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Comparative Assessment of Antibacterial and Cytotoxic Effects of Colistin (CST), Colistin Methanesulfonate (CMS) and Polymyxin B (PMB) Using *Pseudomonas aeruginosa*, Macrophages, Renal cells and Liposomes.

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Abstract

Objectives: Colistin (CST) causes bacterial lysis by gross changes in membrane fluidity and permeability. To better assess its selectivity towards bacteria *vs.* eukaryotic cells, we directly compared CST, its prodrug colistin methanesulfonate (CMS) and polymyxin B (PMB) for bacterial killing, cell toxicity and membrane permeabilization.

Methods: Bacterial killing: 4 strains of Psedomonas aeriginosa (*P.a.*) with distinct resistance phenotypes (See table). Cytotoxicity: lactate dehydrogenase (LDH) release and apoptosis in murine J774, human THP-1 macrophages and porcine renal LLC-PK1 cells, with drug uptake measured by LC-MS-MS. Membrane permeabilization: release of calcein (self quenched fluorescence tracer) from liposomes with composition mimicking the eukaryotic cell membrane.

Results: CST (i) was bactericidal (3 log cfu decrease) for 3 strains but at higher concentrations than the reported steady state C_{max}; (ii) showed onset of cytotoxicity (10% LDH release) at concentration > 20 x higher than the MIC (but 5-10 x higher than the bactericidal concentration) for macrophages, and much larger for renal cells (iii) did not permeabilize liposomes. CMS was less potent but showed similar cytotoxicity (due to fast conversion in CST). PMB was more potent but also more cytotoxic (with an approximately 5 x larger accumulation in cells) and destabilized liposomes. Apoptosis was not detected for any compound (positive control: gentamicin).

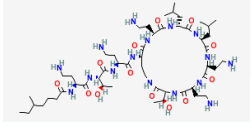
Conclusions: Colistin showed a large safety margin in the models used if considering MIC but the larger doses needed to obtain a bactericidal effect may reduce it. This model can be used for screening novel derivatives of colistin or polymyxin B.

drug	concentration (mg/L) causing										
	bacterial killing (3 log cfu decrease) [a]				cytotoxicity (% LDH release) [b] from					liposome permeabilization (% calcein release) [c]	
					J774		THP1		LLC-PK1		
	PAO1	PA50	PA117	PA434	10 %	50 %	10 %	50 %	10 %	10 %	25 %
CST	7.9	6.2	12.6	> 25	78	> 100	62	> 100	> 100	> 500 [d]	> 500 [d]
CMT	> 16	15.8	> 16	> 16	49	> 100	75	> 100	100	not done	
PMB	2.1	9.5	8.9	13.2	33	77	40	47	> 100	25	50

MIC (mg/L): PAO1: CST = 2; CMT = 4; PMB = 0.5; GEN = 2; MEM = 0.5; PTZ = 2; CAZ = 2; CIP = 0.5
PA50: CST = 2; CMT = 4; PMB = 2; GEN = 4; MEM = 8; PTZ = 8; CAZ = 4; CIP = 16
PA117: CST = 4; CMT = 8; PMB = 4; GEN = 8; MEM = 1; PTZ = 8; CAZ = 4; CIP = 0.25
PA434: CST = 4; CMT = 8; PMB = 4; GEN > 64; MEM = 8; PTZ > 128; CAZ = 16; CIP > 16

[a] from an initial inoculum of 1 x 10^6 CFU in MH broth after 24 h; determined by intrapolation from concentration-effect experiments (Hill equation)
[b] 24 h incubation; determined by graphical intrapolation (each experiment made in triplicate; coeff. var. < 7 %)
[c] liposomes made of phosphatidylcholine:sphingomyelin:phosphatidylinositol:cholesterol (4:4:1:1) incubated at pH 7.4 and 37°C for up to 40-250 min (values are taken at plateau); 100 % release: exposure to 0.2 %Triton-X-100
[d] max. release: 8.1 %

Colistin Sulfate



Cyclic **amphipathic polycationic peptide** with a short aliphatic side chain

- that **interacts with the lipopolysaccharide (LPS)** of the outer membrane of Gram-negative bacteria, triggering a "self-promoted uptake" process
- and **displaces Ca^{++} and Mg^{++}**, which further destabilizes microbial outer membranes and helps conferring more specificity towards proacarotic cells

Background and Aim

The worldwide increase in antibacterial resistance among Gram-negative bacteria is a serious threat to the management of the infections caused particularly by multi-drug resistant oragnisms, including *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (1).

In this context, colistin, a polypeptide antibiotic (see the structure in the left) available since 1960s is now increasingly used for treatment of patient suffering from infections caused by MDR-GNB(2)

Colistin is a peptide antibiotic comprising two main components, colistin A (polymyxin E1) and colistin B (polymyxin E2) (1) and it is administered in the form of colistin methanesulfonate (CMS), a prodrug of colistin that is less toxic than colistin it-self but is quickly hydrolyzed into colistin both *in vivo* and *in vitro*.

According to CLSI (3) and EUCAST (4) the clinical breakpoint for colistin for *P. aeruginosa* and *A. baumannii* is defined as ≤ 4 mg/L.

Considering these facts we were interested to compare antimicrobial activity to cytotoxicity using *P. aeruginosa*, different cultured cell lines and liposomes mimicking eukaryotic cell membranes.

Methods

Bacterial susceptibility and kill-curves: *P. aeruginosa* strain ATCC PAO1 was used as reference. Clinical isolates resistant to ciprofloxacin (PA50) and aminoglycosides (PA117), as well as multi-drug resistant (PA434) were also used. MICs were measured by microdilution in cation adjusted Mueller-Hinton broth (MHB; pH 7.4, 24 h). For kill-curves, bacteria (10^6 CFU/mL) were cultivated in MHB in presence of increasing concentration of antibiotics for 24 h.

Measurement of necrosis: Necrosis was assessed by measuring the amount of the lactate dehydrogenase activity released in the medium (5).

Measurement of apoptosis: Apoptosis (J774 macrophages, THP-1 macrophages, LLC-PK1 cells) was assessed in incubated cells by staining with DAPI and counting of fragmented nuclei (5, 6).

Assessment of membrane permeabilization: Membrane permeabilization was assessed by measuring the amount of calcein (a self quenched fluorescence tracer) released from the liposome with composition mimicking eukaryotic cell membrane (7).

Drug accumulation: Human THP-1 cells were incubated with CST, CMS, PMB (25 mg/L) for 24h at 37°C and the intracellular antibiotic content was then measured by mass spectrometry.

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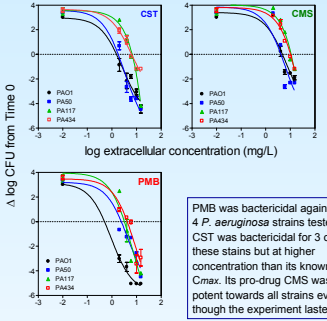
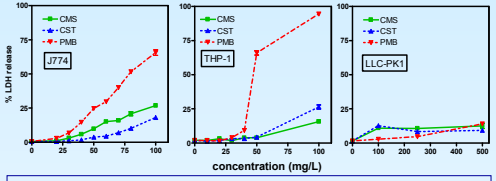
Results

P. aeruginosa killing induced by CMS, CST and PMB in MHB



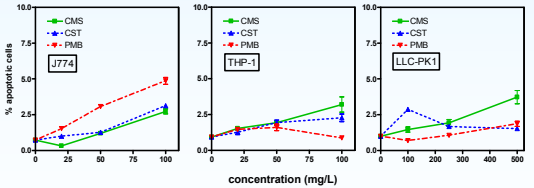
PMB was bactericidal against the 4 *P. aeruginosa* strains tested. CST was bactericidal for 3 of these stains but at higher concentration than its known C_{max}. Its pro-drug CMS was less potent towards all strains even though the experiment lasted 24 h

Necrosis induced by CMS, CST and PMB in murine J774 macrophages, human THP-1 macrophages and porcine renal LLC-PK1 cells (24 h incubation)



- Neither CST nor its prodrug CMS caused severe necrosis in J774 and THP-1 macrophages compared to PMB (the latter caused massive LDH release in both cells, reaching ~100% in THP-1 cells t 100 mg/L.
- In contrast, LLC-PK1 cells proved very resistant to all 3 drugs, even when tested at much larger concentrations (up to 500 mg/L).
- Of note, the cellular accumulation of polymyxin B (measured at an extracellular concentration of 25 mg/L (i.e. in the absence of necrosis) was systematically about twice that of colistin.

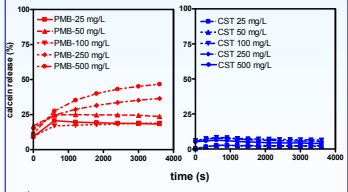
Apoptosis induced by CST, CMS and PMB in murine J774 macrophages, human THP-1 macrophages and porcine renal LLC-PK1 cells (24 h incubation)



With this protocol, gentamicin is known to cause apoptosis up to 20-25 % of apoptosis in J774 and LLC-PK1 cells (see ref. 8 and Denamur et al. ECCMID 2007). In contrast, all values here were considerably lower. A clear-cut effect-concentration relationship was seen for PMB with J774 macrophages, but not for THP1 cells where a max. was reached at 50 mg/L followed by a decline at larger concentrations (perhaps in relation to the massive necrosis). CST and CMS had less effect (but no decline in THP-1 cells). LLC-PK1 cells showed much resistance to PMB and CST, with tendency concentration-dependent effect for CMS.

Please, note that all those changes are actually minimal, involving max. 5 % of cells.

Membrane permeabilization from liposome with composition mimicking eukaryotic cell membrane (*)



*Phosphatidylcholine:phosphatidylethanolamine:sphingomyelin:phosphatidylinositol:cholesterol=4:4:3:1:1

100% release: exposure to 0.05% Triton-X-100

- PMB destabilized the liposomes with ~50% of calcein release after 1h at higher concentrations
- In contrast, CST caused only minimal changes with no or little progression over time.

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

Conclusions

- **Colistin is considerably less cytotoxic compared to PMB and, interestingly enough, showed a wide safety range considering its MIC.**
- **PMB was more potent than CST, which means that the larger doses of CST required to obtain a bactericidal effect should also be considered prior its use in terms of toxic potential**
- **The lower cytotoxicity of CST *vs.* PMB could be due to its lower accumulation. However, a higher intrinsic toxicity of PMB must also be considered in view of the results obtained with liposomes (membrane destabilizing effect).**
- **CMS was less potent against bacteria than CST although supposedly fully converted into CST during the time of the experiment, but showed almost a similar cytotoxicity.**
- **This model can be used for screening novel derivatives of colistin or polymyxin B and could be used also to design appropriate animals studies comparing the toxicity of CST to its antibacterial activity.**

References

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