Overcoming the Mucosal Barrier: Tetraether Lipid-Stabilized Liposomal Nanocarriers Decorated with Cell-Penetrating Peptides Enable Oral Delivery of Vancomycin

Philipp Uhl, Max Sauter, Tobias Hertlein, Dominik Witzigmann, Flavia Laffleur, Götz Hofhaus, Veronika Fidelj, Anja Tursch, Suat Özbek, Elisa Hopke, Uwe Haberkorn, Andreas Bernkop-Schnürch, Knut Ohlsen, Gert Fricker, and Walter Mier*

Despite the high medical need for oral peptide delivery, instability in the gastrointestinal tract and low mucosal permeation still impede this preferred route of administration. Herein, a liposomal nanocarrier combining two self-reliant strategies to overcome these delivery barriers is reported. This approach enables the design of a nanocarrier system with synergistic properties: tetraether lipids derived from archaea are incorporated into liposomes to provide the particles with the stability required to traverse the stomach. When the surface of the resulting inert particles is modified with cell-penetrating peptides, mucosal permeation can be achieved. The designed nanocarrier is proven effective by the high mucosal uptake of the glycopeptide antibiotic vancomycin in Ussing chamber studies. Efficacy in vivo is demonstrated in naïve rats, where a highly increased oral bioavailability is obtained for vancomycin, a drug known to be minimally absorbed. In contrast, administration of liposomes with single modification (tetraether lipids) leads to a substantially lower bioavailability. Therapeutic efficacy is proven by the antimicrobial activity of vancomycin in a Galleria mellonella and a systemic infection mouse model. The high oral bioavailability in absence of cytotoxic effects demonstrates that this nanocarrier delivery strategy might boost the oral application of macromolecular drugs in general.

1. Introduction

The number of macromolecular drugs, in particular peptides and antibodies, steadily increases.[1] Despite the benefits of macromolecular drugs, their troublesome application still represents a major limitation.[2] Attempts for their oral administration are hampered by degradation in the acidic milieu of the stomach and low mucosal permeation.[1,4] Even approaches, that succeeded with oral delivery to the intestine, failed due to the enzymatic degradation of the drug.[5] Therefore, the practicable administration routes remain subcutaneous or intravenous application, both causing high medical costs and low patient compliance.[6] To circumvent these limitations, several approaches for macromolecular drug delivery have been studied with focus on nanocarriers, including solid lipid nanoparticles,[7,8] nano- or microemulsions,[9,10] polymeric micelles[11] or surface-modified liposomes.[12-14]
Unfortunately, none of these formulations could provide results sufficient for further clinical application. This failure can be explained by the tightness of the intestinal barrier, designed to protect the entry of macromolecules that potentially endanger the health.

A successful carrier system that allows the selective oral uptake should combine the protection of the drug while allowing for mucosa penetration. This could be achieved by the nanocarrier approach illustrated in Figure 1, which is based on three steps: first, a high drug load is provided by liposomal formulation using dual asymmetric centrifugation; second, the liposomes are stabilized using membrane spanning tetraether lipids (TELs) and third, mucosa interaction and permeation of the resulting inert liposomes is achieved by surface modification with cell-penetrating peptides (CPPs).

The TELs used in this study are able to stabilize bilayer membranes and thus confer strongly enhanced stability of the liposomes. As components of the membrane of archaea, these lipids contain ether bonds and are therefore less susceptible to hydrolysis and oxidation than normal ester phospholipids. Previous studies demonstrated an increase in the oral availability of the octapeptide octreotide using TEL-liposomal formulations, while subsequent studies established a liposomal formulation for the hepatitis B entry inhibitor Myrcludex B containing glycerylcaldyltetreather lipid (GCTE), an isolated tetraether lipid from Sulfolobus islandicus. Moreover, this liposomal formulation showed increased oral availability of the glycopeptide antibiotic vancomycin. A further study demonstrated that liposomes containing a mixture of tetraether lipids derived from Sulfolobus islandicus can withstand intestinal bile salts.
Despite this improvement, the low mucosal permeation still represents the main hurdle to obtain sufficient resorption levels in the intestine. Specifically, the incorporation of TELs leads to inert liposomes that are less prone to interaction with the mucosal endothelium. Therefore, more expedient methods for enhancement of mucosal permeation have to be addressed. For this purpose, in this study, the liposomal nanocarrier, was modified on its surface with CPPs. The class of CPPs consists of short peptides of less than 30 amino acids and increasingly gains attention in noninvasive delivery technologies for macromolecules. In general, CPPs are able to penetrate cell membranes and to translocate different cargos into cells. It has been previously shown, that the oral coadministration of insulin and the linear CPP penetratin could increase insulin blood levels in mice. Based on these findings, in this study, CPP-phospholipid conjugates were synthesized and incorporated into tetraether lipid containing liposomes. With respect to the enzymatic stability of the cell-penetrating peptide presented on the liposomal surface, special, cyclic CPPs were used. Cyclic CPPs are known to be less susceptible to hydrolysis by peptidases. The idea beyond this strategy was the assumption, that the CPPs presented on the liposomal surface enhance the mucosal permeation while GCTE stabilizes the liposomes both in the gastric milieu and also against enzymatic degradation in the intestine (Figure 1).

The synthesis strategy of the conjugates (preparation and purification of the conjugates prior to liposomal preparation) shows various advantages in comparison with the common method of liposomal surface modification (performance after liposomal production): the CPP-phospholipid conjugates can be purified by preparative high performance liquid chromatography (HPLC) after the synthesis which enables the incorporation of highly purified conjugates into the liposomes during liposomal production. Due to this procedure, it can be ensured that no unrelated head group-modified phospholipid is contained in the liposomes. This avoids possible side-reactions, e.g., between the lipid and the incorporated drug. The manufacturing of these modified liposomes was performed by the dual asymmetric centrifugation (DAC) technology as described previously. In a proof of concept study, the glycopeptide antibiotic vancomycin was incorporated into the liposomes. In rats, the vancomycin blood levels were found to be substantially increased after oral administration. The applicability for therapeutic purposes was confirmed in a systemic infection mouse model. An oral single dose administration showed a reduction of the colony forming units (CFU) in the kidneys.

2. Results

Liposomes are still considered to be one of the most promising drug delivery systems. Encouragingly, some liposomal formulations such as AmBisome or Doxil have received approval by both Food and Drug Administration (FDA) and European Medicines Agency (EMEA) and are commonly used in clinics all over the world. However, until now, nanocarrier approaches have not succeeded in establishing oral formulations of macromolecular drugs in general. Therefore, we attempted to establish an oral liposomal formulation applicable for these drugs.

First, the phospholipid conjugate of the cyclic, arginine-rich cell-penetrating peptide (R9K) was synthesized (for synthesis strategy see Figure S1 in the Supporting Information) and purified by preparative HPLC (MS analytics see Figures S2 and S3 in the Supporting Information). After the synthesis was completed, this conjugate was incorporated into GCTE-stabilized liposomes (for liposomal compositions and preparation method see Tables S1 and S2 in the Supporting Information) in a molar ratio of 0.1–3 mol% and liposomal characteristics were determined by dynamic light scattering (Zetasizer) and nanoparticle tracking analysis (NanoSight). While the size of the liposomes remained nearly constant in this molar ratio of the conjugate (Figure 2A), with increasing concentrations of the conjugate, the zeta potential strongly increased (Figure 2B), demonstrating the successful incorporation of the CPP-conjugate into the GCTE-stabilized liposomes.

The overlay of the results obtained by NanoSight measurements (Figure 2C) clearly indicates that the size distribution of the CPP-modified liposomes is slightly broadened in comparison to control liposomes (containing only lecithin and cholesterol) and the GCTE-modified ones. For an overview of liposomal characteristics determined by nanoparticle tracking analysis, see Table 1 Analysis of the CPP-modified liposomes by Cryo-TEM (Figure 2D) revealed no relevant differences in their morphology in comparison to unmodified liposomes. Additionally, the CPP-liposomes displayed low lamellarity, the prime prerequisite for high drug loading capacity.

Due to the hypothesis that the positively charged cell-penetrating peptides on the surface of the liposomes might interact with negatively charged components of the intestinal mucosa, in the second step of development, two cell binding assays using the colon carcinoma cell line Caco-2 and in vitro model were performed. One assay was based on fluorescence measurements. Therefore, 1 mol-% of a rhodamine-modified phospholipid was incorporated into the CPP-modified liposomes and binding on Caco-2 cells was compared with binding of control liposomes. The control liposomes showed no obvious binding on the Caco-2 cells.

In contrast, the CPP-liposomes showed a strong binding already after five minutes, which was once again enhanced after 15 minutes (Figure 3A). One further assay was based on binding and uptake studies of radiolabeled vancomycin (radiolabeled with 125I), which was incorporated into the liposomes. Both, the membrane binding and also the internalization of the purified CPP-modified liposomes on Caco-2 cells were examined after removal of free vancomycin by size exclusion chromatography (SEC) and again compared to control liposomes. As shown before, the CPP-liposomes enabled a strongly enhanced membrane binding and also the internalization rate of 125I-vancomycin in comparison with the control liposomes was increased (Figure 3B). Additionally, the encapsulation efficiency of vancomycin in the different liposomal formulations was determined by HPLC measurements before and after the purification by SEC. Comparing the different formulations, no relevant difference with respect to the encapsulation efficiency could be observed (Table S3, Supporting Information), demonstrating that the modification with CPP-phospholipid conjugates does not influence the encapsulation rate of APIs.

To evaluate the permeation of vancomycin across rat intestine, Ussing chamber studies were conducted. Therefore, vancomycin was incorporated into CPP-modified liposomes and
Figure 2. Characterization of CPP-modified liposomes. A) Size and PDI of CPP-modified liposomes after incorporation of different amounts of the CPP-phospholipid conjugate; mean ± SD; n ≥ 3. B) Zeta potential analysis of CPP-modified liposomes (1.0 mol% of CPP—phospholipid conjugate) in comparison to control and GCTE-modified liposomes; mean ± SD; n ≥ 3. Statistical significance was analyzed with one-way ANOVA, followed by the Tukey’s multiple comparison test: *p < 0.05, **p < 0.01, ***p < 0.001. C) NanoSight-analysis (overlay) of control, GCTE-, and CPP-modified liposomes. D) Cryo-TEM analysis of CPP-modified (D1) and control (D2) liposomes; scale bar = 100 nm.

Table 1. Nanoparticle tracking analysis of different liposomal formulations (n ≥ 3).

|                | Control liposomes | GCTE-liposomes | CPP-modified liposomes |
|----------------|-------------------|----------------|------------------------|
| Mean [nm] ± SE | 125.5 ± 0.7       | 159.6 ± 1.8    | 157.8 ± 1.2            |
| D10 [nm] ± SE  | 87.3 ± 0.3        | 106.7 ± 0.7    | 104.3 ± 0.7            |
| D90 [nm] ± SE  | 170.8 ± 1.9       | 220.8 ± 4.1    | 223.2 ± 5.4            |

Free vancomycin was removed by size exclusion chromatography. These studies showed a highly increased transport of vancomycin across the rat intestine for the CPP-modified liposomes in comparison with free vancomycin (Figure 3C).

A coadministration of vancomycin and the free cell-penetrating peptide (cyclic R9) could also not provide similar results, demonstrating that the CPP-modified liposomes are required for the increased uptake of vancomycin.

Cytotoxicity studies were also performed on Caco-2 cells in different liposomal concentrations using the alamarBlue assay. These studies showed no relevant cytotoxicity for all eligible liposomal concentrations (<1 mg liposomes mL−1; Figure S4A, Supporting Information). Higher concentrations (>1 mg liposomes mL−1) showed a moderate increase in liposomal toxicity, demonstrating that the toxicity might be dependent on the amount of CPP–phospholipid conjugate. Nevertheless, these concentrations of the liposomes (>1 mg mL−1) are far beyond liposomal concentrations achievable in a clinical setting. With respect to one of the most limiting factors of liposomal suspensions, namely the release of drug out of the liposomes during storage, in this study, a method for freeze-drying of this liposomal formulation was tested according to Uhl et al.[17] It is clearly shown that freeze-drying is successful using 500 × 10⁻³ m sucrose as lyoprotector (Figure S4C, Supporting Information). High recovery rates (> 80%) for vancomycin encapsulated in the CPP-modified liposomes after freeze-drying and subsequent rehydration with phosphate buffered saline could be demonstrated (Table S4, Supporting Information). These studies also revealed high stability (size, PDI) of the CPP-modified liposomes in the rehydration media for a period of one week. However, slow leakage of vancomycin was observed, indicating that...
lyophilization is the prime prerequisite for storage (Table S5, Supporting Information).

In contrast to sucrose, other commonly used lyoprotectors such as trehalose did not provide comparable results (Figure S4D, Supporting Information). Interestingly, there was no relevant difference between solving the lyoprotector in phosphate buffer and the addition just before the freeze-drying process (Figure S4B, Supporting Information). Upon completion of in vitro analysis, the established liposomal formulation was tested in an animal model using female Wistar rats. First, the pharmacokinetics of the CPP-modified liposomes after oral administration by gavage were examined (Figure 4B). This study showed, that the highest blood level of oral liposomal vancomycin was reached 60 min post administration. For this reason, this time point was chosen for comparing the formulations with controls and it could be demonstrated that the CPP-modified liposomes showed a high increase in the blood levels of vancomycin (Figure 4C).

To ensure that liposomal encapsulation of vancomycin does not affect its activity, the minimal inhibitory concentration (MIC) of liposomal vancomycin in comparison to free vancomycin on different bacterial strains was determined. As expected, the liposomal formulation of vancomycin showed high and comparable activity as free vancomycin (Table 2). To verify that the absorbed vancomycin after oral administration remained its functionality, two in vivo infection models were performed. Both models highlighted the therapeutic efficacy of liposomal vancomycin against methicillin-resistant *Staphylococcus aureus* (MRSA) strain LAC<sup>*</sup> lux. In the first model, *Galleria mellonella* larvae were infected in

### Table 2. MIC determination of CPP-modified liposomal vancomycin in comparison to free vancomycin on different *Staphylococcus aureus* strains (n = 4 individual triplicates).

| Bacterial strain | MIC CPP-modified liposomal vancomycin [µg mL<sup>−1</sup>] | MIC free vancomycin [µg mL<sup>−1</sup>] |
|------------------|----------------------------------------------------------|------------------------------------------|
| LAC<sup>*</sup> lux | 0.5                                                       | 1                                        |
| LAC              | 0.5–1                                                     | 1                                        |
| JE2              | 1                                                         | 1                                        |

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**Figure 3.** Characterization of CPP-modified liposomes using Caco-2 cells and rat intestinal tissue. A) The CPP-modified liposomes (containing 1 mol% of a rhodamine-modified phospholipid) showed a strong binding on Caco-2 cells in comparison to the control liposomes, which showed no binding at all; scale bar = 100 µm. B) A radiolabeled cell binding assay (125<sup>I</sup>-vancomycin) showed a strongly increased membrane binding and also an increased internalization rate of the radiolabeled compound incorporated in the CPP-modified liposomes; mean ± SD; n = 3. C) Ussing chamber studies using rat intestine revealed a highly increased transport of vancomycin incorporated into the CPP-modified liposomes in comparison to control groups; mean ± SD; n ≥ 3.
Figure 4. Blood concentration of vancomycin after oral administration in female Wistar rats. A) Schematic illustration of the proof of concept study in rats. B) Vancomycin blood levels after oral administration of CPP-modified liposomes (orange) are compared with vancomycin i.v. (blue) over 5 h. The liposomal formulation reaches the highest blood levels of vancomycin after 60 min ($\text{AUC}_{0–300} = 1919$), while vancomycin i.v. is rapidly excreted ($\text{AUC}_{0–300} = 4758$). C) Blood levels of $125^I$-vancomycin 1 h post oral administration of the CPP-modified liposomal formulation in contrast to free vancomycin, control liposomes (consisting solely of lecithin and cholesterol) and GCTE- liposomes. The CPP-modified liposomes enabled an up to five-fold increase in vancomycin blood levels (mean $\pm$ SD; $n = 3$). Statistical significance was analyzed with one-way ANOVA, followed by the Tukey’s multiple comparison test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and n.s. = not significant.

Figure 5. A) Galleria mellonella in vivo infection model. Both, CPP-modified liposomal and free vancomycin significantly decreased ($p < 0.001$) mortality of Galleria mellonella infected with S. aureus strain LAC$^+$ lux in comparison to the control (PBS) group. Between both vancomycin groups, no significance could be observed ($n = 40$ larvae per group). Statistical significance was analyzed by Gehan–Breslow–Wilcoxon test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and n.s. = not significant. B) Resistance induction testings. In contrast to the positive control (fusidic acid) which showed rapid development of resistance, none could be observed for both vancomycin formulations over a period of nine passages on S. aureus strain LAC$^+$ lux in order to investigate whether treatment with CPP-liposomal vancomycin or free vancomycin enhanced the survival of the maggots in comparison to PBS as control treatment (Figure 5a).

While less than 10% of the larvae treated with PBS survived the first 38 h after infection, more than 70% of those treated with CPP-liposomal or free vancomycin were alive. Overall, treatment with free or liposomal vancomycin led to a significantly improved survival of the G. mellonella larvae in this experiment, with importantly, no significant difference between free and liposomal vancomycin.

In parallel, resistance development testings were performed comparing the CPP-liposomal and the free vancomycin formulation. The results presented in Figure 5B demonstrate that the bacteria were able to adapt up to concentrations of 2 or $4 \mu g \, mL^{-1}$.
Figure 6. MRSA systemic infection mouse model. The CFU in the kidneys are shown. A) Schematic illustration of the conventional therapeutic infection model. B) In the therapeutic study (drug administration 1 h post infection), a reduction of the CFU in the kidneys could be shown for the CPP-modified liposomes in contrast to control groups (PBS, vancomycin oral). Shown are the values of each individual mouse and the median values of the respective group; \( n = 6 \). C) In the prophylaxis study (drug administration 1 h prior infection), again the CFU in the kidneys could be reduced in the CPP-liposomal group in comparison to all other groups. Shown are the values of each individual mouse and the median values of the respective group \( n \geq 9 \). One included value of an individual mouse in the vanco i.v. — group is not depicted, as it does not fit the Y-axis scaling (100 CFU).

of liposomal or free vancomycin but could not grow at higher concentrations during steadily increasing antibiotic concentrations for 9 passages.

In contrast, challenging the bacteria with steadily increasing concentrations of fusidic acid as a positive control led to fast resistance development, elevating the tolerated concentration of this antibiotic up to 64 µg mL\(^{-1}\) at passage 9. These findings highlight the challenges for resistance development to vancomycin for \( S. aureus \), which was not altered by the liposomal encapsulation.

As second in vivo infection model, investigations in a (MRSA) systemic infection mouse model were performed. In the first part of the study, vancomycin encapsulated in the CPP-modified liposomes was examined in a conventional “treatment” schedule in comparison to intravenous vancomycin and two control groups (PBS and free vancomycin oral; for schematic illustration see Figure 6A). The success of this formulation and delivery strategy could be shown by the reduction of the CFU in the kidneys of the infected mice in comparison to the negative control groups (Figure 6B). A second prophylactical study was performed in order to demonstrate the influence of the different pharmacokinetics of the CPP-modified liposomes in comparison to intravenously administered vancomycin. The liposomal formulation reached the highest plasma concentration of vancomycin about 1 h post administration (Figure 4B). Therefore, in this study, the mice were infected ultimately after dosing. Again, a reduction of the CFU in the kidneys was observed in contrast to vancomycin i.v. and the further control groups (Figure 6C).

3. Discussion

The development of formulations enabling the oral administration of macromolecular drugs is still considered to be a difficult issue. To stop this unsustainable trend, the aim of this study was the development of a formulation based on cell-penetrating liposomes for oral delivery of vancomycin. Cell-penetrating peptides showed promising effects in co-administration studies before.\([24,27–29]\) Nevertheless, this coadministration approach seems to exhibit several disadvantages such as the fact that the CPP, which should permeate the adsorption, is not directly linked to the macromolecular drug. Furthermore, the enzymatic environment in the intestine may inactivate both the CPP and also the incorporated drug. It has also to be considered that cross-reactions between the CPP and the drug could occur. Therefore, in this project, the cyclic CPP was directly linked to the
Figure 7. Ways to overcome the permeation barrier in the intestine. Up to now, the mucosal barrier can only be overcome by drugs that cross the barrier a) by passive diffusion or b) by the use of specific transporters. In contrast, the liposomal nanocarrier (c) enables the transfer of encapsulated substances through the permeation barrier independently of the substance characteristics. Therefore, this nanocarrier might represent a platform technology for oral delivery of macromolecular drugs.

liposome, which should protect the drug in the GIT. That CPPs can be covalently linked to liposomes was reported previously.[30,31] But in most cases, the CPP was linked to the liposome after liposomal preparation. In this study, another approach was applied to enable the incorporation of the CPPs during liposomal preparation, preventing the subsequent purification steps. Inspired by this hypothesis, a class of CPP-phospholipid conjugates was established. The feasibility of the CPP-phospholipid synthesis and purification could be demonstrated by HPLC/MS-analysis. Additionally, these conjugates could be successfully incorporated into GCTE-containing liposomes during liposomal production as shown by the high increase in the zeta potential of the CPP-modified liposomes. This strategy shows several benefits in comparison with the modification of the liposomal surface post manufacturing.[30,32] Because by the use of these conjugates, a defined amount of CPP can be attached to the liposomal surface. However, it should be considered, that the stability of the CPP in the GIT is a must for CPP-derived adsorption in the intestine. Poor stability of linear CPPs was previously reported.[24] Therefore, in recent studies, we investigated linear and cyclic cell-penetrating peptides with respect to their stability in simulated gastric and intestinal fluid.[25] It could be clearly shown by HPLC/MS analysis, that the cyclic CPP was more stable than the linear ones, which matches the findings of Mandal et al.[31]

Based on these results, the cyclic CPP–phospholipid conjugate was chosen to be incorporated into the liposomes for the sub-sequent studies. The assumption, that the CPP-liposomes will strongly bind to the Caco-2 cells in the intestine, was evidenced by two cell binding assays, which clearly showed the prolonged mucosa retention and also the enhanced drug uptake for the CPP-liposomes in contrast to the control groups (for schematic illustration, see Figure 7). The mechanism of this effect is still unknown. However, Kamei et al.[29] performed mechanistic studies for a linear R8 peptide by evaluating the uptake across membranes of Caco-2 cells and an intestinal epithelial transport via energy-independent pathways was postulated. An interesting further aspect would be the determination of the localization of the CPP–phospholipid conjugate to gain more information of possible uptake mechanisms. Regarding the alamarBlue assay and with respect to the dilution of the particles after oral administration by gastric and intestinal fluid, the liposomes showed high cytocompatibility up to the calculated target concentration (<1 mg mL⁻¹). This could be previously shown for CPPs in similar concentrations.[34–36] For the transfer of the in vitro results to in vivo, Ussing chamber studies were performed and a strong increase in the vancomycin-uptake for the CPP-modified liposomes could be shown, even in comparison with the simulated coadministration strategy of API and CPP, highlighting the strong benefit of our nanocarrier approach. As result of this observation, it was decided to perform in vivo experiments using female Wistar rats. Wistar rats represent an established and trustworthy test system for such bioavailability experiments.[16–18,37] The pharmacokinetics of the liposomal formulation was determined in comparison to vancomycin after intravenous administration and it could be shown that the highest blood level of the CPP-liposomes after oral administration were reached one hour post administration. Consequently, this time point was regarded as the most descriptively one for the comparison of the CPP-liposomes with control groups. Again, the administration of CPP-liposomes resulted by far in the highest blood levels of vancomycin (Figure 4). This highlights the efficacy of the liposomal nanocarrier, as the free vancomycin is known to be minimally absorbed.[38]

Based on these promising results, it was decided to perform in vivo infection studies. Both models used highly demonstrated the therapeutic efficacy of liposomal vancomycin against S. aureus LAC® lux. Such systemic infection mouse models have been performed previously several times, few times for liposomal formulations, but mostly for intravenous and intraperitoneal routes of administration.[39,40] Further studies had been performed using a macrophage infection model.[41] Therefore, this is to our knowledge, the first successful attempt of oral administration of liposomal encapsulated vancomycin in such an infection mouse model. In the first part of the study, a conventional (therapeutic) treatment scheme was chosen.[42,43] Encouragingly, the
CPP-liposomal group, which obtained vancomycin incorporated into the CPP-modified liposomes, could show a reduction of CFU in the kidneys. These findings clarify that intact vancomycin reaches the blood after oral administration of the CPP-modified liposomes enabling the treatment of systemic MRSA-infections by the oral route. The inferior efficacy in comparison to intravenous vancomycin is not unexpected due to the substantially lower C_{\text{max}} values after oral administration (Figure 4B), especially due to the single dose administration scheme.

However, because the utilized MRSA strain shows a susceptibility of 1 µg mL^{−1}, the observed reduction of the CFU in the kidneys is a demonstration of the high oral bioavailability reached with the presented liposomal drug delivery system bearing in mind that this effect could be obtained after single dose administration.

The reduction of the CFU in the kidneys and superiority of liposomal to intravenous vancomycin in the prophylactic setup confirms the high bioavailability and that therapeutic blood concentrations could be reached. It is in line with the expected time to reach maximum blood concentration for intravenous and oral liposomal administration. The higher efficacy in comparison to the conventional treatment schedule fits furthermore to the relatively earlier effect on bacterial growth. These results look very promising and might facilitate new strategies in oral peptide delivery in future.

4. Conclusion
The highly increased oral availability of vancomycin in combination with the therapeutic efficacy in a systemic infection mouse model demonstrates the great potential of this nanocarrier delivery system. The in vivo results are in line with the in vitro experiments that showed strongly enhanced binding on Caco-2 cells and improved uptake of CPP-liposomal encapsulated vancomycin in Ussing chamber studies.

This demonstrates, that the synergistic combination of two self-reliant modification strategies can be used to achieve unprecedented delivery properties.

5. Experimental Section
Materials: Lecithin (EPC) was obtained from AppliChem GmbH (Darmstadt, Germany), all kinds of head group-modified phospholipids from Avanti Polar Lipids (Alabaster, Alabama, USA) while cholesterol was obtained from Sigma Aldrich (Steinheim, Germany) and Amicon Ultra-4 centrifugal filters from Merck Millipore (Tullagreen, Ireland) while Fitropol’s 0.2 sterile filters were purchased from Sarstedt (Numbrecht, Germany). Dulbecco’s phosphate buffered saline was applied from Gibco by life technologies (Paisley, UK); Atto dyes from Atto-tec GmbH (Siegen, Germany), NAP-S columns were obtained from GE Healthcare (Buckinghamshire, UK) and radioiodine^{121}I was purchased from Hartmann Analytic GmbH (Braunschweig, Germany), while Triton X-100, chloroform, methanol, and all other solvents were obtained from Sigma Aldrich (Taufkirchen, Germany). Vancomycin was obtained from Sigma Aldrich (Steinheim, Germany) and the cyclic R9-PP was synthesized by solid phase synthesis in the laboratory. For the cell binding assay, DMEM medium (high glucose), fetal bovine serum, and Trypsin-EDTA was purchased from Thermo Fisher (Waltham, Massachusetts, USA) while glass coverslips were obtained from Greiner-Bio one (Frickenhausen, Germany), The dye DAPI was obtained from Sigma Aldrich (Steinheim, Germany), Mowioli 4–88 from Roth (Karlsruhe, Germany), and alamarBlue from Bio-Rad antibodies (Puchsheim, Germany).

Methods—Synthesis of CPP: The cyclic cell-penetrating peptide Lys-(Arg)_{9} (referred to as cyc R9-CPP) was synthesized via solid phase peptide synthesis (SPPS) on a chlorotriyl resin (2-CTC) employing the Fmoc/tBu strategy as described previously.44) 94 mg (0.2 mmol) Fmoc-Lys(Boc)-OH dissolved in dichloromethane (DCM) with 5 eq. diisopropylethylamine (DIPEA) were loaded onto 250 mg of 2-CTC for 90 min at RT in order to yield a loading of 0.8 mmol g^{−1}. The resin was preswelled in DCM. After the coupling of Fmoc-Lys(Boc)-OH to the resin, the uncoupled part of the amino acid was removed by washing with DCM for three times. Subsequently, free reactive sites on the resin were blocked by the addition of DCM/methanol/DIPEA (17/2/1 v/v/v) for 30 min. Then, the resin was washed with DCM and DMF followed by nine consecutive steps of coupling Fmoc-Arg(Pbf)-OH in DCM for 20 min at RT, using an excess of 5 eq. amino acid, 4.75 equivalents HBTU, and 4 equivalents DIPEA. Fmoc-groups were removed before each coupling and after final coupling by treatment with 20% piperidine in DMF. In between steps, the resin was washed rigorously with DMF. The resulting peptide was then cleaved from the resin with 10% acetic acid and 20% trifluoroethanol in DCM. The procedure was repeated two times for 3 h. The cleavage solution was subsequently evaporated with an excess of toluene for three times for quantitative removal of acetic acid. The side chain protected peptide was dissolved in DMF in a concentration of 3 mg mL^{−1}. Subsequently, the cyclization was performed with 4 equivalents of PyAOP and DIPEA at RT overnight. After stopping the reaction with water (10% of reaction volume), the solution was concentrated to a fifth of hundredth of the starting volume and the side chain protected cyclic peptide precipitated by pouring into cold tert-butyl methyl ether. The precipitated protected cyclic peptide was dried and subsequently deprotected with a mixture of 5% ethanedithiol in TFA. Finally, the peptide was precipitated in diethyl ether and centrifuged for 5 min at 3000 × g. The pellet was dried under vacuum and the peptide was purified via preparative HPLC (Reprosil Pur 120 C18-AQ, 5 µm (250 × 25 mm), 0–30% acetonitrile + 0.1% TFA in 25 min) and the purity of the peptide was confirmed by LC-MS.

Methods—Synthesis of Mal-PEG_{12}-cR9K: 20 mg of Mal-PEG_{12}-NHS (Iris Biotech GmbH, Marktredwitz, Germany) and 20 mg of cR9K were dissolved in 2 mL DMF containing 4 eq. DIPEA. Reaction took place at RT for 2 h. After dilution with water (1:10), the Mal-PEG_{12}-cR9K was purified by preparative HPLC as described previously.

Methods—Synthesis of CPP-Phospholipid Conjugate: To 5 mL of a 1 × 10^{-3} M solution of the thiol-modified phospholipid in a 2:1 mixture of DCM/DMSO, 20 mg of Mal-PEG_{12}-cR9K pre-dissolved in 200 µL DMSO were added. The reaction mixture was stirred overnight at room temperature. The solvents were evaporated and the crude product was dissolved in 20 mL of a 4:1 mixture of ACN/H_{2}O. Purification was performed on a LaPrep P 110 (WVR International) HPLC system equipped with a Resprosil Gold 120 C-18 column (4 µm, 150 × 20 mm). Water and acetonitrile containing 0.1% TFA were used as eluents with a flow rate of 20 mL min^{−1}. Analytical analyses were performed on an Agilent 1100 HPLC system equipped with a Chromolith Performance RP-C18 column (100 × 3 mm). Water and acetonitrile containing 0.1% TFA were used as eluents with a flow rate of 2 mL min^{−1}. HPLC–MS analyses were performed on an Agilent 1200 HPLC system equipped with a Waters Hypersil Goldaq column (200 × 2.1 mm) followed by a Thermo Scientific Exactive mass spectrometer. Water and acetonitrile containing 0.05% TFA were used as eluents with a flow rate of 200 µL min^{−1}.

Methods—Preparation of Liposomes: Liposomes were prepared by the DAC-method using a SpeedMixer (DAC150FVZ Hauschild Engineering GmbH & Co. KG, Hamm, Germany). First of all, the lipids were dissolved in chloroform/methanol 9:1 to obtain 100 nmol stock solutions while the CPP was dissolved in chloroform/methanol 1:1 (1 mmol stock). The required amount of the CPP stock solution (0.1–3.0 mol%) was added to the lipid mixture and the organic solvent was evaporated by a nitrogen stream. Afterward, the resulting lipid film was dried for 1 h in a vacuum chamber. Before starting the speed mixing process, 20 mg of 0.075–1.00 mm glass beads were added. The liposomes were prepared by speed mixing in 3 runs to obtain a total volume of 250 µL (see Table S2 in the Supporting
Information). For comparison, control liposomes (85 mol% EPC; 15 mol% cholesterol) were prepared in the same way.

**Methods—Liposomal Characterization:** The particle size, PDI and zeta potential of all liposomal formulations were determined at room temperature using a Zetasizer Nano ZS from Malvern (Malvern Instruments Ltd., Worcestershire, United Kingdom). Size and PDI were measured after the dilution to a concentration of 0.076 mg mL\(^{-1}\) with PBS while the zeta potential was determined after the dilution to a concentration of 0.95 mg mL\(^{-1}\) by a 50 mmol phosphate buffer. The measurements were conducted by using the automatic mode and the average of three measurements. The size is specified in nm and the zeta potential in mV, while the PDI is a dimensionless value. The encapsulation efficiency of vancomycin in different liposomal samples was determined by size exclusion chromatography and HPLC quantification of vancomycin as described previously.[117]

**Methods—Nonparticle Tracking Analysis (NTA):** NTA analysis of liposomal size distribution and concentration was performed using the Nanosight NS300 equipped with a 488 nm laser (Malvern Instruments Ltd, Instrumat AG, Switzerland). To confirm the absence of particles, the chamber was filled with fresh PBS (Sigma-Aldrich, PBS, D8537) and analyzed. Liposome samples were diluted 1:1000 in PBS and injected using 1 mL sterile syringes. The syringe pump was set to 50 for all video recordings and the temperature was monitored all the time during the measurement. The camera level was set to 11 and detection threshold was set to 5. A total number of 1498 frames were recorded using a CMOS camera and analyzed using the NTA 3.1 Build 3.1.54 software. Five video recordings of 60 s for each sample were performed.

**Methods—Cryo-TEM:** As known in the art, in order to determine the size and lamellar structure of the CPP-modified liposomes, samples were frozen using a FEI Vitrobot on 2/2 Quantifoil grids. Afterwards, each sample was glow discharged for 3 s and blotted at 4 °C and 100% humidity for 8–10 s. The grids were observed in a Krios microscope operated at 200 kV and liquid nitrogen temperature. The pictures of the CPP-liposomal samples were taken at 64 000× magnification.

**Methods—Freeze-Drying of liposomes:** In order to enable long-term storage, the CPP-modified liposomes were freeze dried (main drying was carried out at −20 °C for 2 days followed by a secondary drying step at 0 °C for at least 6 h) in a Delta 1–20 KD from Martin Christ (Osterode, Germany). Sucrose and trehalose in concentrations of (100–500) × 10\(^{-5}\) M were used as lyoprotect. Briefly, the liposomes were prepared and sucrose respectively trehalose was added to the aliquots (50 µL each). Afterward, the aliquots were shock frozen in liquid nitrogen and freeze dried. In order to assess the quality of the freeze-dried products, the liposomes were rehydrated with 50 µL phosphate buffered saline and the liposomal characteristics (size, PDI) were determined by Zetasizer measurements as described above.

**Methods—Cytotoxicity Assay:** The cultivation of the Caco-2 cells took place in DMEM (20% fetal bovine serum, 1 × 10\(^{-3}\) M sodium pyruvate, GlutaMAX, 4 × 10\(^{-3}\) M L-alanyl-glutamine, and 1% nonessential amino acids as supplement). The cells were cultured at 37 °C in an atmosphere of 95% air and 5% CO\(_2\). When cells reached 80% confluence, subcultures were taken. Afterward, the Caco-2 cells were seeded into 96 well plates and grown for 14 days after the formation of a monolayer. Therefore, the medium was changed every 2 days. The liposomal suspensions were added in appropriate concentrations and incubated for 3 h. At this stage, the medium was replaced by growth medium (supplemented with 10% alamarBlue) and the cells were incubated for 3 h. Fluorescence measurement was performed on an Infinite Tecan Plate reader (wavelength of 590 nm with an excitation wavelength of 560 nm). Normalization of the cell viability was done with respect to wells containing untreated cells and wells without cells as blank.

**Methods—Cell Binding Assay:** Caco-2 cells were kept in culture medium comprising DMEM medium (high glucose, Life Technologies) supplemented with 20% fetal bovine serum (Life Technologies) at 37 °C. When cells reached a confluency of 50%, they were washed in prewarmed DPBS (Life Technologies) for 10 min. Dissociation of the cells was performed using Trypsin-EDTA (0.25%; Life Technologies) at 37 °C. The cell suspension was counted using a Neubauer counting chamber. 100 000 cells were seeded into each well of a 48-well plate already containing glass coverslips (Greiner-Bio-one). On the next day, cells were washed in DPBS and kept in 50% v/v culture medium/50% v/v DPBS during the experiment. Cells were incubated with a liposomal suspension diluted 1:40 for the time periods indicated. Afterward, CaCo-2 cells were washed four times in DPBS for 5 min each and subsequently fixed in 4% formaldehyde/DPBS for 10 min on ice. Fixative was removed by washing four times in DPBS at RT. Cell nuclei were stained using DAPI (Roche) diluted 1:1000 in DPBS for 10 min at RT. Cells were mounted in Mowiol 4–88 and analyzed using a Nikon Eclipse Ti.

**Methods—Ussing Chamber Experiments:** For the permeation studies, rat intestine was collected. The tissue was rinsed free of luminal contents with NaCl solution. The intestine was cut into strips of 1.5 to 2 cm and mounted in Ussing chambers (0.64 cm² surface area). Preheated incubation medium KRB (37 °C) was added to the donor and acceptor chambers in a volume of 1 mL, respectively. The temperature within the chambers was maintained during the 360 min of experiment at 37 ± 1 °C. After an equilibration period of 30 min, incubation medium was removed and subsequently the samples as well as controls were added to the donor chamber (1 mL). Furthermore, KRB was applied in a volume of 1 mL to the acceptor chamber. Over a time period of 6 h, 200 µL of samples were withdrawn at predetermined time points (0, 30, 60, 120, 180, 360 min) and immediately replaced by 200 µL KRB. The vancomycin concentration was determined by immunoassay measurements (ADVIA Centaur VANC ReadyPack, Siemens, Tarrytown, USA) in the accredited analysis center of Heidelberg University Hospital.

**Methods—Radioiodination of Vancomycin:** To 25 µL of vancomycin (1 × 10\(^{-3}\) M stock solution in 0.25 m phosphate buffer pH 7.5) ≤ 3 mgbecquerel (MBq) of radioactive \(^{125}\)I were added. The radiolabeling was performed using the chloramine T method according to Crim et al.[45] The reaction mixture was purified by semi preparative HPLC as described by Schiek et al.[46] Afterward, the purity of the radiolabeling was determined by radio-HPLC (Agilent 1100 series) using a Chromolith Performance RP-18e 100–3 mm column applying a linear gradient of 0.1% TFA in water (eluent A) to 0.1% TFA in acetonitrile (eluent B) within 5 min; flow rate 2 mL min\(^{-1}\); UV absorbance λ = 214 nm; γ-detection.

**Methods—In Vivo Bioavailability Studies:** The procedures of this study were approved by the Animal Care and Use Committee at Regierungspräsidium Karlsruhe (Karlsruhe, Germany). For this study, female Wistar rats with a body weight of about 200–250 g were used. The model substance vancomycin was labeled with \(^{125}\)I and incorporated into the modified liposomes as described previously.[117] Post oral administration, at determined time points, the vancomycin uptake was measured by direct counting of the radioactivity in the blood samples. Briefly, four groups (n = 3) of female Wistar rats were formed. Prior to 12 h of the experiment, the rats were kept without food, but with free access to water. Oral application of the radiolabeled samples took place by gavage. Rat group 1 obtained a dose corresponding to 0.5 mbecquerel (MBq) of labeled free vancomycin (negative control), while each rat of group 2 obtained a dose corresponding to 0.5 MBq of vancomycin incorporated into the control liposomes. The rats of group 3 obtained a dose corresponding to 0.5 MBq of vancomycin incorporated into the CPP-modified liposomes. The rats of group 4 obtained a dose corresponding to 0.5 MBq of vancomycin incorporated into the CPP-modified liposomes. The rats were sacrificed 1 h post oral administration and the radioactivity of all blood samples was measured using a Cobra Auto γ-Counter (Packard Bioscience, USA) in comparison with standards. The radioactivity of the blood samples was related to the total injected dose (ID) and expressed as a percentage of the total injected dose as described previously.[117]

**Methods—In Vivo Vancomycin Blood Levels:** For the determination of vancomycin blood levels over 5 h in female Wistar rats, a vancomycin immunoassay (ADVIA Centaur VANC ReadyPack, Siemens, Tarrytown, USA) of the CPP-modified liposomal formulation (vancomycin incorporated) was performed. Background radioactivity and vancomycin administered i.v. at determined time points, the blood samples were taken, plasma was separated, and the vancomycin concentration was determined by the immunoassay described above in the accredited analysis center of Heidelberg University Hospital.

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Methods—Antimicrobial Activity of Liposomal Vancomycin: To determine the minimum inhibitory concentration of liposomal vancomycin in comparison to free drug, an overnight culture of the selected bacterial strain was initially prepared by picking a single bacterial colony on a B agar plate and incubating it overnight in 3 mL of MH media. On the second day, 200 µL of overnight culture were added to 20 mL of MH media and incubated at 37 °C for 4 h, until the bacteria reached the logarithmic growth phase. In the meantime, dilutions of the compounds in MH medium were prepared in 96 well plates with a round bottom. Bacterial concentration was then adjusted in MH media to reach a final concentration of 2 × 10^6 CFU mL^-1. 100 µL of this dilution was added to each well of the plate. Subsequently, the plate was checked for bacterial growth after incubation for 18 h at 37 °C.

Methods—Resistance Development Studies: To examine the bacteria capability of developing resistance to liposomal and free vancomycin, they were cultivated at increasing antibiotic concentrations. In short, bacteria were grown overnight in MH medium at 37 °C supplemented with different concentrations of either fusidic acid, free or liposomal vancomycin. The culture with the lowest concentration of antibacterial compound, which showed no growth, was designated as MIC value for the respective passage while the culture with the highest concentration which showed growth of the bacteria was used for the cultures of the following passage.

Methods—in Vivo Studies Using C. mellonella: As first in vivo model, experiments on the therapeutic efficacy of vancomycin were conducted in. mellonella. Therefore, larvae (40 per group) were infected with 2 × 10^6 CFU S. aureus LAC® lux in PBS. One hour after start of infection, the maggots received either 2 mg free or liposomal vancomycin per kg body weight in a volume of 20 µL or the same volume of sterile PBS. Survival of the maggots was monitored every 4–6 h within the first 48 h of the experiment, afterward every 8 h until the end of the experiment.

Methods—in Vivo Infection Experiments: Ethics statement: animal studies were approved by the local government of Lower Franconia, Germany (approval number 55.2-2532-2-57) and performed in strict accordance with the guidelines for animal care and experimentation of German Animal Protection Law and the DIRECTIVE 2010/63/EU of the EU. For the in vivo experiments, S. aureus strain USA300 LAC® lux, which was constructed by transducing the luxABCDE operon from S. aureus Xen29 (PerkinElmer, Waltham, MA, USA) with phage ϕ11 into S. aureus LAC® (AH2163) was applied [47]. Overnight cultures of S. aureus USA300 LAC® lux in BHI medium were diluted to a final OD_600 of 0.05 in 50 mL fresh BHI medium and grown for 3.5 h at 37 °C. After centrifugation, the cell pellet was resuspended in BHI with 20% glycerol, aliquoted, and stored at −80 °C. For the generation of in vivo infection inocula, aliquots were thawed and washed twice with PBS. The desired bacterial concentration (4.2 × 10^7 CFU/100 µL) was then adjusted based on the respective OD_600 values. A sample of the infection inoculum was plated on TSB agar plates in order to control the infection dose.

Female Balb/c mice (9 for the PBS treated negative control group, 10 for each of the test groups, 6 weeks, Janvier Labs, Le Genest-Saint-Isle, France) were used for all experiments. The vancomycin i.v. group received 100 µL of vancomycin (6 mg mL^-1) in PBS; corresponding to a vancomycin concentration of 30 mg kg^-1 body weight i.v., mice from vancomycin gavage group 100 µL of vancomycin (50 mg mL^-1 in PBS) by gavage, while the liposomal vancomycin group received 100 µL of the respective liposomal formulation by gavage. The PBS group received 100 µL sterile filtered PBS by gavage orally. For the therapeutic treatment, mice were first infected with 5 × 10^7 CFU S. aureus LAC® lux and treated 1 h post infection with the respective regimens. For the prophylactic approach, intravenous infection with 4.2 × 10^7 CFU bacteria via the tail vein was performed 60 min after the single dose treatment.

The mice were housed in individually ventilated cages under normal diet in groups of five throughout the experiment with ad libitum access to food and water. During infection, mice were scored twice a day. Mice were sacrificed and kidneys were harvested. Organs were homogenized and plated in serial dilutions on LB agar plates in order to determine colony-forming units (CFU).

Statistical Analyses: Statistical analysis was performed by the specific tests indicated in each figure caption using the Prism software (GraphPad Software, San Diego, CA, USA). p-values *p < 0.05, **p < 0.01, ***p < 0.001 were considered significant for all statistical analysis.

For Figure 4B, the oral concentration–time curve of CPP-modified liposomal vancomycin was fitted (nonlinear regression) with Y = Span1 × exp(−K1 × X) − Span2 × exp(−K2 × X), the i.v. concentration–time curve of free vancomycin was fitted as one phase exponential decay with Y = Span × exp(−K × X) + Plateau.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements
The authors acknowledge K. Leotta and L. Dreher for support with animal experiments. In addition, the authors thank D. Palomino and G. Badic from Instrumag AG (Switzerland) for assistance with NTA measurements. Furthermore, the authors thank the “Deutsche Forschungsgemeinschaft” (Project 363770907) and the Herbert-Kienzle-Stiftung for financial support. D.W. acknowledges the support from the Novartis Foundation for Medical-Biological Research (#19004).

Author Contributions
The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflict of Interest
The authors declare no conflict of interest.

Keywords
cell-penetrating peptides, liposomal nanocarrier, oral peptide delivery, tetraether lipids, vancomycin

Received: November 2, 2020
Revised: December 14, 2020
Published online: January 27, 2021

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