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Pulmonary Delivery of the Kv1.3-Blocking Peptide HsTX1[R14A] for the Treatment of Autoimmune Diseases

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ABSTRACT

HsTX1[R14A] is a potent and selective Kv1.3 channel blocker peptide with the potential to treat autoimmune diseases. Given the typically poor oral bioavailability of peptides, we evaluated pulmonary administration of HsTX1[R14A] in rats as an alternative route for systemic delivery. Plasma concentrations of HsTX1[R14A] were measured by liquid chromatography coupled with tandem mass spectrometry in rats receiving intratracheal administration of HsTX1[R14A] in solution (1-4 mg/kg) or a mannitol-based powder (1 mg/kg) and compared with plasma concentrations after intravenous administration (2 mg/kg). HsTX1[R14A] stability in rat plasma and lung tissue was also determined. HsTX1[R14A] was more stable in plasma than in lung homogenate, with more than 90% of the HsTX1[R14A] remaining intact after 5 h, compared with 40.5% remaining in lung homogenate. The terminal elimination half-life, total clearance, and volume of distribution of HsTX1[R14A] after intravenous administration were 79.6 ± 6.5 min, 8.3 ± 0.6 mL/min/kg, and 949.8 ± 71.0 mL/kg, respectively (mean ± SD). After intratracheal administration, HsTX1[R14A] in solution and dry powder was absorbed to a similar degree, with absolute bioavailability values of 39.2 ± 5.2% and 44.5 ± 12.5%, respectively. This study demonstrated that pulmonary administration is a promising alternative for systemically delivering HsTX1[R14A] for treating autoimmune diseases.

Introduction

The overexpression of voltage-gated potassium Kv1.3 channels in activated effector memory (T_{EM}) lymphocytes is implicated in the development of autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis.[1,2] Kv1.3 channels have therefore been recognized as a promising target for these diseases, with the use of Kv1.3 channel blockers considered a valuable therapeutic strategy.[3-5] Indeed, the sea anemone–derived peptide *Stichodactyla helianthus* (ShK) and its analogues, which have high affinity for Kv1.3 channels, are being developed as therapeutic agents for these autoimmune disorders.[5-7] Blockade of Kv1.3 channels in activated T_{EM} cells by ShK peptides significantly reduces disease severity in 3 animal autoimmune disease models (delayed-type hypersensitivity, chronic relapsing-remitting experimental autoimmune encephalomyelitis, and pristine-induced arthritis), preventing disease-associated tissue damage.[4,5] As a result, one of the highly effective ShK analogues, ShK-186, has undergone phase I human clinical trials following subcutaneous injection, which demonstrates that blockade of Kv1.3 channels is indeed a viable approach for these autoimmune diseases.

The successful development of naturally occurring peptides such as ShK and its analogues has led to efforts to discover alternative potent Kv1.3 blockers including HsTX1, a 34-residue peptide isolated from the scorpion *Heterometrus spinnifer*.[8-10] An analogue of HsTX1, HsTX1[R14A], was recently developed in our laboratory, which not only retains high affinity for Kv1.3 channels, with an IC_{50} value of 45 pM, but also exhibits greater selectivity for Kv1.3 over

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Kv1.1 and other channels.[10] Given its superior selectivity, it is likely that HsTX1[R14A] can effectively block Kv1.3 channels without compromising the physiological function of other channels, making HsTX1[R14A] a novel attractive therapeutic candidate for the treatment of autoimmune diseases. In this early stage of discovery and development, the efficacy of HsTX1[R14A] in a rodent model of autoimmune disease has yet to be investigated, in part because of a lack of understanding of its absorption and pharmacokinetic properties, which are crucial to the design of appropriate dosing regimens for future efficacy studies in relevant disease models. Owing to the presence of abundant peptidases and proteases in the stomach and gastrointestinal tract, together with the hydrophilic characteristics of peptides,[11-13] oral absorption of peptides such as HsTX1[R14A] is expected to be low. Therefore, regular self-injections would be necessary, which can lead to low patient compliance.[14,15] Given the limitations associated with oral and parenteral administration, various alternative noninvasive routes have been explored for the systemic delivery of peptides and proteins, including the buccal mucosal and transdermal routes.[13,16-18] Indeed, we have recently demonstrated that buccal administration of an ShK analogue to mice results in detectable plasma concentrations of this peptide, confirming this route as feasible for the systemic delivery of this particular immunomodulatory peptide.[19]

Pulmonary delivery is another noninvasive route that has attracted increasing attention for the delivery of macromolecules.[14,20] Similar to the buccal mucosal and transdermal routes, the pulmonary route has the advantages of low enzymatic activity and extensive vascularization.[21] Moreover, it offers a large absorptive surface area (~140 m^2), mainly at the alveolar regions, and an extremely thin alveolar epithelium (<1 μm) which can lead to high rates of absorption and a rapid onset of pharmacological action.[22,23] Numerous studies have been conducted to assess the systemic delivery of peptides and proteins through the lung in the form of aerosol and dry powder formulations in both animals and humans.[24-31] The bioavailability of macromolecules via the pulmonary route, however, is variable, largely due to differences in the administration method (i.e. insufflation vs. instillation) and the properties of the macromolecule.[24] However, with an optimized formulation and administration method, the bioavailability of peptides after pulmonary administration can be comparable to that of subcutaneous administration and as high as 80%.[32-34] Although it has been suggested that dry powder inhalers have some advantages over wet nebulization owing to superior chemical stability, greater ease of storage, portability, and use,[35,36] their manufacturing process is substantially more complicated than the use of solutions, and this should be considered when deciding on which formulation should be advanced in the clinic.

The purpose of this study, therefore, was to provide preclinical insight into the pharmacokinetics and pulmonary disposition of HsTX1[R14A], which is crucial information for future studies to be conducted in a relevant rodent model of autoimmune disease. The stability of HsTX1[R14A] in both rat plasma and lung tissue was assessed. The pharmacokinetics of HsTx[R14A] after intravenous administration and the bioavailability of HsTX1[R14A] in rats after intratracheal administration of both solution and dry powder formulations were subsequently determined.

Materials and Methods

Materials

HsTX1[R14A] was synthesized and purified as described previously.[10] Trifluoroacetic acid was purchased from Merck KGaA (Darmstadt, Germany). Trichloroacetic acid was obtained from Sigma-Aldrich (St. Louis, MO). Solid-phase extraction cartridges (tC_{18} Sep-Pak®, 50 mg) were purchased from Waters (Waters Corporation, MA). Calu-3 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's Modified Eagle's Medium and Ham's F-12 nutrient mixture, fetal bovine serum, and nonessential amino acid solution were obtained from Life Technologies Australia Pty Ltd. (Scoresby, Victoria, Australia). All other reagents were of HPLC grade and water was obtained from a Millipore purification system (Millipore Corporation, Billerica, MA).

Stability of HsTX1[R14A] in Rat Plasma and Lung Homogenate

Animal experiments were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee and performed in accordance with the National Health and Medical Research Council guidelines for the care and use of animals for scientific purposes. To obtain rat lung homogenate, lungs from male Sprague-Dawley rats (330-360 g) were homogenized in a volume of phosphate-buffered saline (PBS) (in mL) equal to twice the weight (in g) of the tissue. An aliquot of 150 μL of HsTX1[R14A] stock solution (267 μM) was spiked into 1.35 mL of rat lung homogenate to a final concentration of 80 nmol/g. The homogenates were then incubated at 37°C and 300 μL of each sample was collected at various time points over 24 h. Similarly, to assess stability in rat plasma, an aliquot of HsTX1[R14A] working solution (150 μL at 0.40 or 13.3 μM) diluted from the stock solution was spiked into blank rat plasma (1.35 mL) to obtain a final concentration of 40 or 1330 nM (concentrations reflecting plasma concentrations observed after intravenous administration of HsTX1[R14A]). The plasma was then incubated at 37°C and 250 μL of plasma was collected at various time points over 24 h. Rat plasma and lung homogenate samples were prepared as described in the following section for the subsequent liquid chromatography (LC) tandem mass spectrometry (MS/MS) analysis of HsTX1[R14A].

Intravenous and Pulmonary Administration of HsTX1[R14A] to Rats

The day before pharmacokinetic studies, rats were anesthetized with gaseous isoflurane (Abbott Animal Health, Abbott Park, IL) (5% vol/vol oxygen to induce and 2% vol/vol to maintain anesthesia during surgery). The right carotid artery of each rat was cannulated for collection of blood (after both intravenous and pulmonary administration) and the jugular vein was cannulated (for intravenous administration). After cannulation, rats were individually housed in metabolic cages and allowed to recover from surgery overnight.

For intravenous administration, HsTX1[R14A] was freshly dissolved in sterile PBS, pH 7.4, and a 200-μL aliquot (equivalent to a dose of peptide at 2 mg/kg) was delivered via the jugular vein cannula; the cannula was then flushed with approximately 0.5 mL of PBS. For intratracheal dosing, rats were anesthetized with gaseous isoflurane and rested in a supine position against a restraining board. The tongue was pulled outward gently using forceps and the blade of a small animal laryngoscope model LS-2-R (PennCentury Inc., Wyndmoor, PA) was guided and positioned in the mouth to visualize the vocal cords.[37-39] The tongue was released, the dosing tube coupled with a high pressure syringe model FMJ-250 (PennCentury Inc.) containing freshly prepared HsTX1[R14A] solution was inserted to the trachea just above the carina (the first trachea bifurcation), and an aliquot of HsTX1[R14A] solution (100 μL in PBS, pH 7.4) was delivered into the lung (at doses equivalent to 1, 2, and 4 mg/kg). Powder consisting of HsTX1[R14A] and mannitol (at a ratio of 1 to 19) was produced by spray drying modified from a previous study.[40] According to routine scanning electron microscopy undertaken in the laboratory, the size of all particles was <5 μm

(a representative scanning electron micrograph is included in the Supplementary Material). For intratracheal dosing of the HsTX1[R14A] powder, a dry powder insufflator™ model DP-4 connected with air pump model AP-1 (PennCentury Inc.) was inserted into the trachea and powder (~7 mg) was aerosolized and delivered to the lung at a dose of 1 mg/kg. After each administration, blood samples (500 μL) were collected at 5, 15, 30, 60, 120, 180, 240, and 300 min via the carotid artery and were immediately centrifuged to obtain plasma. Plasma samples were stored at −20°C until analysis by LC MS/MS.

Preparation of Calibration Standards, Rat Plasma, and Lung Homogenate Samples

For plasma samples, a stock solution of HsTX1[R14A] (267 μM) was prepared in MilliQ water. Working standard solutions with concentrations of 67, 133, 267, 400, and 667 nM were prepared by serial dilution of stock solution in MilliQ water. The low, medium, and high quality control (QC) solutions of 67, 267, and 667 nM, respectively were prepared in the same manner using an independently prepared stock solution. Calibration and QC samples were prepared by spiking an aliquot of the working solutions (25 μL) into blank plasma (225 μL), followed by vortex mixing for 10 s. Plasma samples were prepared similarly, except that an aliquot of MilliQ water (25 μL) was added into the plasma sample (225 μL) instead of the working solution. Each mixture was diluted with MilliQ water (500 μL) and then transferred to a solid-phase extraction cartridge, which had been conditioned by washing with acetone (1 mL) and methanol (1 mL), followed by MilliQ water (1 mL). Samples were then eluted in trifluoroacetic acid/milliQ water/acetonitrile (1:70:30, vol/vol, 750 μL) and evaporated under a constant stream of N_2. The residue was reconstituted in MilliQ water (130 μL) and analyzed by the LC MS/MS method described in the following section.

To quantify the concentration of HsTX1[R14A] in lung homogenate, a stock solution of HsTX1[R14A] (814 μM) was first prepared in MilliQ water, and working solutions with concentrations of 54, 81, 133, 267, and 407 μM were prepared by serial dilution of stock solution in MilliQ water. The low, medium, and high QC solutions of 54, 133, and 407 μM, respectively were prepared in the same manner using an independently prepared stock solution. Calibration and QC samples were prepared by spiking 30 μL of each of the working solutions into a 270 μL aliquot of blank lung homogenate to achieve concentrations of 16.2, 24.4, 40.0, 80.3, and 121.0 nmol/g, followed by vortex mixing for 10 s. Lung homogenate samples were similarly prepared, except that 30 μL of MilliQ water was added to 270 μL of lung homogenate. To each mixture was added 300 μL of 5% vol/vol trichloroacetic acid followed by 1 min vortex mixing before centrifugation at 16,100 × *g* for 5 min. The supernatant was then collected and analyzed by the LC MS/MS method described in the following section.

For each LC MS/MS method, intraday assay precision and accuracy were determined by comparative analyses of each of the 4 QC samples at each concentration. Precision was expressed as relative standard deviation (in %) and accuracy was expressed as the difference between the experimentally determined and nominal concentration in terms of percentage.

LC MS/MS Analysis

Before analysis, samples were diluted to an appropriate concentration of HsTX1[R14A] within the linear range of the standard curve. Samples were then injected onto an Ascentis® Express C_{18} column (2.7 μm particle size, 2.1 × 50 mm internal diameter) with a Phenomenex SecurityGuard™ C_{18} guard column (2.0 × 4.0 mm). LC was performed on a Shimadzu HPLC system consisting of 2 LC-30AD pumps, a SIL-30AC autoinjector, and a DGU-20A_5 degasser (Shimadzu, Kyoto, Japan). Mobile phase A consisted of 0.1% (vol/vol) formic acid in MilliQ water and mobile phase B was methanol. Concentrations of HsTX1 [R14A] were determined using the following gradient profile: 0-1 min, 90% A; 1-1.5 min, 75% A; 1.5-2 min, 70% A; 2-2.5 min, 60% A; 2.5-3.5 min, 60% A; and 3.5-4.5 min, 90% A followed by a 1.5 min equilibration period at the initial conditions. Mass spectrometry was performed on a Shimadzu LCMS-8030 quadrupole mass spectrometer (Shimadzu) using electrospray ionization in the positive mode. Mass detection was performed by multiple reaction monitoring (m/z 747.0 to 84.10). Nitrogen was used as the nebulizing gas and drying gas with flow rates set at 3.0 and 15.0 L/min, respectively. The desolvation line and heat block temperatures were 250°C and 400°C, respectively, and the dwell time was set at 500 ms.

Impact of HsTX1[R14A] and Mannitol on Pulmonary Epithelial Cell Viability

To evaluate the impact of HsTX1[R14A] and its dry powder formulation on the viability of the pulmonary epithelium, Calu-3 cells were exposed to various treatments, and viability was measured by the thiazolyl blue tetrazolium bromide (MTT) test. The cells were seeded on 96-well plates at a density of 40,000 cells/well and cultured in 1:1 Dulbecco's Modified Eagle's Medium/Ham's F-12 supplemented with 10% fetal bovine serum and nonessential amino acid solution 100 × (1% vol/vol). The plates were then incubated at 37°C in an atmosphere of 5% CO_2 and 95% O_2 for 2 days. Once confluent, the cells were treated with medium, 10% vol/vol dimethyl sulfoxide (DMSO) in medium (as a positive control known to induce cellular death), HsTX1[R14A] in medium (50, 100, and 200 nM), mannitol in medium (1.6 M), and HsTX1[R14A] (200 nM) with mannitol (1.6 M) in medium for 5 h. This concentration of mannitol is expected to be similar to that encountered in the lung with the doses administered to rats. After 5 h, the cells were washed twice with 150 μL of prewarmed PBS and then treated with 100 μL of MTT (5.0 mg/mL) for 4 h at 37°C. Subsequently, the MTT solution was removed and replaced with 150 μL of DMSO. The plates were then wrapped in aluminum foil and incubated for 30 min at 37°C. The absorbance of each well at 540 nm was recorded using a microplate reader (EnSpire® Multimode Plate Reader, Perkin Elmer, Waltham, MA). The absorbance in the treated cells was compared to control (cells incubated with medium alone) and expressed as a percentage viability (relative to control).

Data Analysis

Noncompartmental analysis was performed using WinNonlin software (version 5.2.1, Pharsight Corporation, Mountain View, CA) to estimate the terminal elimination half-life ($t_{1/2}$), volume of distribution (V_z), and total plasma clearance (CL). The area under the curve from zero to infinity ($AUC_{0\text{-inf}}$) was calculated using the linear trapezoidal rule based on the plasma concentration-time profile. Statistical comparisons of $AUC_{0\text{-inf}}$ between intratracheal administration of solution at various doses and powder were made using a one-way ANOVA followed by Tukey's multiple comparisons test (IBM SPSS Statistics for Windows, version 23.0; Armonk, NY). Statistical comparisons of cell viability after various treatments were also made using a one-way ANOVA followed by Tukey's multiple comparisons test. All results are presented as mean ± SD, unless stated otherwise.

Results

LC MS/MS Assay Validation

For the plasma assay, a linear correlation between peak area and HsTX1[R14A] concentration was observed over the concentration range 6.7-67 nM ($R^2 > 0.998$). The precision coefficient of variation (CV) and accuracy of the assay were determined and validated at the lowest, medium, and highest QC concentrations of HsTX1[R14A]. The intraday coefficients of variation were 3.6%-7.9% and the accuracy ranged from 94.0% to 106.7%, as detailed in Table 1. For the lung homogenate assay, a linear correlation between peak area and HsTX1[R14A] concentration was observed over the concentration range 16.2-121.0 nmol/g ($R^2 > 0.998$). The intraday coefficients of variation were 4.5%-8.0%, and the accuracy ranged from 92.9% to 105.2%, as detailed in Table 2. The LC MS/MS assays were demonstrated to be robust and reliable for the determination of HsTX1 [R14A] concentration in plasma and lung homogenate and were therefore deemed suitable for the subsequent stability and *in vivo* pharmacokinetic studies.

Stability of HsTX1[R14A] in Rat Plasma and Lung Homogenate

As shown in Figure 1, HsTX1[R14A] was stable in the plasma, as more than 90% of the initial spiked amount was detected after 5 h at both the low and high concentrations, representative of those observed after intravenous dosing. After 24-h incubation in plasma, the amount of peptide remaining in the plasma ranged from 69.7% to 83.4%. After incubation in lung homogenate, 40.5% of HsTX1 [R14A] remained intact after 5 h and 14.6% of the initial amount was detected after 24 h, suggesting significant degradation in the lung homogenate. However, the degradation in lung homogenate is likely to be an overestimate of degradation observed *in vivo* following intratracheal administration, as *in vivo* exposure of the peptide to the lung would be for a shorter period of time than what was assessed in these *in vitro* stability studies.

Intravenous and Pulmonary Pharmacokinetics of HsTX1[R14A]

After intravenous administration, the plasma concentrations of HsTX1[R14A] decreased rapidly (Fig. 2) and the pharmacokinetic parameters including C_{max}, T_{max}, and area under the curve from time 0 extrapolated to infinite time ($AUC_{0\text{-inf}}$) are shown in Table 3. The $t_{1/2}$, V_z, and CL of HsTX1[R14A] after intravenous administration were 79.6 ± 6.5 min, 949.8 ± 71.0 mL/kg, and 8.3 ± 0.6 mL/min/kg, respectively.

HsTX1[R14A] was rapidly absorbed following intratracheal dosing of the solution (Fig. 3), with plasma concentrations being detected in the first post-dose sample (i.e. 5 min) and then gradually increasing and peaking at 120 min, after which they declined over the subsequent 3 h. The pharmacokinetic parameters of HsTX1[R14A] are shown in Table 3. The average $AUC_{0\text{-inf}}$ values after intratracheal administration of the solution at doses of 1, 2, and 4 mg/kg were 13.3, 22.2, and 57.2 min·μM, respectively. The $t_{1/2}$, V_z, and CL of HsTX1[R14A] after intratracheal administration were not presented as it was uncertain whether the complete elimination phase had been reached at the end of 5 h. To determine whether the absorption of HsTX1[R14A] over this dose range followed a passive diffusion process, the relationship between dose and $AUC_{0\text{-inf}}$ was plotted; as shown in Figure 4, a linear relationship was observed, suggesting passive diffusion over this dose range.

Having shown that HsTX1[R14A] was absorbed when administered as a solution, the plasma concentrations after administration of peptide formulated as a mannitol-based powder were also assessed. As shown in Figure 3, administration of the powder led to faster absorption, with C_{max} occurring at 15 min post dose. The average $AUC_{0\text{-inf}}$ value after intratracheal administration of the powder was 13.0 min·μM. The bioavailability of HsTX1[R14A] when administered as a solution and powder after intratracheal dosing was similar, with average values of 39.2% and 44.5%, respectively (Table 3).

Table 1
Precision and Accuracy of the LC MS/MS Assay for Quantification of HsTX1[R14A] in Rat Plasma (n = 4)

Target Concentration (nM)	Measured Concentration (nM), Mean ± SD	Precision (%)	Accuracy (%)
6.7	6.3 ± 0.5	7.9	94.0
26.8	28.6 ± 1.1	3.8	106.7
67	66.4 ± 2.4	3.6	99.1

Table 2
Precision and Accuracy of the LC MS/MS Assay for Quantification of HsTX1[R14A] in Rat Lung Homogenate (n = 4)

Target Concentration (nmol/g)	Measured Concentration (nmol/g), Mean ± SD	Precision (%)	Accuracy (%)
16.2	15.6 ± 0.7	4.5	96.3
40.3	42.4 ± 3.4	8.0	105.2
121	112.5 ± 5.8	5.2	92.9

Impact of HsTX1[R14A] and Mannitol on Pulmonary Epithelial Cell Viability

As shown in Figure 5, only 8.8% of Calu-3 cells survived after the 5-h treatment with 10% DMSO ($p < 0.05$). However, compared with medium-treated cells, other treatments including HsTX1[R14A] alone (50, 100, and 200 nM), mannitol alone (1.6 M) or HsTX1 [R14A] (200 nM) with mannitol (1.6 M) did not significantly affect the viability of the cells ($p > 0.05$), suggesting that HsTX1[R14A] and the dry powder formulation consisting of mannitol does not induce considerable cytotoxicity on the lung epithelium at these concentrations.

Discussion

Treatment of autoimmune diseases such as multiple sclerosis and rheumatoid arthritis through the blockade of Kv1.3 channels in

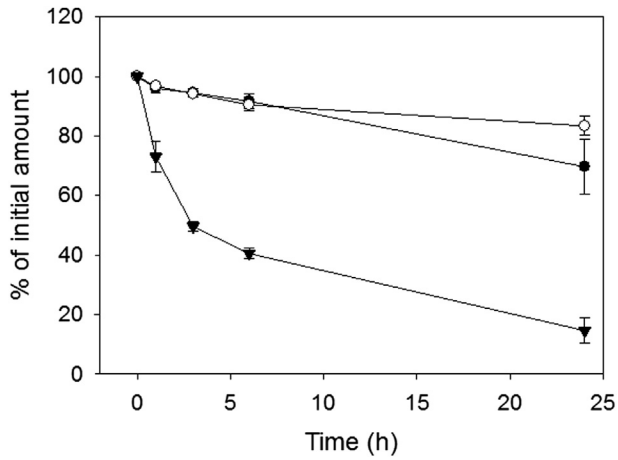

Figure 1. Percentage of initial amount of HsTX1[R14A] in rat plasma after spiking at 40 nM (closed circles) or 1330 nM (open circles) or in lung homogenate after spiking at 80 nmol/g (closed triangle). Data are presented as mean ± SEM (n = 4).

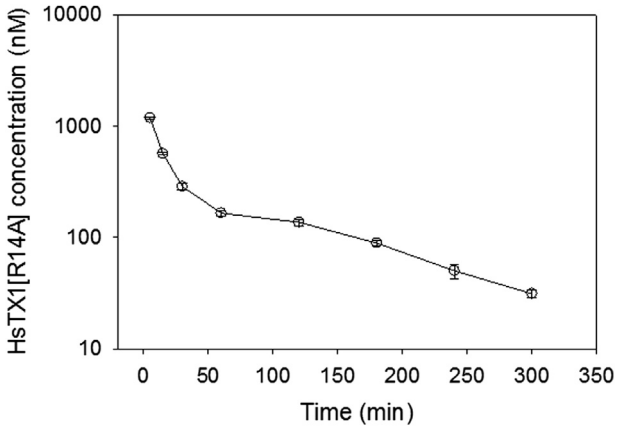

Figure 2. Plasma concentration of HsTX1[R14A] in rats after an intravenous dose of 2 mg/kg. Data are presented as mean ± SEM (n = 3).

activated T_{EM} cells has recently attracted attention, as this approach minimizes the impact on other immune cells and maintains most of the host's protective immune response.[3] The scorpion toxin, HsTX1, potently blocks Kv1.3 channels at pM levels, and therefore could be a potentially attractive candidate for treatment of various autoimmune diseases.[8] Furthermore, HsTX1[R14A], an analogue of HsTX1, exhibits a selectivity of more than 2000-fold for Kv1.3 over Kv1.1 channels,[10] which significantly reduces the potential toxicity associated with loss of Kv1.1 channel function, including cardiac arrhythmias or seizures.[41,42] In this study, we therefore undertook an initial pharmacokinetic evaluation of HsTX1[R14A] after intravenous administration to rats and assessed the potential of the pulmonary route for systemic delivery of HsTX1[R14A] in the form of solution and dry powder, given the limitations associated with parenteral injections.

To determine the *ex vivo* plasma stability of HsTX1[R14A], plasma was spiked with concentrations that reflected maximum and minimum plasma levels after intravenous administration to rats at 2 mg/kg. Similarly, it was important to determine the *ex vivo* lung stability of HsTX1[R14A], and the concentration chosen for this study was based on the likely concentration to be exposed to the entire lung (around 2.5 g in a 350 g rat) with a total dose of 2 mg/kg. Under these conditions, HsTX1[R14A] appeared to be less stable in the lung homogenate (relative to plasma), suggesting a rapid rate of degradation of the peptide in lung homogenate, which is in line with what has been reported for peptides with similar properties, including insulin and salmon calcitonin.[43] However, absorption of HsTX1[R14A] through the lung epithelium is likely to occur mainly via the paracellular route,[44] in which case there would be minimal intracellular exposure of HsTX1[R14A] to cytosolic enzymes (such as aminopeptidase and serine peptidases) that would have been released by epithelial cells during homogenization. Therefore, the degradation of HsTX1[R14A] observed *ex vivo* is likely to be an overestimation of that occurring *in vivo*, given that it is unlikely that HsTX1[R14A] would be exposed to such intracellular enzymes if permeating by the paracellular route. Furthermore, it could be envisaged that, if there was this significant amount of degradation of HsTX1[R14A], then minimal plasma exposure of HsTX1[R14A] would be observed after pulmonary administration. In contrast, absorption of HsTX1[R14A] after an intratracheal dose was appreciable as the bioavailability via this route was up to 42.4%. Similar observations have been made with other peptides such as insulin, where there is significant degradation in lung homogenate stability studies, but high bioavailability after pulmonary administration,[43] supporting the hypothesis that paracellular absorption (and minimal intracellular trafficking) is involved in macromolecular transport across the lung epithelium. It should be noted that there may be some adsorption of HsTX1[R14A] to lung tissue when administered at therapeutically relevant doses, as this process may have been saturated at the higher doses administered in this study; if this occurs, it may decrease the pulmonary bioavailability of HsTX1[R14A].

With respect to degradation in plasma, HsTX1[R14A] was more stable than ShK-186 (an analogue of ShK peptide with high affinity for the Kv1.3 channel and similar molecular mass and charge to HsTX1[R14A]).[5,45] Moreover, the *in vivo* plasma elimination of HsTX1[R14A] after intravenous dosing was much slower than that of ShK-186 after subcutaneous dosing, with HsTX1[R14A] exhibiting a longer elimination $t_{1/2}$ and lower CL than ShK-186 but being similar to that of salmon calcitonin after intravenous dosing.[5,46] The average volume of distribution for HsTX1[R14A] of 950 mL/kg after intravenous administration is in line with that reported for salmon calcitonin after intravenous dosing but smaller than that of ShK-186 after subcutaneous dosing at a dose of 1 mg/kg.[5,46] Therefore, HsTX1[R14A] did not exhibit any unexpected pharmacokinetic properties for a peptide of its size and physicochemical properties, and showed the expected plasma profile generally observed for macromolecules.

Both solution and dry powder formulations of drugs for inhalation have been widely evaluated in animals[29,47,48] and some of these have been tested in clinical trials or are currently on the market.[49-52] Compared with administration as a solution, pulmonary absorption of HsTX1[R14A] was faster when administered as a powder, as the T_{max} of powder and solution were 15 min and 120 min, respectively. Given the limited volume of the fluid lining the alveolar epithelium (~3-12 μL),[25] the concentration of the administered powder at a dose of 1 mg/kg in the alveolar region could be 10 times higher than that of the administered solution (in a volume of 100 μL) at the same dose (i.e., same amount of HsTX1[R14A] dissolved in much less fluid), which could potentially increase the rate of absorption. Secondly, the concentration of mannitol included in the powder formulation may have resulted in increased paracellular absorption of the peptide as concentrations of mannitol of 1.6 M and beyond have been reported to enhance paracellular permeability.[53] Although it could be considered that HsTX1[R14A] or mannitol, or their combination, may cause generalized toxicity of the lung epithelium, we have demonstrated using the MTT assay that these

Table 3
Pharmacokinetic Parameters of HsTX1 [R14A] in Rats After Intravenous (i.v.) and Intratracheal (i.t.) Administration at Various Doses

Pharmacokinetic Parameter	i.v. Solution	i.t. Solution			i.t. Powder
	2 mg/kg	1 mg/kg	2 mg/kg	4 mg/kg	1 mg/kg
C_{max} (nM)	1372.1 ± 26.3	58.4 ± 3.0	83.9 ± 6.3	178.1 ± 28.0	115.8 ± 41.0
T_{max} (min)	5	120	120	120	15
$AUC_{0\text{-inf}}$ (min·μM)	63.8 ± 4.1	13.3 ± 2.4	22.2 ± 0.8	57.2 ± 7.4	13.0 ± 5.4
Absolute bioavailability (%)	N.A.	37.8 ± 7.2	37.4 ± 1.1	42.4 ± 5.6	44.5 ± 12.5

Data are presented as mean ± SD (n = 3).

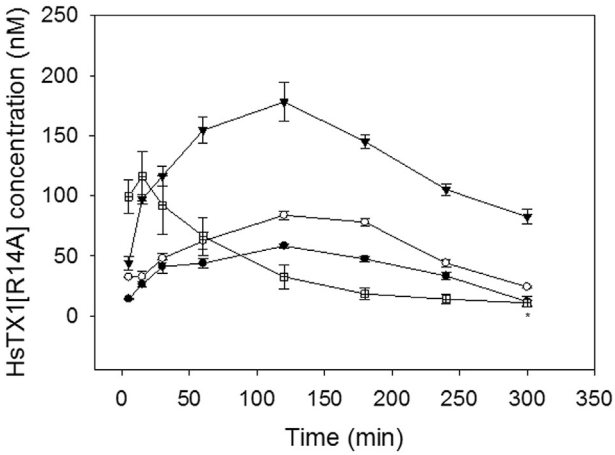

Figure 3. Plasma concentration of HsTX1[R14A] in rats after intratracheal administration of solution at doses of 1 (closed circle), 2 (open circle), and 4 (closed triangle) mg/kg and dry powder (open square) at a dose of 1 mg/kg. Data are presented as mean ± SEM (n = 3-4). *The average HsTX1[R14A] plasma concentration after intratracheal administration of dry powder at 300 min was based on 3 animals as the concentration in one of the animals was below the detection limit (i.e., 6.7 nM).

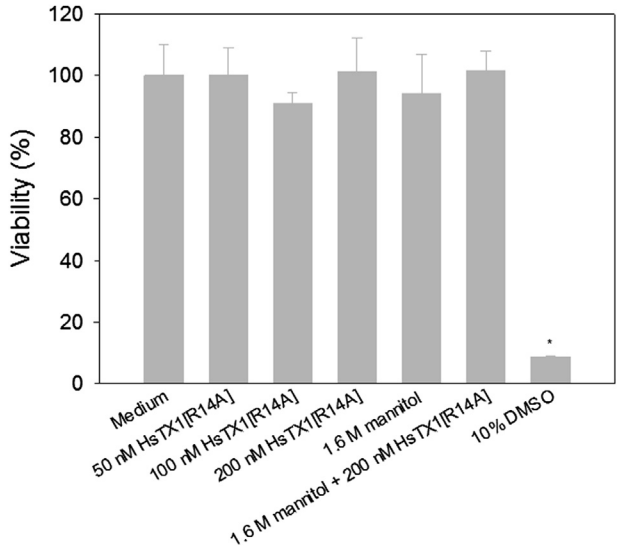

Figure 5. Viability of Calu-3 cells after a 5-h exposure to medium, HsTX1[R14A] alone (50, 100, and 200 nM in medium), mannitol (1.6 M) in medium, HsTX1[R14A] (200 nM) with mannitol (1.6 M) in medium, and 10% vol/vol DMSO in medium. Data are presented as mean ± SD (n = 5). *Indicates a significant difference in viability induced by 10% vol/vol DMSO using a one-way ANOVA followed by a Tukey's multiple comparison test ($p < 0.05$).

agents did not affect the viability of Calu-3 cells, a human adenocarcinoma cell model. Although there may be increased paracellular permeability with the concentrations of mannitol used, this does not appear to result in actual changes to epithelial viability.

Despite a slower rate of absorption of HsTX1[R14A] from the solution formulation, the plasma levels of HsTX1[R14A] detected after intratracheal administration of both solution and dry powder far exceeded the plasma concentration required for therapeutic activity, which is in the pM range.[10] This is due to the fact that much larger doses of HsTX1[R14A] were administered than would be required in the clinical setting (i.e. mg/kg vs. μg/kg); however, given the limitations of the LC MS/MS assay, this proof-of-principle study was conducted with these higher doses. It should also be noted that our preliminary study showed that, after incubation with rat plasma for up to 5 h, HsTX1[R14A] maintained its Kv1.3-binding activity as measured using surface plasmon resonance (Supplementary Fig. 2).[54] Therefore, our studies demonstrate that

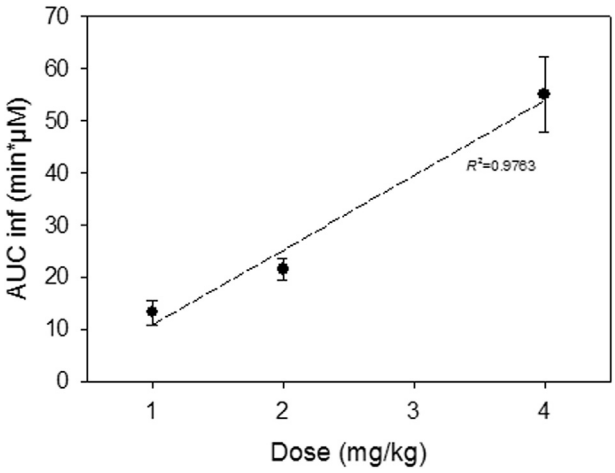

Figure 4. Relationship between $AUC_{0\text{-inf}}$ and dose of HsTX1[R14A] after intratracheal administration of HsTX1[R14A] solution. Data are presented as mean ± SD (n = 3-4). The line represents the linearity between $AUC_{0\text{-inf}}$ and doses with $R^2 = 0.9763$.

HsTX1[R14A] can be absorbed rapidly through the pulmonary route and it is extremely likely that plasma concentrations in the high pM range will be reached when lower (and more therapeutically relevant) doses are administered. Furthermore, the activity of this peptide is likely to be maintained when present in the plasma for up to at least 5 h.

Both solution and powder formulations exhibited a similar bioavailability of approximately 40%, despite a more rapid absorption rate with the powder formulation, suggesting a similar extent of absorption, which is in line with peptides of similar properties.[25,28,47] This relatively high pulmonary bioavailability could be a consequence of factors such as the large absorption area, extensive vasculature, thin alveolar epithelium, and function of various transporters.[55] It has been suggested that some transporters located at the apical side of the lung alveolar epithelium including proton-coupled peptide transporter may be involved in the pulmonary absorption of peptides in both animals and humans.[56,57] For this reason, we assessed linearity between doses and $AUC_{0\text{-inf}}$ to evaluate whether any transporter might contribute to the pulmonary absorption of HsTX1[R14A]. After dose-escalating studies with pulmonary administration of HsTX1[R14A] solutions (i.e., 1, 2, and 4 mg/kg), a linear relationship was found between the dose and $AUC_{0\text{-inf}}$ ($R^2 = 0.9763$) suggesting the pulmonary absorption of HsTX1[R14A] is likely via passive diffusion, at least within the current dose range (Fig. 4), which is in line with paracellular diffusion across the lung epithelium. Whether the same *in vivo* behavior occurs at more clinically relevant doses (i.e., μg/kg) needs to be assessed once a more sensitive assay is established.

In conclusion, our study has provided insight into the pharmacokinetic properties of a novel peptide with potential for the treatment of autoimmune diseases and demonstrated that intratracheal administration of HsTX1[R14A] in rats leads to rapid absorption and considerable bioavailability. This study suggests that pulmonary administration is a promising alternative route for delivery of this potent peptide, and provides key information necessary for the design of efficacy studies with HsTX1[R14A] in a relevant rodent model of autoimmune disease.

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