

Cell-Penetrating Antimicrobial Peptides – Prospectives for Targeting Intracellular Infections

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Received: 30 June 2014 / Accepted: 9 October 2014 / Published online: 17 March 2015
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ABSTRACT

Purpose To investigate the suitability of three antimicrobial peptides (AMPs) as cell-penetrating antimicrobial peptides.

Methods Cellular uptake of three AMPs (PK-12-KKP, SA-3 and TPk) and a cell-penetrating peptide (penetratin), all 5(6)-carboxytetramethylrhodamine-labeled, were tested in HeLa WT cells and analyzed by flow cytometry and confocal microscopy. Furthermore, the effects of the peptides on eukaryotic cell viability as well as their antimicrobial effect were tested. In addition, the disrupting ability of the peptides in the presence of bilayer membranes of different composition were analyzed.

Results AMP uptake relative to penetratin was ~13% (PK-12-KKP), ~66% (SA-3) and ~50% (TPk). All four peptides displayed a punctate uptake pattern in HeLa WT cells with co-localization to lysosomes and no indication that clathrin-mediated endocytosis was the predominant uptake mechanism. TPk showed the highest antibacterial activity. SA-3 exhibited selective disruption of liposomes mimicking Gram-positive and Gram-negative membranes.

Conclusion PK-12-KKP is an unlikely candidate for targeting intracellular bacteria, as the eukaryotic cell-penetrating ability is poor. SA-3, affected the cellular viability to an unacceptable

degree. TPk showed acceptable uptake efficiency, high antimicrobial activity and relatively low toxicity, and it is the best potential lead peptide for further development.

KEY WORDS antibiotics · antimicrobial peptides · cell-penetrating peptides · peptide-membrane interaction · *Staphylococcus aureus*

INTRODUCTION

Cell-penetrating peptides (CPPs) are short, often cationic peptides capable of internalizing into eukaryotic cells without damaging the plasma membrane at effective internalization concentrations, and therefore they are actively pursued as potential drug delivery vehicles (1). Cationic antimicrobial peptides (AMPs) comprise a class of peptides structurally similar to cationic CPPs, and AMPs constitute an essential part of the innate immune system in almost all living organisms (2). Most known AMPs exert their antibacterial effect via selective membrane disruption thereby killing the bacteria, but also intracellular targets have been identified for some AMPs (e.g. buforin II (3), drosocin (4) and apidaecin (5)). The two peptide classes, cationic CPPs and cationic AMPs, share features such as: (i) a relatively short chain length (10–40 residues), (ii) a positive net charge, important for initial interaction with phospholipid membranes possessing an overall negative charge, and (iii) an amino acid composition with a significant content of non-polar residues considered to promote their interaction with, and in the case of AMPs, insertion into the hydrophobic core of the membrane bilayer (6). The dominant biological effect of such highly cationic peptides may be altered from cell-penetrating to antimicrobial or vice versa by even small modifications of their sequence. Thus, examples of successful development of both AMP-based CPPs (e.g. SynB1 (7) and Bac7 (15–24) (8)) and CPP-based AMPs (e.g. Pep-1-K) (9) have been reported. The increasing interest

Electronic supplementary material The online version of this article (doi:10.1007/s11095-014-1550-9) contains supplementary material, which is available to authorized users.

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in the dual effects of these types of peptides has uncovered several examples of peptides exhibiting both eukaryotic cell membrane penetration and antimicrobial effects (10).

During the last decade, a massive increase in community-associated and nosocomial multidrug-resistant *Staphylococcus aureus* infections has been registered, now reaching an alarming level; combined with a high rate of recurrent infections this poses a serious healthcare problem (11). Increasing evidence infers that the high incidence of recurrent infections is caused by bacteria being internalized into epithelial cells via phagocytosis at the site of infection (12). Thus, complete eradication is not obtainable through treatment with commonly applied antibiotics (13). We propose that development of a suitable cell-penetrating antimicrobial peptide could provide an alternative treatment regime against such persistent intracellular infections either in itself or as part of a combination therapy. In a previous study (14), we found that the well-characterized CPP, penetratin, and a number of its analogs in fact possess antimicrobial properties. In the present study, we investigated whether bacteria-penetrating AMPs could be applied as CPPs.

Zhu and coworkers (9) showed that modification via Gly→Lys substitution in the synthetic CPP, Pep-1, afforded a designed AMP, Pep-1-K (also denoted PK) that efficiently inhibited growth of both Gram-negative and Gram-positive bacteria. Further optimization towards a shorter analog was based on a 12 amino acid truncated version of PK with two lysine residues added to the C-terminus and three Thr→Pro substitutions (15). The resulting 14-mer, PK-12-KKP, displayed a more pronounced positive charge, low hydrophobicity, and only partial folding into a well-defined secondary structure due to the presence of the proline residues. Although the antimicrobial activity of PK and PK-12-KKP were comparable, their mode of action was indicated to be different as the latter exhibited a very low membrane depolarization of *S. aureus* similar to that of buforin 2 known to have an intracellular target. Joshi and coworkers (16) reported a range of synthetic peptides designed from an amphipathic template displaying mainly hydrophobic and cationic amino acids. Amongst these, a 12-mer peptide, SA-3 composed mainly of lysine and tryptophan, was found to exhibit efficient inhibition of both Gram-negative and Gram-positive bacteria as well as low hemolytic activity. Based on uptake of FITC-labeled peptide into *E. coli* and strong DNA binding properties of the peptide the authors suggested an intracellular target as the primary mode of action for SA-3 (16). In addition, Zhu and co-workers reported that TPK, a peptidomimetic analog of tritrypticin, displayed high activity against Gram-positive and Gram-negative bacteria as well as low cytotoxicity against eukaryotic cells (17). The design of this analogue involved replacement of two proline residues with peptoid lysine residues (NLys), thereby increasing the overall molecular charge, while preserving the lack of amide protons required for

hydrogen bonding to the backbone. Additionally, TPK depolarized the membrane potential of *S. aureus* to a low degree and only caused weak leakage of a fluorescent dye entrapped within negatively charged vesicles modeling the bacterial membrane. Confocal laser-scanning microscopy of bacteria exposed to FITC-labeled TPK showed clear accumulation of fluorescence in the cytoplasm, whereas the parent tritrypticin remained outside or associated to the cell membrane. These findings suggest that the mode of action for TPK involves inhibition of intracellular components after crossing the bacterial cell membrane.

The common feature of the three AMPs selected for this study is that investigations of their bacterial killing mechanism have inferred that they act via intracellular targets. By applying advanced artificial membrane systems to study these peptides, we expect to gain a more thorough understanding of their effects on the membranes of both bacteria and eukaryotic cells.

MATERIALS AND METHODS

Materials

Reagents, resin and amino acid building blocks for solid-phase peptide synthesis were purchased from Iris Biotech (Merkredwitz, Germany) while DMF was acquired from VWR (Herlev, Denmark). Chemicals utilized were: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (AppliChem, Darmstadt, Germany), Hanks Balanced Salt Solution (HBSS) (Gibco, Paisley, UK), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-phenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) (Promega, Madison, WI, USA), phenazine methosulphate (PMS), sodium dodecyl sulfate (SDS), Eagle's minimal essential medium (EMEM), Triton X-100, calcein, diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBt), dimethylformamide (DMF) (all Sigma-Aldrich, Buchs, Switzerland), 5(6)-carboxytetramethylrhodamine (TAMRA) (Fluka, Buchs, Switzerland), DRAQ7® (Biostatus, Leicestershire, UK), Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Paisley, UK) and phosphate buffered saline (PBS) (Invitrogen, Paisley, UK) Sephadex G-100 (GE Healthcare, Little Chalfont, UK), 2,2,2-trifluoroethanol (TFE) (Acros organics, Geel, Belgium) Mueller Hinton Broth (MHB) (BD, Le Pont de Claix, France). The lipids: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), cholesterol, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (POPG), 1',3'-bis[1,2-dimyristoyl-*sn*-glycero-3-phospho]-*sn*-glycerol (cardiolipin 14:0; CL), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Lipids, Alabaster, AL, USA.

Methods

Solid Phase Peptide Synthesis and Labeling

Peptide synthesis was performed by Fmoc-based solid-phase peptide synthesis (SPPS) employing a microwave-assisted automated CEM Liberty synthesizer (CEM, Matthews, NC, USA); peptides were purified to >95% and 90% for TAMRA-labeled peptides as described earlier (14). For the fluorophore labeling, TAMRA (2.5 equivalents) was used in a mixture of DIC (2.5 equivalents), and (HOBt; 2.5 equivalents) in (DMF) overnight at room temperature. The identity of the peptides was confirmed by HRMS spectra obtained by using a Bruker micrOTOF-Q II Quadrupole MS detector; analyses were performed as ESI-MS (m/z): $[M+nH]^{n+}$ (Bruker, Bremen, Germany). An overview of the synthesis products can be found in Table I.

Cell Culture

HeLa WT cells from the American Type Culture Collection (ATCC, Manassas, VA, USA) were maintained in EMEM supplemented with 100 IU/mL penicillin, 100 ug/mL streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids (all from Sigma-Aldrich, St. Louis, MO, USA), 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA, USA), and 10% (*v/v*) fetal bovine serum (FBS) (Fisher Scientific, Waltham, MA, USA). Cells were kept under tissue culture conditions (5% CO₂, 37°C) and detached from culturing flasks at 80% confluency by treatment with trypsin-EDTA, and subsequently subcultured once weekly. For the MTS/PMS assay HeLa WT cells were seeded at a density of ~30,000 cells/cm² in flat-bottomed 96-well Plates (Corning Costar, Sigma-Aldrich, Brøndby, Denmark). NIH 3T3 cells for MTS/PMS assay were acquired from ATCC (Manassas, VA, USA), maintained in

DMEM supplemented with 10% (*v/v*) newborn bovine serum (NCS) (Gibco, Paisly, U.K.), 100 IU/mL penicillin, 100 ug/mL streptomycin and 2 mM L-glutamine and seeded in a flat-bottomed 96-well plates (Corning Costar, Sigma-Aldrich, Brøndby, Denmark) at a density of ~69,000 cells/cm². HUVEC cells were acquired from the European Collection of Cell Cultures (ECACC) (Salisbury, UK) and maintained in Ham's F-12 K (Kaighn's) medium supplemented with 10% (*v/v*) FCS, 0.1 mg/mL heparin, 0.03 mg/mL endothelial cell growth supplement as well as 100 IU/mL penicillin, 100 ug/mL streptomycin and 2 mM L-glutamine and seeded in a flat-bottomed 96-well plates (Corning Costar, Sigma-Aldrich, Brøndby, Denmark) at a density of ~47,000 cells/cm². HeLa WT cells for confocal microscopy visualization were cultured on (poly-D-lysine)-coated, 35 mm imaging dishes (MatTek Glass Bottom Microwell Dishes, MatTek, Ashland, MA, USA) at a density of ~140,000 cells/cm² and for flow cytometry cells were seeded at a density of ~110,000 cells/cm² in 12-well plates (NUNC, Roskilde, Denmark). For all experiments, the cells were cultured for 22–24 h to obtain a subconfluent cell monolayer (confluency 80–90%).

MTS/PMS Viability Assay

The cells were washed twice with 37°C HBSS supplemented with 10 mM HEPES (denoted: hHBSS) and exposed to 100 µL of peptide solution in hHBSS (concentration range 1–256 µM) for 1 h under tissue culture conditions. A 0.2% (*w/v*) SDS solution and hHBSS were used as positive (OD_{pos.ctrl}) and negative (OD_{neg.ctrl}) control, respectively. The peptide solution was removed by inverting and gently tapping the back of the plate. Subsequently, a mixture of 240 µg/mL MTS and 2.4 µg/mL PMS in test buffer was applied. The plate was kept shielded from light (37°C, 50 rpm) for ~2 h until the cells exposed to buffer (the negative control) reached an absorbance of ~1. Absorbance was measured on a POLARstar Optima ($\lambda = 492$ nm) (GMB Labtech, Offenburg,

Table I Amino Acid Sequences, Number of Residues and Molecular Weight of the Investigated Peptide Variants

Compound	Sequence	Residues	Mw	Ref
PK-12-KKP	KKPWWWKPKWKK-amide	14	2008.46	(15)
SA-3	IKWAGKWWWKLFK-amide	12	1589.97	(16)
TPk	VRRFkWWWkFLRR-amide ^a	13	1963.38	(17)
Pen	RQIKIWFQNRRMKWKK-amide	16	2245.74	(18)
TAMRA-PK-12-KKP	TAMRA-KKPWWWKPKWKK-amide	14	2420.89	
TAMRA-SA-3	TAMRA-IKWAGKWWWKLFK-amide	12	2002.40	
TAMRA-TPk	TAMRA-VRRFkWWWkFLRR-amide	13	2375.81	
TAMRA-Pen	TAMRA-RQIKIWFQNRRMKWKK-amide	16	2658.17	

^a k refers to NLys [H₂N-(CH₂)₄-NH-CH₂-COOH]

Germany). The relative cell viability (%) was calculated according to Eq. (1):

$$\text{Viability}(\%) = (\text{OD}_{\text{peptide}} - \text{OD}_{\text{pos.ctrl.}}) / (\text{OD}_{\text{neg.ctrl.}} - \text{OD}_{\text{pos.ctrl.}}) \quad (1)$$

Confocal Laser-Scanning Microscopy (CLSM)

The cells were rinsed with DMEM supplemented with 10% (*v/v*) heat-inactivated fetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin (37°C). The medium was replaced with 200 µL of 10 µM TAMRA-labeled peptide in PBS and incubated for 1 h under tissue culture conditions. Subsequently, the test solutions were discarded, the cells washed twice with DMEM, once with imaging medium (DMEM without phenol red), and finally resuspended in 300 µL of imaging medium containing 0.3% (*v/v*) DRAQ7®. Immediately hereafter, CLSM was performed using an inverted Leica SP5 confocal laser-scanning microscope (Leica Mikrosystems, Wetzlar, Germany) equipped with an Ar and HeNe laser and a 63× 1.4 NA oil immersion objective. Live-cell microscopy was performed at room temperature and with a fixed instrument configuration across all samples. Furthermore, the endocytosis pathway markers dextran and transferrin were applied. In lysosome labeling experiments the cells were incubated with 0.1 mg/mL 10 kDa dextran Alexa Fluor® 647 (Life Technologies, Basel, Switzerland) for 210 min, and the label subsequently chased into lysosomes via a further incubation in dextran-free medium for 20 h, after which the cells were incubated with peptide as described above. To investigate uptake by clathrin-mediated endocytosis, the cells were incubated for 50 min with 100 nM transferrin Alexa Fluor® 647 (Life Technologies, Basel, Switzerland), rinsed with complete DMEM. PBS with 100 nM transferrin Alexa Fluor® 647 was supplemented with TAMRA-labeled peptides (200 µL) at various concentrations and incubated under tissue culture conditions for 10 min before proceeding with the washing and imaging procedure stated above.

Flow Cytometry

Cellular internalization of TAMRA-labeled peptides was quantified by flow cytometry. Initially, the cells were washed once with 1 mL PBS (37°C) containing 10 µM peptide solution (700 µL in PBS), and then incubated for 1 h under tissue culture conditions. The cells were subsequently rinsed three times with ice-cold PBS. Detachment of the cells from the wells was done by addition of 0.01% (*w/v*) trypsin-EDTA (200 µL, 10 min, 37°C, 5% CO₂), and after centrifugation (5 min, 1076×*g*) the cells were washed twice with ice-cold PBS containing 10% (*v/v*) FBS. Lastly, the cells were resuspended in

500 µL PBS with 10% (*v/v*) FBS containing 0.3% (*v/v*) DRAQ7®. Flow cytometry analysis was performed shortly after the preparation by using a Gallios flow cytometer (Beckman Coulter, Fullerton, CA, USA). The fluorescence mean of 10,000 viable cells was recorded gating for forward and sideways scattering as well as for DRAQ7® fluorescence intensity.

Minimal Inhibitory Concentration (MIC)

The inhibitory effect of peptides on bacterial growth was determined by microdilution broth assay according to Clinical Laboratory Standard Institute (CLSI) guidelines (19). In brief, the peptides were assayed against *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) suspended in MHB to a turbidity of 0.5 McFarland units. The bacterial suspension was added in a volume of 100 µL to wells containing 100 µL 2-fold serial dilutions of peptides in MHB, yielding final peptide concentrations in the range 1–128 µM (experiments performed in triplicate on at least two separate days). The polypropylene microtiter plates (Nunc) were incubated at 35°C in ambient air for ~18 h, after which the MIC values were determined as the lowest concentration showing no visible growth as compared to the controls either without antibacterial compound or bacteria added.

Liposome Preparation

Liposomes were prepared by using a procedure previously described (14). In brief, large unilamellar vesicles (LUV) were prepared from POPC and cholesterol in a molar ratio of 10:1 in order to mimic an eukaryotic cell membrane composition (20–22), while a liposome composition with POPG and synthetic CL in a molar ratio of 3:1 was used to mimic Gram-positive *S. aureus* membrane composition (20–22). POPG, CL, and DOPE in a molar ratio of 2:7:1 were employed as a model for Gram-negative *Escherichia coli* membranes (20–22). From chloroform solutions of the lipids, the solvent was removed by rotary evaporation. The lipid film was stripped with ethanol three times and dried overnight on a rotary evaporator to remove trace amounts of chloroform. Calcein-loaded liposomes were formed in Tris buffer (10 mM, pH 7.4) containing 150 mM NaCl, 0.1 mM EDTA and 70 mM calcein. The solutions were vigorously vortexed six times over 1 h, and the resulting multilamellar vesicles were extruded once through a 100 nm polycarbonate membrane filter followed by nine extrusions through a 50 nm polycarbonate membrane filter. The particle size distribution was measured on a Zetasizer Nano ZS (Malvern, Worcestershire, UK) using a 633 nm laser and 173° detection optics. Malvern DTS v. 5.10 software was used for acquisition and analysis. Subsequently, excess calcein was removed by passing the liposomes twice through a Sephadex G-50 column collecting 3 mL fractions.

Calcein Release Assay

To measure leakage from calcein-loaded liposomes, these were added to black 96-well plates (MicroWell 96 optical bottom plates, NUNC, Roskilde, Denmark) at a concentration of 1.2 μM lipid estimated from inductively coupled plasma mass spectrometry. The fluorescence was monitored by utilizing a FLUOstar OPTIMA plate reader (GMB Labtech, Offenburg, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm to determine F_0 . A volume of 20 μL of peptide solution was added and measurement of calcein leakage was initiated within 10 s from the addition of peptide, and the peptide-induced calcein leakage was recorded for a period of 680 s (340 s for PK-12-KKP with POPG/CL liposomes) in order to determine F_t . Subsequently, the liposomes were disrupted completely by addition of 20 μL 20% (v/v) Triton X-100, and then the signal from the total amount of calcein was recorded (F_t). The relative release was calculated according to Eq. (2):

$$\text{Calcein leakage}[\%] = [(F_t - F_0)/(F_t - F_0)] \times 100\% \quad (2)$$

Data Analysis

Results are presented as the mean of three measurements \pm SD, and unless otherwise stated experiments were performed in triplicate. Statistical significance was determined by the students *t*-test where $P < 0.05$ is considered significant. For the viability assays the 50% cellular viability (EC_{50}) was estimated by using Prism 6 for Mac OS X vs. 6.0 and by non-linear regression according to Eq. (3):

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + (X/\text{EC}_{50})^{-\text{Hill Slope}}). \quad (3)$$

RESULTS

In the present study, three AMPs (PK-12-KKP, SA-3 and TPk) and a CPP (penetratin) were investigated with respect to their: (i) ability to internalize into eukaryotic cells, (ii) subcellular localization inside eukaryotic cells, (iii) ability to inhibit bacterial growth, (iv) influence on eukaryotic cell viability, and (v) ability to disrupt artificial membranes.

Uptake of Short Cationic AMPs and CPP in HeLa WT Cells

To determine whether the three AMPs were capable of internalizing into eukaryotic cells, TAMRA-labeled variants of the peptides were used. The uptake efficiency of the three AMPs

was compared to that of the similarly labeled well-characterized CPP, penetratin. As expected, penetratin displayed a higher total cellular uptake when compared to the AMPs as determined by flow cytometry (Fig. 1). The Pep-1 derived analog PK-12-KKP displayed a significantly lower uptake ($\sim 10\%$ relative to penetratin) than the other compounds tested ($P < 0.01$). SA-3 showed the highest uptake as compared to the other tested AMPs reaching a level of $\sim 65\%$ of the uptake of penetratin, which was significantly better than that of TPk ($P < 0.05$). TPk exhibited intermediate uptake efficiency with about half the uptake found for penetratin.

Intracellular Distribution and Co-Localization With Endocytosis Pathway Markers

Microscopy analysis confirmed the flow cytometry data showing that the TAMRA-labeled variants of the AMPs and penetratin gave rise to significant uptake resulting in subcellular punctate distribution patterns throughout the cytoplasm of the HeLa WT cells without affecting the viability of the cells (data not shown) as determined by addition of DRAQ7®; a DNA-binding dye that selectively stains dead and permeabilized cells. Peptide uptake was also imaged in cells that first were stained with fluorescently labeled pulse/chased 10 kDa dextran that accumulates in lysosomes. As expected, peptide incubations in lysosome-labelled cells gave rise to a punctate pattern of fluorescence for the four peptides (Fig. 2), and in the case of PK12-KKP and penetratin there was clear evidence of delivery to lysosomes manifested as co-localization with the dextran fluorophore. Whether clathrin-dependent endocytosis of the peptides occurred to a significant extent was studied by testing uptake in cells that were pre-incubated with fluorescent transferrin present in the media during these short peptide incubations. This resulted in an overall low uptake of

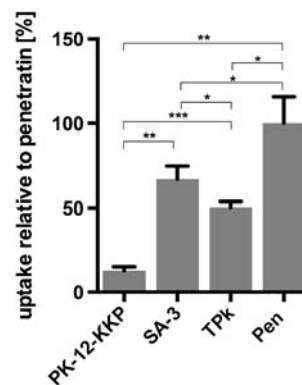


Fig. 1 Cellular uptake of three TAMRA-labeled AMPs versus that of TAMRA-penetratin measured by flow cytometry in HeLa cells. Prior to analysis, the cells were incubated with 10 μM of each peptide for 1 h at 37°C. The plot represents the mean of the medians of the raw data in triplicate; error bars representing \pm SD and normalized against the mean of penetratin. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ statistically significant differences.

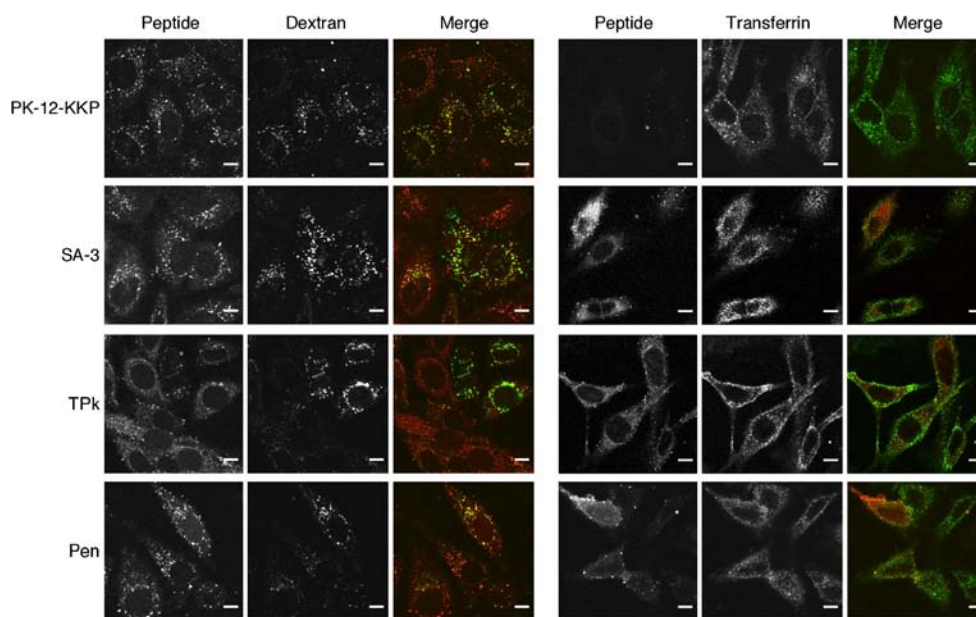


Fig. 2 Cellular distribution of three selected AMPs (PK-12-KKP, SA-3, TPK) and penetratin (Pen) (all TAMRA labeled). For each photo montage: Left, grayscale: peptide; middle, grayscale: dextran/transferrin; right, red: peptide, green: transferrin/dextran, yellow: dextran/transferrin co-localized with peptide; blue: DRAQ7®. **Dextran:** The cells were incubated with 0.1 mg/mL dextran for 210 min and further incubated in media for 20 h before exposure to 10 μ M peptide for 1 h (all at 37°C). **Transferrin:** The cells were incubated for 50 min with 100 nM transferrin and further incubated with both 100 nM transferrin and 10 μ M of the peptides for 10 min (all at 37°C). Media for all samples contained 0.3% (v/v) DRAQ7®. Images are representative single cross sections from the middle of cells. Scale bars correspond to 10 μ m.

PK-12-KKP with no discernible co-localization with transferrin suggesting that clathrin-mediated endocytosis is not utilized by this peptide. With respect to transferrin there was minimal co-localization with SA-3 and TPK, however, these cells also showed diffuse cytosolic staining. Penetratin localization was confined to punctate structures that did not overlap to any extent with transferrin. For all four peptides DRAQ7® positive cells were not observed indicating that at a 10 μ M concentration the plasma membrane was not compromised to a degree that resulted in leakage of the dye to label nuclear DNA.

Cellular Viability and Antimicrobial Effect

To assess the relative effects of the peptides on eukaryotic cell viability and bacterial growth, the results of the tetrazolium dye-based viability assay MTS/PMS and the determined minimal inhibitory concentrations on *S. aureus* were compared. To gain insight regarding a possible selectivity of the peptides toward specific eukaryotic cells, the viability of HeLa WT cells as well as of fibroblast cells (NIH 3T3) and human umbilical vein endothelial cells (HUVEC) in the presence of the three AMPs and penetratin was investigated (Table II). Several cell lines were tested as earlier studies have shown large variations in the influence of AMPs on the viability of different cell lines (23,24). No significant reduction in viability of all three cell lines upon exposure to PK-12-KKP was detected within the concentration range tested (i.e. up to

256 μ M). By contrast, cell viability was severely lowered by SA-3 with EC₅₀ values against HeLa WT and NIH 3T3 cell lines at concentrations 25–30 μ M. HUVEC cells were generally much less sensitive to the peptides, and SA-3 was the only peptide that affected these cells at the tested concentrations. TPK reduced the viability of HeLa WT cells with an EC₅₀ value only slightly higher than that of SA-3, however, its cytotoxicity against NIH 3T3 cells was at least four times lower than that of SA-3. Also, for penetratin no significant decrease in cell viability of any of the cell lines was observed within the tested concentration range. Table II shows that overall, the antimicrobial activity of the AMPs was more

Table II Viability of Mammalian Cells and Minimal Inhibitory Concentrations

	Eukaryotic cell viability ^a			MIC (μ M)	
	NIH 3T3	HUVEC	HeLa WT	<i>S. aureus</i> ^b	<i>E. coli</i> ^c
PK-12-KKP	>256	>256	>256	32	16–32
SA-3	30 \pm 4.5	128–256 [#]	27 \pm 1.7	32	16–32
TPK	128–256 [#]	>256	41 \pm 4.0	8–16	2
Pen	>256	>256	>256	32	8–16

^aEC₅₀ values are given in μ M ($n=3$ in 3 passages; mean \pm SD) as the concentration required to give a 50% reduction in viability of the cells after incubation with peptide for 1 h. [#] EC₅₀ listed as an interval as the value was too close to the maximum concentration to allow accurate fitting

^bStrain: ATCC 25923

^cStrain: ATCC 25922

pronounced toward the Gram-negative *E. coli* than against the Gram-positive *S. aureus*, and that TPk was the most effective AMP.

Membrane Interactions

Calcein-containing liposomes of different compositions were exposed to varying peptide concentrations and the change in fluorescence over time was measured in order to assess both the degree and the kinetics of the membrane disruption induced by the peptides. The liposomes prepared with calcein displayed monomodal size distributions, namely POPG:CL (molar ratio 3:1, 48.8 nm, PdI =0.3), POPG:CL:DOPE (molar ratio 2:7:1, 51.6 nm, PdI =0.2) and POPC:cholesterol (molar ratio 10:1, 39.1 nm, PdI <0.1). When comparing the specific model membranes, clear differences were found regarding the maximal amount of calcein released upon exposure to the three peptides for up to 680 s (Table III). Considering the individual peptides, PK-12-KKP gave rise to a low partial calcein leakage of 13–25% for all three liposomal compositions. Overall, SA-3 resulted in a higher relative leakage, but with a higher degree of selectivity towards the liposomes modeling the bacterial membranes; namely the POPG:CL modeling Gram-positive bacteria and POPG:CL:DOPE modeling Gram-negative bacteria. Thus, SA-3 induced a high relative leakage (82% to completion) from the bacteria-mimicking liposomes, whereas the eukaryotic model liposomes only released 33% of the encapsulated calcein at the same peptide concentration. TPk had only a minimal effect on the POPG:CL:DOPE (2:7:1) liposomes mimicking the *E. coli* membrane as only ~3% of the calcein was released. In contrast, TPk disrupted both POPG:CL (3:1) liposomes mimicking *S. aureus* membranes and liposomes corresponding to a mammalian membrane composition releasing more than 30% of the encapsulated calcein. Penetratin exhibited a selective disruption of the *S. aureus* model liposomes with low disruptive capacity for the other two liposome compositions.

Table III Maximum Calcein Release After Exposure to a Concentration Of 1.6 μM Peptide Expressed as Percent of Total Amount of Calcein Released (mean \pm SD)

	POPG:CL (3:1) ^a <i>S. aureus</i> model	POPG:CL:DOPE (2:7:1) ^b <i>E. coli</i> model	POPC:cholesterol (10:1) ^c Eukaryotic model
PK-12-PPK	23.6 \pm 6.1 ^d	13.1 \pm 1.7	24.3 \pm 2.4
SA-3	82.2 \pm 1.4	101.7 \pm 1.7	33.2 \pm 2.2
TPk	32.5 \pm 1.3	2.7 \pm 0.5	36.6 \pm 4.5
Pen	99.6 \pm 0.6	13.3 \pm 1.4	8.4 \pm 0.8

^a *S. aureus* membrane model, (n = 3)

^b *E. coli* membrane model, (n = 2)

^c Eukaryotic membrane model (n = 3). Run time 680 s

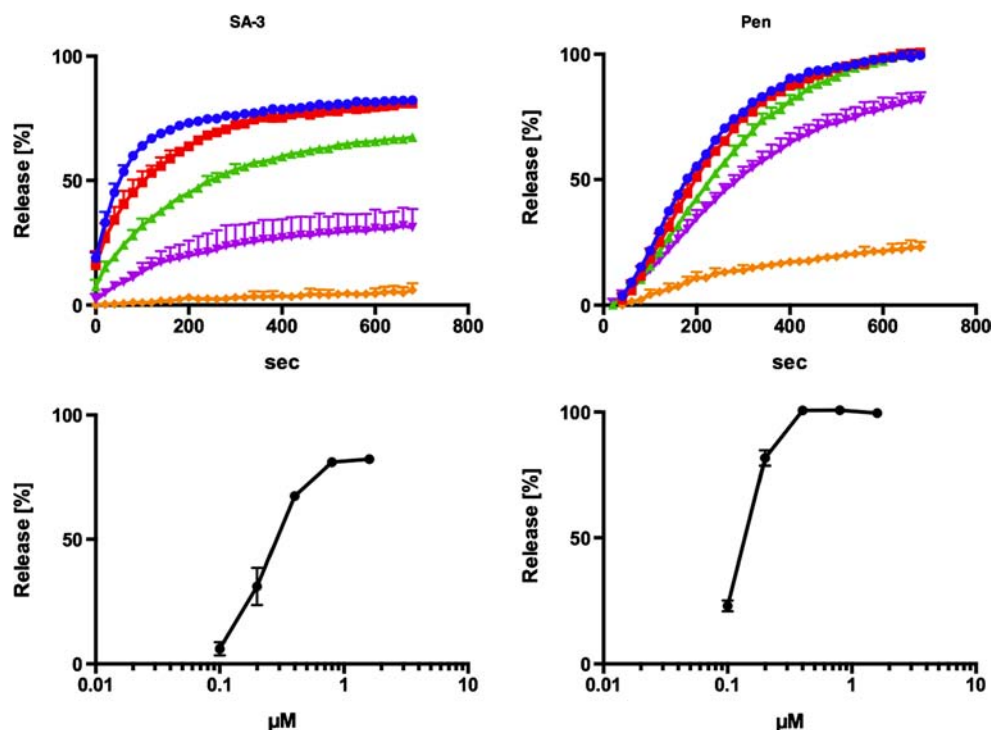
^d Run time 340 s

More detailed insight into the effects of SA-3 and penetratin on Gram-positive model membranes (Fig. 3) and Gram-negative model membranes (Figure S1, supplementary information) was gained by depicting comparative time-resolved and concentration-dependent profiles. For both model membranes, the release of calcein triggered by SA-3 was immediate, as e.g. the first measurement of 1.6 μM peptide together with the Gram-positive membrane model showed ~20% calcein release (Fig. 3). For penetratin, a slow initial calcein release was observed with a lag period of 40 s, however, after 10 min full liposomal disruption was observed, while SA-3 reached a total release of 82%. Inspection of the concentration-dependent release of calcein from the POPG:CL model membranes revealed that these vesicles are more sensitive to penetratin than to SA-3 over the entire concentration range, even though the initial release rate is more rapid for SA-3.

DISCUSSION

The peptides investigated in this study have previously been tested for their antibacterial activity (15–17), whereas the objective of the current study was to assess their potential to target bacteria internalized in eukaryotic host cells. Quantification of the cellular uptake of the peptides allowed a ranking of these sequences according to their internalization efficiency in comparison to that of penetratin, a well-characterized CPP that has previously been shown to internalize efficiently into several eukaryotic cell lines (25,26) including HeLa WT cells (27). Further, the distribution pattern of the AMPs in HeLa WT cells was assayed by confocal microscopy with and without fluorophore-labeled dextran as an endocytic probe that can be used to label the entire endolysosomal pathway or specifically lysosomes (28), and which under the applied experimental conditions accumulate in the late vacuolar system, particularly in the lysosomes. Fluorophore-labeled transferrin known to be taken up by clathrin-dependent endocytosis (29) was included to investigate a potential contribution to the uptake via this pathway. Expectedly, penetratin showed a punctate uptake pattern with some co-localization with dextran, and no or poor co-localization with transferrin indicating that its uptake is not receptor-mediated. This confirms earlier reports supporting a non-receptor mediated endocytosis of penetratin (30). Although reports have indicated that direct translocation (31) or clathrin-dependent endocytosis are responsible for penetratin uptake (32), this was not evident under the conditions applied in the present study. It has also been suggested that penetratin uptake most likely involves a combination of direct translocation and endocytotic pathways, while the dominant mechanism seems to be highly dependent on the specific experimental conditions (33). Similarly, the exact subcellular

Fig. 3 Release of calcein from POPG:CL liposomes modelling the membrane composition of Gram-positive bacteria upon incubation with the antimicrobial peptide, SA-3, and the cell-penetrating peptide, penetratin. **Top:** Time-resolved release of calcein starting within 10 s of peptide addition is depicted yellow: 0.1 μM ; purple: 0.2 μM ; green: 0.4 μM ; red: 0.8 μM and blue: 1.6 μM . **Bottom:** The relative release after 680 s versus the total calcein entrapped. Each point represents the mean \pm SD ($n=3$).



localization of several intracellular bacteria is not well documented; while initially internalized by phagocytosis their final location may vary; for example they may hide in membrane-bound compartments such as vacuoles or escape into the cytosol (34).

To determine the influence of the peptides on immediate cell toxicity (8), various cell lines were exposed to the peptides, and their viability determined. Penetratin showed no impact on cell viability within the tested concentration range in accordance with our earlier findings (14). A general trend was that HeLa WT cells were more sensitive towards the tested compounds as compared to the HUVEC and NIK 3T3 cells. This is in accordance with earlier observations on the membrane toxicity of short cationic AMP mimics towards various cell lines (23).

To test the effect of the peptides on the integrity of different model membranes the corresponding liposomes containing a self-quenching solution of calcein were prepared and exposed to the AMPs and CPP. Penetratin showed negligible calcein release from the model liposomes mimicking eukaryotes and *E. coli* (8% and 13% release, respectively), whereas complete disruption of the liposomes mimicking *S. aureus* membranes indicated selectivity toward this membrane model composition. Interestingly, in the bacterial inhibition assay, penetratin showed a stronger inhibition of *E. coli* than of *S. aureus*, indicating a mode of action against *E. coli* other than simple membrane disruption. The MIC values were within the range of earlier findings for penetratin (14,35–37).

Surprisingly, the amount of PK-12-KKP taken up by HeLa WT cells was significantly less than that seen for

penetratin even though its design is based on a CPP template. Apparently, the modifications of the CPP aimed at conferring antimicrobial properties to the analogue concomitantly reduced its capability for eukaryotic internalization. Visualization of cell uptake by confocal microscopy showed that despite relatively low internalization of PK-12-KKP, some intracellular presence was clearly detectable. The peptide exhibited a punctate uptake pattern that showed some co-localization with internalized dextran, indicating uptake by endocytosis and trafficking of the peptide to the late stages of the endocytic pathway. No co-localization with transferrin was observed, suggesting that uptake is not mediated via clathrin-coated endocytosis. Exposure of mammalian cells to PK-12-KKP only gave rise to a minor negative effect on their viability indicating low affinity for eukaryotic membranes in accordance with its low propensity for internalization. Interestingly, induction of dye leakage from the calcein-containing liposomes by PK-12-KKP only differed slightly between the three liposomal compositions implying a lack of bacteria-specific membrane disruption. Despite this fact, the potency of PK-12-KKP was modest (MIC of 32 μM and 16–32 μM against *S. aureus* and *E. coli*, respectively) deviating significantly from an earlier investigation of its antimicrobial activity with a MIC of 2 μM against both *S. aureus* (KCTC 1621) and *E. coli* (KCTC 1682) tested in a low-nutrition 1% (*w/v*) peptone solution (15), which has been shown to drastically diminish bacterial survivability (38). However, as the present MIC determinations were performed in MHB according to CLSI recommendations (19), this may explain the observed discrepancies. Overall, the low uptake of PK-12-

KKP into eukaryotic cells combined with a modest antibacterial activity does not favor a potential application in targeting intracellular bacteria.

SA-3 is a synthetic designed AMP that showed the highest cellular uptake of the three investigated AMPs, most likely due to its high content of both cationic and hydrophobic residues; this feature is known to facilitate CPP uptake (39). The intracellular distribution of SA-3 was punctate, and thus similar to that seen for penetratin and PK-12-KKP. Also for this AMP, co-localization with markers of the endocytotic pathway indicated an uptake mechanism distinct from that utilized by transferrin. A small increase in peptide concentration would likely have revealed DRAQ7® positive cells as the concentrations used for microscopy were close to that showing toxicity of SA-3 in the MTS/PMS assay. Thus, SA-3 was the most toxic peptide against eukaryotic cells, however, the antimicrobial potency of this peptide are in the same range as that of PK-12-KKP (*E. coli* 16–32 μM ; *S. aureus* 32 μM), which is significantly lower than reported in literature (i.e. MICs of 1.2 μM and 0.6 μM for *S. aureus* and *E. coli*, respectively), however, different strains were tested (16). When interacting with calcein-containing liposomes SA-3 completely disrupted those mimicking *E. coli* membranes, while the liposomes mimicking *S. aureus* membranes released ~80% of the encapsulated calcein. By contrast, SA-3 induced only ~33% disruption of the liposomes mimicking eukaryotic membranes. Thus, despite a relatively high release, this indicates that SA-3 displays some degree of selectivity towards bacterial membrane compositions. Comparison of the calcein release kinetics of SA-3 interacting with liposomes mimicking *S. aureus* with that of penetratin revealed an immediate onset of membrane disruption, with a critical concentration of SA-3 of ~0.1 μM , in contrast to the slower release triggered by penetratin. While SA-3 shows capacity for eukaryotic internalization its significant effect on cellular viability is problematic in relation to a potential use against internalized bacteria. Nevertheless, the indication of secondary structure and its apparent selectivity towards bacterial membranes suggest that SA-3 may constitute a useful design template allowing for optimization of cellular uptake over cytotoxicity.

The peptidomimetic tritriptin analog TPk exhibited ~50% uptake efficiency compared to penetratin and slightly lower than that of SA-3. The relatively high uptake of TPk is likely a result of its high content of cationic and non-polar amino acids. The intracellular localization of this peptidomimetic was marginally more pervasive than seen for the other peptides. However, there was still a clear punctate intracellular distribution, but with significantly less co-localization with dextran as compared to that observed for the other peptides. This could indicate that TPk is trafficked to a different subcellular localization or that there is loss of peptide into the cytosol via endosomal escape. The antimicrobial activity of TPk was up to 8-fold higher against *E. coli* and up

to 4-fold higher against *S. aureus* than that of the other tested AMPs. However, these MIC values are higher than reported in the literature by Zhu and coworkers (17), who determined inhibitory concentrations of 2 μM for *E. coli* (KCTC 1682) and 1 μM for *S. aureus* (KCTC 1261) in a 1% (*w/v*) peptone solution. Surprisingly, TPk only gave rise to a minor calcein leakage when tested at 1.6 μM on the membrane model of *S. aureus*, releasing 32.5% of the encapsulated calcein, which is less than the release induced by both SA-3 ($P < 0.001$) and penetratin ($P < 0.001$), and only slightly higher than that of PK-12-KKP ($P < 0.001$). From the liposomes mimicking *E. coli* membranes only 2.7% calcein were released, and the peptide was thus practically inactive towards this membrane model, while the *in vitro* antibacterial activity was pronounced. This confirms that the antibacterial effects do not rely exclusively on membrane disruption involving phospholipids. The interaction of TPk with the eukaryotic membrane model gave results comparable to those of the other AMPs. Moreover, TPk had a less negative effect on the viability of the three cell lines as compared to SA-3, but overall it was more membrane-disrupting than PK-12-KKP and penetratin. Zhu and coworkers reported an EC_{50} value $> 100 \mu\text{M}$ for TPk when assayed for viability of both HeLa and NIH 3T3 cells as determined by the MTT assay (17), which is in agreement with our findings for NIH 3T3 cells, but somewhat higher than the 41 μM towards HeLa cells found in the present study. The intermediate uptake efficiency and notable effect on viability are less desirable for this peptide as a lead candidate aiming at targeting intracellular infections, but a high antimicrobial effect and enhanced selectivity increases its potential value as a template for further optimization.

CONCLUSION

In summary, the present study reports on the applicability of three different AMPs for targeting intracellular infections. Thus, the peptides were ranked against the well-known CPP penetratin with respect to cellular uptake and distribution. Also, their effect on eukaryotic and prokaryotic cell viability and leakage-inducing effect on liposomal membranes was compared. It was shown that the uptake mechanism for all tested peptides is very unlikely to involve clathrin-mediated endocytosis, and that they all aside from TPk are trafficked to lysosomes. Furthermore, TPk showed a stronger inhibitory effect on both Gram-positive and Gram-negative bacteria as compared to the other tested AMPs. One of the peptides, SA-3 reported to have an intracellular target, exhibited potent disrupting effects on bacteria-mimicking membranes of both Gram-positive and Gram-negative bacteria. PK-12-KKP was found to be well tolerated in eukaryotic cell models and with good antimicrobial properties, however, with weak eukaryotic

internalization ability. Based on these investigations, TPK appears to be the best candidate for further investigations as an AMP capable of targeting intracellular infections as it may potentially be optimized for cell-penetration yielding an effective tool for eradication of bacteria residing inside epithelial cells.

ACKNOWLEDGMENTS AND DISCLOSURES

The authors would like to thank Karina Juul Vissing, Maria Læssøe Pedersen, Thara Qais Hussein, Fabrice Rose and Uraivan Adamsen for excellent technical assistance, Kenneth Johansen for performing HRMS (all from University of Copenhagen) and Statens Serum Institut, Copenhagen, Denmark for providing bacterial strains for testing. We acknowledge Brodrene Hartmanns Fond, Danish Agency for Science Technology and Innovation (DanCARD, grant no. 06–097075), The Alfred Benzon Foundation and Drug Research Academy for financial support.

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