Novel derivatives of polymyxins

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Polymyxin B and colistin (polymyxin E) are bactericidal pentacationic lipopeptides that act specifically on Gram-negative bacteria, first by disrupting their outermost permeability barrier, the outer membrane (OM), and then damaging the cytoplasmic membrane. Both were discovered in the mid-1950s and subsequently used in intravenous therapy, but soon largely abandoned because of nephrotoxicity. The emergence of extremely multi-resistant strains has now forced clinicians to reinstate them in the therapy of severe infections caused by such strains. This article reviews recent attempts to develop novel derivatives of polymyxins that exhibit less toxicity and greater potency than the existing drugs. In addition, studies of novel des-fatty acyl-polymyxin derivatives that display activity against Pseudomonas aeruginosa are included. The review also covers recent studies of derivatives that lack potent bactericidal action, but which disrupt the OM, which increases bacterial permeability to other antibiotics, facilitating their entry into the cell.

Keywords: Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii, NAB739, des-fatty acyl-polymyxin, nephrotoxicity

Introduction

The crisis due to escalating antibiotic resistance in Gram-negative bacteria combined with the absence of novel antibiotics is worrisome and might turn disastrous in the coming years.1,2 The major concern is the pandemic caused by multiple drug-resistant (MDR) strains of Gram-negative enteric bacteria. Escherichia coli and Klebsiella pneumoniae cause almost 40% of community-acquired bacteraemias and approximately one-third of healthcare-associated bacteraemias.3,4 They cause ~60%–75% of all Gram-negative bacteraemias. We have already witnessed the stunning pace at which enterobacterial strains that express extended-spectrum β-lactamases (ESBLs) and especially CTX-M-type enzymes have spread worldwide, first in hospitals and then in the community.5 Now, several ESBL-producing clones produce carbapenemases too, and are resistant to practically all antibiotics except polymyxins (polymyxin B and colistin) and tigecycline. Their ongoing spread might follow the path of the CTX-M strains.

The pipeline for novel agents against Gram-negatives has dried up in the last two decades.1,2,6 The reasons include over-optimism in genomics, other omics, combinatorial chemistry and high-throughput screening, as well as difficulties in getting drugs inside the Gram-negative cell and in keeping them there in the presence of effective efflux mechanisms. Furthermore, the majority of large pharmaceutical companies have abandoned work on antibiotic discovery. The lack of new antibiotics against Gram-negative bacteria has, however, prompted attempts to improve known classes of antibiotics, such as aminoglycosides, quinolones and β-lactam/β-lactamase inhibitor combinations.6

Polymyxins7–9 are cyclic lipodecapeptides that are strongly cationic, due to the presence of five free amino groups (Figure 1). They were discovered as early as 1947. In 1948, a Washington newspaper10 wrote ‘Time will tell, however, whether polymyxin will work in human beings. After all, many of the wonder drugs have reached this stage only to fail when put to the acid test’. Polymyxins were subsequently used in intravenous therapy, but largely abandoned in the 1960s owing to toxicity issues and the discovery of better-tolerated drugs. The MDR pandemic has now forced clinicians to reinstate them as the last-line therapy for severe Gram-negative infections.

Polymyxin B and colistin are rapidly bactericidal and act specifically on Gram-negative bacteria. Gram-positive bacteria, eukaryotic microbes and mammalian cells are typically resistant. The antibacterial activities of polymyxin B and colistin are identical11. Polymyxins interact with the anionic lipopolysaccharide (LPS) molecules exclusively present in the Gram-negative bacterial outer membrane (OM).12,13 The interaction of polymyxin with LPS results in disruption of the OM and the loss of its function as a permeability barrier.12 As shown for polymyxin B nonapeptide (PMBN; see below), their OM-disrupting action is dependent on their three-dimensional conformation, since the enantiomer of PMBN is completely inactive.14 The final and lethal action of polymyxins is damage to the cytoplasmic membrane.

Currently, the nephrotoxicity of polymyxins complicates therapy or may even require its discontinuation.8,15,16 Accordingly, the risk of nephrotoxicity must be weighed against the beneficial
Acquired polymyxin resistance in clinical isolates is still rare. In the worldwide SENTRY programme, rates of susceptibility to colistin (interpreted as MIC ≤2 mg/L) of strains of E. coli, K. pneumoniae, Acinetobacter spp. and Pseudomonas aeruginosa, all isolated in 2009, were 99.8%, 98.2%, 97.9% and 99.5%, respectively.\(^1\) Polymyxin B displayed almost identical susceptibility rates for polymyxin B (interpreted as above) were 99.8%, 96%, 97% and 99.5%, respectively.\(^1,\)\(^7\) However, amongst imipenem-negative strains are spreading and the empty pipeline of novel drugs against them has quite recently led to interest in developing novel derivatives of polymyxins, in the hope that the old polymyxins could be replaced by less toxic and/or more potent derivatives. Here, recent approaches to the development of such derivatives are reviewed. The review also covers recent studies of derivatives that lack potent bactericidal action, but which disrupt the OM.

**NAB739**

The cyclic core of NAB739 is identical to that of polymyxin B, but its side chain consists of octanoyl-threonyl-d-serinyl (Figure 1).\(^3\)\(^5\) Accordingly, its linear region lacks the two positively charged diaminobutyryl (Dab) residues present in the linear region of polymyxin B and colistin. As first reported by Vaara et al., more than 30 years ago, polymyxin-resistant strains of Enterobacteriaceae and P. aeruginosa elaborate less-anionic LPS, due to increased incorporation of 4-aminoarabinose and phosphoryl ethanolamine.\(^12,\)\(^13,\)\(^22\)–\(^26\) The altered LPS compromises the function of the OM as a permeability barrier and increases susceptibility to other agents, such as many antibiotics and bile acids.\(^27\)–\(^30\) In A. baumannii, polymyxin resistance is mediated by at least two alternative mechanisms.\(^21\) One involves decoration of lipid A with phosphoryl ethanolamine. As recently shown by Moffatt and Boyce and their collaborators,\(^31\) the other is a result of complete loss of LPS. Polymyxin-resistant strains of A. baumannii are susceptible to many antibiotics normally active exclusively on Gram-positive organisms and have reduced in vivo fitness and decreased virulence.\(^32\)–\(^34\)

The worrisome speed at which extremely resistant Gram-negative strains are spreading and the empty pipeline of novel drugs against them has quite recently led to interest in developing novel derivatives of polymyxins, in the hope that the old polymyxins could be replaced by less toxic and/or more potent derivatives. Here, recent approaches to the development of such derivatives are reviewed. The review also covers recent studies of derivatives that lack potent bactericidal action, but which disrupt the OM.

**Figure 1.** Structures of polymyxin B and colistin, as well as their recent derivatives. Boxed parts indicate locations where the compounds are not identical. Abbreviations for non-trivial amino acyl residues: Dab, diaminobutyryl; Abu, aminobutyryl; Ada, aminodecanoyl; Dap, diaminopimelyl. Other abbreviations: Cpnd, Compound; MHA/MOA, mixture of methyl octanoyl and methyl heptanoyl; OA, octanoyl; NA, nonanoyl; Ac, acetyl; cy, cyclic portion indicated with brackets. The positive charge of the free α- and γ-amino group is also shown.
Thus, against strains, whereas polymyxin B inhibited 84.3% of the strains. NAB739 at a concentration of 1 mg/L inhibited 74.5% of the active, NAB740, which carries decanoyl as the fatty acyl tail but is otherwise identical to NAB739, is more active against otherwise identical to NAB739, is more active against A. baumannii and K. pneumoniae (nine strains, including KPC-, OXA-48-, VIM- and IMP-producing strains), the MIC ranges of NAB739 and polymyxin B were 1–4 and 1–2 mg/L, respectively. For A. baumannii (49 strains), the MIC90 of NAB739 was 8 mg/L and that of polymyxin B was 2 mg/L. On the other hand, low subinhibitory concentrations of NAB739 sensitized A. baumannii to other antibiotics by facilitating their entry into the cell. At a concentration of 0.5 mg/L, NAB739 reduced the MIC of rifampicin for the two studied strains from 4–12 to 0.05–0.1 mg/L. The MIC of clarithromycin was reduced from 6–8 to 0.5 mg/L and the MIC of vancomycin was reduced from 256 to 3 mg/L. For P. aeruginosa (49 strains), the MIC90 of NAB739 was 16 mg/L while that of polymyxin B was 2 mg/L. Another derivative, NAB740, which carries decanoyl as the fatty acyl tail but is otherwise identical to NAB739, is more active against P. aeruginosa (MIC for P. aeruginosa ATCC 27853, 4 mg/L), but its activity against Enterobacteriaceae is inferior to that of NAB739.

Inherently polymyxin-resistant bacterial species, as well as strains of E. coli and K. pneumoniae that have acquired resistance to polymyxin, are also resistant to NAB739. The MICs of NAB739 are also high for polymyxin-non-susceptible mutants of Acinetobacter spp. and P. aeruginosa. Staphylococcus aureus and Candida albicans are resistant, indicating that the activity of NAB739 is as specific as that of polymyxin B and colistin.

NAB739 is effective in treating experimental E. coli peritoneal infection in mice. A >4.0 log10 reduction in bacterial load compared with saline control was achieved 6 h after initiation of treatment with 1 mg/kg of NAB739 twice (at an interval of 2 h). To avoid inactivation of the polymyxins by mucin, the polyanionic polymer commonly used to suppress phagocytosis in the mouse peritonitis model, the K1 capsule-elaborating strain IH3080 (O18:K1:H7) was used. It is virulent for mice in the peritonitis model even in the absence of mucin.

The affinity of NAB739 for the isolated rat kidney brush border membrane (BBM) is approximately one-seventh that of polymyxin B.

In non-polarized porcine renal proximal tubular LLC-PK1 cells, which express an active polymyxin uptake mechanism (megalin) only poorly, polymyxin B elicits half-maximal necrosis at 0.5 mM (corresponding to ~600 mg/L), whereas NAB739 is inert even at 1 mM. In artificially permeabilized (electroporated) LLC-PK1 cells, polymyxin B induces total necrosis at a concentration as low as 0.016 mM (~20 mg/L), whereas an ~8-fold concentration of NAB739 is required for the same effect.

In another study, the cytotoxicity of NAB739 was compared with that of polymyxin B by using human renal proximal tubular HK-2 cells (Figure 2). The assays were performed without any artificial permeabilization. The calculated half-maximal cytotoxic concentrations (CC50) for NAB739 and polymyxin B were 337 and 13 mg/L, respectively. The CC50 for colistin was 45 mg/L. Hence, NAB739 was 26-fold less toxic than polymyxin B and 7.5-fold less toxic than colistin to the HK-2 cells. In this assay, colistin appears to be better tolerated than polymyxin B. Similar results using macrophages have recently been published. Therefore, it would be interesting to determine the CC50 value of the ‘colistin analogue’ of NAB739 (i.e. octanoyl-Thr-o-Ser-(Dab-Dab-o-Leu-Leu-Dab-Dab-Thr)), which might be even less cytotoxic than NAB739.

In rats, the serum half-life of NAB739 after a single intravenous dose of 1 mg/kg is very close to that of colistin sulphate, i.e. 69 min versus 75 min. After this dose, the urinary recovery rate of NAB739 is 19% and that of colistin sulphate almost nil (0.18%), and the renal clearance of NAB739 is ~50-fold that of colistin sulphate.

Other NAB peptides that possess notable antibacterial activity (as tested against E. coli) include NAB734, NAB737, NAB748, NAB749 and NAB782. All carry three positive charges only and have hydrophilic amino acyl residues in the linear peptide chain.

Monash University compounds

Li, Velkov, Nation and their colleagues at Monash University have synthesized polymyxin derivatives that have activity against polymyxin-resistant strains of A. baumannii and P. aeruginosa. Polymyxin-resistant K. pneumoniae strains remain resistant. In one of them (Compound 2 in WO2010/130007, Figure 1), the hydrophobicity of polymyxin is increased by substituting 2-amidodecanoyl (Aa) for leucyl in the heptapeptide ring portion, to yield octyl instead of iso-butyl as the side chain at position R7 (numbering according to the standard numbering of amino acid residues in the polymyxin decapeptide). The fatty acyl tail consists of an octanoyl residue. For polymyxin-susceptible reference strains K. pneumoniae ATCC 13883, A. baumannii ATCC 19606 and P. aeruginosa ATCC 27853, its MICs are 2, 4 and 4 mg/L, respectively. The MIC for

![Figure 2. Comparison of the activity of NAB739 (filled symbols) and polymyxin B (open symbols) against E. coli (cumulative MICs for 51 strains shown as triangles) and against human renal proximal tubular HK-2 cells (cytotoxicity shown as squares). Adapted and compiled from publications by Vaara et al. and Vaara and Vaara.](image-url)
E. coli was not disclosed. Compound 1 in the same publication lacks the positive charges in the linear peptide part and hence carries three positive charges only. Its activity appears to be similar to that of Compound 2. The MIC values for other microorganisms, such as Gram-positive bacteria and yeasts, were not given.

Compounds 1 and 2, belonging to another series of polymyxins with increased hydrophobicity (WO2012/051663), carry Ada instead of oPhe at position R6, and hence acyl instead of phenyl as the side chain in this position. In Compound 1 the fatty acyl tail is octanoyl and in Compound 2 biphenylcarboxyl. For K. pneumoniae ATCC 13883, A. baumannii ATCC 19606 and P. aeruginosa ATCC 27853, the MIC values of Compound 1 are 4, 2 and 1 mg/L, respectively. No data were given on the MICs for E. coli. For Compound 2, the corresponding values are 4, 2 and 2 mg/L, respectively. For polymyxin-resistant strains of P. aeruginosa and A. baumannii, both compounds display rather low MIC values (2–16 mg/L), but for polymyxin-resistant K. pneumoniae the MIC is >32 mg/L. Furthermore, they are also rather active against Gram-positive bacteria, with MIC values of 4 and 4–16 mg/L for Enterococcus faecium and S. aureus, respectively.

Accordingly, the mode of action of the derivatives with increased hydrophobic properties differs from the classic and quite specific mode of action of polymyxins. It has long been known that the polymyxin-resistant pmrA mutants of Salmonella Typhimurium and their analogues in E. coli with identical alterations in lipid A are as susceptible as their parents to octapeptin A (EM49). Furthermore, polymyxin-resistant K. pneumoniae is fully susceptible to octapeptin A. Octapeptin A is structurally very similar to polymyxins but is more hydrophobic, since it lacks two hydrophilic amino acids in the linear peptide portion and carries a fatty acyl tail that is two methylene units longer than that of polymyxin B. In contrast to polymyxins, octapeptin A is also remarkably active against Gram-positive bacteria and fungi. Accordingly, the action of octapeptin A is rather nonspecific and perhaps resembles that of cationic detergents. The pmrA mutants are even more susceptible than their parents to the conventional cationic detergents benzalkonium chloride and cetlytrimethylammonium chloride.

**Anti-pseudomonal des-fatty acyl derivatives**

Polymyxin derivatives that lack the fatty acid tail (i.e. des-fatty acyl derivatives) are significantly less active against species such as E. coli and K. pneumoniae than polymyxin B. On the other hand, as first shown by Vaara and Vaara in 1983, they still possess a notable OM-disrupting activity and, hence, act as permeabilizers. The classic model compound PMBN (Figure 1) lacks the fatty acyl tail and the N-terminal amino acyl residue (Dab). However, in conditions where the MIC of PMBN for E. coli and K. pneumoniae was >500 mg/L, its MIC for P. aeruginosa was reported to be rather low (8 mg/L).

Significantly more active anti-pseudomonal des-fatty acyl derivatives have recently been published by Sakara and Sato and their colleagues at Kanazawa University. They comprise the peptide ring portion of polymyxin B and the linear peptide portion consisting of 1–3 hydrophilic amino acyl residues (Figure 1). The compounds with the linear peptide sequence Ser-Dap (diaminopimelyl) and Ser-Thr-Dab, respectively, displayed MICs as low as 1 mg/L for P. aeruginosa, whereas their MICs for E. coli were 64 mg/L. Both were much better tolerated than polymyxin B in an acute toxicity assay in mice. This was quite expected in the light of previous studies conducted with PMBN. PMBN is 150-fold less active than polymyxin in causing neuromuscular blockade, 15-fold less toxic in an acute toxicity assay in mice and 25-fold less active in releasing histamine from rat mast cells. Hopefully, there will be future studies on the toxicology of these novel anti-pseudomonal peptides. If they release less histamine than the old polymyxins they might offer advantages in the inhalation therapy of P. aeruginosa infections in cystic fibrosis patients.

**Permeabilizer compounds NAB7061 and NAB741**

As described above, PMBN has retained the ability to permeabilize the OM. Consequently, even at low concentrations (1–3 mg/L) it makes E. coli and several other enterobacterial species up to 100-fold more susceptible to lipophilic and amphi-

phile compounds, including many antibiotics. The combination of PMBN with erythromycin administered intraperitoneally in multiple doses was shown to protect mice infected with K. pneumoniae or P. aeruginosa in conditions where PMBN alone or erythromycin alone were inactive. Even though toxicity studies in dogs indicated that PMBN might be less nephrotoxic than polymyxin B, experiments with rats PMBN was considered to be too nephrotoxic, and its development for therapeutic purposes was discontinued. However, PMBN has been widely exploited as a useful tool to increase the permeability of the OM in various cellular studies, including those dealing with the discovery and development of novel antibacterial drugs.

NAB7061 and NAB741 have their cyclic part identical to that of polymyxin B and NAB739, but their side chain consists of octanoyl-Thr-Abu (aminobutyryl) and acetyl-Thr-D-Ser, respectively (Figure 1). Hence, they carry only three positive charges. Both compounds lack potent direct antibacterial activity, but by disrupting the bacterial OM they facilitate the access of hydrophobic antibiotics and the large hydrophilic antibiotic vancomycin inside the Gram-negative cell. At 4 mg/L, NAB7061 decreased the MIC of rifampicin for E. coli (11 strains), other polymyxin-susceptible Enterobacteriacae (12 strains) and A. baumannii (three strains) by factors of 85–750, 10–2000 and 25–125, respectively. With clarithromycin, the corresponding factors are 90 to >750, 10–1000 and 40–100, respectively. NAB7061 is able to sensitize even the polymyxin-resistant K. pneumoniae strain CL5762B to rifampicin and clarithromycin by factors of 24 and 12, respectively. The antibacterial properties of NAB741 are similar to those of NAB7061. Several other structurally related sensitizer compounds have been described.

The sensitizer activity of NAB7061 has been demonstrated also in vivo in the therapy of experimental E. coli peritoneal infection in mice. In contrast to NAB7061 or erythromycin alone, the combination of NAB7061 (5 mg/kg body weight, twice, at an interval of 2 h) and erythromycin (10 mg/kg) was effective in therapy.

Vaara and co-workers showed in the mid-1980s that PMBN acts synergistically with the complement system of fresh serum against Enterobacteriaceae and P. aeruginosa. The
combination of PMBN with human, guinea pig, rabbit and rat serum is strongly bactericidal, but no synergy can be found with mouse serum.\textsuperscript{55} NAB7061 and NAB741 also sensitize the smooth, encapsulated \emph{E. coli} strain to complement-mediated bactericidal action.\textsuperscript{54,57}

The affinity of NAB7061 for the isolated rat kidney BBM is approximately one-fifth that of polymyxin B.\textsuperscript{35} The affinity of NAB741 has not been determined. In non-polarized electroporated LLC-PK1 cells (see above under NAB739), the cytotoxicities of NAB7061 and NAB741 are approximately one-eighth and one-thirtieth, respectively, of that of polymyxin B.\textsuperscript{39}

In rats, the serum half-life of NAB7061 after a single intravenous dose of 1 mg/kg is 66 min and that of NAB741 is 33 min.\textsuperscript{42,54} The corresponding urinary recovery rates are 7% and 51%, respectively. The renal clearance of NAB7061 is \(\sim 30\)-fold and that of NAB741 \(\sim 400\)-fold that of colistin sulphate.\textsuperscript{42,54}

Remarks and conclusions

NAB739 is undergoing preclinical studies. Indirect \textit{in vitro} evidence suggest that it might be less nephrotoxic than the old polymyxins. However, NAB739 has not yet been subjected to \textit{in vivo} efficacy and toxicity studies that will determine whether its therapeutic window is wider than that of the old polymyxins in the therapy of infections caused by the most common Gram-negative organisms, such as \emph{E. coli} and \emph{K. pneumoniae}.

Promising agents may also evolve from the compounds under development by Monash University, provided that their wider antibacterial spectrum is not accompanied by increased toxicity to mammalian cells.

There is notable synergy between the existing old polymyxins and several other antibiotics.\textsuperscript{7–9} Combination therapies can therefore be expected to be useful, and clinical evidence for their advantages is currently accumulating.\textsuperscript{7–9} In this light, any successful novel polymyxin brought through the clinical phases of development should probably be used in a synergistic combination with another suitable antibacterial agent (a partner antibiotic), to enhance its potency and minimize the risk of resistance development. Even though it would be advisable that the partner antibiotic itself possesses notable antibacterial activity against the main target pathogens, lack of such agents is evident. Therefore, partner antibiotics such as rifampicin that alone are not considerably active against Gram-negative bacteria are currently used with polymyxins. Because polymyxins damage the OM permeability barrier and enhance the entry of compounds normally active on Gram-positive bacteria only,\textsuperscript{12} potential partners for the novel polymyxins may include some of the compounds currently under development by the pharmaceutical industry against Gram-positive organisms, such as the novel oxazolidinones, ketolides (such as solithromycin), lipoglycopeptides (telavancin, oritavancin, dalbavancin), peptide deformylase inhibitors, pleuromutilins and inhibitors of FabI enzymes. As an example, colistin has recently been reported to be synergistic with telavancin against several Gram-negative isolates,\textsuperscript{56} but the combination might be too nephrotoxic. Telavancin could be a better partner to a novel, less nephrotoxic polymyxin. Finally, the partner drugs may include compounds under development that inhibit targets such as LpxC, present in Gram-negative organisms only, but against which the intact OM is a permeability barrier.

In addition, the sensitizer compounds that lack any direct activity may find value when used as a combination with a suitable partner antibacterial agent. As suggested previously,\textsuperscript{59} it should be evaluated whether the NAB compounds could be used as co-vasoulently linked carriers in getting antisense oligonucleotides inside the Gram-negative cell. They might be less toxic and/or more active than the classic (KKF)\textsubscript{K} carrier and the other cationic peptides used.\textsuperscript{57,59} As noted above, the cytotoxicity of NAB741 to the porcine kidney proximal tubular cell line LLC-PK1 is only one-thirtieth that of polymyxin B. This warrants an \textit{in vivo} nephrotoxicity study. Furthermore, whether the acute toxicity of the ‘tail-less’ NAB741 is as low as that of PMBN and the other des-fatty acyl polymyxins (see above) remains to be studied.

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