto bacterial cell

560 membranes, they have demonstrated selective membrane permeabilization and lysis of Gram-

561 positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) cells compared to

562 mammalian cells [79]. Cyclic peptide nanotubes have great potential as synergistic

563 antimicrobial therapies when used in conjunction with conventional antibiotics. They act as

564 delivery systems increasing antibiotic concentration, hence antimicrobial activity, within the

565 bacterial cell [80].

566

567 **III. Targeting Gram-negative pathogens with an engineered phage lytic enzyme**

568 Phages are viruses that demonstrate activity against bacterial cells, including multi-drug

569 resistant Gram-negatives. They were originally studied as potential antimicrobial therapies in

570 the United States in the 1930s and more extensively over the past 80 years in Eastern Europe

571 [81]. Phages have been reported to be effective in resolving a variety of infections including:

572 skin infections caused by *Pseudomonas*, *Staphylococcus*, *Proteus*, *Escherichia coli*, surgical

573 wound infections, staphylococcal lung and pleural infections, and *Pseudomonas aeruginosa*

574 infections in cystic fibrosis patients [82]. There are several reports that show that enzymes

575 isolated from phages, termed lysins, may be considered as therapeutic agents. Lysins

576 produced by bacteriophages are recombinant proteins designed to make "holes" in the cell

577 wall of a bacterium causing rapid cell lysis and death [83]. Until recently the action was

578 mainly restricted to Gram-positive bacteria. Applying the same strategy to Gram-negative

579 pathogens was considered difficult because their enzymatic target, peptidoglycan, is

580 sequestered beneath a protective outer membrane where the lytic enzyme cannot reach.

581 However, research by Lukacik and colleagues demonstrated that phage lytic enzymes can be

582 engineered to cross the outer envelope of Gram-negative bacteria. This is achieved by

583 production of hybrid lysins that have the ability to travel across the outer membrane of Gram-

584 negatives such as *Yersinia pestis* and pathogenic *Escherichia coli* strains, breaking down the

585 peptidoglycan layer in the periplasm. Hybrid lysin demonstrated cidal action against these

586 strains without disrupting the outer membrane. [84]. Variations to this theme also exist.

587 Artilysins are engineered lysins conjugated to cationic peptides extending the bactericidal

588 activity against Gram-negatives, including *Pseudomonas aeruginosa* and *Acinetobacter*

589 *baumannii*. The inclusion of a cationic peptide disturbs the LPS outer membrane layer,

590 allowing lysins to enter the periplasmic space resulting in degradation of peptidoglycan, cell

591 lysis and death [83].

592

593 **Conclusions**

594 The increasing resistance of Gram-negative bacteria to a multitude of currently available

595 antibiotics requires urgent action. Research regarding novel alternative therapies has focused

596 on a variety of strategies, many of which have failed to progress successfully to clinical

597 translation and utilisation for patient benefit. The majority of approaches have focused on

598 extending the spectrum of activity of current Gram-positive targeting molecules to Gram-

599 negatives. Whilst this warrants attention and should not be dismissed, a narrow spectrum,

600 species targeted approach is likely to be more beneficial with greater consideration of a

601   healthy commensal microbiota. This approach requires increased ability to rapidly diagnose

602   and detect specific causative microorganisms implicated infection so that optimal targeted

603   therapy can be provided. The research strategies outlined in this review contribute to

604   expanding potential future therapeutic options at a time when clinical choices are becoming

605   increasingly limited. Currently there are clinical trials involving several antimicrobial

606   peptides. This diverse group of molecules display selective antimicrobial activity against

607   bacteria relative to mammalian cells. Whilst *in vitro* results have demonstrated promise, *in*

608   *vivo* toxicity and biostability has restricted their progress. Other successful laboratory

609   research, involving attenuation of LPS and inhibition of RND efflux, display promise in

610   limiting the severe clinical implications of Gram-negative infection. Indeed many compounds

611   that display a LPS neutralizing ability may be suitable for future clinical trials as they have

612   demonstrated both *in vitro* and animal model efficacy. RND efflux pumps inhibitors are

613   attractive compounds that improve the clinical efficacy of antibiotics in resistant bacterial

614   pathogens. Understanding biochemical pathways in Gram-negative bacterial metabolism and

615   resistance will complement the development of novel and tailored therapies. For example,

616   targeted inhibition of DsbA-DsbB enzymes prevents disulfide bond formation and the

617   formation of stable protein tertiary structures within bacterial virulence factors. Despite the

618   promise shown by such an approach no compounds have yet transferred from the laboratory

619   into clinical trials highlighting the importance of pharmaceutical formulation in advancing

620   molecular targets. Improving antibiotic delivery using liposomes or nanotubes is another

621   encouraging approach to extend therapeutic activity of conventional antibiotics against Gram-

622   negatives. There is real hope for progress within this area especially as liposomal approaches

623   have successfully resulted in licensed formulations for a variety of drugs including the

624   antifungal amphotericin B. Engineered lysins have proven to be a truly alternative approach

625   resulting in a new class of antimicrobials. However, this approach still requires further

626   investigation particularly with regard to patient safety and their likelihood to develop

627   resistance.

628

629   **Future Perspectives**

630   As outlined, the need to eradicate multi-drug resistant bacteria and reduce the impending

631   threat of resistance is an increasing challenge not only for the scientific community but

632   society as a whole. It is everyone's responsibility to use existing antibiotics wisely in order to

633   delay an antimicrobial crisis and allow time for the development of effective novel

634   compounds. The research community has a key role to play in breaking down the microbial

635   processes that lead to resistance and developing strategies to combat such biomolecular

636   pathways. Collaboration is key for successful clinical translation. There is widespread

637   acceptance that a targeted isolate-specific approach to eradicate multi-drug resistant bacteria

638   is necessary to prevent treatment failure and risk of an increased number of antimicrobial

639   resistant strains. Some of the strategies outlined in this review provide great potential for

640   future therapeutics against Gram-negative pathogens. Key to future drug development in this

641   area is repeating the success of the early to mid-20th century boom in antibiotic discovery.

642   Bacteria are the most successful and innovative organisms on earth. Just as mother nature

643   provides infectious microorganisms with the tools for survival, so too does she hold the key

644   to solving the riddle of antimicrobial resistance. Scientists at Northeastern University Boston

645   recently uncovered a new antibiotic molecule, teixobactin, produced by bacteria (*Eleftheria*

646   *terrae*) present in soil. This molecule displays activity against *Methicillin resistant*

647   *Staphylococcus aureus* and bacteria implicated in tuberculosis infections but lacks effective

648   action against Gram-negatives. Similarly, "The Drugs from Dirt" project is a worldwide

649   initiative aiming to harness the capability of soil bacteria and the antimicrobial compounds

650   they produce. Microorganisms have long been known to be capable of producing such

651   molecules. They serve as weapons for survival facilitating destruction of competitive

652   microbial species and enabling survival in natural environments. Therefore their ability to

653   produce Gram-negative selective compounds seems logical. Chemically the most promising

654   of these naturally occurring compounds are peptides. Present throughout the animal and plant

655   kingdoms as part of the immune response, peptides are one the most effective molecules in

656   the fight against multi-drug resistant infection. Most promising, and in contrast with many

657   current therapies, is their ability to attack infectious microorganisms by multiple mechanisms.

658   The ability of bacteria to develop resistance against peptides is thus significantly limited. A

659   mining-like approach is an encouraging strategy to unlock innovative peptide antimicrobials

660   and may eventually lead to an era of discovery and a 21st century "antimicrobial rush."

661   Creating patient friendly therapies, for example oral dose formulations, from the most

662   promising of these molecules will require input from experts within the pharmaceutical

663   industry, healthcare workers and patients themselves. Only this way will such discoveries

664   create true value and easily translate from the laboratory to hospitals, communities and the

665   patient.

666

667   **Executive summary**

668   **Introduction**

669   • Resistance to standard therapies employed in Gram-negative bacterial infection have

670   increased to worrying levels over the last 30 years.

671   • There are a multitude of reasons for the declining clinical translation of antimicrobial

672   drugs in the past 20 years, including safety issues highlighted in clinical trials and

673 concerns from the pharmaceutical industry that investment in novel therapies would not

674 warrant a significant financial return.

675

676 **The Gram-negative outer membrane as a barrier to therapy**

677 **I. Bacterial cell wall structure**

678 • The outer membrane of Gram-negative bacteria acts as a selective barrier to the entry of a

679 vast range of currently available antibiotic molecules.

680 **II. Antimicrobial resistance mechanisms of Gram-negative bacterial cell wall**

681 • Alteration of lipid A, phospholipids and/or protein composition of the outer membrane

682 contribute to increased resistance to antimicrobial/antiseptic molecules that target the

683 bacterial cell membrane.

684

685 **Strategies for extending therapeutic activity against Gram-negatives**

686 **I. Antimicrobial peptides**

687 • Antimicrobial peptides exist throughout nature as mediators of the innate immune

688 response.

689 • Most cationic antimicrobial peptides target the bacterial cell membrane, leading to rapid

690 cell lysis and bacterial death. They also possess multiple intracellular targets.

691 • Cyclic antimicrobial peptides, which are among the most promising antimicrobial agents,

692 provide a starting point for designing low molecular mass anti-LPS compounds.

693 **II. Combinational antibiotic treatment for Gram-negative bacteria**

694 • Combination therapy is recommended for patients at high risk of being infected with

695 multidrug-resistant Gram-negative bacteria, demonstrating lower mortality rates and

696 improved clinical outcomes.

697 **III. The activity of silver against Gram-negative bacterial infection**

698 • Silver increases the permeability of Gram-negative bacterial membranes and can

699 potentiate the activity of a broad range of antibiotics against these microorganisms.

700 • Silver nanoparticles have attracted interest due to their potential applications within

701 wound dressings, medical device coatings, and drug delivery.

702

703 **Specific methods to target Gram-negative pathogens**

704 **I. Negating the biological effects of Gram-negative lipopolysaccharide**

705 • An important consideration when treating suspected or confirmed Gram-negative

706 infection is preventing the biological effects of Gram-negative lipopolysaccharide. This

707 potent molecule signals bacterial invasion and triggers defensive host responses to release

708 pro-inflammatory mediators, cytokines, chemokines, and lipoproteins.

709 **II. Targeting disulfide bond formation by the bacterial DsbA-DsbB enzyme system of**
710 **Gram-negative bacteria**

711 • DsbA-DsbB system in Gram-negative bacteria is a key target for the development of new

712 drug molecules. Inhibition of disulfide bond formation has been demonstrated to prevent

713 the assembly of key bacterial virulence factors.

714

715 **III. Inactivating Gram-negative efflux pumps**

716 • Inactivating Gram-negative efflux pumps has the potential to restore resistant antibiotics

717 activity.

718

Methods to extend the spectrum of activity of existing narrow spectrum Gram-positive antibiotics to Gram-negatives

I. Fusogenic liposomes

- Encapsulating narrow spectrum Gram-positive selective antibiotics within fusogenic liposomes has been shown to broaden their spectrum of activity to cover Gram-negative infections by enabling transversion across the outer membrane.

II. Carbon and peptide nanotubes

- Single-walled carbon nanotubes may be useful in molecules as future antimicrobials due to their inherent antimicrobial properties and ability to deliver existing and future antibiotic molecules via nanoparticle-based drug delivery.
- Cyclic D, L-alpha peptides are able to selectively target bacterial cell membranes, including the outer membrane of Gram-negatives. They are able to self-assemble, forming peptide nanotubes with the potential to act as biofunctional nanomaterials and improve intracellular delivery of antibiotics.

III. Targeting Gram-negative pathogens with an engineered phage lytic enzyme

- Phage lytic enzymes can be engineered to cross the outer envelope of targeted Gram-negative bacteria. This is achieved by production of a "hybrid lysin" and "artilysin" that have the ability to kill pathogenic *Escherichia coli* strains and *Pseudomonas aeruginosa*.

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960   engineered to target Gram-negative bacteria.

961

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