

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository:<https://orca.cardiff.ac.uk/id/eprint/67643/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Bahnsen, Jesper Soborg, Franzyk, Henrik, Sayers, Edward John and Jones, Arwyn Tomos 2015. Cell-penetrating antimicrobial peptides - perspectives for targeting intracellular infections. *Pharmaceutical Research* 32 (5) , pp. 1546-1556.
10.1007/s11095-014-1550-9

Publishers page: <https://doi.org/10.1007/s11095-014-1550-9>

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies. See <http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.



Cell-penetrating antimicrobial peptides – prospectives for targeting intracellular infections

Jesper S. Bahnsen^a, Henrik Franzyk^b, Edward J. Sayers^c, Arwyn T. Jones^c and Hanne M. Nielsen^a.

^aDepartment of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark.

^bDepartment of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark

^cCardiff School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, Wales, CF10 3NB, United Kingdom

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 effect of the peptides on eukaryotic cell viability as well as their antimicrobial effect were tested. In addition, the secondary structure and 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 anti-bacterial activity. Only penetratin displayed clear secondary structure in the presence of membrane-mimicking liposomes. SA-3 exhibited selective disruption of liposomes mimicking Gram-positive and Gram-negative membranes.

1
2
3
4
5 **Conclusion.** PK-12-KKP is an unlikely candidate for targeting intracellular bacteria, as the eukaryotic cell-
6
7 penetrating ability is poor. SA-3, affected the cellular viability to an unacceptable degree. TPk showed
8
9 acceptable uptake efficiency, high antimicrobial activity and relatively low toxicity, and it is the best potential
10
11 lead peptide for further development.
12
13

14
15 **Key words:** antimicrobial peptides; cell-penetrating peptides; antibiotics; *Staphylococcus aureus*; peptide-
16
17 membrane interaction.
18
19

20 **INTRODUCTION**

21
22
23 Cell-penetrating peptides (CPPs) are short, often cationic peptides capable of internalizing into eukaryotic cells
24
25 without damaging the plasma membrane at effective internalization concentrations, and therefore they are
26
27 actively pursued as potential drug delivery vehicles [1]. Cationic antimicrobial peptides (AMPs) comprise a class
28
29 of peptides structurally similar to cationic CPPs, and AMPs constitute an essential part of the innate immune
30
31 system in almost all living organisms [2]. Most known AMPs exert their antibacterial effect via selective
32
33 membrane disruption thereby killing the bacteria, but also intracellular targets have been identified for some
34
35 AMPs (e.g. buforin II [3], drosocin [4] and apidaecin [5]). The two peptide classes, cationic CPPs and cationic
36
37 AMPs, share features such as: (i) a relatively short chain length (10–40 residues), (ii) a positive net charge,
38
39 important for initial interaction with phospholipid membranes possessing an overall negative charge, and (iii) an
40
41 amino acid composition with a significant content of non-polar residues considered to promote their interaction
42
43 with, and in the case of AMPs, insertion into the hydrophobic core of the membrane bilayer [6]. The dominant
44
45 biological effect of such highly cationic peptides may be altered from cell-penetrating to antimicrobial or vice
46
47 versa by even small modifications of their sequence. Thus, examples of successful development of both AMP-
48
49 based CPPs (e.g. SynB1 [7] and Bac7 (15-24) [8]) and CPP-based AMPs (e.g. Pep-1-K) [9] have been reported.
50
51 The increasing interest in the dual effects of these types of peptides has uncovered several examples of peptides
52
53 exhibiting both eukaryotic cell membrane penetration and antimicrobial effects [10].
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5 During the last decade, a massive increase in community-associated and nosocomial multidrug-resistant
6 *Staphylococcus aureus* infections has been registered, now reaching an alarming level; combined with a high
7 rate of recurrent infections this poses a serious healthcare problem [11]. Increasing evidence infers that the high
8 incidence of recurrent infections is caused by bacteria internalizing into epithelial cells at the site of infection
9 [12]. Thus, complete eradication is not obtainable through treatment with commonly applied antibiotics [13]. We
10 propose that development of a suitable cell-penetrating antimicrobial peptide could provide an alternative
11 treatment regime against such persistent intracellular infections either in itself or as part of a combination
12 therapy. In a previous study [14], we found that the well-characterized CPP, penetratin and a number of its
13 analogs in fact possess antimicrobial properties. In the present study, we investigated whether bacteria-
14 penetrating AMPs could be applied as CPPs.

15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
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 internal target as
the primary mode of action for SA-3 [16]. In addition, Zhu and co-workers reported that TPk, a peptidomimetic

1
2
3
4
5 analog of tritrypticin, displayed high activity against Gram-positive and Gram-negative bacteria as well as low
6 cytotoxicity against eukaryotic cells [17]. The design of this analogue involved replacement of two proline
7 residues with peptoid lysine residues (NLys), thereby increasing the overall molecular charge, while preserving
8 the lack of amide protons required for hydrogen bonding to the backbone. Additionally, TPk depolarized the
9 membrane potential of *S. aureus* to a low degree and only caused weak leakage of a fluorescent dye entrapped
10 within negatively charged vesicles modeling the bacterial membrane. Confocal laser-scanning microscopy of
11 bacteria exposed to FITC-labeled TPk showed clear accumulation of fluorescence in the cytoplasm, whereas the
12 parent tritrypticin remained outside or associated to the cell membrane. These findings suggest that the mode of
13 action for TPk involves inhibition of intracellular components after crossing the bacterial cell membrane.
14
15
16
17
18
19
20
21
22
23
24
25

26 The common feature of the three AMPs selected for this study is that investigations of their bacterial killing
27 mechanism have inferred that they act via intracellular targets. By applying advanced artificial membrane
28 systems to study these peptides, we expect to gain a more thorough understanding of their effects on the
29 membranes of both bacteria and eukaryotic cells.
30
31
32
33
34
35
36
37
38

39 **MATERIALS AND METHODS:**

40 **Materials**

41
42 Reagents, resin and amino acid building blocks for solid-phase peptide synthesis were purchased from Iris
43 Biotech (Merkredwitz, Germany) while DMF was acquired from VWR (Herlev, Denmark). Chemicals utilized
44 were : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (AppliChem, Darmstadt, Germany), Hanks
45 Balanced Salt Solution (HBSS) (Gibco, Paisley, UK), 3-(4,5-dimethylthiazol-2-yl)- 5-(3-carboxy-phenyl)-2-(4-
46 sulphophenyl)-2H-tetrazolium (MTS) (Promega, Madison, WI, USA), phenazine methosulphate (PMS), sodium
47 dodecyl sulfate (SDS), Eagle's minimal essential medium (EMEM), Triton X-100, calcein,
48 diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBt), dimethylformamide (DMF) (all Sigma-
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5 Aldrich, Buchs, Switzerland), 5(6)-carboxytetramethylrhodamine (TAMRA) (Fluka, Buchs, Switzerland),
6
7 DRAQ7® (Biostatus, Leicestershire, UK), Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Paisley,
8
9 UK) and phosphate buffered saline (PBS) (Invitrogen, Paisley, UK) Sephadex G-100 (GE Healthcare, Little
10
11 Chalfont, UK), 2,2,2-trifluoroethanol (TFE) (Acros organics, Geel, Belgium) Mueller Hinton Broth (MHB) (BD,
12
13 Le Pont de Claix, France). The lipids: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), cholesterol,
14
15 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (POPG), 1',3'-bis[1,2-dimyristoyl-*sn*-glycero-3-
16
17 phospho]-*sn*-glycerol (Cardiolipin 14:0; CL), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were
18
19 obtained from Avanti Lipids, Alabaster AL, USA.
20
21
22
23

24 **Methods**

27 *Solid phase peptide synthesis and labeling*

28
29
30 Peptide synthesis was performed by Fmoc-based solid-phase peptide synthesis (SPPS) employing a microwave-
31
32 assisted automated CEM Liberty synthesizer (CEM, Matthews, NC, USA); peptides were purified to >95% and
33
34 90% for TAMRA-labeled peptides as described earlier [14]. For the fluorophore labeling, TAMRA (2.5
35
36 equivalents) was used in a mixture of DIC (2.5 equivalents), and (HOBt; 2.5 equivalents) in (DMF) overnight at
37
38 room temperature. The identity of the peptides was confirmed by HRMS spectra obtained by using a Bruker
39
40 micrOTOF-Q II Quadropol MS detector; analyses were performed as ESI-MS (m/z): [M + nH]ⁿ⁺ (Bruker,
41
42 Bremen, Germany). An overview of the synthesis products can be found in Table I.
43
44
45

47 *Cell culture*

48
49
50 HeLa WT cells from the American Type Culture Collection (ATCC, Manassas, VA, USA) were maintained in
51
52 EMEM supplemented with 10,000 IU/mL penicillin, 10 mg/mL streptomycin, 2 mM L-glutamine, 0.1 mM non-
53
54 essential amino acids (all from Sigma-Aldrich, St. Louis, MO, USA), 1 mM sodium pyruvate (Invitrogen,
55
56 Carlsbad, CA, USA), and 10% (v/v) fetal bovine serum (FBS) (Fisher Scientific, Waltham, MA, USA). Cells
57
58 were kept under tissue culture conditions (5% CO₂, 37°C) and detached from culturing flasks at 80% confluency
59
60
61
62
63
64
65

1
2
3
4
5 by treatment with trypsin-EDTA, and subsequently subcultured once weekly. For the MTS/PMS assay HeLa WT
6
7 cells were seeded at a density of $\sim 30,000$ cells/cm² in flat-bottomed 96-well Plates (Corning Costar, Sigma-
8
9 Aldrich, Brøndby, Denmark). NIH 3T3 cells for MTS/PMS assay were acquired from ATCC (Manassas, VA,
10
11 USA), maintained in DMEM supplemented with 10% (v/v) newborn calf serum (NCS) (Gibco, Paisly, U.K.),
12
13 10,000 IU/mL penicillin, 10 mg/mL streptomycin and 2 mM L-glutamine and seeded in a flat-bottomed 96-well
14
15 Plates (Corning Costar, Sigma-Aldrich, Brøndby, Denmark) at a density of $\sim 69,000$ cells/cm². HUVEC cells
16
17 were acquired from the European Collection of Cell Cultures (ECACC) (Salisbury, U.K.) and maintained in
18
19 Ham's F-12K (Kaighn's) medium supplemented with 10% (v/v) FCS, 0.1 mg/mL heparin, 0.03 mg/mL
20
21 endothelial cell growth supplement as well as 10.000 IU/mL penicillin, 10 mg/mL streptomycin and 2 mM L-
22
23 glutamine and seeded in a flat-bottomed 96-well Plates (Corning Costar, Sigma-Aldrich, Brøndby, Denmark) at
24
25 a density of $\sim 47,000$ cells/cm². HeLa WT cells for confocal microscopy visualization were cultured on (poly-D-
26
27 lysine)-coated, 35 mm imaging dishes (MatTek Glass Bottom Microwell Dishes, MatTek, Ashland, MA, USA)
28
29 at a density of $\sim 140,000$ cells/cm² and for flow cytometry cells were seeded at a density of $\sim 110,000$ cells/cm² in
30
31 12-well plates (NUNC, Roskilde, Denmark). For all experiments, the cells were cultured for 22-24 h to obtain a
32
33 subconfluent cell monolayer (confluency 80-90%).
34
35
36
37
38
39

40 *MTS/PMS viability assay*

41
42
43 The cells were washed twice with 37°C HBSS supplemented with 10 mM HEPES (hHBSS) and exposed to 100
44
45 μ L of peptide solution in hHBSS (concentration range 1–256 μ M) for 1 h under tissue culture conditions. A
46
47 0.2% (w/v) SDS solution and hHBSS were used as positive (OD_{pos.ctrl}) and negative (OD_{neg.ctrl}) control,
48
49 respectively. The peptide solution was removed by inverting and gently tapping the back of the plate.
50
51 Subsequently, a mixture of 240 μ g/mL MTS and 2.4 μ g/mL PMS in test buffer was applied. The plate was
52
53 incubated shielded from light (37°C, 50 rpm) for approx. 2 h until the cells exposed to buffer (the negative
54
55 control) reached an absorbance of ~ 1 . Absorbance was measured on a POLARstar Optima ($\lambda = 492$ nm) (GMB
56
57 Labtech, Offenburg, Germany). The relative cell viability (%) was calculated according to Eq. (1):
58
59
60
61
62
63
64
65

1
2
3
4
5 (1) Viability (%) = $(OD_{\text{peptide}} - OD_{\text{pos.ctrl.}}) / (OD_{\text{neg.ctrl.}} - OD_{\text{pos.ctrl.}})$
6
7
8
9

10
11 *Confocal laser-scanning microscopy (CLSM)*
12

13
14 The cells were rinsed with DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 IU/mL
15 penicillin and 100 µg/mL streptomycin (37°C). The medium was replaced with 200 µL of 10µM TAMRA-
16 labeled peptide in PBS and incubated for 1 h under tissue culture conditions. Subsequently, the test solutions
17 were discarded, the cells washed twice with DMEM, once with imaging medium (DMEM without phenol red),
18 and finally resuspended in 300 µL of imaging medium containing 0.3% (v/v) DRAQ7®. Immediately hereafter,
19 CLSM was performed using an inverted Leica SP5 confocal laser-scanning microscope (Leica Mikrosystems,
20 Wetzlar, Germany) equipped with an Ar and HeNe laser and a 63× 1.4 NA oil immersion objective. Live-cell
21 microscopy was performed at room temperature and with a fixed instrument configuration across all samples.
22 Furthermore, endocytosis pathway markers: dextran and transferrin were applied: In lysosome labeling
23 experiments the cells were incubated with 0.1 mg/mL 10 kDa dextran Alexa Fluor® 647 (Life Technologies,
24 Basel, Switzerland) for 210 min, and the label subsequently chased into lysosomes via a further incubation in
25 dextran-free medium for 20 h, after which the cells were incubated with peptide as described above. To
26 investigate uptake by clathrin-mediated endocytosis, the cells were incubated for 50 min with 100 nM transferrin
27 Alexa Fluor® 647 (Life Technologies, Basel, Switzerland), rinsed with Complete DMEM. PBS 100 nM
28 transferrin Alexa Fluor® 647 was supplemented with TAMRA- peptides (200 µL) at various concentrations and
29 incubated under tissue culture conditions for 10 min before proceeding with the washing and imaging procedure
30 stated above.
31
32

33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53 *Flow cytometry*
54

55
56 Cellular internalization of TAMRA-labeled peptides was quantified by flow cytometry. Initially, the cells were
57 washed once with 1 mL PBS (37°C), containing 10 µM peptide solution (700 µL in PBS) and incubated for 1 h
58
59
60
61
62
63
64
65

1
2
3
4
5 under tissue culture conditions. The cells were subsequently rinsed three times with ice-cold PBS. Detachment
6
7 of the cells from the wells was done by addition of 0.01 % (w/v) trypsin-EDTA (200 μ L, 10 min, 37°C, 5%
8
9 CO₂), and after centrifugation (5 min, 1076 \times g) the cells were washed twice with ice-cold PBS containing 10%
10
11 (v/v) FBS. Lastly, the cells were resuspended in 500 μ L PBS with 10% (v/v) FBS containing 0.3% (v/v)
12
13 DRAQ7®. Flow cytometry analysis was performed shortly after the preparation by using a Gallios flow
14
15 cytometer (Beckman Coulter, Fullerton, CA, USA). The fluorescence mean of 10,000 viable cells was recorded
16
17 gating for forward and sideways scattering as well as for DRAQ7® fluorescence intensity.
18
19
20
21

22 *Minimal inhibitory concentration (MIC)*

23
24

25 The inhibitory effect of peptides on bacterial growth was determined by micro dilution broth assay according to
26
27 Clinical Laboratory Standard Institute (CLSI) guidelines [19]. The peptides were assayed against *S. aureus*
28
29 (ATCC 25923) and *E. coli* (ATCC 25922) in the dilution range 1-128 μ M in MHB (experiments performed in
30
31 triplicate on at least two separate days). The bacteria were inoculated to 5×10^5 CFU/mL and incubated at 35°C
32
33 in ambient air for ~18 h, after which the MIC values were determined as the lowest concentration showing no
34
35 visible growth as compared to the control without antibacterial compound added.
36
37
38

39 *Liposome preparation*

40
41

42 Liposomes were prepared by using a procedure previously described [14]. In brief, large unilamellar vesicles
43
44 (LUV) were prepared from POPC and cholesterol in a molar ratio of 10:1 in order to mimic an eukaryotic cell
45
46 membrane composition [20–22], while a liposome composition with POPG and synthetic CL in a molar ratio of
47
48 3:1 was used to mimic Gram-positive *S. aureus* membrane composition [20–22]. POPG, CL, and DOPE in a
49
50 molar ratio of 2:7:1 were employed as a model for Gram-negative *Escherichia coli* membranes [20–22]. For
51
52 liposomes, the lipid film was dispersed in Tris buffer (10 mM, pH 7.4), whereas calcein-loaded liposomes were
53
54 formed in Tris buffer (10 mM, pH 7.4) containing 150 mM NaCl, 0.1 mM EDTA and 70 mM calcein. Excess
55
56 calcein was removed by passing the liposomes through a Sephadex G-50 column.
57
58
59
60
61
62
63
64
65

1
2
3
4
5 *Circular dichroism (CD) spectroscopy*
6
7

8 CD spectra were measured as previously described [14]. In brief, peptide and liposomes were added to a quartz
9 cell with 1 mm light path, and 5 consecutive scans were collected in the range 190–260 nm. The scans were
10 averaged and the background contribution from buffer or liposome-containing buffer was subtracted. An Olis
11 DSM 10 spectrophotometer (Olis, Bogart, GA, USA) was used for all measurements except the measurements
12 with the POPG:CL:DOPE (2:7:1 molar ratio) which was performed on a J-815 CD spectrometer (Jasco, Easton,
13 MD, USA).
14
15
16
17
18
19
20
21

22 *Calcein release assay*
23
24

25 To measure leakage from calcein-loaded liposomes, these were added to black 96-well plates (MicroWell 96
26 optical bottom plates, NUNC, Roskilde, Denmark) at a concentration of 1.2 μM lipid determined from the
27 strongest fluorescent signal from the lysed calcein-containing vesicles in a volume of 180 μL . The fluorescence
28 was monitored by utilizing a FLUOstar OPTIMA plate reader (GMB Labtech, Offenburg, Germany) at an
29 excitation wavelength of 485 nm and an emission wavelength of 520 nm to determine F_0 . A volume of 20 μL of
30 peptide solution was added and measurement of calcein leakage was initiated within 10 sec from the first
31 addition of peptide, and the peptide-induced calcein leakage was recorded for a period of 680 sec (340 sec for
32 PK-12-KKP with POPG/CL liposomes) in order to determine F . Subsequently, the liposomes were disrupted
33 completely by addition of 20 μL 20% (v/v) Triton X-100, and then the signal from the total amount of calcein
34 was recorded (F_t). The relative release was calculated according to Eq. (2):
35
36
37
38
39
40
41
42
43
44
45
46
47
48

49 (2) Calcein leakage [%] = $[(F-F_0)/(F_t-F_0)] \cdot 100\%$
50
51

52 *Data analysis*
53
54

55 Results are presented as the mean of three measurements \pm SD, and unless otherwise stated experiments were
56 performed in triplicate. Statistical significance was determined by the students *t*-test where $P < 0.05$ is considered
57
58
59
60
61
62
63
64
65

1
2
3
4
5 significant. For the viability assays the 50% cellular viability (EC_{50}) was estimated by using Prism 6 for Mac OS
6
7 X vs. 6.0 and by non-linear regression according to Eq. (3):
8
9

10 (3) $Y = \text{Bottom} + (\text{Top}-\text{Bottom}) / (1 + (X/EC_{50})^{-\text{HillSlope}})$.
11
12
13
14
15
16

17 **RESULTS**

18
19

20 In the present study, three AMPs (PK-12-KKP, SA-3 and TPk) and a CPP (penetratin) were investigated with
21 respect to their: (i) ability to internalize into eukaryotic cells, (ii) subcellular localization inside eukaryotic cells,
22 (iii) ability to inhibit bacterial growth, (iv) influence on eukaryotic cell viability, and (v) interaction with
23 different membrane models.
24
25
26
27

28 *Uptake of short cationic AMPs and CPP in HeLa WT cells*

29
30
31
32

33 To determine whether the three AMPs were capable of internalizing into eukaryotic cells, TAMRA-labeled
34 variants of the peptides were used. The uptake efficiency of the three AMPs was compared to that of the
35 similarly labeled well characterized CPP, penetratin. As expected, penetratin displayed a higher total cellular
36 uptake as compared to the AMPs as determined by flow cytometry (Figure 1). The Pep-1 derived analog PK-12-
37 KKP displayed a significantly lower uptake (~10% relative to penetratin) than the other compounds tested
38 ($P < 0.01$). SA-3 showed the highest uptake as compared to the other tested AMPs reaching a level of ~65% of the
39 uptake of penetratin, which was significantly better than that of TPk ($P < 0.05$). TPk exhibited intermediate
40 uptake efficiency with about half the uptake found for penetratin.
41
42
43
44
45
46
47
48
49
50
51

52 *Intracellular distribution and co-localization with endocytosis pathway markers*

53
54

55 Microscopical analysis confirmed the flow cytometry data showing that the TAMRA-labeled variants of the
56 AMPs and penetratin gave rise to significant uptake resulting in subcellular punctate distribution patterns
57
58
59
60
61
62
63
64
65

1
2
3
4
5 throughout the cytoplasm of the HeLa WT cells without affecting the viability of the cells (data not shown) as
6 determined by addition of DRAQ7[®]; a DNA binding dye that selectively stains dead and permeabilized cells.
7
8 Peptide uptake was also imaged in cells that first were stained with fluorescently labeled pulse/chased 10 kDa
9 dextran that accumulates in lysosomes. As expected peptide incubations in lysosome-labelled cells for the four
10 peptides gave rise to punctate fluorescent structures (Figure 2), and in the case of PK12-KKP and penetratin
11 there was clear evidence of delivery to lysosomes manifested as co-localization with dextran fluorophore.
12
13 Whether clathrin-dependent endocytosis of the peptides occurred to a significant extent was studied by testing in
14 cells that were pre-incubated with fluorescent transferrin present in the media during these short peptide
15 incubations. This resulted in an overall low uptake of PK-12-KKP with no discernible co-localization with
16 transferrin suggesting that clathrin-mediated endocytosis is not utilized by the peptide. With respect to
17 transferrin there was minimal co-localization with SA-3 and TPk, however, these cells also showed diffuse
18 cytosolic staining. Penetratin localization was confined to punctate structures that did not overlap to any extent
19 with transferrin. For all four peptides DRAQ7[®] positive cells were not observed indicating that at this 10 μ M
20 concentration the plasma membrane was not compromised to a degree that resulted in leakage of the dye to label
21 nuclear DNA.
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39

40 *Cellular viability and antimicrobial effect*

41
42 To assess the relative effects of the peptides on eukaryotic cell viability and bacterial growth, the results of the
43 tetrazolium dye-based viability assay MTS/PMS and the determined minimal inhibitory concentration on *S.*
44 *aureus* were compared. To gain insight regarding a possible selectivity of the peptides towards specific
45 eukaryotic cells, the viability of HeLa WT cells as well as of fibroblast cells (NIH 3T3) and human umbilical
46 vein endothelial cells (HUVEC) in the presence of the three AMPs and penetratin was investigated (Table II).
47
48 No significant reduction in viability of all three cell lines upon exposure to PK-12-KKP was detected within the
49 concentration range tested (i.e. up to 256 μ M). By contrast, cell viability was severely lowered by SA-3 with
50 EC₅₀ values against HeLa WT and NIH 3T3 cell lines at concentrations 25-30 μ M. HUVEC cells were generally
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5 much less sensitive to the peptides, and SA-3 was the only peptide that affected these cells at the tested
6
7 concentrations. TPk reduced the viability of HeLa WT cells with an EC₅₀ value only slightly higher than that of
8
9 SA-3, however, its cytotoxicity against NIH 3T3 cells was at least four times lower than that of SA-3. Also, for
10
11 penetratin no significant decrease in cell viability of any of the cell lines was observed within the tested
12
13 concentration range. Table II shows that overall, the antimicrobial activity of the AMPs was more pronounced
14
15 towards the Gram-negative *E. coli* than against the Gram-positive *S. aureus*, and that TPk was the most effective
16
17 AMP.
18
19

20 21 22 *Membrane interactions*

23
24
25 The folding behavior of the four peptides was examined by circular dichroism (CD) spectroscopy in the presence
26
27 of liposomes at a peptide to lipid molar ratio of 1:100; a ratio that was previously used to successfully study CPP
28
29 secondary structure in the presence of unilamellar vesicles [23]. Different liposome compositions were employed
30
31 in order to investigate interactions with specific types of membranes. As a model for Gram-positive bacteria
32
33 membranes POPG:CL liposomes at a molar ratio of 3:1 (70.0 nm, PdI < 0.1) was utilized, while for Gram-
34
35 negative bacterial membranes POPG:CL:DOPE liposomes at a molar ratio of 2:7:1 (80.84 nm, PdI < 0.1), and
36
37 for eukaryotic cell membranes POPC:cholesterol liposomes at a molar ratio of 10:1 (75.9 nm, PdI = 0.1),
38
39 respectively, were employed.
40
41

42
43
44 The CD spectrum of the proline-rich PK-12-KKP (Figure 3a) displayed uncharacteristic spectra in the presence
45
46 of all liposomal model membranes and in 90:10 (v/v) TFE-water, and thus no specific secondary structure of
47
48 PK-12-KKP could be deduced. SA-3 appeared to possess some degree of α -helicity in 90% TFE with minima
49
50 around 208 nm and 222 nm. The minimum at 222 nm was also observed in the presence of both POPG:CL and
51
52 POPC:cholesterol liposomes, although it was more distinct for the latter. In the presence of both of these
53
54 liposomal membrane variants, the 208 nm minimum flattens out (Figure 3b). TPk showed almost no variation in
55
56 the secondary structure upon interaction with different liposomal model membranes as well as in 90% TFE. Due
57
58
59
60
61
62
63
64
65

1
2
3
4
5 to the small changes in the CD spectra, it was not possible to assign any change in specific secondary structure
6
7 by analysis of the spectra obtained for TPk (Figure 3c). Penetratin exhibited a highly variable secondary
8
9 structure depending on the different model membranes (Figure 3d). The CD spectrum of penetratin recorded in
10
11 90% TFE corresponds to a classical α -helix conformation with minima at 208 nm and 222 nm along with a
12
13 positive ellipticity in the range below 200 nm. In the presence of POPG:CL liposomes, penetratin seems to adopt
14
15 an antiparallel β -sheet-like secondary structure with a minimum just below 220 nm and a maximum around 200
16
17 nm, while in the presence of the POPG:CL:DOPE liposomes no unambiguous secondary structure was observed.
18
19 However, in the presence of POPC:cholesterol liposomes penetratin remained unstructured.
20
21
22
23
24
25
26

27 Calcein-containing liposomes of the same compositions as described above were exposed to varying peptide
28
29 concentrations and the change in fluorescence over time was measured in order to assess both the degree and the
30
31 kinetics of the membrane disruption induced by the peptides. The liposomes prepared with calcein displayed
32
33 monomodal size distributions, yet overall they displayed a slightly smaller size than the ones without calcein;
34
35 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)
36
37 and POPC:cholesterol (molar ratio 10:1, 39.1 nm, PdI < 0.1). When comparing the specific model membranes,
38
39 clear differences were found regarding the maximal amount of calcein released upon exposure to the three
40
41 peptides for up to 680 sec (Table III). Considering the individual peptides, PK-12-PPK gave rise to a low partial
42
43 calcein leakage of 13-25 % for all three liposomal compositions. Overall, SA-3 resulted in a higher relative
44
45 leakage, but with a higher degree of selectivity towards the liposomes modeling the bacterial membranes. Thus,
46
47 SA-3 induced a high relative leakage (82% to completion) from the bacteria-mimicking liposomes, whereas the
48
49 eukaryotic model liposomes only released 33% of the encapsulated calcein at the same peptide concentration.
50
51 TPk had only a minimal effect on the POPG:CL:DOPE (2:7:1) liposomes mimicking the *E. coli* membrane as
52
53 only ~3% of the calcein was released. In contrast, TPk disrupted both POPG:CL (3:1) liposomes mimicking *S.*
54
55 *aureus* membranes and liposomes corresponding to a mammalian membrane composition releasing more than
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5 30% of the encapsulated calcein. Penetratin exhibited a selective disruption of the *S. aureus* model liposomes
6
7 with low disruptive capacity for the other two liposome compositions.
8
9

10 More detailed insight into the effects of SA-3 and penetratin on the different model membranes was gained by
11 depicting comparative time-resolved and concentration-dependent profiles (Figure 4). The release of calcein
12 from POPG:CL liposomes triggered by SA-3 is immediate, as the first measurement in the presence of 1.6 μM
13 peptide showed ~20% calcein release (Figure 4). For penetratin, a slow initial calcein release was observed with
14 a lag period of 40 sec, however, after 10 min full liposomal disruption was observed, while SA-3 reached a total
15 release of 82%. Inspection of the concentration-dependent release of calcein from the POPG:CL model
16 membranes revealed that these vesicles are more sensitive to penetratin than to SA-3 over the entire
17 concentration range, even though the initial release rate is more rapid for SA-3.
18
19
20
21
22
23
24
25
26
27
28
29
30
31

32 **DISCUSSION**

33
34
35 The peptides investigated in this study have previously been tested for their antibacterial activity [15–17],
36 whereas the objective of the current study was to assess their potential to target bacteria internalized in
37 eukaryotic host cells. Quantification of the cellular uptake of the peptides allowed a ranking of these sequences
38 according to their internalization efficiency in comparison to that of penetratin, a well-characterized CPP that has
39 previously been shown to internalize efficiently into several eukaryotic cell lines [24,25] including HeLa WT
40 cells [26]. Further, the distribution pattern of the AMPs in HeLa WT cells was assayed by confocal microscopy
41 with and without fluorophore-labeled dextran as an endocytic probe that can be used to label the entire
42 endolysosomal pathway or specifically lysosomes [27], which under the applied experimental conditions
43 accumulate in the late vacuolar system, particularly in the lysosomes. Fluorophore-labeled transferrin known to
44 be taken up by clathrin-dependent endocytosis [28] was included to investigate a potential contribution to the
45 uptake via this pathway. Expectedly, penetratin showed a punctate uptake pattern with some co-localization with
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5 dextran, and no or poor co-localization with transferrin indicating that its uptake is not receptor-mediated. This
6
7 confirms earlier reports supporting a non-receptor mediated endocytosis of penetratin [29]. Although reports
8
9 have indicated that direct translocation [30] or clathrin-dependent endocytosis are responsible for penetratin
10
11 uptake [31], this was not evident under the conditions applied in the present study. It has also been debated that
12
13 penetratin uptake most likely involves a combination of direct translocation and endocytotic pathways, while the
14
15 dominant mechanism seems to be highly dependent on the specific experimental conditions [32].
16
17
18

19
20 The secondary structure of the peptides in the presence of three different liposomal compositions was examined
21
22 by CD spectroscopy. Penetratin showed a classical α -helical conformation in TFE-water 90:10, but in the
23
24 presence of POPG:CL:DOPE (molar ratio 2:7:1) liposomes mimicking the membrane of *E. coli* it did not show
25
26 any clear secondary structure, however, its CD spectrum might represent a mix of partial α -helix and β -sheet
27
28 conformations. To determine the influence of the peptides on immediate cell toxicity [8], various cell lines were
29
30 exposed to the peptides, and their viability determined. Penetratin showed no impact on cell viability within the
31
32 tested concentration range in accordance with our earlier findings [14]. A general trend was that HeLa WT cells
33
34 were more sensitive towards the tested compounds as compared to the HUVEC and NIH 3T3 cells. This is in
35
36 accordance with earlier observations on the membrane toxicity of short cationic AMP mimics towards various
37
38 cell lines [33].
39
40
41

42
43 The propensity of penetratin to adopt an α -helical conformation while interacting with membranes mimicking
44
45 eukaryotic cells was very low in disagreement with earlier findings [14]. This confirms that the presence of
46
47 POPG is important for induction of secondary structure in penetratin corroborating earlier studies demonstrating
48
49 that unstructured penetratin conformations are predominant in the presence of purely PC-based lipid systems
50
51 [22]. To test the effect of the peptides on the integrity of different model membranes the corresponding
52
53 liposomes containing a self-quenching solution of calcein were prepared and exposed to the AMPs and CPP.
54
55 Penetratin showed negligible calcein release from the model liposomes mimicking eukaryotes and *E. coli* (8%
56
57 and 13% release, respectively), whereas complete disruption of the liposomes mimicking *S. aureus* membranes
58
59
60
61
62
63
64
65

1
2
3
4
5 indicated selectivity toward this membrane model composition. Interestingly, in the bacterial inhibition assay,
6 penetratin showed a stronger inhibition of *E. coli* than of *S. aureus*, indicating a mode of action against *E. coli*
7 other than simple membrane disruption. The MIC values were within the range of earlier findings for penetratin
8 [14,34–36].
9

10
11
12
13
14 Surprisingly, the amount of PK-12-KKP taken up by HeLa WT cells was significantly less than that seen for
15 penetratin even though its design is based on a CPP template. Apparently, the modifications of the CPP aimed at
16 conferring antimicrobial properties to the analogue concomitantly reduced its capability for eukaryotic
17 internalization. Visualization of cell uptake by confocal microscopy showed that despite relatively low
18 internalization of PK-12-KKP, some intracellular presence was clearly detectable. The peptide exhibited a
19 punctate uptake pattern that showed some co-localization with internalized dextran, indicating uptake by
20 endocytosis and trafficking of the peptide to the late stages of the endocytic pathway. No co-localization with
21 transferrin was observed, suggesting that uptake is not mediated via clathrin-coated endocytosis. To understand
22 the initial peptide-membrane interaction, PK-12-KKP was investigated by CD spectroscopy in the presence of
23 the three different model membranes, and the spectra of the peptide in all cases indicated a very high presence of
24 unstructured conformations confirming earlier studies done in SDS micelles [15]. Since secondary structure (e.g.
25 α -helical folding) often has been shown to be a prerequisite for efficient CPP uptake [37,38], the primarily
26 unstructured conformation of PK-12-KKP is a plausible cause of its low uptake. Exposure of mammalian cells to
27 PK-12-KKP only gave rise to a minor negative effect on their viability indicating low affinity for eukaryotic
28 membranes in accordance with its low propensity for internalization. Interestingly, induction of dye leakage
29 from the calcein-containing liposomes by PK-12-KKP only differed slightly between the three liposomal
30 compositions implying a lack of bacteria-specific membrane disruption. Despite this fact, the potency of PK-12-
31 KKP was modest (MIC of 32 μ M and 16-32 μ M against *S. aureus* and *E. coli*, respectively) deviating
32 significantly from an earlier investigation of its antimicrobial activity with a MIC of 2 μ M against both *S. aureus*
33 (KCTC 1621) and *E. coli* (KCTC 1682) tested in a low-nutrition 1% (w/v) peptone solution [15], which has been
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5 shown to drastically diminish bacterial survivability [39]. However, as the present MIC determinations were
6 performed in MHB according to CLSI recommendations [19], this may explain the observed discrepancies.
7
8 Overall, the low uptake of PK-12-KKP into eukaryotic cells combined with a modest antibacterial activity do not
9 favor a potential application in targeting intracellular bacteria.
10
11

12
13
14
15 SA-3 is a synthetic designed AMP that showed the highest cellular uptake of the three investigated AMPs, most
16 likely due to its high content of both cationic and hydrophobic residues; this feature is known to facilitate CPP
17 uptake [40]. Furthermore, the peptide displays a slight propensity for adopting an α -helical secondary structure
18 that also may promote cellular internalization. The intracellular distribution of SA-3 was punctate, and thus
19 similar to that seen for penetratin and PK-12-KKP. Also for this AMP, co-localization with markers of the
20 endocytotic pathway indicated an uptake mechanism distinct from that utilized by transferrin. A small increase
21 in peptide concentration would likely have revealed DRAQ7[®] positive cells as concentrations used for
22 microscopy were close to that showing toxicity using the MTS/PMS assay for this peptide. This was the most
23 toxic peptide against eukaryotic cells, however, the antimicrobial potency of this peptide are in the same range as
24 PK-12-KKP (*E. coli* 16-32 μ M; *S. aureus* 32 μ M), which is significantly lower than reported in literature (i.e.
25 MICs of 1.2 μ M and 0.6 μ M for *S. aureus* and *E. coli*, respectively), however, different strains were tested [16].
26
27 The effect of the two peptides, PK-12-KKP and SA-3, on the viability of NIH 3T3 cells and HeLa WT cells were
28 almost equal. When interacting with calcein-containing liposomes SA-3 completely disrupted those mimicking
29 *E. coli* membranes, while the liposomes mimicking *S. aureus* membranes released ~80% of the encapsulated
30 calcein. By contrast, SA-3 induced only ~33% disruption of the liposomes mimicking eukaryotic membranes.
31
32 Thus, despite a relatively high release, this indicates that SA-3 displays some degree of selectivity towards
33 bacterial membrane compositions. Comparison of the calcein release kinetics of SA-3 interacting with liposomes
34 mimicking *S. aureus* with that of penetratin revealed an immediate onset of membrane disruption, with a critical
35 concentration of SA-3 of ~0.1 μ M, in contrast to the slower release triggered by penetratin. While SA-3 shows
36 capacity for eukaryotic internalization its significant effect on cellular viability is problematic in relation to a
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5 potential use against internalized bacteria. Nevertheless, the indication of secondary structure and its apparent
6
7 selectivity towards bacterial membranes suggest that SA-3 may constitute a useful design template allowing for
8
9 optimization of cellular uptake over cytotoxicity.
10

11
12 The peptidomimetic tritrypticin analog TPk exhibited ~50% uptake efficiency compared to penetratin and slightly
13
14 lower than that of SA-3. The relatively high uptake of TPk is likely a result of its high content of cationic and
15
16 non-polar amino acids. The intracellular localization of this peptidomimetic was marginally more pervasive than
17
18 seen for the other peptides. However, there was still a clear punctate intracellular distribution, but with
19
20 significantly less co-localization with dextran as compared to that observed for the other peptides. This could
21
22 indicate that TPk is trafficked to a different subcellular localization or that there is loss of peptide into the
23
24 cytosol via endosomal escape. No clear secondary structure was apparent from the CD spectra of TPk. The
25
26 antimicrobial activity of TPk was up to 4-fold higher than that of the other tested AMPs. However, these MIC
27
28 values are higher than what was reported in the literature, by Zhu and coworkers [17] who determined inhibitory
29
30 concentrations of 2 μM for *E. coli* (KCTC 1682) and 1 μM for *S. aureus* (KCTC 1261) in a 1% (w/v) peptone
31
32 solution. Surprisingly, TPk only gave rise to a minor calcein leakage when tested at 1.6 μM on the membrane
33
34 model of *S. aureus*, releasing 32.5% of the encapsulated calcein, which is less than the release induced by both
35
36 SA-3 ($P < 0.001$) and penetratin ($P < 0.001$), and only slightly higher than that of PK-12-PPK ($P < 0.001$). From the
37
38 liposomes mimicking *E. coli* membranes only 2.7% calcein were released, and the peptide was thus practically
39
40 inactive towards this membrane model, while the *in vitro* antibacterial activity was pronounced. This confirms
41
42 that the antibacterial effects do not rely exclusively on membrane disruption involving phospholipids. The
43
44 interaction of TPk with the eukaryotic membrane model gave results comparable to those of the other AMPs.
45
46 Moreover, TPk had a less negative effect on the viability of the three cell lines as compared to SA-3, but overall
47
48 it was more membrane-disrupting than PK-12-KKP and penetratin. Zhu and coworkers reported an EC_{50} value
49
50 $> 100 \mu\text{M}$ for TPk when assayed for viability of both HeLa and NIH 3T3 cells as determined by the MTT assay
51
52 [17], which is in agreement with our findings for NIH 3T3 cells, but somewhat higher than the 41 μM towards
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5 HeLa cells found in the present study. The intermediate uptake efficiency and notable effect on viability are less
6
7 desirable for this peptide as a lead candidate aiming at targeting intracellular infections, but a high antimicrobial
8
9 effect and enhanced selectivity increases its potential value as a template for further optimization.
10
11

12 13 14 15 16 **CONCLUSION**

17
18
19 In summary, the present study reports on the applicability of three different AMPs for targeting intracellular
20
21 infections. Thus, the peptides were ranked against the well-known CPP penetratin with respect to cellular uptake
22
23 and distribution. Also, their effect on eukaryotic and prokaryotic cell viability and leakage-inducing effect on
24
25 liposomal membranes was compared. It was shown that the uptake mechanism for all tested peptides is very
26
27 unlikely to involve clathrin-mediated endocytosis, and that they all aside from TPk are trafficked to lysosomes.
28
29 Furthermore, TPk showed a stronger inhibitory effect on both Gram-positive and Gram-negative bacteria as
30
31 compared to the other tested AMPs. One of the peptides, SA-3 reported to have an intracellular target, exhibited
32
33 potent disrupting effects on bacteria-mimicking membranes of both Gram-positive and Gram-negative bacteria.
34
35 PK-12-KKP was found to be well tolerated in eukaryotic cell models and with good antimicrobial properties,
36
37 however, with weak eukaryotic internalization ability. Based on these investigations, TPk appears to be the best
38
39 candidate for further investigations as an AMP capable of targeting intracellular infections as it may potentially
40
41 be optimized for cell-penetration yielding an effective tool for eradication of bacteria residing inside epithelial
42
43
44
45
46 cells.
47
48
49
50
51

52 **ACKNOWLEDGEMENTS**

53
54
55 The authors would like to thank Karina Juul Vissing, Maria Læssøe Pedersen, Thara Qais Hussein, Fabrice Rose
56
57 and Uraiwan Adamsen for excellent technical assistance, Associate Professor Peter W. Thulstrup for use of CD
58
59
60
61
62
63
64
65

1
2
3
4
5 spectrometer, Kenneth Johansen for performing HRMS (all from University of Copenhagen) and Statens Serum
6
7 Institut, CPH Denmark for providing bacterial strains for testing. We acknowledge Brødrene Hartmanns Fond,
8
9 Danish Agency for Science Technology and Innovation (DanCARD, grant no. 06-097075), The Alfred Benzon
10
11 Foundation and Drug Research Academy for financial support.
12
13
14
15
16
17

18 REFERENCES

- 21 1. Patel LN, Zaro JL, Shen W-C. Cell penetrating peptides: intracellular pathways and pharmaceutical
22 perspectives. *Pharm Res.* 2007;24(11):1977–1992.
23
24
- 25 2. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature.* 2002;415(6870):389–395.
26
27
- 28 3. Park CB, Kim HS, Kim SC. Mechanism of action of the antimicrobial peptide buforin II: buforin II kills
29 microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochem Biophys*
30 *Res Commun.* 1998;244(1):253–257.
31
32
- 33 4. Otvos L, O I, Rogers ME, Consolvo PJ, Condie BA, Lovas S, Bulet P, Blaszczyk-Thurin M. Interaction
34 between heat shock proteins and antimicrobial peptides. *Biochemistry.* 2000;39(46):14150–14159.
35
36
- 37 5. Castle M, Nazarian A, Yi SS, Tempst P. Lethal effects of apidaecin on *Escherichia coli* involve
38 sequential molecular interactions with diverse targets. *J Biol Chem.* 1999;274(46):32555–32564.
39
40
- 41 6. Almeida PF, Pokorny A. Mechanisms of antimicrobial, cytolytic, and cell-penetrating peptides: from
42 kinetics to thermodynamics. *Biochemistry.* 2009;48(34):8083–8093.
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

7. Rousselle C, Clair P, Lefauconnier JM, Kaczorek M, Scherrmann JM, Temsamani J. New advances in the transport of doxorubicin through the blood-brain barrier by a peptide vector-mediated strategy. *Mol Pharmacol.* 2000;57(4):679–686.
8. Sadler K, Eom K, Yang J, Dimitrova Y. Translocating proline-rich peptides from the antimicrobial peptide bactenecin 7. *Biochemistry.* 2002;41(48):14150–14157.
9. Zhu WL, Lan H, Park I-S, Kim J Il, Jin HZ, Hahm K-S, Shin SY. Design and mechanism of action of a novel bacteria-selective antimicrobial peptide from the cell-penetrating peptide Pep-1. *Biochem Biophys Res Commun.* 2006;349(2):769–774.
10. Splith K, Neundorf I. Antimicrobial peptides with cell-penetrating peptide properties and vice versa. *Eur Biophys J.* 2011;40(4):387–397.
11. Boucher HW, Corey GR. Epidemiology of methicillin-resistant *Staphylococcus aureus*. *Clin Infect Dis.* 2008;46 Suppl 5:S344–S349.
12. Garzoni C, Kelley WL. *Staphylococcus aureus*: new evidence for intracellular persistence. *Trends Microbiol.* 2009;17(2):59–65.
13. Clement S, Vaudaux P, Francois P, Schrenzel J, Huggler E, Kampf S, Chaponnier C, Lew D, Lacroix J-S. Evidence of an intracellular reservoir in the nasal mucosa of patients with recurrent *Staphylococcus aureus* rhinosinusitis. *J Infect Dis.* 2005;192(6):1023–1028.
14. Bahnsen JS, Franzyk H, Sandberg-Schaal A, Nielsen HM. Antimicrobial and cell-penetrating properties of penetratin analogs: Effect of sequence and secondary structure. *Biochim Biophys Acta.* 2012;1828(2):223–232.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

15. Zhu WL, Hahm K-S, Shin SY. Cell selectivity and mechanism of action of short antimicrobial peptides designed from the cell-penetrating peptide Pep-1. *J Pept Sci.* 2009;15(9):569–575.

16. Joshi S, Bisht GS, Rawat DS, Kumar A, Kumar R, Maiti S, Pasha S. Interaction studies of novel cell selective antimicrobial peptides with model membranes and *E. coli* ATCC 11775. *Biochim Biophys Acta.* 2010;1798(10):1864–1875.

17. Zhu WL, Lan H, Park Y, Yang S-T, Kim J Il, Park I-S, You HJ, Lee JS, Park YS, Kim Y, Hahm K-S, Shin SY. Effects of Pro --> peptoid residue substitution on cell selectivity and mechanism of antibacterial action of tritrypticin-amide antimicrobial peptide. *Biochemistry.* 2006;45(43):13007–13017.

18. Derossi D, Joliot a H, Chassaing G, Prochiantz A. The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem.* 1994;269(14):10444–10450.

19. CLSI. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically* ; Approved Standard — Ninth Edition. CLSI document M07-A9. Wayne, PA: Clinical and Laboratory Standards Institute; 2012.

20. Bi X, Wang C, Ma L, Sun Y, Shang D. Investigation of the role of tryptophan residues in cationic antimicrobial peptides to determine the mechanism of antimicrobial action. *J Appl Microbiol.* 2013;115(3):663–672.

21. Epanand RF, Schmitt MA, Gellman SH, Epanand RM. Role of membrane lipids in the mechanism of bacterial species selective toxicity by two alpha/beta-antimicrobial peptides. *Biochim Biophys Acta.* 2006;1758(9):1343–1350.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
22. Ishibashi J, Saido-Sakanaka H, Yang J, Sagisaka A, Yamakawa M. Purification, cDNA cloning and modification of a defensin from the coconut rhinoceros beetle, *Oryctes rhinoceros*. *Eur J Biochem*. 1999;266(2):616–623.
 23. Magzoub M, Eriksson LEG, Gräslund A. Comparison of the interaction, positioning, structure induction and membrane perturbation of cell-penetrating peptides and non-translocating variants with phospholipid vesicles. *Biophys Chem*. 2003;103(3):271–288.
 24. Thoren P. Uptake of analogs of penetratin, Tat(48-60) and oligoarginine in live cells. *Biochem Biophys Res Commun*. 2003;307(1):100–107.
 25. Tréhin R, Krauss U, Beck-Sickinger AG, Merkle HP, Nielsen HM. Cellular uptake but low permeation of human calcitonin-derived cell penetrating peptides and Tat(47-57) through well-differentiated epithelial models. *Pharm Res*. 2004;21(7):1248–1256.
 26. Letoha T, Gaál S, Somlai C, Venkei Z, Glavinas H, Kusz E, Duda E, Czajlik A, Peták F, Penke B. Investigation of penetratin peptides. Part 2. In vitro uptake of penetratin and two of its derivatives. *J Pept Sci*. 2005;11(12):805–811.
 27. Al-Taei S, Penning NA, Simpson JC, Futaki S, Takeuchi T, Nakase I, Jones AT. Intracellular traffic and fate of protein transduction domains HIV-1 TAT peptide and octaarginine. Implications for their utilization as drug delivery vectors. *Bioconj Chem*. 2006;17(1):90–100.
 28. Al Soraj M, He L, Peynshaert K, Cousaert J, Vercauteren D, Braeckmans K, De Smedt SC, Jones AT. siRNA and pharmacological inhibition of endocytic pathways to characterize the differential role of macropinocytosis and the actin cytoskeleton on cellular uptake of dextran and cationic cell penetrating peptides octaarginine (R8) and HIV-Tat. *J Control Release*. 2012;161(1):132–141.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

29. Jones SW, Christison R, Bundell K, Voyce CJ, Brockbank SM V, Newham P, Lindsay MA. Characterisation of cell-penetrating peptide-mediated peptide delivery. *Br J Pharmacol.* 2005;145(8):1093–1102.

30. Alves ID, Bechara C, Walrant A, Zaltsman Y, Jiao C-Y, Sagan S. Relationships between Membrane Binding, Affinity and Cell Internalization Efficacy of a Cell-Penetrating Peptide: Penetratin as a Case Study. Gasset M, editor. *PLoS One.* 2011;6(9):e24096.

31. Duchardt F, Fotin-Mleczek M, Schwarz H, Fischer R, Brock R. A comprehensive model for the cellular uptake of cationic cell-penetrating peptides. *Traffic.* 2007;8(7):848–866.

32. Alves ID, Jiao C-Y, Aubry S, Aussedat B, Burlina F, Chassaing G, Sagan S. Cell biology meets biophysics to unveil the different mechanisms of penetratin internalization in cells. *Biochim Biophys Acta.* 2010;1798(12):2231–2239.

33. Jahnsen RD, Sandberg-Schaal A, Vissing KJ, Nielsen HM, Frimodt-Møller N, Franzyk H. Tailoring Cytotoxicity of Antimicrobial Peptidomimetics with High Activity against Multidrug-Resistant *Escherichia coli.* *J Med Chem.* 2014;57(7):2864–2873.

34. Zhu WL, Shin SY. Antimicrobial and cytolytic activities and plausible mode of bactericidal action of the cell penetrating peptide penetratin and its lys-linked two-stranded peptide. *Chem Biol Drug Des.* 2009;73(2):209–215.

35. Alves ID, Goasdoué N, Correia I, Aubry S, Galanth C, Sagan S, Lavielle S, Chassaing G. Membrane interaction and perturbation mechanisms induced by two cationic cell penetrating peptides with distinct charge distribution. *Biochim Biophys Acta.* 2008;1780(7-8):948–959.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

36. Palm C, Netzereab S, Hällbrink M. Quantitatively determined uptake of cell-penetrating peptides in non-mammalian cells with an evaluation of degradation and antimicrobial effects. *Peptides*. 2006;27(7):1710–1716.

37. Caesar CEB, Esbjörner EK, Lincoln P, Nordén B. Membrane interactions of cell-penetrating peptides probed by tryptophan fluorescence and dichroism techniques: correlations of structure to cellular uptake. *Biochemistry*. 2006;45(24):7682–7692.

38. Lindberg M, Jarvet J, Langel U, Gräslund A. Secondary structure and position of the cell-penetrating peptide transportan in SDS micelles as determined by NMR. *Biochemistry*. 2001;40(10):3141–3149.

39. Hein-Kristensen L, Knapp KM, Franzyk H, Gram L. Selectivity in the potentiation of antibacterial activity of α -peptide/ β -peptoid peptidomimetics and antimicrobial peptides by human blood plasma. *Res Microbiol*. 2013;164(9):933–940.

40. Almeida PF. Membrane-active peptides: Binding, translocation, and flux in lipid vesicles. *Biochim Biophys Acta*. 2014;1–12.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Legend to figures

Figure 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 hr at 37°C. The plot represents the mean of the medians of the raw data in triplicate error bars representing $\pm\text{SD}$ and normalized against the mean of penetratin. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ statistically significant differences.

Figure 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 μM 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 μM 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 μm .

Figure 3 - Circular dichroism spectra of (a) PK-12-PPK; (b) SA-3; (c) TPk and (d) penetratin in TFE-water 90:10 (solid line) or in the presence of liposomes representing the membranes of Gram-positive bacteria, POPG:CL (3:1 molar ratio; dashed line); Gram-negative bacteria, POPG:CL:DOPE (2:7:1 molar ratio; dotted line); and eukaryotic cells, POPC:cholesterol (10:1 molar ratio; dashed and dotted line). Measurements were performed with a peptide concentration of 10 μM peptide to a lipid concentration of 1 mM yielding a peptide-to-lipid molar ratio of 1:100.

Figure 4 – Release of calcein from liposomes consisting of POPG:CL upon incubation with the antimicrobial peptide, SA-3, and the cell-penetrating peptide, penetratin. **Top:** Time-resolved release of calcein starting within 10 sec 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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

μM . **Bottom:** The relative release after 680 sec versus the total calcein entrapped. Each point represents the mean \pm SD (n=3).

Table I. Amino acid sequences, number of residues and molecular weight of the investigated peptide variants.

Compound	Sequence	Residues	Mw	Ref
PK-12-KKP	KKPWWKPWWPKWKK-amide	14	2008.46	[15]
SA-3	IKWAGKWWKLFK-amide	12	1589.97	[16]
TPk	VRRFkWWWkFLRR-amide*	13	1963.38	[17]
Pen	RQIKIWFQNRRMKWKK-amide	16	2245.74	[18]
TAMRA-PK-12-KKP	TAMRA-KKPWWKPWWPKWKK-amide	14	2420.89	
TAMRA-SA-3	TAMRA-IKWAGKWWKLFK-amide	12	2002.40	
TAMRA-TPk	TAMRA-VRRFkWWWkFLRR-amide	13	2375.81	
TAMRA-Pen	TAMRA-RQIKIWFQNRRMKWKK-amide	16	2658.17	

*k refers to Nlys [$\text{H}_2\text{N}-(\text{CH}_2)_4-\text{NH}-\text{CH}_2-\text{COOH}$].

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

^a EC₅₀ 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.

^b Strain: ATCC 25923. ^c Strain: ATCC 25922.

Table III – Maximum calcein release after exposure to a concentration of 1.6 μ M 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*	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 sec; *Run time 340 sec.

Figure 1
[Click here to download high resolution image](#)

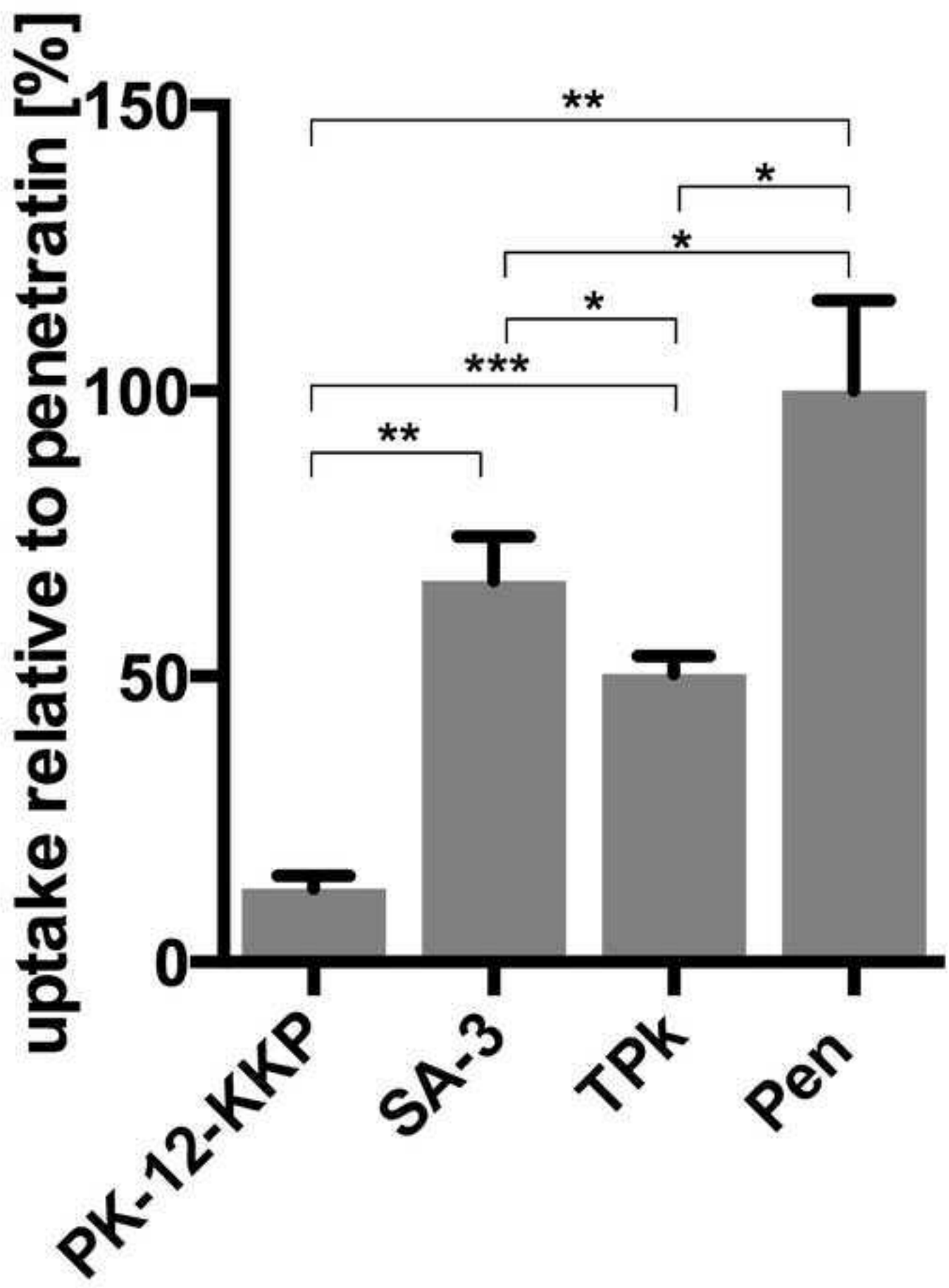


Figure 2
[Click here to download high resolution image](#)

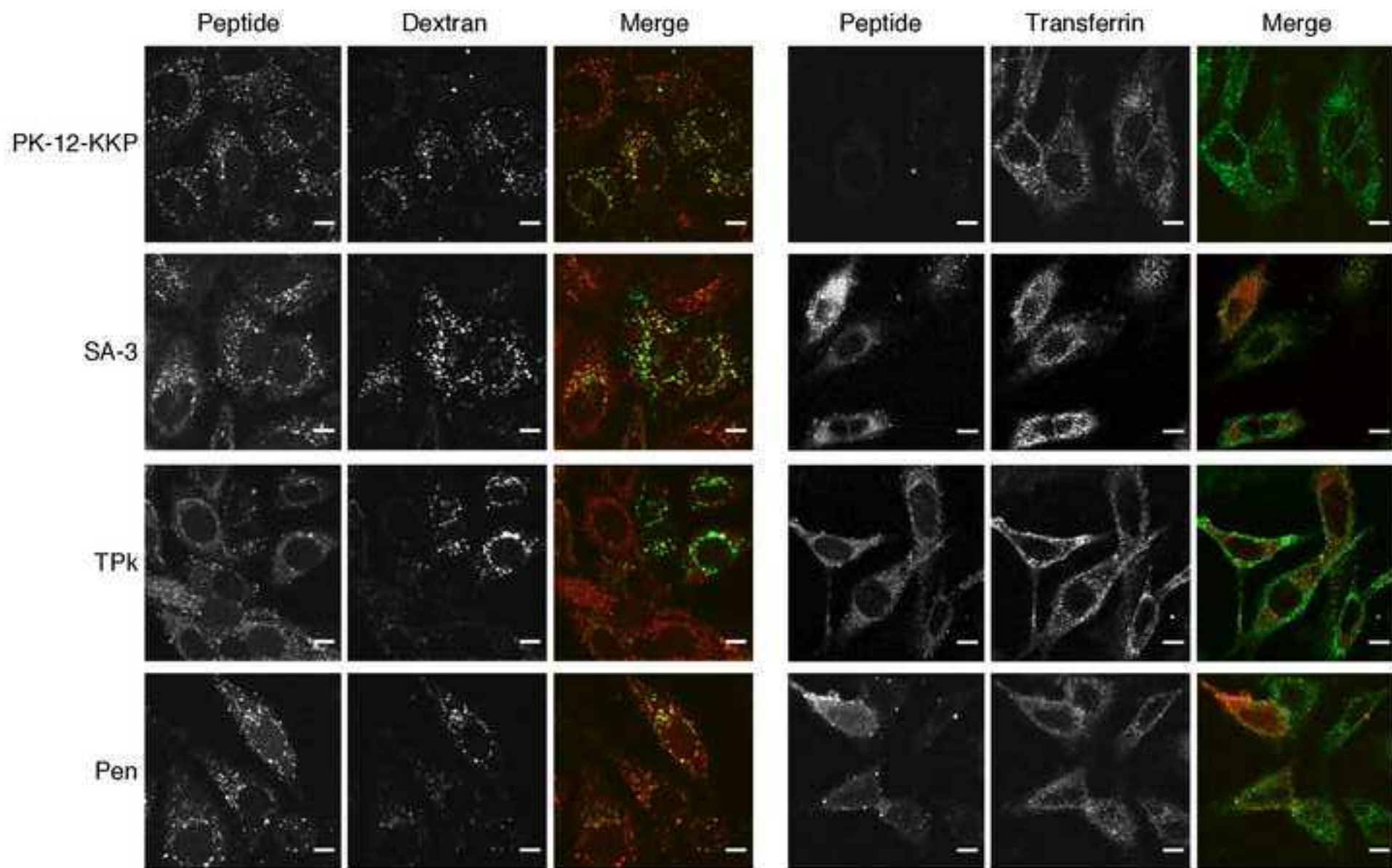


Figure 3
[Click here to download high resolution image](#)

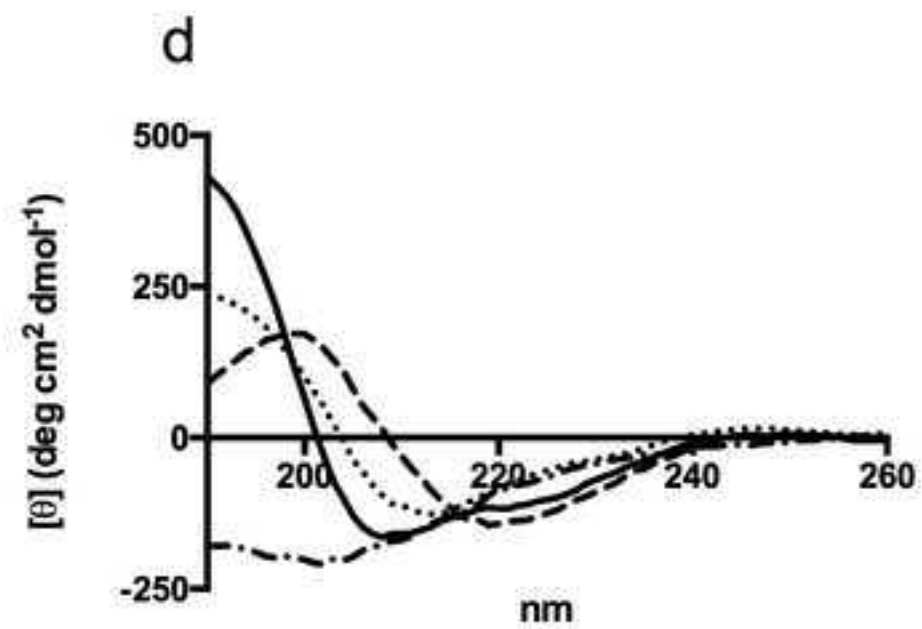
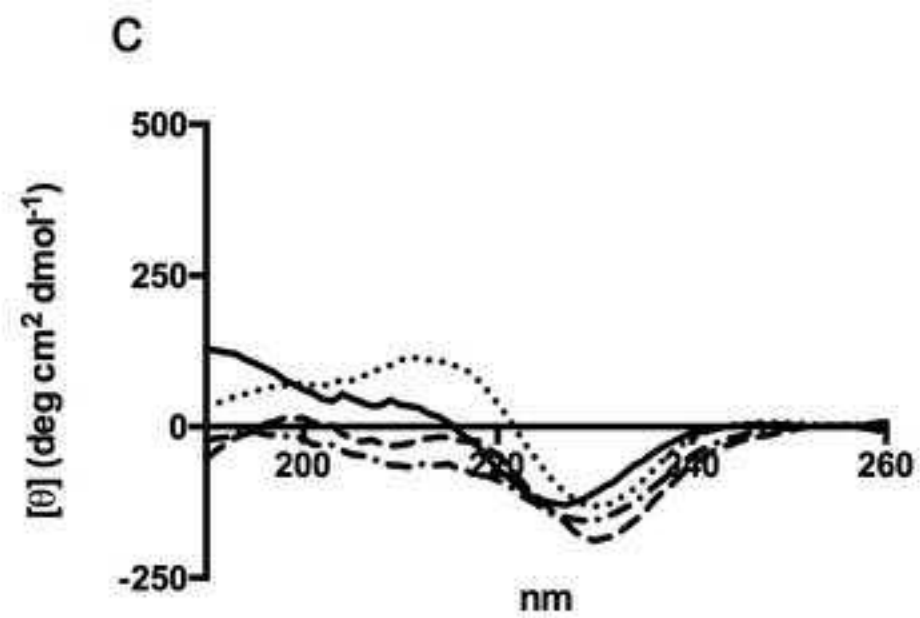
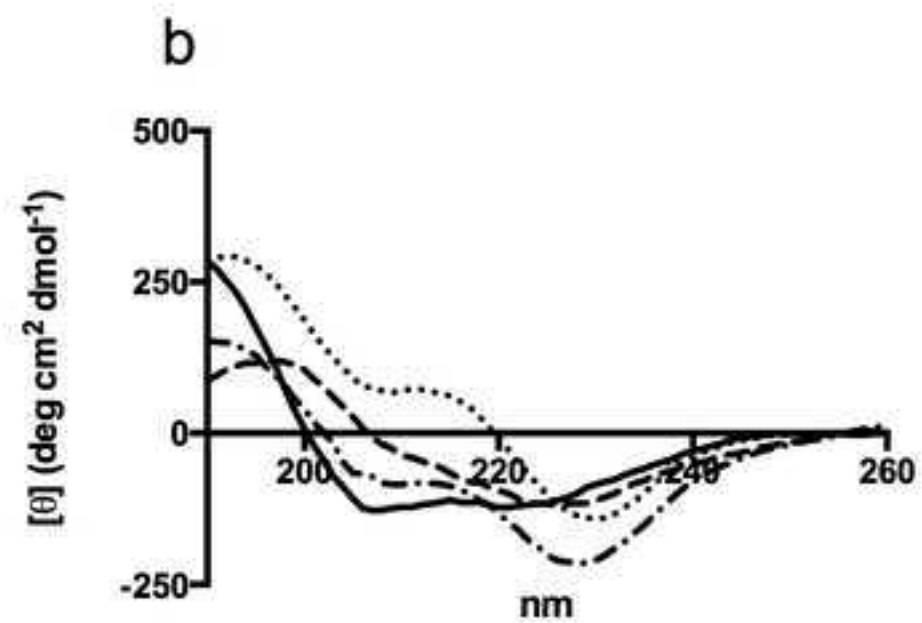
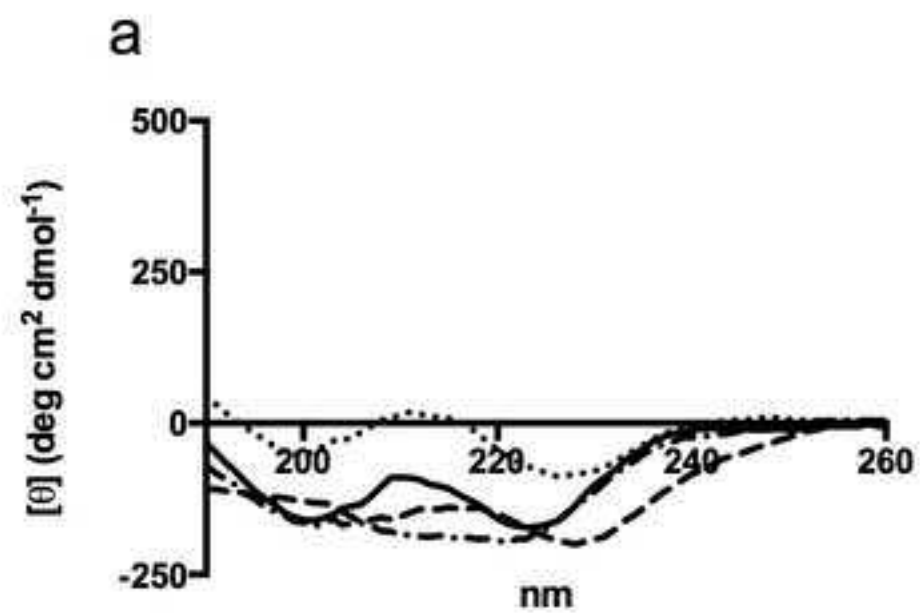


Figure 4
[Click here to download high resolution image](#)

