PEGylated Liposome Encapsulation Increases the Lung Tissue Concentration of Vancomycin

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Pneumonia due to methicillin-resistant Staphylococcus aureus (MRSA) often cannot be cured by vancomycin treatment. Poor lung tissue and intracellular penetration limits the ability to achieve effective bactericidal levels, particularly in alveolar macrophages, where MRSA can evade phagocytic killing. Compared to standard formulations, liposome encapsulation has been shown to enhance vancomycin intracellular killing of MRSA. In this murine pharmacokinetic and biodistribution study, PEGylated liposomal vancomycin, compared to standard and non-PEGylated formulations, significantly prolonged blood circulation time and increased deposition in lung, liver, and spleen and yet reduced accumulation in kidney tissue. As a result of optimizing antimicrobial targeting of infected lung tissue and limiting renal parenchymal exposure, administration of PEGylated liposomal vancomycin may improve the efficacy of treatment of MRSA pneumonia and reduce the risk of nephrotoxicity.

MATERIALS AND METHODS

(i) Materials. DSCP (1,2-distearoyl-sn-glycero-3-phosphocholine) and MPEG-2000DSPE (methylpolyethylene glycol-1,2-distearoyl-phosphatidyl ethanolamine conjugate) were obtained from Genzyme Pharmaceuticals (Cambridge, MA). Cholesterol, vancomycin hydrochloride, sodium chloride, phosphate-buffered saline (PBS [pH 7.4]), trifluoroacetic acid, sucrose, and heparin sodium salt were obtained from Sigma Chemicals (St. Louis, MO). Norvancomycin was obtained from Northern China Pharmaceutical Corporation (Shijiazhuang, Hebei, China). Potassium chloride and dibasic sodium phosphate were obtained from Baker (Phillipsburg, NJ). Monobasic potassium phosphate was procured from Fisher Scientific (Houston, TX). High-performance liquid chromatography (HPLC)-grade chloroform, acetonitrile, and methanol were obtained from EMD Chemicals (Gibbstown, NJ). Isoflurane was obtained from Piramal Health Care Limited (AP, India). Male CF-1 mice were obtained from Charles River Laboratories (Wilmington, MA).

(ii) Preparation of liposomal vancomycin formulations. The liposomes were initially prepared using the thin-film hydration method (8) and the ammonium sulfate gradient method (20). Due to poor encapsulation efficiency and poor stability of the prepared formulations, we decided to prepare subsequent formulations using a modified dehydration-rehydration method (4). Both conventional and PEGylated liposomal vancomycin formulations were prepared using DSPC, cholesterol, and polyethylene glycol (PEG) in 3:1:0 and 3:1:0.02 molar ratios, respectively. The detailed procedure for the preparation of liposomal vancomycin formulations has been published recently (35).

(iii) Pharmacokinetic and biodistribution study. Prior to our undertaking the study, the protocol was approved by the Institutional Animal Care and Use Committee at the Western University of Health Sciences. Vancomycin (5 mg/kg of body weight) was administered in a tail vein injection under isoflurane anesthesia. A simple protein precipitation procedure was used to extract vancomycin from the plasma sample. As an internal standard, 10 μl of a solution of norvancomycin (100-μg/ml) was added to 200 μl of plasma and the mixture was subjected to a vortex procedure for 1 min. Then, 250 μl of acetonitrile and 250 μl of methanol were added to the mixture, which was subjected to a vortex procedure for 1 min and centrifuged at 14,000 rpm for 10 min. Supernatant (400 μl) was then transferred into a new tube and subjected to evaporation to dryness by the use of a stream of filtered air for 1 to 2 h. The residue was reconstituted with 200 μl of nanopure water, and 20 μl of the resulting solution was analyzed
to determine the vancomycin concentration by the use of a sensitive, validated HPLC method and a C18 column. The standard calibration curve was linear in the concentration range of 0.1 to 20 μg/mL, with a correlation coefficient (r) higher than 0.995. The lower limit of detection (LLOD; signal-to-noise ratio [S/N], 5) of vancomycin was 0.05 μg/mL, and the lower limit of quantitation (LLOQ; S/N, 10) was 0.1 μg/mL. The upper limit of quantitation was 20 μg/mL. The coefficients of variance ranged from 1.7 to 9.5% for intraday and 6.3 to 9.4% for interday precision.

(i) Pharmacokinetics. After the administration of a single dose of vancomycin (5 mg/kg) in standard solution and conventional and PEGylated liposomal formulations, plasma samples were analyzed using a validated HPLC assay to determine the levels of vancomycin. The pharmacokinetic profiles for all three formulations are shown in Fig. 1. Vancomycin plasma concentrations rapidly declined within the first 2 h of injection of the standard vancomycin formulation (half-life [t1/2], 22 min); after 2 h, no measurable amount could be found. However, following injection of conventional and PEGylated liposomal formulations, plasma vancomycin concentrations remained at >1 μg/mL until 4 h and 12 h, respectively. With both liposomal formulations, vancomycin was detectable in plasma samples at 48 h, when the experiment was terminated.

Pharmacokinetic parameters were calculated using the non-compartmental method and are reported in Table 1. The peak vancomycin plasma concentrations at 15 min for the PEGylated liposomal formulation and at 5 min for the conventional liposomal formulation were 2.3- and 1.4-fold higher, respectively, than that at 5 min for the standard vancomycin formulation. The AUC for conventional liposomes was 4-fold higher than that of the standard vancomycin formulation, and the AUC of the PEGylated liposomes was a further 1.7-fold higher than that of conventional liposomes. The vancomycin concentrations were sustained when administered as liposomal formulations compared to the results seen with standard vancomycin. Clearance from plasma was decreased by liposomal encapsulation of vancomycin. Since plasma concentrations were determined from samples that included both released and still-encapsulated liposomal vancomycin, it was difficult to fit the data into a specific compartmental model and it was not possible to accurately determine all the pharmacokinetic parameters.

(ii) Biodistribution. The biodistribution of vancomycin into major organs following administration of a single intravenous dose (5 mg/kg) of standard, conventional, and PEGylated liposomal vancomycin formulations was determined, and results from liver, spleen, lung, and kidney are presented in Fig. 2. No significant levels of vancomycin were detected in thigh muscle tissue treated with all three formulations. Liposome encapsulation enhanced reticuloendothelial system uptake, as evidenced by increased levels of vancomycin in spleen and liver.

**RESULTS**

**TABLE 1. Main pharmacokinetic parameters of vancomycin in plasma after intravenous administration of a dose (5-mg/kg) of standard, conventional, and PEGylated liposomal vancomycin formulations**

<table>
<thead>
<tr>
<th>Vancomycin formulation</th>
<th>Cmax (μg/mL)</th>
<th>AUC0-48 (μg · h/mL)</th>
<th>CL (ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV</td>
<td>6.8 ± 0.4</td>
<td>6.8 ± 0.3</td>
<td>18.3 ± 0.7</td>
</tr>
<tr>
<td>CLV</td>
<td>9.8 ± 1.1**</td>
<td>25.9 ± 2.8***</td>
<td>4.9 ± 0.6***</td>
</tr>
<tr>
<td>PLV</td>
<td>15.5 ± 1.7***</td>
<td>47.2 ± 2.4***</td>
<td>2.7 ± 0.1***</td>
</tr>
</tbody>
</table>

*SV, standard formulation; CLV, conventional formulation; PLV, PEGylated formulation. Symbols *, **, and *** denote P values of <0.05, 0.01, and 0.001, respectively, versus parameters for the standard solution.
tissues. However, compared to the levels of uptake seen with conventional liposomes, the uptake of vancomycin from the PEGylated liposomes at 4 h and 24 h was significantly less ($P < 0.001$). Maximum concentrations of vancomycin in liver from all three formulations were found at 4 h. The distribution of vancomycin from conventional and PEGylated liposomal formulations into liver tissue was significantly greater than that from standard vancomycin at 1, 4, and 24 h ($P < 0.001$). Furthermore, compared to conventional liposome results, a statistically significant increase in vancomycin distribution from PEGylated liposomes was observed at 5 min ($P < 0.001$) and 1 h ($P < 0.01$). The AUC and MRT values for all three formulations in liver, kidneys, lungs, and spleen tissue were calculated and are reported in Table 2.

**DISCUSSION**

Vancomycin remains an important part of a restricted treatment armamentarium for MRSA pneumonia, and yet concerns have been raised about its effectiveness. Retrospective analysis from two multinational, prospective, randomized, double-blind studies revealed that the clinical cure rate for vancomycin treatment of documented MRSA pneumonia was 36% compared to 59% for linezolid (52). Inherent pharmacokinetic limitations such as slow, time-dependent killing and poor penetration into lung tissue and alveolar macrophages (10, 23, 26, 30, 33, 35, 43, 47) have been blamed for high rates of vancomycin failure (28). Nevertheless, vancomycin is the comparator drug for randomized, double-blind, prospective trials evaluating novel agents (e.g., telavancin) for treatment of hospital-acquired pneumonia due to MRSA (38). Although vancomycin MICs $\leq 2$ $\mu$g/ml are considered to represent susceptibility, treatment of invasive infections due to MRSA strains with drug
TABLE 2. Biodistribution of vancomycin in liver, kidney, lung, and spleen tissue after intravenous administration of a dose (5-mg/kg) of standard, conventional, and PEGylated liposomal vancomycin formulations

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Standard (SV)</th>
<th>Conventional (CLV)</th>
<th>PEGylated (PLV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{AUC}_{0-24}}$ (μg/ml)</td>
<td>21.9 ± 5.3</td>
<td>42.6 ± 4.3</td>
<td>78.9 ± 5.4</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (μg/ml)</td>
<td>11.1 ± 0.2</td>
<td>11.0 ± 0.9</td>
<td>11.2 ± 3.1</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>1.6 ± 0.2</td>
<td>1.3 ± 0.9</td>
<td>1.3 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>1.563 ± 0.9**</td>
<td>0.9 ± 0.5</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>AUC$_{0-24}$/H9262</td>
<td>4.9 ± 10.5</td>
<td>4.0 ± 13.5</td>
<td>4.0 ± 13.5</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{AUC}_{0-24}}$ (μg/ml)</td>
<td>9.4 ± 1.2</td>
<td>5.4 ± 0.7</td>
<td>4.4 ± 0.9</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (μg/ml)</td>
<td>9.9 ± 0.9</td>
<td>9.9 ± 0.8</td>
<td>9.9 ± 0.9</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1.5 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>AUC$_{0-24}$/H9262</td>
<td>8.5 ± 12.6</td>
<td>6.6 ± 17.5</td>
<td>6.6 ± 17.5</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{AUC}_{0-24}}$ (μg/ml)</td>
<td>3.0 ± 0.2</td>
<td>3.0 ± 0.2</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (μg/ml)</td>
<td>8.5 ± 1.2</td>
<td>8.5 ± 1.2</td>
<td>8.5 ± 1.2</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1.5 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>AUC$_{0-24}$/H9262</td>
<td>13.5 ± 2.5</td>
<td>13.5 ± 2.5</td>
<td>13.5 ± 2.5</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{AUC}_{0-24}}$ (μg/ml)</td>
<td>1.2 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (μg/ml)</td>
<td>1.0 ± 0.2</td>
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<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1.5 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>AUC$_{0-24}$/H9262</td>
<td>10.1 ± 3.5</td>
<td>10.1 ± 3.5</td>
<td>10.1 ± 3.5</td>
</tr>
</tbody>
</table>

* SV, standard formulation; CLV, conventional formulation; PLV, PEGylated formulation. Symbols *, **, and *** denote $P$ values of $0.05, 0.01, and 0.001, respectively, versus parameters for the standard solution.

More recently, data from a large U.S. multicenter study suggested that reports of MIC creep may be exaggerated and that it perhaps poses less of a widespread problem than the reports have indicated (40). Pharmacodynamic studies suggest that curative vancomycin treatment of MRSA infection requires achieving a ratio of the area under the concentration-time curve for 24 h to MIC ($\text{AUC}_{24}/\text{MIC} \geq 400$ (30)). Administration of higher-than-standard doses of vancomycin is needed to achieve an $\text{AUC}_{24}/\text{MIC} \geq 400$ (16). However, higher serum vancomycin levels increase the risk of nephrotoxicity (14, 31).

The vancomycin plasma concentration-time profile is complex and can be characterized in the form of one, two, and three compartment pharmacokinetic models (39), with 80% to 90% of the vancomycin recovered unchanged in the urine within 24 h after administration of a single dose (29). Vancomycin displays ~45% protein binding to immunoglobulin A and albumin (48), and the concentration in alveolar lining fluid is only one-sixth that in plasma (26). In our study, the curves representing the plasma profiles for both liposomal formulations as shown in Fig. 1 were not smooth, with apparent rapid decreases between 2 h and 6 h followed by relative stabilization to 48 h. This phenomenon is intriguing and remains unexplained. One possibility is that, during the first 6 h of bloodstream circulation, liposome stability became compromised, thus increasing the ratio of unencapsulated to encapsulated vancomycin. Since we analyzed only total vancomycin concentrations, we could not directly determine the amount of vancomycin still trapped within liposomes.

Epithelial lining fluid (ELF) measurements have previously been used to reflect antibiotic activity for cases of pneumonia (7). However, ELF levels more specifically reflect extracellular concentrations that may be better suited for evaluation of upper respiratory tract infections or infections by pathogens whose activity is primarily extracellular (42). ELF measurements are typically performed via bronchoalveolar lavage (BAL), and data may be compromised by the presence of lavage fluid, cell contamination, protein binding, or incomplete lysis (22). Homogenized tissue has been used for measurement of pulmonary vancomycin levels (10) in humans; although others have criticized this technique, the correlation of vancomycin ELF concentrations with clinical outcomes has not been defined (42). Since BAL would have been technically difficult to perform in our mouse model and since lung tissue levels may be the most reliable predictor of treatment efficacy (21), we decided to utilize lung tissue homogenates in this study to determine the levels of biodistribution of the different formulations of vancomycin.

Liposomes were first described in 1965 and have already been in use as effective drug delivery systems (8, 19). Similar in structure and composition to host cell membranes, liposomes are biodegradable and are low in toxicity and immunogenicity (49). Liposomes can carry both hydrophilic and lipophilic drugs through encapsulation within the aqueous core and lipid bilayer, respectively (19). Liposomes lacking surface
modification ("conventional liposomes") are rapidly engulfed by intravascular and hepatosplenic phagocytes of the monocyte-macrophage line (11). Conversely, surface modification through PEGylation creates "stealth" liposomes that evade opsonization and delay hepatosplenic clearance, thus allowing prolonged circulation time (34) and extravasation into infected tissues. *S. aureus* can survive inside neutrophils (50) and alveolar macrophages (13) and persist in phagolysosomes for 3 to 4 days before escaping into the cytoplasm, resulting in host cell lysis and dissemination (24). We recently confirmed earlier observations indicating that, compared to the standard formulation, conventional liposome encapsulation enhances vancomycin intracellular killing of MRSA (33, 35).

The present report demonstrates that, compared to the standard formulation, liposome encapsulation increases vancomycin distribution into lung, liver, and spleen tissue while decreasing accumulation within kidneys. This preferential distribution may lead to improved treatment efficacy and reduced renal toxicity. However, despite vancomycin not being historically known as a hepatotoxic agent, the marked liver distribution of the drug resulting from PEGylated liposome encapsulation raises concerns regarding the potential for an additional adverse effect. PEGylated liposomes, compared to conventional liposome encapsulation, further increase vancomycin lung tissue concentrations, perhaps due to a longer circulation time that allows greater release within the targeted tissue.

This *in vivo* model does present some important limitations. First, although overall lung tissue concentrations were measured, we did not determine the percentages of intracellular and interstitial vancomycin. Therefore, we can merely infer that drug deposited in the interstitium would ultimately gain entry into alveolar macrophages. Second, levels of biodistribution of liposomal vancomycin may differ in the setting of lung infection. The local tissue effects of edema, vasconstriction, ischemia, and necrosis may variably and unpredictably alter the kinetics of drug delivery. Lung inflammation has been suggested to increase passive diffusion of (free and protein-bound) vancomycin, but as inflammation subsides with treatment of infection, tissue permeability may normalize (42). Third, we assume that persistent intracellular MRSA infection plays a prominent role in the potential for clinical relapse following a course of vancomycin treatment. Although this is a reasonable assumption, in reality, there are likely multiple factors that play a role affecting the clinical response to therapy. Furthermore, theoretical concerns are raised that MRSA pneumonia episodes complicated by ongoing bacteremia not due to intracellular parasitism may be inadequately treated with the administration of a "stealth" liposomal vancomycin formulation. Fourth, we opted to use a murine model for our biodistribution and pharmacokinetic studies on the basis of cost and space required for procurement and maintenance. Although murine models have been used for other antimicrobials (e.g., telavancin) targeting MRSA (36), concerns have been raised about the applicability to humans (32), particularly in cases of pneumococcal pneumonia.

Nevertheless, further studies are warranted to determine the *in vivo* efficacy of the use of liposomal vancomycin in treating MRSA lung infection. To address this topic, we are preparing to study these same formulations by the use of a murine model of pneumonia and methods published elsewhere (36) to measure outcome variables such as CFU counts, survival rates, and histopathological changes. We expect such an investigation to shed more light on the potential for development of PEGylated liposomal vancomycin as a novel delivery system that may improve treatment outcomes by targeting MRSA infections complicated by intracellular parasitism.

**REFERENCES**


**ACKNOWLEDGMENTS**


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34. Reference deleted.