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An Insect Antibacterial Peptide-Based Drug Delivery System

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Abstract: The ability of the short, proline-rich native antibacterial peptides to penetrate bacterial and host cells suggests the utility of these transport systems in delivering peptidic cargo into cells. We studied the uptake of pyrrhocoricin and its most potent dimeric analogue by bacteria as well as human dendritic cells and fibroblasts. Native pyrrhocoricin entered the susceptible organism *Escherichia coli* very efficiently and the nonsusceptible bacterium *Staphylococcus aureus* to a significant degree. The antibacterial peptide also penetrated human monocyte-derived dendritic cells. It failed, however, to enter fibroblasts, whereas the designer analogue Pip-pyrr-MeArg dimer penetrated all the cell types that were studied. When glucoincretin hormone Glp-1 fragment 7−36 was cosynthesized with the dimer, the antibacterial peptide derivative lost its ability to cross the bacterial membrane layer. In contrast, a chimera of the Pip-pyrr-MeArg dimer and two copies of a shorter (nine residues) class I major histocompatibility complex epitope successfully entered bacterial and mammalian cells. While the Pip-pyrr-MeArg dimer was not immunogenic when inoculated into mice, the chimera elicited a strong cytotoxic T-cell response, indicating the maintenance of the antigenic integrity of the cargo in the peptide conjugate. The chimera when tested for its immunological properties activated human dendritic cells significantly more strongly than any of the two independent fragments alone, yet lacked mammalian cell toxicity. These results confirm the utility of designed pyrrhocoricin analogues for delivery of peptidic cargo across cell membranes in general, and their potential as carriers for epitope-based vaccines in particular.

Keywords: Cell penetration; dendritic cells; membrane; pyrrhocoricin; T-cell epitope

Introduction

Epithelial tissues represent a major barrier through which molecules must be absorbed. The phospholipid bilayer of the plasma membrane of epithelial cells is considered the major factor restricting the free movement of substances from the lumen to the bloodstream through the transcellular pathway.[1] While hydrophobic and small molecules can penetrate cell membranes, these barriers are almost impermeable to large hydrophilic biopolymers such as peptides.[2] Strategies therefore need to be developed that apply the principle of the "Trojan Horse". Peptide cargo is to be transported into appropriate cells using physiologically acceptable carrier systems.[3]

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Currently, there are three potential ways of delivering peptides into cells and across epithelial layers.

(1) Using the active transport mechanism of the cells, we can target cell surface receptors that recognize and internalize specific molecules. These include carbohydrate receptors, lipoprotein receptors, transferring receptors, and receptors involved in cell adhesion. Delivery vehicles explored so far for this purpose include antibodies, carbohydrates, viral proteins, and some molecules of endogenous origin.[4]

(2) In a process of passive transport, the binding of polycationic substances to the negatively charged cell surfaces leads to endocytosis. While receptor-mediated uptake is characterized by K_d values in the low nanomolar range with maximum binding capacities of <1 pmol/mg of membrane protein, absorption-mediated uptake features micromolar saturation constants but binding capacities of several nanomoles per milligram.[5] Enzymatic treatments reveal that the anionic microdomains on the luminal side of endothelial capillaries consist of negatively charged glycoproteins with sialic acid residues, while the abluminal anionic binding sites contain heparan sulfates.[6]

(3) Particle delivery involves the uptake of biodegradable polymers by phagocytic cells, and if the particles are sufficiently small, they can also be sampled by constitutive macropinocytosis which is exhibited by many cell types. Particles currently used for peptide delivery fall into two classes: nanoparticles, ranging in size from 10 to 1000 nm, and microparticles, ranging in size from 1 μm to 1 mm.[7] In general, the smaller the particle, the better the peptide is absorbed due to the difficulty in engulfing larger particles and also the improved hydrophobic to hydrophilic ratio.

Vaccines are one of the most cost-effective public health interventions.[8] Peptide-based approaches to vaccine design offer potential advantages over conventional whole protein or DNA vaccination in terms of purity, batch-to-batch consistency, cost of production, and the high specificity of the resulting immune responses.[9,10] There are, however, a number of problems associated with peptide-based vaccine design and development. Synthetic peptides alone are not usually sufficiently immunogenic,[11] mostly because of their poor penetration into antigen-presenting cells (APC) and cytotoxic T-lymphocytes (CTL).[12] Additional disadvantages include *in vivo* instability and the lack of a reliable and safe adjuvant for delivery to the immune system as a whole.[13] It is clear that successful immunization requires a robust penetration of the antigens into APC.

The potential to deliver drugs through the anionic surfaces of cells is well represented by the proline-rich native antibacterial peptide family, originally isolated from insects.[14] As with other antimicrobial peptides,[15] the proline-rich peptides are also rich in the cationic residues, arginine, lysine, and histidine. Cationic peptides, just like cationized proteins, are generally able to interact electrostatically with negatively charged phospholipids and be inserted into model membranes of planar bilayers or liposomes.[16,17] In general, peptides penetrate bacteria more easily than they do mammalian cells. The interaction of most peptides with eukaryotic membranes is inhibited by the lack of negatively charged lipids on the cell surface, by the rather low negative membrane potential that exists across the plasma membrane, and also by the presence of cholesterol. In contrast, bacterial membranes are abundant in anionic surface phospholipids, have high negative transmembrane potential, and lack cholesterol.[18] Antibacterial peptides with strong membrane activity may, however, overcome these limitations.

Pyrrhocoricin, a 20-amino acid peptide originally isolated from the European sap-sucking bug *Pyrrhocoris apterus*,[19]

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shows remarkable activity against Enterobacteriaceae *in vitro*[20] and protects mice against moderate systemic *Escherichia coli* infection.[21] This peptide is unable to kill phylogenically unrelated bacteria, such as *Staphylococcus aureus*,[22] due to sequence alterations of the bacterial target protein DnaK.[23] The hypothetical molecular archeology of pyrrhocoricin is constructed of a DnaK-binding amino-terminal domain and a cell-penetrating carboxy-terminal half.[24] In support of this model, fluorescein-labeled pyrrhocoricin enters *E. coli* cells very efficiently, as do analogues in which the DnaK-binding region is mutated (and therefore unable to kill *E. coli* strains), but in which the cell-penetrating C-terminal domain is retained.[24]

Lytic activity, directed toward mammalian cells, is a major concern with peptides used for drug delivery.[25] Pyrrhocoricin analogues, however, show little or no toxicity *in vitro*[26] or *in vivo*.[21] This lack of toxicity coincides with the lack of side effects of the many cationic antibacterial peptides that have been examined in clinical trials.[27] In addition, cationic antibacterial peptides are not strongly immunogenic, a feature that is an advantage for their use as delivery vehicles. These properties of pyrrhocoricin led us to investigate its potential as a peptide drug delivery vehicle with an emphasis on epitope-based vaccine design. The studies were extended to the Pip-pyrr-MeArg dimer, which exhibits improved *in vitro* and *in vivo* antibacterial properties presumably due to its improved membrane penetrating properties and stability against peptidase cleavage.[26] In fact, the Pip-pyrr-MeArg dimer was designed to enter "hard-to-penetrate" cells and, unlike the native molecule, can avoid the resistance that develops following changes in the bacterial membrane structure when pathogens are treated with antimicrobial peptide derivatives.[26] This report examines the scope and limitations of the drug delivery properties of the pyrrhocoricins *in vitro* and provides a proof of principle for enhancement of cell penetration using a subunit vaccine candidate.

Experimental Section

Peptide Synthesis. Amino acids protected with a 9-fluorenylmethoxycarbonyl group (Fmoc)[28] were used for the synthesis of the peptides. The peptide chain assembly was carried out on a Rainin PS3 automated synthesizer. After cleavage, peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) in a water/acetonitrile/trifluoroacetic acid elution system, until matrix-assisted laser desorption ionization (MALDI) mass spectra revealed only single species with the expected molecular ions. Table 1 lists the synthetic peptides used in this study. The detected molecular ions of the C-terminally amidated peptide series were as follows: *m*/*z* 2339 for pyrrhocoricin, *m*/*z* 4842 for Pip-pyrr-MeArg dimer, *m*/*z* 1363 for NPK^d epitope KK, *m*/*z* 7532 for the epitope−dimer chimera, *m*/*z* 3298 for Glp-1 7−36, and *m*/*z* 8106 for the dimer−Glp chimera. The peptides were also N-terminally acylated with 5(6)-carboxyfluorescein. Fluoresceinated peptides were purified, and their structural integrity was verified in a manner similar to that for the unlabeled analogues. The mass spectra were acquired at the Wistar Institute Protein Microchemistry Facility.

Cell Penetration Assays. To study the ability of peptide derivatives to enter *E. coli* or *S. aureus* cells, fluorescein-labeled peptides were added to bacterial cultures at final concentrations of 5 μg/mL. The cells were allowed to acquire the peptides for 1 h at 37 °C; excess substrate was then removed, and the cells were washed extensively with phosphate-buffered saline (PBS, pH 6.8). The cells were fixed with PBS containing 1% paraformaldehyde and visualized using a Leica TCS SPII laser scanning confocal microscope. To determine the total number of bacteria in the same preparations, cells were also detected by differential interference contrast microscopy (DIC) and counted. This technique produces a monochromatic shadow-cast image (Figure 3, right-hand panel) that effectively displays the gradient of optical paths for both high and low spatial frequencies present in the specimens.

Human fibroblast cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% CO_2. The cells were plated in a two-well format chamber slide system

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Table 1. Amino Acids of Sequences of the Peptides Used in This Study[a]

pyrrhocoricin
H-Val-Asp-Lys-Gly-Ser-Tyr-Leu-Pro-Arg-Pro-Thr-Pro-Pro-Arg-Pro-Ile-Tyr-Asn-Arg-Asn-NH_2
Pip-pyrr-MeArg dimer
(H-Pip-Asp-Lys-Gly-Ser-Tyr-Leu-Pro-Arg-Pro-Thr-Pro-Pro-Arg-Pro-Ile-Tyr-Asn-MeArg-Asn)$_2$-Dab-NH_2
NPK^d epitope-KK
H-Thr-Tyr-Gln-Arg-Thr-Arg-Ala-Leu-Val-Lys-Lys-NH_2
epitope−dimer chimera
(H-Thr-Tyr-Gln-Arg-Thr-Arg-Ala-Leu-Val-Lys-Lys-Pip-Asp-Lys-Gly-Ser-Tyr-Leu-Pro-Arg-Pro-Thr-Pro-Pro-Arg-Pro-Ile-Tyr-Asn-MeArg-Asn)$_2$-Dab-NH_2
Glp-1 7−36
H-His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-NH_2
dimer−Glp chimera
(H-Pip-Asp-Lys-Gly-Ser-Tyr-Leu-Pro-Arg-Pro-Thr-Pro-Pro-Arg-Pro-Ile-Tyr-Asn-MeArg-Asn)$_2$-Dab-His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-NH_2

[a] Dab is 2,4-diaminobutyric acid. MeArg is *N*-methylarginine. Pip is 4-aminopiperidine-4-carboxylic acid.

with plastic tissue culture-treated slides; each well contained 2×10^5 cells. Subsequently, the chamber slides were incubated under the same conditions 24 h prior to addition of peptide. Peptides were added to wells containing 1 mL of medium at a final concentration of 5 μg/mL. Following peptide addition, plates were incubated for 1 h at 37 °C in an atmosphere of 5% CO_2. In a control experiment, fibroblasts were incubated with medium alone. The medium was aspirated, and wells were washed twice with PBS for 5 min each. The cells were fixed with 4% paraformaldehyde for 15 min at 4 °C and then washed again with PBS three times. The upper slide structure was removed, and the microscope slides were air-dried and examined using a Leica TCS SPII laser scanning confocal microscope.

Human monocyte-derived dendritic cells were incubated on ice with 1−50 nmol of fluorescein-conjugated pyrrhocoricin, dissolved in 300 μL of PBS, or medium only (no peptide treatment) for 1 h. The cells were then washed twice with cold FACS Wash (PBS containing 1% FBS and 5 mM EDTA) and fixed with 1% formaldehyde in PBS before flow cytometric analysis. The values in the top right corner of the graphs in Figure 1 refer to the mean fluorescence intensities of the analyzed populations.

Antibacterial Assay. Antibacterial growth inhibition assays were performed using sterile 96-well plates (Nunc F96 microtiter plates) in a final volume of 100 μL as described previously.[22] Briefly, 90 μL of a suspension of a mid-logarithmic-phase culture of *E. coli* (strain JC7623) at an initial absorbance at 600 nm of 0.001 in one-quarter strength Muller-Hinton broth was added to 10 μL of serially diluted peptides dissolved in sterilized water. The final peptide concentration was 40 μM. Cultures were then incubated at 37 °C for 24 h with gentle shaking, and growth inhibition was assessed by recording the absorbance at 600 nm using a microplate reader.

Maturation of Dendritic Cells. Human monocyte-derived dendritic cells were generated from $CD14^+$ monocytes that were cultured in RPMI medium supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 for 5 days.[29] Following confirmation of their differentiation into dendritic cells (loss of CD14 and appearance of CD1a surface markers), the cells were incubated with medium only, lipopolysaccharide (5 μg/mL), or test peptides at 50 μM for 48 h at 37 °C followed by surface staining for human leukocyte antigens HLA-DR, CD83, and CD86. The values in each histogram of Figure 6 correspond to the percentage of cells expressing high levels of surface antigen (right side of the histograms).

Immunization Protocol. To examine the ability of peptide antigens to elicit epitope-specific T-cells, BALB/c mice were inoculated subcutaneously (sc) with either 48 μg (10 nmol) of the Pip-pyrr-MeArg dimer or 76 μg (10 nmol) of the NPK^d epitope−dimer chimera (containing 20 nmol of the NPK^d epitope) or 28 μg (20 nmol) of the NPK^d epitope-KK peptide, all emulsified at a 1:1 (v/v) ratio in complete Freund's adjuvant (CFA, from Sigma Chemical Co., St. Louis, MO). Seven days after being primed, mice were sacrificed, and the inguinal and popliteal lymph nodes were removed and single-cell suspensions prepared from these organs. These cells were then used as effectors in the interferon-γ (IFNγ) ELISPOT and cytotoxic T-cell assays.

ELISPOT Assay for IFNγ-Secreting Cells. NPK^d epitope-specific IFNγ-secreting cells were enumerated by an ELISPOT assay modified from that of Murali-Krishna et al.[30] Flat-bottomed polyvinyl chloride microtiter plates (96-well, from Dynatech) were coated overnight with 50 μL of rat anti-mouse IFNγ antibody (clone R4-6A2) at 5 μg/mL in PBS. Unoccupied sites on the wells were then blocked by incubation for 1 h with 10 mg/mL bovine serum albumin

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(BSA) in PBS, and the plates were washed three times with PBS containing 0.05% Tween 20 (PBST). Two-fold dilutions of lymph node cells in T-cell culture medium consisting of RPMI-1640 supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 2 mM sodium pyruvate, 30 μg/mL gentamicin, 100 μg/mL streptomycin, 100 IU/mL penicillin, and 100 μM 2-mercaptoethanol were then added to the wells. In addition, 5×10^5 irradiated (2200 rad, ^{60}Co source) syngeneic spleen cells from nonimmunized mice and 10 units/well of recombinant human interleukin-2 (IL-2) (Pharmingen, San Diego, CA) were also added to these wells. Cells were incubated at 37 °C in 5% CO_2 for 18 h in the presence or absence of the NPK^d epitope at a concentration of 1 μg of peptide/mL. Cells were then lysed and removed by rinsing the plates, initially with distilled water, and then with PBST. Fifty microliters of a 1:500 dilution of biotinylated anti-mouse IFNγ antibody (clone XMG 1.2, Pharmingen) was added, and the plates were incubated at room temperature for 2 h. Plates were again washed, and 50 μL of streptavidin-alkaline phosphatase (Pharmingen; 1:400 dilution in 5 mg/mL BSA in PBST) was added to each well and incubated for a further 2 h. The plates were then washed, and 100 μL of ELISPOT substrate containing 1 mg of 5-bromo-4-chloro-3-indolyl phosphate (5-BCIP) in 1 mL of 2-amino-2-methyl-1-propynol buffer (Sigma) was added to each well. When blue-green spots had developed, the plates were washed with water and dried and the spots counted with the aid of an inverted microscope.

Cytotoxic T-Cell Assays. Secondary effector cells were generated from inguinal and popliteal lymph nodes of mice that had been immunized sc 7 days previously with peptide immunogens emulsified in CFA. Briefly, 4×10^7 lymph node cells, depleted of erythrocytes by treatment with Tris-buffered ammonium chloride (ATC; 0.15 M NH_4Cl in 17 mM Tris-HCl at pH 7.2), were cultured with 1×10^7 irradiated (2200 rad, ^{60}Co source) virus-infected or peptide-pulsed syngeneic spleen cells in 25 cm^2 tissue culture flasks (Falcon) containing 15 mL of T-cell culture medium. The virus-infected spleen cells had been preincubated at 37 °C for 30 min with 3000 HAU of infectious PR8 virus in 1 mL of serum-free RPMI, and washed once prior to being added to the flask. The peptide-pulsed spleen cells had been preincubated at 37 °C for 60 min with 100 μg/mL NPK^d peptide and also washed once prior to being added to the flask. After being cultured for 5 days at 37 °C in a humidified atmosphere containing 5% CO_2, the cells were washed three times and used in ^{51}Cr release assays. The ^{51}Cr release assays were performed in triplicate as described previously[31] using P815 mastocytoma cells (H-2^d, DBA/2) as targets.

Toxicity to COS Cells. Toxicity to mammalian kidney cells was studied as previously described.[26] Briefly, COS-7 cells were grown in DMEM containing 10% FBS at 37 °C in an atmosphere of 10% CO_2. The cells were plated in 24-well tissue culture dishes (5×10^3 cells/well) and incubated for 24 h prior to peptide addition. Peptides, dissolved in water, were added in final concentrations of 150 μM to 1 mM in duplicate. The plates were incubated for an additional 24 h at 37 °C in an atmosphere of 10% CO_2; the medium was aspirated, and the assay was terminated by adding 100 μL of trypsin EDTA (0.25% trypsin/0.1% EDTA in HBSS, Cellgro). One milliliter of fresh medium was added to detached cells, and 50 μL of this solution was mixed with 50 μL of a trypan blue solution (0.4%, Sigma) to stain dead cells. The remaining live cells were counted using a hemacytometer.

Results

Characterization of the Cell Penetrating Ability of Native Pyrrhocoricin. In the first assay of this study, we examined whether pyrrhocoricin penetrated the nonsusceptible organism *S. aureus* and compared the efficacy of cell penetration with that observed with the susceptible bacterium *E. coli*. We aimed first to determine whether the peptide was able to pass through the thickened bacterial membrane of Gram-positive bacterial cells and second to acquire further evidence for our hypothesis that pyrrhocoricin kills bacteria by a mode of action other than simple membrane disintegration. We also wanted to quantitatively compare the level of peptide translocation into bacterial cells that are sensitive or resistant to pyrrhocoricin treatment. To extend the investigations to antigen delivery, we repeated the cell penetration assays with fibroblasts and also with human dendritic cells because antigen uptake by dendritic cells is a prerequisite for vaccine development.[32] Fibroblasts were selected as a model for drug delivery because these cells are easy to grow and exhibit transport properties similar to those of epithelial surfaces. It has been demonstrated that fibroblasts sort newly synthesized proteins into two different pathways for delivery to the cell surface that are equivalent to the apical and the basolateral post-Golgi routes in epithelial cells.[33] Furthermore, fibroblast membranes share ion transport properties with model epithelial layers.[34] For the delivery of extracellular arginine-containing compounds, both fibroblasts and endothelial cells express ubiquitous cationic amino acid transporter-1.[35] Indeed, the cell-penetrating peptide pVEC

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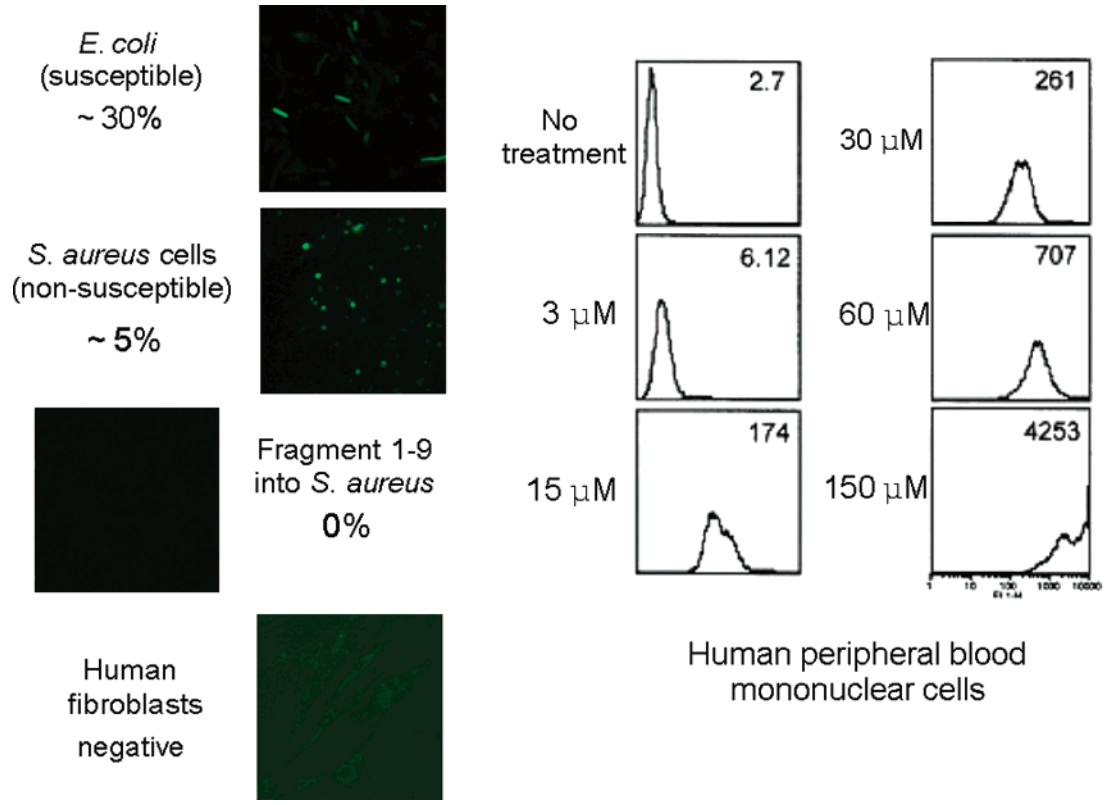

Figure 1. Entry of fluorescein-labeled pyrrhocoricin into cells. *E. coli* and *S. aureus* cells and human fibroblasts were incubated with 2 μM pyrrhocoricin derivative or the analogous C-terminal half of pyrrhocoricin; the full-sized pyrrhocoricin concentration for human PMBC ranged from 5 to 250 μM (1−50 nmol being the total peptide amount in 300 μL of solvent). The incubation time was 1 h throughout. The native peptide was taken up by the bacteria and dendritic cells, but not by fibroblasts. The N-terminal half of pyrrhocoricin failed to enter cells. The percentage of cells containing labeled peptides was calculated on the basis of the total number of cells identified by differential interference contrast.

is found to translocate into aortic endothelial cells and murine fibroblasts by an identical, apparently nonendocytic mechanism.[36] Thus, our fibroblast model represents well the mammalian cell penetrating ability of members of the short, proline-rich peptide family.

N-Terminally fluorescein-labeled pyrrhocoricin entered *E. coli* cells very efficiently (Figure 1, top left). In a comparison with the number of total cells in the same preparation (detected with DIC), pyrrhocoricin penetrated approximately 30% of all bacterial cells. Significantly, pyrrhocoricin stained the cells uniformly, not just the surface membrane layer indicating full distribution in the cytoplasm. Similarly positive albeit less efficient peptide translocation was observed with *S. aureus* cells (Figure 1, second from the top), with a total peptide penetration value of 5%. Once again, if pyrrhocoricin entered the cells, it became evenly distributed in all cellular compartments, indicating that the peptide did not remain attached to the membrane and cell entry did not involve disintegration of the membrane structure. Earlier, we reported that the N-terminal half of pyrrhocoricin (residues 1−9), lacking the domain responsible for cell entry, labels a significantly smaller fraction of *E. coli* cells, and stains only the surface of these bacteria.[24] On the basis of these observations, we did not expect pyrrhocoricin fragment 1−9 to enter *S. aureus*, for which the entry level of full-sized pyrrhocoricin was reduced, and in fact, the amino-terminal half of pyrrhocoricin failed to enter a single *S. aureus* bacterium (Figure 1, third from the top).

Fluoresceinated pyrrhocoricin was unable to penetrate human fibroblasts (Figure 1, bottom left). This finding reflects the inability of native pyrrhocoricin to cross certain mammalian membranes that are generally less permeable to antibacterial peptides.[18] In contrast, fluorescein-labeled pyrrhocoricin was taken up very efficiently by dendritic cells derived from human peripheral blood mononuclear cells (PBMC), which were positively selected using an anti-CD14 antibody and then cultured in the presence of GM-CSF and interleukin-4 (Figure 1, center and right panels). Uptake of peptide was observed at a concentration as low as 15 μM (5 nmol being the total amount in 300 μL of buffer), and the uptake appeared to be concentration-dependent. On the basis of this finding, it seemed to us that pyrrhocoricin derivatives could be considered for the delivery of subunit vaccine candidates to dendritic cells. It has to be added that cell sorting cannot distinguish between true cellular uptake and only binding to the surface of the cells. Nevertheless, as the same labeled pyrrhocoricin derivative was distributed all over

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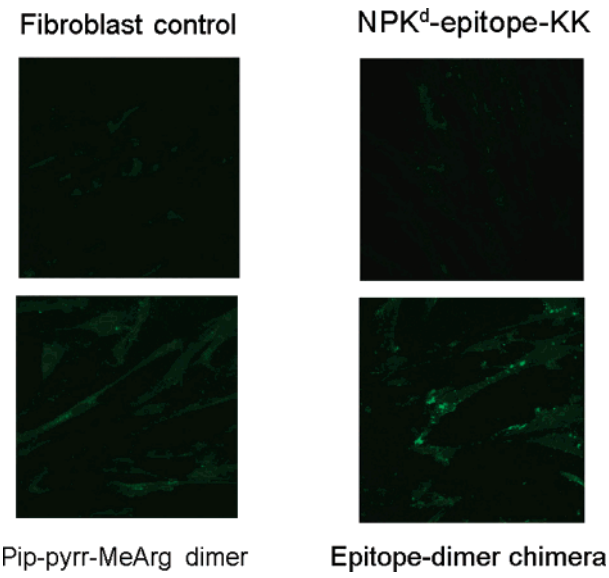

Figure 2. The Pip-pyrr-MeArg dimer penetrates human fibroblasts efficiently and can carry cargo inside the cells. Peptides (5 μg/mL) were incubated with the cells for 1 h; the cells were fixed under mild conditions, and the fluorescence intensity was recorded with a laser scanning confocal microscope. While the NPK^d epitope alone is unable to enter the cells, when conjugated to the pyrrhocoricin dimer, the chimera becomes distributed in all cell compartments, except the nucleus.

in mouse macrophages,[24] we expect true cell entry. Apparently immune cells are more permeable to native pyrrhocoricin than other mammalian cell types, and this may reflect the immunostimulatory properties of native antimicrobial peptides.

To obtain pyrrhocoricin analogues with broader cell penetrating capability, we examined a C-terminally branched pyrrhocoricin dimer. C-Terminally branched pyrrhocoricin derivatives have been shown to have improved antibacterial activity against the Gram-positive bacterium *Micrococcus luteus*,[22] and the Pip-pyrr-MeArg dimer kills those hard-to-penetrate Gram-negative (*Pseudomonas aeruginosa* and *Proteus* strains) and Gram-positive (*Staphylococcus saprophyticus*) strains that are not susceptible to native pyrrhocoricin.[26] Experiments with the fluorescein-labeled Pip-pyrr-MeArg dimer derivative demonstrated that this peptide analogue successfully entered human fibroblasts (Figure 2, bottom left panel). Because of the enhanced activity of the dimeric form of pyrrhocoricin, we used the Pip-pyrr-MeArg dimer in subsequent studies.

Selection of Peptide Cargos for the Pip-pyrr-MeArg Dimer. We have previously shown that when mice with an experimental ascending urinary tract infection (UTI) are treated with two oral doses of the Pip-pyrr-MeArg dimer at a concentration of 3 mg/kg, a cumulative 5-fold reduction in the bacterial count ensues in the kidneys, bladder, and urine.[26] Furthermore, the corresponding bacterial counts are reduced by more than 2 log units when the Pip-pyrr-MeArg dimer is administered intravenously or subcutaneously. While the oral efficacy is not satisfactory for the development of the Pip-pyrr-MeArg peptide as a treatment for UTI, these results are a clear indication that this pyrrhocoricin derivative is bioavailable when given orally and reaches target organs such as those of the urinary tract. For successful drug delivery, we must select cargos that need low systemic concentrations, which can be attained by oral administration as a pyrrhocoricin-based construct, for their activity. Subunit vaccines, for example, are promising peptide-based drugs that are effective at very low doses, but they need penetration enhancers or adjuvants to induce antibody or T-cell production in mice.[13] One of the best studied peptide antigens is the NPK^d peptide, a major histocompatibility complex protein (MHC) class I epitope, capable of eliciting an influenza virus-specific CTL response.[37] CTL epitopes are relatively short (nine amino acid residue) peptides; thus, they represent attractive cargo to be delivered into cells. Another family of peptide drug leads that require only a small dose for activity yet are inactive when administered orally is represented by endogenous hormones. Perhaps most current research involves the glucagon-like peptide 1 (Glp-1), an important glucoincretin hormone that is secreted from intestinal L-cells in response to nutrient ingestion.[38] Peptide hormones, including Glp-1, are usually longer than T-cell epitopes; therefore, they can be used to study the capacity limits of peptidic delivery systems. Glp-1 analogues were made for structure−activity relationship studies and to stabilize the peptide against dipeptidyl-peptidase IV.[38] It has also been shown that the N-terminus can be freely substituted without a major loss in the *in vitro* or *in vivo* biological activities.

On the basis of these considerations, the NPK^d peptide was synthesized at the amino terminus of the Pip-pyrr-MeArg dimer, and the Glp-1 peptide was added to the carboxy terminus. Because the Pip-pyrr-MeArg dimer is branched at the C-terminus, synthesis of additional peptide sequences at the N-terminus results in two copies of the peptide cargo being attached. The functional domains were separated by two lysine residues to facilitate intracellular processing of the chimera following cell penetration.[39,40] Despite the size of the chimera (>7500 Da), synthesis proceeded without difficulty presumably due to the facile synthesis opportunity exhibited by complex pyrrhocoricin analogues.[22]

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Table 2. Activity of Pyrrhocoricin Dimers on Bacterial and Mammalian Cells

peptide	half-inhibitory concentration (IC_{50}) against *E. coli* JC7623 (μM)	percent of COS-7 cell survival upon incubation with peptides at the indicated concentration
Pip-pyrr-MeArg dimer	1	80% at 1 mM
epitope−dimer chimera	4	88% at 150 μM
dimer−Glp chimera	>40	not tested

The biologically active forms of Glp-1 [Glp-1(7−36) amide and Glp-1(7−37)] have been demonstrated to possess multiple functions, including enhancement of glucose-dependent insulin secretion, stimulation of proinsulin gene expression, and suppression of glucagon secretion and gastric emptying.[41,42] For the dimer−Glp chimera, we started the solid-phase synthesis with Glp-1 fragment 7−36 and continued with the Pip-pyrr-MeArg dimer (Table 1). In this design, the antibacterial peptide is located at the N-terminus, and there is consequently no need for a spacer between the two functional units. This construct was even larger (more than 8000 Da) than the epitope−dimer chimera, and posed more synthesis challenges, mostly due to the low yield of the glucoincretin hormone fragment. Nevertheless, we managed to purify the target chimera as a single peak by RP-HPLC with the correct molecular ion in sufficient quantity for detailed functional studies.

Functional Studies of the Integrity of the Component Peptides. Before detailed cell penetration studies were initiated, we wanted to ensure that the individual components retained their intended biological activities, *viz.*, bacterial cell penetration by the Pyp-pyrr-MeArg dimer, elicitation of an immune response with the NPK^d epitope, and induction of insulin secretion by the Glp-1 fragment.

Entry into bacterial cells was assessed by fluorescence microscopy and a standard broth dilution growth inhibition assay using *E. coli* JC7623, an organism that is highly susceptible to pyrrhocoricin analogues.[22] The Pip-pyrr-MeArg dimer without any peptide cargo killed this cell line with an IC_{50} value of 1 μM (Table 2). Some reduction in the antibacterial activity (IC_{50} of 4 μM) was observed when the NPK^d epitope was added (epitope−dimer chimera), indicating that the antimicrobial activity was slightly modulated by extension of the antibacterial peptide with the epitope. This minor loss in activity could be explained by the generally observable loss of antibacterial efficacy when pyrrhocoricins are N-terminally extended.[21] Examination by fluorescence microscopy supported these observations: the epitope−dimer chimera readily penetrated *E. coli* JC7623 cells with an overall cell entry value of 30% and stained the intracellular space homogeneously (Figure 3, left panel). Taken together, the bacterial cell penetrating ability of the Pip-pyrr-MeArg dimer was not significantly compromised when two copies of the NPK^d epitope were attached and suggested that the pyrrhocoricin derivative would be able to deliver the antigenic cargo into mammalian cells as well.

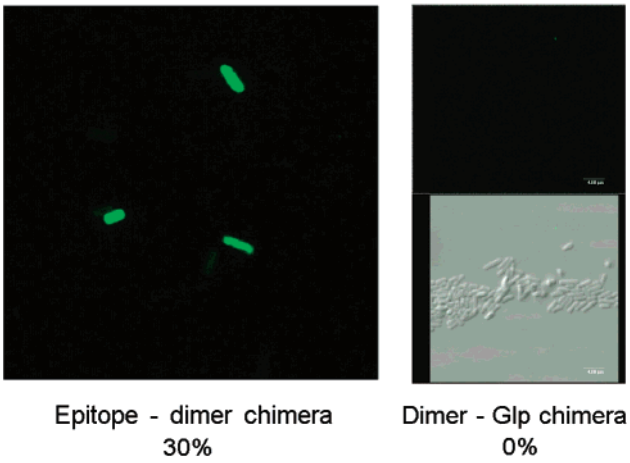

Figure 3. Penetration of fluorescein-labeled peptides into *E. coli* JC7623 cells. The NPK^d epitope−dimer chimera entered the cells and became homogeneously distributed in the intracellular space. The dimer−Glp-1 7−36 construct failed to enter *E. coli* cells, despite a large number of cells that are present according to differential interference contrast (DIC). The percentage of *E. coli* cells containing the labeled epitope−dimer chimera was calculated on the basis of the total number of cells identified by DIC.

No bacterial killing was observed in the case of the dimer−Glp chimera (Table 2), and this construct was unable to penetrate *E. coli* JC7623 cells (Figure 3, top right panel), despite vigorous bacterial growth as measured by DIC (Figure 3, bottom right panel). Pyrrhocoricins are less sensitive to C-terminal modifications than to N-terminal additions.[21] Because bacterial cell penetration is most likely initiated by the amino-terminal charges of the cationic antibacterial peptides[43] which are still intact in the dimer−Glp chimera, it is unlikely that the presence of the C-terminal peptide cargo per se is responsible for this loss of cell penetration. To further demonstrate that Glp-1 peptide 7−36 became nonfunctional in the dimer−Glp chimera, we conducted a limited set of insulin secretion assays. The construct did not induce insulin production *in vitro* using a HIT-1 hamster islet tumor cell line (data not shown). When C3H mice were subjected to an oral glucose tolerance test,[38] the blood sugar levels were identical for mice receiving *per os* 10−50 nmol of Glp-1 peptide alone (73−83 mg/dL) or the dimer−Glp chimera (71−83 mg/dL), once again demonstrating that the dimer−Glp chimera was not bioavailable.

Penetration into Human Fibroblasts. To gauge the general drug delivery properties of pyrrhocoricin-based

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transport systems, entry into human fibroblasts was examined. The cell penetrating ability of the epitope−dimer chimera was compared to that of the NPK^d epitope-KK peptide alone. The Pip-pyrr-MeArg dimer entered the cells efficiently which together with the *in vivo* efficacy of this pyrrhocoricin derivative[26] indicates that the human fibroblast cell line is a valid model for the membrane transport properties of epithelial cells. No fluorescing cells were detected following treatment with the NPK^d epitope C-terminally extended with two lysine spacers (Figure 2, top right). The Pip-pyrr-MeArg dimer−epitope chimera was even more successful in penetrating into fibroblasts than the Pip-pyrr-MeArg analogue alone (Figure 2, bottom right). A possible explanation for these findings is that the cationic NPK^d epitope potentiates the Pip-pyrr-MeArg dimer by strengthening the interaction with the negatively charged eukaryotic cell surface. Remarkably, the epitope−dimer construct stained all cell compartments (except the nucleus) equally well. This property could be highly advantageous for ensuing processing and/or association with intracellular recognition or transport molecules.

Peptide Immunogenicity. An IFNγ ELISPOT assay was used to determine whether the MHC class I binding epitope when part of the chimera retained its antigenic properties and whether immunization of BALB/c mice with the Pip-pyrr-MeArg dimer−epitope chimera could induce NPK^d epitope-specific IFNγ-secreting CD8$^+$ T-cells. The results (Figure 4) demonstrate that 7 days after priming a significant number of specific IFNγ-secreting CD8$^+$ T-cells were detected in the draining lymph nodes of Pip-pyrr-MeArg dimer−epitope primed mice, which is similar to the case in which mice received the NPK^d epitope-KK peptide alone. As a negative control, lymph node cells from mice given the Pip-pyrr-MeArg dimer were also tested, and exhibited insignificant numbers of IFNγ-secreting CD8$^+$ T-cells. The CD8$^+$ T-cells elicited by the epitope−dimer chimera also exhibited lytic activity as measured by their ability to lyse influenza virus-infected P815 target cells (Figure 5). Clearly, the NPK^d epitope within the epitope−dimer chimera was capable of eliciting a potent antigen-specific cytotoxic T-cell response.

Activation of Dendritic Cells. Having established the integrity of the cell penetrating and antigenic properties of the Pip-pyrr-MeArg dimer−epitope chimera, we studied the improvements in the pharmacological properties of drugs upon conjugation to the pyrrhocoricin-based delivery system by examining the ability of the epitope−dimer chimera to activate dendritic cells. Human monocyte-derived dendritic cells were incubated with NPK^d epitope alone, with the Pip-pyrr-MeArg dimer, or with the Pip-pyrr-MeArg dimer−epitope chimera. Bacterial lipopolysaccharide (LPS) was used as a positive control. The NPK^d epitope alone did not significantly stimulate the cells over the untreated control background, as documented by staining for markers indicative of dendritic cell maturation (Figure 6). The approximate 1.5-fold increase in the level of expression of surface markers was similar to the values obtained for the Pip-pyrr-MeArg

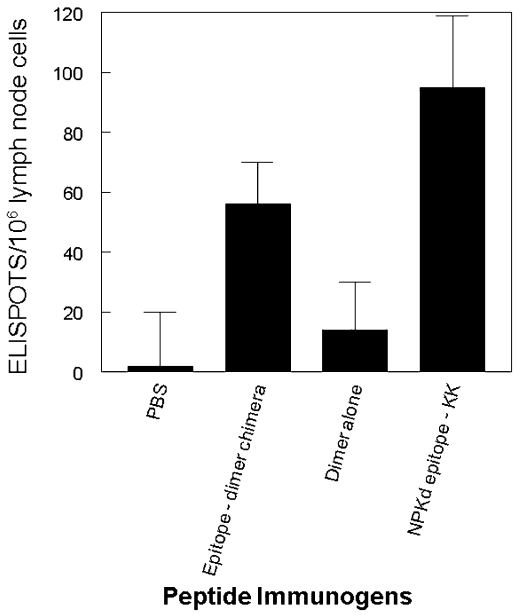

Figure 4. Comparison of the number of NPK^d epitope-specific IFNγ-secreting cells in the NPK^d epitope−dimer chimera, the NPK^d epitope-KK peptide, and Pip-pyrr-MeArg dimer immunized mice. Phosphate-buffered saline was used as a negative control. ELISPOT assays were performed on cells from the inguinal and popliteal lymph nodes of mice primed 7 days previously with the indicated immunogens emulsified in CFA. The data represent the number of ELISPOTs per million lymph node cells, with backgrounds in cultures lacking antigen subtracted. The results are expressed as the mean value obtained from an assay performed in triplicate, and error bars represent one standard deviation of the mean.

dimer alone. Individually, both components of the chimera proved to be weak activators of human dendritic cells. In contrast, the Pip-pyrr-MeArg dimer−epitope chimera effected a 3−3.5-fold (Figure 6) increase in the level of expression of these maturation markers. These data clearly demonstrated that the Pip-pyrr-MeArg peptide facilitated delivery of the epitope into APC.

COS Cell Toxicity. The toxicity of the Pip-pyrr-MeArg dimer and the epitope−dimer chimera to mammalian cells was studied by investigating their effect on COS-7 cells. These cells were originally transformed from monkey kidney, and as such are a better measure of toxic properties of antibacterial drugs than erythrocytes that are relatively resistant to antibacterial peptides.[24] The COS cells were incubated with 150−1000 μM peptide, and the number of surviving cells was counted. The applied concentration range was selected on the basis of the *in vivo* dose generally used for the pyrrhocoricin analogues. The dose of the epitope−dimer chimera for the immunization was 76 μg, which is a concentration of ~7.5 μM peptide in mouse blood. With the cell toxicity studies, we wanted to see whether we could reach a therapeutic index of 20; i.e., the peptide was not toxic at 150 μM. As the results in Table 2 demonstrate, the epitope−dimer chimera caused no measurable toxicity to COS cells at this concentration. Since the Pip-pyrr-MeArg

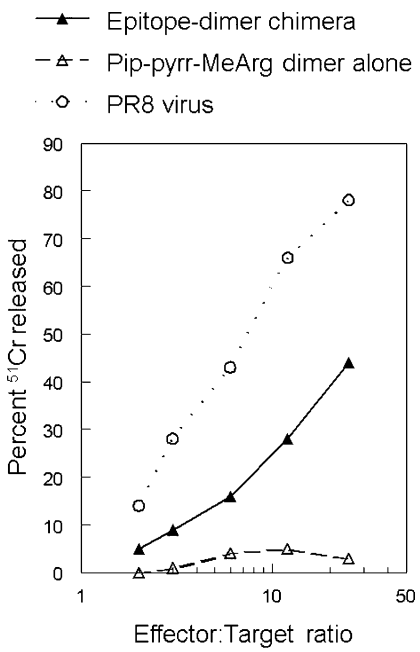

Figure 5. Lysis of target cells infected with influenza virus of the PR8 strain. P815 mastocytoma cells were subsequently labeled with ^{51}Cr and tested for lysis by peptide- or PR8-primed lymphocytes at various effector to target cell ratios.

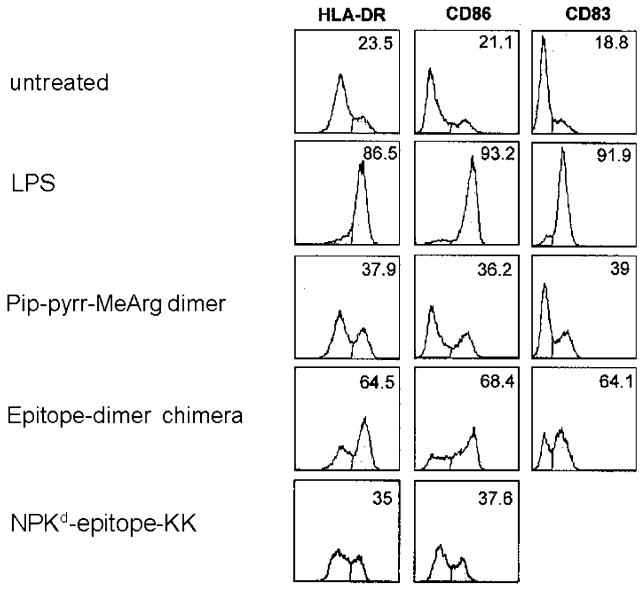

Figure 6. Activation of human dendritic cells by peptide derivatives. Human monocytes were differentiated into dendritic cells and were treated with 50 μM peptides for 2 days. As the surface markers indicate, the epitope−dimer chimera activates the cells significantly better than the NPK^{d} cytotoxic T-lymphocyte epitope alone.

dimer lacked COS cell toxicity up to the highest concentration that was studied (1 mM), we are confident that larger doses of pyrrhocoricin-based constructs will have no measurable toxicity. In contrast, magainin 2, a member of the antibacterial peptide family from *Xenopus* skin[44] known to disintegrate the bacterial membrane, destroys these COS cells at 300 μM.[26]

Discussion

Cellular Activities of Cationic Antimicrobial Peptides. Because of their ability to cross biological membranes, antimicrobial peptides are regularly tested for their drug delivery properties. Generally speaking, peptides penetrate bacteria more easily than they do mammalian cells. Native pyrrhocoricin for example is able to enter both Gram-positive and Gram-negative bacteria but not fibroblasts, although dendritic cells were found to be able to assimilate the peptide. Peptides with strong membrane disintegrating activity are able to overcome barriers to cell penetration, but aggressive activity at the cell membrane may destroy the host cell. It is clear that to make use of membrane-penetrating peptides as delivery vehicles, a balance needs to be struck between efficacious cell penetration and low toxicity. It is noteworthy that the Pip-pyrr-MeArg dimer was not toxic to COS cells either alone or as part of the epitope−dimer chimera. From the point of view of antibacterial activity, the lack of mammalian toxicity is due to the inhibition of a bacteria-specific intracellular protein rather than to activity at bacterial membranes. Magainin 2 is less active against *E. coli* than the Pip-pyrr-MeArg dimer but exhibits remarkably increased toxicity to mammalian cells.[45]

Mechanism of Cell Penetration. In general, cellular uptake of cationic cell-penetrating peptides is described as a process that does not involve endocytosis.[46,47] However, this mechanism is currently being re-evaluated. Using human cervical carcinoma HeLa and fibroblastic TM12 cells, the cellular uptake of fluorescent analogues of two antimicrobial peptides, magainin 2 and buforin II, was studied in comparison with the amino-terminal undecapeptide fragment of the representative cell-penetrating arginine-rich Tat peptide derived from human immunodeficiency virus-1 (HIV-1).[48] As expected from its membrane-disintegrating mode of action, magainin-based translocation is coupled with cytotoxicity. In contrast, the buforin peptide, having a midchain proline residue and acting on intracellular targets in bacteria, translocates to human cells within 10 min by a temperature-independent, less concentration-dependent passive mecha-

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nism without showing any significant cytotoxicity at the highest concentration that was investigated (100 μM). The level of cellular uptake of the fluorescein-labeled Tat peptide is proportional to the peptide concentration, and the concentration dependence is lost upon ATP depletion.[48] While the 11-mer Tat peptide containing no prolines is reported to exhibit a moderate cytotoxicity at higher concentrations,[48] when compared with the Pip-pyrr-MeArg dimer, in our hands the 13-mer Tat 68−80 peptide, the sequence of which includes two proline residues, exhibited no toxicity at all.

Under our mild cell fixation conditions, pyrrhocoricin and our designed dimer were evenly distributed in all bacterial cell compartments. It was recently demonstrated that cell fixation, even under mild conditions, leads to the artifactual uptake of cationic peptides, including that of Arg_9 and HIV-1 Tat 48−60.[49] For these peptides, fluorescence microscopy of live unfixed cells shows a characteristic endosomal distribution, and the kinetics of uptake are similar to the kinetics of endocytosis. The conclusion was made that even mild fixation disrupts the membrane barrier function, and the accumulation of the peptides in the cell nucleus may be fixation-dependent.[49] In our case, fixation does not appear to involve any altered cell distribution. When *E. coli* cells were treated with DnaK-binding pyrrhocoricin fragment 1−9 lacking the cell-penetrating C-terminal module, the peptide labeled only the membrane, as opposed to full-sized pyrrhocoricin that labeled the entire cell interior.[24] Likewise, in the current study, neither did pyrrhocoricin fragment 1−9 enter fixed *S. aureus* cells nor did the arginine-rich Pip-pyrr-MeArg dimer penetrate the cell nucleus of human fibroblasts (Figure 2), indicating that our cell fixation procedure did not modify the cellular distribution pattern of the peptides. These findings find support from recent studies conducted in other laboratories. Dermaseptins are a family of antimicrobial peptides that lyse target bacterial cells by destabilization of their membranes. At nontoxic concentrations, a fluorescently labeled dermaseptin fragment is able to penetrate intact cultured HeLa cells but essentially fails to enter their nuclei.[50] Covalent attachment of nuclear localization signal motifs of the SV40-T antigen and of the HIV-1 Rev protein confers karyophilic properties upon the same dermaseptin fragment.[50] Highly cationic SynB constructs, derived from yet another native antimicrobial peptide, protegrin, penetrate cells by an adsorption-mediated endocytosis process rather than temperature-independent translocation.[51] In these SynB peptides, the membrane-disintegrating Cys bridge component of protegrin is eliminated, and only the translocation-specific arginine-rich domain is retained. Thus, these peptides exhibit a reduced level of cellular uptake compared with more hydrophobic sequences, but also fail to induce cell permeabilization, suggesting that their interaction with the plasma membrane is not destabilizing in nature.

Prospects for Antimicrobial Peptide Design. Pyrrhocoricin entered the nonsusceptible bacterium *S. aureus*, although less efficiently than it entered the susceptible strain *E. coli*. In addition to the finding that a pyrrhocoricin-based peptide can penetrate the thickened cell wall of Gram-positive bacteria, our findings are also significant for the future design of novel antibacterial peptides. Since the DnaK sequences of many pyrrhocoricin nonsusceptible bacteria are known and the general fold of the DnaK multihelical region is identical for all these strains despite the amino acid alterations,[24] novel DnaK inhibitors can be either designed computationally or selected from peptide libraries. Recently, we identified the putative *E. coli* DnaK-binding pyrrhocoricin surface as Asp2, Tyr6, Leu7, and Arg9.[45] While modification of these residues can lead to the identification of novel and increasingly potent antimicrobial peptides for fighting currently susceptible or nonsusceptible pathogens, the rest of the peptide is able to serve as a general cell-penetrating module. Yet, our data also suggest that for efficacious translocation into *S. aureus* cells, the cell-penetrating unit also needs to be modified, perhaps by increasing the number of positive charges.

Antigen Delivery with Cationic Antimicrobial Peptides. Cationic antimicrobial peptides, once thought to have only bacterial killing properties, are also potent stimulators of the innate immune system.[52] For example, peptide LL-37 is shown to induce chemokine production and surface expression of chemokine receptors.[53] β-Defensins, murine antimicrobial peptides, bind murine chemokine receptor CCR6, and they attract bone marrow-derived immature but not mature dendritic cells.[54] Using various defensins fused with otherwise nonimmunogenic tumor antigens, some of the fusion constructs elicit humoral, protective, and therapeutic immunity against various syngeneic lymphomas.[54]

Pyrrhocoricin is a typical representative of the short, proline-rich antibacterial peptide family. Bac-7, another member of the proline-rich antibacterial peptide group (although not of insect origin), was used to deliver cargo

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into cells with a future goal of using this delivery system for peptide-based vaccine development.[55,56] In general, long fragments of Bac-7 containing both the antibacterial and intracellular delivery regions were bactericidal and cell-permeable, whereas short fragments with only a cationic or hydrophobic region were cell-permeant without microbicidal activity.[55] Interestingly, the common characteristic shared by the cell-permeant Bac-7 fragments, irrespective of their number of charged cationic amino acids, is their high proline content. Indeed, even a short 10-residue Bac-7 fragment with two arginine residues was capable of delivering noncovalently linked NeutrAvidin protein into cells. The importance of proline inserts between the arginine residues is supported by a cellular uptake study, in which polyarginine analogues are better absorbed if aminocaproic acid spacers are included.[57] These inserts are thought to work by increasing the degree of conformational freedom of the membrane-interacting arginine side chains. Recently, chimeric peptides consisting of Bac-7 delivery modules of various lengths and both B- and T-cell epitopes from melittin and the coat protein of gp120 of HIV-1 were synthesized.[56] It was noted that the longer the delivery modules were, the better intracellular penetration was observed. It was concluded that chimeras of cationic antibacterial peptides and T-cell epitopes can be preferred constructs for eliciting strong cellular immune responses.

Immunogenicity of the Constructs and Considerations for Vaccine Development. We found that the Pip-pyrr-MeArg dimer activated human dendritic cells only weakly. This correlates with the poor immunogenicity of the proline-rich antibacterial peptide family. Our attempts to elicit monoclonal antibodies in mice against a 20-mer peptide derived from the insect antimicrobial protein diptericin have been unsuccessful despite numerous attempts. The amino-terminal sequence of diptericin used for the immunizations is closely homologous with that of pyrrhocoricin[58] and may indicate that pyrrhocoricin is also poorly immunogenic. The feature of poor immunogenicity in transporting peptides is highly desirable because antibodies specific for the transporting moiety could restrict their use as delivery vehicles. The Pip-pyrr-MeArg dimer possesses other favorable properties. It is functional when administered orally and is nontoxic;[26] it is resistant to serum peptidases, and its synthesis is easily carried out.[22] Due to the limited size, or amino acid composition, of the cargo, the pyrrhocoricin-based drug delivery system is most suitable for short hormones (shorter than 30-mer Glp-1 fragment 7−36) or peptide antigens. Among these, peptide vaccines to cancer offer an immediate and well-studied need.

Despite the initial successes, the lack of a sufficiently vigorous immune response to control cancer growth *in vivo* is in part due to the poor immunogenicity of epitopes that are expressed by tumor cells.[59] In attempts to improve epitope immunogenicity, a variety of methods have been tried, including modifications to prevent proteolytic degradation,[60] optimization of antigen presentation,[61] and increased triggering of the T-cell receptor.[62] Conjugation of a lipid to MHC class I epitopes has been shown to improve immunogenicity.[35,63] The efficacy of lipidated epitope-based vaccines appears to correlate with dendritic cell activation.[13] The use of dendritic cells loaded with peptide antigens is a powerful and increasingly popular method of immunotherapy.[64] Clinically significant responses are observed when cancer patients are treated with dendritic cells loaded with melanoma-derived[65] or prostate-specific[66] MHC class I-restricted tumor-associated peptide antigens. Nevertheless, peptide-based vaccines aimed at the induction of effective T-cell responses against established cancers have so far only met with limited clinical success, and the conclusion was reached that these strategies clearly need to be improved.[67] Cationic, mostly

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polylysine-based delivery enhancers improve the uptake and MHC class I-dependent presentation of CTL peptide antigens *in vitro* and their immunogenicity *in vivo*.[12,68,69]

Conclusions

One of the major problems to be solved in increasing the efficacy of dendritic cell-based vaccines is the optimal loading of the professional antigen-presenting dendritic cells with peptides.[64] As presented here, the Pip-pyrr-MeArg dimer is a powerful delivery system for transporting peptide antigens into dendritic cells, and this cell penetration results in vigorous dendritic cell activation. Thus, the pyrrhocoricin-based epitope delivery system appears to overcome some of the current obstacles of dendritic cell-based immune therapy and may be a valuable addition to a growing arsenal of immune stimulators. Generally, the native sequence of pyrrhocoricin featuring positively charged residues interspersed with prolines, together with our modifications to improve cell penetration, stability, and other pharmacological parameters, presents the Pip-pyrr-MeArg dimer as a strong competitor to the currently used cell permeable peptides considered for drug delivery.

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