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Altered Activity and Physicochemical Properties of Short Cationic Antimicrobial Peptides by Incorporation of Arginine Analogues

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Abstract: The incorporation of nongenetically encoded amino acids is a well established strategy to alter the behavior of several types of promising cationic antimicrobial peptides. Generally, these elements have been improved mimics of the hydrophobic amino acids yielding peptides with increased stability and potency. In this initial study, the effect of systematic replacement of Arg in a well-defined moderately antimicrobial tripeptide library is described. It is shown that the arginine analogues need to display a strong basicity to produce active peptides. It is further revealed that the hydrophobic units needed for activity in these peptides can be effectively incorporated in the direct vicinity of the cationic charge to produce compounds with improved antibacterial properties. A well-defined facial amphiphilic structure, which remains intact upon introduction of hydrophobic elements in the cationic side chains, is seen for the majority of the tested peptides. Microcalorimetric studies revealed a peptide binding to large anionic unilamellar vesicles (LUVs) mimicking the Gram-positive bacterial membrane as well as a potentially competitive binding to human serum albumin in the low- to mid-micromolar range. No considerable alterations in binding to either albumin or the LUVs were seen for the analogue containing peptides. A neutral LUV mimicking the eukaryotic cell membrane showed no significant binding to any of the peptides. The oral absorption of this class of short lactoferricin based peptides was investigated for the first time and revealed that incorporation of weaker bases than Arg produced peptides with much improved permeability in a recently developed permeation model, the phospholipid vesicle based barrier assay. Collectively, the results presented here show that there is ample room to toggle the activity and physical properties of short cationic antimicrobial peptides by incorporation of arginine analogues.

Keywords: Antimicrobial peptides; arginine; arginine analogues; isothermal titration calorimetry; phospholipid vesicle barrier

Introduction

Cationic antimicrobial peptides (CAPs) represents a promising class of molecules in the battle against microorganisms that have developed resistance toward conventional antibiotics.[1] The CAPs are generally short (less than 50 residues), carrying multiple positive charges with well-defined hydrophobic and polar regions.[2] They constitute a part of the innate immune system and several hundred have been identified in a range of organisms.[3,4] Lactoferricin, a truncated 25 amino

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(1) Hancock, R. E. W.; Sahl, H. G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **2006**, *24*, 1551–1557.

(2) Jenssen, H.; Hamill, P.; Hancock, R. E. W. Peptide antimicrobial agents. *Clin. Microbiol. Rev.* **2006**, *19*, 491–511.

(3) Hancock, R. E. W.; Chapple, D. S. Peptide antibiotics. *Antimicrob. Agents Chemother.* **1999**, *43*, 1317–1323.

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acid peptide residue released upon peptidic degradation of lactoferrin, is one such peptide displaying antibacterial, antifungal, and antitumoral actitivties.[5−8] Lactoferrin based peptides have potential as promising pharmaceutics, and several emerging companies base their R&D work on these compounds. The activity of lactoferricin is mainly ascribed to the presence of five arginine and four hydrophobic residues (Phe and Trp) responsible for the interaction with the negatively charged bacterial membrane.[9−12] It has been shown that this sequence can be further truncated to yield very efficient antimicrobial agents, and a minimal antibacterial motif, consisting of two units of hydrophobic bulk and two cationic charges, has been defined.[13] Thus, very small di- and tripeptide derivatives can be designed with high antibacterial activity toward Gram-positive bacteria.[14]

Our group has systematically worked toward minimizing the size of the CAPs while still maintaining a high activity with the ultimate goal of making their synthesis easier and more affordable for a future large scale production and potential pharmaceutical use. One factor that has enabled us to increase the potency of the peptides while reducing the sequence length has been the introduction of unnatural amino acid analogues to Trp and Phe present in the native form of lactoferricin.[15] Selective substitution of amino acids within a peptide sequence with unnatural analogues is a well established strategy to change the properties of the peptide. Reduced hemolytic activity, increased stability to proteolytic degradation, and increased potency are potential effects of amino acid replacements as has been described in several reports over the years.[16−18] Several novel bulky, hydrophobic analogues, either prepared using the Suzuki−Miyaura coupling or from commercial sources, have been introduced in these CAPs, resulting in a drastic increase in antimicrobial activity and also in an increased stability to tryptic degradation *in vitro*.[19,20]

A recent report described the role of the net positive charge in a series of amphipathic α-helical antibacterial peptides, but apart from that surprisingly little is currently known about the role and nature of the cationic contributions except that they are essential for activity.[21] In short lactoferricin based CAPs, the cationic charges are generally provided by arginine, and despite the massive amount of structure−activity relationship (SAR) data available in this field no study has yet been solely focused on the cationic contribution. The arginines are often incorporated by default in these structures, and they have thus not been subjected to systematical structural alterations in the fashion as the hydrophobic elements have. The current project is consequently aimed at investigating, in more detail, the role of arginine in short CAPs through the introduction of arginine analogues (Figure 1). Aspects such as ease of preparation, effect on the antibacterial potency, secondary structure, protein binding, hemolysis, permeability in an oral permeation model and binding to bacterial membrane mimics has been investigated.

(4) Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature (London)* **2002**, *415*, 389–395.
(5) Bellamy, W.; Takase, M.; Wakabayashi, H.; Kawase, K.; Tomita, M. Antibacterial Spectrum of Lactoferricin-B, a Potent Bactericidal Peptide Derived from the N-Terminal Region of Bovine Lactoferrin. *J. Appl. Bacteriol.* **1992**, *73*, 472–479.
(6) Bellamy, W.; Takase, M.; Yamauchi, K.; Wakabayashi, H.; Kawase, K.; Tomita, M. Identification of the Bactericidal Domain of Lactoferrin. *Biochim. Biophys. Acta* **1992**, *1121*, 130–136.
(7) Bellamy, W.; Yamauchi, K.; Wakabayashi, H.; Takase, M.; Takakura, N.; Shimamura, S.; Tomita, M. Antifungal Properties of Lactoferricin-B, a Peptide Derived from the N-Terminal Region of Bovine Lactoferrin. *Lett. Appl. Microbiol.* **1994**, *18*, 230–233.
(8) Eliassen, L. T.; Berge, G.; Sveinbjornsson, B.; Svendsen, J. S.; Vorland, L. H.; Rekdal, O. Evidence for a direct antitumor mechanism of action of bovine lactoferricin. *Anticancer Res.* **2002**, *22*, 2703–2710.
(9) Gifford, J. L.; Hunter, H. N.; Vogel, H. J. Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. *Cell. Mol. Life Sci.* **2005**, *62*, 2588–2598.
(10) Haug, B. E.; Svendsen, J. S. The role of tryptophan in the antibacterial activity of a 15-residue bovine lactoferricin peptide. *J. Pept. Sci.* **2001**, *7*, 190–196.
(11) Strom, M. B.; Haug, B. E.; Rekdal, O.; Skar, M. L.; Stensen, W.; Svendsen, J. S. Important structural features of 15-residue lactoferricin derivatives and methods for improvement of antimicrobial activity. *Biochem. Cell Biol.* **2002**, *80*, 65–74.
(12) Chan, D. I.; Prenner, E. J.; Vogel, H. J. Tryptophan- and arginine-rich antimicrobial peptides: Structures and mechanisms of action. *Biochim. Biophys. Acta* **2006**, *1758*, 1184–1202.
(13) Strom, M. B.; Haug, B. E.; Skar, M. L.; Stensen, W.; Stiberg, T.; Svendsen, J. S. The Pharmacophore of Short Cationic Antibacterial Peptides. *J. Med. Chem.* **2003**, *46*, 1567–1570.
(14) Haug, B. E.; Strom, M. B.; Svendsen, J. S. M. The medicinal chemistry of short lactoferricin-based antibacterial peptides. *Curr. Med. Chem.* **2007**, *14*, 1–18.
(15) Haug, B. E.; Stensen, W.; Stiberg, T.; Svendsen, J. S. Bulky Nonproteinogenic Amino Acids Permit the Design of Very Small and Effective Cationic Antibacterial Peptides. *J. Med. Chem.* **2004**, *47*, 4159–4162.
(16) Gante, J. Peptidomimetics - Tailored Enzyme-Inhibitors. *Angew. Chem., Int. Ed.* **1994**, *33*, 1699–1720.
(17) Leung, D.; Abbenante, G.; Fairlie, D. P. Protease Inhibitors: Current Status and Future Prospects. *J. Med. Chem.* **2000**, *43*, 305–341.
(18) Hicks, R. P.; Bhonsle, J. B.; Venugopal, D.; Koser, B. W.; Magill, A. J. De Novo Design of Selective Antibiotic Peptides by Incorporation of Unnatural Amino Acids. *J. Med. Chem.* **2007**, *50*, 3026–3036.
(19) Haug, B. E.; Stensen, W.; Svendsen, J. S. Application of the Suzuki-Miyaura cross-coupling to increase antimicrobial potency generates promising novel antibacterials. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2361–2364.
(20) Svenson, J.; Stensen, W.; Brandsdal, B. O.; Haug, B. E.; Monrad, J.; Svendsen, J. S. Antimicrobial Peptides with Stability toward Tryptic Degradation. *Biochemistry* **2008**, *47*, 3777–3788.
(21) Jiang, Z.; Vasil, A. I.; Hale, J. D.; Hancock, R. E. W.; Vasil, M. L.; Hodges, R. S. Effects of net charge and the number of positively charged residues on the biological activity of amphipathic -helical cationic antimicrobial peptides. *Pept. Sci.* **2008**, *90*, 369–383.

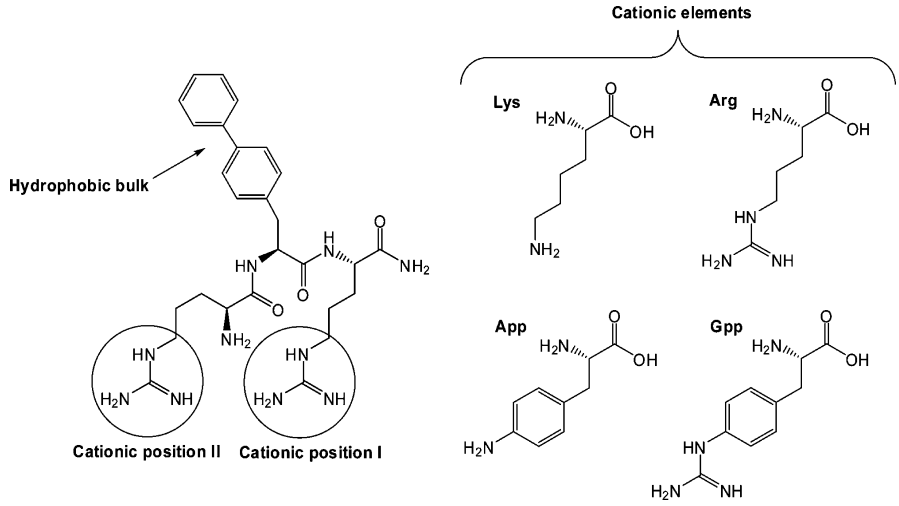

Figure 1. Structures of the cationic elements used to provide variety in the tripeptide library.

Experimental Section

Materials. Fmoc protected L-amino acids, 2-amino-3-(4-guanidinophenyl)propanoic acid(Bis-Boc) (Gpp), Arg(Pbf), and Lys(Boc) were purchased from Fluka (Basel, Switzerland). Biphenylalanine (Bip) and 2-amino-3-(4-aminophenyl)propanoic acid(Boc) (App) were provided by Bachem (Bubenhof, Switzerland). 1-Hydroxybenzotriazole (HOBt), piperidine, and triisopropylsilane (TIS) were purchased from Fluka. *N*,*N*′-Diisopropylcarbodiimide (DIC) was supplied by Aldrich (Oslo, Norway), and Riedel-deHaën provided trifluoroacetic acid (TFA) (Seelze, Germany). The Rink amide resin used was purchased from NovaBiochem (Darmstadt, Germany). Dimyristoylphosphatidylglycerol (DMPG), egg phosphatidylcholine, and lipoid E-80 were obtained from Lipoid (Ludwigshafen, Germany), and dimyristoylphosphatidylcholine (DMPC) and essentially fatty acid free human serum albumin ~99% (HSA) (A3782) were from Sigma-Aldrich (Oslo, Norway). Filter inserts (transwell Ø = 6.5 mm) and plates for the permeability experiments were supplied by Corning Inc. (Corning), while the mixed cellulose filters (0.65 μm pore size) came from Millipore (Billerica). Dimethylformamide (DMF) and methanol (MeOH) were purchased from BDH Prolabo (Briare, France), while dichloromethane (DCM) was supplied by Merck (Darmstadt, Germany). All solvents were used as delivered.

General SPPS Procedure. The peptides were prepared in a Biotage initiator microwave synthesizer employing standard SPPS Fmoc-strategy.[22] For the first coupling, the resin was preswelled in DMF and treated with piperidine (20% in DMF) at room temperature to remove the Nα-protecting group. The resin was further washed with DMF (×5) and transferred to microwave vials (2 mL) for the couplings. All peptide coupling reactions were performed with 3 equiv of amino acid, 4.5 equiv of HOBt, and 3.3 equiv of DIC in enough DMF to dissolve the reagents. Microwave irradiation for 10 min at 60 °C ensured high coupling yields as verified by Chloranil or Kaiser test on a small washed (DMF × 5, DCM × 5) and filtered sample of the resin after each step. Recoupling was performed in those cases where unsatisfactory yields were experienced before deprotection. The resin was washed with DMF (×5), DCM (×2), and MeOH (×2) and then dried after the final coupling and deprotection. Cleavage of the peptides from the resin was performed employing a mixture of TFA/TIS/H_2O (95:2.5:2.5 v/v/v), which yielded a crude that was further purified.

004a: ^{1}H NMR (400 MHz, D_2O) δ 7.64 (dd, J = 7.8, 16.3, 4H), 7.50 (t, J = 7.5, 2H), 7.42 (t, J = 7.4, 1H), 7.36−7.28 (m, 6H), 4.63 (t, J = 8.0, 1H), 4.45 (t, J = 7.3, 1H), 4.00 (t, J = 6.5, 1H), 3.19−3.01 (m, 6H), 1.94−1.77 (m, 2H), 1.60−1.46 (m, 2H). ESMS: calcd for $C_{30}H_{38}N_8O_3$, 558.7; found, 558.3. Purity determined by HPLC: Retention time, 15.70 min; purity, 99.3%. See the Supporting Information for data on remaining compounds.

Peptide Purification and Analysis. The crude peptides were purified by reversed phase HPLC (RP-HPLC) on a Delta-Pak (Waters) C-18 column (100 Å, 15 μm, 25 × 100 mm) with a mixture of water and acetonitrile (both containing 0.1% TFA) as the eluent. The purified peptides were further analyzed by RP-HPLC using an analytical Delta-Pak (Waters) C-18 column (100 Å, 5 μm, 3.9 × 150 mm) to asses the final purity (≥95%). To ensure positive identification of the desired product, positive ion electrospray mass spectrometry on a VG Quattro quadrupole mass spectrometer (VG Instruments Inc., Altringham, U.K.) was performed. Peptide purity was assessed by HPLC.

Microbiological Studies. The antibacterial activity of the peptides was tested against *Staphylococcus aureus* strain ATCC 25923 and methicillin reistant *Staphylococcus aureus* (MRSA) strain ATCC 33591. The studies were perfomed by Toslab AS employing standard methods.[23]

(22) Fara, M. A.; Diaz-Mochon, J. J.; Bradley, M. Microwave-assisted coupling with DIC/HOBt for the synthesis of difficult peptoids and fluorescently labelled peptides - a gentle heat goes a long way. *Tetrahedron Lett.* **2006**, *47*, 1011–1014.

(23) Amsterdam, D. Susceptibility testing of antimicrobials in liquid media. In *Antibiotics in laboratory medicine*, 4th ed.; Lorian, V., Ed.; Williams and Wilkins: Philadelphia, 1996; pp 75−78.

Liposome Preparation. Dimyristoylphosphatidylglycerol (DMPG) and dimyristoylphosphatidylcholine (DMPC) were dissolved in a mixture of chloroform and methanol (1:1 v/v) and chloroform, respectively. The organic solvent was removed under vacuum at 55 °C. The deposited lipid film was exposed to vacuum of 55 hPa at room temperature for an additional period of 3 h to remove traces of solvent before hydration with 10 mM HEPES buffer (100 mM NaCl, pH 7.0) to obtain a 10 mg/mL liposomal dispersion. The liposome dispersions were exposed to five subsequent freeze−thaw cycles, with the use of liquid nitrogen and water bath at 55 °C, to obtain liposomes with a reduced lamellarity.[24] The liposome dispersions were then extruded 20 times at room temperature with a miniextruder (LiposoFast, Avestin Inc., Ottawa, Canada) equipped with a 100 nm pore sized polycarbonate membrane filter. The final liposome suspensions were stored at 25 °C until further use. Analysis was initiated within 1 h of preparation for the ITC studies.

Permeability Experiments. The phospholipid vesicle based barriers were prepared according to the procedure reported by Flaten et al.[25] and were stored for up to 2 weeks at −80 °C in accordance with previous stability studies.[26] In brief, two liposome dispersions extruded through filters (pore size 800 nm and 800 nm followed by 400 nm) were deposited on a filter support by centrifugation. The liposomes were added in consecutive steps allowing the smaller liposomes to be deposited before the larger. Freeze−thaw cycling was employed as a final step to promote fusion of the lipsomes, affording a tight barrier. Permeability experiments were performed at room temperature without agitation, and inserts, containing the peptides at variable concentrations, were moved at certain time intervals to wells containing an equal quantity of fresh buffer as recently described.[25] Samples (200 μL each) were collected from each acceptor compartment and transferred to 96-well UV-transparent plates for a spectrophotometric analysis (Spectramax 190, Molecular Devices, Molecular Device Corporation, Sunnyvale, CA) of the drug concentration. Peptide **004d** was detected at 240 nm, whereas peptides **001a** and **002b** were detected at 255 nm. The electrical resistance across the barrier was measured (Millicell-ERS, Millipore, Billerica, MA) immediately after completion of the permeation studies to ensure a maintained barrier integrity. All barriers displaying a resistance below 1000 Ω were excluded from the study. The experiments were performed with 12 single inserts for each peptide.

Isothermal Titration Calorimetry. Heats of interaction were determined using a CSC 5300 Nano-Isothermal Titration Calorimeter III instrument with a 1 mL cell volume (Calorimetry Sciences Corporation, UT). In a typical HSA titration experiment, the peptide (2.1 mM) was added in 33 aliquots (3 μL) to a stirred (150 rpm) solution of HSA (0.1 mM) in an aqueous buffer at 25 °C. The buffer used was 50 mM Tris-HCl, 10 mM $CaCl_2$ at pH 7.4. An interval of 400 s between the injections was employed to reach equilibrium between the interacting species. The heats of dilution were determined in a similar fashion where the peptides were added to a stirred buffer solution without albumin. Subtraction of the dilution heat yielded the heat of interaction and a binding isotherm from which the association constant and complex stoichiometry were calculated using BindWorks analysis software. For the liposome studies, a 10 mM liposome solution (DMPG or DMPC) was added (25 aliquots, 10 μL each) to a stirred (150 rpm) 50 μM peptide solution maintained at 37 °C. The buffer system employed here was the same as that used for preparation of the liposome dispersions. Reference runs with buffer were done in order to determine the heat of dilution and subtracted from the peptide runs. The data were analyzed in an identical manner for all the titrations.

Hemolysis. The hemolytic activity of the peptides (**001a**, **002b**, **003c**, and **004d**) was tested against human red blood cells (hRBCs) obtained from healthy volunteers. Fresh heparinized hRBCs were rinsed three times with phosphate-buffered saline (PBS) buffer (35 mM, 150 mM NaCl, pH 7.4) by centrifugation (10 min, 1500 rpm), followed by resuspension and dilution in PBS (10% hematokrit). Various concentrations of the peptides were then dissolved in PBS buffer and added to the hRBC solution, yielding a final erythrocyte concentration of 1% v/v. The suspensions were incubated under agitation for 1 h at 37 °C, followed by centrifugation (5 min, 4000 rpm). Release of hemoglobin was monitored by measuring the absorbance of the supernatant at 450 nm. Negative controls for zero hemolysis and positive controls (100% hemolysis) consisted of hRBC suspended in PBS and Triton 1%, respectively. The degree of hemolysis is defined as the ratio of the optical density (OD) of the peptide sample relative to the OD of the difference between the positive and negative control for hemolysis.

Molecular Modeling. Three dimensional structures of the peptides were constructed using the Maestro software from Schrödinger. Conformational space for the peptides was explored using ConfGen[27] with the OPLSAA-2005 force field.[28] A GB/SA continuum solvation model was used to

(24) Mayer, L. D.; Hope, M. J.; Cullis, P. R. Vesicles of Variable Sizes Produced by a Rapid Extrusion Procedure. *Biochim. Biophys. Acta* **1986**, *858*, 161–168.

(25) Flaten, G. E.; Dhanikula, A. B.; Luthman, K.; Brandl, M. Drug permeability across a phospholipid vesicle based barrier: A novel approach for studying passive diffusion. *Eur. J. Pharm. Sci.* **2006**, *27*, 80–90.

(26) Flaten, G. E.; Bunjes, H.; Luthman, K.; Brandl, M. Drug permeability across a phospholipid vesicle-based barrier - 2. Characterization of barrier structure, storage stability and stability towards pH changes. *Eur. J. Pharm. Sci.* **2006**, *28*, 336–343.

(27) *ConfGen*, version 2.0; Schrödinger, LLC: New York, 2008.

(28) Kaminski, G. A.; Friesner, R. A.; Tirado-Rives, J.; Jorgensen, W. L. Evaluation and Reparametrization of the OPLS-AA Force Field for Proteins via Comparison with Accurate Quantum Chemical Calculations on Peptides. *J. Phys. Chem. B* **2001**, *105*, 6474–6487.

describe water as solvent.[29] Nonbonded interactions were truncated at 8 and 20 Å for van der Waals and electrostatic interactions, respectively. Redundant conformers were eliminated using a root-mean-squared deviation of 1.0 Å, and conformers whose energy was more than 100 kJ/mol higher than the lowest energy conformer were also removed. The five conformations with lowest energy for each peptide were subsequently geometry optimized using density functional theory (DFT) with the hybrid functional B3LYP and the 6-311G basis set.[30,31] All DFT calculations were done using the *ab initio* quantum mechanical program Jaguar.[32]

Results

Synthetic Incorporation. Peptides were prepared in a similar fashion to the novel method recently described by Fara et al.[22] Microwave irradiation was employed in combination with the general Fmoc-SPPS methodology on Rink amide resin to generate the peptide library. None of the alternative amino acids called for any alterations in the chemistry employed to prepare the CAPs.

Effect on Antibacterial Potency. The peptide library was based around **001a**, a compound with limited bactericidal properties, as it has been previously shown that Bip alone as hydrophobic element only yields a weak antibacterial compound.[15] Peptide **001a** is thus short of one unit of bulk by design, as the aim of the study was to investigate how well arginine analogues perform compared to arginine and not solely on the preparation of a CAP with a low MIC-value (the MIC value for a C-terminally benzylated derivative of **001a** is 5 μg/mL against *Staphylococcus aureus* strain ATCC 25923 as a comparison[33]). The data from the antibacterial assays are summarized in Table 1.

Molecular Modeling. To investigate if the three-dimensional structures of these compounds could provide insights into the mechanism, the conformational space of the low-energy conformers of the peptides was explored and their potential amphiphilic character investigated. Depending on the peptide, between 14 and 39 conformers were generated using ConGen, all within 100 kJ/mol of the lowest energy conformer. The five conformers with lowest energy for a given peptide spanned approximately 20 kJ/mol. Analysis of these structures shows that most structures indeed are amphiphilic, which is illustrated using **001c** and **003a** in Figure 2. The root-mean-squared deviation between the conformers is in the range 0.5−1.6 and 0.8−2.9 Å for **001c** and **003a**, respectively.

Table 1. Antibacterial Activity

					MIC (μg/mL)	
peptide	sequence	mass (calcd)	bulk[a]	charge[b]	*S. aureus*	MRSA
001a	Arg-Bip-Arg	552.2 (552.7)	1	+3	200	50
001b	Lys-Bip-Arg	524.3 (524.7)	1	+3	>200	200
001c	Gpp-Bip-Arg	600.3 (600.7)	2	+3	75	50
001d	App-Bip-Arg	558.2 (558.7)	2	+2	>200	>200
002a	Arg-Bip-Lys	524.2 (524.7)	1	+3	>200	>200
002b	Lys-Bip-Lys	496.3 (496.6)	1	+3	>200	>200
002c	Gpp-Bip-Lys	572.3 (572.7)	2	+3	>200	150
002d	App-Bip-Lys	530.2 (530.7)	2	+2	>200	>200
003a	Arg-Bip-Gpp	600.3 (600.7)	2	+3	75	50
003b	Lys-Bip-Gpp	572.2 (572.7)	2	+3	100	50
003c	Gpp-Bip-Gpp	648.2 (648.8)	3	+3	50	25
003d	App-Bip-Gpp	606.1 (606.7)	3	+2	150	100
004a	Arg-Bip-App	558.3 (558.7)	2	+2	>200	>200
004b	Lys-Bip-App	530.3 (530.7)	2	+2	>200	>200
004c	Gpp-Bip-App	606.2 (606.7)	3	+2	150	75
004d	App-Bip-App	564.3 (564.7)	3	+1	>200	>200

[a] Isolated hydrophobic units. Bip is regarded as a single separate unit in this study. [b] At physiological pH.

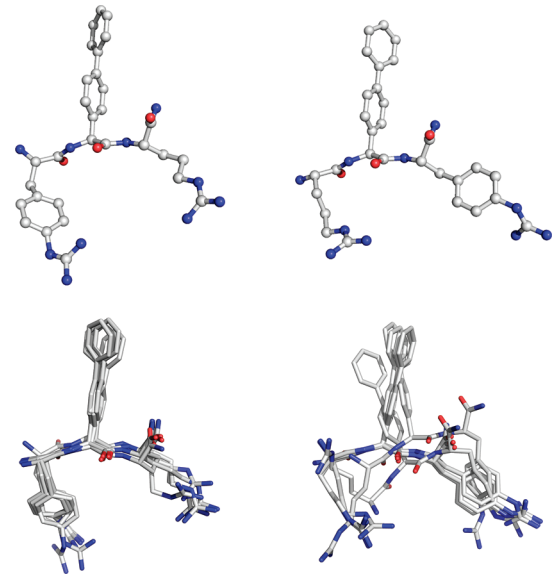

Figure 2. Three dimensional structures and overlays of the five conformers with lowest energy of Gpp-Bip-Arg (**001c**, to the left) and Arg-Bip-Gpp (**003a**, to the right) obtained after molecular modeling and geometry optimization using DFT. Red atoms represent oxygen, blue is nitrogen, and white atoms are carbon. Reversal of the peptide sequence still maintains the structural properties, in particular the amphiphilic nature of the peptides.

Microcalorimetric Studies. The binding of similar short CAPs to HSA have recently been shown to have a big impact on the antibacterial efficiency *in vitro* with a 10-fold increase in MIC reported following inclusion of HSA at physiological

(29) Still, W. C.; Tempczyk, A.; Hawley, R. C.; Hendrickson, T. Semianalytical Treatment of Solvation for Molecular Mechanics and Dynamics. *J. Am. Chem. Soc.* **1990**, *112*, 6127–6129.
(30) Parr, R. G.; Yang, W. Density-functional theory. In *Density Functional Theory of Atoms and Molecules*; Oxford University Press: New York, 1989; pp 47−69.
(31) Becke, A. D. A New Mixing of Hartree-Fock and Local Density-Functional Theories. *J. Chem. Phys.* **1993**, *98*, 1372–1377.
(32) *Jaguar*, version 7.5; Shrödinger, LLC: New York, 2008.
(33) Svenson, J.; Brandsdal, B. O.; Stensen, W.; Svendsen, J. S. Albumin Binding of Short Cationic Antimicrobial Micropeptides and Its Influence on the In Vitro Bactericidal Effect. *J. Med. Chem.* **2007**, *50*, 3334–3339.

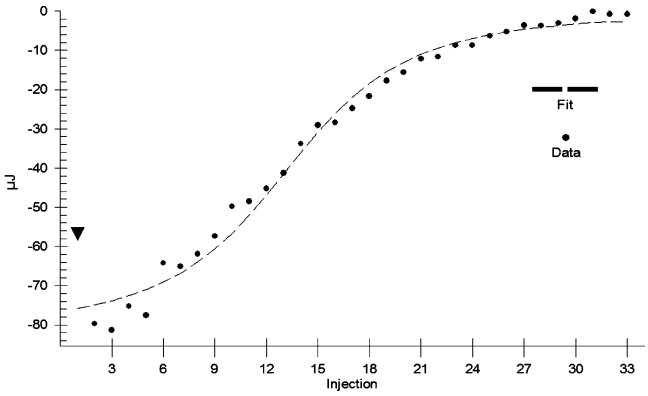

Figure 3. Binding isotherm for **004d** titrated into a solution of HSA and fit to the theoretical one site binding model provided by the software. The data from the first injection (▼) is not included due to slow peptide diffusion from the syringe during the equilibration step prior to the first injection. Standard deviation: 3.48.

Table 2. Data from the Peptide Binding to HSA and DMPG LUVs

peptide	MIC (μg/mL)[a]	n[b,c]	K_d (μM)[c]	ΔG (kJ/mol)[c,d]	ΔH (kJ/mol)[c,d]	$T\Delta S$ (kJ/mol)[c]
HSA						
001a	200	0.9	16.6	−27.3	−14.0	13.3
002b	>200	0.9	6.2	−29.7	−11.1	18.6
003c	50	0.8	13.8	−27.7	−14.1	13.6
004d	>200	0.9	5.1	−30.2	−11.5	18.7
DMPG						
001a	200	25.4	68.9	−23.7	−7.1	16.6
002b	>200	23.3	50.1	−24.5	−7.2	17.4
003c	50	23.5	81.9	−23.3	−9.1	14.2
004d	>200	17.0	21.4	−26.6	−9.7	16.9

[a] Generated using standardized microdilution technique. [b] Interaction stoichiometry. [c] Data from the ITC experiments and the binding isotherms. [d] Fitting error ± 15%.

concentrations in the assay.[33] Identical experiments were therefore performed to evaluate whether the use of arginine analogues could influence the protein binding (Figure 3). The different peptides exhibit comparable binding properties with dissociation constants between 5 and 17 μM, indicating that charge/basicity alone is not the sole contributor to albumin binding. The stronger hydrogen bonding capacity of the guanidino containing **001a** and **003c** is nevertheless apparent as an increase in binding enthalpy. The weaker bases **002b** and **004d** receive larger favorable entropic contributions, making the overall change in free energy for the four peptides similar with ΔG values ranging from −27 to −30 kJ/mol (Table 2). The binding to HSA was stoichiometric, and the site and mode of binding are currently under investigation.

The binding of the same selection of peptides to a Gram-positive bacterial cell membrane mimic (DMPG) and a eukaryotic cell membrane mimic (DMPC) was also assessed by ITC (Figure 4). Binding to the DMPG LUVs was strong for all the tested peptides with dissociation constants in the mid-micromolar range. No significant differences in binding strength were seen for peptides incorporating alternatives to arginine, and the complex stoichiometries compare well with previous reports on similar peptides.[34] The overall change in free energy (ΔG) ranged from −23 to −27 kJ/mol (Table 2) with a significant contribution coming from $-T\Delta S$, presumably due to the incorporation of the biphenyl side chain into the core region of the lipid bilayer, which is a strongly entropy driven event governed by hydrophobic interactions. A reference system encompassing neutral DMPC LUVs was used to mimic eukaryotic cells. As is shown in Figure 4B, the heat generated in those experiments equaled that of the heats of dilution, and no binding was detected for any of the tested peptides in this model.

Permeability Experiments. Since highly basic drugs, which carry a net charge at physiological pH in general, are poorly absorbed,[35] the incorporation of less basic arginine analogues into the peptide sequence should be beneficial from an uptake point of view, and this was thus investigated for a selection of the peptides using the phospholipid vesicle based model. In this system, compounds with P_{app}-values < 0.1×10^{-6} cm/s are generally classified as poorly absorbed (<30% absorbed *in vivo*), compounds with P_{app}-values between 0.1×10^{-6} and 0.9×10^{-6} cm/s are moderately absorbed (30−70% absorbed *in vivo*), while compounds with P_{app}-values of $>0.9 \times 10^{-6}$ cm/s have excellent oral absorption (>70% *in vivo* absorption).[25] The results of permeability studies are shown in Table 3 and Figure 5.

Hemolysis. None of the peptides displayed any significant hemolytic activity (<7%) at relative peptide concentrations up to 1000 μg/mL.

Discussion

L-Arginine represents an important epitope in many biological interactions.[35,36] Several enzymes such as the trypsin-like proteases and nitric oxide synthase (NOS) rely on an interaction between a negatively charged carboxylate in the active site and the positively charged guanidine of an arginine in the substrate to exert their action. Agonists and antagonists for these enzymes have thus historically been based upon ligands incorporating arginine. However, introduction of arginine analogues allows for the design of compounds with improved selectivity, activity and bioavailability, and a virtual plethora of arginine mimics have thus been prepared, beautifully described in the extensive reviews by Mašič[35,36] and in a report from

(34) Andrushchenko, V. V.; Aarabi, M. H.; Nguyen, L. T.; Prenner, E. J.; Vogel, H. J. Thermodynamics of the interactions of tryptophan-rich cathelicidin antimicrobial peptides with model and natural membranes. *Biochim. Biophys. Acta* **2008**, *1778*, 1004–1014.

(35) Mašič, L. P. Arginine mimetic structures in biologically active antagonists and inhibitors. *Curr. Med. Chem.* **2006**, *13*, 3627–3648.

(36) Peterlin-Mašič, L.; Kikelj, D. Arginine mimetics. *Tetrahedron* **2001**, *57*, 7073–7105.

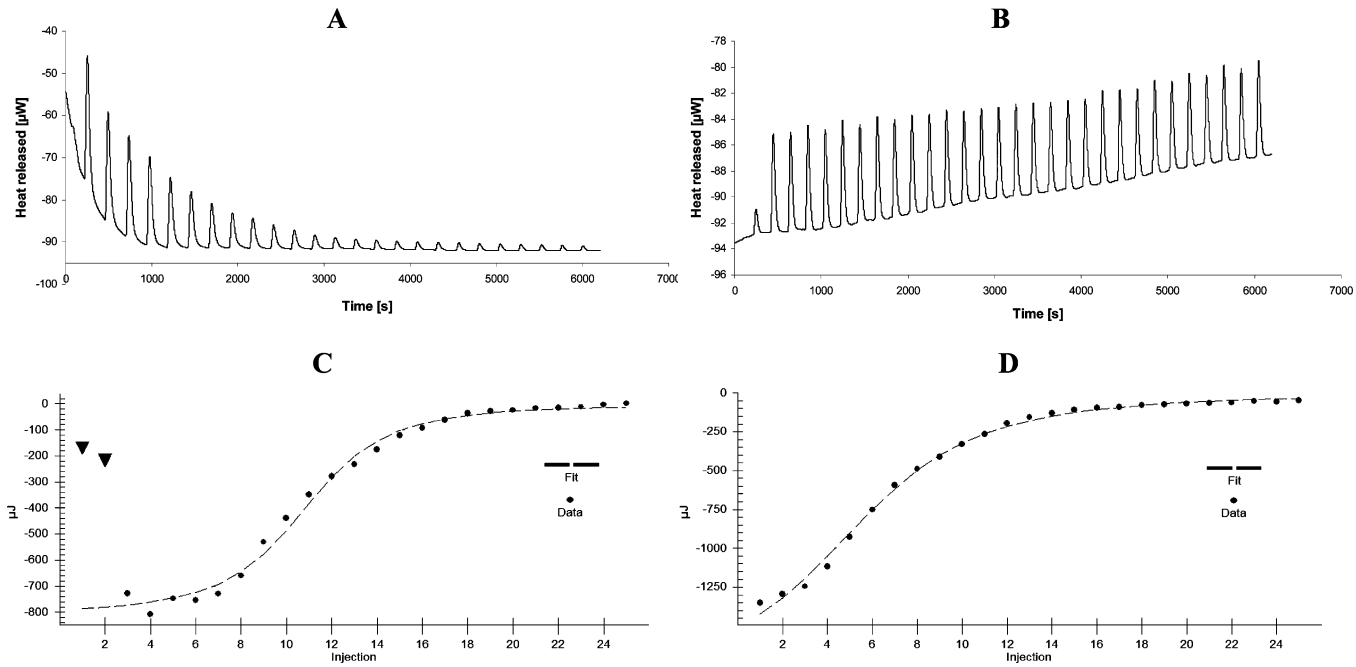

Figure 4. Raw data and examples of binding isotherms from the LUV−peptide ITC experiments. (A) Anionic DMPG LUVs titrated into a solution of **004d**. (B) Neutral DMPC LUVs titrated into **004d** illustrating the negligible interaction between peptide and LUV as compared to the anionic LUV shown in (A). (C) Binding isotherm for DMPG LUVs titrated into **003c** and fit to the theoretical one site binding model provided by the software. The data from the first injections (▼) is not included due to slow LUV diffusion from the syringe during the equilibration step prior to the first injection. Standard deviation: 26.62. (D) Binding isotherm for DMPG LUVs titrated into **004d** and fit to the theoretical one site binding model provided by the software. Standard deviation: 33.64.

Table 3. Physiochemical Parameters and Permeability Data

peptide	log P^a	charge[b]	P_{app} (10^{-6} cm/s)	oral absorption[c]
001a	−0.58	+3	0.007 ± 0.008	poor
002b	−0.03	+3	0.062 ± 0.026	poor
003c	2.08	+3	nd[d]	nd[d]
004d	2.23	+1	0.502 ± 0.325	moderate

[a] Calculated using Chemoffice Ultra 10.0 and Bio-Loom 1.5. [b] At physiological pH. [c] As discussed above.[25] [d] Not determined. Peptide **003c** influenced the integrity of the permeation barrier, yielding a reduced electrical resistance, and the results from the permeation of this peptide were therefore not included in the study.

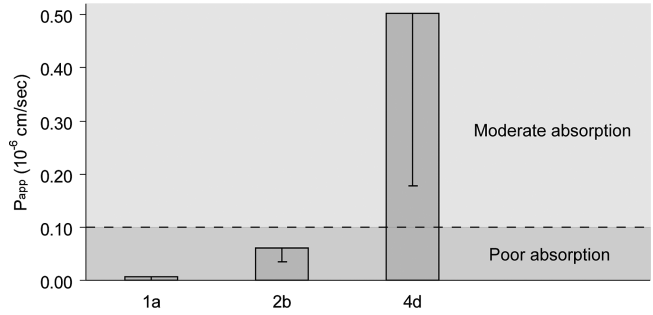

Figure 5. Graphical representation of the apparent permeability values and standard deviation for peptides **001a**, **002b**, and **004d**. The dashed line indicates the border between the classes of drugs displaying moderate and poor absorption. The level displaying excellent absorbed drugs (P_{app} = >0.9 × 10^{-6} cm/s) is outside the range in this figure. Large error bars are due to work in the lower range of the absorbance scale during analysis. Standard curves do show good linearity in the range measured (data not shown).

Kennedy et al.[37] In the CAPs investigated, the cationic charges provided by the arginines are responsible for the initial contact between the negatively charged membrane of the bacteria.[2,38] The bactericidal event taking place after this first interaction is still under debate, but it remains certain that a cationic element is essential for high activity.[39] This is regardless of the final mechanism for bacterial destruction, which may be due to interaction with an intracellular target and/or through membrane disruption via a carpetlike mechanism or through the formation of pores.[40]

(37) Kennedy, K. J.; Lundquist, J. T.; Simandan, T. L.; Kokko, K. P.; Beeson, C. C.; Dix, T. A. Design rationale, synthesis, and characterization of non-natural analogs of the cationic amino acids arginine and lysine. *J. Pept. Res.* **2000**, *55*, 348–358.

(38) Hancock, R. E. W.; Rozek, A. Role of membranes in the activities of antimicrobial cationic peptides. *FEMS Microbiol. Lett.* **2002**, *206*, 143–149.

(39) Vorland, L. H.; Ulvatne, H.; Rekdal, O.; Svendsen, J. S. Initial binding sites of antimicrobial peptides in Staphylococcus aureus and Escherichia coli. *Scand. J. Infect. Dis.* **1999**, *31*, 467–473.

(40) Yeaman, M. R.; Yount, N. Y. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol. Rev.* **2003**, *55*, 27–55.

Design of Peptides. The short cationic peptides developed by our group have historically utilized nongenetically encoded hydrophobic amino acids to provide increased bactericidal efficiencies.[15] For this study, biphenylalanine (Bip) was used on behalf of its ready availability and ease of incorporation. A library containing 16 tripeptides was prepared on a solid support employing Bip as the central residue flanked by any of four arginine-like amino acids. No bulky C-terminal modifications were included in an attempt to evaluate the influence of combining bulk and charge in the same side chain.

Arginine and lysine were included to represent naturally occurring basic amino acids and to generate reference peptides. Lysine has previously been used to produce peptides with somewhat inferior bactericidal potency in some studies,[13] but it has not been thoroughly investigated as a cationic element in other aspects. The two basic phenyl alanine derivatives L-2-amino-3-(4-aminophenyl)-propanoic acid (App) and L-2-amino-3-(4-guanidinophenyl)propanoic acid (Gpp) were included to establish the influence on the antibacterial effect of adding hydrophobic bulk in direct conjunction with the cationic charge and not as a separate amino acid residue or as a C-terminal end-capping. App is further a very interesting building block, as it incorporates a *p*-aminophenyl unit which is a known arginine mimic epitope used for increasing the oral bioavailability of peptidomimetic drugs such as thrombin inhibitors.[41,42] Gpp on the other hand displays a phenylguanidine epitope which also has been used in a range of protease inhibitors.[36,43,44]

Effect on Antibacterial Potency. As the apparent shortage of hydrophobicity and bulk allowed us to evaluate Gpp and App as bulky arginine analogues, most of the peptides investigated in this study incorporate one or two additional hydrophobic elements adjacent to the cationic groups. Those peptides thereby theoretically fulfill the minimum bactericidal motif but may simultaneously yield a class of peptides with a potentially changed secondary structure due to differences in the distribution of hydrophobic bulk.

Lys and App (pK_a 10.5 and 4.9, respectively) are poorer bases than Arg and Gpp (pK_a 12.5 and 10.8, respectively), and it is apparent that these compounds are less suited as cationic elements in these peptides when analyzing the results from the antibacterial study in Table 1. Lys produced poor antibacterial peptides compared to peptides containing Arg in the same position, which is in agreement with previous reports describing incorporation of Lys in antibacterial peptides derived from lactoferricin.[13] Vogel et al. have however shown that certain tritrpticin analogues retain their antibacterial potency upon complete substitution of Arg for Lys, and it is likely that this effect is both peptide and mechanism dependent.[45] Peptides prepared with App were generally poor antibacterial agents. App is too weak a base to be sufficiently protonated at physiological pH which clearly has a big impact. It may be suggested that the extra hydrophobic element supplied by App could improve the antibacterial potential (peptides **001d** and **004a**, for example, as both theoretically fulfill the pharmacophore) but it does not seem to be enough to yield an effective peptide of the type employed in these experiments. This suggests that the antibacterial motif may be more complex than what has been reported earlier.[13] Incorporation of Gpp, displaying both a unit of hydrophobicity and a positive charge, produced the most effective peptides with **003c** being the most potent peptide in this study, with two Gpp units providing both cationic charges and additional hydrophobic bulk (Figure 1). Interestingly, the two second most potent peptides, **001c** and **003a**, also indicate that the location of Arg and Gpp in this sequence is of less importance for the bactericidal effect, an observation which is generally valid for all the peptides included with peptide pair **002c/003b** being the only pair displaying any major differences in MIC. An activity independent of sequence suggests a nonspecific initial mode of binding not requiring a specific sequence or well-defined secondary structure for antibacterial activity, which would be in agreement with what is discussed in the recent study on antibacterial peptides and synthetic peptoid mimics from the Barron group[46] and with previous studies of similar short peptides.[13] For this class of compounds, introduction of hydrophobic bulk in the direct vicinity of the cationic charge increases the antibacterial effect, illustrating that a combination of both functionalities can successfully be combined in the same side chain. This discovery does not only provide insight into the structural motifs governing antibacterial activity, but it also allows for a more versatile design of these and similar peptidiomimetic compounds in the future. Unfortunately, the MIC values of the tested peptides all remain outside the concentration range of direct therapeutic

(41) Menear, K. Progress towards the discovery of orally active thrombin inhibitors. *Curr. Med. Chem.* **1998**, *5*, 457–468.

(42) Rewinkel, J. B. M.; Lucas, H.; Smit, M. J.; Noach, A. B. J.; van Dinther, T. G.; Rood, A. M. M.; Jenneboer, A.; van Boeckel, C. A. A. Design, synthesis and testing of amino-bicycloaryl based orally bioavailable thrombin inhibitors. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2837–2842.

(43) Sperl, S.; Jacob, U.; de Prada, N. A.; Sturzebecher, J.; Wilhelm, O. G.; Bode, W.; Magdolen, V.; Huber, R.; Moroder, L. (4-Aminomethyl)phenylguanidine derivatives as nonpeptidic highly selective inhibitors of human urokinase. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5113–5118.

(44) Pimenta, D. C.; Melo, R. L.; Caliendo, G.; Santagada, V.; Fiorino, F.; Severino, B.; de Nucci, G.; Juliano, L.; Juliano, M. A. Design of inhibitors for human tissue kallikrein using non-natural aromatic and basic amino acids. *Biol. Chem.* **2002**, *383*, 853–857.

(45) Schibli, D. J.; Nguyen, L. T.; Kernaghan, S. D.; Rekdal, O.; Vogel, H. J. Structure-function analysis of tritrpticin analogs: Potential relationships between antimicrobial activities, model membrane interactions, and their micelle-bound NMR structures. *Biophys. J.* **2006**, *91*, 4413–4426.

(46) Chongsiriwatana, N. P.; Patch, J. A.; Czyzewski, A. M.; Dohm, M. T.; Ivankin, A.; Gidalevitz, D.; Zuckermann, R. N.; Barron, A. E. Peptoids that mimic the structure, function, and mechanism of helical antimicrobial peptides. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 2794–2799.

importance, and it is clear that adding the hydrophobic bulk as a C-terminal modification, as has been investigated in numerous studies, yields more efficient peptides. The "standard" C-terminal modification however generally leads to an increase in susceptibility toward tryptic degradation, which further motivates these studies.[20]

Molecular Modeling. The modeling experiments initiated to establish whether these compounds indeed are random, which may be suggested by the antibacterial assay, actually revealed the opposite. Most of the peptides are instead unexpectedly clearly facially amphiphilic with two well-defined faces along the axis of the molecule.[47] This is the common distribution of bulk and charge seen for most antibacterial peptides,[4] but it has not been seen for these small peptides before which have previously been claimed to lack secondary structure in solution.[14] The extra units of hydrophobicity remain separated from the central bulky Bip moiety, which seem to maintain the overall amphiphilic structure. This suggests not only that these small compounds adopt a defined secondary structure, even in the absence of a negatively charged surface, but also that the additional hydrophobic elements incorporated in the cationic side chains do not have to belong directly to the hydrophobic region in the amphiphilic structure to contribute beneficially to the antibacterial activity. Of additional interest is the clear structural resemblance between the reversed structures which would explain their similar antibacterial activities. (See Figure 2.)

Microcalorimetric Studies. The overall binding for the peptides (**001a**, **002b**, **003c**, and **004d** in order to evaluate the different basic residues) to HSA was strong and did not differ much from what has previously been reported by Svenson et al.[33] A similar increase in MIC is thus expected when HSA is present in the antibacterial assay. ITC experiments with LUV membrane mimics were also performed in an attempt to couple the membrane binding strength with the antibacterial activity of the peptides. Since there is no standardized mimic described in the literature for these types of studies, DMPG LUVs served as a simple mimic of the Gram-positive bacterial cell membrane. It is an adequate mimic in the sense that it is anionic as compared to the zwitterionic eukaryotic cells, imitated by DMPC LUVs in this study. The charge density is nevertheless too high to correctly mirror the bacterial cell membrane in detail. The Gram-positive bacterial cell membrane is indeed mainly composed of anionic phosphatidyl glycerol and cardiolipin. However, since a substantial fraction of the polar head groups have a lysine moiety attached, the excess of anionic head groups is only 25%.[48] A range of different systems addressing the issue of more accurate mimicry have been evaluated, described, and compiled in the extensive work by Vogel et al., Lohner et al., and Yeaman and Yount.[34,40,48] The binding behavior of the peptides with DMPG LUVs was consistent with that recently reported for related 15 residue antimicrobial peptides derived from lactoferricin where relatively small ΔH values also were seen upon interaction with an anionic model membrane.[49] All peptides with three positive charges behaved very similar, while **004d** with a single positive charge exhibited a lower complex stoichiometry and a slightly different binding isotherm as is shown in Figure 4C and D. Vogel et al. recently reported the thermodynamics of the interaction between tryptophan rich antimicrobial peptides and similar model membranes as those used here. In their work, it was shown that certain peptides yielded complex ITC traces suggested to arise from simultaneous events such as pore formation, peptide aggregation, and changes to the lipid phases during the experiment.[34] We occasionally experienced similar results, but in our case it was dependent on the specific LUV batch (data not shown) and seemingly not on the properties of the individual peptides. In summary, the binding to DMPG LUVs indicates that these peptides all strongly bind and that this binding is governed by favorable changes in both entropy and enthalpy. The insignificant binding to neutral DMPC LUVs illustrates the negligible interactions between the neutral surface and the peptides. No apparent correlation between the antibacterial activity and the LUV binding is evident, indicating that an intracellular target may be involved in the bactericidal mechanism of these short CAPs. Comparison with the HSA experiments suggests a stronger interaction with albumin than with a bacterial surface under equilibrium conditions. This has been suggested in our recent work and the reader is referred to ref 33 for a comprehensive discussion on the potential implications of this competitive binding.

Permeability Experiments. Sufficient oral bioavailability is a prerequisite for a drug candidate, and it has recently been shown that a tight membrane barrier composed of fused deposited liposomes on a mixed cellulose filter can serve as an excellent model to screen the drug permeability.[25] The passive diffusion of a drug through such a barrier (P_{app}) correlates well both with absorption literature data from human *in vivo* studies and with Caco-2 cell models. Based on their apparent permeability values, the three peptides are categorized into the classes of moderately absorbed drugs for peptide **004d** and poorly absorbed drugs for peptides **001a** and **002b** applying the classification suggested earlier.[25] Both the basic polar side chains in **001a** and **002b** are expected to be fully ionized at pH 7.4, while peptide **004d** should only display a charged N-terminus. This is clearly correlated with the permeation rate, which is in accordance with the generally accepted perception of influence of charge on

(47) Walker, S.; Sofia, M. J.; Kakarla, R.; Kogan, N. A.; Wierichs, L.; Longley, C. B.; Bruker, K.; Axelrod, H. R.; Midha, S.; Babu, S.; Kahne, D. Cationic facial amphiphiles: A promising class of transfection agents. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 1585–1590.

(48) Lohner, K.; Sevcsik, E.; Pabst, G. Liposome-based Biomembrane Mimetic Systems: Implications for Lipid-Peptide Interactions. In *Advances in Planar Lipid Bilayers and Liposomes*; Liu, A. L., Ed.; Elsevier: Amsterdam, 2008; Vol. 6, pp 103−137.

(49) Jing, W. G.; Svendsen, J. S.; Vogel, H. J. Comparison of NMR structures and model-membrane interactions of 15-residue antimicrobial peptides derived from bovine lactoferricin. *Biochem. Cell Biol.* **2006**, *84*, 312–326.

passive diffusion through bilayers as well as experimental studies performed on peptidomimetic thrombin inhibitors where the ionization state of the drug candidates is described as a major limiting factor for oral adsorption.[41] A small but significant difference (Student's *t* test for comparison of two means with a significance level of $p = 0.05$) is however seen when comparing the P_{app}-values for **001a** and **002b**, perhaps further illustrating the effect of differences in base strength. The tripeptides showed increasing permeability with decreasing pK_a values of their hydrophilic side chains (Arg 12.5, Lys 10.5, and App 4.9). The reduced number of hydrogen bonds to the functionalities present in peptide **002b** as compared to **001a** may also serve as an explanation, in agreement with observations of Conradi et al.[50] This difference between **001a** and **002b** is reflected in differences in lipophilicity as well, which is regarded as another key parameter used in the estimation of drug absorption. Obviously, passive permeability of these peptides is facilitated by increasing log *P* values, which is further in accordance with Menear's findings for the thrombin inhibiting peptidomimetics displaying log *P* values in the range of 2−4.[41] Superior uptake of peptide **004d** is most likely due to a combination of higher lipophilicity, a single cationic charge, and a lower number of potential hydrogen bonds with surrounding water. The permeation of peptide **003c**, with three cationic charges and a log *P* value in the vicinity of that of **004d** could have revealed the influence of the individual contributions, but that peptide did unfortunately disrupt the permeation barrier. These results clearly demonstrate that the oral bioavailability for this class of peptides is low but that it can be significantly increased by replacing arginine with weaker bases. However, since the major chemical parameters influencing the uptake also are the ones that dictate the antibacterial effect, careful design is needed to generate a peptide that is both a potent antimicrobial and suitable for oral administration. For these short peptides, it may be detrimental for the antimicrobial activity with too many arginine replacements, but larger antimicrobial peptides carrying multiple charges should be able to benefit from increasing their oral bioavailability without impacting their antimicrobial activity.

Hemolysis. All the tested peptides displayed a very low hemolytic activity. A C-terminally benzylated version of **001a** also lacks hemolytic activity in a similar concentration range, suggesting that these peptides incorporating analogues can be further modified without inducing any significant changes in hemolytic activity.

(50) Conradi, R. A.; Hilgers, A. R.; Ho, N. F. H.; Burton, P. S. The Influence of Peptide Structure on Transport across Caco-2 Cells. *Pharm. Res.* **1991**, *8*, 453–1460.

Conclusion

Sixteen moderately antibacterial peptides with different combinations of cationic elements have been investigated in detail in an attempt to elucidate the role of arginine in short cationic antibacterial peptides. It is shown that charged moieties indeed are needed for activity, but it is not crucial to function that arginine supplies them. These and similar short lactoferricin derived peptides are simple enough to be rapidly synthesized at low cost, which in conjunction with their substantial stability and low hemolytic activity provides them with potential as future pharmaceuticals. Based on previous studies, it is perceived that introduction of hydrophobic bulk adjacent to the cationic charge provides units that will display altered or reduced binding to the S1 pockets of the proteolytic enzymes trypsin and chymotrypsin, and studies are underway where this impact is investigated for a larger library of arginine analogue containing peptides. These peptides will also be improved in terms of their antibacterial properties via different capping strategies.

Abbreviations Used

CAP, cationic antimicrobial peptide; DMPG, dimyristoylphosphatidylglycerol; DMPC, dimyristoylphosphatidylcholine; MIC, minimal inhibitory concentration; ITC, isothermal titration calorimetry; RP-HPLC, reversed phase high performance liquid chromatography; hRBC, human red blood cells; Bip, biphenyl alanine; App, L-2-amino-3-(4-aminophenyl)propanoic acid; Gpp, L-2-amino-3-(4-guanidinophenyl)-propanoic acid; HSA, human serum albumin; ATCC, American Type Culture Collection; LUV, large unilamellar vesicle; P_{app}, apparent permeability.

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Supporting Information Available: Purity data, HPLC-traces, and ^{1}H NMR spectra for all the tested peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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