Tobramycin Disposition in the Rat Lung
Following Airway Administration

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d) Abbreviations:

A, the absorbable amount

\(A_t/A_0\), the fraction of administered dose remaining in the dialysis sac

B, the amount of drug bound to, or sequestered in, tissue

CF, cystic fibrosis

CFC, chlorofluorocarbon

D, the administered dose

Fa, the absorbable fraction

F-Na, sodium fluorescein

Fp, mean fraction of each administered dose reaching the perfusate
IACUC, Animal Care and Use Committee

IPRL, isolated perfused rat lung

\( k_{12}\left( k_{12}'\right)\), the association rate constants for binding and/or retention in the intact IPRL (sliced IPRL; dynamic dialysis)

\( k_{21}\left( k_{21}'\right)\), the dissociation rate constants for binding and/or retention in the intact IPRL (sliced IPRL; dynamic dialysis)

K4, Krebs-Henseleit solution (KHS) containing 4% (w/v) BSA

\( k_a\), the apparent first-order rate constant for absorption

\( k_e\), the apparent first-order rate constant for dialysis

KHS, Krebs-Henseleit solution

MDI, metered dose inhaler

MSC, model selection criterion

NS, normal saline

P, the amount absorbed into the perfusate

R, the amount released into the receiver solution

U, the untransferable (unabsorbable) amount of each dose

e) Recommended section assignment:

Chemotherapy, Antibiotics, and Gene Therapy
Abstract

A realistic ex vivo model, the isolated perfused rat lung, IPRL, was used to investigate tobramycin’s pulmonary disposition at typical therapeutic concentrations. Different nominal doses were administered in aqueous solution to the airways alongside non-binding absorption markers, fluorescein and mannitol. The mean fraction of each administered dose reaching the perfusate, Fp, was determined as a function of time following administration. Dynamic dialysis was also used to quantify the kinetics of tobramycin binding and/or tissue retention in the IPRL immediately after drug administration. While the absorption markers fluorescein and mannitol both showed mono-exponential dose-independent increases in Fp with time, tobramycin’s pulmonary absorption into the perfusate was bi-exponential and dose-dependent due to tissue binding or retention. Best estimates for the first-order rate constants of tobramycin absorption (kₐ) appeared dose-independent (0.065-0.070 min⁻¹), with values close to the mean for fluorescein (0.076 min⁻¹). The rate constant for dissociation from IPRL tissue (k₂₁) was also relatively constant (0.018-0.022 min⁻¹), while that for association (k₁₂) decreased from 0.16 to 0.07 min⁻¹ with increasing airway dose from 0.002 to 2 mg. Dynamic dialysis data from sliced IPRL tissue following identical airway administration were consistent with those from the intact IPRL, confirming tobramycin’s “slow on, slow off” binding and sequestration by rat lung. Overall, tobramycin absorption was fast following airway administration. However, dose-, and concentration-dependent slow onset tissue binding extended the duration of tobramycin’s presence in the rat lung. These findings may explain in part, the apparent success of inhaled tobramycin therapy when treating pulmonary infections.
Introduction and Theory

Tobramycin administration direct to the airways via nebulizer has brought great improvements to the health and wellbeing of cystic fibrosis (CF) patients and others suffering from chronic pulmonary infections with pathogens such as *Pseudomonas aeruginosa* (Chuchalin et al., 2009). The efficacy and safety brought about by topical use of this drug is well-recognized in clinical circles although it remains unclear whether the antibiotic’s physicochemical properties lead to slow absorption, tissue binding and/or lung retention to sustain the drug’s anti-infective properties for periods between dosing (Li and Byron, 2012; Patton et al., 2004). Notably, tobramycin is believed to cross epithelial barriers poorly and it is therefore marketed primarily as an injectable (Jaresko and Alexander, 1995; Phillips and Shannon, 1997). Our recent meta-analysis of pharmacokinetic data in humans following IV and inhalation administration found that it was not possible to come to a statistically sound conclusion about its bioavailability and possible binding to, or sequestration in, lung tissue following inhalation administration due largely to the variance associated with the available data (Li and Byron, 2012), even though tobramycin and other aminoglycoside antibiotics are known to manifest some of their toxicity through tissue binding or sequestration and the creation of “deep compartments” (Mingeot-Leclercq and Tulkens, 1999; Nagai and Takano, 2004; Stepanyan et al., 2011). Notably, and probably because of the physiologic existence of these drugs as polycations, transfer into cells is slow and intracellular sequestration can be persistent. For these reasons we have used the isolated perfused rat lung (IPRL) to systematically investigate the disposition of tobramycin in the lung following its administration directly to the airways. The IPRL has been used similarly before (Byron
et al., 1986; Niven and Byron, 1988; Niven et al., 1990; Sakagami et al., 2002). In the most simple cases, when solutes like fluorescein are administered in solution to the airways of this preparation, they behave in accord with Scheme I. Absorption of fluorescein then occurs in a dose-independent, first-order fashion with no evidence of binding or metabolism. However, the nominal dose, or dose loaded into a dosing cartridge (Figure 1) usually results in a smaller administered dose, \( D \) (Scheme I), only part of which, the absorbable amount, \( A_0 \) (\( A_0 = D - U \)), can be transferred to the perfusate, \( P \). This is because the bronchial circulation in the IPRL is severed and only solute that deposits proximal to the actively perfused pulmonary circulation can be absorbed into the perfusate (Byron et al., 1986; Niven and Byron, 1988). In such a case, solute in perfusate will increase mono-exponentially toward an asymptotic value equal to the initial condition, \( A_0 \). The fraction of the administered dose (\( D \)) transferred to the perfusate, \( F_p \), for a solute described by Scheme I, is given as a function of time, \( t \), by:

\[
F_p = F_a \times (1 - e^{-k_a t})
\]

where \( F_a \) is the absorbable fraction (\( = A_0/D \)) and \( k_a \) is the apparent first-order rate constant for absorption.

In the case of other non-metabolized drugs that are absorbed by apparent first-order kinetics, binding or sequestration to lung tissue can be modeled in accord with Scheme II. This Scheme differs from Scheme I because of the addition of a “bound drug” compartment (\( B \)), and the association and dissociation rate constants, \( k_{12} \) and \( k_{21} \). In Scheme II, which is analogous to a 2-compartment pharmacokinetic model, the drug in \( A \) and \( B \) should behave in a similar fashion to drug in plasma and drug in tissue, respectively, while drug in \( P \) should behave in the same way as cumulative elimination.
Thus, the fraction in the perfusate, $F_p$, for Scheme II is given by a rearranged form of

$$F_p = F_0 \cdot \left(1 - \frac{k_a - \beta}{\alpha - \beta} e^{-\alpha t} - \frac{\alpha - k_a}{\alpha - \beta} e^{-\beta t}\right) \tag{2}$$

where

$$\alpha = \left(k_a + k_{12} + k_{21} + \sqrt{(k_a + k_{12} + k_{21})^2 - 4k_{21}k_a}\right)/2$$

and

$$\beta = \left(k_a + k_{12} + k_{21} - \sqrt{(k_a + k_{12} + k_{21})^2 - 4k_{21}k_a}\right)/2.$$

In this current study, the dynamic dialysis techniques, first described by Meyer and Guttman and used subsequently by other groups to study solute-ligand binding (Bottari et al., 1975; Hashimoto et al., 1984; Hiji et al., 1978; Meyer and Guttman, 1968; 1970; Pedersen et al., 1977; Sparrow et al., 1982), were first employed to support the use of the IPRL binding and sequestration model described in Scheme II. When dynamic dialysis under sink conditions is used to extract solutes from sliced IPRL tissue following initial airway dosing to that tissue, a model analogous to Scheme II, Scheme III applies (Meyer and Guttman, 1968). In contrast to Scheme II, Scheme III lacks an untransferable component, i.e. $U=0$, because all of D that is administered to the airways becomes available for dialysis. Furthermore, the binding or sequestration rate constants, $k_{12}'$ and $k_{21}'$, differ from those in Scheme II because sliced tissue and drug release from the dialysis sac differ from the intact IPRL. The first-order rate constant for solute release, $k_e$, from a dialysis sac into the sink $R$, depends largely on the diffusive properties of the solute and the conditions of dialysis. However, the fraction of administered dose remaining in the sac ($A_t/A_0$), at time $t$, should be a reflection of the solute’s tissue binding, and theoretically conform to Eq.218 from Gibaldi and Perrier (Gibaldi and Perrier, 1975), where
\[ \frac{A_t}{A_0} = \frac{k_2 - \beta}{\alpha - \beta} e^{-\alpha t} + \frac{\alpha - k_2}{\alpha - \beta} e^{-\beta t} \quad \text{Eq. 3} \]

in which
\[ \alpha = \left( k_e + k_{12}' + k_{21}' + \sqrt{(k_e + k_{12}' + k_{21}')^2 - 4k_{21}' k_e} \right)/2 \]

and
\[ \beta = \left( k_e + k_{12}' + k_{21}' - \sqrt{(k_e + k_{12}' + k_{21}')^2 - 4k_{21}' k_e} \right)/2. \]
Materials and Methods

Experimental Design. Tobramycin, mannitol and fluorescein were administered in different aqueous solution formulations, by forced intratracheal instillation, to the airways of the isolated perfused rat lung preparation (Figure 1), using a previously described method (Byron and Niven, 1988). Using this technique, studies were performed to explore the kinetics and mechanisms responsible for tobramycin’s pulmonary disposition. IPRL Absorption Studies were performed to elucidate the effects of concentration on airway-to-perfusate transfer (absorption). Prior to the analysis of data from those studies, IPRL Binding Studies were performed using dynamic dialysis (Meyer and Guttman, 1968). Those binding experiments employed identical surgical preparation, perfusion and airway dosing as those for IPRL Absorption Studies, after which the extent and rate of tobramycin and/or mannitol dialysis from lung tissue was determined.

Animals. Specific pathogen-free, 300–400g, male Sprague-Dawley rats (Hilltop Lab Animals Inc., Scottsdale, PA) were used throughout. The animals were housed with access to water and food at 30-70% RH and 18-26°C, and a 12-hr light/dark cycle for ≥ 2 days before sacrifice. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University.

Chemicals and Materials. Tobramycin (≥98% free base) and bovine serum albumin, BSA, ≥98% (agarose gel electrophoresis) lyophilized powder (Sigma-Aldrich, St Louis, MO) were stored according to USP, 2007. Krebs-Henseleit solution (KHS) containing 4% (w/v) BSA (K4) was freshly prepared and used as perfusate in the isolated perfused rat lung preparation. ³H-tobramycin and ³H-
mannitol solutions (0.8 Ci/mmol, 1 mCi/mL; 10-30 Ci/mmol, 1 mCi/mL, respectively) were purchased from Moravek Biochemicals (Brea, CA) and PerkinElmer (Waltham, MA) while sodium fluorescein (F-Na) was obtained from Arocos Organics (Geel, BE). Ecoscint XR scintillation cocktail (National Diagnostics, Atlanta, GA) was used for liquid scintillation counting. Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA). Dialysis Tubing (Snakeskin®, 10K MWCO; Thermo Scientific, Rockford, IL) was washed with water and immersed in aqueous buffer solutions for ≥30mins prior to use.

**Dosing Solutions.** Aqueous radiolabeled tobramycin solutions were prepared in different concentrations (100, 20, 2, 0.2, 0.04 and 0.02 mg/mL) containing a nominal radiolabel content of 18 µCi/mL in 0.45% w/v NaCl and adjusted to pH 7.4 with H₂SO₄. Radiolabeled mannitol was prepared with and without tobramycin in different concentrations at 12 µCi/mL, while sodium fluorescein (unlabeled) solutions were prepared in pH 7.4 phosphate buffer and used at a concentration of 0.2 mg/mL. While the majority of the experiments involving tobramycin were performed with dosing solutions containing 0.45% w/v NaCl (1/2NS), osmolality was varied in some investigations by using different concentrations of sodium chloride.

**IPRL Binding Studies.** A dynamic dialysis method (Meyer and Guttman, 1968) was modified to investigate solute binding or sequestration to lung tissue. Tobramycin binding was investigated across doses (0.002, 0.004, 0.2 and 2 mg) by comparing its interaction with lung tissue to that of mannitol (a non-binding solute). In binding studies, the IPRL preparation was used and dosed in exactly the same way as described below for **IPRL Absorption Studies.** After dosing, the isolated lung
preparation was taken out of the artificial glass thorax (AGT), sliced into 60-90 rectangular pieces (maximum linear dimension = 4mm) and transferred to a dialysis sac (dry length: 15 cm, washed and immersed in KHS solution prior to use) with 10 mL of K4. To determine the intrinsic diffusive properties of tobramycin and mannitol in this system, solute dialysis was also studied in the absence of lung tissue and BSA, from sacs with identical total volumes of KHS. Sacs were closed, with minimal headspace, by ligating with cotton thread, after which they were immersed at time zero in the perfusate reservoir containing 200 mL magnetically-stirred receiver solution (KHS or K4) maintained at 37°C and pH 7.4. The solute mass enclosed in the sac at time zero ($A_0$) was determined by subtracting the solute lost during lung slicing (by assay of rinse solutions used in the procedure) from the administered dose, D (determined as described in IPRL Absorption Studies). One mL samples were removed for assay from the receiver solution at 5, 10, and 20 minutes after which a 100 mL samples was withdrawn at each time point of 30, 60, 90, 120, 180 minutes and hourly thereafter. An equal volume of solute-free receiver solution (37°C) was added to replace each sampling aliquot and restore the receiver volume to 200 mL. The amount of solute remaining in the sac as a function of time ($A_t$) was calculated assuming mass balance.

**IPRL Absorption Studies.** Use of the IPRL preparation (Figure 1) to study solute absorption was carefully controlled as described previously (Byron and Niven, 1988; Sun et al., 1999). Briefly, a rat lung was surgically removed and housed in an artificial glass thorax (AGT) maintained at 37°C. Krebs-Henseleit solution (KHS) with 4% (w/v) BSA was used as perfusate (K4; 200 mL) and recirculated through
the pulmonary circulation via the pulmonary artery at a constant flow rate of 15 mL/min. A metal dosing cartridge containing 0.1 mL dosing solution was inserted into the trachea via a tracheal cannula. A metered dose inhaler (MDI, 25 μL drug-free CFC propellants per actuation) was connected to the dosing cartridge and actuated once. The dosing solution was propelled into the lung as a coarse spray, and the lung was inflated simultaneously to about 6 mL. The dosing cartridge was removed and the lung allowed to deflate (Byron and Niven, 1988). The *administered dose* (to the airways of the IPRL) was determined from the initial weight of the primed dosing cartridge, solution density and concentration after subtracting the mass remaining in the dosing cartridge after administration (determined by assay). The perfusate samples were taken from the well-mixed reservoir at time 0 (blank sample, immediately prior to dosing) and subsequently at 1, 3, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 105 and 120 min following dosing. Solute concentrations were determined according to *Assay Methods* described below. Sufficient IPRL studies were performed to yield more than four fully viable preparations for each dosing solution, as evidenced by the absence of any signs of edema onset over 120 min (preparations that were “non-viable” at times ≤ 120min were discarded). We have shown previously, and observe consistently with this preparation, that “signs of edema onset” occur when the (blood-free) lungs change in outside color and texture from smooth white to a grey and/or patchy appearance; this appearance change is an early indicator of the preparation’s declining viability shortly after which values for the wet lung/dry lung weight ratio, epithelial permeability to solutes and other effects change markedly (Byron et al., 1986; Niven et al., 1990). At the end of each IPRL
absorption study, the lung tissues were collected, homogenized and assayed for solute remaining in both the airways and the lung tissue.

**Assay Methods.** $^3$H-Tobramycin and $^3$H mannitol concentrations were determined by scintillation counting relative to standards prepared freshly for each experiment (Liquid Scintillation Analyzer, Tri-Carb 2800-TR, PerkinElmer, Waltham, MA) after first validating the radioactivity assay for tobramycin with high performance liquid chromatography–mass spectrometry (HPLC-MS) to ensure that chemical degradation and/or metabolism did not occur in lung tissue or perfusate over the duration of a typical experiment. Briefly, perfusate samples containing radiolabeled solutes were used neat or diluted in K4. One mL aliquots were added to 5 mL scintillation cocktail (Ecoscint XR, National Diagnostics, Atlanta, GA) in 7 mL polypropylene scintillation vials and well mixed. Total radioactivity was expressed as disintegrations per minute (DPM). DPMs from accurately prepared $^3$H-tobramycin or $^3$H-mannitol standard solutions in K4 were measured independently for each assay series in order to calculate solute concentrations in unknown test solutions. Fluorescein concentrations in perfusate were assayed by spectrofluorophotometer (RF-5301 PC, Shimazu Corporation, Kyoto, JP; $\lambda_{ex}$ and $\lambda_{em}$ = 490 and 520 nm, respectively), as described earlier (Byron and Niven, 1988; Byron et al., 1986; Sakagami et al., 2002).

**Data analysis.** Kinetic Analysis was performed according to the theory described above and Schemes I-III. The data from IPRL Binding Studies using dynamic dialysis were expressed in accord with Scheme III, as the fraction of the solute dose ($A_t/A_0$) remaining in the sac as a function of time. The value for $k_e$ for tobramycin
was first determined by studying its dialysis kinetics in the absence of IPRL components (tissue and protein). In this case, linear regression analysis of the first-order data for $\ln \left[ \frac{A_t}{A_0} \right]$ vs $t$ was performed to determine its value (Meyer and Guttman, 1968). The binding rate constants, in the presence of lung tissue, $k_{12}'$ and $k_{21}'$, were then determined by fitting data for $\left[ \frac{A_t}{A_0} \right]$ vs $t$ to Eq.3 by least mean square non-linear regression analysis. The value for $k_e$ was fixed while $k_{12}'$ and $k_{21}'$ were allowed to float. Goodness-of-fit was assessed using the calculated $r^2$ and model selection criterion (MSC) by SCIENTIST 3.0 (MicroMath Scientific Software, Salt Lake City, UT). Data from IPRL Absorption Studies were grouped in accord with the nominal dose initially added to the dosing cartridge. Each nominal dose resulted in a mean value for the “administered dose”, $D$ (Scheme I), due to solution retention in the cartridge. Fluorescein absorption into perfusate was expressed as the mean fraction of each administered dose reaching the perfusate, $F_p$, versus time. Best estimates for fluorescein’s mean absorbable fraction, $F_a = \frac{A_0}{D}$ (Scheme I) and its apparent first-order rate constant for absorption, $k_a$, were obtained by fitting the unweighted data for $F_p$ vs time to Eq.1 using Scientist 3.0. Because fluorescein that penetrates the airways proximal to the circulating perfusate is known to be completely absorbed, this provided a mean value for the solute’s eventually absorbable fraction, $F_a$, that could be used across solutes. Tobramycin data was analyzed according to Scheme II where best estimates for the rate constants describing binding and absorption ($k_{12}$, $k_{21}$, $k_a$) at different nominal doses were determined by curve-fitting tobramycin $F_p$ (fraction of administered dose in perfusate) vs time data to Eq. 2.
Results

**IPRL Binding Studies.**

Tobramycin dialysis from the sac is shown as mean \( A_t / A_0 \) (fraction of administered dose remaining in the sac) vs time in Figure 2A. Biphasic (biexponential) profiles resulted from experiments in which drug release from sliced lung tissue occurred after airway dosing to the IPRL at 5 different dose levels; smaller doses showed greater values for tissue retention with time. In the absence of lung tissue, control experiments showed mono-exponential (apparent first-order) release of tobramycin from the dialysis sac under sink conditions (Meyer and Guttman, 1968). Following Data Analysis, the mean dialysis rate constant, \( k_e \) (Scheme III), for tobramycin was found to be dose-independent \((0.0107±0.0021\text{min}^{-1}; \text{Figure 2A})\). Mannitol, a non-binding solute, produced mono-exponential and dose-independent \( A_t / A_0 \) profiles in the presence and absence of IPRL tissue (Figure 2B). Tobramycin dialysis data from IPRL tissue (Figure 2A) was fitted to Eq.3 to produce the best estimates of the rate constants in Scheme III. These are shown in Table 1 for each dose; dashed curves in Figure 2A were produced by simulation using the rate constant values shown in the table. Tobramycin's dissociation rate constant \((k_{21}')\) was effectively dose-independent while \( k_{12}' \) appeared saturable and decreased with ascending dose.

**IPRL Absorption Studies**

Fp vs time data for fluorescein (Figure 3) was fitted to Eq.1 to obtain best estimates of the absorbable fraction \((Fa)\) and the first-order rate constant for absorption \((k_a)\) for this non-binding solute; the values were 0.75 and 0.076 min\(^{-1}\), respectively (Table 2). While mannitol absorption was too slow to reach an asymptote within the IPRL's viable lifetime
(Sun et al, 2009), its $k_a$ values shown in Table 2 were derived by fitting mannitol data to Eq. 1 assuming that the absorbable fraction, $F_a$, was the same as that of fluorescein ($F_a = 0.75$). The approach was supported by the absence of a statistical difference between mannitol’s $F_p$ (fraction of administered dose in perfusate) vs time profiles at different nominal doses (0.02 and 2 mg; t-test, $p < 0.05$; data not shown) and the agreement of $k_a$ values (Table 2) with reports in the literature for mannitol in the in situ rat lung (Brown and Schanker, 1983). In contrast to fluorescein and mannitol, tobramycin’s absorption was clearly dose-dependent, the rate and extent of its pulmonary absorption increasing with the magnitude of the administered dose (Figure 3). At 120 minutes following tobramycin administration, the values for mean $F_p$ ($\pm$ SD; nominal dose) were 0.449 ($\pm$ 0.024; 0.002 mg); 0.505 ($\pm$ 0.021; 0.02 mg); 0.587 ($\pm$ 0.028; 0.2 mg) and 0.612 ($\pm$0.050; 2 mg). Tobramycin’s $F_p$ vs time data showed good agreement with Scheme II and Eq. 2, as indicated by the continuous curves shown in Figure 3. Best estimates of the rate constants used to generate these curves are reported in Table 2 alongside the values for the coefficient of determination, $r^2$, and the model selection criterion, MSC. The rate constant for absorption appeared to be dose-independent (range from 0.065 to 0.070 min$^{-1}$) and close to that of fluorescein (0.076 min$^{-1}$). The rate constant for dissociation from intact IPRL tissue ($k_{21}$) was also relatively constant (0.018-0.022 min$^{-1}$), while that for association ($k_{12}$) decreased from 0.164 to 0.072 min$^{-1}$ with increasing airway dose from 0.002 to 2 mg. As a result, the ratio of $k_{12}/k_{21}$ decreased from 8.9 to 3.4 when the nominal dose was increased from 0.002 to 2 mg.
Discussion

Binding of tobramycin and other aminoglycosides to various tissues has been used to explain the formation of deep compartments in pharmacokinetic studies (Schentag et al., 1978; Vozeh et al., 1979; Winslade et al., 1987), some epithelial cells of which (e.g. kidney proximal tubule and inner ear hair cells) are associated with nephro- and oto-toxicity (Hiel et al., 1993; Just and Habermann, 1977; Nagai and Takano, 2004; Todd and Hottendorf, 1995). Notably, and probably because of the physiologic existence of these drugs as polycations, transfer into cells is slow and intracellular sequestration can be persistent. In the hope of elucidating the drug’s binding to and/or intracellular sequestration by lung tissue, dynamic dialysis was performed both in the absence of tissue, and from the sliced IPRL, after dosing the airways using the same technique as that used for IPRL absorption studies. The open circles in Figure 2A show the apparent first-order, dose-independent dialysis of tobramycin in the absence of lung tissue in which there was no evidence of drug binding. The comparator data shown in Figure 2B, for the non-binding solute mannitol, was mono-exponential and dose-independent ($r^2 >0.98$) both in the presence and absence of tissue, indicating only that the presence of sliced lung tissue slightly reduced the value of $k_e$ for diffusive release from the sac (Scheme III; $k_e$ for mannitol = 0.025 and 0.019 min$^{-1}$ in the absence and presence of sliced IPRL tissue, respectively) by hindering diffusion. Most notably, the tobramycin data for $A_t/A_0$ (fraction of administered dose remaining in the sac) versus time at different doses in the presence of lung tissue were described well by Eq.3 and Scheme III ($r^2>0.998$) showing clear evidence of dose-dependent lung tissue binding or
sequestration, as summarized by the decreasing values for the forward binding rate constant, $k_{12}'$ and the ratio $k_{12}'/k_{21}'$ as doses were increased (Scheme III, Table 1). These values for $k_{12}'$ and $k_{21}'$ implied that tissue binding occurred relatively slowly after airway administration and that all three rate constants in Scheme III had a similar order of magnitude. Efforts to determine whether significant binding/sequestration was due to tissue or the constituents of the IPRL airway lining fluid implied that lung tissues themselves were largely responsible for antibiotic sequestration. Bronchoalveolar lavage samples; diluted with KHS containing low tobramycin concentrations (consistent with doses of approximately 0.002 mg) showed no difference in drug dialysis kinetics from the IPRL tissue-free control data for $A_t/A_0$ in Figure 2A.

This “slow-on” and “slow-off” binding behavior seen during dynamic dialysis supported the kinetic analysis of Fp vs time data in IPRL Absorption Studies. When the ex vivo IPRL was used, the values of the aminoglycoside’s binding constants in Scheme II (Table 2) were quantitatively different to those from the dialysis experiments (association and dissociation occurred faster under conditions involving absorption into perfusate), although the overall trend in the rate constant values was the same as that seen during dialysis. Neither fluorescein nor mannitol showed dose-dependent binding behavior and both showed mono-exponential absorption properties ($k_a$ and $F_a$ values) consistent with reports in the literature (Brown and Schanker, 1983; Byron and Niven, 1988). Tobramycin however, showed decreasing rates of fractional absorption (transfer from airways to perfusate) as a function of decreasing dose and increasing binding/sequestration. Notably,
dosing solution osmolality appeared to have no effect on Fp vs time data. For example, Fp at 120 min for a tobramycin dose at 0.2 mg in 0.9% w/v NaCl showed no statistical difference to that from 0.2 mg in 0.45% w/v NaCl (t test, p<0.05). However, the data for Fp vs time at different doses were described well by Eq. 2 and Scheme II (r^2>0.998) with clear evidence of dose-dependent and possibly saturable lung tissue binding summarized by the decreasing values for the forward binding rate constant, k_{12} and the ratio k_{12}/k_{21} as doses were increased (Table 2). The significant difference between the retention of this solute in lung tissue was related to tobramycin’s slow binding or sequestration following its administration. Values for k_{21} appeared unrelated to dose, but values of k_{12} fell as the dose was increased, but without evidence of capacity limitation. While the exposure of different tissue sites was clearly possible for absorption and dialysis experiments (IPRL was sliced immediately following dosing), the trend in the data for the lung tissue binding or sequestration constant, k_{12} (increases with decreasing dose) and k_{21} (effectively dose-independent), was consistent with the rate constant data for the sliced IPRL.

To the best of our knowledge, this is the first work to investigate tobramycin binding to and/or sequestration by lung tissue and explore its effects on the drug’s pulmonary disposition following airway administration. Dynamic dialysis, initially used for protein binding studies, was successfully employed to study tissue binding and sequestration and the results were consistent with those from the realistic ex vivo IPRL model. Based on the results, it was possible to calculate the apparent elimination half life from the IPRL (equal to 0.693/β; Eq. 2) as 2.3, 1.8, 1.3 and 1.2
hr for nominal doses of 0.002, 0.02, 0.2 and 2 mg, respectively. Notably, if binding did not occur, absorption into the perfusate (and thus, elimination from the IPRL) should occur with a half-life of about 10 minutes (0.693/kₐ; Table 2), similar to that for fluorescein anions (Table 2 and Niven and Byron, 1988). The 1.8 hr half life at a nominal dose of 0.02 mg is consistent with the in vivo lung elimination half life of 2.1 hr reported by Valcke, Y.J and Pauwels, R.A in rat alveolar lining fluid following aerosol administration of a similar dose (Valcke and Pauwels, 1991).

In clinical treatment of humans, tobramycin inhalation solution (TOBI®, Novartis), the commercial nebulizer formulation used for cystic fibrosis patients, is prescribed as an intermittent (28-day on/28-day off) treatment with 300 mg/5 mL nebulized and inhaled twice daily regardless of age (≥6 yrs) or body weight. Since the percent of each nebulizer dose reaching the lung via the recommended PARI LC PLUS™ nebulizer has been reported to be around 15% (Lenney et al., 2011), a lung dose of about 45 mg could be expected in a 70 kg human. Based on weight scaling, this corresponds approximately to an 0.2 mg nominal dose to the airways of the rat lungs used in the IPRL studies described here, where the k₁₂/k₂¹ ratio was 4.06 (Table 2). While species differences may well exist, similar cell constituents and concordance with alveolar surface area, lung volume, capillary volume and body weight have been reported across many species including rats and humans (Crapo et al., 1983; Cryan et al., 2007; Plopper, 1983). In conclusion therefore, these studies in rat lung appear to support the existence of tobramycin retention in lung tissue following airway administration at doses likely to produce airway concentrations seen in humans. As a result of this slow on and slow off tissue
binding or sequestration, the antibiotic’s longevity in the lung is extended. It is possible that this may account, at least in part, for the apparent success of tobramycin inhalation therapy seen in clinical practice.
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Authorship Contributions

The authors are faculty and students of Virginia Commonwealth University. No conflicts of interest exist.

Participated in research design: Li and Byron

Conducted experiments: Li

Contributed new reagents or analytic tools: Byron

Performed data analysis: Li and Byron

Wrote or contributed to the writing of the manuscript: Li and Byron
References:


Footnotes:

ML received a stipend and tuition and fees during her graduate studies from School of Pharmacy, Virginia Commonwealth University. Supplies and equipment were provided by The Medical College of Virginia Foundation, Richmond, VA.
Scheme Legends.

**Schemes I and II**: Kinetic models describing solute (fluorescein, tobramycin) dosing and absorption from the airways of the IPRL in the presence and absence of tissue binding or sequestration. Compartmental abbreviations show the administered dose, D, the untransferable (unabsorbable) amount of each dose, U, and the absorbable amount, $A_0$ ($D=U+A_0$), while $P$ and $B$ are the amount absorbed into the perfusate and the amount of drug bound to, or sequestered in, tissue at time $t$, respectively. The apparent first-order rate constant for absorption from the airways is designated as $k_a$, while $k_{12}$ and $k_{21}$ are first-order association and dissociation rate constants for binding or sequestration.

**Scheme III**: Kinetic model describing solute dosing and dialysis from the IPRL in the presence of tissue binding or sequestration; the dialysis sac is shown diagrammatically as a dashed line. Compartmental abbreviations show the administered dose, D, the dialyzable amount, A ($A=D$), while $R$ and $B$ are the amount released into the receiver solution and the amount of drug bound to or sequestered in tissue at time $t$, respectively. The apparent first-order rate constant for dialysis is designated as $k_e$, while $k_{12}'$ and $k_{21}'$ are the association and dissociation rate constants for binding/sequestration to the sliced tissue.
Figure Legends.

**Figure 1:** The IPRL preparation showing perfusate circulation. The lung was suspended horizontally in the artificial glass thorax (AGT) after cannulation of the pulmonary artery and trachea (Sakagami, 2006).

**Figure 2:** Mean fraction of solute remaining in the dialysis sac, $A_t/A_0$, vs. time. (A) Tobramycin at different nominal doses. Dashed lines show the best fits to Eq. 3. Error bars are sample standard deviations ($n \geq 3$). Key: -●-: 0.002 mg; -▲-: 0.004 mg; -▼-: 0.2 mg; ■-: 2 mg; ◆-: 10 mg; -O-: control (tobramycin 2 mg and 0.002 mg in absence of IPRL). (B) Mannitol at two nominal doses. Key: -△-: 0.02 mg; -◇-: 2 mg; -□-: control, mannitol (0.02 mg in absence of IPRL).

**Figure 3:** Mean $F_p = \text{mean (±SD)}$ fraction of administered dose, $D$, transferred to perfusate vs time for fluorescein (nominal dose = 0.02 mg; open symbols) and tobramycin (closed symbols) in the IPRL. Key: Nominal doses were 0.002mg (●; n=6); 0.02mg (▼; n=5); 0.2mg (▲; n=5) and 2mg (◆; n=4). Solid curves are the best fits for all profiles to Eq. 1 (fluorescein) and 2 (tobramycin) based on Scheme I and II, respectively. $F_a$, the absorbable fraction of the administered dose, was fixed at 0.75 while $k_a$, $k_{12}$ and $k_{21}$ were allowed to float to produce the best estimates shown in Table 2.
Tables.

Table 1: Best estimates of tissue binding/sequestration rate constants in Scheme III (Eq. 3) from dynamic dialysis alongside the coefficient of determination, $r^2$, and “model selection criterion” (MSC). The value of $k_e$ was fixed at 0.01069 min$^{-1}$, its value in the absence of lung tissue.

<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>$k_{12}'$ min$^{-1}$</th>
<th>$k_{21}'$ min$^{-1}$</th>
<th>$k_{12}'/k_{21}'$</th>
<th>$r^2$</th>
<th>MSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.002</td>
<td>0.0174</td>
<td>0.0041</td>
<td>4.29</td>
<td>0.9999</td>
<td>5.74</td>
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<td>0.0037</td>
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<td>0.0043</td>
<td>2.17</td>
<td>0.9999</td>
<td>7.03</td>
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<td>2</td>
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<td>1.83</td>
<td>0.9997</td>
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<tr>
<td>10</td>
<td>0.0052</td>
<td>0.0039</td>
<td>1.3</td>
<td>0.9982</td>
<td>4.29</td>
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</table>
Table 2: Estimated parameters and goodness-of-fit for different solutes in accord with Schemes I (fluorescein and mannitol) and II (tobramycin) in IPRL Absorption Studies with Fa=0.75.

<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>$k_a$ (min$^{-1}$)</th>
<th>$k_{12}$ (min$^{-1}$)</th>
<th>$k_{21}$ (min$^{-1}$)</th>
<th>$k_{12}/k_{21}$</th>
<th>$r^2$</th>
<th>MSC</th>
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<td>Tobramycin</td>
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<td>0.0905</td>
<td>0.0223</td>
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<td>0.0721</td>
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<td>0.9999</td>
<td>6.82</td>
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<td>Mannitol</td>
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*Model selection criterion. Blank cells for $k_{12}$ and $k_{21}$ indicate data consistent with Scheme I.
Scheme III

$D \xrightarrow{k_e} A \xrightarrow{k_{12}'} B \xrightarrow{k_{21}'} A \xrightarrow{k_e} R$