Cell-penetrating Peptides Targeting Intracellular Infections

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ABSTRACT SUMMARY

A well-known cell-penetrating peptide (CPP), penetratin, that previously had been shown to possess antibacterial properties was tested for a putative antibiotic effect against *Staphylococcus aureus* internalized into Calu-3 human airway epithelial cells. The results indicate that highly cationic peptides exhibiting such dual biological effects indeed constitute an interesting compound class that may be applied in a novel treatment strategy for such infections. Expectedly, higher concentrations of penetratin were required for achieving an inhibitory effect on internalized bacteria than necessary to kill extracellular bacteria.

INTRODUCTION

The emergence of multiresistant microorganisms poses a severe threat to public health leading to increased treatment costs, as well as increased morbidity and mortality. Successful eradication of bacterial infections can in some cases be further complicated by the fact that the bacteria localize inside mammalian cells. Thereby the bacteria evade the host immune response as well as a chemotherapeutic treatment with an ensuing high risk for a relapse of infection.

One class of new potential antibiotics is the antimicrobial peptides. These often short and cationic peptides constitute a part of the innate immune system in most living organisms from plants to highly developed mammals and have thus been evolutionarily conserved through millions of years without development of bacterial resistance1.

Recently, another type of membrane-interacting peptides, namely CPPs, has been shown to possess antimicrobial activity2. Penetratin is a well-known 16-residue CPP capable of crossing the eukaryotic membrane and efficiently internalize in eukaryotic cells: 3

H-RQIKIWFQNRRMKWKK-NH2.

Penetratin was recently shown to possess some antimicrobial activity towards both Gram-negative and Gram-positive bacteria,4 and may thus be a candidate for eradication of internalized bacteria in human cells.

EXPERIMENTAL METHODS

Penetratin was synthesized by Fmoc solid-phase peptide synthesis on a Rink-amide resin and purified by preparative RP-HPLC. The purity and identity of the peptide was determined by RP-HPLC and high resolution LC-MS, respectively.

The growth-inhibitory effect of penetratin against *S. aureus* (ATCC 6538) was determined by microtiter MIC determination according to CLSI guidelines. Cell viability and hemolysis were determined as described previously5.

Calu-3 cells were maintained in DMEM medium. Cells were grown in 5% CO2, 37 °C and detached from culturing flasks at 80% confluency by exposure to trypsin. A seeding density of ∼2.5×103 cells/cm2 was used, and the cells were grown in 24-well plates (NUNC, Roskilde, DK) for 22-24 h to a confluence of 80–90%. The cells were incubated for 2 h (37°C, 5% CO2) with approximately 10⁸ *S. aureus* in DMEM effectively infecting the Calu-3 cells. Subsequently, the cells were rinsed with PBS and extracellularly residing bacteria were removed by incubation for 45 min with 50 µg/mL gentamicin. The cells were rinsed four times with PBS buffer and incubated for 22-24 h with penetratin (4-256 µM, 1 mL). Hereafter, the cells were rinsed with PBS x 4, and then the cells were lysed in a 0.01 % Triton-X 100 solution. The lysate was diluted and spread on TSA agar plates to determine the intracellular colony count.

For confocal microscopy cells were seeded on Poly-D-lysine coated, 35 mm dishes, (MatTek, Ashland, MA, US) following the same procedure as described above. *S. aureus* were labeled with FITC according to Krut 20046. Approximately 10⁷ bac/mL FITC labeled *S. aureus* was added to the cell cultures and
incubated for 120 min. The cells were treated with 50 µg/mL gentamicin for 45 min and incubated with penetratin for 60 min before microscopy.

RESULTS AND DISCUSSION
Penetratin was tested against *S. aureus* to determine the minimal inhibitory concentration (MIC), against Calu-3 cells in a viability assay and against human red blood cells in a hemolysis assay (Table 1).

Table 1. Penetratin tested against *S. aureus* (MIC), Calu-3 cells (viability, EC50) and human red blood cells (hemolysis, EC50).

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<th>MIC [µM]</th>
<th>Viability [µM]</th>
<th>Hemolysis [µM]</th>
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The significant difference in concentrations needed to inhibit bacterial growth compared to the concentrations needed to affect Calu-3 cell viability shows that penetratin may have potential as an effective antibiotic compound.

To test the intracellular killing efficiency of penetratin, different concentrations of peptide were applied to a Calu-3 cell culture, which had initially been infected with *S. aureus*. After 24 h of incubation, no apparent inhibition of bacterial growth by penetratin is observed at lower concentrations (Figure 1). It is clear that the eukaryotic cellular membrane reduces the antimicrobial effect of the cell-penetrating peptide considering the MIC value determined against non-internalized bacteria. However, at concentrations above 32 µM there is a clear antibacterial effect with a bacteriostatic concentration of about 100µM indicating transport of the peptide to the site of the internalized bacteria followed by growth inhibition and some killing of the bacteria.

To test the co-localization of the bacteria with peptide, a TAMRA labeled variant of penetratin and FITC labeled *S. aureus* was studied by confocal microscopy after internalization of the bacteria into Calu-3 cells. Manders’ overlap coefficient showed 62% of the internalized bacteria to be co-localized with peptide.

CONCLUSION
A cell-penetrating peptide was effectively used to inhibit or kill *S. aureus* internalized into Calu-3 cells. Thus this proves the concept that a peptide displaying a combination of cell-penetrating and antimicrobial activity may be optimized towards new tools for eradicating internalized bacteria.

REFERENCES

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