

Lipidation of the Kv1.3 blocking peptide HsTX1[R14A] alters its pharmacokinetics and biodistribution

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ABSTRACT

Upregulation of the voltage-gated potassium channel Kv1.3 in effector memory T cells has been implicated in several autoimmune diseases, making selective Kv1.3 blockade an attractive therapeutic strategy. HsTX1[R14A], a 34-residue peptide with picomolar potency and high selectivity for Kv1.3, is effective in a rodent model of rheumatoid arthritis. However, the *in vivo* half-life of HsTX1[R14A] can be improved to enhance its potential as a therapeutic candidate. Here we explore how conjugation to C14, C16 or C18 acyl chains affects HsTX1[R14A] binding to serum albumin and lipoproteins, potency at Kv1.3, pharmacokinetics (PK), and biodistribution to key organs, tissues, and lymph nodes. The fluorophore Cy5 was conjugated to the HsTX1[R14A] analogs for biodistribution studies. LC-MS/MS assays were developed to quantify the lipidated peptides in mouse plasma. Conjugation of palmitic and stearic acids to HsTX1[R14A] caused over 20-fold loss in potency but significantly enhanced its binding to albumin, while showing minimal association with plasma lipoproteins. This modification extended the plasma exposure by 9-fold and prolonged the elimination half-life by 2-fold after subcutaneous (SC) injection to mice. Biodistribution studies demonstrated that Cy5-labelled lipidated analogs enhanced accumulation at the dosing site and draining lymph nodes at 4 and 12 h after SC injection compared to Cy5-HsTX1[R14A]. Cy5-labelled lipidated analogs of HsTX1[R14A] also accumulated in the gastrointestinal tract more than HsTX1[R14A], while having significantly less exposure in the kidneys at 4 and 12 h after SC administration. Thus, lipidation is a promising approach to optimize the PK, biodistribution and therapeutic potential of HsTX1[R14A].

1. Introduction

Autoimmune diseases are characterized by a breach of immune tolerance, resulting in overactive immune cells that mount a response against endogenous antigens, causing significant mortality and morbidity in the host (Wang et al., 2015; Abramson and Husebye, 2016; Wulff and Zhorov, 2008). Autoimmune diseases affect approximately 5–10% of the population worldwide (Cooper et al., 2009; Shapira et al., 2010), and their prevalence has been rising in the past decade (Miller,

2023). Current treatments primarily target symptom relief or immunosuppression (Macaron and Ontaneda, 2019; Mohamed et al., 2019; Li et al., 2017), but they are often associated with drug resistance, severe side effects or a high risk of disease relapse (Ethgen et al., 2013). There is thus a significant need for the development of more effective and targeted therapeutics that can provide better long-term management for autoimmune patients. Previous studies have demonstrated upregulation of Kv1.3 channels in multiple cell types from patients with a range of autoimmune diseases, including multiple sclerosis, rheumatoid arthritis

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and inflammatory bowel diseases (Wulff et al., 2003; Nielsen et al., 2017; Beeton and Chandy, 2005). Accordingly, inhibition of Kv1.3 has been investigated as a potential therapeutic strategy for autoimmune disorders. Dalazatide (ShK-186), a Kv1.3 blocker derived from sea anemone venom, demonstrated efficacy in improving skin lesions in patients with plaque psoriasis in a Phase 1 clinical trial using twice-weekly SC dosing (Tarcha et al., 2017; Pennington et al., 2009; Chi et al., 2012). Dalazatide is currently scheduled to start a Phase 2 clinical trial for the treatment of multiple sclerosis. The development and early success of dalazatide have prompted the development of new Kv1.3-targeted therapeutics, including venom-derived compounds from diverse species (Cheng et al., 2024).

The scorpion-derived peptide HsTX1[R14A] is a promising candidate for autoimmune disease treatment owing to its potent activity against the voltage-gated potassium channel Kv1.3 ($IC_{50} \sim 45$ pM) and high target selectivity (Rashid et al., 2014). HsTX1[R14A] is highly stable and resistant to a range of proteases *in vitro* studies (Rashid et al., 2014). The peptide at 0.1 mg/kg was also effective in reducing inflammation at the time of immunization and at the time of challenge in a rodent delayed-type hypersensitivity (DTH) model, and reduced disease severity in a rat pristane-induced arthritis (PIA) model when dosed weekly at the same dose (Tanner et al., 2017). HsTX1[R14A] has a short *in vivo* half-life (~ 80 min) following intravenous (IV) administration (Jin et al., 2016), which poses a limitation to its potential as a candidate for autoimmune disease treatment, where patients often require long-term medication for continuous management. Extending the *in vivo* half-life of HsTX1[R14A] would reduce the required frequency of injections and improve patient convenience and adherence.

Lipidation is a common approach to modify therapeutics to improve their physiological and pharmacological profiles (Cherepanov et al., 2019; Coppens et al., 2021; De Vrieze et al., 2019; Hu et al., 2016; Wang et al., 2019; Liu et al., 2014; Osborn and Khvorova, 2018). Conjugation of peptides to lipid moieties results in association with endogenous plasma proteins such as albumin and lipoproteins (Abdallah et al., 2020), extending the plasma exposure of the peptide by protecting against rapid degradation and/or renal clearance (Zhang and Bulaj, 2012; Jensen et al., 2011). Moreover, lipidation of peptides may extend their plasma exposure by forming a depot at the SC injection site that provides slow release of the peptide to the systemic circulation (Zhang and Bulaj, 2012; Jensen et al., 2011). In addition to the alteration of PK profiles, lipidation has the potential to alter biodistribution to specific organs and tissues (Gracia et al., 2020). Following SC administration, lipidated peptides can hitchhike on endogenous albumin or lipoprotein trafficking pathways into lymphatics, thus increasing the potential for lipidated compounds to interact with immune cells in lymphoid tissues (Gracia et al., 2020; Trevaskis et al., 2015). Enhancing delivery to the lymphatic system may thus improve the efficacy of immunomodulators like HsTX1[R14A] (Liu et al., 2014; Khan et al., 2013; Gracia et al., 2022).

In the present study, we modified HsTX1[R14A] by conjugating different lipid moieties on the N-terminus of the peptide, aiming to increase the circulation time and optimize the biodistribution of the peptide by integrating into natural lipid trafficking pathways. The three lipidated HsTX1[R14A] analogs were conjugated to saturated fatty acids with 14, 16 and 18 carbons (myristic acid, palmitic acid and stearic acid, respectively). Conjugation to myristic and palmitic acid has been shown to increase the half-life of various molecules, with palmitic acid being the most commonly used in lipidated peptide therapeutics on the market and in clinical trials (Kurtzhals et al., 2022). Stearic acid was also included due to its higher affinity for albumin compared to myristic acid and palmitic acid (Yamasaki et al., 2013), and to evaluate the impact of chain length on half-life extension and biodistribution profiles. We developed bespoke liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods for the quantification of lipidated HsTX1[R14A] analogs in plasma to facilitate evaluation of the PK profiles of the lipidated peptides. These studies are essential for optimizing the efficacy

and safety of lipidated HsTX1[R14A] analogs by characterizing how the analogs are absorbed, distributed, and eliminated in the body.

2. Materials and methods

2.1. Materials

Cy5 carboxylic acid was purchased from W&J PharmaChem (MD, USA). Solid-phase extraction cartridges (tC18 Sep-Pak® 1 cc Vac, 50 mg) were purchased from Waters (Waters Corporation, MA). Human serum albumin (HSA), low-binding Eppendorf tubes and pipette tips, trifluoroacetic acid (TFA) and all other reagents of LC-MS grade were purchased from Merck (Darmstadt, Germany). The CM5 sensor chip was from Biacore. Water was obtained from a Millipore purification system (Millipore Corporation, Billerica, MA). 4.2 mL open-top polypropylene tube for ultracentrifuge was purchased from Beckman Coulter (Victoria, Australia). Bicinchoninic acid (BCA) protein assay kit, Infinity® cholesterol reagent and Infinity® triglyceride reagents were purchased from Thermo Fisher (Victoria, Australia). Paraformaldehyde (PFA) was purchased from ProSciTech (Queensland, Australia). Axon Digidata 1440 A data acquisition hardware and pClamp 10 software package were from Molecular Devices (CA, USA). GC 150F-15 borosilicate glass capillaries were from Harvard Apparatus (MA, USA). Histopaque-1077, phytohemagglutinin A, fetal calf serum, and bovine serum albumin (BSA) were from Sigma-Aldrich (Budapest, Hungary and MO, USA). TRANSIL HSA binding kit was purchased from Sovicell GmbH (Leipzig, Germany).

2.2. Peptide synthesis

Synthesis and purification of HsTX1[R14A] were performed as described previously (Wai et al., 2022). For lipidated HsTX1[R14A] analogs, myristic, palmitic or stearic acid was coupled to the N-terminus of HsTX1[R14A] by agitation of the resin with 3 eq. fatty acid and 3 eq. O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) in 7% (v/v) N,N-diisopropylethylamine (DIPEA)/N,N-dimethylformamide (DMF) for 1 h. The reduced peptide was cleaved from the resin using 85% (v/v) trifluoroacetic acid (TFA), 5% H₂O, 5% thioanisole, 2.5% triisopropylsilane (TIPS) and 2.5% ethane-1,2-dithiol (EDT), as previously described (Wai et al., 2022). Precursors for Cy5-labelled lipidated HsTX1[R14A] analogs were synthesized by coupling Fmoc-Lys(N₃)-OH (3 eq.) to the N-terminus of HsTX1[R14A] on-resin using HCTU/DIPEA as above. The Fmoc group was removed by agitation in 20% piperidine/DMF (2 × 5 min), the resin washed with DMF (3×), and the fatty acid coupled as above to yield Myr-, Palm- or Stear-KN₃-HsTX1[R14A] after cleavage from resin. All reduced peptides were purified by reversed phase high performance liquid chromatography (RP-HPLC) prior to oxidation.

Purified reduced peptides were dissolved in either 50 mM Tris pH 8 (HsTX1[R14A] and KN₃-HsTX1[R14A]) or 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.5, 30% (v/v) acetonitrile (Myr-/Palm-/Stear-(KN₃)-HsTX1[R14A]) containing reduced and oxidized glutathione at a ratio of 1:2 molar equivalents relative to the peptide. The peptides were folded by air oxidation with gentle rocking overnight, then purified by RP-HPLC.

2.3. Reversed phase high performance liquid chromatography (RP-HPLC)

Crude and oxidized peptides were purified by RP-HPLC on an Agilent 1260 Infinity II liquid chromatography system using a Vydac C18 column (250 × 10 mm, 10 µm), with 0.1% (v/v) TFA in H₂O as buffer A and 0.1% (v/v) TFA in acetonitrile as buffer B. The column was eluted at a flow rate of 4 mL/min using linear gradients as follows: KN₃-HsTX1[R14A] (reduced and oxidized) and KN₃(Cy5)-HsTX1[R14A], 15–30% B over 15 min; Myr-HsTX1[R14A] and Myr-KN₃-HsTX1[R14A] (reduced and oxidized), 30–50% B over 20 min; Myr-KN₃(Cy5)-HsTX1[R14A],

35–50% B over 30 min; Palm-HsTX1[R14A] and Palm-KN₃-HsTX1[R14A] (reduced), 35–55% B over 20 min; Palm-HsTX1[R14A] and Palm-KN₃-HsTX1[R14A] (oxidized), 35–50% B over 15 min; Palm-KN₃(Cy5)-HsTX1[R14A], 30–55% B over 25 min; Stear-HsTX1[R14A] and Stear-KN₃-HsTX1[R14A] (reduced), 35–85% B over 50 min; Stear-HsTX1[R14A] (oxidized), 40–55% B over 15 min; Stear-KN₃-HsTX1[R14A] (oxidized) and Stear-KN₃(Cy5)-HsTX1[R14A], 55–75% B over 30 min.

The identity and purity of peptide fractions were analyzed by liquid chromatography mass spectroscopy (LC-MS) using a Phenomenex Luna C8(2) reverse-phase column (100 Å, 3 µm, 100 × 2.0 mm) operated on a Shimadzu 2020 LC-MS. Analysis was conducted at a flow rate of 0.2 mL/min, with 0.05% (v/v) TFA in H₂O as buffer A and 0.05% (v/v) TFA in ACN as buffer B. Fractions containing pure peptide were lyophilized and stored at –20 °C.

2.4. Synthesis of Cy5-alkyne and conjugation to peptides

Cy5 carboxylic acid was coupled to propargylamine (3 eq.) using 3 eq. HCTU in 7% (v/v) DIPEA/DMF and agitation for 1 h. Excess diethyl ether was then added and the reaction mixture centrifuged at 3000 g for 10 min. The precipitate was purified by RP-HPLC as above (10–70% B over 30 min) and fractions containing pure Cy5-alkyne were lyophilized and stored at –20 °C.

Purified oxidized peptides were dissolved in 50 mM HEPES (pH 7.5) to a final concentration of 0.1–0.3 mM, and 2 eq. of Cy5-alkyne and aminoguanidine (3 mM) was added. Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) was pre-mixed with CuSO₄ in a 5:1 molar ratio and added to the reaction mixture. The reaction was incubated with sodium ascorbate (5–7.5 mM) at ambient temperature overnight.

2.5. Nuclear magnetic resonance (NMR) spectroscopy

Purified peptides (0.1–0.5 mg) were dissolved in 550–600 µL 5% ²H₂O in MilliQ water and the pH was measured. A separate sample of Stear-KN₃(Cy5)-HsTX1[R14A] was also dissolved in 30% DMSO-²H₆/65% water/5% ²H₂O before pH measurement. One-dimensional ¹H NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer at 298 K. ¹H chemical shifts were referenced to the dioxane peak at 3.75 ppm. Spectra were processed and analyzed in Topspin (version 4.5.0).

2.6. Patch-clamp electrophysiology study

The patch-clamp study to quantify K_v1.3 inhibition by the HsTX1[R14A] analogs was performed as described previously (Reddiar et al., 2021; Naseem et al., 2022). The study was approved by Ethical Committee of University of Debrecen, (DE RKEB/IKEB 6627-2023). Informed consent was obtained from each participant. The investigation conformed to the principles outlined in the Declaration of Helsinki. Heparinised human peripheral venous blood was obtained from healthy volunteers. Mononuclear cells were separated through Histopaque-1077 density gradient centrifugation. Cells were washed twice with Ca²⁺- and Mg²⁺-free Hanks' solution containing 25 mM HEPES buffer, pH 7.4. Cells were cultured for 4–5 days in 24-well culture plates in a 5% CO₂ incubator at 37 °C, in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine (cell density, 0.5 × 10⁶ cells/mL). The culture medium also contained 2, 5 or 10 µg/mL phytohemagglutinin A, to increase K⁺ channel expression.

Whole-cell currents were measured in voltage-clamped cells according to standard protocols (Naseem et al., 2022; Hamill et al., 1981), using an Axopatch 200B or Multiclamp 700B amplifier connected to a personal computer with Axon Digidata 1440 A and for data acquisition, Clampex 10.7 software was used (Molecular Devices, CA, USA). Pipettes

were pulled from GC 150F-15 borosilicate glass capillaries using a Sutter laser puller (P-2000) with tip resistance generally ranging from 3 to 6 MΩ in the bath. For measurements of K_v1.3 channel, the bath solution consisted of 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, 5.5 mM glucose, and 10 mM HEPES, pH 7.35, supplemented with 0.1 mg/mL bovine serum albumin. Equimolar substitution of Na⁺ for tetraethylammonium-Cl was used in TEA⁺-containing positive control solution. The measured osmolarity of the external (bath) solution was between 302 and 308 mOsm. The internal solution contained 140 mM KF, 2 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES, and 11 mM ethylene glycol-bis(2-aminoethylether)-N,N,N', N'-tetraacetic acid (EGTA), pH 7.22 and its measured osmolarity was approximately 295 mOsm. Lyophilized peptide analogs were dissolved in external solution supplemented with 1 mg/mL BSA to obtain 100 µM stock concentration.

To evoke the K_v1.3 currents in activated T lymphocytes, 15-ms voltage pulses to +50 mV from a holding potential of –120 mV were applied every 15 s. All recordings were carried out at room temperature (20–25 °C). Control and test solutions were perfused to the cells via a gravity-driven micro-perfusion system and AutoMate Perfusion Pencil Multi-Barrel Manifold Tip (AutoMate Scientific), and the excess fluid was removed continuously. The complete exchange of solutions in the bath chamber i.e., the proper operation of the perfusion apparatus was confirmed frequently using 10 mM TEA⁺, a reversible inhibitor of K_v1.3 as positive control at a concentration equivalent to its IC₅₀ value.

For data analysis, the pClamp 10.7 software package was used. In general, currents were low-pass filtered using the built-in analog four-pole Bessel filters of the amplifiers and were sampled (20 kHz) at least twice the filter cut-off frequency. Before analysis, whole-cell current traces were digitally filtered (three-point boxcar smoothing). For the concentration response curves, remaining current fractions (RCF = I/I₀, where I₀ is the peak current in the absence of peptide and I is the peak current at equilibrium block in the presence of peptide) at different peptide concentrations were determined and fitted by unweighted ordinary least-squares nonlinear regression using the four-parameter Hill equation in GraphPad Prism software (version 8.0.1):

$$RCF = I/I_0 = \frac{IC_{50}^H}{IC_{50}^H + [peptide]^H}$$

where IC₅₀ is the concentration of the peptide that gives 50% block, [peptide] is the molar concentration of peptide, and H is the Hill coefficient. The fitting was parametrized with *Top* (highest RCF) and *Bottom* (minimum RCF at full channel block) parameters constrained to 1 and 0, respectively. Each data point represents the mean RCF value ± SEM from 3 to 6 individual recordings.

2.7. Surface plasmon resonance

Surface plasmon resonance (SPR) studies were performed to assess the binding affinity of the HsTX1[R14A] analogs to HSA. All glassware was rinsed with water, ethanol and acetone, and dried overnight at 60 °C to remove detergent residues, which can bind HSA. HEPES-buffered saline (HBS) running buffer containing 10 mM HEPES and 150 mM NaCl was prepared in MilliQ water (MQW) under constant stirring. The pH was adjusted to 7.4 using NaOH, and the buffer was filtered through a 0.22 µm cellulose membrane and degassed by stirring slowly under vacuum for 1 h. A CM5 sensor chip was rinsed with MQW and dried under a stream of N₂. The chip was subsequently docked to the Biacore T200 SPR instrument and equilibrated with detergent-free HBS overnight. The surfaces of both reference and target flow cells of the chip were activated using 0.05 M NHS and 0.2 M EDC. HSA was injected into the target flow cell at a flow rate of 5 µL/min with a contact time of 1200 s. After 1825 response units (RU) of HSA were immobilized, residual surface-bound carboxyl groups of the chip were capped using 1 M ethanolamine-HCl.

Concentrated peptide stock solutions were prepared in Milli-Q water

(MQW), and two-fold serial dilutions into HBS were performed using 20 μM and 15 μM starting concentrations to achieve a total of 22 points per experiment. Blanks were run at the beginning and end of each SPR run, and a wash was run after each experiment. A flow rate of 30 μL per minute was used with a contact time of 60 s and a dissociation time of 120 s. R_{max} was calculated as $(\text{RU of HSA immobilized}) \times (\text{MW of peptide})/(\text{MW of HSA})$.

2.8. Ultracentrifugation method to determine HsTX1[R14A] analog association with proteins and lipoproteins

The association of HsTX1[R14A] analogs with proteins and different lipoproteins in mouse plasma was further investigated using a single-step potassium bromide (KBr) density gradient ultracentrifugation method (Gracia et al., 2020; Humberstone et al., 1998; Gracia et al., 2020) with minor changes. Briefly, KBr solutions of different densities (1.02 g/mL, 1.15 g/mL and 1.21 g/mL) were prepared by dissolving different amounts of KBr into the background solution. The background solution was prepared by adding NaCl into TE buffer (containing 10 mM Tris-HCl, 1 mM disodium EDTA at pH 7.4) to adjust the density to 1.0063 g/mL. The density of the plasma background solutions and different KBr solutions was confirmed by measuring the refractive index (RI) with a refractometer, then converting the normalized RI value to density using a standard salt table (NaCl and KBr, respectively) (Rodkey, 1966).

The different Cy5-labelled HsTX1[R14A] analogs were added at concentrations of 267 nM or 1069 nM into mouse plasma and incubated for 30 min at 37 °C. Subsequently, 0.31 mL of 1.21 g/mL KBr solution, 1.25 mL of 1.15 g/mL KBr solution and 1 mL of plasma containing the Cy5-labelled HsTX1[R14A] analogs were gently layered from the bottom to top of a 4.2 mL open-top polypropylene tube. The 1.02 g/mL KBr solution was layered on top of the sample to make up the final volume to around 4–4.2 mL. The tubes were placed in the buckets of the Beckman Coulter SW 60 Ti Swinging-Bucket rotor, then ultracentrifuged at 453,300 g at 15 °C for 19 h. After ultracentrifugation, the samples were separated into plasma protein (PP), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and chylomicron/very-low-density lipoprotein (CM/VLDL) fractions based on density. A 27-gauge needle was used to pierce the bottom of the tubes, then a series of ~200 μL liquid aliquots were collected in low-binding Eppendorf tubes to obtain around 20 fractions in each 4.2 mL tube. The lipoprotein subtypes in each fraction were classified by density: (1) PP at 1.18 g/mL or above; (2) HDL at 1.06–1.18 g/mL; (3) LDL at 1.06–1.02 g/mL; (4) CM/VLDL lower than 1.02 g/mL (Feingold, 2000).

A 100 μL aliquot of each eluted fraction was transferred to a 96-well black polystyrene microplate. Fluorescence intensity was measured by an EnSight™ multimode plate reader, using 642 nm and 665 nm as excitation and emission wavelengths for Cy5, respectively. Data acquisition of the fluorescence and absorbance readings was performed using Kaleido™ (version 3.0) software (PerkinElmer, Inc., Waltham, MA, USA). Cholesterol, triglyceride, and total protein content in each fraction were measured by Infinity® cholesterol reagent, Infinity® triglyceride reagent and BCA assay kit according to the manufacturer's instructions.

2.9. Albumin binding of HsTX1[R14A] analogs

The binding of HsTX1[R14A], Palm-HsTX1[R14A] and Stear-HsTX1[R14A] to HSA was additionally assessed using a TRANSIL HSA binding kit based on the manufacturer's protocol. Briefly, after thawing the plate, 15 μL of HsTX1[R14A] and the two lipidated analogs in 6% MeOH in MQW were added to wells containing different concentrations of HSA (in a range of 3.2 μM –61.7 μM HSA) to achieve a final peptide concentration of 267 nM. The selected concentration was within the plasma concentration range of HsTX1[R14A] and its lipidated analogs *in vivo*. After incubation for 12 min at room temperature on a plate shaker at

1000 rpm, the plate was centrifuged for 10 min at 750 g. Supernatant was taken and analyzed by the developed LC-MS/MS assay mentioned below to quantify the amount of unbound peptide. Dissociation constants for peptide binding to HSA (K_d HSA) and the bound peptide drug fraction (% Bound) were calculated using the spreadsheet provided by the manufacturer.

2.10. Animal experiments

Animal studies were approved by the Monash Institute of Pharmaceutical Sciences (MIPS) Animal Ethics Committee (Animal Ethics No. 35465) and were conducted in accordance with the Australian and New Zealand Council for the Care of Animals in Research and Teaching guidelines. Animals were purchased from the Monash Animal Research Platform, Monash University, and had free access to water and a standard rodent diet prior to studies.

2.11. Pharmacokinetics of HsTX1[R14A], Palm-HsTX1[R14A] and Stear-HsTX1[R14A]

Female C57BL/6J mice (8–12 weeks old) were used to conduct PK studies of HsTX1[R14A], Palm-HsTX1[R14A] and Stear-HsTX1[R14A]. All mice had access to food and water during the study. A 2 mg/kg dose was used in PK studies to ensure that the plasma concentration of HsTX1[R14A] analogs remained above the lower limit of quantification of the LC-MS/MS assay throughout most of the sampling period. This dosage was also consistent with previous PK studies of HsTX1[R14A] in rodents (Reddiar et al., 2021). 71.2 μM HsTX1[R14A]; 67 μM Palm-HsTX1[R14A] and 66.7 μM Stear-HsTX1[R14A] in saline were prepared in separate low-binding Eppendorf tubes. The pH of the dosing solution was measured immediately after preparation and before dosing to ensure a near physiological pH to avoid the pain and irritation associated with the injection of an acidic solution. Each dosing stock solution was visually inspected to confirm the absence of turbidity or precipitation prior to administration to animals. The concentration of each dosing stock was confirmed by LC-MS/MS analysis to ensure dose accuracy. The ^1H NMR shown in Figs. 3 and 4 indicate that the lipidated analogs are soluble and not aggregated. The dosing volume was 150 μL per 20 g mouse, with the volume adjusted based on the body weight of each mouse.

After an acclimatization period of at least 7 days, each mouse was administered HsTX1[R14A], Palm-HsTX1[R14A] or Stear-HsTX1[R14A] subcutaneously in the hind leg area via a 27 G needle, at a volume of around 150 μL as a bolus dose. SC injection was chosen over IV to avoid anaphylaxis and to give more sustained and prolonged absorption of the peptide to the systemic circulation (Datta-Mannan, 2019). Each syringe was weighed before and after dosing to confirm the volume and thus the dose of peptide delivered. Blood samples were collected via terminal cardiac puncture at pre-dose and various time points (0.5, 1, 2, 3, 4, 6, 8, 10, 16, 24 h) after dosing, with 3–4 animals used at each time point. Plasma samples were separated via centrifugation at 4000 g for 5 min, then stored at -80°C freezer until analysis.

The concentration of the peptide in plasma was measured using the LC-MS/MS assay described below. Samples collected at different time points were diluted with blank mouse plasma to appropriate concentrations within the linear range of the standard curve. Concentrations of peptide in plasma samples were back-calculated from their peak area in comparison to the standard curve, followed by back-calculation from any dilution factors and dose normalization. The plasma concentration versus time data were analyzed with a non-compartment PK model in PK Solver (a Microsoft Excel add-in program) using the linear trapezoidal method with no time delay for absorption ($T_{\text{lag}} = 0$) and automatic determination of the number of points for the terminal slope. Analysis was performed on data points at varying time range (0–6 h for HsTX1[R14A], 0–16 h for Palm-HsTX1[R14A] and 0–10 h for Stear-HsTX1[R14A]) to obtain PK parameters including the maximum plasma

concentration (C_{max}), time of the maximum plasma concentration (T_{max}), plasma elimination half-life ($t_{1/2}$), and plasma exposure from time zero to infinity (AUC_{0-inf}) (Foster et al., 2007; Zhang et al., 2010).

2.12. LC-MS/MS assay optimization and validation for detection and quantification of HsTX1[R14A] in solvent and mouse plasma

Low-binding Eppendorf tubes and pipette tips were used for the following sample preparation steps. To prepare HsTX1[R14A] standards for albumin binding assay quantification, duplicates of standards with concentrations of 1.3, 2.7, 6.7, 13.3 and 26.7 nM were prepared in 30% MeOH in MQW using a simple dilution method. Triplicates of quality control (QC) samples were prepared in low (1.3 nM), medium (6.7 nM) and high (26.7 nM) concentrations in the same matrix using the same dilution method from an independently prepared stock solution.

To prepare HsTX1[R14A] standards in plasma, a stock solution of HsTX1[R14A] in MQW was first prepared, then working solutions with concentrations of 27, 67, 133, 267, 400, 667 nM were prepared in MQW in duplicate using simple dilution from the stock solution. Triplicates of the high, medium and low concentration QCs at 27 nM, 133 nM and 667 nM were prepared in MQW using the same dilution method from an independently prepared stock solution. Standards and QC were prepared by spiking 25 μ L of the working solutions into 225 μ L of blank mouse plasma. For blank plasma samples, 25 μ L of MQW was added to 225 μ L of blank plasma. All standards and QCs were centrifuged at 21,100 g for 10 min at room temperature to remove bubbles induced during vortexing and to maximize the sample volume for solid-phase extraction. tC18 cartridges were conditioned using 1 mL of methanol (MeOH) and washed with 1 mL of MQW prior to sample loading. After 250 μ L of plasma samples were loaded, each cartridge was washed with 500 μ L of MQW, and then the samples were eluted with 750 μ L of 70:30:1 (vol/vol/vol) MQW/acetonitrile (ACN)/trifluoroacetic acid (TFA). Eluted samples were briefly vortexed, centrifuged and then evaporated using a nitrogen evaporator at 40 °C. Sample residues were reconstituted with 80 μ L of MQW for LC-MS/MS analysis.

Development and validation of the LC-MS/MS assay of HsTX1[R14A] in solvent were conducted on a HPLC system coupled to a Shimadzu 8050 tandem quadrupole mass spectrometer through an electrospray ionization (ESI) source. Chromatographic separation was achieved with an Agilent Polaris 180 Å C18-A column (2.0 $\times$ 50 mm, 5 μ m) at a flow rate of 0.4 mL/min at room temperature. The injection volume of each sample was 10 μ L. 0.1% formic acid (FA) in MQW was used as mobile phase A, 0.1% FA in MeOH was used as mobile phase B and isopropyl alcohol (IPA) was used as the needle rinse solution. The chromatography gradient of HsTX1[R14A] in solvent assay used: 0–1 min, 10% B; 1–1.5 min, 25% B; 1.5–2 min, 30% B; 2–2.5 min, 40% B; 2.5–3.5 min, 95% B; 3.5–4.5 min, 95% B; 4.5–4.6 min, 10% B; 4.6–5.6 min, 10% B. MS quantification of HsTX1[R14A] was conducted in positive mode ESI with multiple reaction monitoring (MRM) parameters optimized by direct infusion. The precursor ion to product ion mass transition used for HsTX1[R14A] was m/z 747.5 > 84.1. The dwell time used was 30 ms for each transition. The ESI source conditions used for the mass spectrometer were interface temperature at 300 °C, desolvation temperature at 526 °C, desolvation line (DL) temperature at 250 °C, heat block temperatures at 400 °C, heating gas flow rate at 10 L/min, drying gas flow rate at 10 L/min and nebulizing gas flow rate at 3 L/min.

Optimization and validation of LC-MS/MS assay of HsTX1[R14A] in mouse plasma used the same conditions as above, except that a Phenomenex C18 guard column (2.0 mm) was used, with the following chromatography gradient: 0–1 min, 10% B; 1–1.5 min, 25% B; 1.5–2 min, 30% B; 2–2.5 min, 40% B; 2.5–3.5 min, 100% B; 3.5–5.5 min, 100% B; 5.5–6.5 min, 10% B; 6.5–8.5 min, 10% B.

To validate the HsTX1[R14A] LC-MS/MS assays, accuracy and precision were determined by analyzing QC samples in solvent or in plasma. The accuracy was expressed as the mean and standard deviation

percentage of the observed concentration compared to the nominal concentration, which was calculated using observed concentration/nominal concentration $\times$ 100%. Precision was expressed as relative standard deviation (%RSD). To validate the assay, accuracy and precision of the QCs would need to lay within 85–115% of the nominal concentration (80–120% for low concentration QCs). Assay validation data are provided in the [Supplementary Information Document](#) (Fig. S1–S3 and Table S1–S2).

2.13. LC-MS/MS assay development and validation for detection and quantification of Palm-HsTX1[R14A] and Stear-HsTX1[R14A] in solvent and mouse plasma

Low-binding Eppendorf tubes and pipette tips were used for the following sample preparation steps. To prepare Palm-HsTX1[R14A] and Stear-HsTX1[R14A] standards for albumin binding assay quantification, duplicates of standards were prepared in 50% MeOH in MQW using a simple dilution method (1.3, 2.5, 6.3, 12.6, 25.1 nM for Palm-HsTX1[R14A] and 1.3, 2.5, 6.3, 12.5, 25 nM for Stear-HsTX1[R14A]). Triplicates of quality control (QC) samples were prepared in low, medium and high concentrations (1.3, 6.3, 25.1 nM for Palm-HsTX1[R14A] and 1.3, 6.3, 25 nM for Stear-HsTX1[R14A]) in the same matrix using the same dilution method from an independently prepared stock solution.

To prepare standards of Palm-HsTX1[R14A] and Stear-HsTX1[R14A] in mouse plasma, stock solutions of Palm-HsTX1[R14A] and Stear-HsTX1[R14A] were dissolved in 50% MeOH in MQW, then working solutions with concentrations of 200, 267, 317, 400, 444, 667 nM were prepared in water in duplicates using simple dilution from the stock solution in 50% MeOH in MQW. Standards were prepared by spiking 25 μ L of working solution into 225 μ L of blank mouse plasma. For blank plasma samples, 25 μ L of MQW was added to 225 μ L of blank plasma. All standards and QCs were centrifuged at 21,100 g for 10 min at room temperature to remove bubbles induced during vortex and to maximize the sample volume for solid-phase extraction. tC18 cartridges were conditioned using 1 mL of MeOH and washed with 1 mL of MQW prior to sample loading. After samples were loaded, each cartridge was washed with 500 μ L of MQW, then the samples were eluted with 750 μ L of 50:50:1 (v/v/v) and 30:70:1 (v/v/v) MQW/ACN/TFA for Palm-HsTX1[R14A] and Stear-HsTX1[R14A], respectively. Eluted samples were briefly vortexed, centrifuged and then evaporated using a nitrogen evaporator at 37 °C. Sample residues were reconstituted with 80 μ L of 50% MeOH in MQW, then transferred to glass inserts for LC-MS/MS analysis.

LC-MS/MS method development and validation for lipidated peptides in solvent were conducted using an HPLC system coupled to a Shimadzu 8050 tandem quadrupole mass spectrometer with an ESI source. Chromatographic separation was achieved with a Phenomenex Kinetex C8 100 Å Column (50 $\times$ 2.1 mm, 2.6 μ m) at a flow rate of 0.4 mL/min at room temperature. The injection volume of each sample was 10 μ L. 0.1% v/v FA in MQW was used as mobile phase A, 0.1% v/v FA in MeOH was used as mobile phase B and IPA was used as needle rinse solution. The chromatography gradient used for both Palm-HsTX1[R14A] and Stear-HsTX1[R14A] was the following: 0–1 min, 5%B; 1–4 min, 5–95%B, 4–5 min, 95%B; 5–5.1 min, 95–5%B; 5.1–6 min, 5%B. MS quantification of Palm-HsTX1[R14A] and Stear-HsTX1[R14A] was conducted in positive mode ESI with multiple reaction monitoring (MRM) parameters were optimized by direct infusion. The precursor ion to product ion mass transition used for Palm-HsTX1[R14A] was m/z 795.3 > 70.2. Stear-HsTX1[R14A] used the mass transitions of m/z 800.70 > 83.95. The dwell time used was 30 ms for each transition. The ESI source conditions used for the mass spectrometer were interface temperature at 300 °C, desolvation temperature at 526 °C, DL temperature at 250 °C, heat block temperatures at 400 °C, heating gas flow rate at 10 L/min, drying gas flow rate at 10 L/min and nebulizing gas flow rate at 2 L/min.

LC-MS/MS methods for Palm-HsTX1[R14A] and Stear-HsTX1

[R14A] in mouse plasma used the same conditions as in the solvent, except for: 1: A C8 guard column (2.1 mm) was added to the column; 2: The injection volume of each sample was 5 μ L; 3. The chromatography gradient used for Palm-HsTX1[R14A] was: 0–1 min, 10%B; 1–4 min, 10–95%B; 4–6.5 min, 95%B; 6.5–6.75 min, 95–10%B; 6.75–8 min, 10%B. The chromatography gradient used for Stear-HsTX1[R14A] was: 0–1 min, 10%B; 1–4 min, 10–95%B; 4–6 min, 95%B; 6–6.2 min, 95–10%B; 6.2–8 min, 10%B.

To validate the Palm-HsTX1[R14A] and Stear-HsTX1[R14A] LC-MS/MS assays, intra-day accuracy and precision were determined by analyzing QC samples. The accuracy and precision were determined using the same methods as the HsTX1[R14A] assay. Data for the assay validations are provided in the [Supplementary Information Document](#) (Fig. S1–S4 and Table S1–S2).

2.14. Biodistribution of KN₃(Cy5)-HsTX1[R14A], Palm-KN₃(Cy5)-HsTX1[R14A] and Stear-KN₃(Cy5)-HsTX1[R14A]

Female C57BL/6J mice (8–12 weeks old) were used to conduct biodistribution studies for KN₃(Cy5)-HsTX1[R14A], Palm-KN₃(Cy5)-HsTX1[R14A] and Stear-KN₃(Cy5)-HsTX1[R14A]. All mice had access to food and water during the study. KN₃(Cy5)-HsTX1[R14A], Palm-KN₃(Cy5)-HsTX1[R14A] and Stear-KN₃(Cy5)-HsTX1[R14A] were prepared at concentrations of 71.2 μ M in saline in low-binding Eppendorf tubes. The dosing volume was 150 μ L per 20 g mouse. The volume was adjusted based on the body weight of each mouse to meet the 2 mg/kg dose.

After acclimatization, each mouse was administered saline (control), KN₃(Cy5)-HsTX1[R14A], Palm-KN₃(Cy5)-HsTX1[R14A] or Stear-KN₃(Cy5)-HsTX1[R14A] subcutaneously at the inner thigh area. At 4 h or 12 h after dosing, mice were perfused intracardially with saline, followed by 4% w/v paraformaldehyde in PBS. Organs and tissues were harvested from the mice, including the brain, heart, lungs, liver, kidneys, spleen, pancreas, stomach, small intestine, large intestine, bladder, inguinal adipose tissue, as well as skin and muscle from the dosing site and the corresponding contralateral site (Fig. 1). Lymph nodes were also collected to evaluate the lymphatic uptake of Cy5-labelled peptides: inguinal, axillary, brachial and iliac lymph nodes from the dosing and non-dosing side, and mesenteric lymph nodes.

2.15. Analysis of the organ and tissue distribution using an *in vivo* imaging system

The harvested mouse tissues and organs were placed in Costar® 24-well microplates (clear tissue culture-treated). Each well was pre-filled with 1.5 mL 4% w/v paraformaldehyde in PBS. The fluorescence intensity of each organ was measured using the AMI HT optical imaging

system (Plymouth, USA) and emission filters were set at 605 nm and 670 nm for Cy5, respectively. The excitation power was 10% and the exposure was 5 s with low binning mode. The field of view (FOV) was 15, and the object height was 1.5 cm. The background fluorescence of different tissues, organs and lymph nodes was measured using tissues and organs harvested from control mice (injected with saline). Background fluorescence intensity was subtracted from the tissues and organs of mice injected with Cy5-labelled peptides. The region of interest (ROI) analysis for each organ of each mouse was conducted via manual outlining using the ImageJ v1.54r software (National Institutes of Health, Bethesda, Maryland, USA) (Fig. S5) and the fluorescence of each ROI was measured.

2.16. Statistical analysis

For electrophysiology, LC-MS/MS and ultracentrifugation studies, *n* are technical replicates prepared independently from the same stock solution. For PK and biodistribution studies, *n* are biological replicates in different animals. Statistical analysis was conducted using GraphPad Prism 10.2.2 software (GraphPad Software Inc., CA, USA). Outliers were identified using robust regression and the outlier removal (ROUT) method with *Q* = 1%. Statistical differences were performed on cleaned data using either Student's *T* test or one-way analysis of variance (ANOVA) tests, followed by Tukey's post-hoc test. A *P* value < 0.05 was considered statistically significant. Graphs were made in R (4.3.2) with tidyverse and ggplot2 libraries, and Graphpad Prism.

3. Results

3.1. Characterization of lipidated analogs of HsTX1[R14A]

Myristoylated (Myr-), palmitoylated (Palm-) and stearyl (Stear-) HsTX1[R14A] were produced by conjugation of myristic, palmitic and stearic acid, respectively, to the N-terminus of HsTX1[R14A]. These fatty acids were selected based on their binding affinity for albumin and prevalent use in other lipidated peptide therapeutics and clinical candidates (Myskova et al., 2023). HsTX1[R14A] contains four disulfide bonds and requires oxidative refolding to achieve its functional, folded form (Fig. 2A) (Rashid et al., 2014; Wai et al., 2022). Distinct from oxidative refolding conditions previously used for HsTX1[R14A], an oxidation buffer with pH < 8 and containing 30% acetonitrile was found to be essential for maintaining peptide solubility during oxidative folding of the N-terminally lipidated analogs, which proceeded with yields of 10–25% (Figs. S6–8). The peak dispersion in the amide-aromatic region of the ¹H NMR spectrum of each analog was consistent with adoption of the HsTX1 fold (Fig. 3), and further indicated that the lipidated peptides were monomeric (rather than micellar) at

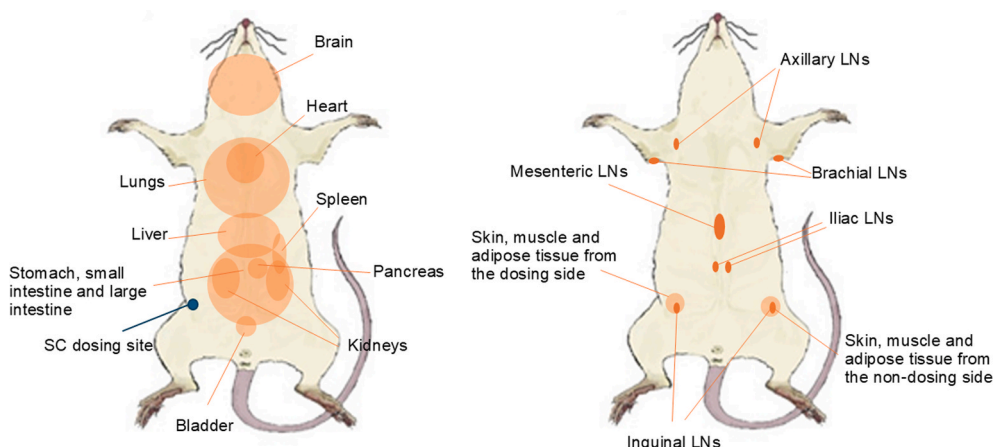


Fig. 1. Tissues and organs collected in the biodistribution study of Cy5-labelled HsTX1[R14A] analogs.

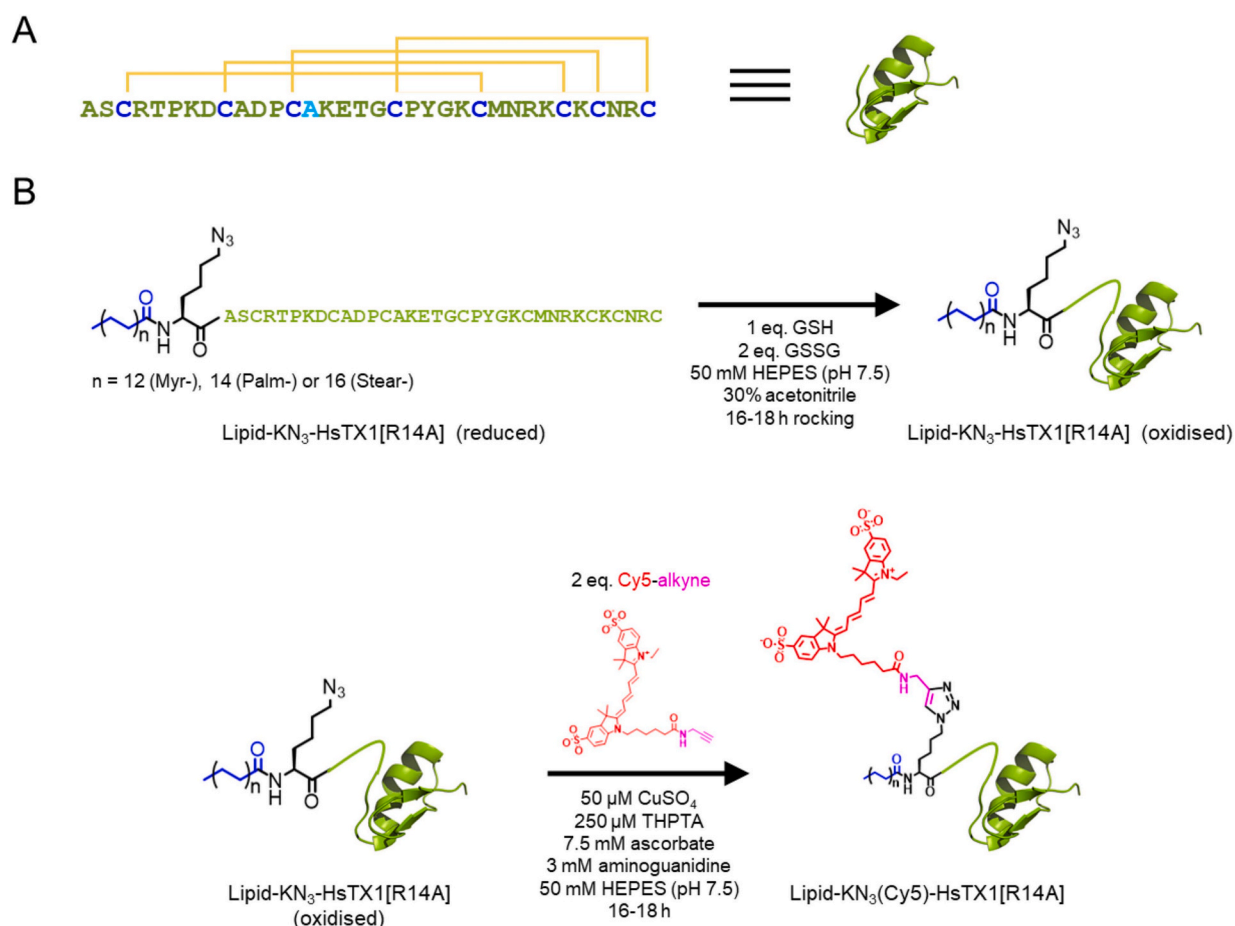


Fig. 2. (A) Sequence of HsTX1[R14A] (with disulfide connectivities indicated in yellow and Arg14 to Ala14 substitution in cyan) and the solution structure of HsTX1 (PDB 1QUZ). (B) Reaction scheme for oxidation of lipid-KN₃-HsTX1[R14A] and CuAAC conjugation with Cy5-alkyne. GSH, reduced glutathione; GSSG, oxidized glutathione.

concentrations of up to 1 mg/mL.

To generate fluorescent lipidated analogs, our initial approach involved adding a lysine to the N-terminus of HsTX1[R14A], then conjugating lipid to the N-terminus and Cy5 to the Lys sidechain via amide coupling. However, the yield from oxidative refolding of these analogs was very low. Therefore, we substituted the N-terminal lysine for azidolysine (hereafter KN₃) to generate Myr-, Palm- and Stear- KN₃-HsTX1[R14A], which could be refolded using the same conditions as for Myr-, Palm- and Stear- HsTX1[R14A]. Subsequently, Cy5-alkyne was conjugated to oxidized Myr-, Palm- or Stear- KN₃-HsTX1[R14A] using copper-catalyzed azide-alkyne cycloaddition (CuAAC) to generate Myr-, Palm- or Stear-KN₃(Cy5)-HsTX1[R14A], respectively (Fig. 2B). KN₃(Cy5)-HsTX1[R14A] was synthesized in an analogous manner. LC-MS analysis confirmed the identity and purity of all peptides synthesized (Figs. S9–15).

Once fully oxidized, Palm-, Stear- and Palm- KN₃(Cy5)-HsTX1[R14A] were fully soluble in water. The ¹H NMR spectrum of Stear-KN₃(Cy5)-HsTX1[R14A] in water, although suggestive of folded peptide, displayed broad peaks that could be attributable to aggregation (Fig. S16). In 30% ²H₂-DMSO/65% water/5% ²H₂O, Stear-KN₃(Cy5)-HsTX1[R14A] exhibited sharp, well-resolved peaks comparable to those observed for the other analogs in water (Fig. 4).

3.2. Potency of Kv1.3 blockade

The potency of blockade of Kv1.3 by all three lipidated HsTX1[R14A] analogs was assessed by whole-cell patch-clamp electrophysiology. Current traces in Fig. 5 A–C show that each of the lipidated

analog at 1 nM concentration reduce the whole-cell K⁺ current without drastically affecting the current kinetics, i.e., the currents in the presence of the lipidated analogs are the scaled down versions of the control corresponding control currents. Tetraethylammonium (TEA⁺) was used as a positive control for the perfusion throughout the experiments. Traces similar to Fig. 5A–C were obtained at various concentrations of the lipidated analogs and the concentration–response relationships were obtained. Myr-KN₃(Cy5)-HsTX1[R14A], Palm-KN₃(Cy5)-HsTX1[R14A] and Stear-KN₃(Cy5)-HsTX1[R14A] inhibited Kv1.3 with IC₅₀ values of 0.59, 1.27 and 6 nM, respectively (Fig. 5D–F and Fig. S17), corresponding to ~13-, ~28- and ~133-fold losses of potency as compared to the IC₅₀ of 45 pM for HsTX1[R14A] mediated inhibition of Kv1.3 (Rashid et al., 2014; Wai et al., 2022). These results indicate that increasing the chain length of the lipid moiety decreases the potency of the lipidated peptide against Kv1.3.

3.3. Lipidated HsTX1[R14A] analogs display strong association with the protein fraction of plasma

In the ultracentrifugation studies, where 267 nM and 1069 nM concentrations of Cy5-labelled HsTX1[R14A] analogs were incubated with plasma that was subsequently separated into protein and lipoprotein fractions, all three lipidated analogs showed significantly increased distribution in the plasma protein (PP) fraction compared to the parental analog at both concentrations (Fig. 6B–C). Palm-KN₃(Cy5)-HsTX1[R14A] and Stear-KN₃(Cy5)-HsTX1[R14A] were around 70% distributed in the PP fraction, demonstrating strong plasma protein association. While Palm-KN₃(Cy5)-HsTX1[R14A] and Stear-KN₃(Cy5)-HsTX1

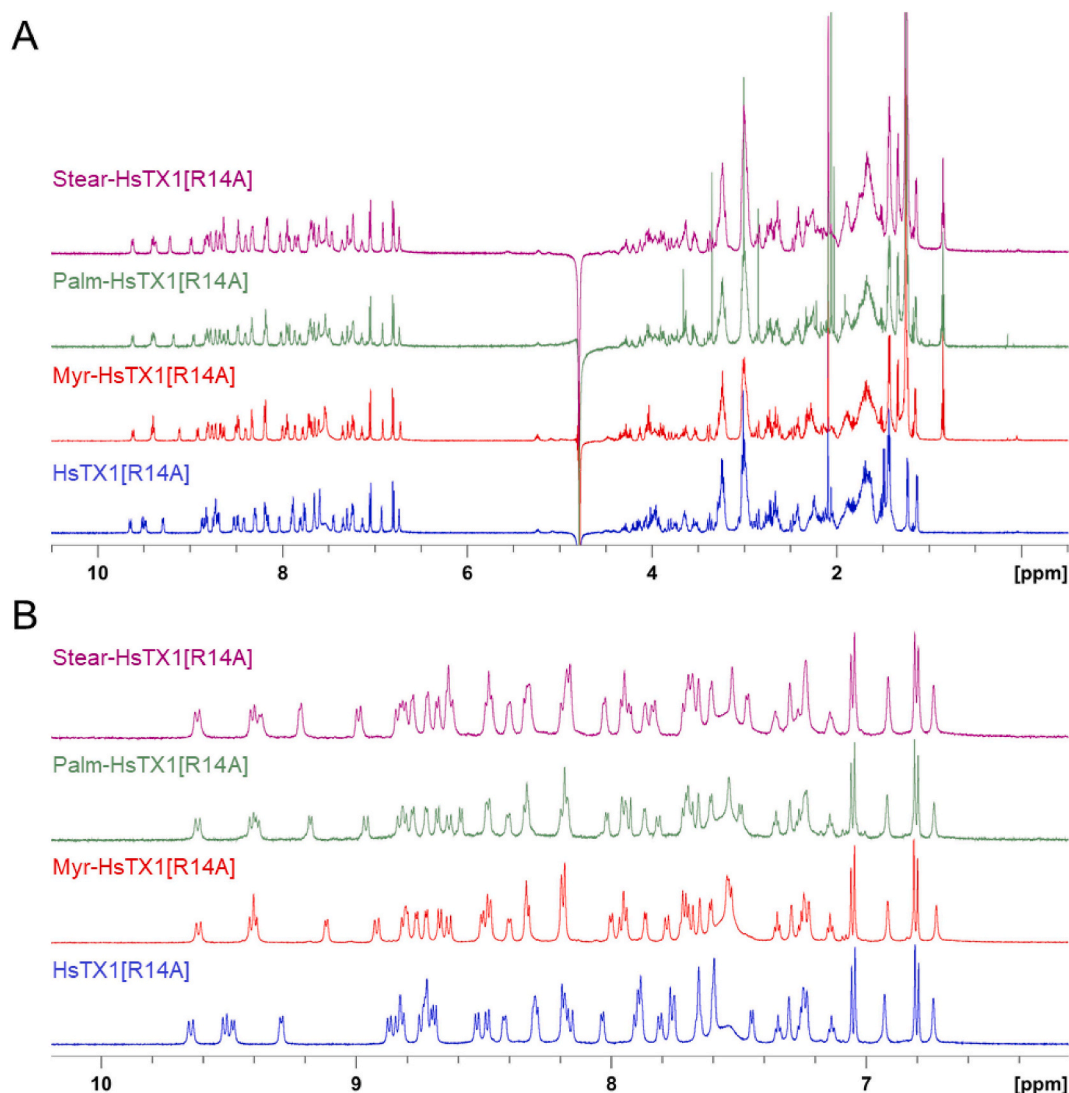


Fig. 3. (A) One-dimensional ^1H NMR spectra of HsTX1 [R14A] (blue), Myr-HsTX1 [R14A] (red), Palm-HsTX1 [R14A] (green) and Stear-HsTX1 [R14A] (purple) in 95% $\text{H}_2\text{O}/5\%$ $^2\text{H}_2\text{O}$ (HsTX1 [R14A], pH 4.0; Myr-HsTX1 [R14A], pH 4.0; Palm-HsTX1 [R14A], pH 4.0; Stear-HsTX1 [R14A], pH 4.7) at 600 MHz and 298 K. (B) Expanded amide-aromatic region of spectra in (A).

[R14A] showed similar distribution across plasma fractions at both 267 nM and 1069 nM concentrations, the distribution of Myr- $\text{KN}_3(\text{Cy}5)$ -HsTX1 [R14A] in the PP fraction increased by 20% when its concentration increased from 267 nM to 1069 nM (Fig. 6A), suggesting that the plasma protein association of Myr- $\text{KN}_3(\text{Cy}5)$ -HsTX1 [R14A] is concentration-dependent. The distribution of Myr- $\text{KN}_3(\text{Cy}5)$ -HsTX1 [R14A] in HDL was significantly lower than $\text{KN}_3(\text{Cy}5)$ -HsTX1 [R14A] and higher than Palm- $\text{KN}_3(\text{Cy}5)$ -HsTX1 [R14A] and Stear- $\text{KN}_3(\text{Cy}5)$ -HsTX1 [R14A] at 267 nM. In LDL and CM/VLDL fractions, Myr- $\text{KN}_3(\text{Cy}5)$ -HsTX1 [R14A] had the highest distribution percentage compared to the other three peptides (Fig. 6B).

Ultracentrifugation of $\text{KN}_3(\text{Cy}5)$ -HsTX1 [R14A] analogs in KBr solution confirmed the distribution of peptides in the absence of plasma protein and lipoproteins. In the absence of mouse plasma, $\text{KN}_3(\text{Cy}5)$ -HsTX1 [R14A] was distributed within the 20–40% cumulative volume fraction of the KBr gradient, overlapping with the HDL region, while both lipidated analogs were distributed within the 40–60% fraction, corresponding to the HDL and LDL region (Fig. S18). Notably, neither $\text{KN}_3(\text{Cy}5)$ -HsTX1 [R14A] nor the lipidated analogs distributed into the plasma protein region (0–20% cumulative volume), confirming that the association with this fraction observed in the presence of plasma is driven by plasma protein binding. This validates the enhanced plasma

protein association observed for the lipidated analogs compared to the $\text{KN}_3(\text{Cy}5)$ -HsTX1 [R14A].

3.4. Lipidation increases the binding of HsTX1 [R14A] to albumin

SPR was used to evaluate the association of HsTX1 [R14A] analogs with human serum albumin (HSA) immobilized on a CM5 chip. Unmodified HsTX1 [R14A] exhibited minimal binding, with slow association that did not reach equilibrium over a contact time of 60 s, although a concentration-dependent relationship could still be observed (Fig. 7). All three lipidated analogs exhibited overbinding to HSA, as evidenced by maximal responses greater than the theoretical R_{max} value (~110 RU) (Fig. 7). The extent of overbinding increased with increasing lipid chain length. Binding profiles of Myr-HsTX1 [R14A] and Palm-HsTX1 [R14A] showed fast association and dissociation with HSA, whereas Stear-HsTX1 [R14A] exhibited slower rates of both association and dissociation. This is likely due to stronger association of Stear-HsTX1 [R14A] with HSA, supported by the ~1700 RU intensity observed at 20 μM being approximately 16 times the R_{max} value.

The TRANSIL HSA binding kit was used to further assess the affinity of HsTX1 [R14A] and Palm- and Stear-HsTX1 [R14A] to HSA. Myr-HsTX1 [R14A] was not evaluated in this experiment as we found the LC-MS/MS

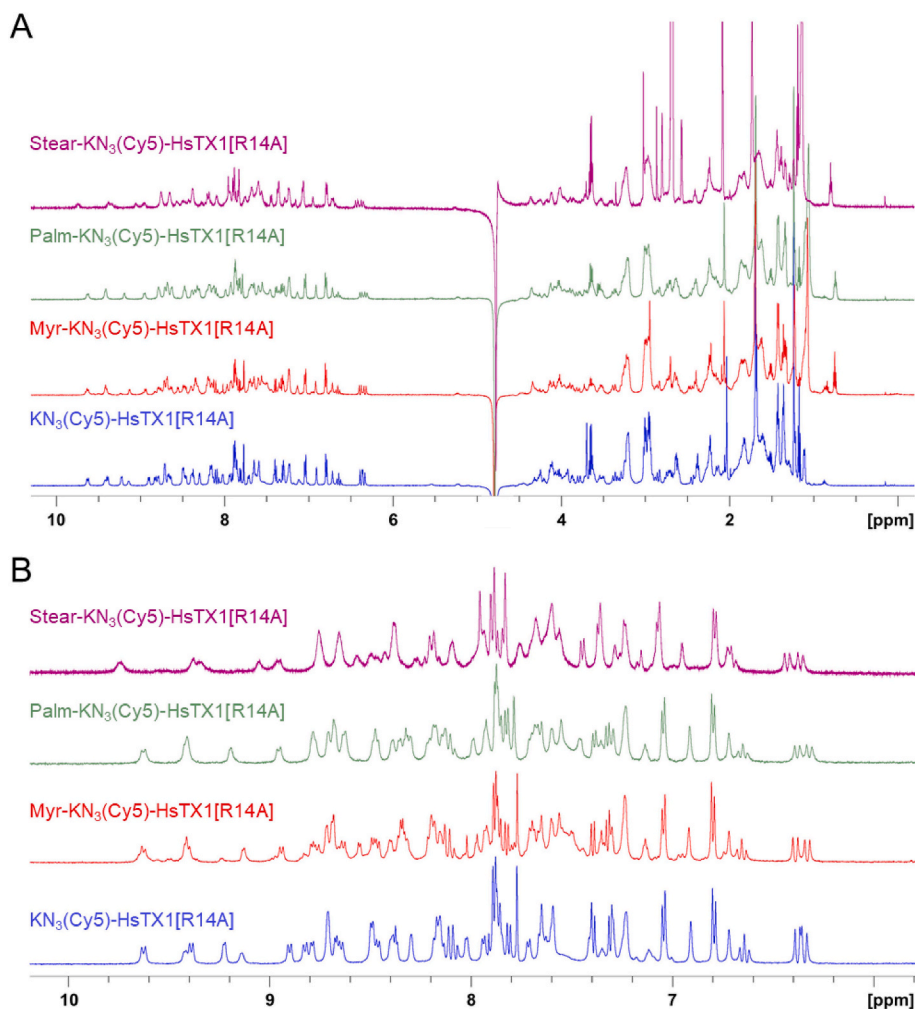


Fig. 4. (A) One-dimensional ^1H NMR spectra of $\text{KN}_3(\text{Cy}5)\text{-HsTX1[R14A]}$ (blue), $\text{Myr-KN}_3(\text{Cy}5)\text{-HsTX1[R14A]}$ (red) and $\text{Palm-KN}_3(\text{Cy}5)\text{-HsTX1[R14A]}$ (green) in 95% $\text{H}_2\text{O}/5\% \text{ } ^2\text{H}_2\text{O}$ ($\text{KN}_3\text{-HsTX1[R14A]}$, pH 4.8; $\text{Myr-KN}_3(\text{Cy}5)\text{-HsTX1[R14A]}$, pH 4.2; $\text{Palm-KN}_3(\text{Cy}5)\text{-HsTX1[R14A]}$, pH 4.6) and $\text{Stear-KN}_3(\text{Cy}5)\text{-HsTX1[R14A]}$ (purple) in 30% $\text{DMSO-}^2\text{H}_6/65\% \text{H}_2\text{O}/5\% \text{ } ^2\text{H}_2\text{O}$, (pH 4.6). All spectra were acquired at 600 MHz and 298 K. (B) Expanded amide-aromatic region of spectra in (A).

assay too difficult to develop. Consistent with the SPR results, HsTX1[R14A] showed negligible binding to HSA, with its bound fraction close to zero even at saturating concentrations of HSA (Fig. S19). Both Palm-HsTX1[R14A] and Stear-HsTX1[R14A] showed strong HSA binding, with percentages bound being 98.6% and 99.3%, respectively (Table 1).

3.5. Lipidation prolongs the plasma exposure and extends the elimination half-life of HsTX1[R14A]

The PK profiles of HsTX1[R14A] , Palm-HsTX1[R14A] and Stear-HsTX1[R14A] following SC injection are shown in Fig. 8. The PK parameters calculated using non-compartmental analysis for the three peptides are summarized in Table 2. Following SC injection, HsTX1[R14A] was rapidly absorbed, with a peak plasma concentration achieved within 30 min; the plasma concentration declined rapidly over 4 h and remained at a similar low concentration from 4–10 h. The mean maximum plasma concentration (C_{max}) of HsTX1[R14A] in plasma after SC injection was 415 nM. The absorption of Palm-HsTX1[R14A] and Stear-HsTX1[R14A] was delayed compared to HsTX1[R14A] . The C_{max} of the lipidated analogs were statistically different and around 3-fold higher than HsTX1[R14A] . The plasma exposure ($\text{AUC}_{0-\text{inf}}$) was increased more than 9-fold when HsTX1[R14A] was conjugated to palmitic and stearic acid. The two lipidated analogs exhibited a significant decrease in clearance and volume of distribution compared to HsTX1[R14A] , indicating that lipidation protected the peptide from

rapid elimination and reduced its ability to distribute out of the blood.

3.6. In vivo biodistribution of Cy5-labelled HsTX1[R14A] analogs

The biodistribution to each organ or tissue is expressed as a % of the total fluorescence in all the organs and tissues collected (%FI). This analysis approach reflects the proportion of the total fluorescence signal in each organ, providing an overall distribution of the compound within the same animal. Fluorescence intensity normalized to tissue weights, which reflects the drug concentration in different tissues rather than total organ distribution, is provided in Figs. S20–S22.

Fig. 9 summarizes the biodistribution pattern of $\text{KN}_3(\text{Cy}5)\text{-HsTX1[R14A]}$, $\text{Palm-KN}_3(\text{Cy}5)\text{-HsTX1[R14A]}$ and $\text{Stear-KN}_3(\text{Cy}5)\text{-HsTX1[R14A]}$ into different tissues and organs at the 4 h and 12 h time points. At the 4 h time point, the top three organs to which $\text{KN}_3(\text{Cy}5)\text{-HsTX1[R14A]}$ distributed were the kidney, skin and liver, receiving 26%, 20% and 17% of the total fluorescence, respectively. Surprisingly, at 12 h, the skin and the adipose tissue had over 40% of the total fluorescence of $\text{KN}_3(\text{Cy}5)\text{-HsTX1[R14A]}$ distributed. The kidneys, skin and liver receive 26%, 20% and 17% of the total fluorescence of the Cy5-labelled native peptide, respectively.

The two lipidated analogs showed highly similar biodistribution patterns to each other at both 4 h and 12 h post-dosing. For lipidated analogs, the GI tract had almost 50% of the total fluorescence at both timepoints. At 4 h, the small intestine had around one-third of the total

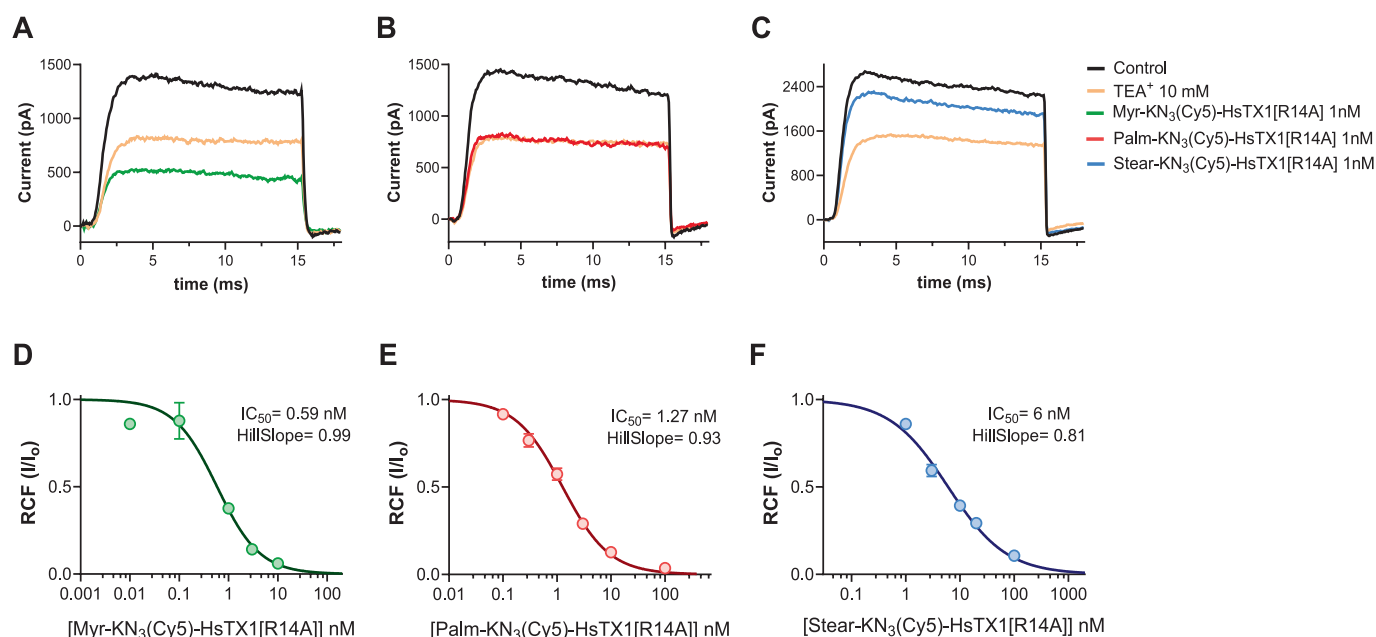


Fig. 5. (A) Electrophysiology assays. (A–C) Whole-cell K_v1.3 currents were recorded in an activated human T lymphocyte by applying 15-ms depolarization pulses to +50 mV from a holding potential of –120 mV every 15 s. Representative current traces show the K⁺ current in control solution (black), at steady-state block in the presence of 10 mM TEA⁺; tetraethylammonium (orange) as a perfusion control and at equilibrium block in the presence of 1 nM of (A) Myr-KN₃(Cy5)-HsTX1[R14A] (green) or (B) Palm-KN₃(Cy5)-HsTX1[R14A] (red) or (C) Stear-KN₃(Cy5)-HsTX1[R14A] (blue). (D–F) Concentration-dependence of Kv1.3 current block by (D) Myr-KN₃(Cy5)-HsTX1[R14A] (E) Palm-KN₃(Cy5)-HsTX1[R14A] or (F) Stear-KN₃(Cy5)-HsTX1[R14A]. The mean values of remaining current fractions (RCF = I/I₀, where I₀ and I are the peak currents in the absence and presence of the indicated concentration of the peptides, respectively) were plotted as a function of peptide concentration. The data points were fitted to the Hill equation (see Materials and Methods) with parameters IC₅₀ and H indicated in the panels. Error bars indicate SEM, n = 3–6.

fluorescence for both peptides. The overall %FI in the GI tract was increased at 12 h compared to 4 h for both lipidated analogs. This change was characterized by a decrease in %FI in the small intestine and a corresponding increase in the large intestine. The %FI in the skin, muscle and adipose tissue from the non-dosing side, as well as spleen, heart, brain and bladder, remained low for all three peptides across both timepoints.

Fig. 10 shows the %FI found in the skin, muscle and adipose tissue on the dosing side (DS) and non-dosing side (NDS) after injection of the peptides. Palm-KN₃(Cy5)-HsTX1[R14A] had significantly increased accumulation in the muscle compared to KN₃(Cy5)-HsTX1[R14A] at 4 h post-dosing. Surprisingly, KN₃(Cy5)-HsTX1[R14A] had significantly higher distribution to the adipose tissue around the injection site compared to the lipidated analogs at 12 h post-dosing (Fig. 10). Stear-KN₃(Cy5)-HsTX1[R14A] also had higher accumulation in the skin and adipose tissues of the non-dosing side compared to KN₃(Cy5)-HsTX1[R14A], and higher accumulation at the skin of the non-dosing side when compared to Palm-KN₃(Cy5)-HsTX1[R14A] (Fig. 10), which could be due to higher affinity for these tissues. When comparing fluorescence in the skin and adipose tissues (Fig. 11), all three peptides were found to have significantly higher % fluorescence accumulation at the dosing side than the non-dosing side at both 4 h and 12 h time points. When comparing muscles from the dosing and non-dosing sides, KN₃(Cy5)-HsTX1[R14A] and Palm-KN₃(Cy5)-HsTX1[R14A] had higher accumulation on the dosing side at 4 h, whereas Stear-KN₃(Cy5)-HsTX1[R14A] had higher %FI on the dosing side compared to the non-dosing side up to 12 h post-dosing.

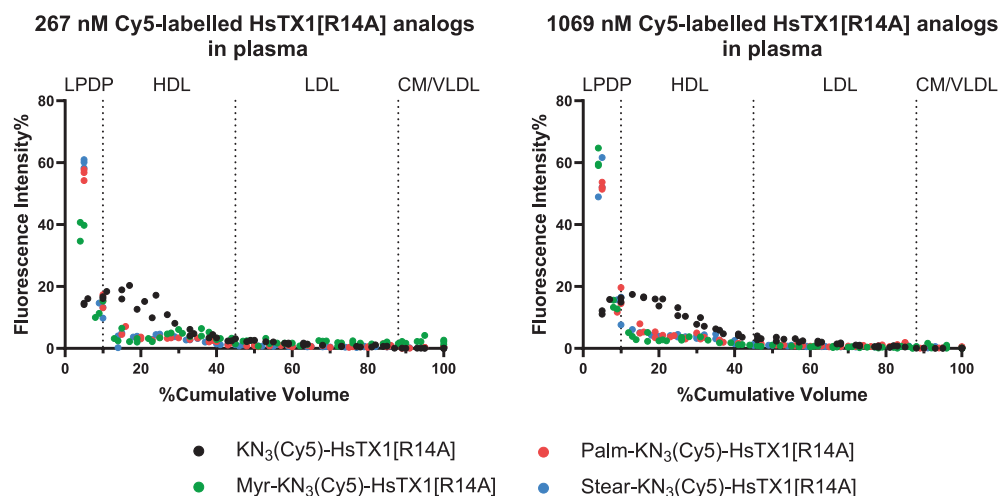
Fig. 12 shows the % of the total fluorescence found in kidneys, spleen, liver, lungs, heart, brain, pancreas, stomach, small intestine, large intestine and bladder after SC injection of the peptides. KN₃(Cy5)-HsTX1[R14A] had significantly increased % total fluorescence in the kidneys compared to Palm-KN₃(Cy5)-HsTX1[R14A] and Stear-KN₃(Cy5)-HsTX1[R14A] at both 4 h and 12 h post-dosing (Fig. 12), which is consistent with the rapid elimination and shorter elimination

t_{1/2} observed in the PK profile for this peptide. The two lipidated analogs showed higher accumulation in the liver (15% total FI) than in the kidneys (<5% total FI). However, the difference in hepatic accumulation across different peptides was not statistically significant. There was also no significant difference in spleen, lungs, heart, brain, pancreas and bladder distribution when comparing KN₃(Cy5)-HsTX1[R14A] and the two lipidated analogs. Palm-KN₃(Cy5)-HsTX1[R14A] and Stear-KN₃(Cy5)-HsTX1[R14A] had significantly higher % fluorescence in the stomach and the small intestine compared to KN₃(Cy5)-HsTX1[R14A] at 4 h post-dosing (Fig. 12). Stear-KN₃(Cy5)-HsTX1[R14A] also had higher % fluorescence in the stomach at 12 h post-dosing when compared to KN₃(Cy5)-HsTX1[R14A]. Both lipidated analogs also showed significantly higher accumulation in the large intestine compared to the parent peptide at 12 h post-dosing (Fig. 12).

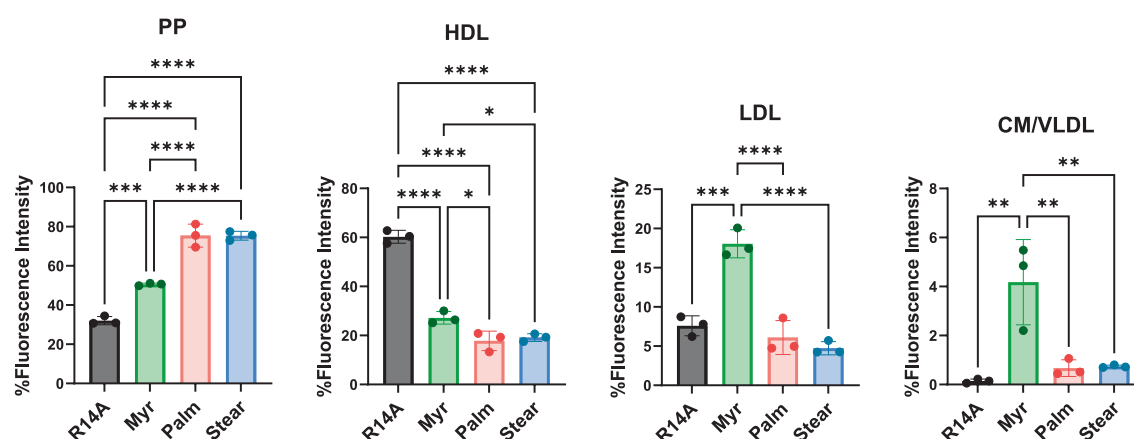
3.7. Lymph node accumulation of Cy5-labelled HsTX1[R14A] analogs

The biodistribution patterns of the KN₃(Cy5)-HsTX1[R14A], Palm-KN₃(Cy5)-HsTX1[R14A] and Stear-KN₃(Cy5)-HsTX1[R14A] into different draining lymph nodes from the dosing side and non-dosing side at the 4 h and 12 h time points are shown in Fig. 13. Both inguinal and axillary lymph nodes showed a higher concentration on the dosing side than on the non-dosing side, whereas no significance was found in brachial and iliac lymph nodes, suggesting that inguinal and axillary are the primary lymph nodes draining most of the fluid from the injection site. Increased accumulation in the lymph nodes on the dosing side compared to the non-dosing side was observed not only for Palm-KN₃(Cy5)-HsTX1[R14A] and Stear-KN₃(Cy5)-HsTX1[R14A], but also for the control peptide Cy5-HsTX1[R14A]. Comparison of fluorescence intensities between lymph nodes on the dosing and non-dosing sides showed significantly higher signals in the inguinal and axillary lymph nodes, which are the primary and secondary draining nodes related to the injection site. For inguinal lymph nodes, the dosing side lymph node had significantly higher fluorescence signal compared to the non-dosing

A.



B. 267 nM



C. 1069 nM

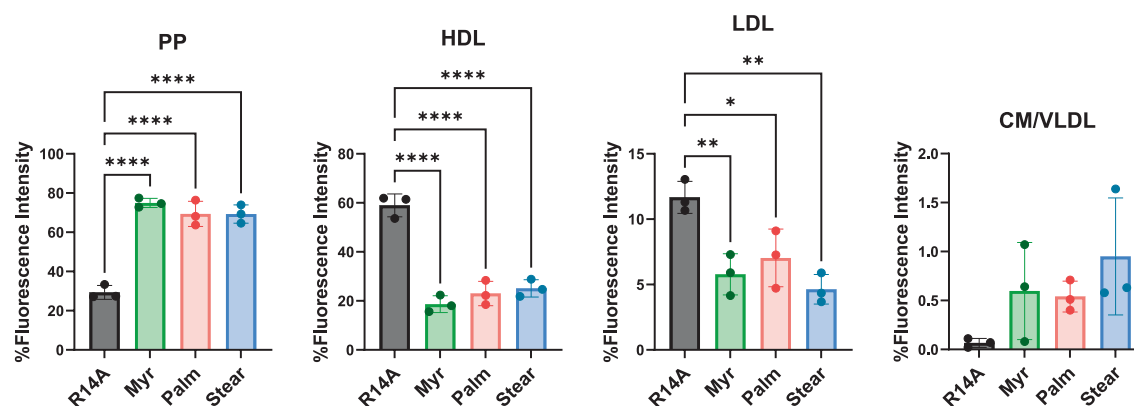


Fig. 6. Association of KN₃(Cy5)-HsTX1[R14A] and its lipidated analogs Myr-KN₃(Cy5)-HsTX1[R14A], Palm-KN₃(Cy5)-HsTX1[R14A] and Stear-KN₃(Cy5)-HsTX1[R14A] with plasma protein (PP) and lipoprotein fractions in mouse plasma. (A) Distribution of 267 nM and 1069 nM KN₃(Cy5)-HsTX1[R14A], Myr-KN₃(Cy5)-HsTX1[R14A], Palm-KN₃(Cy5)-HsTX1[R14A] and Stear-KN₃(Cy5)-HsTX1[R14A] in different fractions of mouse plasma after ultracentrifugation. (B) % of total fluorescence intensity of 276 nM Cy5-labelled HsTX1[R14A] analogs distributed in different plasma protein and lipoprotein fractions. (C) % of total fluorescence intensity of 1069 nM Cy5-labelled HsTX1[R14A] analogs distributed in different plasma protein and lipoprotein fractions. Data presented as mean $\pm$ SD, n = 3. Data analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. *: P < 0.05, **: P $\leq$ 0.01, ***: P $\leq$ 0.001, ****: P $\leq$ 0.0001. PP: plasma protein, HDL: high-density lipoprotein, LDL: low-density lipoprotein, CM: chylomicron, VLDL: very-low-density lipoprotein.

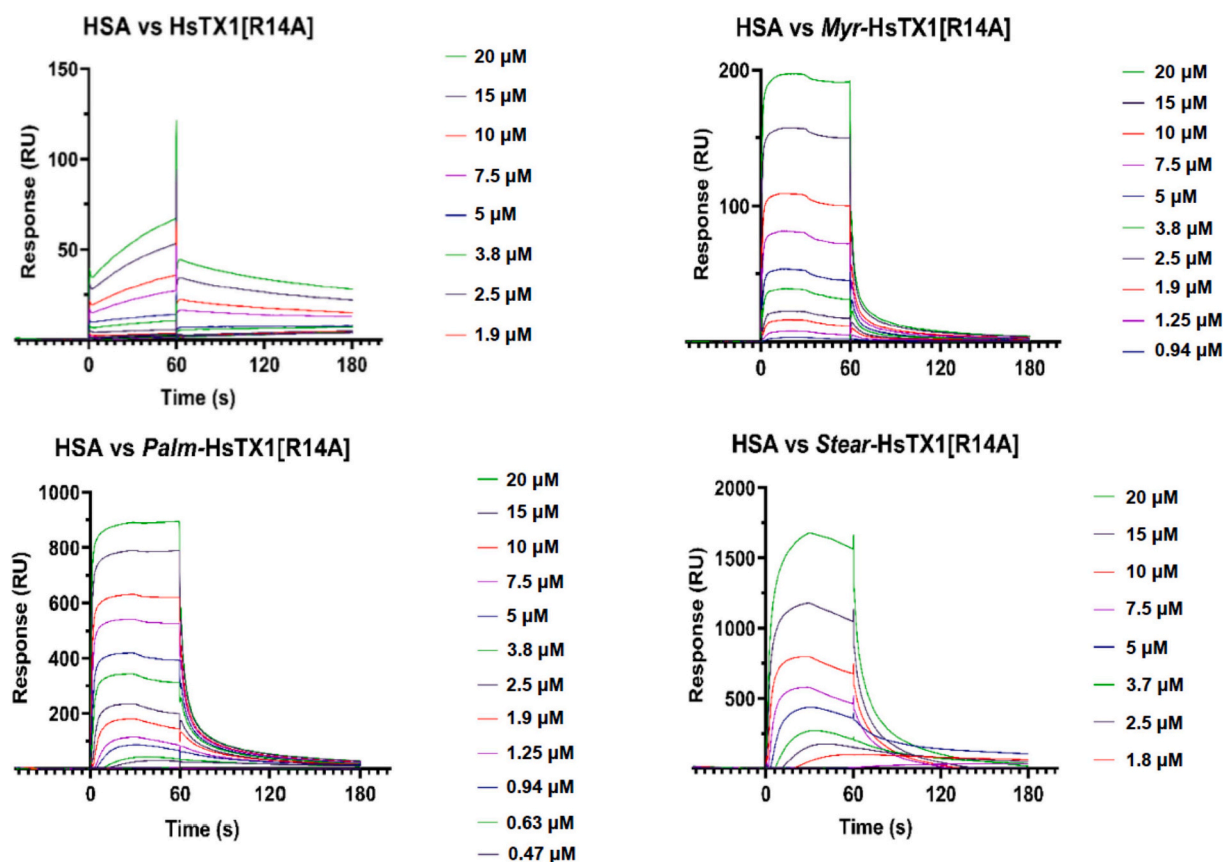


Fig. 7. Reference-subtracted surface plasmon resonance sensorgrams of HsTX1[R14A], Myr-HsTX1[R14A], Palm-HsTX1[R14A] or Stear-HsTX1[R14A] binding to HSA immobilized on a CM5 sensor chip.

Table 1

Binding of HsTX1[R14A], Palm-HsTX1[R14A] and Stear-HsTX1[R14A] to HSA. ND: Not detectable.

Peptide	K_d HSA	% Bound
HsTX1[R14A]	ND	ND
Palm-HsTX1[R14A]	8.2 μ M	98.6% $\pm$ 0.01
Stear-HsTX1[R14A]	4.4 μ M	99.3% $\pm$ 0.01

side at 12 h post-dosing for all three peptides, suggesting that the peptides directly traffic into the lymphatics from the injection site. There

was no significant difference when comparing brachial and iliac lymph nodes across different groups. However, the fluorescence detected in these nodes may reflect redistribution of the peptide from the systemic circulation rather than direct drainage from the injection site.

We also evaluated the ratio of fluorescence intensity of the dosing-side lymph node over the non-dosing-side lymph node as an indication of direct lymphatic uptake from the injection side. The ratio was greater than one for Palm-KN₃(Cy5)-HsTX1[R14A] and Stear-KN₃(Cy5)-HsTX1[R14A] in the inguinal and axillary lymph node, suggesting that they may directly enter these lymph nodes from the injection site. The lymph node ratios were not, however, significantly different between the

SC PK Profiles

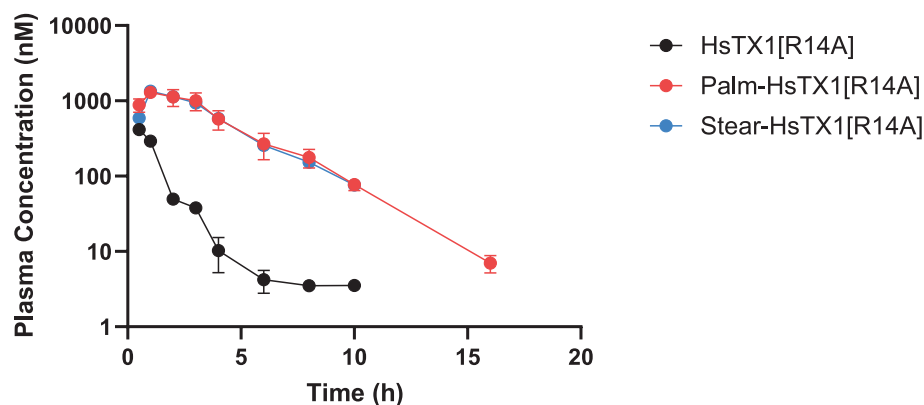


Fig. 8. PK profiles of HsTX1[R14A], Palm-HsTX1[R14A] and Stear-HsTX1[R14A] in mice after SC injection of a 2 mg/kg dose. Data are presented as mean $\pm$ SD, n = 3–4 animals.

Table 2

The SC PK parameters of HsTX1[R14A], Palm-HsTX1[R14A] and Stear-HsTX1[R14A] calculated using non-compartmental analysis. N = 3–4, data expressed as mean $\pm$ SD. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test. AUC_{0-inf}: total area under the plasma concentration–time curve from time zero to infinity.

PK parameter	HsTX1 [R14A]	Palm-HsTX1 [R14A]	Stear-HsTX1 [R14A]
C _{max} (nM)	415 $\pm$ 37	1315 $\pm$ 32 ^a	1544 $\pm$ 240 ^a
T _{max} (h)	0.5	1.33 $\pm$ 0.58	1.33 $\pm$ 0.58
Half-life (h)	0.90 $\pm$ 0.24	1.75 $\pm$ 0.26 ^a	2.25 $\pm$ 0.47 ^a
AUC _{0-inf} (nmol $\cdot$ h/L)	548 $\pm$ 3	4416 $\pm$ 11 ^a	5126 $\pm$ 12 ^a
Clearance/F (L/kg/h)	0.99 $\pm$ 0.05	0.09 $\pm$ 0.02 ^a	0.09 $\pm$ 0.01 ^a
Volume of Distribution/F (L/kg)	1.28 $\pm$ 0.26	0.23 $\pm$ 0.07 ^a	0.30 $\pm$ 0.07 ^a

^aSignificantly different parameter compared with HsTX1[R14A] ($p < 0.05$). F is fraction bioavailable, which is unknown as no IV studies were performed.

lipidated and parental analogs, perhaps due to the high variability in the ratios for the lipidated peptides (Fig. 14).

4. Discussion

Approaches to extend the lifetime of peptides in physiological

settings are important in enhancing their therapeutic applications, and lipidation is one of the most commonly used methods to achieve this (Zhang and Bulaj, 2012; Trevaskis et al., 2015; Bech et al., 2018; Shrivastava et al., 2020). In this study, we demonstrate that conjugation of HsTX1[R14A] with lipid moieties of different chain lengths leads to increased association with albumin but not lipoproteins in plasma, extends the plasma PK profile and substantially changes the bio-distribution profile, including reduced distribution to the kidney and increased distribution to the GI tract and potentially lymph nodes. The changes in biodistribution are likely linked to the altered plasma PK profile and could be useful in the treatment of inflammatory diseases of the GI tract.

Developing sensitive and robust methods for peptide quantification in biological matrices is a crucial step in assessing their PK profile. Whilst LC-MS/MS methods for HsTX1[R14A] have been reported previously (Jin et al., 2016; Reddiar et al., 2021), we further optimized the sensitivity of the method by 2.5-fold by using low-binding Eppendorf tubes and tips, as well as changing the dilution method to reduce non-specific peptide adsorption. HsTX1[R14A] analogs displayed varying extents of non-specific binding to plasticware, which introduced variability to the measured peptide concentration over time (Wai et al., 2022; Reddiar et al., 2021; Dong et al., 2018; Tsumagari et al., 2024; Ziebarth et al., 2024; Verbeke et al., 2020). We observed inconsistent

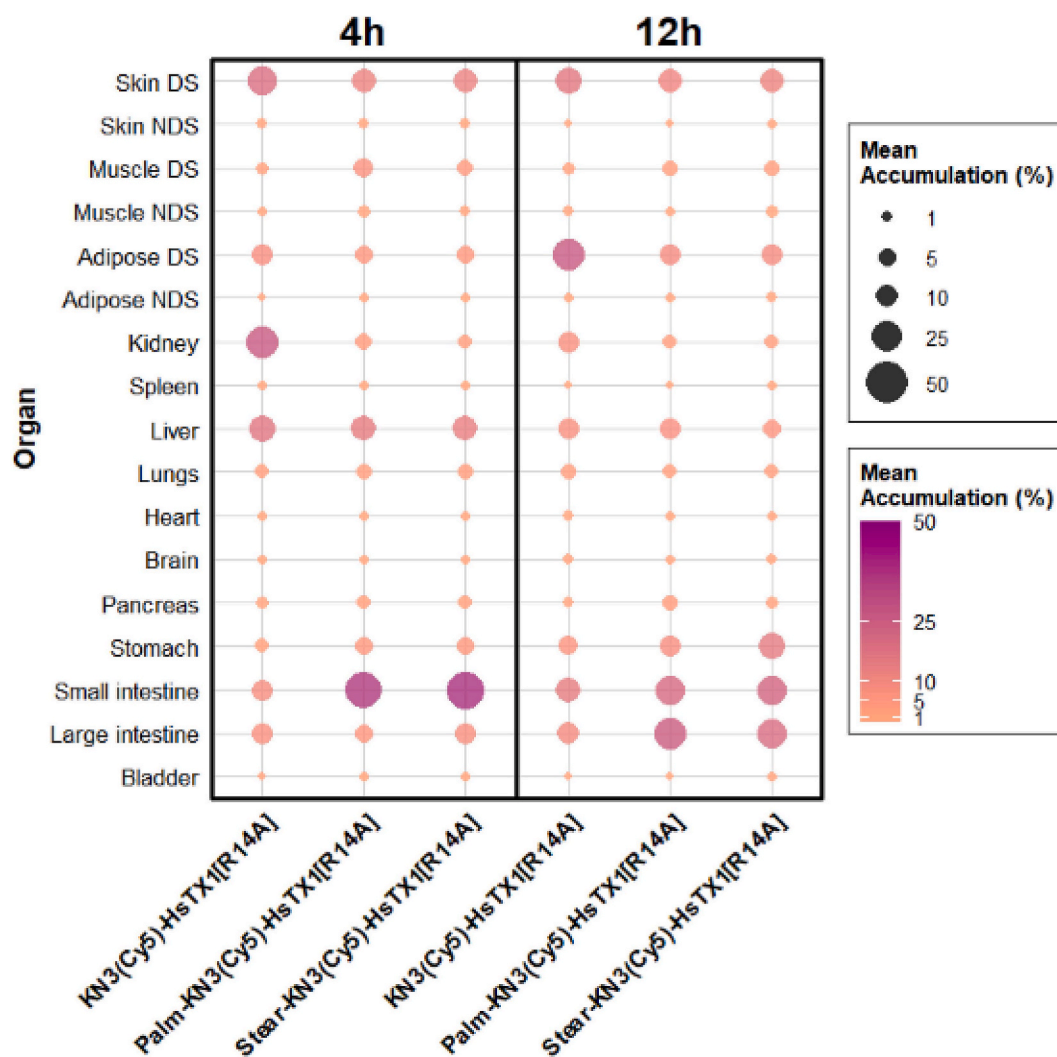


Fig. 9. Bubble plot showing the % of the total fluorescence intensity found in each of the harvested tissues and organs from mice at 4 and 12 h after SC dosing of KN₃(Cy5)-HsTX1[R14A], Palm-KN₃(Cy5)-HsTX1[R14A] and Stear-KN₃(Cy5)-HsTX1[R14A]. Data presented as mean percentage, n = 5–8 animals. DS: dosing site; NDS: non-dosing site.

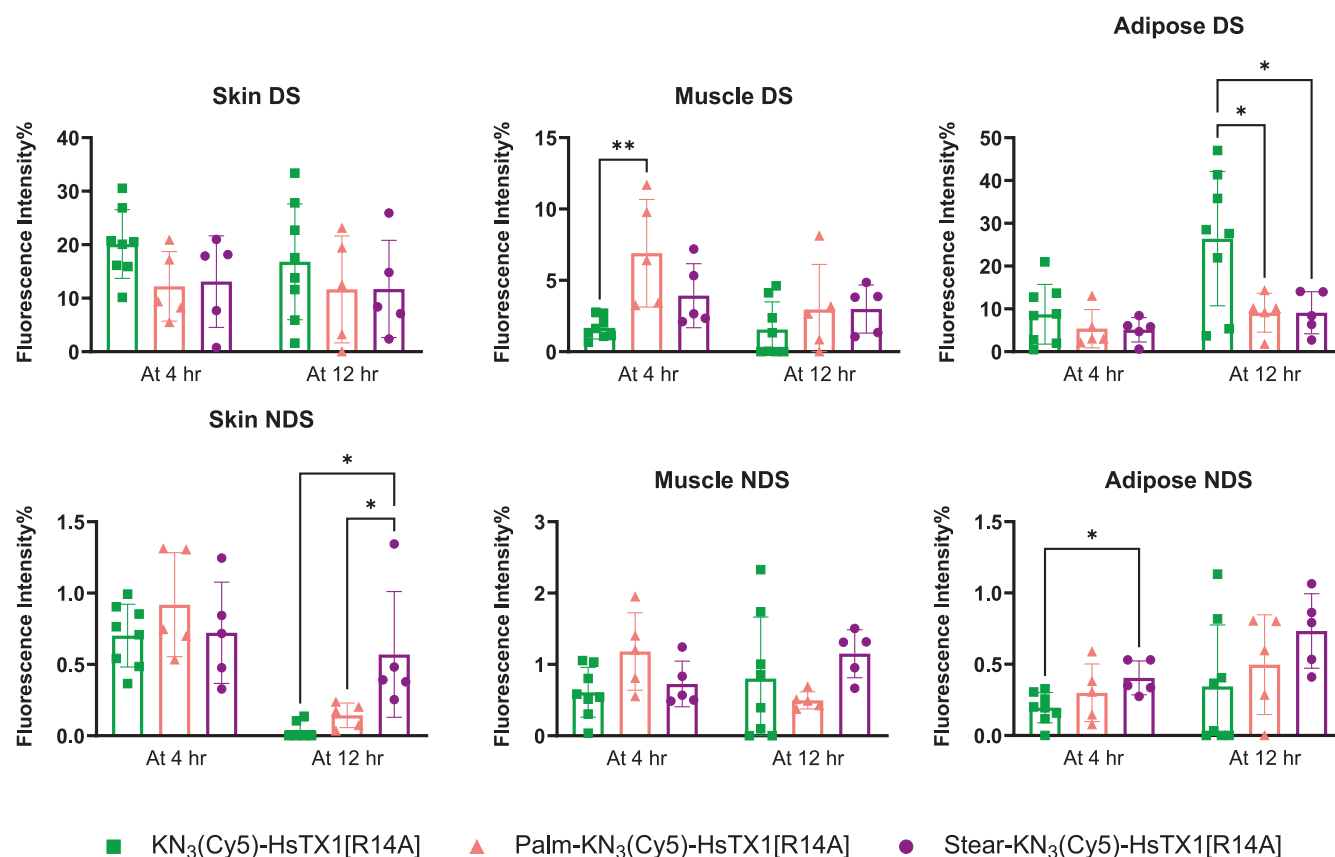


Fig. 10. The % of the total fluorescence intensity found in each of the harvested tissues from mice on the dosing side (DS) and non-dosing side (NDS) at 4 and 12 h after SC dosing of $\text{KN}_3(\text{Cy5})\text{-HsTX1[R14A]}$, $\text{Palm-KN}_3(\text{Cy5})\text{-HsTX1[R14A]}$ and $\text{Stear-KN}_3(\text{Cy5})\text{-HsTX1[R14A]}$. Data presented as mean $\pm$ SD, $n = 5\text{--}8$ animals. Data analysis was performed by One-way analysis of variance (ANOVA), followed by Tukey's multiple comparison analysis. *: $P < 0.05$, **: $P \leq 0.01$.

and unreliable results when the peptides were stored in polypropylene LC-MS vials for intra-day measurements of the same sample. Following optimization of the LC-MS/MS method for HsTX1[R14A], we developed and validated LC-MS/MS assays for the two lipidated peptides by tailoring solid phase extraction (SPE) methods to the hydrophobicity of the peptides. We also used the peptide elution profiles in the LC-MS/MS system to determine the optimal organic solvent matrix for efficient plasma extraction. Thus, the developed method is robust and reliable for the quantification of lipidated peptides in plasma.

Using the validated LC-MS/MS methods, we obtained the SC PK profile for HsTX1[R14A] and the two lipidated analogs. The plasma concentration for HsTX1[R14A] was consistent with previous PK studies using healthy mice at the same dose (Reddiar et al., 2021). Interestingly, we found that HsTX1[R14A] remains at a low concentration in plasma for up to 10 h following SC dosing, whereas prior studies described the complete disappearance of the peptide from plasma from 6 h post-dosing (Jin et al., 2016). Indeed, HsTX1[R14A] was still detectable via LC-MS/MS in plasma samples up to 24 h post-dosing, although it fell below the quantification limit after 10 h. Given that HsTX1[R14A] has an IC_{50} of ~ 45 pM for $\text{Kv}1.3$, which is much lower than the detection limit of the LC-MS/MS method, the peptide could potentially remain effective in blocking $\text{Kv}1.3$ even after 24 h. This could explain why HsTX1[R14A] remained effective in a rat PIA model at a weekly dose of 0.1 mg/kg, despite its short PK half-life following either IV or SC administration (Tanner et al., 2017; Bergmann et al., 2017). It is important to note that the PK of HsTX1[R14A] have not yet been evaluated in animal models of disease for a time frame greater than 2 h post-dosing. It would be valuable to compare the PK profiles of the peptide from healthy and diseased animals (e.g. Crohn's disease) to further evaluate the factors that may impact absorption, distribution and elimination of the peptide.

The PK study of Palm-HsTX1[R14A] and Stear-HsTX1[R14A] showed that fatty acid conjugation significantly extended the *in vivo* plasma exposure and elimination half-life of the peptide, although there was no significant difference between the two lipidated peptides. The observed alteration of PK parameters aligns with other palmitoylated and stearoylated peptides (Bellmann-Sickert et al., 2011; Blanchfield et al., 2007; Wang et al., 2016; Chaudhuri et al., 2023). For example, liraglutide, a palmitoylated GLP-1 agonist peptide currently on the market as a treatment for type 2 diabetes, has an extended circulation time of hours compared to minutes for natural GLP-1 (Wang et al., 2016). Previous studies have shown that conjugating peptides or polymers with diacyl lipids or cholesterol can extend the plasma exposure even further than single-chain fatty acids (Abdallah et al., 2024). Future studies could explore conjugation of HsTX1[R14A] to other lipid types to modulate the PK profile.

While lipid conjugation to HsTX1[R14A] reduced its potency against $\text{Kv}1.3$ by approximately 100-fold compared to the parent compound, this modification substantially improved its PK profile, especially the systemic exposure. Notably, the plasma concentration of the lipidated analogs remained more than 20-fold higher than HsTX1[R14A] at 10 h post-dosing, while the plasma concentration of HsTX1[R14A] fell below the limit of quantification beyond this time point. Despite the reduction in the *in vitro* potency, the lipidated analogs maintained systemic exposure well beyond that achievable by HsTX1[R14A], which is particularly relevant for chronic autoimmune conditions requiring sustained management. Moreover, lipidated peptides, such as human pancreatic polypeptides and long-acting GLP-1 agonists demonstrate that the benefits of lipidation can compensate for potency loss in a therapeutic context (Mu et al., 2026). Lipidation of human pancreatic polypeptide (hPP) was reported to lead to compromised binding affinity,

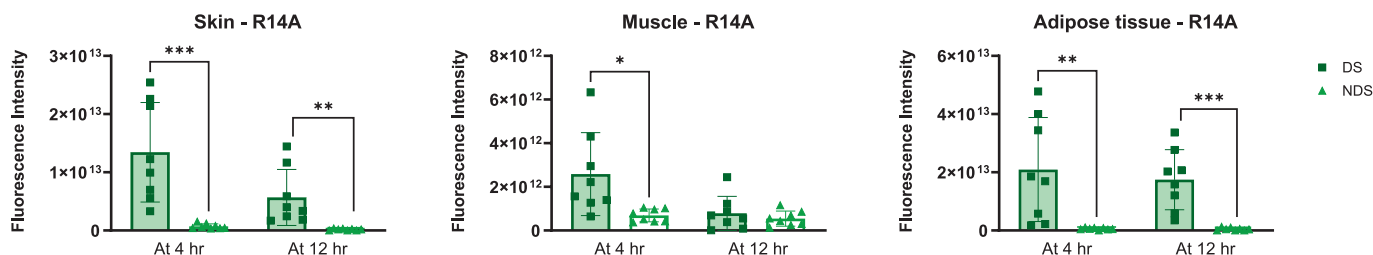
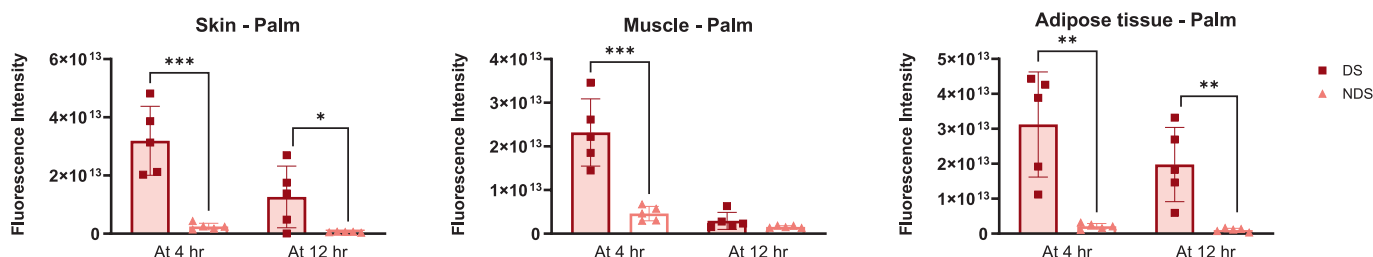
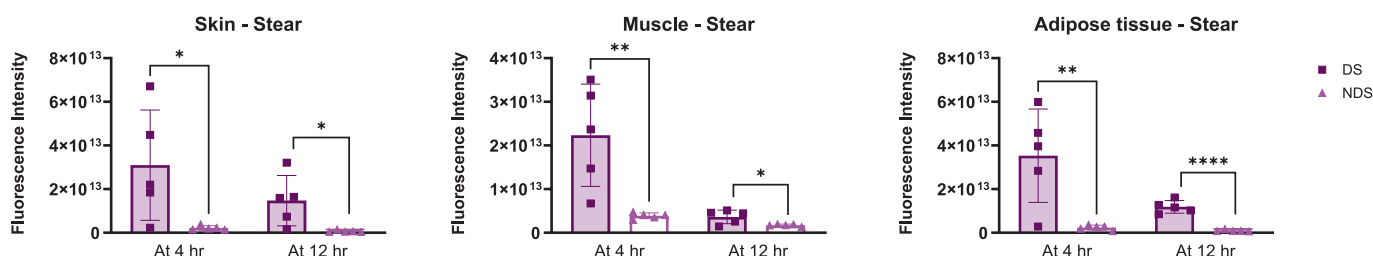
A. KN₃(Cy5)-HsTX1[R14A]**B. Palm-KN₃(Cy5)-HsTX1[R14A]****C. Stear-KN₃(Cy5)-HsTX1[R14A]**

Fig. 11. Comparison of the % of the total fluorescence intensity found in each of the harvested tissues from the dosing side (DS) versus non-dosing side (NDS) at 4 and 12 h after SC dosing of KN₃(Cy5)-HsTX1[R14A] (A), Palm-KN₃(Cy5)-HsTX1[R14A] (B) and Stear-KN₃(Cy5)-HsTX1[R14A] (C). Data presented as mean $\pm$ SD, $n = 5-8$ animals. Data analysis was performed by Student's T test. *: $P < 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****: $P \leq 0.0001$.

while improving PK properties and resulting in prolonged suppression of food intake in efficacy studies (Bellmann-Sickert et al., 2011). Similarly, the GLP-1 analog semaglutide had diminished potency at the GLP-1 receptor, but its prolonged plasma exposure translated into a sustained duration of action, contributing to its market success (Knudsen and Lau, 2019; Lau et al., 2015). Therefore, the prolonged plasma exposure and half-life of lipidated analogs of HsTX1[R14A] are expected to translate into a more sustained pharmacodynamic effect, including prolonged K_v1.3 inhibition and suppression of T_{EM} cell activity, thereby extending the period of autoimmune disease management.

In the biodistribution study, the two lipidated peptides exhibited higher lymph node accumulation on the dosing than the non-dosing side following SC administration. This is consistent with their observed albumin-binding profiles, as prior studies have found that vaccine antigens or adjuvants conjugated to lipids can 'hitchhike' on albumin trafficking pathways into the lymphatics following SC injection at the tail base (Liu et al., 2014). The same study found that vaccine adjuvants conjugated with diacyl lipids displayed significantly increased retention in draining lymph nodes after SC delivery compared with those conjugated to monoacyl lipids or cholesterol, consistent with their higher albumin binding affinity (Liu et al., 2014). Similarly, our group demonstrated that diacyl lipid conjugated polymers have increased lymphatic uptake compared to monoacyl lipid conjugated polymers (Abdallah et al., 2024). The PK and distribution pattern of peptides like HsTX1[R14A] to lymph nodes and other tissues can thus be controlled

through careful choice of the conjugated lipid type. Conjugation of HsTX1[R14A] with different lipid moieties, such as cholesterol and diacylglycerol, could be valuable to further extend the plasma exposure and enhance lymph node delivery.

When comparing the current mouse PK profiles for HsTX1[R14A] to previous rat IV and SC PK profiles of HsTX1[R14A] determined using [⁶⁴Cu]Cu-NOTA-HsTX1[R14A] in positron emission tomography (PET) studies, the elimination pattern of HsTX1[R14A] was similar, demonstrating rapid clearance of the peptide over the first 6 h (Bergmann et al., 2017). The same PET study also found high distribution of HsTX1[R14A] in the kidney, consistent with its rapid clearance via the renal pathway (Tanner et al., 2017; Bergmann et al., 2017). Additionally, other K_v1.3 blocking peptides, EgK5 and ShK-211, also accumulated at high levels in the kidney and bladder in rodent biodistribution studies using radiolabeled analogs, suggesting urine is a major route of excretion (Ong et al., 2020; Tarcha et al., 2012). This was consistent with our finding that HsTX1[R14A] exhibited much higher accumulation in the kidney when compared to Palm-HsTX1[R14A] and Stear-HsTX1[R14A]. This suggests that the kidney may be the major clearance pathway for HsTX1[R14A] and that the lipidated analogs may extend the elimination half-life through avoidance of kidney clearance. We hypothesized initially that Palm-HsTX1[R14A] and Stear-HsTX1[R14A] may be cleared predominantly via the liver, as lipidated conotoxin (MII conjugated with C10) was previously shown to exhibit high distribution to the liver (Blanchfield et al., 2007). However, our data demonstrated a

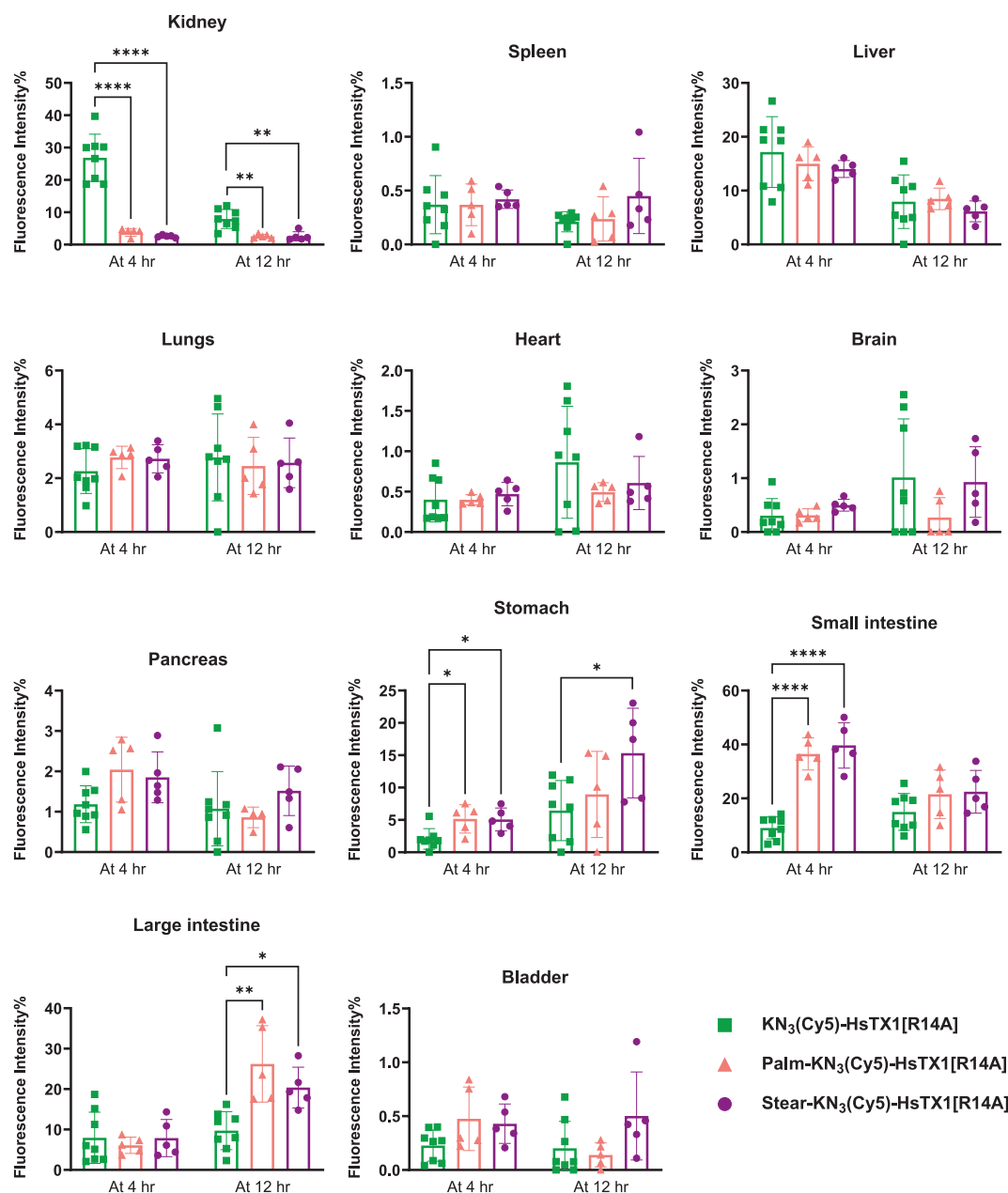


Fig. 12. Comparison of % of the total fluorescence intensity found in the kidneys, spleen, liver, lungs, heart, brain, pancreas, stomach, small intestine, large intestine and bladder at 4 and 12 h after SC dosing of $\text{KN}_3(\text{Cy5})\text{-HsTX1[R14A]}$, $\text{Palm-KN}_3(\text{Cy5})\text{-HsTX1[R14A]}$ and $\text{Stear-KN}_3(\text{Cy5})\text{-HsTX1[R14A]}$. Data presented as mean $\pm$ SD, n = 5–8 animals. Data analysis was performed by one-way ANOVA followed by Tukey's post-hoc analysis. *: $P < 0.05$, **: $P \leq 0.01$, ****: $P \leq 0.0001$.

similar distribution of the lipidated peptides and HsTX1[R14A] in the liver.

The lipidated peptides displayed particularly high accumulation in the GI tract, suggesting that these peptides may have potential applications in the treatment of GI tract disorders such as inflammatory bowel diseases, where enhanced tissue exposure could support improved therapeutic efficacy (Unterwieser et al., 2021). The mechanism behind GI tract accumulation of the lipidated analogs is unclear at this stage. The peptides may enter the intestine from either the blood side or the mucosal side, although the former is more likely, given the subcutaneous route of administration. We recognise the value of understanding the distribution profile of HsTX1[R14A] analogs within the gastrointestinal tract, and we plan to use fluorescence microscopy in future studies to explore the mechanism by which the peptides access intestinal tissue. *In vitro* cell-based assays, such as Caco-2 permeability assays, can also be employed in future studies to evaluate the intestinal

absorption potential of lipidated HsTX1[R14A] analogs. Further investigation into metabolic pathways and the extent to which hepatobiliary excretion contributes to the clearance and GI tract accumulation of the lipidated peptides would be an important next step to provide more insights into the GI tract accumulation and clearance mechanism of the lipidated analogs. We note the limitations of employing Cy5-labelled HsTX1[R14A] analogs for biodistribution studies, which could include tissue-dependent quenching, signal attenuation, and the potential influence of Cy5 on the physicochemical properties of HsTX1[R14A]. In principle, the effect of Cy5 conjugation is expected to be minimal, as the conjugation site was selected to avoid interference with binding activity, and the molecular weight of Cy5 represents only a small fraction of the overall conjugate. Nevertheless, to determine whether Cy5 labelling alters tissue distribution, additional studies using non-fluorescence-based analytical techniques like LC-MS/MS would be valuable, as this can quantify peptides in tissues directly and provide a more accurate

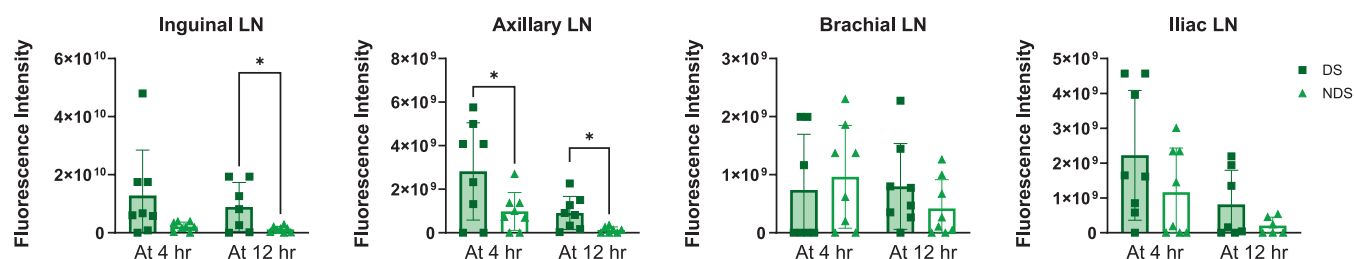
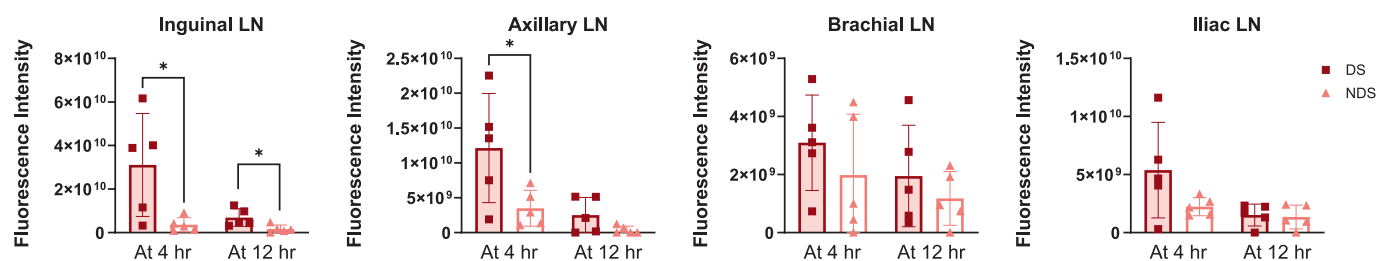
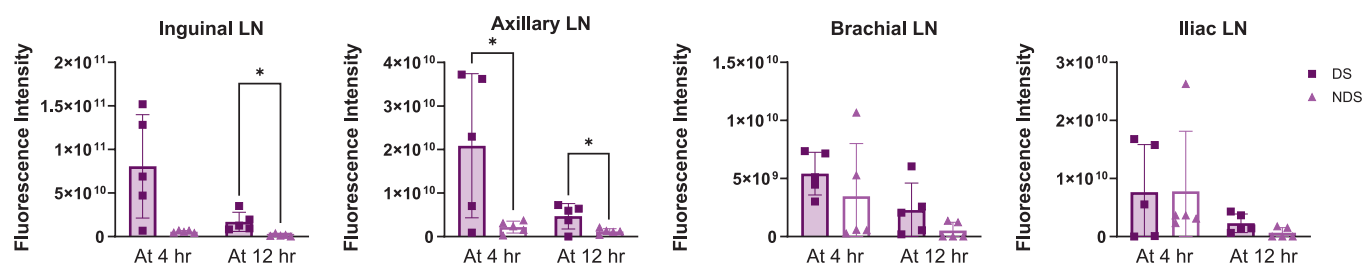
A. KN₃(Cy5)-HsTX1[R14A]**B. Palm-KN₃(Cy5)-HsTX1[R14A]****C. Stear-KN₃(Cy5)-HsTX1[R14A]**

Fig. 13. The fluorescence intensity of harvested lymph nodes from mice, including inguinal, axillary, brachial and iliac lymph nodes from the dosing side (DS) and non-dosing side (NDS) at 4 and 12 h after SC dosing of (A) KN₃(Cy5)-HsTX1[R14A], (B) Palm-KN₃(Cy5)-HsTX1[R14A] and (C) Stear-KN₃(Cy5)-HsTX1[R14A]. Data presented as mean $\pm$ SD, n = 5–8 animals. Data analysis was performed by Student's T-test. *: $P < 0.05$.

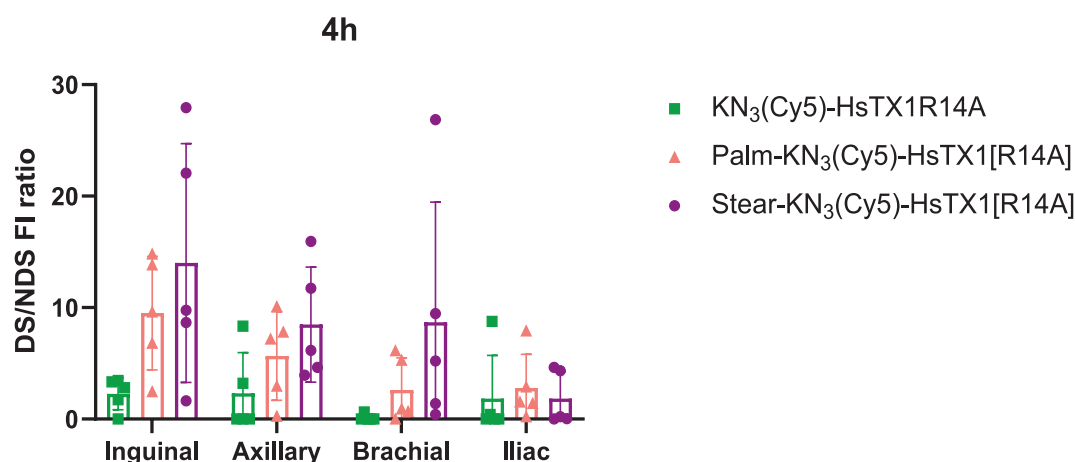


Fig. 14. Ratio of fluorescence intensity of harvested lymph nodes from dosing side over non-dosing side at 4 and 12 h after SC dosing of KN₃(Cy5)-HsTX1[R14A], Palm-KN₃(Cy5)-HsTX1[R14A], and Stear-KN₃(Cy5)-HsTX1[R14A]. Data presented as mean $\pm$ SD, n = 5–8 animals. Data analysis was performed by one-way ANOVA. No significant difference was found across the three peptides.

assessment of the biodistribution profile of HsTX1[R14A] and its analogs.

In this study, we observed prolonged plasma exposure, altered elimination pathways and tissue distribution of peptide HsTX1[R14A]

following lipid conjugation. To explore the mechanisms that contribute to the altered PK, we determined the association between the lipidated peptides and albumin and lipoproteins using semi-quantitative and quantitative methods. Both studies supported that conjugation of palmitic and stearic acid to HsTX1[R14A] promotes association with albumin, while having minimal impact on lipoprotein association. Albumin contributes ~60% of the total plasma protein pool and also has nine lipid-binding pockets, which likely results in its dominant role in binding to lipidated peptides in plasma and prolongation of the peptide circulation half-life by altering the biodistribution and reducing clearance (Chaudhury et al., 2003; Bhattacharya et al., 2000). Furthermore, macromolecules in the systemic circulation can extravasate to interstitial spaces and slowly return via lymphatic vessels following the unidirectional flow of lymph (Trevaskis et al., 2015; Oliver et al., 2020). Albumin-bound lipidated peptides may similarly circulate between the blood and lymphatics, contributing to the prolonged circulation time. The PK changes we observed on lipidation of HsTX1[R14A] were therefore highly likely to be driven by the association between the lipidated peptides and endogenous albumin (Abdallah et al., 2020; Abdallah et al., 2024).

We cannot, however, rule out that the lipidated peptides formed a self-assembled peptide oligomer depot at the injection site following administration, resulting in slow release and absorption into the systemic circulation (Jensen et al., 2011; Hutchinson et al., 2017; Khan et al., 2012; van Witteloostuijn et al., 2017). A previous study found that lipidated incretin peptides formed stable oligomers that had no association with serum albumin at mg/mL concentration, but dissociated into monomers and bound strongly with albumin in the ng/mL range (Wang et al., 2016). However, our ^1H NMR spectra showed no evidence of micelle formation for either Palm- or Stear-HsTX1[R14A] in water. Another possibility is that our lipidated analogs associate with tissues such as adipose tissue at the injection site, resulting in a delayed release into the systemic circulation compared to HsTX1[R14A]. This aligns with the increased accumulation of the lipidated analogs in the skin, skeletal muscle and adipose tissue at the dosing compared to the non-dosing side. Further studies incorporating a more comprehensive time-course evaluation of peptide concentration at the site of injection would be valuable to confirm the absorption kinetics of the lipidated analogs.

5. Conclusion

In summary, we demonstrated that lipidation of HsTX1[R14A] could be a useful strategy to alter the PK and biodistribution of the peptide, especially by prolonging the plasma exposure and reducing the rapid clearance of the peptide. Future studies will explore conjugation of HsTX1[R14A] to other lipid moieties that can offer longer circulation time and tissue-specific targeting. Lipidation of HsTX1[R14A] with palmitic and stearic acid resulted in high accumulation in the GI tract. Further studies will focus on characterizing distribution within specific regions of the GI tract to elucidate the basis for the high peptide accumulation observed in this tissue. Studies in autoimmune disease animal models, particularly inflammatory bowel disease models, will provide important insight into how pathological states impact the PK and biodistribution of lipidated HsTX1[R14A] analogs, as well as how lipidation impacts the efficacy of HsTX1[R14A] in autoimmune disease treatment. This will in turn inform the rational design of analogs with optimal dosing strategies for treating autoimmune and inflammatory conditions.

CRediT authorship contribution statement

Lihuan Lin: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Thomas R. Mitchell:** Writing – review & editing, Methodology, Investigation. **Karoline Sanches:** Writing – review & editing,

Methodology, Investigation. **Mohammad Abdallah:** Writing – review & editing, Methodology, Investigation. **Yuxiang Ren:** Writing – review & editing, Methodology, Investigation. **Muhammad Asim Farooq:** Writing – review & editing, Methodology, Investigation. **Hefeng Song:** Writing – review & editing, Methodology, Investigation. **Jinghan Qu:** Writing – review & editing, Methodology, Investigation. **Zengke Chen:** Writing – review & editing, Methodology, Investigation. **William De Nardo:** Writing – review & editing, Methodology, Investigation. **Roisin McCague:** Writing – review & editing, Methodology, Investigation. **Muhammad Umair Naseem:** Writing – review & editing, Methodology, Investigation. **Gyorgy Panyi:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Sanjeevini Babu Reddiar:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Dorothy C.C. Wai:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Raymond S. Norton:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Natalie L. Trevaskis:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpharm.2026.127198>.

Data availability

Data will be made available on request.

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