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Research paper

Lymphatic uptake of the lipidated and non-lipidated GLP-1 agonists liraglutide and exenatide is similar in rats

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ABSTRACT

Peptides, despite their therapeutic potential, face challenges with undesirable pharmacokinetic (PK) properties and biodistribution, including poor oral absorption and cellular uptake, and short plasma elimination half-lives. Lipidation of peptides is a common strategy to improve their physicochemical and PK properties, making them viable drug candidates. For example, the plasma half-life of peptides has been extended via conjugation to lipids that are proposed to promote binding to serum albumin and thus protect against rapid clearance. Recent work has shown that lipid conjugation to oligodeoxynucleotides, polymers and small molecule drugs results in association not only with albumin, but also with lipoproteins, resulting in half-life prolongation and transport from administration sites via the lymphatics. Enhancing delivery into the lymph increases the efficacy of vaccines and therapeutics with lymphatic targets such as immunotherapies. In this study, the plasma PK, lymphatic uptake, and bioavailability of the glucagon-like peptide-1 (GLP-1) receptor agonist peptides, liraglutide (lipidated) and exenatide (non-lipidated), were investigated following subcutaneous (SC) administration to rats. As expected, liraglutide displayed an apparent prolonged plasma half-life (9.1 versus 1 h), delayed peak plasma concentrations and lower bioavailability (~10 % versus ~100 %) compared to exenatide after SC administration. The lymphatic uptake of both peptides was relatively low (<0.5 % of the dose) although lymph to plasma concentration ratios were greater than one for several early timepoints suggesting some direct uptake into lymph. The low lymphatic uptake may be due to the nature of the conjugated lipid (a single-chain C16 palmitic acid in liraglutide) but suggests that other peptides with similar lipid conjugations may also have relatively modest lymphatic uptake. If delivery to the lymph is desired, conjugation to more lipophilic moieties with higher albumin and/or lipoprotein binding efficiencies, such as diacylglycerols, may be appropriate.

1. Introduction

As therapeutics, peptides possess desirable properties such as high potency and selectivity, which can enhance therapeutic response while reducing off-target toxicities.[1] However, peptides face challenges in achieving adequate oral bioavailability due to their inherent physicochemical properties and instability.[2] As a result, peptides are mainly administered via parenteral injection, with subcutaneous (SC) injection being the most convenient and preferred mode of injection for patients.[2] Naturally occurring peptides, like glucagon-like peptide-1 (GLP-1) and somatostatin, have short plasma half-lives due to rapid degradation and bioconversion in the systemic circulation, lasting only minutes.[3,4] This short half-life limits the use of endogenous peptide forms, even with parenteral administration. To overcome these limitations, lipidation is a common strategy used to enhance the physicochemical, pharmacokinetic (PK), and/or pharmacodynamic properties of peptides.[5,6] Lipidation generally prolongs the plasma elimination half-life of peptides by enhancing binding to serum albumin, protecting peptides against degradation and clearance processes.[7] Recent studies have shown that lipidation can also prolong the serum half-life of polymers by increasing association with both plasma lipoproteins and albumin.[8].

Lipidated peptides are used clinically to treat diabetes, including lipidated insulin analogues and GLP-1 agonists like liraglutide and semaglutide.[9] Intensive research on GLP-1 analogues aims to develop

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therapeutic peptides with improved physicochemical and PK properties, including oral bioavailability.[10,11] While endogenous GLP-1 effectively regulates postprandial hyperglycemia, its short *in vivo* half-life due to degradation by dipeptidyl peptidase 4 (DPP4) and neutral endopeptidase (NEP) poses a challenge to providing effective therapy.[12] Modifications preserving key amino acids and reducing degradation by DPP4 and NEP have led to various clinically approved GLP-1 analogues like exenatide. The conjugation of C16, C18 or C20 chain length fatty acids or fatty diacids to GLP-1 and/or glucose-dependent insulinotropic polypeptide (GIP) analogues such as liraglutide (C16 fatty acid, GLP-1 agonist), semaglutide (C18 fatty di-acid, GLP-1 agonist) and tirzepatide (C20 fatty-diacid, dual GLP-1 and GIP agonist) extends their plasma elimination half-life after SC injection through enhanced albumin binding leading to reduced clearance.[13] An oral formulation of semaglutide (Rybelsus, NovoNordisk) has also been recently approved where the GLP-1 agonist is formulated with salcaprozate sodium (SNAC) as an absorption enhancer.[14].

In liraglutide, the long-acting GLP-1(7–37) analogue clinically in use as a type 2 diabetes treatment, Lys-34 is replaced by Arg and Lys-26 is acylated with a C16 palmitic acid connected to a γ-glutamic acid spacer (Fig. 1). [15] Palmitic acid conjugation delays liraglutide's absorption in humans after SC injection, and prolongs its elimination half-life to around 13 h, compared to about 2 min for native GLP-1.[15,16] This occurs via enhanced binding to serum albumin by the fatty acid moiety shielding the peptide from degradation by DPP4 and clearance via the kidney.[17,18] Lipidation significantly enhances the PK profile of liraglutide, making it suitable for once-daily SC administration.[15,19] Another significant GLP-1 mimetic peptide for type 2 diabetes treatment is exenatide, which received FDA approval in 2005.[20] Exenatide is based on exendin-4, a hormone found in the *Heloderma suspectum* (Gila monster) lizard, and mimics the effects of incretins such as GLP-1 and GIP.[21–23] While exenatide's amino acid structure has been modified to enhance its plasma half-life, it still requires twice-daily SC injections before meals due to its relatively short half-life (2–3 h).[24].

Endogenous C10-C20 fatty acids bind to albumin at seven sites. The combination of high plasma concentration, multiple binding sites, and a long half-life gives albumin an extensive capacity to transport fatty acids in systemic compartments.[25,26] This property has been leveraged in drug discovery, where various ligands, including small molecules, peptides, and proteins, bind to albumin. The structure of lipids attached to bioactive peptides and the spacer between the peptide and lipid significantly impact the peptide's activity and elimination half-life, primarily due to differences in affinity for serum albumin.[27–30] As is seen above, binding to circulating albumin extends residence time, reduces enzymatic degradation, and acts as a reservoir for slow drug release, thereby maintaining a continuous therapeutic plasma concentration. [31–36].

Additionally, recent work has taken advantage of the natural trafficking of albumin from tissues into the lymphatics following extravasation. For example, Liu et al. first described an "albumin hitchhiking" approach to enhance delivery of molecular vaccines to the lymphatics. These comprised of an antigen or adjuvant cargo linked to a lipophilic albumin-binding tail where conjugation to di-acyl lipid was found to lead to higher binding to albumin and lymphatic uptake than mono-acyl lipid or cholesterol.[37] Recent work from our group and others reported that conjugation of brush polyethylene glycol (PEG) polymers with di-acyl lipids (e.g. 2C12 and 2C18) and cholesterol prolongs plasma half-life more than conjugation with single-chain lipids (e.g. 1C2 and 1C12), consistent with their albumin binding affinity.[8,38] However, when the uptake of these polymers into thoracic lymph was explored after SC injection, higher uptake was seen for brush PEG polymers conjugated to 1C2, 2C12 or 2C18 lipids compared to cholesterol or 1C12, inconsistent with their albumin binding affinity.[39] This led us to suggest that the polymers traffic not only on albumin but also lipoproteins.

Like albumin, lipoproteins such as high-density lipoprotein (HDL) and low density lipoprotein (LDL) continuously extravasate from the bloodstream into the interstitial space of tissues, from where the majority is subsequently absorbed into the lymphatic system before re-entering the systemic circulation.[40,41] Consequently, it can be assumed that molecules that exhibit an affinity for albumin or lipoproteins follow a similar route, draining into the initial lymphatic vessels following extravasation from the blood or after administration into tissues via SC, intradermal (ID), or intramuscular (IM) injection.[42] In support, cholesterol-conjugated CpG oligodeoxynucleotide showed higher lymphatic uptake than free CpG, mostly associated with HDLs in lymph suggesting that it entered the lymph with HDL.[43].

While several marketed therapeutic peptides are linked to lipids to optimize their plasma PK and extend plasma half-life,[44] no previous study has examined whether lipid attachment promotes the uptake of therapeutic peptides into the lymphatics after SC injection. This potential is particularly significant considering the crucial physiological roles of the lymphatic system in lipid metabolism, immune cell trafficking, immune activation, and fluid homeostasis.[42,45] Enhancing lymphatic delivery of immunotherapies[46] as well as vaccines[47] and therapeutics for cancer,[46] metabolic[48] and infectious disease[49] can thus enhance efficacy of treatment. The present study focuses on the question of whether lipid conjugation to peptides enables lymphatic uptake following SC injection. The plasma PK and lymphatic uptake of the lipid-conjugated GLP-1 agonist liraglutide and the non-lipid-conjugated GLP-1 agonist exenatide are compared following intravenous (IV) and SC administration to rats. While some lymphatic uptake is observed following SC injection, with lymph to plasma concentration ratios greater than one for several timepoints suggesting direct

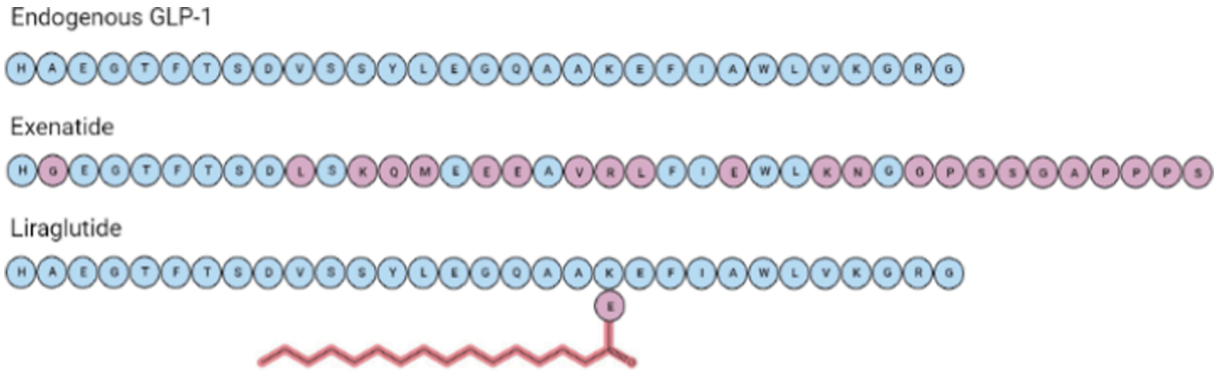

Fig. 1. The amino acid sequences of endogenous GLP-1 and two GLP-1 analogues, liraglutide and exenatide. Modified amino acids are indicated in pink. Clinically approved GLP-1 analogues contain modifications that preserve key amino acids (His^{7}, Gly^{10}, Phe^{12}, Thr^{13}, Asp^{15}, Phe^{28}, and Ile^{29}) to maintain their potency and reduce degradation by DPP4 and NEP, as is the case with exenatide. Liraglutide has been modified by fatty acid conjugation, which promotes albumin binding and extends its plasma half-life.

lymphatic uptake, the lymphatic uptake is similar for both peptides.

2. Methods

2.1. Materials

Exenatide (hor-246-b) was procured from Prospec Protein Specialist, while liraglutide (AG-CP3-0034) was obtained from Adipogen Life Science. Ultrapure water (MilliQ® 18.2 MΩ*cm) was utilized for liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis, in conjunction with LC-MS grade acetonitrile (1.00029), methanol (1.06035), formic acid, and isopropanol, all sourced from Merck (Bayswater VIC 3153, Australia). For *in vivo* experimentation, cOmplete® mini protease inhibitor cocktail tablets were acquired from Roche (Sydney, NSW 2000, Australia). Sterile saline was provided by Baxter (Old Toongabbie, NSW 2146, Australia), and heparin and pentobarbitone were purchased from Clifford Hallem Healthcare (Keysborough, VIC 3173, Australia). Polyethylene (PE) (0.5 mm ID, 0.8 OD) and polyvinyl chloride (PVC) (0.58 mm ID, 0.96 OD) tubes were procured from Microtube Extrusions (NSW, Australia). Isoflurane was purchased from Pro-vet (VIC, Australia).

2.2. Animal studies

The Monash Institute of Pharmaceutical Sciences (MIPS) Animal Ethics Committee (AEC) granted approval for all animal experiments. Male Sprague-Dawley (SD) rats aged between 7 and 15 weeks (330–350 g, Monash Animal Services, Clayton, VIC, Australia) were used to evaluate the lymphatic uptake and PK profiles of exenatide and liraglutide. After cannulation, rats had continuous access to water but were fasted overnight and for 4 h after dosing on the following day. During the experimental phase, the animals were housed individually in BASi Culex Raturn cages at the MIPS facilities.

2.3. Lymphatic transport, plasma pharmacokinetic and bioavailability studies

Two cohorts of surgically prepared rats were used to evaluate the PK and lymphatic uptake of liraglutide and exenatide: 1) lymph-cannulated rats, where the lymphatic uptake of liraglutide (2 mg/kg) and exenatide (1 mg/kg) were examined after SC administration, 2) lymph-intact rats, in which PK evaluations were conducted for both liraglutide and exenatide at a dose of 1 mg/kg following SC or IV administration.

All surgical cannulation procedures were performed according to previously established methods.[50–52] In the lymph-cannulated cohort, a PVC cannula was inserted into the thoracic lymph duct under isoflurane anesthesia to collect thoracic lymph, which collects the majority of lymph in the body, including that from the SC injection site. PE cannulae were inserted into the carotid artery and jugular vein for blood sampling and saline infusion (to compensate for fluid loss resulting from lymph collection), respectively. Lymph flow rate was approximately 1–2 mL/h consistent with past literature[53–55], and IV saline infusion was performed at 1.2 mL/h (in addition, animals had unrestricted access to water). For the non-lymph-cannulated (*i.e.* lymph-intact) cohort, a cannula was implanted into the carotid artery to facilitate blood sampling and jugular vein for IV dosing. All cannulae were guided to the nape of the neck and through a spring and harness mechanism, enabling sampling external to the cage.

The day after surgery, rats were administered either SC or IV doses of liraglutide or exenatide. In the lymph intact cohort, rats were anesthetized using isoflurane (5 % induction, 2.5 % maintenance) for SC administration and received a 1 mg/kg injection of the peptides in saline into the medial aspect of the right hind leg. IV administration comprised an infusion at a rate of 2 mL/min over 45 s, with a 1 mg/kg dose delivered in sterile saline via the jugular vein cannula, followed by a saline flush to ensure complete dose administration. Blood samples (250 μL) were collected at predetermined time points. For SC-dosed rats, time points included pre-dose, 15 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr, 8 hr, 10 hr, 24 hr, and 30 hr. For IV-dosed rats, time points were pre-dose, 1 min, 5 min, 15 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr, 10 hr, and 24 hr. A protease inhibitor cocktail (Roche) in heparin (2.5 IU/mL in saline) was added to all sampling tubes (10 μL per tube) to prevent degradation of the peptides in the blood samples. Following collection, the samples were centrifuged at 4500 g for 5 min at 4 °C, plasma was separated and flash-frozen on dry ice, and subsequently stored at −20 °C until analyzed. Blood glucose levels were monitored at each time point to confirm that liraglutide and exenatide did not induce hypoglycemia. After sample collection, rats were anesthetized for exsanguination and euthanasia. Exsanguination was performed via the carotid artery cannula, and euthanasia was achieved through pentobarbitone injection (100 mg/kg) via the same cannula.

In the lymph-cannulated cohort, dose administration and blood sample collection were performed as described above, using a dose of 2 mg/kg for liraglutide and 1 mg/kg for exenatide, respectively. Lymph was collected continuously into tubes at specified time intervals: pre-dose, 0–15 min, 15–30 min, 30–45 min, 45–60 min, 1–1.5 hr, 1.5–2 hr, 2–3 hr, 3–4 hr, 4–6 hr, 6–8 hr, 8–10 hr, 10–24 hr, and 24–30 hr. Lymph was collected into tubes containing a protease inhibitor cocktail (Roche) in heparin 2.5 IU/mL (10 μL per 150–200 μL of lymph, with anticoagulant volumes scaled to accommodate larger collection volumes) to reduce degradation of the peptides. Tubes were weighed before and after sample collection to determine the volume of lymph collected. Subsequently, samples were flash-frozen on dry ice and stored at −20 °C until analyzed.

2.4. Lymph and plasma sample preparation and analysis by LC-MS/MS

First, stock solutions of liraglutide in methanol and exenatide in sterile water were prepared at 1 mg/mL. Subsequently, spiking solutions were generated through further dilution of these stock solutions in methanol. Plasma and lymph standards were freshly prepared, with each standard set containing a minimum of six distinct analyte concentrations (25, 50, 100, 500, 1000, 2500, 5000 ng/mL). Plasma and lymph calibration standards were prepared by spiking blank plasma or lymph (45 μL) with solution standards (5 μL, varying concentrations) and an internal standard (IS) – liraglutide served as IS for exenatide, while exenatide served as IS for liraglutide (5 μL of a 5 μg/mL solution). Quality control (QC) samples were generated in the same manner at concentrations of 25, 500, and 2500 ng/mL, respectively. The 50 μL plasma or lymph standards or actual study plasma or lymph samples were subsequently treated with 150 μL of a 75 % acetonitrile/25 % methanol mixture (1:3 dilution), followed by gentle vortexing for 2 min and brief centrifugation (5000 RPM for 10 min at 4 °C) to precipitate the plasma proteins and ensure an optimal sample volume. The supernatant (50 μL) was transferred to LC-MS vials for analysis.

The concentration of the peptide in the dosing solutions used in the study was analyzed by comparison with standard curves prepared from peptides in solvent. To create matrix-matched standards, a blank formulation vehicle (saline) was diluted 100-fold in methanol, which served as the assay diluent. A 1 mg/mL stock solution of the peptide (10 μL) was added to 90 μL of this diluent, resulting in a concentration of 100 μg/mL. Subsequently, this 100 μg/mL solution was serially diluted in assay diluent to generate a concentration range from 250 to 7500 ng/mL. A mixture of 150 μL 75 % acetonitrile/25 % methanol was combined with 5 μL of a 50 μg/mL IS solution (also prepared in assay diluent – liraglutide functioned as the IS for exenatide, while exenatide served as the IS for liraglutide), vortexed, and transferred to LC-MS vials for analysis. The study stock solutions for analysis were diluted 100-fold (across two steps) in assay diluent, in duplicate.

LC–MS/MS assay development, validation, and sample analysis were performed using an ultra-high-performance liquid chromatography (UHPLC) system coupled to a Shimadzu 8050 tandem quadrupole mass

spectrometer. Chromatographic separation of analytes was accomplished on a Kinetex C8 100 Å, LC Column 50 x 2.1 mm, employing a gradient of 0.1 % formic acid in water (mobile phase A) and 0.1 % formic acid in acetonitrile (mobile phase B). The gradient encompassed an initial hold at 15 % B for 0.5 min, a linear increment to 50 % B over 2.5 min, 95 % B for 1.7 min, a secondary hold at 95 % B for 0.2 min, a decline to 60 % B over 0.1 min, and re-equilibration at 15 % B for 1.0 min, culminating in a total chromatographic run time of 7.0 min at a flow rate of 0.3 mL/min. The column temperature was maintained at 40 °C, and an injection volume of 4 μL was used. The detection of analytes and IS was performed in positive ion mode, with mass spectrometer conditions including a nitrogen gas temperature of 300 °C, a drying gas flow rate of 10 L/min, and a nebulizing gas flow rate of 3 L/min. The desolvation line and heat block temperatures were set at 250 and 400 °C, respectively, while the dwell time was established at 100 ms. Ion monitoring was performed through multiple reaction monitoring, Q1 → Q3 transitions of m/z 938.7 → m/z 136.25 (cone voltage = 50 V) for liraglutide and m/z 1047.5 → m/z 299.15 (cone voltage = 47) for exenatide.

2.5. LC-MS/MS assay validation

Calibration curves were generated using linear regression with 1/x weighting and by plotting the plasma and lymph standard concentrations against the corresponding chromatographic peak area ratios of liraglutide or exenatide relative to the IS. The calibration curve's linear range spanned from 25 to 5000 ng/mL for both peptides. Intraday accuracy and precision were determined by analyzing four replicates (n = 4) of each of the three QC levels (25, 500, and 2500 ng/mL). The assays were found to be accurate and precise (within ± 16 % at all concentrations). See supplementary Tables 1-2.

2.6. Peptide stability in collected lymph and plasma

A mini protease inhibitor cocktail (Roche) was added to the plasma and lymph samples to prevent degradation by this enzyme. A stability study was performed to ensure the stability of the peptides in collected and stored lymph and plasma. For this, a stock solution was prepared by dissolving a complete tablet in 1 mL of potassium fluoride (4 mg/mL) and then mixing with 200 μL of 1000 IU heparin. For the stability studies, 2000 ng/mL of both liraglutide and exenatide were added directly to plasma and lymph samples, with or without the addition of 10 μL of the anticoagulant cocktail to each vial. One aliquot was analyzed immediately after centrifugation, while the remaining five aliquots were stored at temperatures below −20 °C (between −25.5 °C and −21.1 °C) for 2, 4, 6, 8, and 12 weeks (±3 days), respectively. All peptide stability samples, and the fresh calibration standards were subjected to LC-MS/MS analysis according to protocol described above. As shown in supplementary Fig. 1, the peptides were stable in lymph and plasma in the presence and absence of the protease inhibitor cocktail over an extended period of time.

2.7. Data analysis

Non-compartmental PK analysis was conducted utilizing the PKSolver program.[54] For lymphatic transport investigations, the peptide mass transport in the lymph was determined by multiplying the collected lymph volume by the corresponding peptide concentration measured within the lymph. The ratio of lymph to plasma concentration was ascertained by dividing the mean peptide lymph concentration for each hourly collection interval by the peptide plasma concentration assessed at the end of the hourly collection period. When determining the lymph-to-plasma ratio, the plasma concentration was set to the lower limit of quantification (LLOQ) for time points where the plasma concentration was below the LLOQ.

2.8. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) unless otherwise stated. An unpaired *t*-test was performed to compare groups. A p-value ≤ 0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism 9.0.1 software (GraphPad Software Inc, CA, USA).

3. Results

3.1. Plasma pharmacokinetics of exenatide and liraglutide after SC and IV administration to lymph-intact rats

The lipidated GLP-1 mimetic peptide liraglutide displayed prolonged plasma exposure and a longer elimination half-life after both IV and SC administration compared to the non-lipidated peptide exenatide, which had a very short elimination half-life of < 1 h (Fig. 2 and Table 1). The estimated elimination half-life of liraglutide was longer after SC compared to IV delivery, although not significantly so, which may have resulted from a prolonged absorption phase (Fig. 2, Tables 1 and 2). Indeed, after SC administration the time to reach maximum plasma concentration appeared to be longer for liraglutide than for exenatide, although this difference was not significant as it was quite variable for liraglutide (Table 2). The absolute bioavailability of exenatide after SC administration was complete (>100 %), whereas the SC bioavailability of liraglutide was much lower (~10 %).Table 2

3.2. Lymph uptake of exenatide and liraglutide after SC administration

The cumulative transport of exenatide into the lymph over time was relatively low after SC administration (~0.4 % of the dose, Fig. 3 and Table 3). Plasma concentrations of exenatide were also not lower in lymph cannulated compared to lymph-intact rats after SC administration. This data suggests minimal lymphatic transport of exenatide. The lymph:plasma concentration ratio of exenatide was, however, relatively high (up to 20). This may be a result of the short plasma half-life of exenatide, which results in a substantial drop in plasma concentration over the lymph collection period, while lymph is collected slowly as it drips from the cannula. Therefore, there is a delay before peptide concentrations are detected to decrease in lymph relative to plasma.

The lymphatic transport of liraglutide was also low after SC administration (mean < 0.2 % dose) and plasma concentrations of the peptide were not lower in lymph cannulated compared to lymph-intact rats (Fig. 4 and Table 3). The lymph:plasma concentration ratio had an early peak of 5, which may indicate a brief direct uptake into the lymph, but at later time points the lymph:plasma ratio was low, suggesting only indirect access to the lymph.Fig. 4

4. Discussion

Lipid conjugation is a widely used approach to improve the PK and delivery properties of therapeutic peptides.[55] It can prolong the plasma half-life of peptides by enhancing their binding to serum albumin, thus providing protection against degradation and renal clearance. Conjugation to albumin-binding moieties, including lipids, dyes, and peptides, has also been used to deliver vaccine antigens, adjuvants, polymers, and oligodeoxynucleotides into the lymph after interstitial injection.[38,56,56,58] This process, known as 'albumin hitchhiking', exploits the natural movement of albumin from blood to tissue and back to the circulation via lymphatic vessels.[55,56].

Recent studies from our lab have shown that the conjugation of lipids to CpG oligodeoxynucleotide and brush PEG polymers enhances their uptake into the lymph after SC injection. In this case, the lipid conjugated CpG and brush PEG polymers were found to be predominantly associated with lipoproteins in lymph rather than proteins such as albumin.[39,43] This led us to conclude that association of these lipidated

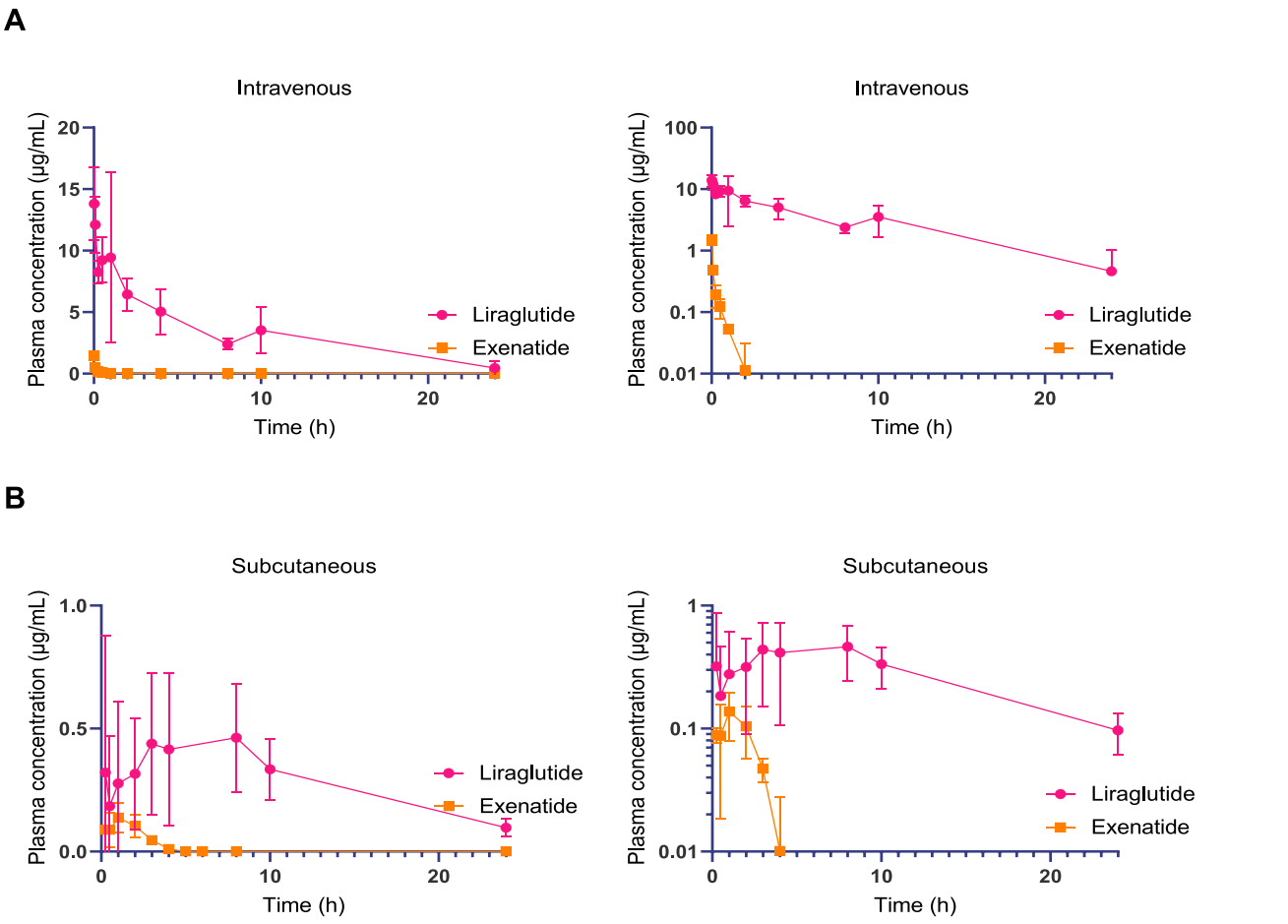

Fig. 2. Plasma concentrations versus time profiles of liraglutide and exenatide are presented on a linear and logarithmic scales following IV and SC administration to conscious rats (at 1 mg/kg). Panel **A** depicts data for liraglutide and exenatide after IV administration, respectively, whereas Panel **B** depicts data for liraglutide and exenatide after SC administration. The mean ± SEM values are presented for n = 3 rats.

Table 1
Pharmacokinetic data for liraglutide and exenatide following IV administration at 1 mg/kg to conscious rats. Values are expressed as mean ± SEM for n = 3 rats. Statistical analysis was performed by comparing differences between the groups using unpaired *t*-test. AUC: area under the plasma-concentration time curve, CL: clearance and Vd: volume of distribution.

Parameter	Unit	Liraglutide (IV)	Exenatide (IV)
$t_{1/2}$	h	5.3 ± 1.5	0.5 ± 0.1
$AUC_{0\text{-}24}$	mg/L*h	77.8 ± 9.0 [a]	0.2 ± 0.03[a]
$AUC_{0\text{-}inf\_obs}$	mg/L*h	82.7 ± 11.7 [a]	0.3 ± 0.02[a]
CL	(L/h)	0.004 ± 0.001 [a]	1.2 ± 0.06 [a]
Vd	(L)	0.03 ± 0.004 [a]	0.9 ± 0.2 [a]

[a] Significantly different parameter compared with Liraglutide (IV) and Exenatide (IV) ($P \leq 0.05$).

Table 2
Pharmacokinetic data for liraglutide and exenatide following SC administration at 1 mg/kg to conscious rats. Values are expressed as mean ± SEM for n = 3 rats. Statistical analysis was performed by comparing differences between the groups using unpaired *t*-test but were not significant. AUC: area under the plasma-concentration time curve.

Parameter	Unit	Liraglutide (SC)	Exenatide (SC)
$t_{1/2}$	h	9.1 ± 2.9	1.03 ± 0.10
T_{max}	h	3.4 ± 2.4	0.8 ± 0.2
C_{max}	mg/L	0.6 ± 0.2	0.2 ± 0.005
$AUC_{0\text{-}24}$	mg/L*h	6.9 ± 1.7	0.30 ± 0.04
$AUC_{0\text{-}inf\_obs}$	mg/L*h	8.2 ± 1.6	0.4 ± 0.05
Bioavailability	(%)	9.9	128.7

molecules with lipoproteins at the injection site may facilitate the trafficking of these molecules through the lymphatics.[59] However, the impact of lipidation on the lymphatic uptake and PK of therapeutic peptides such as GLP-1 agonists have not been quantified.

In the current study, the lipidated peptide liraglutide exhibited a prolonged plasma half-life compared to the non-lipidated peptide exenatide after SC administration in non-lymph cannulated rats (~9 vs ~ 1 h). The determined half-life is consistent with previously published data in rats (13 vs 1–1.5 h).[28] The absorption of liraglutide into the blood after SC injection appeared to be delayed compared to exenatide, with a substantially lower absolute bioavailability (~10 vs ~ 100 %). This delay in absorption may lead to a more sustained effect leading to the requirement for less frequent administration, although it may also result in lower total bioavailability due to retention at the injection site and loss of some of the peptide.

Surprisingly, the lymphatic uptake of both lipidated liraglutide and non-lipidated exenatide was relatively low (<0.5 % dose) after SC injection in thoracic lymph cannulated rats. For both peptides, there were 5–20 fold higher concentrations of peptide in lymph compared to plasma at early timepoints suggesting that there may be some direct lymph uptake from the injection site. Previous studies have suggested that endogenous GLP-1 is present in mesenteric lymph at higher concentrations than in plasma, indicating direct uptake into lymph following secretion from the intestine.[60] However, the higher concentration of GLP-1 in lymph may also result from greater stability in

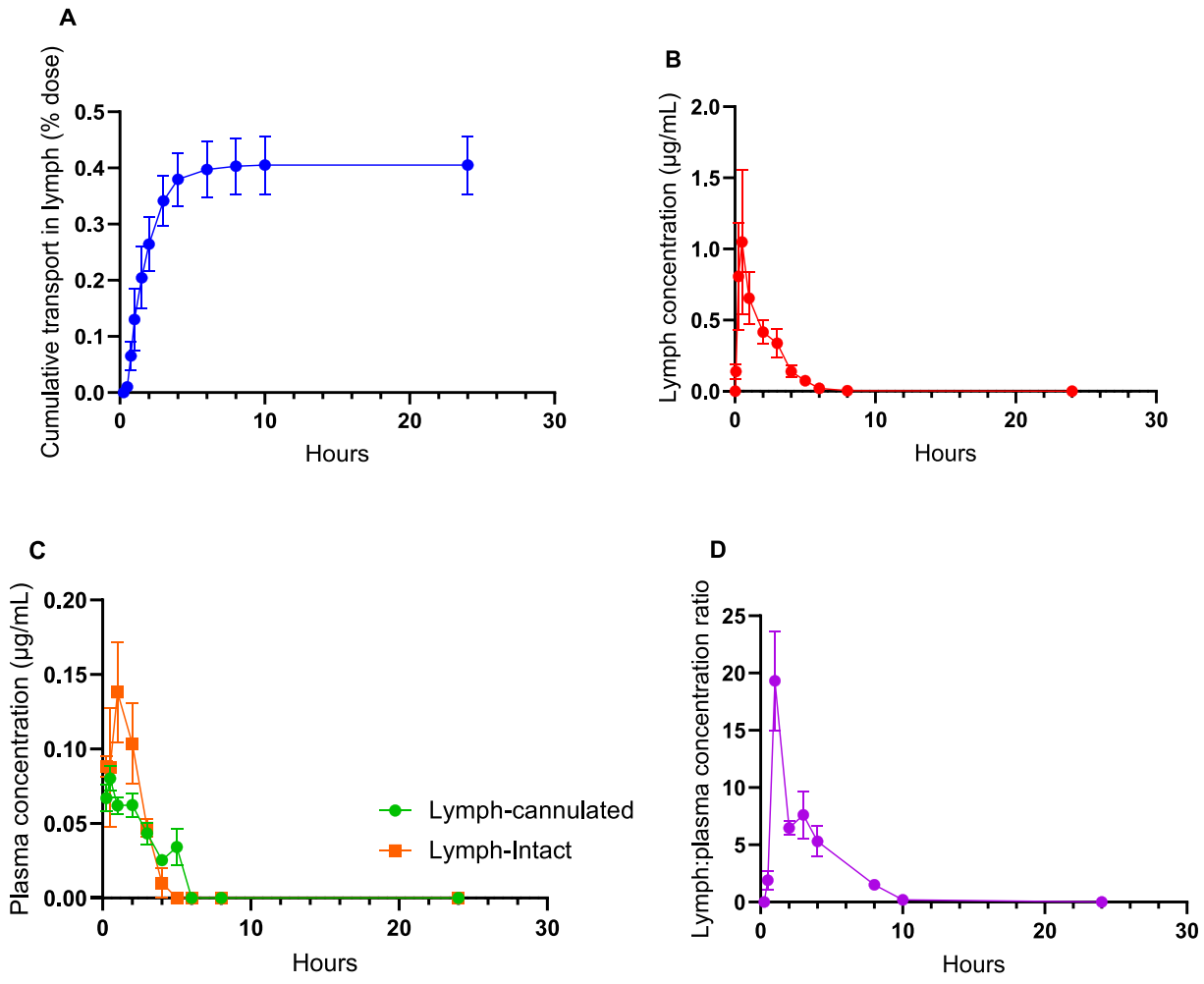

Fig. 3. Thoracic lymph transport and plasma PK of exenatide after SC administration of 1 mg/kg to rats with the thoracic lymph-duct cannulated. Panel **A** illustrates the cumulative lymphatic transport (% dose) of exenatide over time. Panel **B** shows the lymph concentration versus time profile of exenatide. Panel **C** displays the plasma concentration versus time profiles of exenatide in lymph cannulated and lymph-intact rats. Panel **D** shows the ratio of lymph to plasma concentrations of exenatide over time. The data are presented as mean ± SEM for n = 3 rats.

Table 3
PK data for liraglutide and exenatide following SC administration at 2 mg/kg and 1 mg/kg, respectively, to conscious rats with the thoracic lymph duct cannulated. Values are expressed as mean ± SEM for n = 3 rats. Statistical analysis was performed using an unpaired *t*-test. AUC: area under the plasma-concentration time curve

Parameter	Unit	Liraglutide (Lymph-cannulated)	Exenatide (Lymph-cannulated)
$t_{1/2}$	h	12.61 ± 5.45	10.26 ± 6.51
T_{max}	h	3 ± 0.58	0.67 ± 0.17
C_{max}	mg/L	0.33 ± 0.02 [a]	0.08 ± 0.01 [a]
$AUC_{0\text{-}30}$	mg/L*h	1.76 ± 0.73	0.33 ± 0.01
$AUC_{0\text{-}inf\_obs}$	mg/L*h	3.45 ± 0.82	1.07 ± 0.61

[a] Significantly different parameter compared with Liraglutide (Lymph-cannulated) or Exenatide (Lymph-cannulated) ($P \leq 0.05$).

lymph due to a lower concentration of the degradative enzyme DPP4.

Prior studies have inferred the uptake of lipidated vaccines or polymers into lymphatics from injection sites through observing higher concentrations in draining lymph nodes compared to non-lipidated forms. However, these studies did not directly quantify the mass recovery of lipidated peptide vaccines in lymph. For example, Liu et al. initially described the concept of albumin hitchhiking to enhance lymph node delivery of vaccine antigens and adjuvants.[37] They found a higher lymph node recovery of vaccines composed of peptide antigens and adjuvant CpG upon conjugation to diacyl lipids compared to monoacyl lipids or cholesterol, which display lower albumin binding affinity, although conjugation to all lipids appeared to increase lymph uptake relative to unconjugated CpG. Similarly, the De Geest group explored the albumin-binding affinity of a linear PEG polymer (3 kDa) conjugated with cholesterol or medium-chain (2C8 or 2C12) or long-chain (2C18) dialkyl groups.[38] Interestingly, linear PEG conjugated with long-chain dialkyl groups had lower lymph node uptake after SC injection, which was attributed to solubility issues.

Recent research by our group has quantified the lymphatic uptake of cholesterol conjugated CpG and lipidated brush PEG polymers after SC administration in rats. Approximately 2 % of the dose of cholesterol-conjugated CpG was recovered in thoracic lymph over 4 h after SC injection, which was > 10-fold higher than the recovery of non-lipidated CpG.[43] On the other hand, the brush PEG polymers, conjugated with different lipid components, showed moderate lymphatic uptake (up to 6 % dose), with some variations depending on the lipid moiety.[39] Both the cholesterol-conjugated CpG and lipidated brush PEG polymers were predominantly recovered in the lipoprotein fraction within the collected lymph suggesting to us that they enter lymph in association with endogenous lipoproteins as well as albumin. The lymph uptake of brush PEG polymer conjugated to a medium chain monoalkyl hydrocarbon tail (1C12) was particularly low (~1% dose), which is consistent

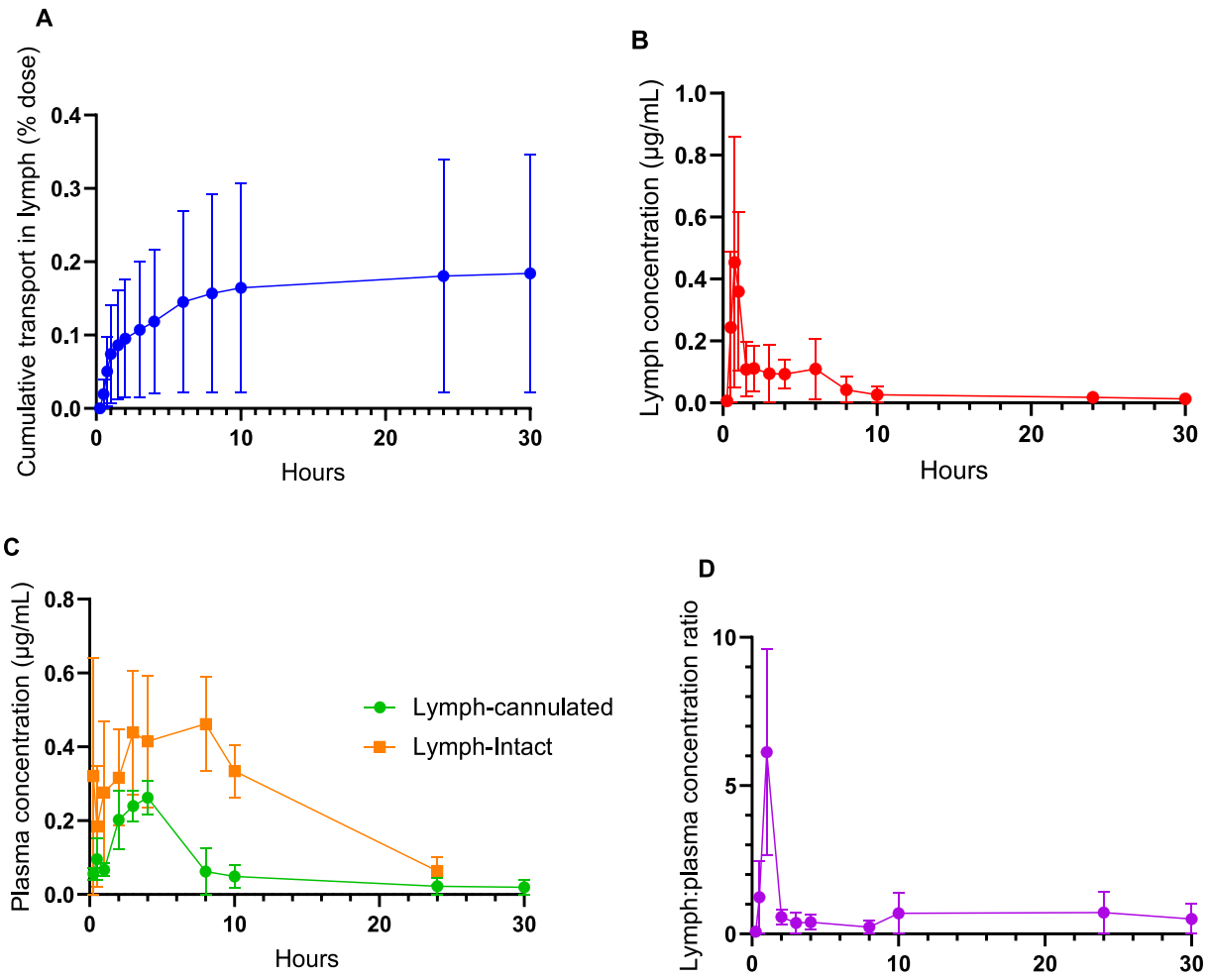

Fig. 4. Thoracic lymph transport and plasma PK of liraglutide after SC administration of 2 mg/kg to rats with the thoracic lymph-duct cannulated. Panel **A** illustrates the cumulative lymphatic transport of liraglutide over time. Panel **B** shows the lymph concentration versus time profile of liraglutide. Panel **C** displays the plasma concentration versus time profiles of liraglutide in lymph cannulated and lymph-intact rats. Panel **D** shows the ratio of lymph to plasma concentrations over time. The data show mean ± SEM for n = 3 rats.

with the lymphatic uptake of liraglutide conjugated to palmitic acid (C16).

The lymphatic uptake of liraglutide is lower than anticipated based on its albumin binding. Previous studies have shown that liraglutide is 99.5 % bound to proteins in plasma, ~99 % bound to proteins in 16.7 % plasma diluted in buffer, and ~ 90 % bound to proteins in 0.2 % plasma. [61] Considering that the concentration of proteins like albumin in the interstitial space and lymph is ~ 30 % of that in plasma, [56] depending on the tissue and condition, we would expect most of liraglutide in the interstitial space to be bound to proteins in a static condition and transported into lymph in association with albumin. We believe that the lower than expected lymphatic uptake of liraglutide is due to the dynamic environment at the injection site. It is possible that liraglutide is transported from the injection site into blood before there is an opportunity to bind to albumin and lipoproteins. Even if liraglutide is 99 % bound to proteins or lipoproteins at the injection site, the bound fraction will be in equilibrium with a free fraction. That free fraction will be available to diffuse into blood (which flows at 100–500 fold higher rates than lymph). As the free fraction enters blood, the equilibrium will be restored such that a lower mass of the liraglutide is bound to proteins. So, whilst the protein bound fraction of liraglutide may be transported into lymph, perhaps the majority enters blood due to dynamics at the injection site. Based on our data with lipidated brush PEG polymers, it is possible that the lymphatic uptake of therapeutic peptides could be enhanced further through conjugation to more lipophilic moieties such as monoalkyl or dialkyl lipids with longer hydrocarbon tails or dicarboxylic acids that have a higher affinity for albumin and/or lipoproteins.

5. Conclusion

Conjugation of GLP-1 peptide agonists with fatty acids (such as palmitic acid in liraglutide) prolongs the plasma elimination half-life and modifies the SC absorption profile. However, liraglutide displayed modest lymphatic uptake suggesting that conjugation to C16 fatty acid does not enable substantial hitchhiking on either the albumin or lipoprotein trafficking pathways into the lymph following SC injection. Future studies could investigate how the conjugation of peptides to different lipid types impacts lymphatic uptake after SC injection. It is also of interest how the molecular weight and structure of the peptide influence the propensity for lymph uptake upon conjugation to different lipids. This strategy could be used to enhance delivery to targets within lymph such as immune cells, cancer cells or microorganisms for immunotherapies, vaccines, chemotherapeutics, and anti-infective peptides.

CRediT authorship contribution statement

Sanjeevini Babu Reddiar: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Mohammad Abdallah:** Writing – review & editing, Methodology,

Investigation. **Ian K. Styles:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Olivia O. Müllertz:** Writing – review & editing, Methodology, Investigation. **Natalie L. Trevaskis:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2024.114339.

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