Novel polymyxin derivatives are effective in treating experimental Escherichia coli peritoneal infection in mice

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Objectives: Novel synthetic polymyxin derivatives including NAB737 and NAB739 are as effective as polymyxin B in vitro against the common opportunistic pathogen Escherichia coli. Another derivative, NAB7061, lacks direct antibacterial action but sensitizes E. coli to several other antibacterial agents including macrolides. The renal handling of NAB739 and NAB7061 in rats differs from that of polymyxin B. Furthermore, the affinities of NAB739 and NAB7061 for isolated rat kidney brush border membrane are significantly lower than that of polymyxin B. Here we investigate the in vivo antibacterial effect of these compounds.

Methods: The polymyxin derivatives were evaluated in an experimental murine peritonitis model. Immuno-competent mice were infected intraperitoneally with E. coli IH3080 and were subcutaneously treated with NAB737, NAB739 or NAB7061.

Results: 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 or 4 mg/kg of NAB737 twice. Combination therapy with NAB7061 (5 mg/kg) twice and erythromycin (10 mg/kg) resulted during the same time course in an 2.0 log10 reduction in bacterial load compared with saline control. Neither NAB7061 nor erythromycin was effective as monotherapy. Together with the ability to reduce bacterial load, the NAB compounds also improved the clinical status of the mice.

Conclusions: We found that the three novel synthetic polymyxin B derivatives had a potent in vivo bactericidal effect against E. coli.

Keywords: NAB7061, NAB737, NAB739, erythromycin

Introduction

The emergence and spread of extremely multiresistant (XMR) or even pan-resistant Gram-negative bacteria is an alarming global threat to public health. Amongst the most worrisome are the XMR strains of Klebsiella pneumoniae and Escherichia coli.1,2 This is especially alarming, since very few new antibiotics effective against Gram-negative bacteria are being developed.

The lack of novel antibiotics against Gram-negative bacteria has reinstated polymyxins (polymyxin B, colistin), polycationic cyclic lipopeptides, as the last resort to treat serious infections caused by XMR Gram-negative bacteria.3–7 However, concerns about the toxicity, especially nephrotoxicity, of polymyxins have restricted their use. Development of novel derivatives of polymyxins with lesser nephrotoxicity would be valuable.

Numerous efforts have been made to make polymyxins less toxic. A recent study investigated the structure–function relationships of novel synthetic polymyxin derivatives NAB737, NAB739 and NAB7061.7 Reducing the number of positive charges from five to three maintained the antibacterial properties of the derivatives but significantly (by a factor of five to seven) decreased the affinity of the compounds for isolated rat kidney brush border membrane. Furthermore, the renal handling of NAB739 and NAB7061 in rats differs from that of polymyxin B.8 These findings might indicate that the novel derivatives are better tolerated than the old polymyxins.

NAB737 and NAB739 are very effective antibacterial agents. The MIC90 of both NAB739 and polymyxin B for E. coli is 1 mg/L.7,8 On the other hand, NAB7061 lacks any potent direct antibacterial activity but has strong synergism with macrolides,
rifampicin and other antibacterial agents against which the intact outer membrane (OM) of E. coli and many other Gram-negative bacteria acts as an effective permeability barrier.\textsuperscript{7,9}

In the present investigation, we verified these in vitro findings in vivo, by using the E. coli murine peritoneal infection model. To avoid actinization of the cationic compounds by mucin, the poly-anionic polymer commonly used to suppress phagocytosis, we used a K1 capsule-elaborating strain, IH3080 (O18:K1:H7).\textsuperscript{10} It is virulent to mice in the peritonitis model even in the absence of mucin.\textsuperscript{11} Since the plasma half-life of colistin in mice and those of polymyxin B, NAB739 and NAB7061 in rats have been reported to be short\textsuperscript{8,12,13} (see the Discussion section for more details), we administered the compounds twice with an interval of 2 h.

### Materials and methods

#### Chemicals

NAB compounds were synthesized as described by Vaara et al.\textsuperscript{7} Their purity, as estimated by reversed phase HPLC, was >95%. Polymyxin B sulphate was from Sigma-Aldrich (P-0972; St Louis, MO, USA) and erythromycin (Abbottin) was from Abbott (Copenhagen, Denmark).

#### Bacterial strain and the inoculum

The smooth, encapsulated E. coli strain IH3080 (O18:K1:H7) is a clinical isolate from the CSF of a human neonate with meningitis.\textsuperscript{15} The MICs of polymyxin B, NAB739 and NAB7061 for this strain, as determined by the agar dilution method using Mueller–Hinton agar according to the CLSI,\textsuperscript{14} are 0.5 mg/L, 1 mg/L and >32 mg/L, respectively.\textsuperscript{7} Bacterial suspensions for inoculation of mice were prepared at room temperature from fresh overnight cultures on 5% blood agar plates produced by the Statens Serum Institut (Copenhagen, Denmark). The inoculum was prepared by picking up colonies and suspending them in sterile 0.9% saline to an optical density of 0.12 at 540 nm, giving a density of 10^8 cfu/mL. Dilutions of this suspension were made in 0.9% saline. If not otherwise indicated, the suspension with 10^6 cfu/mL was used in the challenge studies. Preparation of the inoculum and inoculation were performed within 1 h. For each experiment, the size of the inoculum was determined by making 10-fold dilutions of the suspension used for the inoculum in 0.9% saline, of which 20 μL was plated on 5% blood agar plates with subsequent counting of colonies after incubation overnight at 35°C in ambient air.

#### Animals

All animal experiments were approved by the National Committee of Animal Ethics – Animal Experiment Inspectorate under the Danish Ministry of Justice. Outbred female NMRI mice 7–9 weeks old (Harlan, the Netherlands) were housed at the animal facility at the Statens Serum Institut. The mice had ad libitum access to domestic quality drinking water and food (2016 16% Protein Rodents Diet; Harlan, USA). The mice were housed in Type 3 macrolone cages with four to eight mice/cage. The bedding was Aspen Wood (Tapvei, Estonia) and the animals were offered paper strands (Sizzle-Nest; Datesand, UK) as nesting material.

#### Mouse peritonitis model

Inoculation was performed by intraperitoneal injection of 0.5 mL of the E. coli suspension. After the inoculation, the mice were observed for 5 h for clinical signs of infection such as lack of curiosity, social withdrawal, changes in body position and pattern of movement, distress and pain. Cfu values in the peritoneum were determined at 1, 4 and 7 h post-inoculation, if not otherwise indicated. After the mice had been sacrificed by cervical dislocation, peritoneal washes were performed by injecting 2 mL of sterile saline intraperitoneally, followed by gentle massage of the abdomen and opening the peritoneum to collect fluid. Peritoneal fluids were serially diluted (10-fold dilutions) and 20 μL was plated on selective blue agar plates produced at the Statens Serum Institut with subsequent counting of colonies after incubation overnight at 35°C in ambient air. No antibiotic carry-over effect in terms of growth inhibition was observed in the spot 10-fold denser than the spot that was counted. All cfu values are averages (of raw cfu values) ±SD from determinations performed from three or four animals.

NAB737 and NAB739 (1, 2 and 4 mg/kg body weight) and polymyxin B (2 mg/kg) were administered at 1 and 3 h post-inoculation as subcutaneous injections in the neck region in a volume of 0.2 mL per dose. Control mice received saline. NAB7061 (5 mg/kg subcutaneously) and erythromycin (10 mg/kg subcutaneously) were administered at 1 h post-inoculation and a second dose of NAB7061 (5 mg/kg subcutaneously) was given at 3 h post-inoculation. Controls included treatment with either drug alone, as well as treatment with saline only.

#### Statistics

Data were analysed with unpaired two-tailed t-test (unequal variance) in GraphPad-Prism. P values <0.05 were considered significant.

### Results

#### Virulence of E. coli IH3080 in a murine peritonitis model

During the first hour post-inoculation the mice were not clinically affected by the infection. At 3 h post-infection all mice in the two groups infected with higher doses, 10^7 or 10^8 cfu, were clearly affected by the infection. The low-dose (10^6 cfu) group did not show any clinical signs of infection until 5 h after inoculation. At this time, the clinical symptoms were apparent at a similar severity regardless of the infection dose. This is an acute infection model where mice do not survive 24 h (data not shown).

The onset of clinical symptoms of the infection coincided with increased bacterial burden in the peritoneum. At 3 h post-inoculation, the cfu levels had increased in all groups and during the subsequent 2 h the cfu levels in the peritoneum continued to increase in all groups (Figure 1). The total increase in cfu levels over 5 h was 1.16, 0.57 and 0.83 log_{10} for the 10^6, 10^7 and 10^8 cfu doses, respectively. The infection also spread to the bloodstream where cfu levels were ∼1 log_{10} lower than in the peritoneum (data not shown).

#### Treatment of murine peritonitis with NAB737 and NAB739

The mean change in cfu levels in the peritoneum 6 h after initiation of treatment in each experiment is presented in Table 1. An increase in cfu levels in the saline-treated control mice ranged from 1.12 to 1.79 log_{10}. Treatment with either NAB737 or NAB739 resulted in a reduction in cfu levels in a dose-dependent manner.

For NAB739 a dose response was observed at 3 h after initiation of the treatment (Figure 2a). Treatment with 1, 2 or 4 mg/kg NAB739 resulted in a 0.82, 3.08 and 3.78 log_{10} reduction in initial cfu levels, respectively (n=3, P<0.01). During the

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subsequent 3 h, the cfu levels remained constantly low in the mice treated with 2 or 4 mg/kg of NAB739. Six hours after initiation of the treatment, the cfu values were 4.8, 4.5 and 5.3 log$_{10}$ lower in the 1, 2 and 4 mg/kg treatment groups, respectively, than in the saline-treated control group ($P$, 0.05).

Treatment with 4 mg/kg NAB737 resulted in a potent bactericidal effect 3 h after initiation of the treatment (Figure 2b). Furthermore, the cfu levels remained low for the subsequent 3 h. At 6 h after initiation of the treatment, the mean reduction in cfu was 3.73 log$_{10}$ ($n$=3, $P$$<$$0.05$) and the cfu value was 4.9 log$_{10}$ lower than in the saline-treated control group ($P$, 0.05).

### Table 1. Activity of NAB compounds against E. coli IH3080 in vivo 6 h after initiation of treatment

<table>
<thead>
<tr>
<th>Compound (dose)</th>
<th>Clinical symptoms</th>
<th>Log$_{10}$ cfu change$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>+</td>
<td>+1.12 ±0.19</td>
</tr>
<tr>
<td>Saline</td>
<td>+</td>
<td>+1.79 ±0.21</td>
</tr>
<tr>
<td>Saline</td>
<td>+</td>
<td>+1.34 ±0.13</td>
</tr>
<tr>
<td>NAB737 (4 mg/kg)</td>
<td>–</td>
<td>3.73 ±0.24</td>
</tr>
<tr>
<td>NAB739 (4 mg/kg)</td>
<td>–</td>
<td>3.48 ±0.60</td>
</tr>
<tr>
<td>NAB739 (2 mg/kg)</td>
<td>–</td>
<td>2.75 ±0.42</td>
</tr>
<tr>
<td>NAB739 (1 mg/kg)</td>
<td>–</td>
<td>3.04 ±0.28</td>
</tr>
<tr>
<td>NAB7061 (5 mg/kg)</td>
<td>+</td>
<td>1.86 ±0.34</td>
</tr>
<tr>
<td>NAB7061 (5 mg/kg)</td>
<td>–</td>
<td>1.26 ±0.71</td>
</tr>
<tr>
<td>+-erythromycin (10 mg/kg)</td>
<td>+</td>
<td>1.54 ±0.45</td>
</tr>
<tr>
<td>Polymyxin B (2 mg/kg)</td>
<td>–</td>
<td>4.26 ±0.11</td>
</tr>
<tr>
<td>Polymyxin B (2 mg/kg)</td>
<td>–</td>
<td>4.21 ±0.11</td>
</tr>
</tbody>
</table>

$^a$Mean $±$ SD ($n$=3–4) change in cfu levels from start of treatment.

Treatment of murine peritonitis with NAB7061 and erythromycin

Treatment with erythromycin or NAB7061 alone resulted in increased cfu levels indistinguishable from the saline control (Figure 3). The increases in cfu levels from 1 to 7 h post-inoculation for the erythromycin, NAB7061 and saline groups were 1.5, 1.9 and 1.3 log$_{10}$, respectively (Table 1). In contrast,
treatment with NAB7061 in combination with erythromycin resulted in reduced cfu levels. At 3 h after initiation of the treatment, a 2.0 \log_{10} cfu reduction (P<0.05) was observed and the cfu levels remained low during the subsequent 3 h (Figure 3). Six hours after initiation of the treatment, the mean cfu value was 2.6 \log_{10} lower in the NAB7061–erythromycin treatment group than in the saline-treated control group (P<0.05).

Discussion

In this paper, we were able to show that the novel polymyxin derivatives NAB739 and NAB737 are effective in treating experimental E. coli peritoneal infection in mice. Furthermore, we showed that the in vitro synergy of NAB7061 with macrolides can be demonstrated also in vivo.

The murine peritonitis model is an excellent model for initial studies of interactions of pathogens and novel antibiotics in vivo. It is relatively easy to use compared with other animal infection models. The experiments are of short duration and the results are reproducible with low variability. It is thus a cost-effective model for the generation of robust animal data.

The limitations of the present study obviously include that the pharmacokinetics and pharmacodynamics of the compounds in mice were not investigated. However, several lines of evidence suggest that the plasma half-lives of the NAB compounds in mice are short. In mice at a dose of 5 mg/kg subcutaneously, the plasma half-life of the fraction of colistin not bound to plasma proteins is 18 min and the C_{max} of total plasma colistin is \sim 1.8 mg/L.\textsuperscript{12} In rats at a dose of 1 mg/kg intravenously, the plasma half-life is 75 min.\textsuperscript{13} Furthermore, following intravenous administration of 1 mg/kg to rats, the plasma half-lives of NAB739 and NAB7061 are 69 ± 22 min and 66 ± 12 min, respectively, and the mean plasma concentrations after 10 min are 5.01 ± 0.70 mg/L and 4.08 ± 0.84 mg/L, respectively (mean ± SD).\textsuperscript{8} It is evident that further studies with other dosing and timing regimens as well as using other infection models, such as urinary tract infection, thigh infection, pneumonia and sepsis models in several animal species, are needed in order to evaluate the potential applicability of the NAB compounds in clinical settings.

Many cationic peptides, originally planned to be used in therapy of bacterial infections, have a non-specific mode of action against cell membranes.\textsuperscript{15} They are generally toxic to Gram-negative and Gram-positive bacteria, yeasts and mammalian cells, often susceptible to serum proteases and, if derived from host tissues, may additionally elicit harmful immunomodulatory responses.\textsuperscript{15} In contrast to them, polymyxins are notably specific in their action. Polymyxins bind to and damage the OM of Gram-negative bacteria.\textsuperscript{16} Even though polymyxin B nonapeptide (PMBN) and NAB7061 lack the bactericidal activity of polymyxins, they render the OM permeable.\textsuperscript{17} The enantiomer of PMBN lacks the OM-permeabilizing activity.\textsuperscript{13} This further substantiates our original view that the antibacterial activity of polymyxins is determined not only by their cationic nature but also by proper conformation.\textsuperscript{16}

Somewhat surprisingly, the combination of NAB7061 and erythromycin, a drug generally regarded as bacteriostatic, was found to decrease the bacterial count. At 3 h after initiation of the treatment, the initial cfu values were reduced by 2.0 \log_{10}. It would be interesting to study the effect of NAB7061 with other antibiotics against which the OM is an effective permeability barrier. Such drugs include the bactericidal antibiotic rifampicin, which in vitro has strong synergism with NAB7061 against E. coli.\textsuperscript{7}

The potential value of the NAB compounds in therapy remains to be elucidated. As presented in the Introduction section, there is indirect evidence suggesting that the NAB compounds, all with three positive charges only, might be less nephrotoxic in rats than polymyxin B and colistin, both carrying five positive charges. Studies are now underway to compare the nephrotoxicity of the NAB compounds and polymyxin B in appropriate animal models.

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Transparency declarations

M. V. and T. V. are employees and shareholders of Northern Antibiotics Ltd. Other authors: none to declare.

References


