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Summary (150 words)

The global challenges presented by drug-resistant bacteria infections has stimulated much activity in finding new treatments. This review summarizes the progress, and set-backs, of non-traditional approaches intent on circumventing bacterial drug-resistance. These approaches include targeting virulence via toxin production and virulence factor secretion, impeding bacterial adhesion to host cells and biofilm formation, interrupting/inhibiting bacterial communication and down regulating virulence. Other strategies include immune evasion, microbiome modifying therapies and the employment of phages as treatments or carriers. Finally, the prospects of nanoparticles, immunotherapy, antisense RNA and drug-resistance modulation approaches are discussed. The development of non-traditional treatments suffers similar challenges faced by developers of conventional antibiotics, however most of these new strategies have additional and considerable hurdles before it can be shown that they are safe and efficacious for patient use. For the foreseeable future, it is likely that most of these treatments, if approved, will be used in combination with antibiotics.

Key words: Anti-virulence, Quorum-sensing, Microbiome, Phage, Nanoparticles, Immunotherapy, Antisense RNA, non-traditional antimicrobials

Introduction

The global crisis of drug resistance, few new drugs to treat infections by the most resistant pathogens and the scientific challenges in discovery and early development of new antibiotics has inspired researchers to explore new ways to treat bacterial infections. The traditional antibiotic approach of treating infections is to find small molecules that either inhibit growth or kill Gram-positive or Gram-negative bacteria or both (broad-spectrum antibiotics). Non-traditional approaches explore many ways to influence the disease beyond inhibiting or killing

34   pathogens through small molecules. It should be noted that in this article, non-traditional does

35   not imply an alternative therapy to antibiotics, which means replacing the use of these drugs.

36   Due to the wide diversity of research activities that could lead to the discovery of new

37   therapies this article is not exhaustive. It focuses on the areas that are most frequently

38   explored and have achieved some late preclinical or clinical experience. We have not

39   discussed vaccines, devices, exclusively topical drugs or directly acting small molecule drugs

40   such as potentiators of antibiotics including ß-lactamase and efflux inhibitors, combinations

41   of small molecule drugs or conjugates or antimicrobial peptides. Instead, we have focused this

42   article on the most promising non-traditional antibacterial treatments under development, their

43   uses, and hurdles that must be overcome to provide safe and efficacious new medicines.

44   Anti-virulence

45   Anti-virulence approaches aim to inhibit the production or activity of Virulence Factors

46   (VFs), typically these have no effect on bacterial growth *in vitro*. The number of VFs is

47   growing (Virulence Factor Database, http://www.mgc.ac.cn/VFs/ (Dickey et al., 2017) and

48   includes toxins, adhesins, quorum sensing molecules, virulence-dedicated secretion and

49   regulation, siderophores, and immune evasion factors (Heras et al., 2015; Totsika, 2016). VFs

50   are often species- or even strain-specific and variably conserved between and/or within a

51   bacterial species. Furthermore, virulence gene expression may depend on the environment or

52   site of infection or on the time course of a pathophysiological process. The complex

53   biological variety constitutes a major challenge to translating early discovery to the clinical

54   situation, especially as anti-VFs usually have a narrow spectrum of activity.

55   Numerous anti-virulence strategies are in the discovery or preclinical development phase

56   (Garland et al., 2017). Progress in understanding pathogenesis and preclinical development

57   efforts has progressed a few anti-virulence drugs to the clinical phase of development, and

58 some that block exotoxins have been approved as treatments. Here, we provide an overview

59 of the anti-virulence strategies that are more advanced in development as a new treatment.

60 Toxins

61 Exotoxins are produced by pathogenic bacteria without which the microbe does not elicit

62 symptoms in the infected individual. Therefore, they are obvious targets for anti-virulence

63 therapies. Many bacterial toxins are released into the environment and are thus, amenable to

64 antibody therapy. Antibodies approved for clinical use are active against toxins of

65 *Clostridium botulinum*, *Bacillus anthracis* and *Clostridium difficile*. The recently FDA and

66 EMA approved human monoclonal antibody (mAb) Bezlotoxumab (Merck) binds to the *C.*

67 *difficile* toxin B and is indicated to prevent recurrent *C. difficile* infection (CDI) in at-risk

68 adults.

69 *Staphylococcus aureus* harbors many VFs to facilitate tissue adhesion, immune evasion, and

70 host cell injury. *S. aureus* α-toxin is a pore-forming toxin that plays an important role in

71 staphylococcal infection. In general, exotoxins and surface-localized structures provide good

72 targets for monoclonal antibodies (Table 1). The mAb suvratoxumab (MEDI4893,

73 Medimmune) binds to and neutralizes *S. aureus* α-toxin (Yu et al., 2017). It is in Phase 2

74 clinical trials in colonized and mechanically ventilated patients to prevent ventilator-

75 associated pneumonia (VAP) caused by *S. aureus*. Other companies are pursuing a similar

76 strategy with α -toxin-binding mAbs (e.g. AR-301; Salvecin; tosatoxumab, Aridis). In

77 contrast to suvratoxumab that is aimed at preventing symptomatic infection, Aridis plans to

78 investigate AR-301 in a Phase 3 trial as an adjunctive therapy in combination with standard of

79 care antibiotics to treat VAP caused by *S. aureus*. The monoclonal antibody preparation

80 ASN100 (Arsanis), that neutralizes six *S. aureus* cytotoxins, failed to reach the primary

81 endpoint in a Phase 2 study.

VF secretion

Type III secretion systems (T3SS) are complex structures embedded in the bacterial inner and outer membranes of many Gram-negative bacteria that are used to deliver virulence effector proteins into host cells and facilitate the establishment and dissemination of infections (Anantharajah et al., 2016; Deng et al., 2017). The interruption of toxin secretion or structural proteins of T3SS, especially the needle tip protein assembly is a focus of current research for small molecule inhibitors (Aiello et al., 2010; Gu et al., 2015, Fasciano et al, 2019) and mAb (Table 1). A bispecific mAb (MEDI3902, Medimmune) targets the *P. aeruginosa* T3SS needle tip protein PcrV to prevent T3SS mediated injection of toxins in host cells and Psl exopolysaccharide to prevent attachment of bacteria to epithelial cells (DiGiandomenico et al., 2014). MEDI3902 is in Phase 2 clinical development in mechanically ventilated patients for the prevention of VAP caused by *P. aeruginosa*. An earlier antibody approach did not progress to late stage clinical trials (Fasciano et al, 2019). Small molecules have the potential for a broader spectrum of activity than mAbs against T3SS, which are target specific and hence have a narrow spectrum.

Adhesion and biofilm formation

S. aureus expresses a number of cell surface adherence and immune evasion proteins, many of which are anchored to the cell wall by the transpeptidase sortase A (SrtA) enzyme (Cascioferro et al., 2015; Dickey et al., 2017). Several antibacterial drug discovery programs have found SrtA inhibitors that are believed to promote the disruption of the structure of mature biofilm; however, early preclinical projects have not advanced.

P. aeruginosa produces the surface polysaccharide alginate in response to environmental conditions. It enhances adhesion, biofilm formation and resistance to human leukocyte killing; this is most apparent in the environment of the affected lung in patients with CF. AR-105 is a

106   mAb directed at alginate and is currently being tested in a Phase 2 clinical trial as adjunctive

107   treatment in mechanically ventilated patients with VAP caused by *P. aeruginosa*.

108   The first step of colonization of the bladder and artificial surfaces such as catheters is to avoid

109   clearance during urine voiding by binding of bacteria via an available epithelial receptor

110   (Spaulding and Hultgren, 2016). Therefore, recognition and attachment of the bacteria to the

111   uroepithelium plays a key role in anti-virulence strategies that target uropathogenic *E. coli*

112   (UPEC) and lower urinary tract infections (UTI). Adhesion is mediated through the

113   expression of pili and their tip adhesin FimH that binds to mannosylated residues on the

114   bladder epithelial surface (Spaulding et al., 2018). Several strategies to block adhesion have

115   been pursued including developing mannose analogs that bind within the mannose-binding

116   pocket of FimH and block pilus binding of FimH to host receptors and thus prevent

117   attachment of UPEC (Maddirala et al., 2018) (Fimbrion). Other FimH inhibitors were

118   effective in the mouse model, but it is not known if these animal models are predictive and

119   whether data from them will translate into clinical efficacy (Kalas et al., 2018). UPEC

120   colonize the intestinal space and establish a bacterial reservoir for infecting the urinary tract.

121   The same adhesion mechanisms and binding principles apply to preventing selectively

122   intestinal colonization of UPEC by treatment with mannosides. The oral and non-systemic

123   small molecule drug EB8018 designed to block the FimH adhesin from overabundant

124   Enterobacteriaceae (adherent-invasive *E. coli*) in Crohn's disease patients is in Phase 1

125   clinical studies (Enterome NCT03709628).

126   Bacterial communication

127   Quorum-sensing (QS) is a molecular communication system to synchronize the expression of

128   certain genes affecting a global change in bacterial gene expression and cell physiology.

129   There is a wide variety of signaling molecules that may serve as attractive and potentially

130   broad-spectrum anti-virulence targets (Rémy et al., 2018). Various QS-interfering agents

131   (called quorum quenchers) including natural and synthetic compounds, enzymes and

132   antibodies that target each step of the QS pathway have been described. These have been

133   tested *in vitro* and *in vivo* (Defoirdt, 2018). *P. aeruginosa* has been studied most and serve as

134   a model for targeting QS. The *P. aeruginosa* QS pathways LasR-LasI, RhlR-RhlI, IQS, MvfR

135   have been explored in both acute and persistent infection models (Dickey et al., 2017). The

136   *las* system controls LasB elastase, a pivotal VF in pseudomonal infection and target of drug

137   discovery projects (Antabio). LasB is secreted at the site of infection, where it exerts a

138   proteolytic action including broad tissue destruction and subtle action on components of the

139   host immune system (Cathcart et al., 2011). The discovery of the role of the multiple VF

140   regulator MvfR in mediating antibiotic tolerance and persister cell formation in *P. aeruginosa*

141   inspired the research and discovery of inhibitors of MvfR (Maura et al., 2017) (Spero). In *S.*

142   *aureus* QS is mainly regulated by the *agr* operon. Although several small molecule inhibitors

143   have been described, none have advanced to the optimization phase of discovery (Salam and

144   Quave, 2018). Based on considerable basic research, QS inhibiting strategies are increasingly

145   included in discovery projects (Haque et al., 2018; Williams, 2017).

146   Counteracting Immune evasion

147   Many bacteria deploy factors to prevent detection by or to escape the host immune response.

148   Therefore, strategies to neutralize such tactics are under development. Monoclonal antibodies

149   targeting bacterial surface epitopes are hypothesized to increase bacterial clearance through

150   enhancing antibody-dependent phagocytosis, and/or complement-mediated bactericidal

151   activity, or via immune system-independent bacterial killing (Wang-Lin and Balthasar, 2018).

152   The *S. aureus* Protein A (SpA) defends the bacterium against the adaptive immune response

153   by resisting phagocytosis and inducing apoptosis of B cells. This protein also facilitates nasal

154   colonization and cell adhesion (Hong et al., 2016). The mAb 514G3 neutralizes the SpA

155   mediated immune evasion of *S. aureus* (Varshney et al., 2018). An on-going a Phase 1/2

156 clinical study to treat *S. aureus* blood stream infections as an adjunctive therapy is due to

157 complete in June 2020 (XBiotech NCT02357966).

158 Regulating virulence

159 Clp proteases suppress the expression of multiple unrelated VFs in *S. aureus* by impacting on

160 central processes such as virulence gene expression, cell wall metabolism, survival in

161 stationary phase, and cell division. The simultaneous suppression of multiple VFs or

162 pathways using small-molecule compounds is a promising approach to reducing the virulence

163 of *S. aureus* (Gao et al., 2018). Many inhibitors have been discovered and are currently being

164 optimized (Aviru). However, whether targeting a master regulator of virulence translates into

165 clinically relevant benefits remains to be seen.

166 Advantages and Disadvantages of anti-virulence approaches

167 As anti-virulence drugs interact with non-essential targets, there is a general assumption that

168 they do not select for resistance; however, this has been challenged (Allen et al., 2014).

169 Experimental data revealed the emergence of resistance showing the complex evolution and

170 resistance selection by some anti-virulence drugs; this was dependent on the importance to the

171 pathogen of the targeted VF (Totsika, 2016). Whether such resistance will impact therapy and

172 the potential of transmissible resistance is unknown (Rezzoagli et al., 2018).

173 There are numerous challenges to translating anti-virulence strategies to new treatments for

174 patients (Table 2). Given the complexities of virulence systems, the likely specificity of single

175 VFs, and the lack of research data, it is not surprising to find extremely long timelines for the

176 preclinical development of anti-virulence approaches and the failure of clinical trials.

177 Development of anti-virulence drugs requires an in-depth understanding of these factors and

178 the roles that specific VFs have in disease processes. Traditional measurement of growth

179 inhibitory activity, the Minimum Inhibitory Concentration (MIC) test, does not apply to anti-

180 virulence drugs as by definition they do not inhibit growth or kill bacteria. In most cases,

181   alternative *in vitro* methods are not developed and the predictability of animal models for

182   clinical outcome are poorly described.

183   Potential clinical indications for anti-virulence therapies are prevention of disease such as

184   CDI, HAP/VAP and recurrent uncomplicated UTI. However, as some VFs are specific for a

185   pathogen and rely upon of their expression, which is influenced by the condition of the patient

186   and disease state, accurate diagnostic tests will be necessary to identify the pathogen and

187   presence/expression of the targeted VF. Therefore, chronic or non-life-threatening infections

188   are likely to be targeted as these will provide the opportunity to carefully select the patient,

189   enabling a tailored patient-specific approach with less time pressure to start therapy. Most

190   anti-virulence therapies are developed as adjunctive therapies in addition to standard therapy,

191   usually antibiotics. This is the only ethically acceptable pathway in clinical practice in

192   patients needing fast acting antibacterial therapy. This poses special challenges for drug

193   development and clinical use as it is not possible to prove clinical efficacy of an adjunctive

194   therapy with a conventional non-inferiority clinical trial design. Superiority design in a

195   combination therapy versus stand-alone antibiotic therapy would be the most convincing way

196   to show a clinically relevant effect of an add-on therapy. Selection of the appropriate

197   indication, patient population, clinical endpoints and clinical trial sites are enormous

198   challenges for late stage clinical studies. Relevant secondary endpoints may support the

199   clinical therapy decisions (Maura et al., 2016).

200   Anti-virulence approaches will not replace antibiotics, thus may not contribute much to

201   resolving the resistance problem and insufficient antibiotics pipelines. Nonetheless, they may

202   complement the action of antibiotics; however, evidence that they provide benefit for patients

203   in high-quality clinical trials is needed. Open discussion and analysis of failed clinical trials

204   would enhance this field.

Microbiome modifying therapies

Recent advances in metagenomic, computational and synthetic biology tools inspired the revival of research into the human microbiome and has provided a deeper understanding of its interactions with the host (Wilson et al., 2019). Manipulating and engineering the human microbiome is an attractive option to prevent and resolve infection and so is generating considerable activity in academia and industry (Timmis et al., 2019) and attracts broad interest among public funders and private investors (Boers et al., 2016). The US National Microbiome Initiative had an important impact and advanced standards for the use of the next-generation technologies in metagenomic studies and generated quality-controlled data (Group et al., 2009). Not only bacterial microbiota but growing knowledge about the role of the human phageome has highlighted the impact of bacteriophages in shaping a healthy intestinal microbiome (Anonye, 2018; Paule et al., 2018; Rohde et al., 2018; Zuo et al., 2018). The gut microbiota is intricately connected to the host's immune system through a reciprocal developmental relationship. Specifically, the microbiome is critical for the appropriate development of the immune system, and in turn, the immune system helps modulate the microbiome community through a balance of pro- and anti-inflammatory pathways (Cammarota et al., 2017).

More than 10 small companies are developing microbiome therapies for infectious diseases (Table 3). So far, most experience has been gained on the impact of the intestinal microbiota on the physiology of *C. difficile* in the gut and recurrent CDI (Young, 2016). The underlying assumption is that rebuilding the microbiome after infection or preserving the microbiome to prevent infection will translate into clinical benefit. The strategies to restore an unbalanced microbiome are based on the experience of clinically successful transfers of a full natural microbiota in form of stool from healthy donors (Fecal Microbiome Transplantation, FMT) (Cammarota et al., 2017). This procedure has a typical cure rate of 90% and has encouraged

230 research groups to modify this principle and focus on production of stool banks, standardized

231 products, engineering and oral delivery strategies.

232 Although no microbiome-modifying therapy has been officially approved, the US FDA

233 allows FMT without filing an Investigational New Drug (IND) application exercising

234 enforcement discretion when using the stool bank OpenBiome to treat *C. difficile* infection

235 not responsive to standard therapy (FDA, 2016). FMT reintroduces a complete, stable

236 community of gut microorganisms (Bakken et al., 2011; Borody and Campbell, 2012) and is

237 the most advanced form of microbiota therapy with a large body of experience (Ooijevaar et

238 al., 2018). Guidelines for clinical use are available (Mullish et al., 2018). In the UK, large

239 clinical trials of FMT are underway (ISRCTN 74072945). Most experience has been gained

240 so far with the application via enema but freeze-dried capsule-based formulations of the

241 microbiota or mixture of spores from several bacteria isolated from healthy donor fecal

242 samples are in clinical development (Baker et al., 2018). There are no high-quality studies

243 available yet to show the efficacy and safety of the oral route (Iqbal, 2018). Two companies

244 are currently conducting a randomized Phase 3 trial enrolling patients with recurrent CDI

245 (Rebiotix NCT03244644, Seres NCT03183128), another company is enrolling patients in a

246 Phase 2 trial to test a therapy that contains microbiota produced from pure, clonal bacterial

247 cell banks (Vedanta NCT03788434) (Table 3).

248 Companies are also developing a wide range of microbiome strategies including rationally

249 selected cocktails of bacteria or bacterial spores containing the "active components" of the

250 complex microbiota (Khanna et al., 2016; Orenstein et al., 2016). The most extremely reduced

251 approach is the use of a single non-toxic strain of *C. difficile* that is hypothesized to

252 outcompete the toxigenic strains in the gut. Another approach is to assemble a synthetic

253 microbiome comprising well characterized individual strains in pure cultures with

254 standardized properties (Timmis et al., 2019). Other strategies build on the gastrointestinal

255 metabolic balance and subsequent changes made during disturbance and restoration to a

256 healthy microbiota (Koropatkin et al., 2012). In another approach, genetically engineered

257 bacteria produce antibacterial compounds that selectively remove key disease-causing species

258 from the microbiota. Increasingly, bacteriophages or nanoparticles serve as vehicles to

259 selectively target pathogenic bacteria or resistance and virulence determinants in the gut flora

260 and thus can be used to manipulate the microbiota. Current approaches also target intestinal

261 colonization with pathogenic bacteria such as carbapenem-resistant *Enterobacteriaceae*

262 (CRE) in critically ill patients. However, it should be noted that there are conflicting data on

263 the causality between CRE colonization and increased mortality in ICU patients (McConville

264 et al., 2017). Studies with FMT to test for the effect of decolonization in high-risk patients

265 were not conclusive (Huttner et al.; Relman and Lipsitch, 2018). Although the gut microbiota

266 is the most common target for microbiome-modifying therapies, other concepts focus on

267 manipulating the skin or lung microbiome. These approaches are at very early stages and

268 correlations with the clinical situation are less clear.

269 The key challenges of developing new simplified microbiome therapies are the incomplete

270 understanding of the complex genomic and phylogenetic diversity of the human microbiome.

271 Indirect testing of hypotheses and statistical correlations may not prove pathophysiological

272 causation. If innovative microbiome therapies beyond FMT and other complex microbiota

273 strategies are to translate into clinical benefit a deeper understanding of the complex role of

274 the microbiota in the pathogenesis of a specific disease is necessary. The extent to which the

275 complexity of the therapeutic approach can be reduced while retaining efficacy remains to be

276 seen. The high variability of the microbiome composition yields inconsistent and

277 contradictory results that are difficult to interpret. Simple preclinical models may not be

278 predictive. Selecting appropriate microbiota is important but proving that live bacteria

279 constitute a coherent community and are incorporated into the recipient's gut and remain after

280   being ingested is similarly essential (Smillie et al., 2018). Additionally, the manufacture of

281   live bacterial products is complex and expensive. There is no good rationale or model to

282   support finding the appropriate or optimum dose and so doing reflects a trial and error

283   strategy rather than characterizing an effective dose. Although microbiome treatments with

284   complex microbiota for preventing recurrent CDI are in late stage clinical trials, the

285   development of next generation treatments rely upon reducing unpredictable factors and

286   filling the gaps in the basic understanding of underlying processes. Currently, reduction of the

287   complexity of the microbiota substantially increases the risk of clinical failure.

288   ## Phages

289   Although therapy of bacterial infections with bacterial viruses (bacteriophages, phages) has

290   been practiced in Eastern Europe for nearly a century, the interest in phage R&D has only

291   gained traction elsewhere in the last 10-15 years as a response to the emergence of multidrug-

292   resistant pathogens (Kortright et al., 2019). Synthetic biology and other modern tools have

293   revived the field of phage research (Pires et al., 2016). They enable the modification of

294   phages, the characterization and careful screening for and removal of genes coding for toxins

295   and VFs to avoid the risk of transfer from one bacterium to another. In general, phages are

296   regarded as safe because they do not infect mammalian cells.

297   There are at least 30 companies pursuing a treatment strategy that involves phages (table 4).

298   Recent and ongoing trials focus on infections by *P. aeruginosa*, *S. aureus* and *E. coli.* Besides

299   anecdotal case studies, case reports of compassionate use programs and poor quality non-

300   controlled clinical studies, only one randomized placebo-controlled Phase 1/2 clinical trial

301   with topical treatment of chronic otitis caused by *P. aeruginosa* has been successfully

302   conducted (Wright et al., 2009). A recently completed Phase 1/2 clinical trial in infected burn

303   wounds (phagoburn) failed to demonstrate efficacy and exemplifies the challenges described

304   below when translating phage approaches to the clinical environment (Servick, 2016). A

305 Phase 1/2 trial with a *S. aureus* phage cocktail for i.v. administration is being prepared to start

306 in 2019 (Ampliphi).

307 Phage therapy is characterized by its specificity to single bacterial species and usually to a

308 subset of strains within that species (Kortright et al., 2019). To be active against >90% of

309 strains within a bacterial species and to prevent rapid emergence of bacterial resistance to a

310 single phage, mixtures (cocktails) of different phages, often more than 10 phages, are used for

311 therapy. Phage resistance can evolve within hours, independently of the use of bacteriophage

312 combinations. Although the combination of multiple phages in a cocktail compensates for a

313 limited host range the increased complexity of such a cocktail not only dilutes the

314 concentration of the individual phages but can promote potential unfavorable interactions

315 between phages and cause manufacturing and quality control issues. This is the reason why

316 companies try to reduce the number of phages in fixed cocktails and some produce mini-

317 cocktails ($\leq$5 phages). The downside of such mini-cocktails may be a smaller host range.

318 Currently, there is a trend towards patient specific cocktails based on libraries of pre-approved

319 phages. Phage banks containing purified or pre-purified phages allow the quick assembly of

320 patient-specific cocktails that contain only the most appropriate phages against the infecting

321 bacterium. The choice of phages is based on new diagnostic tools that are not yet available in

322 clinical practice. Based on modern genetic engineering tools, recent and current research

323 focuses on engineered phages with improved or specific features (Barbu et al., 2016). In

324 contrast to the above-mentioned strategies that use lytic phages, non-lytic phages are utilized

325 as vehicles to express antibacterial proteins or genes (Krom et al., 2015).

326 There are several challenges to the clinical use of phage treatment. These include

327   1) the prerequisite of selecting appropriate phages to achieve an appropriate range of

328   activity and prevent development of bacterial resistance. Such fixed phage cocktails

329   need to consider bacterial isolates from different infections and geographic locations.

330   2) manufacturing phages under good manufacturing practices (GMP) and chemistry,

331   manufacturing, and control (CMC) guidance. Some progress has been achieved when

332   tackling the insurmountable challenge regarding CMC, especially production,

333   stability, purity and quality control.

334   3) Considering phage biology in the design of phage treatment is a prerequisite of any

335   successful approach (Bull and Gill, 2014). Unique pharmacokinetics (PK) and

336   pharmacodynamics (PD) of phages means that dose finding processes are challenging.

337   The immense size of phages when compared to small molecule antibiotics results in a

338   wide range of PK challenges and is the reason why many sites of infection are not

339   accessible by phages. Questions of basic PK such as distribution, dilution in the blood

340   compartment, rapid clearance (Inchley, 1969), as well as kinetics of phage infection

341   and other PK/PD characteristics are not well defined. Development of methods to

342   access these parameters needs to proceed in parallel with clinical work to assess

343   exposure and efficacy relationships. The concept of phage therapy is based on

344   localized amplification in the presence of the specific susceptible bacteria. High

345   bacterial loads are necessary for amplification and their localization is a complex

346   pathophysiological issue (Rose et al., 2014). Though localized amplification is a key

347   concept, the initially applied dose and the fate of the phages in the systemic circulation

348   is not well understood. Bacterial loads and decreased availability of active phages in

349   the circulation or localized infection sites may be responsible for a potentially slow

350   onset of activity. The success of phage therapy ultimately depends on the optimal

351   dose, dosing regimen, timing, formulation and administration, with PK and PD

352   characterized for each phage or phage cocktail.

353   4) showing efficacy in clinical trials, and thus, gaining regulatory approval. Although

354   case reports in compassionate use programs indicate the possibility of systemic use,

355   due to access issues, topical delivery has been much more common. However, in this

356 setting, concomitantly used treatments (e.g. wound care products, disinfectants and

357 antibiotic topical treatments) may affect the local stability of phages (Merabishvili et

358 al., 2017). Inhalation treatment with phages, potentially also in combination with an

359 antibiotic for specific indications such as CF seems to be feasible (Chang et al., 2018).

360 Another aspect of phage therapy is their natural immunogenicity which is stimulated

361 by bacteria (bacteria can highjack the innate immunity of hosts to inhibit phage).

362 Interacting elements of adaptive and innate immunity are contributing to the clearance

363 of phages with consequences on phage PK (Hodyra-Stefaniak et al., 2015). To use in

364 patients, the immune response to phage therapy need to be assessed (Dąbrowska et al.,

365 2014; Krut and Bekeredjian-Ding, 2018). At present, there are no high-quality data to

366 show that phage therapy works routinely in clinical settings. The future and potential

367 clinical use of phage therapeutics depends on the careful selection of phage-accessible

368 infections and to be able to show a clinical benefit for patients (Harper, 2018). The

369 current challenges of regulatory pathways, especially for patient-specific but also fixed

370 phage cocktails include the need for constantly adjusting the preparation as the

371 bacteria evolve and requires an appropriate legal and regulatory framework

372 (Fauconnier, 2017; Pirnay et al., 2018) with recent progress in approval to conduct

373 clinical trials.

374 5) developing and implementing appropriate diagnostics is essential to support use in

375 patients.

376 For the most common bacterial infections it is unlikely that phage therapy will replace use of

377 antibiotics (Rohde et al., 2018). However, synergy with antibiotics has been seen in vitro and

378 in animal models (Oechslin et al., 2017). Therefore, phage therapy may be a promising

379 adjunctive treatment in specific indications or salvage therapy for patients with infections that

380   have not responded to any other treatment. Convincing clinical efficacy in well-designed

381   randomized controlled clinical trials needs to be demonstrated.

382   Phages as carriers

383   Genetically engineered non-replicative phages are designed to serve as specific nano-delivery

384   vehicles and carry payloads that exert antibacterial activity beyond direct lysis of the cell

385   (Krom et al., 2015). Synthetic biology approaches enable the use of a wide range of gene

386   expression systems to target bacteria. Phage delivery systems are usually specific and so

387   render the delivered therapeutics narrow spectrum and pathogen-specific. The delivered genes

388   may be DNA sequence-independent and cause a rapid bactericidal effect (Phico) or utilize the

389   clustered regularly interspaced short palindromic repeat (CRISPR) RNA-guided genome

390   editing systems to engineer novel functions (Locus Biosciences, Eligo Bioscience) (Hatoum-

391   Aslan, 2018). CRISPR Cas9 has developed into a new powerful technology to regulate gene

392   expression in bacteria (Bikard et al., 2013). The RNA-guided nuclease Cas9 may serve as a

393   sequence-specific antibacterial (Bikard et al., 2014) or may target a specific DNA sequence to

394   inactivate antibiotic resistance or virulence genes (Nemesis Bioscience). Another CRISPR–

395   Cas system is used to insert CRISPR-Cas3 constructs into the phage genome that specifically

396   degrade the DNA of target bacterial cells (Locus Biosciences). The CRISPR/Cas mediated

397   technology can also be used to modify phages and optimize favorable characteristics

398   (Hatoum-Aslan, 2018).

399   As such phage vehicles are not self-replicative the dose must be very high to target bacteria in

400   an infection. PK and dose finding are not well understood. New antibacterial approaches that

401   use phages as carriers are faced with two big challenges, (1) the new technology (e.g.

402   CRISPR-Cas) and (2) the phages themselves as discussed above. This doubles the risk and

403   will need considerable time to progress to clinical studies to show a clinical benefit for

404   patients.

Phage-derived products

406 Though phage-derived enzymes have been explored since the late 1990s, renewed interest

407 emerged to address the current drug-resistance issues. Endolysins are the best studied phage-

408 derived peptidoglycan-degrading enzymes. They are encoded by phages to liberate progeny

409 phage from inside of infected bacterial cells, resulting in fast osmotic lysis and bacterial cell

410 death (Fischetti, 2018). Endolysins are bacteriolytic on contact, independent of resistance

411 pattern to conventional antibiotics, and are highly specific for a bacterial species or genus

412 (Fernandes and São-José, 2018). Naturally, endolysins work from inside the cell but purified,

413 recombinant lysins enabled enzymes are lytic from the outside. When developed as drugs,

414 lysins must be stable, soluble and able to hydrolyze peptidoglycan from the outside.

415 Numerous endolysins against Gram-positive bacteria have been studied in vitro and in animal

416 models (Gutiérrez et al., 2018; Haddad Kashani et al., 2017). The opportunities to customize

417 endolysin properties such as specificity, activity, stability and solubility are currently being

418 explored and extensive protein engineering efforts have expanded (Oliveira et al., 2018). The

419 first two products that have reached Phase 2 clinical development are recombinant lysins

420 directed against *S. aureus* (SAL200/tonabacase from Roivant in-licensed from iNtRON

421 Biotechnology (Kim et al., 2018) and CF-301/exebacase from Contrafect) (Schuch et al.,

422 2014). A topically applied endolysin for inflammatory conditions due to *S. aureus* in atopic

423 dermatitis is already available (Micreos Human Health BV). Another topical endolysin

424 against staphylococci is being studied in Phase 1/2 clinical trials for nasal decontamination

425 (GangaGen). In contrast to Gram-positive bacteria, the outer membrane of Gram-negative

426 bacteria represents a difficult barrier to reach the peptidoglycan layer. Therefore, discovery of

427 endolysins against Gram-negative bacteria has faced extensive challenges and projects are

428 still in preclinical research (Bioharmony Therapeutics) (Briers and Lavigne, 2015). Based on

429 protein engineering techniques some progress has been made but translating results into

430 formal development programs requires more research (Lukacik et al., 2012; Schirmeier et al.,

431   2018). One approach for Gram-negative bacteria is fusing a natural antimicrobial peptide

432   (AMP) to an endolysin (Artilysin) (São-José, 2018). Other options explored include

433   combining endolysins with outer membrane-permeabilizing agents (Oliveira et al., 2018).

434   Via protein engineering, there is the potential to generate enzymes with several improved

435   features; these include altered catalytic activities and binding specificities, solubility, and

436   other physicochemical properties (Gutiérrez et al., 2018; São-José, 2018). Great progress has

437   been achieved improving large scale production, purification, formulation, delivery, stability

438   and acceptable shelf life. However, some studies have shown that in vitro antibacterial

439   activity is not translated in vivo. It is unclear if discrepancies in antibacterial activity of some

440   endolysins is due to the influence of bacterial cell growth conditions or growth stage (Oliveira

441   et al., 2018). Pharmacokinetics are not well understood. Endolysins have a relatively short

442   half-life which may be explained by proteolysis via plasma proteases and degradation of

443   enzyme aggregates (Jun et al., 2017). PK/PD characteristics and dose finding are a new field

444   for the first lysins in development. As lysins are proteins they are immunogenic in mammals.

445   In vitro and animal studies with lysins have shown that non-neutralizing antibodies are

446   generated (Pastagia et al., 2013), so could allow for repeated use in humans, but the

447   development of antibodies has raised concern and requires further study (Jun et al., 2017).

448   The potential for resistance to lysins is unknown as only simple serial passage experiments

449   have been done so far. Although biologics are a growing part of authorized medicines

450   (Cooper et al., 2016), the regulatory pathway of new antibacterial biologics still needs some

451   clarification. Endolysins are likely to be suitable for classical clinical trials procedures due to

452   their similarities with conventional antibiotics.

453   If developers succeed with defining an appropriate dosing schedule (beyond a single dose) as

454   a basis for successful clinical studies for patients with confirmed infection due to drug-

455   resistant pathogens, or who experience recurrent or relapse infections, endolysins may provide

456 an adjunctive therapy option. Endolysins are very large molecules and their distribution in the

457 body is restricted to the bloodstream. Therefore, their clinical use will be limited to systemic

458 infections or to infections with topical application. Synergistic activity with antibiotics and in

459 vitro and in vivo (Schuch et al., 2014) and activity against biofilms may open opportunities to

460 treat infections of infected implanted devices and endocarditis. In addition to human health,

461 endolysin- based technologies are applied in many areas, including food safety, animal health

462 and agriculture.

Other approaches

464 Recent advances in genome editing, gene regulation and systems biology has inspired a wide

465 variety of other innovative discovery projects including $\geq$ 100 discovery and preclinical

466 projects on approaches including nanoparticles, immunotherapy, anti-sense RNA, resistance

467 modulation and removal of drug-resistance plasmids (Theuretzbacher et al., 2017).

Nanoparticles

469 Nanoparticles are 1–100 nm with ill-defined multiple simultaneous modes of action against

470 Gram-positive and Gram-negative bacteria. Nanoparticles have been used for many years as

471 antibacterial coatings for implantable devices and medicinal materials, wound dressings, bone

472 cement, dental materials and vaccines (Wang et al., 2017). Several types of nanoparticles

473 (especially liposomes) are currently available for drug delivery and extended release forms

474 (Kwon et al., 2017). Nanoparticles have been studied as toxin binders in various infections

475 including intestinal infections e.g. cholera (Das et al., 2018). One company is developing

476 liposomes that mimic domains targeted by toxins so neutralizing many toxins, e.g.

477 phospholipase C, pore-forming toxins, T3SS and can be used for a range of different

478 infections (Combioxin, Phase 1/2) (Azeredo da Silveira and Perez, 2017). Nanoparticles also

479 serve as delivery vehicle for synthetic oligonucleotides that function as transcription factor

480 decoys and thus, control gene regulation (Mamusa et al., 2017) (Procarta). The potential of

481   nano-strategies as adjunctive therapy in addition to existing antibiotics is discussed in the

482   recent review by Baptista et al (Baptista et al., 2018). Despite offering promising solutions,

483   translational studies and development of nanoparticles for severe infections is in its infancy

484   and several challenges remain.

485   Immunotherapy

486   Host-directed therapies utilize small-molecule drugs and proteins to target critical host

487   signaling enzymes exploited by bacteria for their intracellular invasion, replication, and/or

488   dissemination and virulence (Chiang et al., 2018; Pirofski and Casadevall, 2006). Potential

489   immunomodulating therapeutics may encompass a great diversity of drug classes targeting a

490   variety of biological processes that modify host cell function. This complexity of the immune

491   response challenges the selection of suitable targets. Immunotherapeutics are thriving in other

492   therapeutic areas but are relatively unexplored for bacterial infections (Baker et al., 2018).

493   Like other non-traditional approaches, a clear correlation between the immunomodulating

494   drug and clinical outcome of bacterial infection needs to be shown. Furthermore, if

495   therapeutics stimulate the immune system, they may be associated with the risk of excessive

496   inflammation leading to a cytokine storm or systemic inflammatory response syndrome

497   (Chiang et al., 2018).

498   Nonetheless, some immunomodulating agents are being tested in preclinical or clinical trials.

499   The most advanced drug, Reltecimod, is in Phase 3 clinical trials (Atox Bio). The short

500   peptide immunomodulator attenuates excessive severe acute inflammation and is

501   hypothesized to protect from superantigen toxins. It is tested in patients with necrotizing soft

502   tissue infections in addition to the current standard of care (broad-spectrum antibiotics, wide

503   surgical debridement, and supportive care). A phase 1/2 clinical trial of recombinant plasma

504   gelsolin in community-acquired pneumonia has been completed (BioAegis Therapeutics).

505   Plasma gelsolin is a highly abundant plasma protein in healthy individuals that enhances

macrophage activity and limits the excessive spread of inflammation. Its decline in a wide

range of infections is correlated with poor clinical outcome (Self et al., 2018).

Antisense RNA

Antisense antimicrobial therapeutics are synthetic sequence-specific oligomers that silence

expression of specific genes including essential, non-essential or resistance genes. (Sully and

Geller, 2016). Broad functional genes are investigated as targets but still need to be validated.

Many different chemical structures have been explored but all need a delivery system to

penetrate bacterial cells. The most common approach is coupling antisense oligomers to cell-

penetrating peptides (Sully and Geller, 2016). Such conjugates have not progressed into

clinical trials. They face considerable challenges, including choice of target, potential

emergence of resistance, carrier and translational issues but may benefit from advanced

research to deliver improved approaches (Good and Stach, 2011).

Drug-resistance modulation

Resistance has inspired research to explore mechanisms to switch off drug-resistance without

affecting bacterial growth as well as preventing horizontal gene transfer between bacteria.

Several ways of silencing drug-resistance genes have been described, including CRISPR-Cas

or synthetic oligomers (Good and Stach, 2011; Yosef et al., 2015). These methods aim at

inactivating or deleting specific genes to re-establish the susceptibility of the bacteria to the

antibiotic. The major challenge is the delivery of the genetic construct to and inside the

bacteria (Vila, 2018). Commonly described delivery systems are phages, cell-penetrating

peptides, nanoparticles or transmissible plasmids.

Although some technologies allow for simultaneous targeting of various drug-resistance

genes, bacteria can express a great variety of different resistance mechanisms, requiring the

identification of the target resistance mechanisms before administration. A few companies are

530   pursuing such resistance modulating approaches to re-sensitize bacteria to existing antibiotics

531   and preventing horizontal drug-resistance-gene transfer in bacteria (see: phages as carriers).

532   Plasmid curing (recently reviewed by Buckner et al., 2018) is another approach, either by

533   reducing transmission of plasmids to new bacterial hosts or reducing the stability of plasmids

534   within bacterial cells. Agents that remove plasmids carrying antimicrobial resistance and/or

535   virulence genes could be used to decolonize humans, animals and/or the environment of these

536   transmissible elements. Adequate animal models to test these agents need to be developed.

537   However, should there be a currently licensed drug that could be re-purposed for this role,

538   analysis of drug-resistance surveillance data in patients taking such a drug and/or a clinical

539   trial could be carried out.

Discussion

541   The increasing number of drug-resistant bacteria especially Gram-negative bacteria, the

542   growing awareness of few new drugs and the scientific challenges to find novel antibiotics

543   without cross-resistance to existing classes has stimulated discovery and early development of

544   new antimicrobials. To contribute to re-stocking the pipelines with new treatments, non-

545   traditional therapies have been proposed. However, it is very unlikely that they will replace

546   the use of antibiotics as their use will mostly depend on concomitant use of active antibiotics

547   (Czaplewski et al., 2016). Therefore, most non-traditional treatments will not solve the drug-

548   resistance problem. Furthermore, the additional clinical benefit of such adjunctive therapies is

549   unknown. It will be very difficult to show a meaningful clinical benefit in hospitalized

550   patients for add-on therapies or preventive approaches for specific high-risk patient groups

551   (the problem is to show that the new treatment works, rather than regulatory issues).

552   In addition to the R&D hurdles that antibiotics face, non-traditional antibacterials share some

553   common challenges:

- Most anti-virulence and simplified microbiome approaches are indirect-acting strategies that do not inhibit or kill bacteria, and act by intervening or interacting with complex biological processes that may not be well understood. Furthermore, a causal relationship with clinical outcomes may not be known.

- Treatments that do not affect bacterial growth cannot use traditional MIC measurements that correlate reasonably well with outcome measures. Alternative in vitro tests need to be developed and validated. It remains unclear if current animal tests can predict outcome of non-traditional treatments in humans.

- The immense challenge of late stage clinical trials applies to all non-traditional therapies that must be administered with an active antibiotic. To demonstrate their efficacy and utility, until recently non-inferiority clinical studies (to show that the experimental treatment is no worse than the comparator) of adjuvants (active antibiotic with add-on therapy versus active antibiotic alone) were sufficient for regulatory approval. However, the FDA has recently indicated that they will prefer superiority studies (to show that the experimental treatment is better than standard of care) in relevant clinical endpoints for regulatory approval and future acceptance in clinical practice.

- In the case of biologics such as live bacterial preparations (Live biotherapeutic products), standardized and well characterized production processes as well as quality controls i.e. CMC requirements remain challenging, but progress is being made.

- Dose finding is a well characterized procedure in the antibiotic field and relies heavily on validated preclinical models that correlate PK and PD. Such predictive models are not usually available for non-traditional approaches and correlates to outcome effects may not be known. This is most apparent in the phage and microbiome fields.

578   • Many non-traditional therapies are pathogen-specific, or specific for a subset of strains

579   of a species, or only active in a specific host niche or specific phase of infection. Such

580   tailored approaches will require knowledge of underlying mechanisms and patient

581   factors, and so high financial resources. Although most experts would agree that

582   patient-specific antibacterial therapy is desirable, its translation into the clinical

583   routine beyond highly specialized settings is unlikely to occur for many years.

584   Therefore, without additional tailored diagnostics some of the therapies may not be

585   useful.

586   Very few non-traditional therapies have advanced to late stage clinical trials. The most

587   advanced non-traditional antibacterials are exotoxin targeting therapies, mostly mAbs (*C.*

588   *difficile*, *S. aureus*). Similarly, microbiome therapies based on complex characterized

589   microbiota are promising and likely to provide new options to treat or prevent CDI.

590   However, most therapies are in the discovery or preclinical development stages and so may

591   not be available for at least 10 years. Commonly, non-traditional approaches require

592   sophisticated diagnostics beyond pathogen identification and are patient-tailored approaches.

593   The development and implementation of such specific companion diagnostics is further

594   contributing to the challenges.

595   In conclusion whilst there is a considerable interest in the opportunities that non-traditional

596   approaches will bring to treating bacterial infections, it is likely that effective treatments will

597   be limited to healthcare settings with the best diagnostic and financial resources, and to

598   healthcare systems that are able to financially support a strong growth of high-cost individual

599   (personalized) medicines, and thus to high-income countries. The highest burden of drug-

600   resistant infections is in babies and children in low-medium income countries. These are

601   unlikely to have the resources for basic traditional antimicrobial treatments and in the near

602   future extremely unlikely to be able to implement personalized medicine.

Author contributions

Ursula Theuretzbacher and Laura J.V. Piddock wrote this article.

Declaration of Interests

LJVP is currently seconded to the Global Antibiotic Research & Development Partnership.

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913 **Table 1. Monoclonal antibodies in clinical development**

Monoclonal antibody, company	Clinical development	Anti-virulence target	Indication
Suvratoxumab (MEDI4893), Medimmune	Phase 2	*S. aureus* α-toxin	Prevention of VAP caused by *S. aureus*
AR-301 (Salvecin, tosatoxumab), Aridis	Phase 3	*S. aureus* α-toxin	Adjunctive therapy for VAP caused by *S. aureus*
MEDI3902, Medimmune	Phase 2	*P. aeruginosa* T3SS needle tip protein PcrV and Psl exopolysaccharide	Prevention of VAP caused by *P. aeruginosa*
AR-105 (Aerucin), Aridis	Phase 2	*P. aeruginosa* alginate	Adjunctive treatment of VAP caused by *P. aeruginosa*
514G3, XBiotch	Phase 1/2	*S. aureus* Protein A	Adjunctive treatment of bloodstream infections caused by *S. aureus*
ASN-100, Arsanis	Phase 2	*S. aureus* α-toxin and five leukocidins	Failed to prove its effectiveness in high-risk, mechanically ventilated patients with *S. aureus* pneumonia

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915 For information about the status of R&D of antibodies, small molecules and other approaches

916 to new treatments, please see www.clincialtrials.gov or the developing organizations website.

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918   **Table 2. Translational challenges of anti-virulence strategies**

Challenge	Factor influencing translation
Selection of target	Selection of the most relevant anti-virulence target according to their distribution and contribution to disease
Coverage	VFs are not expressed uniformly in all strains. May differ geographically and gene not present in all strains (genetic variation)
Effectiveness	May be effective only in specific disease states (e.g. chronic, dormant), a specific time point in the infectious process, at a specific infection site, or in specific patient groups (e.g. immunocompetent)
Diagnostics	Diagnostics beyond species identification may be necessary to guide use
Dose finding	Predictive models to support decisions to find the optimum dose are mostly lacking
Predictive models of efficacy	For most approaches there are no validated models that predict clinical outcome and preclinical studies may not be a meaningful guide to clinical development
Resistance	Resistance development has been shown for some anti-virulence approaches. It is not known how to predict the likelihood of developing anti-virulence drug resistance and any relevance in patients
Activity in patients	For some approaches it is not known if the selected approach has enough clinically relevant impact on the disease in humans
Clinical development	For adjunctive therapies non-inferiority studies will not prove that the therapy works in patients. Superiority studies are essential to show that the adjunctive therapy provides a benefit to patients. Superiority studies (and preventive studies) are very difficult to do.

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Table 3. Current approaches to manipulate the microbiome

Approach	Indication	Comments
Transfer of human intestinal microbiota	Prevention of recurrent CDI	• FMT: Transfer of stool suspension from a donor via colonoscopy, enema, nasogastric routes or pills (Finch, Open Biome) • Fecal microbiota suspension: Standardized number of live bacteria from stool suspension from donors via enema or capsules, GMP produced (Rebiotix/Ferring) • Rationally selected microbiota: Well characterized selection of bacterial strains via capsules (Seres, Finch Therapeutics, Vedanta) • Spore suspension: Spores, fractionated from stool specimens from donors via capsules (Seres)
Synthetic microbiota	Intestinal, dermatological (e.g. atopic dermatitis), lung conditions (e.g. CF)	• Selected live bacteria producing specific metabolites or cocktail of secondary metabolites • Selected live bacteria from skin microbiota (MatriSys Bio) • Selected live bacteria for balancing the lung microbiota
Manipulating the metabolism of microbiota		Manipulating the metabolic balance through specific bacterial nutrients, e.g. Glycans (Kaleido)
Competition	Prevention of recurrent CDI, catheter associated UTI	• Non-toxinogenic *C. difficile* that is assumed to outcompete the toxic strain (Microbiotica) • Apathogenic E. coli introduced into the bladder via catheter coating (Atterx)
Engineering probiotics to deliver	Various indications (Bäumler and Sperandio, 2016)	• Engineered *Lactobacillus* to express bacteriocin against *P. aeruginosa* (inhaled, CF) and *C. difficile* (SciBac)

antibacterial proteins		• Engineered *Lactobacillus* to express SagA protein that promotes tolerance to enteric infections incl. *C. difficile* infection *(*Rise Therapeutics) • R-type bacteriocins against *C. difficile*
Prevention of disbalance of microbiome due to antibiotic therapy	Prevention of recurrent CDI	• Hydrolysing specific beta-lactam antibiotics in the gut (beta-lactamase, Synthetic Biologics, DaVolterra)
Decolonisation of MDR Gram-negative pathogens in high risk patients	Various indications	• Decolonisation of asymptomatic carriers with live bacteria consortia (e.g. *C. difficile*, MDR Gram-negative pathogens in high risk patients, *Salmonella* Typhi)

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Table 4. Current approaches using phages

Approach	Composition
Fixed phage cocktails	Fixed composition of lytic phages to achieve a broad host range of a bacterial species
Individualized phage cocktail	The lytic phages are stored individually in a phage bank with established QC. Only the best active phages based on rapid diagnostic tests are selected for an individual patient
Genetically engineered phages	Engineered phages with improved or specific characteristics
Genetically engineered non-replicating phages as vehicles	Engineered phages to express additionally antimicrobial peptides or protein toxins leading to rapid, nonlytic bacterial death. May deliver CRISPR CAS3 genes directly into bacteria
Phage products, e.g. endolysins	Natural or recombinant cell wall hydrolyzing phage-based enzymes. Endolysins against *S. aureus* are in clinical development

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