Lymphatic absorption, metabolism and excretion of a therapeutic peptide in dogs and rats

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Lymphatic absorption and metabolism of a PEGylated peptide

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ABBREVIATIONS: PEG, poly(ethylene glycol); LDC, lymph duct-cannulation; HPLC, high-performance liquid chromatography; SC, subcutaneous; IV, intravenous; EDTA: ethylenediaminetetraacetic acid; LSC, liquid scintillation counting; AUC, area under the concentration-time curve.
Abstract

The objective of the current study was to evaluate the mechanism of absorption and metabolism of a PEGylated peptide, MRL-1 (46 kDa), following subcutaneous (SC) dosing in dogs and rats. Thoracic lymph duct-cannulated (LDC) dog and rat models were developed which allowed continuous collection of lymph for up to 8 days. When \(^{3}\text{H}\)MRL-1 was administered SC to LDC dogs, ~73% of the administered radioactivity was recovered in pooled lymph over a period of 120 hr, suggesting that lymphatic uptake is the major pathway of SC absorption for this peptide. In agreement with this data, the systemic exposure of \(^{3}\text{H}\)MRL-1 related radioactivity in LDC dogs was decreased proportionately when compared to that in non-cannulated control dogs. Following IV dosing with \(^{3}\text{H}\)MRL-1 in LDC dogs, 20% of the administered radioactivity was recovered in pooled lymph over 168 hr, suggesting some level of recirculation of \(^{3}\text{H}\)MRL-1 related radioactivity from plasma compartment into the lymphatic system. Experiments conducted in LDC rat model also resulted in similar conclusions. Analysis of injection site subcutaneous tissue showed significant metabolism of \(^{3}\text{H}\)MRL-1, which provides an explanation for the <100% bioavailability of therapeutics proteins and peptides following SC dosing. Following SC dosing, major circulating components in plasma were the parent peptide and the PEG-linker \(^{3}\text{H}\)MRL-2. Metabolism profiles in lymph were similar to those in plasma, suggesting that loss of peptide was minimal during lymphatic transport. Following IV dosing in rats, \(^{3}\text{H}\)MRL-1 was metabolized and excreted primarily in the urine as metabolites.
Introduction

Subcutaneous (SC) administration is the patient-friendly route of delivery for biological drugs such as peptides and proteins. Due to poor membrane permeability and high enzymatic degradation in the gastrointestinal tract, their bioavailability following administration via non-parenteral routes is low and variable (Tang et al., 2004; Lin, 2009). Even though SC delivery has been utilized for biological drugs for many years, little is known about the processes that govern the absorption of macromolecules from the subcutaneous space and the impact on bioavailability and pharmacokinetic profiles in various species. Based on studies conducted in thoracic lymph-duct cannulated sheep model, it is suggested that the lymphatic system plays an important role in the absorption of large molecules following SC administration. It is demonstrated that the proteins larger than 16-20 kDa are taken up primarily by the lymphatic system and transported to the systemic circulation via the thoracic duct whereas smaller molecules primarily diffuse directly into blood capillaries (Supersaxo et al., 1990; Porter and Charman, 2000; Tang et al., 2004). The walls of blood capillaries are composed of a sealed endothelium with tight junctions that only allow small molecules but limit large molecules to diffuse. On the other hand, the lymphatic capillary has more open structure with an incomplete basal lamina and an absence of tight junctions which allow free passage for large molecules. Once administered through SC injection, large proteins travel from the local extracellular space in the subcutis into the peripheral lymphatics and regional lymph nodes, collecting at the central lymphatics before the proteins enter the vascular circulation (Swartz, 2001; McDonald et al., 2010). The studies conducted in sheep model have also contributed to our understanding of the lymphatic uptake following central vs. peripheral lymph duct-
cannulation, impact of injection site on lymphatic uptake, and potential for drug clearance during lymphatic transport (Supersaxo et al., 1988; Charman et al., 2000; Charman et al., 2001; McLennan et al., 2005; McLennan et al., 2006; Kota et al., 2007).

However, it is not clear if the molecular weight dependency for SC lymphatic absorption established in sheep is also applicable to other animals. In the process of drug discovery and development, preclinical models for pharmacokinetics and nonclinical safety studies usually consist of one rodent (usually rat) and one large animal species (typically dog or monkey). Therefore, developing lymph duct-cannulation models in relevant animal species such as rat and dog is of great interest to better understand the absorption, biodistribution and metabolism of biological drugs following SC administration. There are conflicting reports on the lymphatic absorption studies conducted in a rat model (Porter et al., 2001; Edwards et al., 2001; Kaminskas et al., 2009). Lymphatic absorption was a minor component in the SC absorption of bovine insulin (5.6 kDa), recombinant human erythropoietin alfa (30.4 kDa) and bovine albumin (66 kDa) in the rat (Kagan et al., 2007). In another study in rats, the thoracic lymph recovery of 5 proteins (MW 7.5 to 75 kDa) was low after subcutaneous administration (Xie and Hale, 1996). Reason for the discrepancy between the results obtained in rat and those in sheep are not clearly understood.

In the present study, we utilized a 46 kDa pegylated peptide, a neuromedin-U receptor agonist (MRL-1), to investigate the following: 1) mechanism of absorption following SC administration, 2) potential for recirculation of systemic MRL-1 to the lymphatic system following IV administration, 3) drug loss at the injection site and during lymphatic transport, and 4) mechanism of elimination. Thoracic lymph duct-
cannulated dog and rat models were developed which allowed continuous lymph collection for up to 8 days. Results obtained on the lymphatic absorption, in vivo metabolism, and elimination mechanism of \(^{3}H\)MRL-1 related radioactivity are discussed.

**Materials and Methods**

**Chemicals.** The PEGylated peptide MRL-1, \(^{3}H\)MRL-1 and \(^{3}H\)MRL-2 (Figure 1) were prepared at Merck Research Labs, Rahway, NJ, as described before (Ingallinella et al., 2012). Briefly, the free (unconjugated) peptide was dissolved in 8 M urea, 4 mM EDTA, 0.1 M sodium phosphate (pH 7.1), and was reacted with 40 kDa PEG-maleimide linker (1.1 eq., dissolved in water) for 1 hr at room temperature. MRL-1 was purified by sequential cation exchange and size-exclusion chromatography. The purity of MRL-1 was >99% as determined by HPLC. The specific activity of \(^{3}H\)MRL-1 was 22 Ci/mmol and the radiochemical purity was >99%, as determined by HPLC. The calculated isoelectric point was 8.7 for the native peptide. The 40 kDa amino-PEG intermediate was obtained from NOF Corporation, Japan. Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ).

**Thoracic lymph duct-cannulated animal models and study design.** All experiments were performed according to procedures approved by the Merck Institutional Animal Care and Use Committee. A parallel study design with thoracic lymph duct-cannulated and sham operated control dogs and rats was used in which a single dose of \(^{3}H\)MRL-1 was administered to each animal either intravenously or subcutaneously. In
both animal models, the subcutaneous injection sites are located at similar hind leg region in order to minimize potential variation in dose recovery from lymph due to the location of injection site (Tilney, 1971; Porter and Charman, 2000). The treatment groups, administered doses and collected samples are listed in Table 1. \[^{3}H\]MRL-1 was formulated as a solution in 5% mannitol buffer containing 6 mM sodium acetate (pH 5).

**The LDC dog model.** Adult male beagle dogs (~5 kg) were surgically prepared ~24 hr prior to dosing (Khoo et al., 2001; Shackleford et al., 2003). Dogs were fed with lipid meal prior to surgery to facilitate identification and isolation of the thoracic duct. Dogs were anesthetized and positioned in dorsal recumbency, with the forelimbs pulled caudally to expose the ventral neck and thoracic inlet region. An incision was made over the left external jugular vein, and the cephalic vein and the omocervical artery were ligated and divided. This facilitated exposure of the thoracic duct ampulla at its entrance into the jugular vein, as well as the portion of the duct traveling into the thoracic inlet. Any branches entering the duct in this region were ligated. The duct was cannulated close to the ampulla with a 0.03" inner diameter silastic catheter which was tunneled under the skin to a small incision over the animal's shoulder region and exteriorized. Similarly for the sham operated control animals, the same procedures were performed to separate the thoracic lymph duct without the actual cannulation. Following anesthetic recovery, the cannula was connected to a sterile fluid collection bag containing 1000 IU heparin. Saline solution (200-400 mL) was given subcutaneously to facilitate lymph flow and to prevent dehydration. The frequency of the saline supplement was determined by the veterinary staff. At the conclusion of the study, catheters were removed percutaneously with no adverse effects on the animal. The lymph flow rate in dogs...
ranged between 8-40 mL/hr with an average flow of 23.1 mL/hr (Edwards GA et al., 2001).

The lymph duct-cannulated dogs and the corresponding control dogs were administered $[^{3}H]$MRL-1 subcutaneously (3 mg/kg, 0.5 mL/kg) at the popliteal region of the dog’s hind limb, on the caudal aspect of the knee, or as a bolus IV (1 mg/kg, 0.5 mL/kg) via a jugular vein cannula. The carotid artery of all animals was also cannulated for blood collection. Blood samples were collected pre-dose, 5 (IV only), 15, 30 min and at 1, 2, 4, 8, 24, 48, 72, 96, 120 hr in heparinized tubes, and were spun in a centrifuge at 1600 $\times$ g at 4°C for 10 min to obtain plasma. Lymph fluid samples were collected over the following periods: 0-1, 1-2, 2-4, 4-6, 6-8, 8-24, 24-26, 26-28, 28-30, 30-32, 32-48, 48-72, 72-96, 96-120 hr. Urine and feces samples were collected at 0-4, 4-8, 8-24 hr then once a day during the study period. The weights of lymph fluid, urine samples were also recorded. The plasma, lymph and urine samples were stored at -80°C until analysis.

The LDC rat model. Male Sprague-Dawley rats (~350 g) were surgically prepared ~48 hr prior to dosing (Edwards et al., 2001; Ionac, 2003; Kagan et al., 2007). Rats were anesthetized in a 5% isoflurane induction chamber with oxygen flow rate at 1 L/min. The surgical areas were shaved and prepared for aseptic surgery with Durprep® (3M). After transferring it to a nose cone, the isoflurane concentration was reduced to 2%. Rats were given 0.5-1.0 ml of olive oil by gavage ~0.5 hour prior to the operation for visualization of lymph vessels. After setting up a sterile surgical field, a midline abdominal incision was made approximately two thirds of the length of the abdomen to the xiphoid cartilage. The liver and the gut were mobilized (pulled upwards and to the right, respectively), and were kept moist using warm, saline-soaked gauze. The thoracic
duct and dorsal aorta were located using cotton-tipped applicators to gently pull apart the connective tissue. The dorsal aorta was separated from the psoas muscle, exposing the thoracic duct. The duct was then separated from the psoas muscle and dorsal aorta using fine forceps and cotton-tipped applicators. The duct was cannulated (3-5 mm) with a heparin (500 units/ml) saline filled catheter (described as below). Once the lymph flow had been established, the cannula was tied down by 5-0 silk ligatures. If a proper seal could not be achieved with ties, tissue adhesive would be placed over the ligatures. The cannula was then tunneled subcutaneously around the back and exteriorized at the back of the neck. A spring tether system was installed and attached to a swivel to allow free movement of the animal. The abdominal muscles were then closed by using 4-0 PDS-II sutures. The skin was closed using sterile wound clips or non-absorbable sutures (3-0 Silk). For the sham operated control animals, the same procedures were performed to separate the thoracic lymph duct without the actual cannulation.

A modified catheter system was used which has two catheters combined with a mixing chamber (Wang et al., 2010). Lymph fluid flows from the cannula to the perforation in catheter one, then enters the mixing chamber, which encases the perforated portion of catheter one. Micro infusion of heparin (500 units/ml) was applied from the second catheter into the mixing chamber at a constant flow rate of 50 μL/hr to prevent the lymph fluid coagulation and maintain a continuous flow. Following the surgical procedures, the rats were monitored during the recovery period until they regained full consciousness. Electrolyte replacement solution (PRANG, Bio-Serv) along with water was supplied to the rats in order to encourage fluid intake and to facilitate overall lymph flow. Rats were allowed to recover and stabilize for approximately 48 hours prior to
dosing. Lymph fluid was continuously collected during this period; the lymph flow rate in rats ranged between 0.8-3.4 mL/hr with an average flow of 2.2 mL/hr (Edwards GA et al., 2001).

The lymph duct-cannulated rats and the corresponding control rats were administered $[^{3}H]$MRL-1 subcutaneously (3 mg/kg, 0.5 mL/kg) at the lateral lower part of the left hind limb, or IV administered (1 mg/kg, 0.5 mL/kg) as a bolus via a femoral vein cannula. The carotid artery of all animals was also cannulated for blood collection. Blood samples were collected pre-dose, at 5 (IV only), 15, 30 min and 1, 2, 4, 8, 24, 48, 72, 96, 120, 144, 168 hr in heparinized tubes. The blood samples were spun in a centrifuge at 1600×g at 4°C for 10 min to obtain plasma. Lymph fluid samples were collected over the following periods: 0-1, 1-2, 2-4, 4-6, 6-8, 8-24, 24-26, 26-28, 28-30, 30-32, 32-48, 48-72, 72-96, 96-120, 120-144 and 144-168 hr. Urine and feces samples were collected at 0-4, 4-8, 8-24 hr then once a day for a week in pre-weighed plastic bottles. The plasma, lymph, urine and feces samples were stored at -80°C until analysis.

In vivo metabolism in plasma and at the site of injection in dog and rat. In order to determine the metabolism at the injection site and plasma, intact dogs and rats (n=1/time point/species) were dosed with $[^{3}H]$MRL-1 SC at (3 mg/kg, 0.5 mL/kg) and plasma and injection site skin samples were obtained at predefined time points at 1, 7 and 48 hr post dose in dogs, and 1, 8, 48 and 432 hr post dose in rats. Dogs scheduled for termination were used in this study. Skin samples at the injection site were cut in 3 (rat) or 5 (dog) inch diameter along with subcutaneous tissue. The plasma and skin samples were stored at -80°C until further analysis.
Sample preparation for metabolite analysis in plasma, lymph and injection site. Plasma and lymph samples (2 mL) from dog and rat were mixed with equal volumes of acetonitrile. The mixture was vortex-mixed and spun in a centrifuge at 14,000 x g for 10 min. The extraction recovery of radioactivity was >90%. All operations involving skin samples were conducted at 4°C. Skin samples were thawed, cut into small slices, and transferred into a clean plastic bottle chilled on ice. Water (w/v; 1:3) was added to it and homogenized using a Polytron PT3100 homogenizer (6 x 30 sec). The skin homogenate was extracted with acetonitrile as described above for plasma. The supernatants of plasma and skin extracts were then concentrated under nitrogen and subjected to HPLC separation (see below). The peak fractions were collected, concentrated under nitrogen and treated with 0.5 M NaOH at ambient temperature for one hour to cleave the peptide from 40 kDa PEG. The reaction mixtures were then neutralized and the cleaved peptides were analyzed by LC/MS.

Measurement of Radioactivity. Aliquots of plasma, lymph fluid, skin homogenate and urine were mixed with Ultima-Flo scintillation fluid (Packard Bioscience) and the total radioactivity in these samples was determined by direct counting in a PerkinElmer Tri-Carb 3100 Liquid Scintillation Analyzer. Aliquots of fecal homogenate (in water, 3:1; v/w) were dissolved in two volumes of Solvable and bleached in two volumes of sodium hypochlorite before mixing with Hionic-Fluor and direct counting as described above.

Instrumentation. A Shimadzu HPLC system equipped with two Series LC-10ADVP micro pumps and Series SIL-10ADVP auto sampler was used to obtain the metabolism profiles. Separation of metabolites was achieved on a Phenominex Jupiter
Proteo C12 column (4 µm particle size, 90Å pore size, 4.6×250 mm). Mobile phases A and B consisted of water and acetonitrile respectively, and contained 0.1% of formic acid. The column was eluted with a gradient of 40-50% B in 40 min with a flow rate of 1.5 mL/min. The column was heated at 45°C to obtain better separation of MRL-1 and its metabolites. The column eluate was directed into a β-Ram radiometric detector (In/US Systems, Inc., FL, USA) for on-line radioactivity profiling. When the sample total radioactivity was too low for on-line detection, the column eluate was collected on 96-well scintillation plates, dried under nitrogen, and then read by a 1450 LSC & Luminescence counter (MicroBeta TriLux, PerkinElmer, Inc., MS, USA). For metabolite identification in dog/rat plasma and at the injection site, an Acquity BEH C18 column (2.1X100 mm, 1.7 um particle size; Waters) was used at a flow rate of 0.15 mL/min and heated at 40°C. Mobile phases A and B consisted of water and acetonitrile, respectively, and contained 0.1% of formic acid. The gradient was 0-35% B from 5 to 55 min. The effluent from the LC column was introduced into the ion source of the LTQ-Orbitrap mass spectrometer operated in positive mode. The MS method consisted of a full scan mass analysis over the range m/z 250 to 2000 at a resolving power of 15,000 followed by three FT MS2 scans for the three most intense ions from scan one at a resolving power of 7,500. The LC-MS/MS data was acquired with Xcalibur 2.0 (Thermo Scientific, Inc.) and processed using SEQUEST search in Bioworks 3.3, and Protein Calculator (Thermo Scientific, Inc.). By adding the sequence of the peptide of interest into an existing database, a user defined database was built and used to search against in order to identify any related peptide metabolites.
Results


Figure 2 shows the cumulative recovery of radioactivity in lymph from LDC dogs following SC or IV dosing of $[^3]H$MRL-1. Approximately 73 and 20% of the administered dose was recovered in lymph following SC and IV dosing, respectively (Figure 2A and Table 2). Majority of the radioactivity was collected in lymph within 24-48 hr following SC and IV administration. Figure 2B shows the concentration of $[^3]H$MRL-1 related radioactivity (nM-equivalents) in lymph and plasma following SC dosing in LDC dogs. The radioactivity concentration in lymph during the absorption phase (within 24 hr) was much higher compared to that in plasma, suggesting that the SC administered radioactivity was absorbed directly into the lymphatic system. Figure 3 shows the mean plasma radioactivity concentration-time profiles following SC and IV dosing in LDC dogs, and the exposure (AUC) data is shown in Table 3. Consistent with lymphatic absorption, the systemic exposure ($AUC_{0-120hr}$) to $[^3]H$MRL-1 related radioactivity was reduced by 82% when compared that in sham-operated control dogs following SC dosing (Figure 3A). Following IV dosing, $AUC_{0-120hr}$ to radioactivity was reduced by only 16% in LDC dogs compared to that in sham-operated control dogs (Figure 3B).


Figure 4 shows the cumulative recovery of radioactivity in lymph from LDC rats following SC or IV dosing of $[^3]H$MRL-1. Approximately 27 and 26% of the administered radioactivity was recovered in lymph following SC and IV administration, respectively (Figure 4A and Table 2). Majority of the radioactivity collected in lymph
was recovered within 24-48 hr following SC and IV administration. Figure 4B shows the concentration of $[{}^{3}\text{H}]$MRL-1 related radioactivity (nM-equivalents) in lymph and plasma following SC dosing. The peak concentration in lymph was only about 2 fold higher than that in plasma, suggesting that the lymphatic uptake in rat was not as significant as that observed in dog following SC dosing. The plasma radioactivity concentration-time profiles following SC or IV dosing of $[{}^{3}\text{H}]$MRL-1 in LDC rats are shown in Figure 5, and the AUC data is summarized in Table 3. Following SC dosing, in agreement with the lymphatic recovery of radioactivity, the systemic exposure (AUC$_{0-168\text{hr}}$) to $[{}^{3}\text{H}]$MRL-1 related radioactivity was 44% of that observed in sham-operated control rats (Figure 5A). Following IV dosing, the exposure to radioactivity was 70% of that observed in control rats (Figure 5B), suggesting some recirculation of systemic radioactivity into the lymphatic system.

**Metabolism profiles of $[{}^{3}\text{H}]$MRL-1 in plasma and at the injection site in dog and rat following SC dosing.** The metabolism profile of $[{}^{3}\text{H}]$MRL-1 was evaluated in intact dogs and rats following SC dosing. Figures 6A and 6B depict representative radiochromatograms of plasma at 48 hours from dog and rat, respectively. The major circulating components in dog and rat plasma were the parent peptide $[{}^{3}\text{H}]$MRL-1 and a metabolite M1 at 30 min. The retention time of M1 matched with that of an authentic sample of PEG-linker $[{}^{3}\text{H}]$MRL-2 (Figure 1). Also, base hydrolysis (see below under Metabolite Identification) of the isolated peak fraction of M1 did not produce any detectable aminoacids (data not shown). Based on this data, it was concluded that structure of M1 is identical or similar to $[{}^{3}\text{H}]$MRL-2. However, minor changes to the linker or PEG, for example hydrolysis of maleimide, are difficult to confirm.
Figures 6C, 6D and 6E depict representative radiochromatograms of extracts of SC injection site in dog at 48 hr, and in rat at 8 and 48 hr post dose, respectively. The metabolite profile in dog at 48 hr was qualitatively similar to that in rats at 8 hr. As described below, there were total of 8 metabolites detected at the injection site in dog and rat. These metabolites, along with $[\text{H}]\text{MRL-1}$ and $[\text{H}]\text{MRL-2}$, were resolved into four broad peaks on HPLC analysis. As shown in Figures 6C and 6D, although metabolite profiles in dog and rat injection site were qualitatively similar, they were significantly different quantitatively. Metabolism of $[\text{H}]\text{MRL-1}$ at the injection site in rat was much faster compared to that in dog; only a small amount of intact $[\text{H}]\text{MRL-1}$ was remaining at 8 hr post dose and mostly PEG-linker $[\text{H}]\text{MRL-2}$ was remaining at 48 hr. Analysis of the skin at the SC injection site in rat showed that 62 and 17% of the administered radioactivity was remaining at 24 and 432 hr post dose, respectively. Similar studies in dog showed that 15% of the SC administered dose was remaining at the injection site 24 hr post dose. In agreement with extensive metabolism occurred at the injection site in dogs and rats, the absolute bioavailability of MRL-1 was found to be low (<30%) in both species following SC doing (data not shown).

**Identification of Metabolites.** Due to high mass and polydispersity of 40 kDa PEG, it is difficult to determine accurate mass of the PEGylated derivatives (Webster et al., 2007). Therefore, base catalyzed cleavage of peptide from PEG followed by identification of the cleaved peptide by LC/MS provided a convenient method for the structural determination of metabolites of $[\text{H}]\text{MRL-1}$. The tritium label on the linker was stable and the tritium loss was not observed in any reaction. As described in Figure 7, retro-Michael reaction of MRL-1 produced dehydroalanine derivative of full length
peptide 1-26, suggesting that the truncated metabolites of MRL-1 can also be analyzed using this procedure. The peak fractions (Fractions 1-4, Figure 6C and 6D), purified using HPLC, were treated with base and the resulting peptides were analyzed by LC/MS as described in methods. Molecular weights of the metabolites detected under each peak are summarized in Table 4, and the LC/MS profiles are shown in Figure 8. Fraction 1 (Figure 8A) primarily consisted of intact PEGylated peptide MRL-1, as the HRMS data agreed well with full length 1-26 peptide. Metabolite with 1-25 sequence was also detected in trace amounts. Figure 8B shows that the fraction 2 was a mixture of 3 metabolites, peptides 1-17, 1-18 and 1-20. As shown in figure 8C, there were two major metabolites under fraction 3, 1-12 and 1-15. In addition, 1-13 and 1-16 were identified in trace amounts under fraction 3. Masses of the metabolites determined by LC/MS matched well with the corresponding theoretical mass for each sequence (Table 4). Summary of metabolic cleavage sites of the peptide MRL-1 is shown in Figure 9.

**Excretion of [3H]MRL-1 in dogs and rats following SC and IV dosing.**

Amount of [3H]MRL-1 related radioactivity collected in the lymph, and excreted in the urine and feces are summarized in Table 2. In both species, majority of the excreted radioactivity was recovered in the urine. Following IV dosing in LDC dogs, 48 and 20\% of the administered radioactivity was recovered in 120 hr in urine and lymph, respectively. Following SC dosing in LDC dogs, total 80\% of the radioactivity was recovered in 120 hr, 73\% in the lymph and 7\% in the urine. Radioactivity in the dog feces was not determined. Following IV dosing in rats, 57 and 8\% of the administered radioactivity was recovered in 144 hr in urine and feces, respectively. However, following SC dosing, only 30 and 9\% of the administered radioactivity was recovered in
144 hr in urine and feces, respectively. Figure 10A depicts the representative radiochromatogram of rat urine extract, and Figure 10B shows the radiochromatogram of authentic standards of [3H]MRL-1 and [3H]MRL-2. Figure 10A shows that [3H]MRL-1 was not excreted intact and there were primarily two peaks, M1 and M2. On the basis of comparison of M1 retention time with that of an authentic standard (Figure 10B), the structure of M1 was tentatively identified as MRL-2 (Figure 1). The structure of M2, which is likely to be a truncated PEGylated peptide, was not further explored.

**Discussion**

We have used a PEGylated peptide [3H]MRL-1 (MW 46 kDa) to determine the lymphatic uptake in LDC dogs and rats. MRL-1 is a 26 amino acid peptide covalently attached to 40 kDa PEG using a maleimide linker. Linker region was selected for tritium labeling because in the event peptide portion is truncated or completely cleaved at the site of injection, the label remains on the PEG-linker (MW ~42 kDa) whose uptake can still be monitored by following the radioactivity. Results obtained using LDC dogs showed that majority of the SC administered radioactivity was absorbed via the lymphatic system. In consistent with this data, radioactivity concentration in the collected lymph was far greater than that detected in plasma, and the plasma AUC decreased proportionately when compared to that in sham-operated control dogs. These results demonstrating predominant lymphatic absorption of a 46 kDa molecule in LDC dogs agree well with those previously reported in LDC sheep and dog (Supersaxo et al., 1990; Charman et al., 2000; McLennan et al., 2005; Kota et al., 2007; Wang et al., 2012).
The results obtained from LDC dogs following IV dosing suggest that the large molecules can travel from systemic circulation to the lymphatic system through interstitial space to a limited extent, as observed previously with pegylated polylysine dendrimers (22-68 kDa) in a rat model (Kaminskas et al., 2009). These results also likely reflect a process during which protein and peptide drugs transport from the blood circulation to the site of action in target tissues to exert their pharmacological activities. Macromolecules in blood can be 'pushed out' across vascular walls along with fluid due to pressure and concentration gradients, and then distribute into the interstitium. Protein drug molecules can then pass through the extracellular matrix by diffusion to the cell surface target, or they are reabsorbed back into the lymph circulation (Audus K.L. and Raub T.J., 1993; Wiig et al., 2008).

While \[^{3}\text{H}\]MRL-1 was primarily absorbed by lymphatic system in dogs following SC dosing, data obtained in rats suggest that there could be SC anatomical differences between these species. Only about 27% of the SC dose administered was collected in thoracic lymph in LDC rats. This apparent discrepancy led us to conduct further analyses of the dose remaining at the injection site in rats to estimate the extent of absorption. The results suggested that only about 50% of the SC dose was absorbed during the lymph collection period of 168 hr in rat. Therefore, 27% of the SC administered dose (or ~54% of the absorbed dose) recovery in thoracic lymph agrees well with the corresponding reduction in AUC (~56%) in lymph duct cannulated rats. These data suggest that lymphatic uptake and vascular absorption are equally feasible in the SC absorption of MRL-1 in rats. Similar results were reported for polylysine dendrimers in LDC rats (Kaminskas et al., 2009). However, in contrast to these results, lymphatic uptake was a
minor component in the absorption of human tumor necrosis factor (17 kDa) and human erythropoietin alfa (30.4 kDa) in LDC rats (Kojima et al., 1988; Kagan et al., 2007). Evidently, experiments conducted in LDC rats by various research groups produced contradictory results on the role of lymphatic system in the SC absorption of large molecules. Additional studies are required to understand the anatomical differences between lymphatic system of rodents and higher species, and to determine if 16 kDa MW cut off, suggested based on sheep model, is also applicable in rodents for predominant lymphatic uptake.

Subcutaneous bioavailability of biologic drugs varies significantly (Tang et al., 2004; Richter et al., 2012). Metabolism in the subcutaneous space and during lymphatic transport have been suggested to be some of the reasons for <100% bioavailability. Results obtained in the present study provide direct evidence for the loss of drug in the subcutaneous space in dogs and rats. It is interesting to note that [³H]MRL-1 was metabolized at significantly higher rate at the injection site in rat compared to that in dog. On the basis of amino acids present at the cleavage sites, we can speculate that trypsin, chymotrypsin and other endopeptidases may be responsible for metabolism. Currently, there is a lack of reliable information on the expression, distribution and substrate specificity of these enzymes in the SC space and species differences in any of these attributes (Woodley JF, 1994; Olsen et al., 2004). Absorption and metabolism data described above seem to suggest that [³H]MRL-1 is absorbed at a faster rate (in dogs) than the metabolites (in rats) which mainly constituted PEG-linker with or without truncated peptide. It is possible that absorption of PEG is influenced by physicochemical properties (such as charge, size etc.) of the attached protein (Richter et al., 2012). Above
data also suggest that the extent of metabolism at the injection site may influence over all absorption rate and route (lymphatic vs. vascular uptake) of large molecules. A limitation of the present work is the inability to assess the bioavailability of MRL-1 based on total radioactivity due to metabolism and other factors. However, in agreement with these findings, a separate PK study indicated low absolute bioavailability of MRL-1 (<30%) in dogs and rats following SC dosing, and bioavailability in rats tended to be even lower than that in dogs (Zou et al., unpublished data). Allometric scaling of preclinical PK is often used to estimate human PK and dose of biologic drugs without consideration of their metabolism in the SC space. Potential species-specific metabolism in the SC space underscores the uncertainty in these extrapolations from preclinical data. Therefore, information obtained from in vitro metabolism studies in tissue preparations such as skin homogenate, hepatocytes and kidney cell membranes, from preclinical species and human may be utilized for cross species comparison. Drug loss within the lymphatic system also has been reported. Based on the experiments conducted in peripheral and central duct-cannulated sheep, it was concluded that loss of human growth hormone within the lymphatics contributed significantly to its reduced bioavailability following SC administration (Charman et al., 2000). However, in the present study, metabolism profiles in lymph and plasma, and in comparison to those at the injection site, suggest that MRL-1 clearance within the lymphatics is not significant.

PEGylation is one of the main approaches explored for improving the plasma half-life of biotherapeutics (Harris et al., 2001; Veronese and Pasut, 2005; Veronese and Mero, 2008). Although currently there are several marketed protein drugs which exploit PEGylation strategy, only limited data is available on the mechanism of their elimination.
Excretion of \(^{3}\text{H}\)MRL-1 related radioactivity was slow in dogs and rats. However, in rats, it was evident that elimination of \(^{3}\text{H}\)MRL-1 was mainly through metabolism followed by excretion of metabolites in the urine. As it can be predicted based on the metabolites described above, the peptide portion of MRL-1 was the main target for the hydrolytic enzymes, ultimately leading to the excretion of 40 kDa PEG. It is difficult to determine if PEG itself was a target for metabolizing enzymes and if any minor changes occurred on the PEG scaffold before excretion (Herold et al., 1989; Veronese and Pasut, 2005). It is well recognized that glomerulus in the kidney filters substances mainly based on their molecular size, shape and charge. Large molecules with molecular weight >70 kDa or molecular sizes >40 Å are mostly restricted from passing through glomerulus and eliminated by other pathways such as liver uptake, proteolytic digestion and by immune system (Brenner et al., 1978; Takakura et al., 1987; Yamaoka et al., 1994; Hamidi et al., 2008). Since peptide in MRL-1 contains only 26 amino acids, 40 kDa PEG was thought to play dominating role in distribution and elimination of the conjugate. Due to extensive hydration, the hydrodynamic volume of 40 kDa is expected to be much higher than that of an equal size globular protein. Nevertheless, high polymer flexibility and deformity, together with its rod-like conformation allow for glomerular filtration, albeit at low rate. Following glomerular filtration, MRL-1 is likely degraded through hydrolysis by brush border enzymes located on the luminal membrane, or endocytosis and subsequent hydrolysis. Excretion details for few PEGylated biologics have been reported. Limited data from SDS-PAGE analysis indicated that only '40 kDa material' was present in the rat urine following 400 mg/kg SC dosing of cerolizumab, a 40 kDa PEGylated TNFα-antibody. Similarly, following IV dosing of \(^{14}\text{C}\)IFNα-2a (Pegasys) in rats, majority of
excreted radioactivity was detected in the urine (51% of the dose), which mostly contained degraded interferon fragments and 40 kDa PEG (Modi et al., 2000). In contrast, PEGylated peptide-based erythropoiesis-stimulating agent peginesatide (45 kDa) was excreted mostly intact in the urine in rats and monkeys (Woodburn et al., 2012; Woodburn et al., 2013).

In summary, this study has described the significance of lymphatic system in SC absorption of a 40 kDa PEGylated peptide MRL-1 in dog and rat. Data showed the loss of drug at the injection site and potential species difference in enzymatic activity in the SC space. MRL-1 peptide was metabolized while still attached to PEG and excreted in the urine as metabolites.
Authorship Contributions

Participated in research design: Reddy, Zou, Bateman, Adreani, Chris Johnson, Cunningham, Shen

Conducted experiments: Zou, Cunningham, Trinh, Shen, Wang, Hong, Christine, Nunes

Contributed new reagents or analytic tools: Reddy, Zou, Bateman, Zhang, Braun, Staskiewicz

Performed data analysis: Reddy, Zou, Bateman, Kumar

Wrote or contributed to the writing of the manuscript: Reddy, Zou, Bateman, Kumar, Adreani, Shen
References


Modi MW, Fulton JS and Buckmann DK (2000) Clearance of pegylated (40 kDa) interferon α-2a (Pegasys) is primarily hepatic. Hepatology 32, No 4, Pt. 2, #848.


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Footnotes

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Figure Legends

Figure 1. Chemical structures of $[^3\text{H}]$MRL-1 and $[^3\text{H}]$MRL-2

Figure 2. Cumulative recovery (2A) and concentration (2B) of $[^3\text{H}]$MRL-1 related radioactivity in dog lymph. Lymph duct-cannulated dogs were dosed with $[^3\text{H}]$MRL-1 at 3 mg/kg SC (▲) or 1 mg/kg IV (■), and the radioactivity in the collected lymph (■) and plasma (□) was measured using Liquid Scintillation analyzer as described in the text.

Figure 3. Mean plasma radioactivity concentration versus time profiles in lymph duct-cannulated (○) and non-cannulated control dogs (●) following administration of $[^3\text{H}]$MRL-1 at 3 mg/kg SC (3A) or 1 mg/kg IV (3B). Radioactivity in plasma was measured using Liquid Scintillation analyzer as described in the text.

Figure 4. Cumulative recovery (4A) and concentration (4B) of $[^3\text{H}]$MRL-1 related radioactivity in rat lymph. Lymph duct-cannulated rats were dosed with $[^3\text{H}]$MRL-1 at 3 mg/kg SC (▲) or 1 mg/kg IV (■), and the radioactivity in the collected lymph (■) and plasma (□) was measured using Liquid Scintillation analyzer as described in the text.

Figure 5. Mean plasma radioactivity concentration versus time profiles in lymph duct-cannulated rats (○) and non-cannulated control rats (●) following administration of $[^3\text{H}]$MRL-1 at 3 mg/kg SC (5A) or 1 mg/kg IV (5B). Radioactivity in plasma was measured using Liquid Scintillation analyzer as described in the text.

Figure 6. Representative HPLC radio chromatograms of plasma and injection site skin extracts from dogs and rats following SC administration of $[^3\text{H}]$MRL-1: (A) dog plasma at 48 hr, (B) rat plasma at 48 hr, (C) injection site in dog at 48 hr, (D) injection site in rat at 8 hr, and (E) injection site in rat at 48 hr. Dogs and rats were dosed SC with $[^3\text{H}]$MRL-1 at 3 mg/kg, and plasma and injection site skin tissues (3-5 inch diameter) were extracted and analyzed as described in the text.
Figure 7. Retro-Michael reaction of MRL-1 resulting in the formation of dehydroalanine peptide derivative.

Figure 8. Extracted Ion Chromatograms of metabolites formed at the injection site in rats at 8 hr following SC dosing of [3H]MRL-1. Fractions 1-3 shown in Figure 6D were purified, and treated with NaOH to cleave PEG. The cleaved peptides were then extracted and analyzed by LC-MS/MS as described in the text.

Figure 9. Metabolic sites of [3H]MRL-1

Figure 10. Representative radiochromatogram of extract of urine from rats following IV dosing of [3H]MRL-1 (10A). Rats were dosed IV with [3H]MRL-1 at 3 mg/kg, and urine was collected up to 168 hr. Urine was extracted and analyzed by HPLC with fraction collection as described in the text. Radiochromatogram of authentic standards of [3H]MRL-1 and [3H]MRL-2 are shown in 10B.
Table 1

Treatment groups, administered doses and collected samples in rats and dogs

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>Dose (mg/kg)</th>
<th>Treatment Group</th>
<th>Rat</th>
<th>Radioactivity in dose (uCi/mL)</th>
<th>Dose volume (mL/kg)</th>
<th>Number of animals</th>
<th>Samples collected</th>
<th>Dog</th>
<th>Radioactivity in dose (uCi/mL)</th>
<th>Dose volume (mL/kg)</th>
<th>Number of animals</th>
<th>Samples collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>3</td>
<td>LDC</td>
<td>75</td>
<td>Plasma, lymph, urine, feces</td>
<td>0.5</td>
<td>6</td>
<td></td>
<td>10</td>
<td>Plasma, lymph, urine</td>
<td>0.5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>75</td>
<td>Plasma, urine, feces</td>
<td>0.5</td>
<td>3</td>
<td></td>
<td>10</td>
<td>Plasma</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1</td>
<td>LDC</td>
<td>75</td>
<td>Plasma, lymph, urine, feces</td>
<td>0.5</td>
<td>8</td>
<td></td>
<td>10</td>
<td>Plasma, lymph, urine</td>
<td>0.5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>75</td>
<td>Plasma, urine, feces</td>
<td>0.5</td>
<td>6</td>
<td></td>
<td>10</td>
<td>Plasma</td>
<td>0.5</td>
<td>2</td>
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Table 2

<table>
<thead>
<tr>
<th>Dose</th>
<th>Group</th>
<th>Percent of administered dose recovered</th>
<th></th>
<th></th>
<th></th>
<th></th>
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<tr>
<td></td>
<td></td>
<td>Rat</td>
<td>Dog</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lymph</td>
<td>Urine</td>
<td>Feces</td>
<td>Total</td>
<td>Lymph</td>
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<tr>
<td>SC (3 mg/kg)</td>
<td>LDC</td>
<td>26.7 ± 9.0</td>
<td>19.1 ± 1.8</td>
<td>5.6 ± 3.9</td>
<td>51.4</td>
<td>72.9</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>/</td>
<td>29.8 ± 0.4</td>
<td>8.6 ± 6.4</td>
<td>38.4</td>
<td>/</td>
</tr>
<tr>
<td>IV (1 mg/kg)</td>
<td>LDC</td>
<td>26.4 ± 3.6</td>
<td>30.5 ± 14.9</td>
<td>4.6 ± 2.9</td>
<td>61.5</td>
<td>20.3 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>/</td>
<td>57.3 ± 22.0</td>
<td>8.0 ± 2.0</td>
<td>65.3</td>
<td>/</td>
</tr>
</tbody>
</table>
## Table 3


<table>
<thead>
<tr>
<th>Dose</th>
<th>Group</th>
<th>Rat AUC(_{0-168\text{hr}}) (µM·hr)</th>
<th>% of Control</th>
<th>Dog AUC(_{0-120\text{h}}) (µM·hr)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>LDC</td>
<td>7.7 ± 2.5</td>
<td>44</td>
<td>6.4 ± 5.6</td>
<td>18</td>
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<tr>
<td></td>
<td>Control</td>
<td>17.4 ± 2.2</td>
<td>100</td>
<td>35.2</td>
<td>100</td>
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<tr>
<td>IV</td>
<td>LDC</td>
<td>4.4 ± 0.8</td>
<td>70</td>
<td>32.3 ± 4.5</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>6.3 ± 0.5</td>
<td>100</td>
<td>38.6</td>
<td>100</td>
</tr>
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</table>
Table 4
Accurate masses of truncated (pegylated) peptide metabolites of MRL-1 identified in vivo*

<table>
<thead>
<tr>
<th>MRL-1 peptide and metabolites</th>
<th>Sequence</th>
<th>Calculated exact mass (Da)</th>
<th>Calculated monoisotopic m/z</th>
<th>Observed monoisotopic m/z</th>
<th>Mass error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-26</td>
<td>CFRVDEEFQS PFASQSRGYF LFRPRN-NH2</td>
<td>3189.5533</td>
<td>798.3956 (4+), 1064.1917 (3+)</td>
<td>798.3919 (4+)</td>
<td>5</td>
</tr>
<tr>
<td>1-25</td>
<td>CFRVDEEFQS PFASQSRGYF LFRPR-OH</td>
<td>3076.4944</td>
<td>770.1309 (4+), 1026.5054 (5+)</td>
<td>770.1314 (4+), 1026.5060 (3+)</td>
<td>1</td>
</tr>
<tr>
<td>1-20</td>
<td>CFRVDEEFQS PFASQSRGYF-OH</td>
<td>2407.0869</td>
<td>803.3696 (3+), 1204.5507 (2+)</td>
<td>803.3700 (3+), 1204.5518 (2+)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>1-18</td>
<td>CFRVDEEFQS PFASQSRG-OH</td>
<td>2096.9552</td>
<td>699.9923 (3+), 1049.4848 (2+)</td>
<td>699.9921 (3+), 1049.4851 (3+)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>1-17</td>
<td>CFRVDEEFQS PFASQSR-OH</td>
<td>2039.9337</td>
<td>680.9852 (3+), 1020.9741 (2+)</td>
<td>680.9853 (3+), 1020.9744 (3+)</td>
<td>1</td>
</tr>
<tr>
<td>1-16</td>
<td>CFRVDEEFQS PFASQS-OH</td>
<td>1883.8326</td>
<td>628.9515 (3+), 942.9236 (2+)</td>
<td>628.9513 (3+), 942.9240 (2+)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>1-15</td>
<td>CFRVDEEFQS PFASQ-OH</td>
<td>1796.8006</td>
<td>599.9408 (3+), 899.4076 (2+)</td>
<td>599.9409 (3+), 899.4079 (2+)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>1-13</td>
<td>CFRVDEEFQS PFA-OH</td>
<td>1581.7080</td>
<td>528.2439 (3+), 791.8623 (2+)</td>
<td>791.8624 (2+)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>1-12</td>
<td>CFRVDEEFQS PF-OH</td>
<td>1510.6729</td>
<td>504.5649 (3+), 756.3437 (4+)</td>
<td>756.3437 (2+)</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* Truncated peptide was cleaved from PEG in each metabolite using a chemical procedure and analyzed by LCMS. Amino acid ‘C’ in each sequence refers to modified cysteine as shown in Figure 7.
Figure 1

MRL-1

\[
\text{FRVDEEFQSPFASQRGYSFRPRN-NH}_2
\]

MRL-2
Figure 2

A

Cumulative recovery of [3H]MRL-1 radioactivity in lymph (% of dose)

Time (hr)

B

[3H]MRL-1 (nM-eq)

Time (hr)
Figure 3

A

B

[\textsuperscript{[3]}H]MR-L-1 radioactivity in plasma (nM eq.

Time (hr)
Figure 4

A

Cumulative recovery of [3H]MRL-1 radiolabelling in lymph (% of dose)

Time (hr)

0 24 48 72 96 120 144 168

B

[3H]MRL-1 (nM-eq)

Time (hr)

0 24 48 72 96 120 144 168
Figure 5

A

B

[Figure description: Graph A shows two lines representing [H]MRL-1 radioactivity in plasma (nM-sec) over time (0-168 hours) with error bars. Graph B shows the same data with a different scale or time frame.]
Figure 6

A. Dog plasma at 48 hr

B. Rat plasma at 48 hr

C. Dog skin at 48 hr

D. Rat skin at 8 hr

E. Rat skin at 48 hr
Figure 7

\[ \text{peptide} \xrightarrow{\text{NaOH}} \text{peptide} + PEG(40\text{kDa}) \]
Figure 8

A  Fraction 1
(RT=16 min)

B  Fraction 2
(RT=21 min)

C  Fraction 3
(RT=25 min)

Relative Abundance

Time (min)