Liposomal tobramycin against pulmonary infections of *Pseudomonas aeruginosa*: a pharmacokinetic and efficacy study following single and multiple intratracheal administrations in rats

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**Objective:** To determine the pharmacokinetics and efficacy of tobramycin against pulmonary infections of *Pseudomonas aeruginosa* in rats after intratracheal administration of conventional and liposomal formulations.

**Methods:** Male Sprague–Dawley rats were inoculated with 10⁶ cfu of a mucoid variant of *P. aeruginosa* (MIC of tobramycin for PA 508 = 1 mg/L) and tobramycin (conventional or liposomal formulations) was administered in single (490 µg) and multiple dose (490 µg during 4 days) experiments. Rats were killed at multiple time points to determine the residual cfu of *P. aeruginosa* and tobramycin amounts in lungs. Pharmacokinetic parameters were calculated using a two-compartment model with NONMEM.

**Results:** Mean (±S.D.) elimination half-life (t¹/₂β) and pulmonary exposure (AUC) of the conventional formulation were 14.0 ± 4.0 h and 663 ± 89 µg·h/lungs, respectively. The pharmacokinetic profile of liposomal tobramycin was markedly different, with a longer t¹/₂β (34.4 ± 5 h, P < 0.05), resulting in an increased AUC (3890 ± 560 µg·h/lungs, P < 0.05). χ² analyses were carried out on cfu data distributed in the following categories: below 10³, 10³–10⁵, and above 10⁵ cfu. In the single dose experiments, approximately 90% of the observations were above 10⁵ cfu for both formulations. Significant differences in cfu distribution were observed after multiple treatments, with approximately 10% of the observations falling below 10³ cfu of *P. aeruginosa* for the conventional formulation versus 30% for the liposomal formulation.

**Conclusion:** The liposomal formulation of tobramycin promoted drug retention in lungs and improved its efficacy after multiple treatments.

Keywords: pharmacokinetics, efficacy, liposomal tobramycin, *P. aeruginosa*

**Introduction**

The usual therapy for cystic fibrosis patients infected with mucoid forms of *Pseudomonas aeruginosa* involves the use of aminoglycosides and a β-lactam.¹ It is recognized that aggressive treatments with antibiotics improve the quality of life of cystic fibrosis patients infected with *P. aeruginosa* by reducing symptoms of acute pulmonary exacerbations and mortality.² However, the frequency of administration and doses of antibiotics in cystic fibrosis patients are high and require parenteral administration to achieve significant serum concentrations and effective penetration in the sputum at the site of infection. As a consequence, nephrotoxic and ototoxic events are frequently observed due to prolonged elevations of peak and trough serum concentrations of antibacterial agents.³,⁴ For the treatment of chronic pulmonary infections, the administration of antibacterial agents in Airways via a nebulizer offers multiple advantages over conventional therapies by delivering high doses of antibacterial agents to the site of infection with reduced risk of toxicity due to minimal absorption of the antibiotic in the systemic circulation. An aerosol formulation of tobramycin (Tobi, tobramycin sulphate for inhalation) was designed for maintenance therapy against *P. aeruginosa* lung infection in cystic fibrosis patients of at least 6 years of age.⁵,⁶ Aggressive treatments with 300 mg of Tobi twice daily for 28 days followed by 28 days off the drug in alternating cycles was associated with improved spirometry and a decreased density of *P. aeruginosa* in the sputum of cystic fibrosis patients.³,⁷ On the other hand, complete eradication of the bacterial pathogen is rarely achieved due to the mucoid bacterial phenotype and high

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sputum production by the host. Specific carriers or modified-release formulations of antibiotics may allow a specific release of the antibiotic at the site of infection and improve the overall uptake and residence time of antibiotics in lungs.

Liposomes are microscopic vesicles composed of one or more phospholipid membranes surrounding an aqueous core. Encapsulation of antibiotics in liposomes allows site-specific delivery of higher doses of drug, improved efficacy and reduced toxicity compared with conventional formulations. The encapsulation of aminoglycosides (gentamicin, amikacin and tobramycin) in liposomes resulted in altered pharmacokinetics and biodistribution as compared with the conventional formulations. Recently, the intratracheal administration of a liposomal formulation of tobramycin in rats decreased its systemic exposure and adverse effects. Although less toxic, the relative efficacy of tobramycin administered as the conventional and liposomal formulations against pulmonary infection of P. aeruginosa remains to be determined in vivo.

The objective of the present investigation was to determine the pulmonary pharmacokinetics of tobramycin after single and multiple intratracheal administrations of conventional and liposomal formulations, and to assess their respective efficacies in an experimental model of P. aeruginosa pulmonary infection in male Sprague–Dawley rats.

Materials and methods

Conventional and liposomal formulations of tobramycin

A formulation of tobramycin sulphate commonly administered to cystic fibrosis patients in the clinic was considered as the ‘conventional formulation’ (Tobi, PathoGenesis Canada Ltd, Montreal, QC, Canada). The liposomal formulation of tobramycin was manufactured at Theralipids (Birmingham, AL, USA). The liposomal formulation of tobramycin was manufactured at Theralipids Canada (Montreal, QC, Canada) in the following manner. Liposomes were prepared with synthetic phospholipids (Avanti Polar Lipids Inc., Birmingham, AL, USA). The liposomal formulation of tobramycin consisted of a 10:1 molar ratio of a non-charged dipalmitoylphosphatidyl-choline (DPPC) phospholipid and a negatively charged dimyristoylphosphatidylglycerol (DMPG) phospholipid. Liposomes were prepared based on a dehydration-rehydration method described elsewhere. Briefly, appropriate amounts of lipid mixture were dissolved in chloroform in a round-bottomed flask and dried to a lipid film by rotovaporation (Bucci Rotavapor-R-144) at 65°C under vacuum. The lipid film was then rehydrated with 0.01 N phosphate-buffered saline (PBS) and lyophilized (FTS-Kinetics, BioPharm Division, Stone Ridge, NY, USA) in vials at 4°C. After rehydration with a concentrated solution of tobramycin (Tobi), liposomes were filtered in an extruder (Lipex Biomembranes, Inc., Vancouver, BC, Canada) with polycarbonate membranes to sterilize and standardize the size of liposomes to between 230 and 400 nm. Control liposomes were prepared similarly, but PBS was used instead of the antibiotic. The final concentrations of the stock solutions for the liposomal and conventional formulations of tobramycin were 4.9 g/L.

Bacterial strain

A stable mucoid strain of P. aeruginosa isolated from the sputum of a patient suffering from cystic fibrosis was used throughout this study (Sainte-Justine Hospital, Montreal, QC, Canada). Identification of the clinical P. aeruginosa strain (PA 508) was confirmed by culture on a selective medium (C-390) with phenanthroline 9-chloro-9-(4-diethylamino)phenyl-9,10-dihydro-10-phenylacridine. Based on broth microdilution assays, the MIC of tobramycin for PA 508 was 1 mg/L.

Bacteria were stored at −70°C in Brain Heart Infusion broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% glycerol and cultured for 18 h in Proteose Peptone broth before all experiments.

Study design

A total of 154 adult male Sprague–Dawley rats weighing between 175 and 225 g (Charles River, Saint-Constant, QC, Canada) were used. Rats were randomized to participate in the single dose (n = 78 rats) or multiple dose (n = 76 rats) experiments to assess differences in pharmacokinetics and efficacy between the liposomal and conventional formulations of tobramycin. All experiments were conducted in accordance with guidelines from the Canadian Council on Animal Care and Use of Laboratory Animals. Pulmonary infections due to P. aeruginosa were induced using a method described elsewhere. Briefly, rats were anaesthetized by intramuscular injection of a 100 µL solution of ketamine/xylazine (10:1). Anaesthetized rats were placed in the supine position, and agar beads each containing 10⁶ cfu of P. aeruginosa per 100 µL were introduced at the bifurcation of the trachea with a 1 mL tuberculin syringe. After 3–5 days, rats were anaesthetized using the same procedures and the mucus in the trachea was collected with a cotton swab. The mucus was plated on a C-390 medium in order to confirm the presence of oropharyngeal colonization with P. aeruginosa. Rats with a pulmonary cfu count higher than 10⁸ were used in the study.

For the single dose experiments, rats were anaesthetized using the same procedures and tobramycin treatments (490 µg of conventional or liposomal tobramycin) or liposomal PBS were administered intratracheally to rats. Following tobramycin treatments, a total of three rats per formulation were killed at the following time points: 0.5, 1, 2, 3, 5, 7, 9, 11, 13, 15, 16 and 18 h and pulmonary samples were taken (n = 36 rats per formulation). For the liposomal PBS control group, a total of three rats were killed at 0.5 and 18 h (n = 6 rats).

For the multiple dose experiments, tobramycin treatments (490 µg of conventional or liposomal tobramycin) or liposomal PBS were administered intratracheally to rats over four consecutive days. A total of six rats per formulation were killed at different trough time points (24, 48, 72 and 96 h) and a total of four rats per formulation were killed at different peak time points (3 and 75 h) following multiple treatments with the liposomal and conventional formulation of tobramycin (n = 32 rats per formulation). For the liposomal PBS control group, a total of three rats were killed at 24, 48, 72 and 96 h (n = 12 rats).

For the single and multiple dose experiments, entire lungs of killed animals were aseptically weighed and immediately homogenized in 2 mL of cold PBS for 30 s with a Polytron homogenizer. Between samples, the homogenizer was rinsed with ethanol, flamed and finally rinsed again with cold PBS. A 100 µL volume of homogenized tissue was immediately used for serial dilutions in cold PBS, resulting in a 20-, 200- or 2000-fold dilution to prevent the killing of P. aeruginosa in the subculture plates. Diluted samples were plated on Proteose Peptone No. 2 agar plates (Difco Laboratories, Detroit, MI, USA) and incubated for 20 to 30 h at 37°C (5% CO₂). The cfu were counted at the dilution where a maximum of 300 cfu were found. The lower limit of detection of cfu was 1 bacterium (in a 20-fold diluted sample) which corresponds to 20 or 1.30 log₁₀ bacteria. Pending the analytical assay of tobramycin, samples of homogenized tissues were stored at −70°C in a methanol solution to extract tobramycin from the phospholipids and to precipitate lung tissues.

Analytical assay

Tobramycin concentrations were determined in lung tissues using an HPLC method described elsewhere. Briefly, the HPLC system consisted of a system controller and chromatographic pump (Waters Alliance 2690), a UV detector set at 350 nm (Waters 996, Photodiode
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array, Waters, Dorval, QC, Canada) and an auto-injector (C2237, Chromatographic Specialties, Inc., Brockville, ON, Canada). The separation was carried out on a Symmetry C-18 column (150 x 4.6 mm, 5 μm, Waters WAT045905) and the mobile phase consisted of a 0.1 N acetic acid–acetic acid (90:10) solution pumped at a flow rate of 1.3 mL/min. Reproducibility was assessed after six injections of a 50 μg/mL standard solution, and the resulting coefficient of variability was less than 2.0%. Linearity was assessed after single injections of standard solutions and the coefficient of correlation (R²) of the responses was higher than 0.990. The limit of quantification was 6.25 μg/mL (corresponding to approximately 10 μg/lungs) with a coefficient of variability of 8.0%. This analytical method for the liposomal formulation gives pulmonary tobramycin concentrations that represent the summation of the encapsulated with the “free” form.

**Pharmacokinetic analysis**

Pharmacokinetic parameters of tobramycin were calculated using a population methodology (NONMEM version 5). Tobramycin amounts from the single and multiple dose experiments were modelled simultaneously. The pulmonary absorption and disposition of tobramycin in its conventional and liposomal formulations were assessed using a first-order absorption rate constant (from a depot compartment) and a two-compartment model with elimination from the central compartment (ADVAN4). Bioavailability in lungs was identifiable since the actual amounts of tobramycin were modelled. The pharmacokinetic model was parameterized in terms of absorption (k₁), transfer (k₂, k₃, and k₄) and elimination (k₅) rate constants. Distributional half-life (t₁/₂d), elimination half-life (t₁/₂e) and the pulmonary exposure (AUC) of tobramycin were derived using standard compartmental and non-compartmental equations. The final population parameters were obtained using the first-order conditional estimation method (FOCE). Observations were fitted using a weighting procedure of 1/SD where the variance (SD) of the error was calculated using a combined additive and proportional model (slope-intercept model). Individual profiles were generated with the software ADAPT II using the NONMEM Bayesian estimates.

**Pharmacodynamic analysis**

The pulmonary cfu of *P. aeruginosa* were counted and distributed in the following categories: below 10⁹ (corresponding to >99.9% kill), between 10⁸ and 10⁹ (corresponding to 90.0–99.9% kill), and above 10⁹ cfu (corresponding to <90.0% kill). χ² tests were used to assess differences in the distribution of pulmonary cfu data between the two formulations (SYSTAT Version 8.0 for Windows, SPSS Inc., Chicago, IL, USA, 1998).

Results

Mean population pharmacokinetic parameters of tobramycin in lungs after the intratracheal administration of the conventional and liposomal formulations are presented in Table 1. The disposition of conventional tobramycin in lungs was described by a rapid rate constant of absorption (k₁: 1.64 ± 0.63 h⁻¹) and an extensive lung bioavailability (F₁: 85.0 ± 10.0%). The distribution half-life (t₁/₂d: 0.13 ± 0.02 h) and elimination half-life (t₁/₂e: 14.0 ± 4.0 h) of the conventional formulation of tobramycin were rapid. Observed and fitted pulmonary amounts of the conventional formulation of tobramycin after simultaneous modelling of the single and multiple dose data are presented in Figure 1A and B, respectively.

The liposomal formulation of tobramycin was associated with a slower rate constant of absorption than that of the conventional formulation (k₁: 0.57 ± 0.13 h⁻¹, P < 0.05), but its lung bioavailability remained unchanged (F₁: 88.0 ± 7.0%, NS). As compared with the conventional formulation, the liposomal formulation of tobramycin displayed a longer distributional (t₁/₂d: 0.68 ± 0.90 h, P < 0.05) and elimination half-life (t₁/₂e: 34.4 ± 5.0 h, P < 0.05). Pulmonary exposure of the liposomal formulation (AUC: 3890 ± 560 μg h/lungs, P < 0.05) was markedly higher than that of the conventional formulation.

**Table 1.** Mean (±S.D.) pharmacokinetic parameters of tobramycin in lungs following the intratracheal administration of conventional and liposomal formulations of tobramycin

<table>
<thead>
<tr>
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<th>Conventional formulation</th>
<th>Liposomal formulation</th>
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<tbody>
<tr>
<td>k₁ (h⁻¹)</td>
<td>1.64 ± 0.63</td>
<td>0.57 ± 0.13*</td>
</tr>
<tr>
<td>F₁ (%)</td>
<td>85.0 ± 10.0</td>
<td>88.0 ± 7.0</td>
</tr>
<tr>
<td>t₁/₂d (h)</td>
<td>0.13 ± 0.02</td>
<td>0.68 ± 0.11*</td>
</tr>
<tr>
<td>t₁/₂e (h)</td>
<td>14.0 ± 4.0</td>
<td>34.4 ± 5.0*</td>
</tr>
<tr>
<td>AUC (μg h/lungs)</td>
<td>663 ± 89</td>
<td>3890 ± 560*</td>
</tr>
<tr>
<td>Residual variability (%)</td>
<td>6.2</td>
<td>11.8</td>
</tr>
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</table>

k₁, rate constant of absorption; F₁, lung bioavailability; t₁/₂d, distribution half-life; t₁/₂e, elimination half-life; AUC, pulmonary exposure. *P < 0.05 versus conventional formulation.

**Figure 1.** Observed and fitted pulmonary amounts (μg/lungs) of tobramycin following the intratracheal administration of the conventional formulation (filled circles) in single and multiple dose experiments (A and B, respectively).

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tion (663 ± 89 µg h/lungs). Observed and fitted pulmonary amounts of the liposomal formulation of tobramycin after simultaneous modelling of the single and multiple dose data are presented in Figure 2 (A and B, respectively). Quality of fits for the conventional and liposomal formulations were very good and residual variabilities were 6.2% and 11.8%, respectively.

The efficacies of the conventional tobramycin formulation against *P. aeruginosa* in the single and multiple dose experiments are plotted in Figure 3 (A and B, respectively). Single treatments with the conventional formulation had no significant impact on the cfu of *P. aeruginosa* since most observations were above 10⁵ cfu. Following multiple treatments with the conventional formulation, the residual cfu of *P. aeruginosa* in lungs were mainly distributed between 10³ and 10⁶ cfu. The efficacies of the liposomal formulation in the single and multiple dose experiments are plotted in Figure 4 (A and B, respectively). Single treatments of liposomal tobramycin resulted in no significant effects on the pulmonary cfu of *P. aeruginosa* since most observations were above 10⁵ cfu. On the other hand, the pulmonary cfu data after multiple treatments with the liposomal formulation were distributed over the 10³ to 10⁶ range. After single and multiple treatments with the liposomal PBS formulation, most of the pulmonary cfu data were above 10⁵.

In order to assess differences in efficacies between the two formulations, non-parametric statistical methods were used due to the highly variable nature of cfu data distribution. Three distinct responses were compared: when residual pulmonary cfu were lower than 10³, between 10³ and 10⁵ and above 10⁵. Data distribution, frequency of observations and statistical comparisons (χ² test) of residual cfu of *P. aeruginosa* in lungs are presented in Table 2. Data distribution between the two formulations were significantly different after multiple treatments only (*P* = 0.037), with 9.4% of the observations falling below 10³ cfu for the conventional formulation against 28.1% for the liposomal formulation.

**Discussion**

Tobramycin pulmonary amounts following single and multiple treatments with the conventional and liposomal formulations of tobramycin were well described using a first-order rate constant of absorption and a two-compartment model to describe the bi-exponential elimination of the product. Rich data from the single dose experiments allowed us to determine the pharmacokinetic model adequately and to assess with robustness the more sparse data in the multiple dose experiments (peaks and troughs). Quality of fits were very good and resulted in residual coefficients of variation of approximately 6.2% and 11.8%
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for the conventional and liposomal formulations of tobramycin. These numbers are representative of variabilities that are not explained by the models and include intra-individual variabilities, experimental 'noise' and errors arising from the pharmacokinetic modelling itself.

Encapsulation of tobramycin in liposomes markedly changed its pulmonary pharmacokinetic profile, with a statistically significant decrease in its rate constant of absorption and a prolongation in both distributional and elimination half-lives. As a result of this, the pulmonary amounts of tobramycin after multiple treatments with the liposomal formulation were sustained at approximately 100 µg/lungs whereas those of the conventional formulation ranged approximately 10–20 µg/lungs. These important amounts of tobramycin in lung tissues might have resulted in an important carryover of the antibiotic to the subculture plates. The carryover effect of tobramycin on the cfu of *P. aeruginosa* in lung homogenates was investigated before the study. Serial dilutions of lung samples with spiked amounts of tobramycin (50, 150, 300, 600 and 1200 µg/lungs) revealed no significant effects on the growth of *P. aeruginosa* (results not shown).

Based on these observations and those of others, we considered that serial dilutions of lung samples minimized the antibiotic carryover to the subculture plates, and resulted in a negligible effect on the growth of *P. aeruginosa.*

Single intratracheal treatments with the conventional or liposomal formulations of tobramycin had no significant effect on the pulmonary cfu of *P. aeruginosa* since approximately 90% of the observations were higher than 10^5 cfu. In the multiple dose experiments, the two formulations displayed statistically significant differences in cfu distribution of *P. aeruginosa*, with approximately 10% of the observations falling below 10^3 cfu for the conventional formulation versus approximately 30% for the liposomal formulation. On the other hand, the cfu of *P. aeruginosa* for the liposomal formulation displayed a wider distribution since approximately 46.9% of the observations were higher than 10^5 compared with only 37.5% for the conventional formulation.

With the understanding of the importance of aggressive treatments for the management of pulmonary infections in cystic fibrosis, it was postulated that higher doses of tobramycin in airways and lungs would improve pulmonary function and reduce hospitalization days. Recently, the rationale for increasing tobramycin doses in aerosol was confirmed in a clinical study where an 80 mg dose twice daily resulted in a preservation of pulmonary functions, whereas a 600 mg dose three times daily increased penetration of the antibiotic in the sputum, reduced the density of *P. aeruginosa* and significantly improved pulmonary functions of cystic fibrosis patients. In the present work, the enhanced pulmonary exposure observed with the liposomal formulation of tobramycin may provide more aggressive treatments for the management of chronic pulmonary infections. On the other hand, important questions must be raised concerning bacterial resistance following aggressive treatments with antibiotics. For this reason, optimal doses and frequency of administration with the liposomal formulation of tobramycin will have to be determined, in

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**Figure 4.** Residual cfu of *P. aeruginosa* in lungs following the intratracheal administration of the liposomal formulation (open circles) of tobramycin in single and multiple dose experiments (A and B, respectively).

**Table 2.** Distribution of residual cfu of *P. aeruginosa* in lungs following the intratracheal administration of the conventional and liposomal formulations of tobramycin in the single and multiple dose experiments

<table>
<thead>
<tr>
<th>Residual cfu</th>
<th>Conventional formulation</th>
<th>Liposomal formulation</th>
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<tr>
<td></td>
<td>single dose</td>
<td>multiple dose</td>
</tr>
<tr>
<td>&gt;10^5</td>
<td>32 (89.0%)</td>
<td>12 (37.5%)</td>
</tr>
<tr>
<td>10^3–10^5</td>
<td>2 (5.5%)</td>
<td>17 (53.1%)</td>
</tr>
<tr>
<td>&lt;10^3</td>
<td>2 (5.5%)</td>
<td>3 (9.4%)</td>
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*P < 0.05 versus conventional formulation.
order to minimize the emergence of bacterial resistance and optimize its cost-effectiveness.

In conclusion, the liposomal formulation of tobramycin has the net advantage of improving drug exposure at the site of infection by prolonging its pulmonary distributional and elimination half-lives. As a result of this, the liposomal tobramycin resulted in a greater efficacy than that of the conventional formulation after multiple treatments. These results support the use of liposomal tobramycin to provide more aggressive treatments for the management of P. aeruginosa pulmonary infections in cystic fibrosis patients. Future clinical studies will need to assess the cost-effectiveness of the liposomal formulation, and its overall efficacy against biofilms, multiple species and resistant strains of bacteria.

Acknowledgements

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References