Effect of Amino Acid Substitutions on 70S Ribosomal Binding, Cellular Uptake, and Antimicrobial Activity of Oncocin Onc112

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Proline-rich antimicrobial peptides (PrAMPs) are promising candidates for the treatment of infections caused by high-priority human pathogens. Their mode of action consists of (I) passive diffusion across the outer membrane, (II) active transport through the inner membrane, and (III) inhibition of protein biosynthesis by blocking the exit tunnel of the 70S ribosome. We tested whether *in vitro* data on ribosomal binding and bacterial uptake could predict the antibacterial activity of PrAMPs against Gram-negative and Gram-positive bacteria. Ribosomal binding and bacterial uptake rates were measured for 47 derivatives of PrAMP Onc112 and compared to the minimal inhibitory concentrations (MIC) of each peptide. Ribosomal binding was evaluated for ribosome extracts from four Gram-negative bacteria. Bacterial uptake was assessed by quantifying each peptide in the supernatants of bacterial cultures. Oncocin analogues with a higher net positive charge appeared to be more active, although their ribosome binding and uptake rates were not necessarily better than for Onc112. The data suggest a complex mode of action influenced by further factors improving or reducing the antibacterial activity, including diffusion through membranes, transport mechanism, secondary targets, off-target binding, intracellular distribution, and membrane effects. Relying only on *in vitro* binding and uptake data may not be sufficient for the rational development of more active analogues.

Introduction

Antibiotics revolutionized medicine in the 20th century and are essential for survival in many clinical situations. However, preventative or inappropriate applications without confirming the pathogen, for example in countries without a prescription requirement, the broad application in life-stock farming, and production with limited environmental regulations favor resistance development, exacerbating the resistance situation.[1] While the cases of life-threatening infections with multi- or pan-resistant pathogens rise globally, only very few new substances relying on novel bactericidal mechanisms of action are approved or in advanced clinical phases.

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Antimicrobial peptides (AMPs) are expressed as part of the innate immune system in all classes of living organisms.[2,3] Thus, they were recognized as promising lead structures for the development of new antibiotics. Proline-rich AMPs (PrAMPs), which represent a subclass of cationic AMPs, show promising antimicrobial activity against several Gram-negative pathogens.[4] Due to the class-defining high proline content, PrAMPs are relatively flexible unstructured peptides with a typically minimal membranolytic activity and intrinsic protease stability. The high content of lysine and arginine residues results in a positive net charge facilitating their attachment to the negatively charged bacterial membrane.[2,5,6] After passive diffusion across the outer membrane of Gram-negative bacteria,[2] PrAMPs of the oncocin-type are internalized into the cytosol by SbmA and MdtM transporter systems.[7–9] Oncocins exert their bactericidal effect by blocking the peptide exit tunnel of the 70S ribosome. They prevent the elongation of the nascent protein chain and destabilize the initiation complex, thereby interrupting protein biosynthesis.[10]

In previous studies, we optimized the sequence and structure reported for the 2-kDa *Oncopeltus* antibacterial peptide 4, a PrAMP identified in *Oncopeltus fasciatus* (milkweed bug),[11] to obtain oncocin and further substituted analogues with broad-spectrum antimicrobial activity.[12–14] Modifications of the C-terminal residues and amidation of the C-terminus increased the antimicrobial activity against Gram-negative bacteria.[12] Efforts to improve the protease stability of peptides resulted in the designer peptide Onc112 providing a serum half-life time exceeding eight hours, which was achieved by substituting arginine at positions 15 and 19 with D-arginine.[15] In addition, a small structure-activity relationship study of

oncocin (Onc18, VDKPPYLPRPRPPRRIYNR-NH_2,) revealed the favorable substitutions Asp2Arg and Pro12Trp enhancing the antimicrobial activity against both *P. aeruginosa* and *S. aureus*.[14]

The recently reported crystal structure of Onc112 (VDKPPYLPRPRPPRrIYNr-NH_2, r: D-arginine) in complex with the 70S ribosome of *Thermus thermophilus* reveals the binding site[16,17] and facilitates rational optimization of the amino acid sequence of Onc112 for stronger ribosomal binding. The interactions of Onc112 with 23S rRNA are mainly based on hydrogen bonds and π-stacking interactions of aromatic side chains. The weak electron density after Pro10 indicates a weaker interaction of the peptide residues beyond position 10 with the ribosome.

Based on these results, monosubstituted Onc112 and [Pro12Trp]-Onc112 (Onc272) analogues were synthesized to evaluate the effects of amino acid substitutions on ribosomal binding and bacterial uptake to extend the antimicrobial potential of PrAMPs. Furthermore, the predictive power of independently collected *in vitro* data regarding the minimal inhibitory concentrations (MIC) as a measure of antibacterial activity in whole cell assays was investigated.

Experimental Section

Materials

AppliChem GmbH (Darmstadt, Germany): HEPES (≥99.5%); Biosolve BV (Valkenswaard, Netherlands): acetonitrile (HPLC gradient grade), formic acid (≥99%); Carl Roth GmbH & Co. (Karlsruhe, Germany): Lysogeny broth (LB) Miller, nutrient broth, agar-agar (Kobe I), sodium dodecyl sulfate (ultrapure, ≥99%); EMD Millipore Calbiochem® (Darmstadt, Germany): Casein (≥95%); Honeywell Fluka™ (Seelze, Germany): ammonium chloride (≥99.8%), magnesium chloride (≥99%); Iris Biotech GmbH (Marktredwitz, Germany): Rink amide resin; Life Technologies (Darmstadt, Germany): penicillin/streptomycin (10,000 units/mL, GIBCO®), phosphate buffered saline (PBS 1X, pH 7.4, GIBCO®), fetal bovine serum, DMEM/F12 medium, trypsin-EDTA (0.05%); Serva Electrophoresis GmbH (Heidelberg, Germany): Tween® 20 (pure); Sigma Aldrich (Steinheim, Germany): 2-mercaptoethanol (≥99%); 5(6)-carboxyfluorescein (Cf, for fluorescence), disodium hydrogen phosphate ×12 H_2O (≥99%), methylthiazolyldiphenyl-tetrazolium bromide (MTT, ≥97.5%), Mueller-Hinton broth 2 (MHB2, for microbiology, cation-adjusted), meropenem trihydrate (≥98%), potassium dihydrogen phosphate (≥99%), potassium hydroxide (>90%), sodium chloride (≥99.5%), trifluoroacetic acid (TFA, for HPLC, ≥99%); Thermo Fisher Scientific Inc. (Darmstadt, Germany): DNase I (RNase-free, 1 u/μL).

Fmoc-protected amino acids for peptide synthesis were purchased from Iris Biotech GmbH or Orpegen Pharma GmbH (Heidelberg, Germany) including 3,5-diiodotyrosine (Dit), 3-nitrotyrosine (Nty), norleucine (Nle), *tert*-leucine (Tle), ornithine (Orn or O), homoarginine (Har), and D-arginine (D-Arg or r).

Bacteria strains: *Escherichia coli* BW25113 (lacI^q rrnB3 ΔlacZ4787 hsdR514 DE(araBAD)567 DE(rhaBAD)568 rph-1, Keio collection), *Escherichia coli* DSM 1103 (or ATCC 25922™), *Escherichia coli* MC4100, *Pseudomonas aeruginosa* DSM 1117 (also called ATCC 27853™), *Klebsiella pneumoniae* DSM 681 (or ATCC 10031™), *Acinetobacter baumannii* DSM 30008 (or ATCC 15308™), *Staphylococcus aureus* DSM 6247 (DSMZ - German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany).

Water was purified on a Purelab Ultra water purification system (electrical resistivity >182 kΩ·m; organic content <2 ppb; ELGA LabWater, Celle, Germany).

Peptide synthesis

Peptides were synthesized on Rink amide resin using the multiple synthesizer SYRO2000[12,18] and purified to >90% by RP-HPLC (Äkta Purifier 10, GE Healthcare Life Science, Solingen, Germany) using a linear gradient of aqueous acetonitrile containing 0.1% TFA. Peptide Onc112 was N-terminally labeled with 5(6)-carboxyfluorescein for fluorescence polarization assays.[10]

Broth microdilution assay

MICs were determined in 96-well plates (flat bottom, ref. 655180, Greiner Bio-One GmbH, Frickenhausen, Germany) using a final volume of 0.1 mL. Peptides were dissolved in water (3 g/L) and serially twofold diluted in 25% Mueller-Hinton-Broth 2 (25% MHB2; 5.5 g/L) to final concentrations of 128 to 1 μg/mL. Overnight cultures of bacteria were diluted 30-fold in fresh 25% MHB2 and incubated for four hours (37 °C, 200 rpm). Cell counts were adjusted using a McFarland Standard (bioMérieux® Deutschland GmbH, Nurtingen, Germany) and aliquots of 50 μL were transferred to each well to obtain a final cell count of 7.5×10^6 cells/mL. Plates were incubated at 37 °C for 18±2 hours before the optical density was measured at 595 nm (Victor3™, PerkinElmer Inc., Waltham, MA, USA). The MIC was defined as the lowest peptide concentration preventing visible bacterial growth. Experiments were performed in triplicates and repeated at least once on another day.

70S ribosome preparation

Bacterial 70S ribosomes were prepared from *E. coli*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *S. aureus* as previously reported.[19] Briefly, bacteria were grown in LB medium (37 °C, 180 rpm) until mid-logarithmic growth phase ($OD_{600}\approx1$) and harvested by centrifugation (5000×g, 4 °C, 15 min). Cell pellets were washed with HEPES buffer (20 mmol/L HEPES, 30 mmol/L NH_4Cl, 6 mmol/L $MgCl_2$, 4 mmol/L 2-mercaptoethanol, pH 7.6) and stored at −80 °C. Prior to lysis, cell pellets were suspended in HEPES buffer (2 mL/g cells) and disrupted with a bead mill homogenizer (BeadBeater, BioSpec Products Inc., Bartlesville, OK, USA) in five intervals of one minute each. *S. aureus* was lysed by addition of 0.4 g/L lysozyme[20] prior to disruption. All lysates were mixed with DNase (5 U/mL) and meropenem (280 μg/mL) and incubated on ice for one hour. Lysates were cleared by two centrifugation steps (16,000×g, 30 min, 4 °C; 32,000×g, 60 min, 4 °C) and ribosomes were pelleted by ultracentrifugation of the supernatant (165,000×g, 17 h, 4 °C). Ribosome pellets were suspended in HEPES buffer (0.05–0.1 mL/g pellet) and stored at −80 °C. The ribosome concentration was determined by measuring the absorbance of RNA at 260 nm and considering that 1 AU corresponds to a ribosome concentration of 28 pmol/mL. The molecular weight of the 70S ribosome was assumed to be 2.3 MDa.

Fluorescence polarization assays

All fluorescence polarization (FP) assays were carried out in black 384 well plates (flat bottom, ref. 781209, Greiner Bio-One GmbH) with a total assay volume of 40 μL. Plates were blocked with 0.5% (w/v) casein in phosphate-buffered saline (PBS; 8.8 mmol/L

$Na_2HPO_4 \times 12\ H_2O$, 1.2 mmol/L KH_2PO_4, 0.3 mol/L NaCl, pH 7.4) containing 0.05% (w/v) Tween® 20 (PBST) at 4 °C overnight and washed three times with PBST. Peptides and ribosomes were diluted in HEPES buffer. FP measurements were performed on a microplate reader at 28 °C with excitation (λ_{exc}) and emission wavelengths (λ_{em}) of 485 nm and 535 nm, respectively. All experiments were performed in triplicate and repeated at least once on another day.

Inhibition constants (K_i) were determined for Onc112 as reported earlier.[21] Briefly, unlabeled Onc112 was serially twofold diluted in HEPES buffer to final concentrations of 82 μmol/L to 20 pmol/L (20 μL). The bacterial ribosome extract was diluted in HEPES buffer (concentration of 1 μmol/L) and an aliquot was transferred to each well (10 μL) to obtain a final concentration of 250 nmol/L. After an incubation period of 90 minutes in the dark, a 5(6)-carboxyfluorescein (Cf)-labeled Onc112 (10 μL, 80 nmol/L) was added to each well (final concentration 20 nmol/L). Fluorescence polarization (FP) was measured after an incubation period of 90 minutes using the PARADIGM™ microplate reader (Beckman Coulter, Krefeld, Germany). Half-maximal inhibitory concentrations (IC_{50}) were calculated by fitting experiments to a dose-response curve with a variable slope parameter $[y = \min + (\max\text{-}\min)/(1 + (x/IC_{50})^{-Hill\ slope})]$ using SigmaPlot 13 (Systat Software Inc., San Jose, CA, USA). Inhibition constants (K_i) were calculated using equations reported by Mathias and Jung.[22] K_i were determined against ribosome extracts of *E. coli* BW25113, *P. aeruginosa* DSM 1117, *K. pneumoniae* DSM 681, *A. baumannii* DSM 30008, and *S. aureus* DSM 6247.

First, all 47 Onc112 and Onc272 analogues were tested for binding to 70S ribosomes isolated from *E. coli* and *A. baumannii.* Three peptide concentrations were chosen based on the IC_{50} curve of reference peptide Onc112 corresponding to approximately 20%, 50%, and 80% inhibition of the competitive binding curve of Onc112 against Cf-Onc112 for each bacterium. Unlabeled peptides were diluted in HEPES buffer to obtain concentrations of 300 nmol/L (*E. coli*) or 400 nmol/L (*A. baumannii*) corresponding to ~80% of the IC_{50} values (Table S1). Aliquots of these peptide solutions (20 μL) were transferred to each well and the bacterial ribosome extract (1 μmol/L; 10 μL) was added (Table S1). After an incubation period of 90 minutes, Cf-Onc112 was added (10 μL, 80 nmol/L). Each plate additionally contained three maximum controls lacking the competing peptide and three minimum controls containing neither the peptide nor the 70S ribosome. Fluorescence polarization was measured after 90 minutes incubation in the dark on a PARADIGM™ multiplate reader (Beckman Coulter). Lower FP values than reference peptide Onc112 indicate a stronger displacement of the labeled competitor and therefore a higher affinity to the 70S ribosome was assumed.

Having established the assay conditions for two bacteria, the peptide binding for the 70S ribosomes of *K. pneumoniae* and *P. aeruginosa* was studied using an automated liquid handling Biomek FX^P workstation (Beckman Coulter GmbH). Final concentrations of unlabeled peptides were 80 nmol/L for *K. pneumoniae* and 40 nmol/L for *P. aeruginosa.* The ribosome concentration was adjusted to the K_d of the competitor Cf-Onc112, i.e., 77 nmol/L for *K. pneumoniae* and 36 nmol/L for *P. aeruginosa.* Unlabeled peptide (20 μL) was mixed with the ribosome solution (10 μL) in the well and incubated for 90 minutes before the Cf-labeled peptide (10 μL, final concentration 6.7 nmol/L) was added. Each plate contained six maximum control wells with 6.7 nmol/L Cf-labeled peptide and 77 nmol/L (*K. pneumoniae*) or 36 nmol/L (*P. aeruginosa*) ribosome and six minimum control wells with 6.7 nmol/L Cf-labeled peptide. After an incubation period of 90 minutes in the dark, fluorescence polarization was measured on an Infinite F500 microplate reader (Tecan, Männedorf, Switzerland). FP values of oncocin analogues were compared to reference peptide Onc112.

Bacterial uptake assay

The bacterial uptake of peptides was studied in a high throughput 96-well plate format as described before.[19] Briefly, aqueous peptide solutions were diluted in 25% MHB2 and aliquots (50 μL, 16 μg/mL) were transferred to a sterile 96-well plate (polystyrene, V-bottom, ref. 651180, Greiner Bio-One GmbH). Overnight cultures of bacteria grown in 25% MHB2 were diluted 30-fold with fresh 25% MHB2 and incubated for 4.0 ± 0.25 hours (37 °C, 200 rpm). The cell density was estimated by recording the optical density at 600 nm assuming that an optical density of 1.0 corresponds to 1.2×10^9 cells/mL. After centrifugation of the cell culture (4 °C, 10 min, 4,000 × g), the pellet was dissolved in a smaller volume of 25% MHB2 to adjust the cell count to 1.5×10^{10} cfu/mL. An aliquot (50 μL) of the cell suspension was added to each well (final concentration: 7.5×10^9 cells/mL). Plates were incubated for 30 minutes (37 °C, 750 rpm; Thermomixer, Eppendorf AG, Hamburg, Germany or Titramax 1000 Incubator 1000, Heidolph Instruments, Schwabach, Germany). After centrifugation (4 °C, 10 min, 1,200 × g), 50 μL of the supernatant were taken for quantitation of the remaining peptide. Residual medium supernatant was removed from the bacterial cell pellets and pellets were stored at −20 °C until lysis. The initial viable cell count was determined by diluting an aliquot (5 μL) of the cell suspension serially hundredfold with 0.9% (w/v) sodium chloride and spreading the dilution series onto nutrient agar plates (10 cm, 50 μL per half plate) to obtain 10 to 100 colonies. Colonies were counted after incubation of the plates under aerobic conditions overnight (37 °C) to determine the colony forming units (cfu). The cell density estimated by the OD_{600} value was corrected accordingly (cfu/mL).

Peptide quantitation

Peptides in the medium supernatants were enriched using solid phase extraction (SPE; Chromabond C18 endcapped 96-well plate, 25 mg sorbent per well, particle size 45 μm, Machery-Nagel GmbH Co. KG, Düren, Germany) as reported with minor adjustments.[19] The stationary phase was conditioned with acetonitrile (500 μL) and aqueous acetonitrile (40%, v/v; 500 μL) containing formic acid (0.1%, v/v) and equilibrated with aqueous TFA (0.1%, v/v; 500 μL). Medium supernatants (50 μL) were mixed with aqueous TFA (0.2% v/v; 50 μL) containing 50 ng of either Onc112 or isotope labelled Onc112 as internal standard and stored on ice before they were diluted with aqueous TFA (0.1% v/v; 150 μL). An aliquot (230 μL) was loaded onto the SPE-plate and washed twice with aqueous formic acid (0.1%, v/v; 2× 1 mL). Samples were eluted with 300 μL of aqueous acetonitrile (30%, v/v) containing formic acid (0.1%, v/v) and stored at −20 °C until evaporation. Eluates were dried in a vacuum centrifuge (60 °C; SpeedVac, Thermo Fisher Scientific Inc.) and stored at −20 °C until further processing. Samples containing polar peptides were reconstituted in 20 μL of aqueous acetonitrile (10% v/v) containing formic acid (0.1% v/v) and sonicated for 5 min (Bandelin electronic GmbH & Co. KG, Berlin, Germany). Samples containing hydrophobic peptides were reconstituted in 6 μL of aqueous acetonitrile (50% v/v) containing formic acid (0.1% v/v), diluted with formic acid (0.1% v/v; 14 μL) and sonicated for 5 min. Aliquots (8 μL) were analyzed on a Waters ACQUITY Ultra Performance Liquid Chromatography (UPLC, M-Class) system coupled online to an electrospray ionization quadrupole time-of-flight mass spectrometer (ESI-QTOF-MS, Synapt G2-Si MS, Waters, MS Technologies, Manchester, UK). Samples were separated on a C_{18}-column (Jupiter C18, Phenomenex®, internal diameter 1 mm, length 150 mm, particle size 5 μm, pore size 30 nm) using a linear gradient from 5 to 25% aqueous acetonitrile containing 0.1% formic acid in 10 minutes, a flow rate of 50 μL/min, and a column temperature of 55 °C. Samples were analyzed using parallel reaction monitoring selecting the most intense precursor for fragmentation and the

most intense fragment ion for quantitation. Further details about transitions and instrument settings are provided as supplementary material (Tables S2 and S3). A serial dilution of each peptide in 25% MHB2 was used for peptide quantitation. Onc112 or isotope-labeled Onc112 with a mass shift of +6 Da[23] was used as internal standard to correct the signal intensities of each peptide before quantitation by the dilution series. Each experiment was repeated on another day.

Cell viability assay

Cells were cultured in DMEM/Ham's F12 medium containing 10% (v/v) fetal bovine serum and 1% (w/v) penicillin/streptomycin at 37 °C and 5% CO_2. Human hepatoma (HepG2) and human embryonic kidney cells (HEK293) were seeded (2×10^5 cells in 200 μL per well) in the same medium in 96-well plates (flat bottom, ref. 655180, Greiner Bio-One GmbH) and incubated for 24 hours (37 °C, 5% CO_2). The medium was discarded, the cells were washed with PBS (100 μL/ well), fresh medium containing the peptide (600 μg/mL) to be tested was added (100 μL/well), and the plate was incubated (37 °C, 5% CO_2, 20 hours). Cell viability was determined using methylthiazolyldiphenyltetrazolium bromide (MTT).[12] Briefly, the medium was replaced by fresh medium (90 μL) and an MTT solution (5 g/L in PBS; 10 μL). After incubation for 4 hours (37 °C, 5% CO_2), the formazan crystals were solubilized with sodium lauryl sulfate (SDS, 10%, w/v) dissolved in hydrochloric acid (10 mmol/L). Absorbance was measured at 570 nm after 20 hours and corrected by the absorbance at 650 nm. Results were normalized to controls with 12% PBS set to 100% cell viability.

Results

Peptide design

Based on the reported X-ray structures of the 70S ribosome of *T. thermophilus* in complex with Onc112,[16,17] we identified certain positions of Onc112 for which amino acid substitutions may allow further interactions with the ribosomal binding site (Table S4). Considering unoccupied space in the binding pocket as well as potential conformational restrictions, the residues Asp2, Lys3, Pro4, Pro5, Tyr6, Leu7, Pro10, Arg11 and Pro12 were selected for our approach due to their interactions with or close proximity to the ribosomal proteins and RNA in the reported structure. Because residues Pro13 to Arg19 were not modeled in the crystal structure, possibly due to inherent flexibility of these residues, a structure-based approach could not be used. Amino acid substitutions were subsequently chosen to increase ionic interactions via basic (Arg, Lys, His, Orn, Har, and D-Arg) or acidic residues (Asp and Glu), hydrophobic interactions (Leu, Ile, Val, Met, Nle, and Tle), and hydrophobic and aromatic (π-stacking) interactions (Phe, Tyr, Dit, and Nty). These substitutions should affect ligand-target interactions based on reported hydrogen bonds (Asp2 and Lys3), π-stacking (Pro5, Tyr6, Leu7, and Arg11), and hydrophobic interactions (Pro4).[16,19] Although no direct interaction has been described for Pro10, it can be speculated that Pro10 guides adjacent residues Arg9 and Arg11 into a proper orientation to interact with nucleotide bases. Amino acids with larger side chains (Arg, Met, Phe, and Trp) were tested for Leu7 and Pro10 as well as isomers of Leu7 to further stabilize the region by presumably stronger interactions with the ribosome. Substitutions of Tyr6 with 3-nitrotyrosine and 3,5-diiodotyrosine were assumed to decrease the antimicrobial activity due to weaker ribosomal interactions. In contrast, ribosomal binding should not be weakened by substituting Tyr17 with 3-nitrotyrosine or 3,5-diiodotyrosine, because the binding affinity of the residues in the C-terminal part of Onc112 appears to be low considering the weak electron density in the X-ray crystal structure C-terminal to Pro10. A previously reported SAR-study indicated an increased activity against *P. aeruginosa* when replacing Pro12 with tryptophan; at least for oncocin.[14] Thus, we assumed that Pro12Trp might add additional binding affinity to the C-terminal region of Onc112, which would allow further optimizations of peptide [Pro12Trp]-Onc112 named Onc272 by substituting position 13 leading to peptides Onc273 to Onc290.

In total, 29 monosubstituted analogues of Onc112 were synthesized to study their potential for 70S ribosome binding and their bacterial uptake for *E. coli*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *S. aureus* (Tables S2 and S4). Furthermore, 18 analogues of Onc272 were evaluated by substituting Pro13 with ornithine or another canonical amino acid except methionine and cysteine.

Antimicrobial activity

Compared to Onc112, several monosubstituted oncocin derivatives were significantly more active against the tested bacteria while most analogues were equally or less active (Table 1, Table S5). For some of the three tested *E. coli* strains,

Table 1. Minimal inhibitory concentrations (MIC) determined for Onc112 and analogues of Onc112 and [Pro12Trp]-Onc112, i.e., Onc272 active against all tested bacteria. MIC values of Onc112 were published earlier.[19]

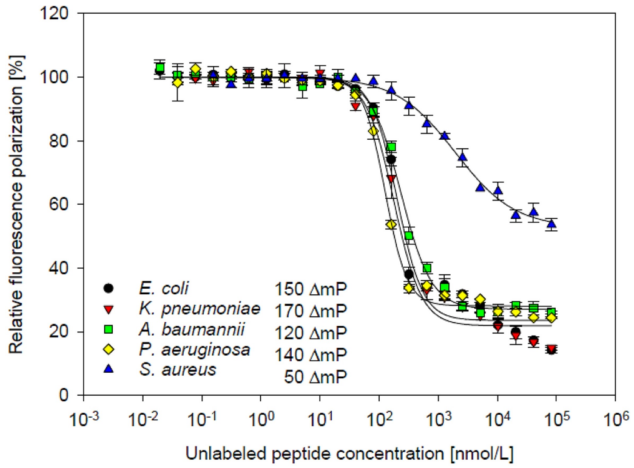
Peptide	MIC [mg/L]				
	E. coli BW25113	*K. pneumoniae* DSM 681	*A. baumannii* DSM 30008	*P. aeruginosa* DSM 1117	*S. aureus* DSM 6247
Onc112	8	2	32	64	64
[Asp2Arg]-Onc112	8	16	8	32	16
[Asp2Trp]-Onc112	8	16	16	32	16
[Pro4Lys]-Onc112	8	8	16	32	32
Onc272	4	4	16	128	64
[13Arg]-Onc272	8	2	16	32	16
[13Lys]-Onc272	8	4	16	32	16
[13Orn]-Onc272	8	4	16	32	8

substitutions Pro4Lys, Tyr6Trp, Tyr6His, Arg11Har, and Pro12Trp increased the antimicrobial activity twofold (MIC = 4 μg/mL) with Onc256 being equally active against all three *E. coli* strains, while the already high activity of Onc112 against *K. pneumoniae* (MIC = 2 μg/mL) was not further increased. Incorporation of the sterically demanding 3,5-diiodotyrosine abolished the activity for both bacteria (MIC ≥ 64 μg/mL). Similarly, [Leu7Phe]-Onc112 was inactive against *K. pneumoniae* (MIC > 128 μg/mL). *P. aeruginosa* was twofold and *A. baumannii* and *S. aureus* were two- to fourfold more susceptible when Asp2 was substituted against arginine or tryptophan. Again, peptides Tyr6Dit and Leu7Phe showed significantly higher MIC values against these bacteria, while other substitutions were better tolerated.

Next, analogues of Onc272 were tested. Expectedly, the high activity against *E. coli* was not further increased, but peptides Onc274, Onc277, Onc282, Onc284, and Onc285 were twofold more active than Onc272 against *K. pneumoniae* reaching the same activity as Onc112 with a MIC value of 2 μg/mL. [13Phe]-Onc272 (Onc279) was twofold more active than Onc272 and fourfold more active than Onc112 against *A. baumannii* (MIC = 8 μg/mL), but eightfold and fourfold less active against *E. coli*, respectively. Substitution Pro13Gly (Onc284) improved the activity twofold against both *P. aeruginosa* and *S. aureus*. Higher antimicrobial activities against *P. aeruginosa* and *S. aureus* were achieved when Pro13 was substituted with basic amino acids arginine, lysine, and ornithine or a second tryptophan. Peptide [13Orn]-Onc272 (Onc290) showed a very promising broad-spectrum activity against all five bacteria with MIC values ≤ 8 μg/mL for the tested *E. coli*, *K. pneumoniae*, and *S. aureus* strains (Table 1).

Determination of inhibition constants

The K_i values determined by fluorescence polarization (FP) for Onc112 and 70S ribosomes prepared from all four Gram-negative bacteria were in the low nanomolar range, i.e., 44 ± 4 nmol/L for *E. coli*, 45 ± 2 nmol/L for *K. pneumoniae*, 56 ± 2 nmol/L for *A. baumannii*, and even 17 ± 1 nmol/L for *P. aeruginosa* (Figure 1). The K_i measured for the 70S ribosome preparation of *S. aureus* was 302 ± 66 nmol/L, which still indicated a strong binding. All K_i values are in the same range as the recently reported dissociation constants (K_d) demonstrating a good reproducibility of the sample preparations and assay conditions.[19] The dynamic range (ΔmP) was at least 100 for 70S ribosomes of Gram-negatives but only 50 for the 70S ribosome of *S. aureus* indicating slight differences in the composition of 70S ribosome extracts from Gram-negative and Gram-positive bacteria. This effect was already observed in the past when measuring K_d values.[19] Presumably, the ribosome concentration in the *S. aureus* ribosome extract determined via the RNA-content was overestimated leading to an excess of Cf-Onc112, which could not bind, decreasing the measured FP values. However, when a threefold higher ribosome concentration was used to determine the K_i, the dynamic range did not increase. In addition, the autofluorescence recorded for the *S. aureus* ribosome extract was ten times higher than for the ribosome extracts of all four Gram-negative bacteria. This is most likely attributed to the presence of small fluorescent molecules in the extract leading to a higher depolarization and thus lower maximum FP values. Due to these encountered problems and the rather low quantities obtained by the ribosome preparation, the *S. aureus* 70S ribosome was not further studied in the competitive FP assay. This should be addressed in future studies, as the K_d values of Onc112 indicate a strong binding that can most likely be further optimized extending the known activity spectrum of PrAMPs from *Enterobacteriaceae* to Gram-positive pathogens including *S. aureus*. Therefore, the K_d of cf-Onc112 was determined using 70S ribosome from *S. aureus* purified by ion exchange chromatography[24] to compensate for autofluorescence. The K_d value of 78 nmol/L measured here for the ribosome extract was slightly lower than the K_d value of 102 nmol/L reported for Onc112.[19] Interestingly, the K_d value increased around twofold



Bacterium	MIC [μmol/L]	K_d [nmol/L]	K_i [nmol/L]
E. coli	3.4	75 ± 4	44 ± 4
K. pneumoniae	0.8	77 ± 1	45 ± 2
A. baumannii	13.4	73 ± 4	56 ± 2
P. aeruginosa	26.8	36 ± 2	17 ± 1
S. aureus	26.8	102 ± 5	302 ± 66

Figure 1. Fluorescence polarization to determine the inhibition constants (K_i) of Onc112 for 70S ribosome preparations obtained from *E. coli* BW25113, *K. pneumoniae* DSM 681, *A. baumannii* DSM 30008, and *P. aeruginosa* DSM 1117 (all 250 nmol/L) as well as *S. aureus* DSM 6247 (750 nmol/L) in the presence of Cf-Onc112 (20 nmol/L) as competitor. MIC and K_d were reported earlier (Kolano et al. 2020).

after purification (158 nmol/L), which still indicates a high binding affinity confirming the ribosome as main target. It remains open if the slight differences obtained for the ribosome extract and the purified ribosome are due to the loss of a second binding partner of Onc112 or slightly different ribosome concentrations, which were calculated from the RNA content. However, this aspect was not further investigated here and should be addressed in future research. Importantly, the dynamic range increased drastically with the purified ribosome (ΔmP = 290), suggesting that autofluorescence of the crude ribosome extract affected the measured fluorescence intensity.

Competitive fluorescence polarization assay

The binding affinity of all monosubstituted Onc112 and Onc272 analogues was studied for 70S ribosome preparations of four Gram-negative bacteria in a competitive fluorescence polarization assay using a 384-well format. Onc112 binding was used as a reference to identify analogues with improved binding characteristics and presumably improved antibacterial activities. First, the optimum concentrations of the ribosome and Cf-Onc112 as competitor were determined for maximum sensitivity and dynamic range ΔmP, which was defined as the difference between the highest and lowest FP values.

The optimized screening conditions for the 70S ribosome preparations of *E. coli* and *A. baumannii* appeared to be a competitor concentration of 20 nmol/L, as in the K_d experiments, and ribosome concentrations of 250 nmol/L, i.e., the concentration corresponding to 80% of the maximum FP observed in the K_d experiment.[19] This ribosome concentration corresponded to three times the K_d providing a large dynamic range exceeding 100 mP and consequently a high sensitivity. The unlabeled analogues of Onc112 and Onc272 were added at a concentration corresponding to the IC_{50} of reference peptide Onc112 enabling identification of stronger and weaker binders in one experiment. Compared to Onc112, higher FP values indicate a weaker binding, as the displacement of competitor Cf-Onc112 from the ribosome is less efficient and thus remains in the bound state, while lower FP values indicate a better binding. The steepness of the K_i curves (Hill slopes of around −2) allowed detection of even small differences in ribosomal binding.

The relative FP values determined for Onc112 (rFP_{Onc112}) competing with Cf-Onc112 for the 70S ribosome binding were used as reference for each bacterium (Figure 2, red solid line). Considering the standard deviation (SD) obtained for Onc112 in this experiment, FP values of analogues differing by at least three times this standard deviation (Figure 2, red dotted line) were considered to identify significantly stronger (rFP(analogue) < rFP(Onc112) - 3x SD) or weaker binder peptides (rFP(analogue) < rFP(Onc112) + 3x SD).

When the *E. coli* BW25113 ribosome was tested first, Onc112 displaced Cf-Onc112 partially from the 70S ribosome leading to a rFP value of 64%. This was slightly lower than expected, but still within the acceptable range allowing the identification of stronger binder analogues. Such differences in the rFP values were also observed for the other ribosome preparations reflecting slightly different experimental conditions (concentrations, temperatures, etc.). Therefore, all experiments of a given ribosome were done in parallel using controls including Onc112 on the same plate. When the *E. coli* ribosome

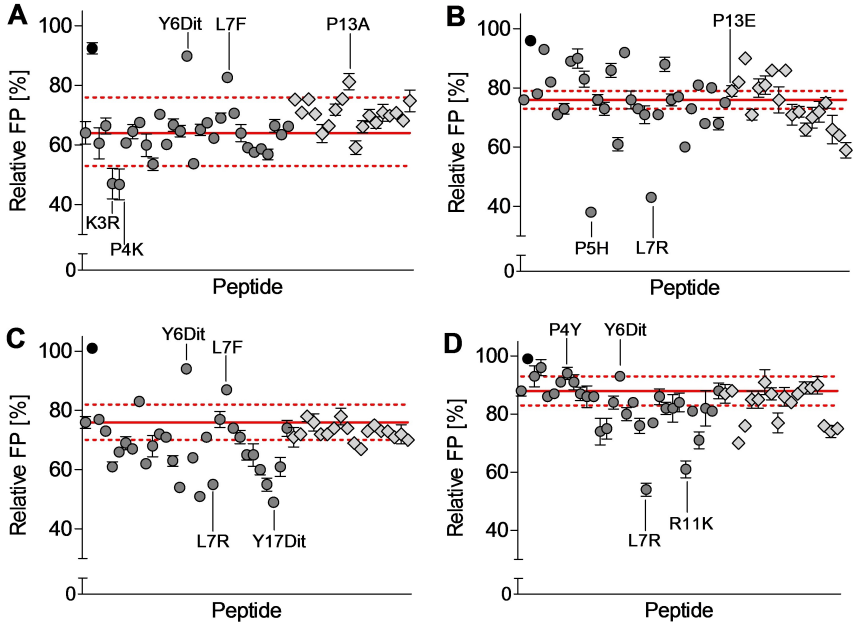


Figure 2. Fluorescence polarization-based screening of Onc112 analogues (circles) and Onc272 analogues (diamonds) using 70S ribosome preparations of *E. coli* BW25113 (A), *K. pneumoniae* DSM 1117 (B), *A. baumannii* DSM 30008 (C) and *P. aeruginosa* DSM 1117 (D). Solid lines correspond to the mean FP value of reference peptide Onc112, dotted lines indicate threefold standard deviation range from the mean. Each circle represents the mean FP value of a triplicate obtained for one peptide with error bars indicating standard deviation. A scrambled Onc18 sequence was used as negative control (black spot).

was probed with all Onc112 analogues, rFP values below 53% and above 76% indicated two stronger and three weaker binding peptides, respectively (Figure 2A). The MIC values of the stronger binder analogues [Lys3Arg]- and [Pro4Lys]-Onc112 were 8 μg/mL for *E. coli* BW25113 and thus identical to Onc112, while the weaker binding analogues [Tyr6Dit]- and [Leu7Phe]-Onc112 and [13Ala]-Onc272 were less active with MIC values of 64, 16, and 16 μg/mL, respectively. [13Ala]-Onc272 was even fourfold less active than Onc272.

Interestingly, about half of the Onc112 analogues bound stronger to the 70S ribosome of *A. baumannii* than Onc112, while only two analogues of Onc272 bound slightly stronger (rFP < 70%). However, the MIC values of four of the five stronger binder analogues (rFP < 60%) were identical to Onc112, only [Leu7Arg]-Onc112 showed a slight improvement (MIC = 16 μg/mL). The two analogues with the lowest MIC values of 8 μg/mL, i.e., [Asp2Arg]-Onc112 and [13Phe]-Onc272 with rFP values of 77% and 74%, respectively, bound as strongly as Onc112 (rFP = 76%). As already mentioned for *E. coli*, substitutions Tyr6Dit (rFP = 94%) and Leu7Phe (rFP = 87%) reduced the binding significantly and also abolished the activity against *A. baumannii* (MIC = 128 μg/mL). Substituting Arg11 with basic (i.e., His, Lys, and Har) or hydrophobic amino acids (i.e., Phe and Trp) similarly increased the binding affinity towards the *A. baumannii* ribosome, as indicated by rFP values ranging from 57% to 64% (Figure 2C). Astonishingly, substitution of Tyr17 with 3,5-diiodotyrosine or 3-nitrotyrosine significantly increased ribosome binding. As the X-ray structure of Onc112 with the 70S ribosome of *T. thermophilus* did not indicate an interaction of Tyr17, it appears likely that the larger side chains of 3,5-diiodotyrosine or 3-nitrotyrosine interact with the 70S ribosome of *A. baumannii*.

Based on the reliable assay conditions achieved for *E. coli* and *A. baumannii* 70S ribosomes, the assay for the 70S ribosomes of *P. aeruginosa* and *K. pneumoniae* was transferred to a liquid handling workstation to increase the sample throughput. Thus, it was necessary to reduce the concentration of Cf-Onc112 threefold to 6.7 nmol/L (Table S1). Consequently, the ribosome concentration was adjusted to obtain a ribosome-to-K_d-ratio of one,[25] i.e., from 250 nmol/L to 77 nmol/L for *K. pneumoniae* and 36 nmol/L for *P. aeruginosa*. Despite significantly reduced quantities of both 70S ribosome and Cf-Onc112, the maximum depolarization and the dynamic range of the assay (ΔmP > 100 mP) were maintained.

The Z′ factor, which reflects dynamic range and precision of an assay, was calculated for each ribosome using the mean and standard deviation of positive and negative controls.[26] Assays with a Z′ factor below 0.5 are not suitable for high-throughput screening, while assays with Z′ factors of 0.5–1.0 are considered high quality assays. The Z′ factors of the manually performed assays described above for 70S ribosome preparations of *E. coli* and *A. baumannii* were 0.91 in both cases. The Z′ factors of the automated screening, which is more relevant for upscaling to high-throughput screening, against 70S ribosomes of *K. pneumoniae* and *P. aeruginosa* were 0.72 and 0.75, respectively, indicating a very good assay quality. The rFP values of Onc112 were 76 ± 0.9% and 88 ± 1.7%, respectively.

The rFP values of nine Onc112- and nine Onc272-analogues were below the threshold of 73% for *K. pneumoniae*. The strongest binder peptides were [Pro5His]- and [Leu7Arg]-Onc112 with rFP values of 38% and 43%, respectively. Indeed, both Onc112 analogues had slightly lower MIC values than Onc112. While 17 analogues of Onc272 were equally active as Onc272 with MIC values of 2 or 4 μg/mL, only [13Glu]-Onc272 was slightly less active (MIC = 8 μg/mL), the rFP values ranged from 59 to 90% indicating stronger and weaker binding peptides. Similarly, all three analogues obtained by substituting Pro5 by Phe, Tyr or His were equally active (MIC = 8 μg/mL), although the rFP values ranged from 38% to 90%.

Several analogues of both Onc112 and Onc272 bound stronger to the *P. aeruginosa* 70S ribosome than Onc112, although Onc112 already bound stronger to this ribosome than to any other ribosome studied here (Fig. 2D). While most analogues showed rFP values above 70%, the two strongest binders were [Leu7Arg]- and [Arg11Lys]-Onc112 with rFP values of 54% and 61%, respectively, and MIC values of 32 μg/mL and 64 μg/mL, respectively. However, some of the other most active analogues (MIC = 32 μg/mL) were only weak binder peptides, including [Asp2Arg]- and [Asp2Trp]-Onc112 with rFP values above or similar to the rFP values of inactive analogues [Pro4Tyr]- and [Tyr6Dit]-Onc112 (MIC = 128 μg/mL).

Bacterial uptake

The antibacterial mechanism of action of PrAMPs requires internalization of the peptides into the bacterial cytoplasm, which strongly depends on the culture medium influencing both bacterial growth and uptake rates.[27] First, PrAMPs attach to the bacterial surface, most likely by ionic interactions in competition with other cations.[6] Therefore, cation-adjusted Mueller-Hinton broth was used in the antimicrobial activity and uptake tests, which relied on the quantitation of PrAMPs in the supernatants and partially the cell pellets. The difference of the peptide quantities added to a cell culture and determined after a certain incubation period in the supernatant (peptide loss) should closely match the peptide quantity in the cell pellet, independently confirming the cellular uptake as long as the peptides are not significantly degraded during incubation. A previous uptake study indicated that these assumptions are true for Onc112.[19,28] Thus, PrAMPs were quantified in medium alone or in the supernatant of a cell culture after a 30-minute incubation. After optimization of sample preparation and LC-MS, a reliable quantitation of peptide concentrations from 1 to 8 μg/mL was achieved in the cell-free supernatant. As the incubation started with an initial peptide concentration of 8 μg/mL, remaining peptide concentrations down to 12.5% could be quantified, which corresponds to a limit of quantitation of 1 μg/mL and a peptide loss of 87.5%. An internal peptide standard was added to further improve the accuracy. The initial peptide concentration of 8 μg/mL was chosen based on the MIC of *E. coli* and retained for all other bacteria. It should be noted that this peptide concentration was fourfold higher than the MIC of Onc112 against *K. pneumoniae* (Table S5). However, the 1000-

fold higher cell counts used in the uptake assay compared to the MIC assay reduced the antibacterial effect of all tested PrAMPs leading to viable *K. pneumoniae* after an incubation period of 30 minutes. The same was true for the other bacteria.

The highest peptide loss in the supernatant was observed for *E. coli* cultures (Figure 3, Figure S1) with most analogues detected at lower quantities than Onc112. The quantities of [Pro5Arg]-, [Leu7Arg]-, and [Pro10Trp]-Onc112 were below the limit of quantitation (LOQ) indicating losses of more than 87.5%. The lowest peptide losses of 40% and 43% were observed for [Arg11Phe]- and [Arg11Trp]-Onc112, respectively. For *K. pneumoniae*, only [Asp2Trp]- and [Asp2Arg]-Onc112 showed higher losses than the 45% detected for Onc112, i.e., 55% and 56%, respectively. In contrast, [Leu7Phe]- and [Tyr17Dit]-Onc112 remained in the medium indicating negligible uptake rates. Peptide losses in *A. baumannii* cultures were lower than in *E. coli* cultures but similar to *K. pneumoniae*. The highest loss was observed for [Asp2Trp]-Onc112 (55%), while all other analogues showed lower losses than Onc112 (50%). Peptides [Leu7Phe]-, [Arg11Lys]-, [Arg11Phe]-, and [Tyr17Nty]-Onc112 completely remained in the supernatants. The losses observed for the monosubstituted Onc112 analogues in *P. aeruginosa* were similar to *K. pneumoniae* and *A. baumannii* samples, while the analogues of Onc272 showed typically higher losses indicating a higher uptake. This was expected as substitution Pro12Trp was identified in oncocin to increase the activity against *P. aeruginosa*.[14] The highest losses of 80% and 77% were detected for [Pro5His]- and [Pro5Arg]-Onc112, respectively, compared to 52% for Onc112. However, the presumably higher uptake did not improve the MIC. No losses were observed when Tyr17 was substituted against 3,5-diiodotyrosine or 3-nitrotyrosine.

Low peptide losses were generally observed for [Tyr6Nty]-, [Leu7Met]-, [Leu7Phe]-, [Tyr17Dit]- and [Tyr17Nty]-Onc112 in all bacteria except *E. coli*. In addition, substitutions of Arg11 decreased peptide losses except for homoarginine. In contrast, peptide losses were typically higher for analogues with a higher net positive charge and more hydrophobic residues than Onc112, which may indicate enhanced cell association and internalization. The influence of net positive charge and hydrophobicity on bacterial uptake is well known,[29] but not yet fully elucidated. While the experiments of all four Gram-negative bacteria were reproducible, the peptide quantities determined in the supernatants of *S. aureus* cultures varied widely (Figure S2). This might be related to the structural differences in the outer membranes of Gram-positive and Gram-negative bacteria or substances secreted by *S. aureus* to the medium. Such differences may appear minor at first glance, but they may have a significant influence on cell adhesion and uptake of PrAMPs.

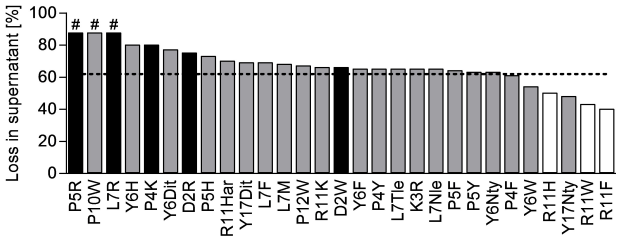


Figure 3. Relative peptide losses in supernatants of an *E. coli* BW25113 culture. Peptides were quantified by RP-HPLC-ESI-QTOF-MS in MRM-mode. *E. coli* cultures were incubated with Onc112 analogues at a concentration of 8 μg/mL in 25% MHB2 medium for 30 minutes. Black, grey, and white bars indicate the peptide loss based on the net positive charge of the peptide, i.e., +7 or +8, +6, and +5, respectively, at neutral pH. Dotted line indicates the loss observed for Onc112 (62%). # indicates peptide quantities below LOQ, which corresponds to losses above 87.5%.

While the quantitation of peptides in supernatants should provide reliable data, quantitation of PrAMPs in pellets is affected by several factors. After lysis of the cell pellets, hydrophobic peptides may strongly bind to bacterial membranes or hydrophobic proteins, while positively charged peptides might bind to negatively charged proteins, DNA, or RNA. In all cases, the peptides would be partially lost after centrifugation leading to an underestimation of the peptide quantities in the cell pellets. In addition, it was not possible to wash the cell pellets extensively to remove remaining medium, as peptides might diffuse out of the cells. The remaining medium will lead to an overestimation of the peptide quantities in the pellet. These factors limit the accuracy of the determined peptide quantities. As peptides are degraded in bacteria, the experimental data do not allow a judgement of whether underestimated peptide quantities are a result of sample losses during the analysis or peptidase and protease activities. It should also be noted that the methods applied here ideally provide the total peptide quantities in the bacteria, either determined by losses in medium or quantities in the cell pellets, but they cannot distinguish if a peptide is indeed internalized or if it either sticks to the cell membrane or remains in the periplasmic space of Gram-negative bacteria. In this respect, the uptake study provides only an information about the peptide quantity in the whole bacterium.

Despite the limitations of the cell pellet analysis, we tried to quantitate the peptides in at least one experiment to confirm the data obtained for the supernatants, especially for peptides completely lost in the supernatants. Again, the pellet data have to be taken with precaution.

Among all Onc112 analogues [Pro4Lys]-, [Leu7Met]-, [Leu7-Phe]-, and [Arg11Lys]-Onc112 were detected in *E. coli* pellets with the highest quantities confirming their high peptide losses in the supernatant (Figure S3). However, the peptide quantities of [Tyr6Dit]-, [Leu7Arg]-, [Arg11Har]-, and [Tyr17Nty]-Onc112 were below the LOQ despite their high losses in the supernatants. [Leu7Arg]-Onc112 was neither detected in the supernatant nor in the pellet and thus was most likely rapidly degraded.

The pellets of *K. pneumoniae* generally contained lower quantities of the tested peptides than *E. coli* pellets, which corresponds to the lower peptide losses obtained in the supernatants. Besides [Pro4Lys]- and [Leu7Met]-Onc112, which were also present in large quantities in the cell pellet of *E. coli*, [Pro5Tyr]- and [Pro5Arg]-Onc112 were also detected in large quantities. This was confirmed in a second experiment also indicating the good reproducibility for a Gram-negative bacterium. For *A. baumannii* pellets, [Pro4Lys]- and [Leu7Phe]-

Onc112 were again present at high levels. In addition to [Tyr6Dit]- and [Arg11Har]-Onc112, which were also not detected in *E. coli*, [Asp2Trp]- and [Tyr6Trp]-Onc112 were below the respective LOQs as well. In none of the *P. aeruginosa* pellet samples was the expected peptide detected, not even Onc112 although it was detected by LC-MS with the expected signal intensity when Onc112 was spiked to an extracted *P. aeruginosa* pellet sample prior to analysis. Thus, interferences with the sample matrix can be excluded. It remains open when the peptides were lost, but we assume that it was during SPE due to saturation of the stationary phase by hydrophobic substances preventing peptide binding, which was not further investigated. Alternatively, the peptides might be rapidly degraded in *P. aeruginosa*, but this appears unlikely as some analogues were moderately active.

In contrast, the pellet samples of *S. aureus* could be analyzed very well (Figure S4). High levels of [Pro4Lys]-, [Pro5His]-, [Pro5Arg]-, and [Arg11Har]-Onc112 were detected, whereas [Tyr6His]-, [Tyr6Nty]-, [Leu7Arg]-, [Leu7Met]-, [Leu7Phe]-, [Arg11Trp]-, and [Arg11Lys]-Onc112 could not be quantified. Remarkably, [Pro4Lys]-Onc112 was present in high quantities in all pellets except *P. aeruginosa*, which suggests a generally enhanced membrane adhesion or internalization that also reflects its broad-spectrum activity (Table 1). Lai and colleagues also reported that substitution Pro4Lys increases the antimicrobial activity, especially in combination with Leu7Lys or Leu7Arg substitutions, presumably due to a higher internalization rate.[30]

The analogues of Onc272 were generally more difficult to analyze due to their higher hydrophobicity related to the substitution of Pro12 by tryptophan. Thus, the LOQs were typically higher, especially when Pro13 was substituted by tryptophan (Onc275), isoleucine (Onc278) or phenylalanine (Onc279) with LOQs of 6 μg/mL in the supernatants. In *E. coli* supernatants only [13Asp]-Onc272 could be quantified, while twelve of the 19 analogues could be quantified in the pellets including [13Phe]-Onc272 with the highest quantity of 626 ng (Figure S3 A). It can be speculated that Onc272 analogues interact strongly with the *E. coli* membrane and cannot be extracted from the cellular debris after lysis. For *K. pneumoniae* samples, the larger portion was found in the supernatant ranging from 57% for [13Arg]-Onc272 to 104% for [13Glu]-Onc272, while only low quantities were detected in the pellets except for [13Trp]-Onc272. These results indicate rather slow uptake rates in contrast to their good antimicrobial activity against *K. pneumoniae*. For *A. baumannii*, a similar peptide distribution was observed in the supernatants ranging from 73% to 109% of the total peptide quantity. The Onc272 quantities in the pellets varied only slightly with [13Phe]-Onc272 present at the highest quantities (475 ng), which correlates well with its higher antimicrobial activity (MIC = 8 μg/mL). In *P. aeruginosa*, substitution of Pro13 with basic amino acids arginine, lysine, and ornithine did not affect the uptake despite their higher antimicrobial activity (MIC = 32 μg/mL). For *S. aureus*, most peptides remained mostly in the supernatant and only 21% to 31% of the original quantity was present in the pellets, which correlated with the low antimicrobial activity. Even peptide [13Orn] with its significantly improved MIC (8 μg/mL), was found only in small quantities (23%).

Cytotoxicity

When HepG2 and HEK293 were incubated with Onc112 (Onc272) at a concentration of 0.6 g/L for 20 hours, the cell viability was reduced to 92% (62 %) and 77% (62 %), respectively. All analogues of Onc112 and Onc272 reduced the cell viability to 36% to 78% (Figure 4), mostly independent of the cell line. Peptide [13Trp]-Onc272 had the strongest effect on HepG2 cells (36% viability) and peptide [Leu7Nle]-Onc112 showed the strongest effect on HEK293 cells (40% viability). In general, Onc112 analogues with a higher hydrophobicity appeared to reduce the cell viability more. Substitution of Pro13 by a hydrophobic amino acid decreased the cell viability

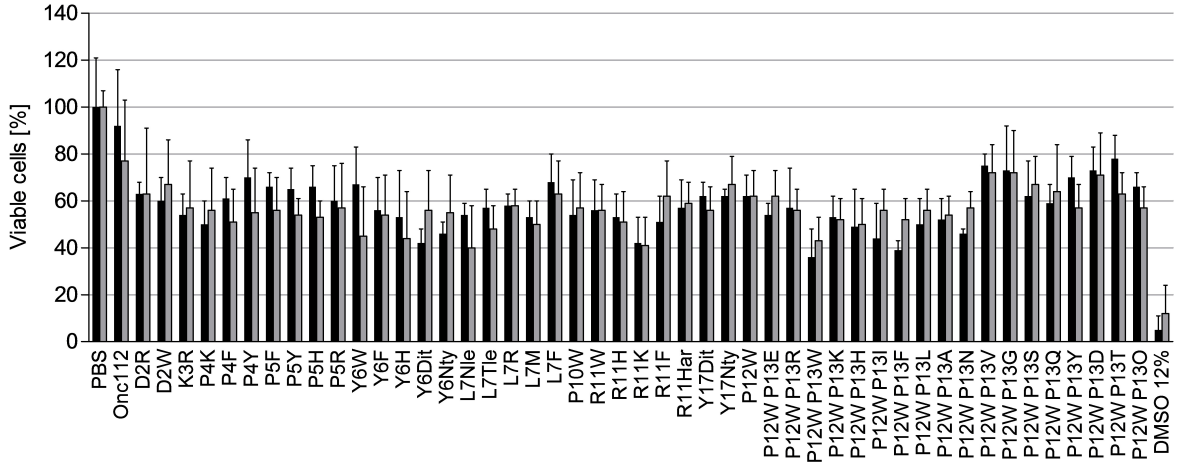


Figure 4. Effect of Onc112 and Onc272 analogues on the cell viability of HepG2 (black) and HEK293 (grey) cells. Cells were grown in DMEM/Ham's F12 medium containing 10% (v/v) fetal bovine serum and the indicated peptide (0.6 g/L) for 20 hours. Each bar represents the mean of a six (HepG2) or nine replicates (HEK293) and error bars indicate standard deviation.

of HepG2 cells further. Similar effects on cell viability of oncocins were observed in a previous study.[14] Generally, substitutions increasing the positive net charge or the hydrophobicity increase nonspecific interactions with mammalian cell membranes reducing cell viability. It should be noted that the tested peptide concentrations are high and exceed the MIC values at least tenfold, indicating a reasonable therapeutic margin of safety.

Discussion

While the high activity of Onc112 against *K. pneumoniae* [MIC = 2 μg/mL] could not be further improved, some substitutions reduced the MIC values twofold against *E. coli*. Assuming that a certain number of oncocin molecules is required to inhibit the ribosomes, it appears unlikely that the MIC value can be further improved. This was also shown for other analogues of apidaecin 1b that could be optimized to MIC values of around 1 μg/mL.[18,31] Although the rather high MIC values of Onc112 against *A. baumannii, P. aeruginosa* and *S. aureus* should offer a great potential for improvement, substitutions increased the activity against *A. baumannii* up to fourfold (MIC = 8 μg/mL) but only twofold for *P. aeruginosa* (MIC = 32 μg/mL). Interestingly, a stronger effect was obtained for *S. aureus*. Substitution Pro12Trp, which was already identified in oncocin as beneficial,[14] improved in combination with Pro13Orn the MIC value of Onc112 eightfold to 8 μg/mL. Promising broad-spectrum activities were typical for analogues with a net positive charge of +7 or +8 compared to Onc112 (net charge of +6), such as Onc244 and Onc245, or +7 compared to Onc272 (net charge of +6), such as Onc274 and Onc290 (Tables 1 and S5). Presumably, a higher net positive charge increases the interaction with the negatively charged bacterial membrane, which may improve the concentration-dependent passive diffusion into the periplasm. Thus, net charge and hydrophobicity might determine the uptake rates, while the substitution site has a weaker effect, except for Onc253. For Onc253 it remains open if the higher peptide loss corresponds indeed to a higher uptake or indicated a degradation due to the Arg substitution in position 5. Additionally, the surface of Gram-negative bacteria is more negatively charged than the surface of Gram-positive bacteria providing better ionic interactions.[32] The highest uptake rates were typically observed for *E. coli*, while peptide losses in the supernatants were at similar levels for all other tested bacteria including *S. aureus*, but significantly changed for different peptides from 0 to 100%. PrAMPs enter the bacteria via SbmA and MdtM transporter proteins, which were both identified in *E. coli*.[7,8] Although analogous transporter proteins are found in other Gram-negative bacteria,[9] minor differences in their structure could drastically alter the transporter selectivity and permeability affecting the uptake rates.

Oncocins with higher net positive charge showed the highest loss in the supernatant, which was most obvious for [Tyr6His]-Onc112 in *E. coli*. This supports the assumed stronger electrostatic membrane interactions. However, the uptake did not always correlate with the activity, for example peptide [Pro12Trp]-Onc112 was twofold more active against *E. coli* than Onc112 despite an only slightly elevated uptake. Analogue [Leu7Arg]-Onc112 was neither detected in the supernatant nor in the pellet of an *E. coli* culture, suggesting a degradation of the peptide. As the peptide was as active as Onc112 (MIC = 8 μg/mL), the peptide is most likely degraded in the cell yielding truncated sequences that are still able to inhibit the 70S ribosome. It will be interesting to identify the degradation products as they may provide further insight into the minimum sequence required for inhibition of the *E. coli* 70S ribosome, as already shown for Onc72.[28]

A high activity against *K. pneumoniae* did not correlate with uptake rates. Presumably, a rather low intracellular PrAMP concentration is sufficient to inhibit the bacterial growth because more peptides reach the ribosome due to a reduced off-target binding. This might also be true for *A. baumannii*, where low uptake rates are compensated by strong ribosome binding resulting in acceptable activity (Figure 5). In contrast, improved uptake in *P. aeruginosa* did not result in higher activities as the ribosome binding was also less efficient. In particular, Pro5-substituted analogues were less active against *P. aeruginosa* despite higher uptake rates, which underlines the importance of this position for ribosome binding.

Despite the high sequence homologies of ribosomal proteins among the studied bacteria and the anticipated high structural similarities, the substituted analogues revealed significantly different binding patterns at the binding site. Most analogues showed a strong binding to the *E. coli* ribosome and a significantly improved binding to the 70S ribosome of *P. aeruginosa*. Generally, the binding constants decreased from the ribosome preparations of *E. coli* to *A. baumannii* and *K. pneumoniae*, which were both on average similar despite distinct differences for some sequences, and finally to *P. aeruginosa*. Overall, [Leu7Arg]-Onc112 showed an improved binding and was also one of the most active Onc112 analogues with MIC values of 8, 4, 16, 32 μg/mL for *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*, respectively. The same MIC values were obtained for [13Arg]-, [13Lys]-, and [13Orn]-Onc272, whereas the most active peptide was [13Arg]-Onc272 with MIC values of 8, 2, 16, and 32 μg/mL, respectively. Analogue [Tyr6Dit]-Onc112 showed lower binding affinity against all ribosomes correlating with weak activity against *E. coli* (MIC = 64 μg/mL) and no antimicrobial activity against the other pathogens.

Conclusion

Several monosubstituted analogues of Onc112 and Onc272 were more active against the tested bacteria. However, the higher antimicrobial activities did not correlate to their improved 70S ribosome binding or an enhanced cellular uptake except for the incorporation of basic amino acids at positions 7 and 13. Thus, further parameters appear to be important for predicting the activity of oncocins. It is tempting to speculate that other targets, such as DnaK, may bind better to the

Figure 5. Correlation of antimicrobial activity (MIC values), peptide uptake (relative peptide loss in supernatant) and 70S ribosome binding (relative fluorescence polarization in a competitive binding assay) of Onc112 and Onc272 analogues against *E. coli* BW25113 (A), *K. pneumoniae* DSM 681 (B), *A. baumannii* DSM 30008 (C), and *P. aeruginosa* DSM 1117 (D). Green color indicates low MIC values, high peptide loss, and low relative FP values. Red color indicates high MIC values, low peptide loss, and high relative FP values. Orange and yellow colors indicate intermediate values, as described in detail in the supplement (Tables S6 and S7).

analogues and thus capture the peptides before they reach the ribosome. Similarly, the analogues could be transported to other cellular compartments protecting the ribosomes. In either case, this limits the possibility of rational approaches to optimize the antibacterial activity based on one or two *in-vitro* assays. Importantly, a loss or ribosome binding abolishes typically the activity confirming that the ribosome is indeed the most important target. However, [Asp2Arg]- and [Asp2Trp]-Onc112 remained active against *P. aeruginosa* despite a weak binding to the ribosome, which may indicate another or at least a second mechanism of action.

Author contributions

Conceptualization, L.K., D.K., and R.H.; methodology, L.K., D.K., A.B., T.B., and R.H.; validation, L.K.; formal analysis, L.K.; investigation, L.K.; resources, T.B. and R.H.; writing and editing, L.K. and R.H.; visualization, L.K.; supervision, D.K. and R.H.; project administration, R.H.; funding acquisition, R.H. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

DK was employed at EnBiotix GmbH. RH was on the advisory board of EnBiotix Inc.

Keywords: fluorescence polarization (FP) · label-free quantitation · Onc112 · oncocin · proline-rich antimicrobial peptide (PrAMP)

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