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Novel polymyxin derivatives are less cytotoxic than polymyxin B to renal proximal tubular cells

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ABSTRACT

The emergence of very multiresistant Gram-negative bacterial strains has reinstated polymyxins (polymyxin B, colistin), pentacationic lipopeptides, in the therapy, in spite of their nephrotoxicity. Extensive tubular reabsorption concentrates polymyxin in proximal tubular cells. The novel polymyxin derivatives NAB739, NAB7061 and NAB741 have their cyclic part identical to that of polymyxin B, but their side chain consists of uncharged octanoyl-threonyl-D-serinyl, octanoyl-threonyl-aminobutyryl, and acetyl-threonyl-D-serinyl respectively. In this study, we compared the toxicities of NAB739, NAB7061 and NAB741 with that of polymyxin B by using the porcine renal proximal tubular cell line LLC-PK1 electroporated or incubated with the selected compound. Both the ability to cause cell necrosis (quantified as the leakage of lactate dehydrogenase) and the ability to cause apoptosis (as quantified by counting apoptotic nuclei) were assessed. In electroporated cells, polymyxin B induced total (>85%) necrosis of the cells at 0.016 mM, whereas an approx. 8-fold concentration of NAB739 and NAB7961 and an approx. 32-fold concentration of NAB741 was required for the same effect. In cells treated without electroporation (incubated), polymyxin B elicited a marked degree (approx. 50%) of necrosis at 0.5 mM, whereas the NAB compounds were inert even at 1 mM. Neither polymyxin B nor the NAB compounds induced apoptosis.

1. Introduction

The pipeline of novel classes of agents for the treatment of infections caused by the emerging very multiresistant Gram-negative bacteria is extremely dry [2,3,5,14,21,35]. Polymyxins (polymyxin B, colistin) were discovered in the late 1940s and used clinically since the late 1950s, but largely abandoned during the 1980s because of toxicities, especially nephrotoxicity. Extensive tubular reabsorption [13,15] concentrates polymyxin B in tubular cells and this may explain, at least partially, the development of this side effect [37]. At present, polymyxins have been reinstated as the last-line therapy to treat infections caused by strains that are resistant to other agents. However, their nephrotoxicity complicates the therapy and may even necessitate its discontinuation [7–9,11,19,20,38]. Less toxic polymyxin derivatives would be a very welcome addition to the clinician's antibacterial arsenal.

The novel polymyxin derivatives NAB739, NAB7061 and NAB741 [28–33] have their cyclic part identical to that of polymyxin B, but their side chain consists of octanoyl-threonyl-D-serinyl, octanoyl-threonyl-aminobutyryl, and acetyl-threonyl-D-serinyl, respectively (for structures, see Fig. 1). Accordingly, they contain only three positive charges whereas polymyxin B and colistin contain five. The MIC_{90} of NAB739 for *Escherichia coli* (17 strains) and for other polymyxin-susceptible *Enterobacteriaceae* (12 strains) are 1 μg/ml and 2 μg/ml, respectively while the corresponding values of polymyxin B are 1 μg/ml and 1 μg/ml, respectively [30]. The MIC range of NAB739 and polymyxin B for the polymyxin-susceptible carbapenemase-producing strains of *E. coli* and *Klebsiella pneumoniae* (9 strains, including KPC-, OXA-48-, VIM-, and IMP-producing strains) is 1–4 μg/ml and 1–2 μg/ml, respectively [32]. NAB739 is also active against *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. NAB7061 and NAB741 lack intrinsic antibacterial activity but, like polymyxin B nonapeptide [27], they strongly synergize the activity of antibiotics toward Gram-negative pathogens, including antibiotics regarded as exclusively anti-Gram-positive drugs such as rifampicin, macrolides, fusidic acid and vancomycin [30–32].

Regarding the renal toxicity, the affinity of NAB7061 and NAB739 for isolated rat kidney brush border membrane (BBM) is lower by a factor of six to seven as compared to polymyxin B [30].

Abbreviations: Abu, aminobutyryl; BBM, brush border membrane; Dab, diaminobutyryl; LDH, lactate dehydrogenase; MIC, minimum inhibitory concentration; MIC_{90}, antimicrobial concentration that inhibited growth of 90% of the strains.

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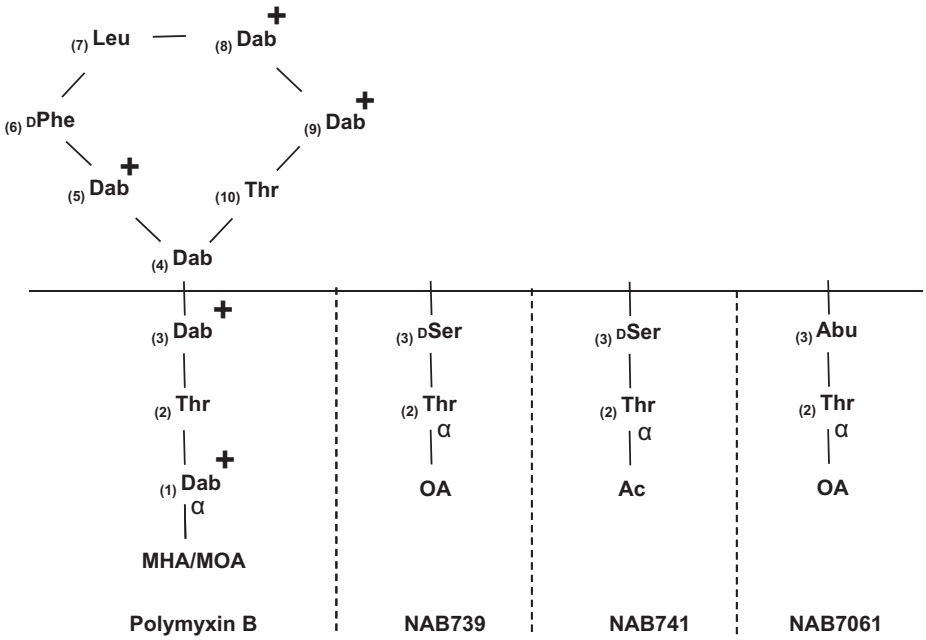

Fig. 1. Structures of polymyxin B, NAB739, NAB7061, and NAB741. Abbreviations for the non-trivial amino acyl residues: Dab, diaminobutyryl; Abu, aminobutyryl. Other abbreviations: MHA/MOA, the mixture of methyl octanoyl and methyl heptanoyl; OA, octanoyl; ac, acetyl. The positive charge of the free amino groups is also shown.

The renal clearance of NAB741 (3.78 ± 1.11 mL/min/kg), NAB739 (0.53 ± 0.30 mL/min/kg), and NAB7061 (0.28 ± 0.16 mL/min/kg) are much higher than that of colistin (0.010 ± 0.008 mL/min/kg) [1,31], suggesting that the positively charged diaminobutyryl (Dab) residues in the side chain of the old polymyxins play an important inhibitive role in the renal elimination.

In this study, we compared the cytotoxicities of NAB739, NAB7061 and NAB741 with that of polymyxin B by using the porcine renal proximal tubular LLC-PK_1 cells. Both the ability to cause cell necrosis and apoptosis were assessed, as these represent two established mechanisms of cellular toxicity induced by gentamicin, another well known nephrotoxic antibiotic [23]. In addition to testing cells exposed to drugs present in the culture medium (conventional drug treatment), the assays were also performed by using electroporated cells. Electroporation allows direct, endocytosis-independent delivery of the compounds into cells, and it has previously been shown for gentamicin, that this procedure induces apoptosis of LLC-PK1 cells at concentrations lower to those required if the cells are conventionally treated [22].

2. Materials and methods

2.1. Compounds

Acetate salts of NAB739, NAB7061 and NAB741 were synthesized by Bachem (Bubendorf, Switzerland). Their purity, as estimated by high performance liquid chromatography and elemental analysis was 96% or more. Polymyxin B sulfate (P-1004) was from Sigma–Aldrich (St. Louis, MO). Gentamicin sulfate was provided as Geomycine by Glaxo-SmithKline Belgium (on behalf of Schering-Plough Corp., Kenilworth, NJ).

2.2. Cells

LLC-PK1 cells (CL-101™), a porcine renal proximal tubular cell line, were obtained from the American Type Culture Collection (Manassas, VA) and were cultivated as described previously [22].

2.3. Drug treatments

For the conventional drug treatment, the cells were continuously exposed to the drugs in complete cell culture medium at the indicated concentrations at 37 °C for 48 h. Controls included cells incubated in the absence of any drugs. Cells treated according to this protocol are referred to as “incubated”.

For the drug treatment using electroporation, the procedure described in detail previously [22] was used. Briefly, subconfluent cells were detached by trypsinization, centrifuged at 1000 rpm. Cells (about 500 μg of protein) were collected in 200 μL of electroporation buffer (10 mM phosphate buffer, pH 7.2, 250 mM sucrose and 1 mM $MgCl_2$), in the presence of increasing drug concentrations. Cells were exposed to 8 pulses (square waves) at 180 V and left thereafter for 15 min at room temperature in the same medium. They were then dispersed in drug-free DMEM, transferred into cell culture dishes, and incubated for 24 h at 37 °C. Controls included cells electroporated in the absence of any drug. Cells treated according to this protocol are referred to as “electroporated”.

2.4. Quantification of necrosis and apoptosis

2.4.1. Necrosis

Cell integrity was assessed by measurement of the release of the cytosolic enzyme lactate dehydrogenase (EC 1.1.1.27) into the culture medium [17]. LDH activity in each sample is measured as NADH consumption during pyruvate to lactate reduction by LDH [34]. At the end of incubation, the culture medium is collected separately and cells are harvested by scrapping in H_2O and sonicated. Release of LDH was expressed as the percentage of activity detected in the media over the sum of the activities in the media and in cells.

2.4.2. Detection and enumeration of apoptotic cells

Cells were detached by trypsinization, pelleted by centrifugation at 1200 rpm for 7 min, washed three times with gentle resuspension and repelleting in ice-cold Phosphate Buffer Saline (PBS). Staining of DNA to reveal apoptotic bodies was made with 4′,6′-diamidine-2′-phenylindole (DAPI). Cells were resuspended in

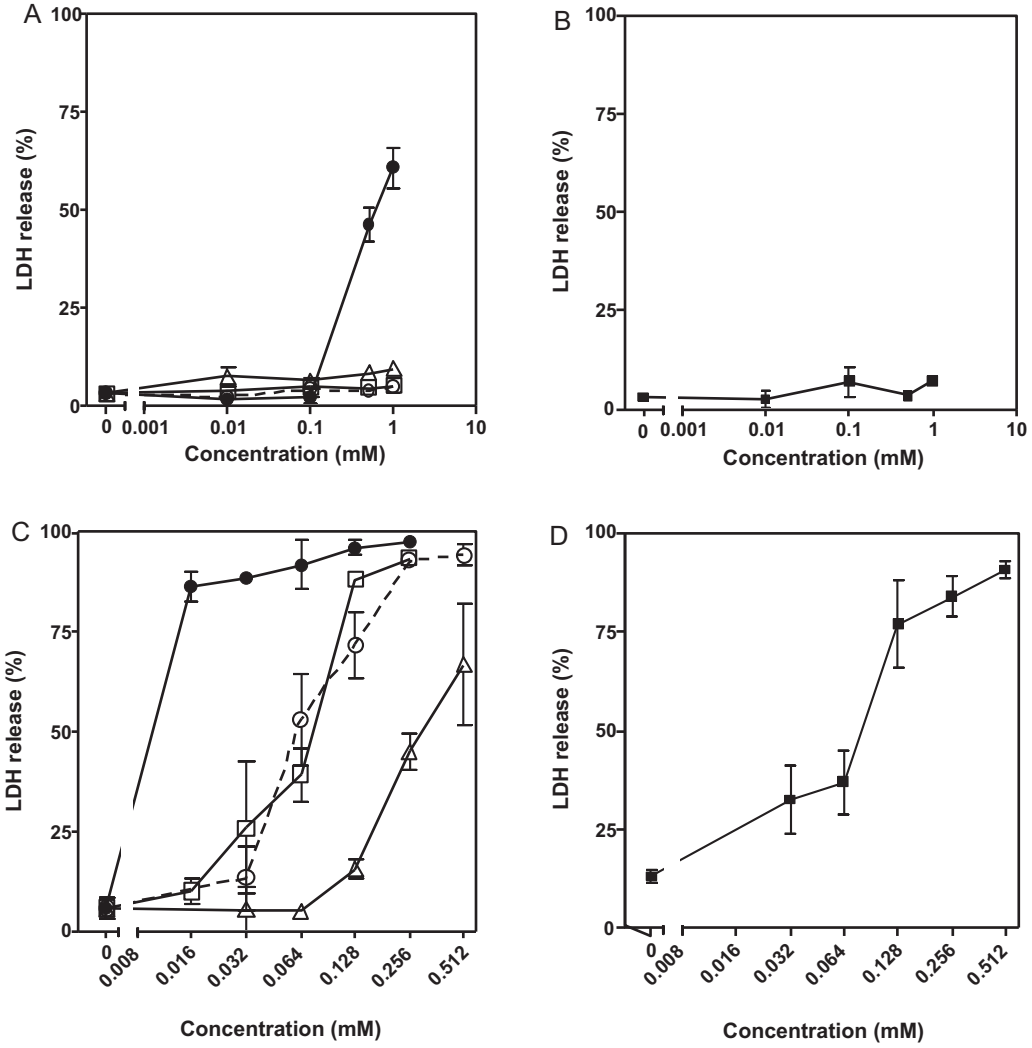

Fig. 2. Drug-induced necrosis induced by polymyxins (Panels A and C) and by gentamicin (Panels B and D) in porcine LLC-PK_1 cells, as assessed by measuring the release of the cytosolic enzyme lactate dehydrogenase (LDH) in the culture medium (shown as percentage of the total amount in cells plus medium). Panels A and B, cells incubated for 48 h in the presence of the drugs. Panels C and D, cells electroporated in the presence of the drugs. Values are means ± standard deviations (two experiments, $n = 3$ in each). Symbols: ●, polymyxin B; −Θ−, NAB739; □, NAB7061; △, NAB741; ■, gentamicin.

PBS, fixed in 4% paraformaldehyde for 30 min, spread on polylysine-coated slides, allowed to dry out for a few hours and incubated with the stain (1 μg/ml in methanol) for 15 min at 37 °C. Samples were then mounted in Mowiol/1, 4-diazabicyclo[2.2.2]octane (DABCO). Enumeration of apoptotic nuclei was made on slides picked up at random, using a Zeiss light microscope with 63× oil-immersion objective. Apoptotic nuclear fragmentation was identified during random counting of 500 cells per condition. Clusters of apoptotic bodies were given as a single count. Data were expressed as the percentage of apoptotic nuclei relative to total number of nuclei counted.

Statistical analyses were made using GraphPad Prism version 4.02 and GraphPad InStat version 3.06 (GraphPad Prism Software, San Diego, CA).

3. Results

3.1. Drug-induced necrosis

The LDH release (±SD) was 46 ± 4%, 4 ± 1%, 5 ± 0%, 8 ± 1%, and 4 ± 1%, when the cells were incubated for 48 h with 0.5 mM polymyxin B, NAB739, NAB7061, NAB741 (Fig. 2A), and gentamicin (Fig. 2B), respectively. At 1 mM, the corresponding values were 61 ± 5%, 5 ± 2%, 5 ± 0%, 10 ± 1%, and 7 ± 0%, respectively. Accordingly, polymyxin B elicited a marked degree of cell necrosis at concentrations at which gentamicin and the NAB compounds were inert.

In electroporated cells, polymyxin B induced total (>85%) necrosis of the cells at 0.016 mM (corresponding to approx. 20 mg/L), whereas an approx. 8-fold concentration of NAB739 and NAB7061 and an approx. 32-fold concentration of NAB741 were required to achieve the same effect (Fig. 2C). Gentamicin showed an effect similar to that observed with NAB739 and NAB7061 (Fig. 2D). Of note, the electroporation procedure used in this study did not cause evidence of gross cell damage or significant leakage of LDH in the absence of drug, and its effects on the permeability of lysosomes and mitochondria have been shown previously to be minimal [22].

3.2. Drug-induced apoptosis

Neither polymyxin B nor the NAB compounds induced apoptosis in incubated (Fig. 3A) or electroporated cells (Fig. 3C), as tested up to 1 mM and 0.5 mM, respectively. As a sharp contrast, gentamicin induced apoptosis, as previously shown [6,22]. Apoptosis

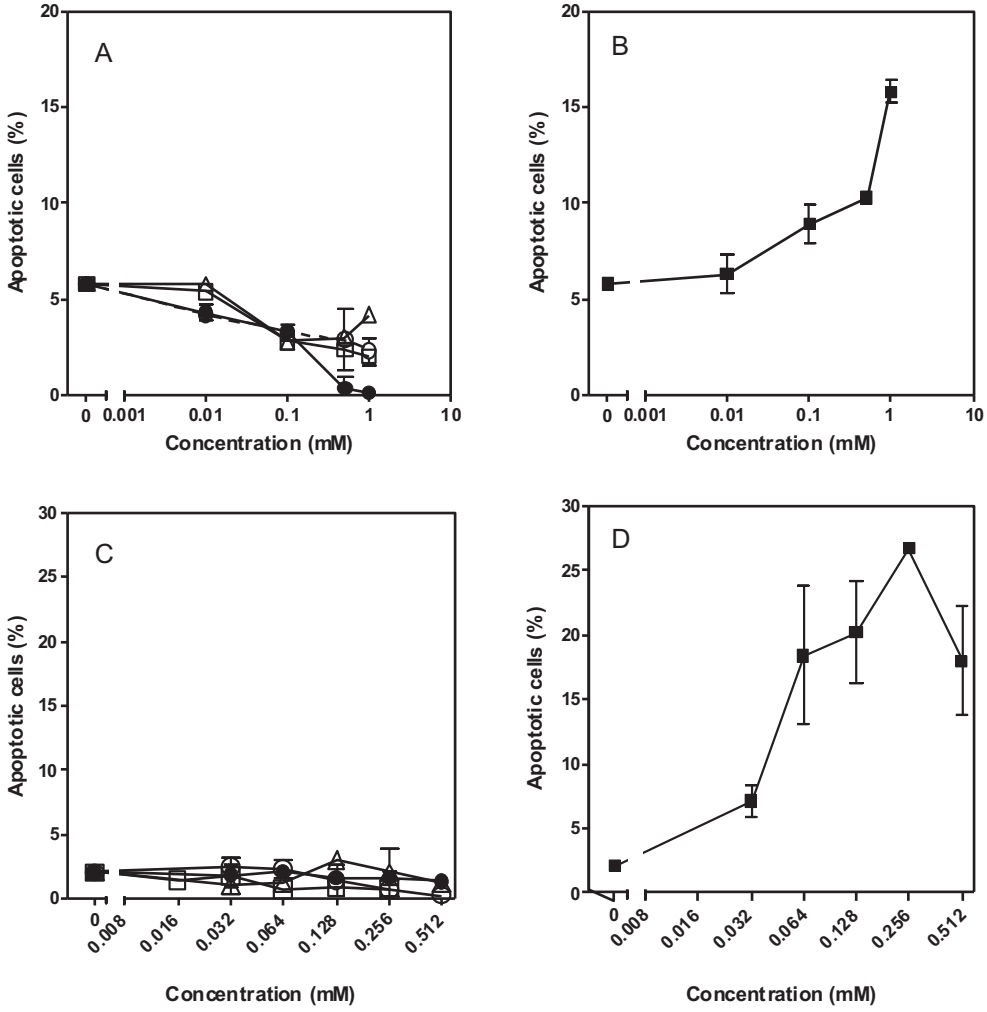

Fig. 3. Drug-induced apoptosis induced by polymyxins (Panels A and C) and by gentamicin (Panels B and D) in porcine LLC-PK_1 cells, as assessed by enumeration of typical apoptotic nuclei. Panels A and B, cells incubated for 48 h in the presence of the drugs. Panels C and D, cells electroporated in the presence of the drugs. Values are means ± standard deviations (two experiments, $n = 3$ in each). Symbols: ●, polymyxin B; –Θ–, NAB739; □, NAB7061; △, NAB741; ■, gentamicin.

manifested in approx. 15% of the cells treated conventionally with 1 mM gentamicin (Fig. 3B) and in approx. 20% of the cells electroporated in the presence of 0.064 mM gentamicin (Fig. 3D).

4. Discussion

The present study showed that NAB739, NAB7061 and NAB741 have a substantially lower necrotic potential toward LLC-PK1 cells than polymyxin B. In assays where the drug treatment was performed using electroporation, polymyxin B induced total (>85%) necrosis of the cells at the concentration of 0.016 mM (corresponding to approx. 20 mg/L), whereas an approx. 8-fold concentration of NAB739 and NAB7061 and an approx. 32-fold concentration of NAB741 was required for the same effect. The most plausible explanation is the difference in the interactions between the different compounds with the lipids from the intracellular leaflet of the plasma membrane resulting in varying loss of membrane integrity. This is likely since, in contrast to polymyxin B with five positive charges, the NAB derivatives have only three positive charges and could therefore be less prone to interact with the negatively charged lipids mostly found in the cytosolic leaflet of the plasma membrane [12]. Moreover, the lower ability of NAB741 to induce LDH release, as compared to NAB739 and NAB7061, should be related with its chemical structure. Indeed, NAB741 has cyclic and linear peptide portions identical to those of NAB739 but differs from NAB739 and NAB7061 by carrying only an acetyl residue in the N-terminus of the peptide instead of a long hydrophobic residue, i.e. octanoyl in NAB739 and NAB7061. Therefore, both the number of positive charges in the linear portion of the peptide and the hydrophobicity of the chain in the N-terminus of the peptide could be critical for the ability to induce necrosis.

Furthermore, neither polymyxin B nor the NAB compounds induced apoptosis whereas this was clearly evidenced for gentamicin as reported earlier [22]. The absence of apoptosis induced by polymyxin B agrees with another publication [4]. Three hypotheses could be suggested for explaining why no apoptosis is observed after treatments with polymyxin B: (i) absence of interactions between polymyxin B and the NAB derivatives with membranes of subcellular organelles impairing loss of membrane integrity and release of pro-apoptotic proteins like cathepsins from lysosomes or cytochrome c from mitochondria, (ii) modulation of pro-and anti-apoptotic proteins like Bax and Bcl-2, respectively, and (iii) huge apoptosis induced at the earliest time and with very low concentrations leading to massive elimination of apoptotic cells and direct observation of necrosis. Accordingly, the nephrotoxicity of polymyxin B appears to be mediated by cellular mechanisms probably quite different from those involved in gentamicin toxicity [24]. Future work is needed to verify this difference in other experimental settings as well as to elucidate the underlying mechanisms.

It should be noted, that LLC-PK_1 cells grown under the conditions of this study represent a simplified model of proximal tubular cells, as megalin, the 600-kDa endocytosis-mediating receptor expressed in several absorptive epithelia including renal proximal tubule belonging to the low-density lipoprotein-receptor family, and involved in the endocytic uptake of a variety of substances including aminoglycosides [16,18], is only poorly expressed. Yet, LLC-PK1 are commonly used for investigation of nephrotoxic compounds [10,26] and apoptosis [25,36]. Because the affinity of polymyxin B to isolated BBM is even higher than that of gentamicin, and the affinities of NAB739 and NAB7061 are significantly lower [30], it is possible that larger differences will be noted in vivo. Yet, even the assay as used in the present study may be a useful tool for the rapid screening of various polymyxin derivatives.

Conflicts of interest

T.V. and M.V. are employees of Northern Antibiotics Ltd. M.-P.M.-L., P.M.T. and S.D. have no conflict of interest.

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