Novel Polymyxin Derivatives are Less Cytotoxic than Polymyxin B in a model of LLC-PK1 Renal Cell Line

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Abstract (Edited)

Background:

Polymyxins, last resource antibiotics against multi-resistant Gram (-) bacteria, cause renal toxicity. NAB739 (mol wt, 1076 d), NAB741 (992 d) and NAB7061 (1074 d) are novel derivatives with the same cyclic peptide part as polymyxin B (PMB; 1189 d), but with linear portion consisting of octanoyl-threonyl-D-serinyl, acetyl-threonyl-D-serinyl and octanoyl-threonyl-aminobutyryl, respectively. Accordingly, they contain only 3 positive charges whereas PMB and colistin contain five. Yet, NAB739 is as or almost as potent as PMB for *E. coli* and other polymyxin-susceptible Enterobacteriaceae (Vaara 2010, Curr Opin Microbiol 13:574). NAB7061 and NAB741 sensitize bacteria to the activity of other antibiotics. We compared the cytotoxicity of these compounds to that of PMB and gentamicin (GEN, 473 d) in a cell culture model.

Methods:

Porcine renal LLC-PK1 cells using cell necrosis (release of lactate dehydrogenase [LDH]) and apoptosis (DAPI staining) with incubated (48h) and electroporated cells as end-points (Servais et al. AAC, 2006, 50:1213-1221).

Results

The Figure (see poster) shows LDH release and apoptosis (±SD, n=3) in incubated (upper row) or electroporated (lower row) cells as a function of the concentration of PMB, NAB739, NAB7061 and NAB741, and GEN.

Conclusions

NAB compounds are less cytotoxic than polymyxin B in this in vitro model and, in contrast to GEN, do not induce apoptosis. These results may justify further in vivo investigations.

Polymyxin B	MHA/MOA	-Dab$^+$	-Thr	-Dab$^+$	-cy[Dab	-Dab$^+$	-bPhe	-Leu	-Dab$^+$	-Dab$^+$	-Thr]
NAB739	OA	-	-Thr	-bSer	-cy[Dab	-Dab$^+$	-bPhe	-Leu	-Dab$^+$	-Dab$^+$	-Thr]
NAB7061	OA	-	-Thr	-Abu	-cy[Dab	-Dab$^+$	-bPhe	-Leu	-Dab$^+$	-Dab$^+$	-Thr]
NAB741	Ac	-	-Thr	-bSer	-cy[Dab	-Dab$^+$	-bPhe	-Leu	-Dab$^+$	-Dab$^+$	-Thr]

Figure 1: structure of polymyxin B and NAB compounds

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Background and Aim

The pipeline of novel classes of agents for the treatment of infections caused by multiresistant Gram (-) negative bacteria is practically empty (Livermore, 2009). Polymyxins (polymyxin B, colistin) are now increasingly used as last resource drugs but are notorious for toxicities that may necessitate discontinuation of the therapy (Falagas *et al.*, 2010).

NAB739, NAB7061 and NAB741 (Vaara, 2009, 2010; Vaara & Vaara, 2010; Vaara *et al.*, 2008, 2010a, 2010b) are polymyxin B derivatives with a side chain made of octanoyl-threonyl-D-serinyl, octanoyl-threonyl-aminobutyryl, and acetyl-threonyl-Dserinyl, respectively (Fig. 1) and carry only 3 positive charges *vs.* 5 for polymyxin B and colistin. The MIC_{90} of NAB739 for *E. coli* and Enterobacteriaceae are similar to those of polymyxin B (1-2 mg/L). NAB739 is also active against *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. NAB7061 and NAB741 strongly synergize the activity of antibiotics (including rifampicin, macrolides, fusidic acid and vancomycin) towards Gram (-) pathogens, (Vaara *et al.*, 2008, 2010a, 2010b).

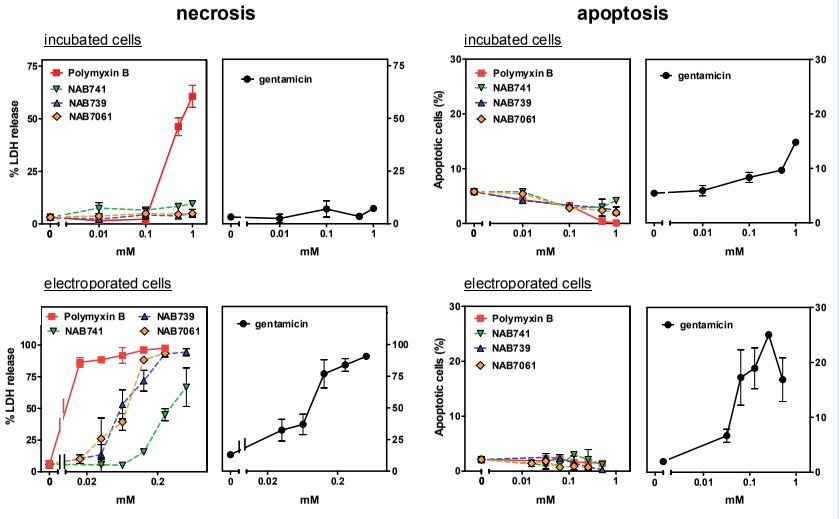
NAB7061 and NAB739 bind 6-7-fold less to isolated rat kidney brush border membrane (BBM) than polymyxin B (Vaara et al., 2008) and their renal clearance is 30 to 300-fold faster (Ali et al., 2009; Vaara *et al.*, 2010a).

Our aim was to compare the intrinsic cytotoxicities of NAB739, NAB7061 and NAB741 with that of polymyxin B using a model of incubated and electroporated LLC-PK1 cells that had been previously validated with gentamicin (Servais et al., 2005; Servais et al., 2006).

Methods

- **Drugs:** NAB739, NAB7061 and NAB741(acetate salts) synthesized by Bachem (Bubendorf, Switzerland) with 96 % purity (HPLC); polymyxin B sulfate from Sigma-Aldrich and gentamicin from Schering-Plough.
- **Cells:** LLC-PK1 cells (CL-101™) from ATCC grown as non-polarized cells to ~80 % confluency (Servais et al., 2005).
- **Treatments**: (i) incubated cells: continuous exposure to drugs for up to 48 h; (ii) electroporated cells: 8 square waves pulses at 800 V/cm (1 msec; 1 Hz repetition frequency) with drug, followed by 15 min rest and incubation in drug-free medium for 24 h (Servais et al., 2006) 2.4. Quantification of necrosis and apoptosis
- **Measures:** lactate dehydrogenase (LDH) activity release for necrosis; counts of condensed/fragmented nuclei after staining with 4',6'-diamidine-2'-phenylindole (DAPI) for apoptosis.

Results



Dug-induced necrosis (left) **:**

- incubated cells (top): LDH release (±SD) was 46±4 %, 4±1 %, 5±0 %, 8±1 %, and 4±1 % with 0.5 mM polymyxin B, NAB739, NAB7061, NAB741, and gentamicin, 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.
- electroporated cells (bottom): 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 (bottom). 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 (Servais et al., 2006).

Drug induced apoptosis (right) **:**

- neither polymyxin B nor the NAB compounds induced apoptosis in incubated or electroporated cells, as tested up to 1 mM and 0.5 mM, respectively. In sharp contrast, gentamicin induced apoptosis, as previously shown (Servais et al., 2006). Apoptosis manifested itself in approx. 15 % of the cells treated incubated with 1 mM gentamicin (top) and in approx. 20 % of the cells electroporated in the presence of 0.064 mM gentamicin (bottom).

This poster will be available for download after the meeting from http://www.facm.ucl.ac.be/posters.htm

Conclusions

- NAB739, NAB7061 and NAB741 have a substantially lower necrotic potential towards LLC-PK1 cells than polymyxin B.
- This was particularly noteworthy in assays where the drug treatment was performed using electroporation, allowing for clear ranking based on dose-response curves with NAB739 and NAB 7061 behaving essentially like gentamicin
- Most importantly, neither polymyxin B nor the NAB compounds induced apoptosis whereas this was clearly evidenced for gentamicin.
- Thus, the nephrotoxicity of polymyxin B could be mediated by cellular mechanisms quite different from those involved in gentamicin toxicity.
- Limitations: Although commonly used for investigation of nephrotoxic compounds, LLC-PK1 cells represent a simplified model of proximal tubular cells and no attempt was made to look for differences in drug uptake and disposition.
- Yet, the assay as used here may help in ranking drugs on basis of their intrinsic toxicity.

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