Pharmacokinetics of Recombinant Human Leukemia Inhibitory Factor in Sheep

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ABSTRACT

The pharmacokinetics of recombinant human leukemia inhibitory factor (rhLIF) were investigated following i.v. and s.c. administration of a wide range of dose levels. Parallel studies were conducted where single i.v. bolus doses of 12.5, 25, 100, 250, 500, or 750 µg/kg rhLIF (n = 2) or s.c. doses of 10, 20, or 50 µg/kg rhLIF (n = 4) were administered to sheep. Blood samples were collected for up to 24 h postdosing, and the plasma concentrations of rhLIF were analyzed by enzyme-linked immunosorbent assay. Non-compartmental analysis demonstrated an increase in the terminal elimination half-life (from 0.27 to 2.29 h) and a decrease in systemic clearance (from 5.18 to 1.09 ml/min/kg) with increasing i.v. doses of rhLIF, suggesting nonlinear pharmacokinetic behavior. A greater than proportional increase in the area under the plasma concentration-time curve with dose also indicated significantly nonlinear pharmacokinetics after s.c. administration. A mechanistic compartmental model was developed to characterize the pharmacokinetics of rhLIF. The key feature of the model accounting for the nonlinear pharmacokinetic behavior of rhLIF was high-affinity, saturable receptor binding and subsequent cellular internalization and degradation. The apparent total density of LIF cell surface receptors and receptor turnover dynamics were included in the model, along with nonspecific binding and linear elimination from the systemic circulation. The absorption of rhLIF from the s.c. injection site into the systemic circulation was characterized by a first-order absorption process via a delay compartment. The proposed model satisfactorily captured the complex pharmacokinetic profiles of rhLIF following both i.v. and s.c. administration.

Leukemia inhibitory factor (LIF) is a 180-amino acid glycoprotein of the interleukin-6-type cytokine family. The name LIF was originally derived from the ability of the cytokine to induce macrophage maturation and suppress the clonogenicity of the murine monocytic leukemia cell line M1 (Gearing et al., 1987). LIF acts on a wide range of cell types and displays remarkable functional diversity. LIF has been demonstrated to play a role, either directly or synergistically, in hematopoiesis, thrombopoiesis, reproduction, bone metabolism, inflammatory responses, and neuroproliferation (Hilton and Gough, 1991a; Waring, 1997); consequently, a number of potential therapeutic uses for LIF have been proposed. Initial clinical interest focused on its use in neurological conditions, particularly chemotherapy-induced peripheral neuropathy (Kurek, 2000). Currently, LIF is under investigation for the treatment of infertility, as it has been shown to enhance embryonic implantation (Stewart et al., 1992).

Native LIF produced by mammalian cells is highly basic (pI = 9.15) and has a reported molecular weight in the range of 32 to 67 kDa. The heterogeneity in molecular weight has been attributed to extensive and variable glycosylation (Hilton, 1992). The recombinant form of human LIF (rhLIF) produced in Escherichia coli is not glycosylated and has a molecular weight of 19.71 kDa. Glycosylation does not seem to be necessary for the biological actions of LIF (Williams et al., 1988), although it may alter the stability of the molecule both in vitro and in vivo (Hilton et al., 1991b; Yamamoto-Yamaguchi et al., 1992). LIF exerts its actions by binding to a specific cell surface receptor complex made up of the LIF receptor β chain and the gp130 receptor chain (Gearing et al., 1992). When LIF binds to the receptor complex, the two receptor components dimerize, initiating signal transduction.

Very few reports describe the pharmacokinetics of LIF. An initial study in mice showed that when administered intravenously at a single dose level, the plasma concentrations of...
murine LIF declined in a biexponential manner, with a rapid initial distribution phase and a more prolonged terminal phase (Hilton et al., 1991b). Recent data obtained in patients with advanced cancer indicated that the absorption of rhLIF after s.c. administration was variable, with maximum plasma concentrations occurring 10 to 120 min postdosing (Gunawardana et al., 2003). The terminal phase half-life of approximately 2 h seemed to be independent of dose; however, there was a disproportionate increase in AUC and $C_{\text{max}}$ with dose indicating nonlinearity in the pharmacokinetics of rhLIF. The authors suggested that the nonlinearity was probably due to increased bioavailability of rhLIF at higher s.c. doses but also recognized that this finding may be a consequence of reduced clearance.

Several studies have attributed nonlinear disposition of cytokines to their high-affinity, low-capacity binding to pharmacological targets on cell surfaces followed by internalization and subsequent degradation (Sugiyama and Hanano, 1989; Mager and Jusko, 2001). Protein clearance by receptor-mediated endocytosis has been demonstrated for a number of proteins that exhibit nonlinear plasma kinetics, including erythropoietin (Chapel et al., 2001), granulocyte colony stimulating factor (Terashi et al., 1999), and hepatocyte growth factor (Liu et al., 1995). Additional mechanisms such as nonspecific renal and hepatic elimination processes and proteolytic degradation may also contribute to the removal of cytokines from the systemic circulation (Ferraiolo et al., 1992).

The purpose of the present study was to evaluate the pharmacokinetics of rhLIF administered intravenously and subcutaneously over a wide range of dose levels. Sheep were chosen as the study species because sheep and human LIF display a high degree of amino acid sequence homology (88%) and structural identity (Willson et al., 1992). This feature is important in obtaining pharmacokinetic data reflective of that in humans, because the binding of cytokines to pharmacological receptors may strongly influence disposition (Sugiyama and Hanano, 1989). Previous studies with recombinant human proteins including human growth hormone (Charman et al., 2000) and leptin (McLennan et al., 2003) have demonstrated the utility of the sheep model to produce pharmacokinetic parameters comparable to those in humans. Using the data generated in the present study, a mechanistic model was developed to describe the pharmacokinetics of rhLIF. The model aided interpretation of the likely mechanisms of absorption and clearance of rhLIF and explored the dose ranges over which the different clearance mechanisms predominate.

**Materials and Methods**

**Animals.** The animal studies were approved by The University of Melbourne Animal Experimentation Ethics Committee and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Adult merino wether sheep weighing between 41 and 69 kg were supplied by the Victorian Institute of Animal Science (Werribee, Australia). During the pharmacokinetic studies, sheep were housed in metabolism cages, and food and water were available ad libitum.

**Experimental Procedures.** A parallel study design was selected to explore the pharmacokinetics of rhLIF after i.v. and s.c. administration. Six groups of two sheep each received a single i.v. bolus rhLIF dose of 12.5, 25, 100, 250, 500, or 750 µg/kg into the jugular vein. Three groups of four sheep received s.c. rhLIF injected into the interdigital space of the hind leg at a single dose of 10, 20, or 50 µg/kg. All animals had a 16-gauge, 135-mm Angiocath i.v. catheter inserted into the jugular vein (contralateral to that used for i.v. injection) to facilitate sampling of systemic blood.

**Formulation of rhLIF.** Recombinant human LIF was provided by Amrad Corporation Limited (Melbourne, Australia) as a stock solution containing 2.78 or 3.08 mg/ml rhLIF in 2 mM phosphate buffer. Undiluted stock solution was used to administer i.v. doses of 100, 250, 500, and 750 µg/kg rhLIF. Dosing solutions for the i.v. administration of 12.5 or 25 µg/kg doses were prepared by diluting rhLIF stock solution 5- or 3-fold, respectively, with 2 mM phosphate buffer. This dilution was necessary to provide an adequate volume to accurately draw up and inject. For s.c. administration, doses were formulated as neutral, isotonic solutions with a constant dosing volume of 1.5 ml. The concentration of rhLIF in the formulations for s.c. injection varied from 0.3 to 1.6 mg/ml depending on the dose administered and the weight of the individual sheep. Dilutions and formulation adjustments were conducted on the morning of the study, and all dosing solutions were sterile-filtered through a 0.22-µm Millex-GV syringe filter (Millipore Corporation, Billerica, MA) immediately prior to administration.

**Sample Collection.** Predosing blood samples were collected from each animal immediately prior to rhLIF administration. After i.v. injection, blood samples were withdrawn via the indwelling jugular vein catheter at 1, 3, 6, 15, 30, and 45 min and 1, 1.5, 2, 2.5, and 3 h. The sampling then continued at 2- to 3-h intervals depending on the dose administered. The total number of sample time points ranged from 10 to 25, corresponding to the lowest and highest i.v. doses, respectively. When rhLIF was given by the s.c. route, blood samples were withdrawn at 0.5, 1, 1.5, 2, 2.5, 3, 4.5, 6, 8, 10, and 12 h postdosing. Blood (2 ml) was initially withdrawn and discarded to flush the catheter and ensure the collection of circulating blood. A subsequent 3-ml sample of blood was withdrawn and transferred to di-potassium EDTA tubes (Sarstedt, Ingle Farm, Australia). Blood samples were centrifuged at 3000 rpm for 10 min; then the plasma was separated and frozen at −20°C until analysis. Between periods of sampling, the jugular vein catheters were kept patent with a heparin saline flush (10 IU/ml).

**Sample Analysis.** Plasma samples were analyzed for immunoreactive active rhLIF using a commercially available ELISA for recombinant human LIF (Quantikine; R&D Systems, Minneapolis, MN). The ELISA was performed according to the manufacturer’s instructions and was validated for use with a sheep plasma matrix. Analysis of predosing sheep plasma samples indicated the absence of cross-reacting species. Comparison of triplicate spiked plasma samples (50, 500, and 2000 pg/ml) to a calibration curve (31–2000 pg/ml) analyzed in one assay and repeated on three different days, indicated that intra- and interassay precision were less than 15%. The measured concentrations of the spiked plasma samples assessed by backcalculation relative to the calibration curve were within 15% of the nominal concentrations. The limit of quantitation for the assay was defined as the lowest spiked plasma sample (50 pg/ml) that demonstrated acceptable accuracy and precision (<15%). Samples containing rhLIF at concentrations above the ELISA calibration range (2000 pg/ml) were diluted with blank pooled sheep plasma prior to analysis.

**Noncompartmental Pharmacokinetic Analysis.** Noncompartmental analysis of rhLIF pharmacokinetics was performed using WinNonlin Professional version 3.2 (Pharsight Corporation, Mountain View, CA). Individual plasma concentrations and sample times for each animal were used in the analysis. For i.v. administration of rhLIF, the initial plasma concentration ($C_{\text{max}}$), initial distribution volume ($V_{\text{d}}$), terminal slope ($\lambda$), terminal elimination half-life ($t_{1/2}$), total area under the plasma concentration-time curve extrapolated to infinity (AUC), systemic plasma clearance (CL), and steady-state volume of distribution ($V_{ss}$) were calculated by standard methods.
Following s.c. administration of rhLIF, the peak plasma concentration ($C_{\text{max}}$) and the time to $C_{\text{max}}$ ($T_{\text{max}}$) were taken directly from individual profiles. The terminal slope, $t_{1/2}$, and AUC were calculated by standard methods as for the i.v. dosing.

Statistical analysis of the calculated noncompartmental parameters was conducted using a standard statistical software package (SigmaStat; SPSS Inc., Chicago, IL). Differences were considered significant at $p < 0.05$. For the s.c. groups, statistical comparisons of the dose-normalized AUC and $t_{1/2}$ were conducted using a one-way analysis of variance with Tukey’s multiple comparisons. Statistical comparisons for $T_{\text{max}}$ were conducted using a Kruskal-Wallis one-way analysis of variance on ranks.

**Pharmacokinetic Model for rhLIF.** Based on the physicochemical properties of rhLIF and the common absorption and clearance pathways for protein drugs, a mechanistic model was proposed to describe the pharmacokinetics of rhLIF after i.v. and s.c. administration (Fig. 1). The model for rhLIF was based on a generalized pharmacokinetic model for drugs exhibiting target-mediated drug disposition as originally described by Mager and Jusko (2001). This generalized model has successfully been applied to describe the pharmacokinetics of several recombinant cytokines, including interferon-β1a (Mager et al., 2003), vascular endothelial growth factor (Eppler et al., 2002), and thrombopoietin (Jin and Krzyzanski, 2004).

The key feature of the model, and that which imparts nonlinearity to the distribution and elimination kinetics, is high-affinity, saturable binding of rhLIF to specific pharmacological receptors on cell surfaces and subsequent internalization and degradation of the entire rhLIF-receptor complex. In vitro studies have demonstrated that LIF displays typical cytokine binding kinetics characterized by a rapid rate of binding and a slow dissociation rate, with an average equilibrium dissociation constant ($K_d$) of approximately 0.1 nM (Hilton et al., 1988; Godard et al., 1992; Tomida, 2000). Because the density of high-affinity receptors on LIF-responsive cells is relatively low, with an average of 300 binding sites per cell (Godard et al., 1992; Tomida, 2000), LIF binding is a low-capacity process that may readily become saturated. Following receptor binding, the entire LIF-receptor complex is subject to internalization and degradation by lysosomal enzymes (Bower et al., 1995).

In the proposed model, rhLIF enters the systemic circulation (central compartment; $A_{\text{c}}$, $V_{\text{c}}$) directly following i.v. bolus administration or is absorbed from the interstitium following s.c. injection (the absorption model is discussed below). Circulating rhLIF binds to pharmacological receptors ($R$) to form rhLIF-receptor complexes (RC) as described by the second-order association rate constant, $k_{on}$.

The initial quantity of receptors ($R_{\text{init}}$) is modeled as a parameter and allowed to vary for different dose levels to account for receptor up- or down-regulation. Unoccupied LIF receptors are subject to a constant turnover governed by zero-order production ($k_{\text{syn}}$) and first-order degradation ($k_{\text{deg}}$). Following binding, the model allows for dissociation of rhLIF from the receptor according to a first-order dissociation rate constant, $k_{off}$, or for the internalization of the entire rhLIF-receptor complex and degradation by lysosomal enzymes. The collective processes of internalization, intracellular transport, and lysosomal degradation are characterized by a first-order rate constant, $k_{\text{int}}$. Because in vitro studies have demonstrated that the LIF receptor is degraded within the cell, a receptor recycling component is not required in this model (Bower et al., 1995).

In addition to clearance by receptor-mediated endocytosis, rhLIF is also likely to be subject to renal elimination, which is common for proteins with a molecular weight below that of albumin (67 kDa) (Maack et al., 1979). Nonspecific degradation processes by circulating proteases or hepatic catabolism are further potential mechanisms for the clearance of rhLIF (Ferraiolo et al., 1992). These nonspecific, high-capacity elimination processes are included in the proposed model as a single first-order elimination process from the central compartment ($k_{\text{e}}$). The model also includes first-order transfer of rhLIF between the central compartment ($A_{\text{c}}$) and a tissue compartment ($A_{\text{t}}$) to account for extravascular distribution and/or nonspecific drug binding. Initially, the intercompartmental distribution rate constants were estimated as separate parameters; however, the estimates were consistently similar and subsequently fixed to be equal ($k_{\text{int}}$), thus reducing the number of parameters in the model.

The s.c. absorption of rhLIF was modeled as a first-order input process into an absorption delay compartment ($A_{\text{d}}$, $V_{\text{d}}$) and subsequent first-order transfer into the systemic circulation ($A_{\text{c}}$, $k_{\text{syn}}$). The delay compartment was included to reflect diffusion of rhLIF through the interstitium, reversible binding at the injection site, and/or possible transport of rhLIF through the lymphatic system. The first-order rate of transfer from the absorption delay compartment into the systemic circulation ($k_{\text{syn}}$) was allowed to vary with dose to account for dose dependence in the absorption process. The bioavailability of rhLIF was initially modeled as a parameter; however, preliminary analysis indicated that the bioavailability was consistently estimated as close to 100% and therefore fixed to this value (data not shown).

The system can be defined by the following differential equations:
\[
\begin{align*}
\frac{dA_{P(t)}}{dt} &= k_{ab} \cdot A_T - (k_{ab} + k_c) \cdot A_P - \left( \frac{k_m}{V_c} \right) \cdot A_P \cdot R + k_{off} \cdot RC \\
\frac{dA_{P(i.v.)}}{dt} &= k_{a2} \cdot A_D + k_{ab} \cdot A_T - (k_{ab} + k_c) \cdot A_P \\
\frac{dA_T}{dt} &= k_{ab} \cdot (A_P - A_T) \\
\frac{dRC}{dt} &= \left( \frac{k_m}{V_c} \right) \cdot A_P \cdot R - (k_{off} + k_m) \cdot RC \\
\frac{dR}{dt} &= k_{syn} - \left( \frac{k_m}{V_c} \right) \cdot A_P \cdot R + k_{off} \cdot RC - k_{deg} \cdot R \\
\frac{dA_{D(s.c.)}}{dt} &= k_{a2} \cdot D_c \cdot e^{-(k_{a1} \cdot t)} - k_{a2} \cdot A_D \\
\end{align*}
\]

where eqs. 1, 3, 4, and 5 describe the disposition of rhLIF following i.v. administration and eqs. 2, 3, 4, and 5 describe the absorption and disposition of rhLIF administered by s.c. injection. The amounts of rhLIF and receptor in the above equations were modeled in units of nanomoles per kilogram, and the rhLIF plasma concentrations were estimated as \(A_P/V_c\), with conversion to units of nanogram per milliliter.

The proposed pharmacokinetic model was simultaneously fitted to the mean plasma drug concentrations for both the i.v. and s.c. routes of administration and all dose levels to obtain a single set of parameters to characterize the entire data set. The parameters estimated included \(k_{a1}, k_{a2}, k_{abh}, k_c, k_{deg}, k_{int}, R_{int}, V_c\), and \(V_c\). The values for \(k_{syn}\) and \(k_{off}\) were determined as secondary parameters according to the relationships \(k_{syn} = k_{deg} \cdot R_{int}\) and \(k_{off} = k_{int} \cdot K_d\), respectively. Initially, both \(k_{a2}\) and \(k_{off}\) were estimated, providing a calculated \(K_d\) value that was similar to the mean reported literature value of 0.1 nM (Hilton et al., 1988; Godard et al., 1992; Tomida, 2000); thus, \(K_d\) was fixed during the modeling process to reduce the number of parameters to be estimated. Initial parameter estimates were derived from the characteristics of the observed plasma concentration-time profiles as previously described for compounds demonstrating target-mediated drug disposition (Mager and Jusko, 2001). Parameters were estimated using the ADAPT II software (D’Argenio and Schumitzky, 1997) by the maximum likelihood method. The variance model was defined as follows:

\[
\text{VAR}_i = \sigma_i^2 \cdot M(\theta, t)^{E_i} 
\]

where \(\text{VAR}_i\) is the variance of the \(i\)th data point, \(\sigma_i\) and \(\sigma_c\) are the variance parameters \((\sigma_c = 2)\), and \(M(\theta, t)\) is the \(i\)th predicted value from the pharmacokinetic model. Using the final parameter estimates, computer simulations were performed to generate profiles of the fraction of receptors involved in binding as a function of time.

The goodness of fit was assessed by model convergence, visual inspection, examination of the residuals, precision of the parameter estimates, Akaike Information Criteria, Schwarz Criterion, and the estimator criterion value for the maximum likelihood method in ADAPT II.

Results

Noncompartmental Pharmacokinetic Analysis. The mean plasma concentration-time profiles following i.v. and s.c. administration of a wide range of rhLIF doses to sheep are shown in Fig. 2. Administration of the lower i.v. doses resulted in a rapid decline in plasma concentrations, whereas higher rhLIF doses produced more complex profiles characterized by regions of convexity and increasingly prolonged terminal phase half-lives. By visual inspection, the time required to reach the maximum plasma concentration after s.c. administration of rhLIF seemed to be prolonged for the lower dose levels, and the peak concentration was followed by an apparent monoexponential decline in plasma concentrations.

Table 1 reports the average \((n = 2)\) pharmacokinetic parameters obtained by noncompartmental analysis following i.v. administration of rhLIF. There was minimal variability between the two sheep at each i.v. dose level, with individual pharmacokinetic parameters generally differing by less than 20% from average values. Table 2 summarizes the mean parameter estimates \((n = 4)\) generated by noncompartmental analysis of individual s.c. profiles. Following i.v. administration, an increase in dose generally resulted in a proportional increase in \(C_p, t\). There were, however, slight departures from linearity at the two lowest doses where less than proportional increases in \(C_p\) were evident. Consistent with this trend, \(V_c\) was relatively consistent across the four highest i.v. dose levels, with a mean value of approximately 38 ml/kg, but again there were deviations at the two lower doses of 12.5

Fig. 2. Plasma concentration-time profiles of rhLIF following i.v. (A) or s.c. (B) administration. The lines represent the predicted profiles when the proposed model (Fig. 1) was simultaneously fit to the observed mean data for all dose levels and both routes of administration; i.v. doses are 12.5 (○), 25 (○), 500 (○), 500 (□), and 750 (□) µg/kg rhLIF; s.c. doses are 10 (△), 20 (△), and 50 (●) µg/kg rhLIF. Error bars represent the standard error of the mean.
and 25 μg/kg, where the calculated values for $V_c$ were 69 and 51 ml/kg, respectively. The AUC increased with i.v. dose in a greater than proportional manner, with a 10-fold increase in dose (from 25 to 250 μg/kg) resulting in a 15-fold increase in AUC, and there was a corresponding dose-dependent decrease in systemic plasma clearance. $V_m$ was variable over the rhLIF dose range investigated but consistently larger than the volume of the central compartment.

Following s.c. administration, the time to reach the maximum plasma concentration ($T_{max}$) was variable, with individual values ranging from 1 to 6 h (Table 2). Although the median $T_{max}$ seemed to be prolonged at lower doses, statistical analysis revealed no significant difference. Both $C_{max}$ and AUC increased in a greater than proportional manner with dose, with a 5-fold increase in s.c. dose resulting in a 35-fold increase in AUC and a 60-fold increase in $C_{max}$. The dose-normalized values for AUC and $C_{max}$ were statistically different between dosing levels. The terminal half-lives were not statistically different across the three s.c. dose levels but were longer than those observed following i.v. administration, presumably reflecting a slow rate of rhLIF absorption from the injection site (flip-flop pharmacokinetics).

**Pharmacokinetic Model.** The pharmacokinetic profiles generated by simultaneously fitting the data after both i.v. and s.c. administration to the proposed model (Fig. 1) are shown in Fig. 2. The model captured the pharmacokinetic data relatively well for all doses and both routes of administration, although there were slight over-estimations of the observed plasma concentrations following administration of the lower i.v. dose levels. The final model parameters were estimated with good precision, with all but one parameter showing CV% values less than 23% (Table 3). The variance model parameter ($\sigma_2$ in eq. 7) was estimated as 0.35 (6.8 CV%). The estimated volume of the central compartment was 53 ml/kg, which is slightly larger than the physiological plasma volume reported for sheep of 39 ml/kg (Adams and McKinley, 1995). Adequate model fitting necessitated the initial quantity of LIF receptors to vary for the highest i.v. rhLIF dosing level (750 μg/kg), designated $R_{m}$. The estimated degradation rate of free receptor (0.566 h$^{-1}$) was approximately 4 times slower than the rate of internalization and degradation of the rhLIF-receptor complex (2.047 h$^{-1}$). This finding agrees with an in vitro study conducted using murine cells, which indicated that unoccupied (free) receptors were internalized or degraded more slowly (0.42 h$^{-1}$) than occupied receptors (1.86 h$^{-1}$) (Hilton and Nicola, 1992).

When rhLIF was administered subcutaneously, the first-order rate of rhLIF transfer from the s.c. injection site into the absorption delay compartment ($k_{a1}$) was assumed to be the rate-limiting step and was estimated as a single value for all three dose levels. An optimal fit of the s.c. data required that the subsequent rate constant describing transfer of rhLIF from the delay compartment into the systemic circulation ($k_{A2}$) be a different value for each s.c. dose. It is important to note that the absorption rate constants $k_{a1}$ and $k_{a2}$ are not uniquely identifiable, and further studies would be required to determine which is the rate-limiting step for absorption. Because a previous study had indicated that a binding protein for LIF may be present in the extracellular matrix (Mereau et al., 1993), a saturable binding process at the s.c. injection site was included in the model in an attempt to enhance physiological relevance. Inclusion of this additional element, however, resulted in over-parameterization of the model given the limited data available (data not shown).

To ensure that all compartments and elimination processes were necessary to adequately describe the disposition of rhLIF, the proposed model was simplified by sequentially excluding either first-order elimination from the central compartment ($k_e$), nonspecific binding ($k_{nsb}$), or internalization and degradation of the rhLIF-receptor complex ($k_{int}$). In all cases, model simplification resulted in unstable fitting and

<table>
<thead>
<tr>
<th>Dose</th>
<th>$C_{max}$</th>
<th>$T_{max}$</th>
<th>AUC</th>
<th>$t_{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>μg/kg</td>
<td>ng/ml</td>
<td>h</td>
<td>ng·h/ml</td>
<td>h</td>
</tr>
<tr>
<td>10</td>
<td>1.02 (0.16)</td>
<td>3.75 [1.5–6.0]</td>
<td>5.36 (0.5)</td>
<td>1.79 (0.16)</td>
</tr>
<tr>
<td>20</td>
<td>9.79 (0.53)</td>
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<td>27.8 (3.77)</td>
<td>1.81 (0.25)</td>
</tr>
<tr>
<td>50</td>
<td>61.9 (6.94)</td>
<td>1.75 [1–2.0]</td>
<td>187 (32.8)</td>
<td>1.26 (0.06)</td>
</tr>
</tbody>
</table>

Values are reported as mean (standard error).

### Table 3: Final estimated model parameters of rhLIF pharmacokinetics in sheep

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Final Estimate</th>
<th>CV %</th>
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<tbody>
<tr>
<td>$k_{m1}$ (h$^{-1}$)</td>
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<tr>
<td>$k_e$ (h$^{-1}$)</td>
<td>1.54</td>
<td>3.3</td>
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<tr>
<td>$V_c$ (ml/kg)</td>
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<tr>
<td>$k_{nsb}$ (nM$^{-1}$·h$^{-1}$)</td>
<td>11.3</td>
<td>15.5</td>
</tr>
<tr>
<td>$k_{int}$ (h$^{-1}$)</td>
<td>2.05</td>
<td>14.7</td>
</tr>
<tr>
<td>$k_{inc}$ (h$^{-1}$)</td>
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<td>14.3</td>
</tr>
<tr>
<td>$R_m$ (nmol/kg)$^a$</td>
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<td>10.8</td>
</tr>
<tr>
<td>$R_{inc}$ (nmol/kg)$^a$</td>
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<td>$k_{a1}$ (h$^{-1}$)</td>
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<td>$k_{a21}$ (h$^{-1}$)$^b$</td>
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<tr>
<td>$k_{a22}$ (h$^{-1}$)$^b$</td>
<td>3.96</td>
<td>22.6</td>
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</table>

$^a$ $R_m$ value for 750 μg/kg i.v. dose.

$^b$ $k_{a2}$ values vary for each s.c. dose level.

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**Table 1**: Pharmacokinetic parameters determined by noncompartmental analysis following i.v. administration of a range of rhLIF doses

<table>
<thead>
<tr>
<th>Dose</th>
<th>$C_{max}$</th>
<th>$V_c$</th>
<th>AUC</th>
<th>CL</th>
<th>$V_m$</th>
<th>$t_{1/2}$</th>
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<tbody>
<tr>
<td>μg/kg</td>
<td>ng/ml</td>
<td>ml/kg</td>
<td>ng·h/ml</td>
<td>ml/min/kg</td>
<td>ml/kg</td>
<td>h</td>
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<td>12.5</td>
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<td>41.5</td>
<td>5.18</td>
<td>90.9</td>
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<td>70.1</td>
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<td>19209</td>
<td>39.1</td>
<td>11592</td>
<td>1.09</td>
<td>81.0</td>
<td>2.29</td>
</tr>
</tbody>
</table>

$CL$, systemic plasma clearance.

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**Table 2**: Pharmacokinetic parameters determined by noncompartmental analysis for individual animals administered s.c. rhLIF

<table>
<thead>
<tr>
<th>Dose</th>
<th>$C_{max}$</th>
<th>$T_{max}$</th>
<th>AUC</th>
<th>$t_{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>μg/kg</td>
<td>ng/ml</td>
<td>h</td>
<td>ng·h/ml</td>
<td>h</td>
</tr>
<tr>
<td>10</td>
<td>1.02 (0.16)</td>
<td>3.75 (1.5–6.0)</td>
<td>5.36 (0.5)</td>
<td>1.79 (0.16)</td>
</tr>
<tr>
<td>20</td>
<td>9.79 (0.53)</td>
<td>2.5 (1–3.0)</td>
<td>27.8 (3.77)</td>
<td>1.81 (0.25)</td>
</tr>
<tr>
<td>50</td>
<td>61.9 (6.94)</td>
<td>1.75 (1–2.0)</td>
<td>187 (32.8)</td>
<td>1.26 (0.06)</td>
</tr>
</tbody>
</table>
Discussion

The pharmacokinetics of recombinant cytokines are inherently complex and frequently demonstrate nonlinearity (Piscitelli et al., 1997). Consequently, comprehensive studies are required to fully characterize the in vivo behavior of cytokines to enable accurate prediction of their pharmacokinetic properties and facilitate the design of rational dose regimens. The objective of the present study was to assess the pharmacokinetics of rhLIF over a wide range of doses administered intravenously and subcutaneously to sheep. In the first instance, the pharmacokinetics were evaluated by noncompartmental analysis. Subsequently, a physiologically relevant, mechanistic model was developed to characterize the kinetic behavior.

Administration of rhLIF by i.v. bolus injection resulted in plasma concentration-time profiles showing trends toward more prominent initial distribution phases, enhanced convexity, and prolonged terminal elimination phases with increasing dose. Noncompartmental analysis revealed a disproportionate increase in AUC and a corresponding decrease in plasma clearance with increasing dose, confirming the existence of nonlinear pharmacokinetics. The clearance of rhLIF was extremely rapid, particularly at the lowest i.v. dose (12.5 μg/kg), where the systemic clearance was 5.2 ml/min/kg. Renal filtration is often considered to be a major clearance pathway for proteins with molecular weights below that of albumin, with the process being most efficient for proteins smaller than 30 kDa (Maack et al., 1979). With rhLIF, however, renal filtration is unlikely to be the predominant mechanism given that the total clearance at low doses far exceeds the glomerular filtration rate in sheep (1.2 ml/min/kg) (Adams and McKinley, 1995). It is therefore evident that additional elimination mechanisms must contribute to the systemic clearance of rhLIF.

Given the nonlinear disposition of rhLIF, traditional models of monophasic or biphasic exponential decline were unsuitable to describe the plasma pharmacokinetics across all dose levels; therefore, a model has been suggested based on the pharmacology of rhLIF and knowledge of the common clearance mechanisms for protein drugs (Fig. 1). In this model, the predominant clearance pathway was high-affinity binding of rhLIF to specific cell surface receptors followed by endocytosis and degradation. Considering the complexity of the plasma concentration-time profiles, the proposed model satisfactorily characterized the pharmacokinetics of rhLIF across the wide range of doses and both i.v. and s.c. routes of administration.

Although target-mediated drug disposition and degradation following endocytosis are the key features of the model, adequate characterization of rhLIF disposition required the inclusion of a parallel first-order elimination process from the central compartment that most likely reflects renal elimination. The relative significance of the linear elimination pathway can be assessed by comparison of the model-predicted clearance via first-order elimination \( k_e \cdot V_c \) of 1.37 ml/min/kg to the apparent total plasma clearance obtained for each dose by noncompartmental analysis of the i.v. data (Table 1). As the dose of rhLIF increases, the linear elimination pathway becomes increasingly important because clearance via receptor-mediated endocytosis is saturated. For example, clearance via the first-order process for the 12.5, 100, and 500 μg/kg rhLIF doses constitutes 26, 60, and 90% of the total clearance of rhLIF, respectively. A further model component that was found to be necessary to adequately characterize the disposition of rhLIF was the reversible transfer of rhLIF between the central compartment and a tissue compartment \( V_T \). This tissue compartment could represent extravascular distribution of rhLIF or, alternatively, reflect nonspecific binding of rhLIF to plasma proteins such as α₁-acid glycoprotein or α₂-macroglobulin.

Optimal characterization of rhLIF kinetics with the present model necessitated that \( R_m \) be a lower value for the highest i.v. dose (750 μg/kg). Without this provision, the model systematically overestimated the observed plasma concentration-time profile following administration of 750 μg/kg rhLIF (data not shown). It is proposed that the pharmacokinetic behavior of rhLIF after administration of very high doses could be altered as a consequence of receptor down-regulation. This proposal is supported by in vitro studies using various cell types that indicated autologous regulation of the LIF receptor within 2 h following treatment with LIF (Bower et al., 1995; Blanchard et al., 2000). Inclusion of a receptor down-regulation process into the current model was not feasible without additional experimental data, such as receptor concentrations or supporting pharmacodynamic data. As an alternative, the empirical approach of allowing \( R_m \) to be a lower value for the highest rhLIF i.v. dose was adopted and resulted in predicted profiles that better reflected the observed pharmacokinetic data with the addition of only one extra model parameter.

The utility of employing a lower \( R_m \) value to reflect receptor down-regulation can be appreciated by examining the predicted changes in free receptor density over time. In Fig. 3, the free receptor density was simulated for a range of rhLIF doses using the parameter estimates for the final model (Table 3). An extremely rapid initial decline in receptor density is apparent after i.v. administration, reflecting the high-affinity binding of rhLIF to the available cell surface.

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**Fig. 3.** Simulation of the free receptor density available for rhLIF binding over time \( R' \) in eq. 5) following i.v. administration of a range of rhLIF doses using the proposed model (Fig. 1) and the final parameter estimates (Table 3). Data are expressed as the proportion of the initial receptor density \( R_m \) or \( R_m' \). Note the immediate initial rapid decline in free receptor density as rhLIF is rapidly acquired by specific cell surface receptors.
receptors. Synthesis of new receptor and dissociation of the rhLIF-receptor complex resulted in a subsequent gradual rise in free receptor density. For the 750 μg/kg rhLIF dose, where $R_{n}$ was estimated as 0.058 mmol/kg, the receptor densities remained at low levels for a sustained period of time, such that even 24 h after dosing, the receptor density had not returned to initial levels.

Although saturable target-mediated drug disposition seemed to adequately describe the plasma concentration-time data in the present study, it is possible that an additional nonlinear binding process could also contribute to the highly complex disposition of rhLIF, since the observed relationship between the administered dose and $V_{ss}$ (Table 1) was not typical of target-mediated drug disposition. For drugs that bind with high affinity to their pharmacological targets, $V_{ss}$ generally decreases with increasing dose to approach plasma volume (Levy, 1994). This has been observed previously with several protein-based drugs including, interferon-β1a and natalizumab (Sheremata et al., 1999; Mager et al., 2003). However, if an additional saturable binding protein, such as a soluble LIF receptor [which has previously been identified in human plasma (Zhang et al., 1998)], was present in sheep plasma, the outcome is less certain, and changes in $V_{ss}$ are difficult to predict.

The development of a model to describe the absorption kinetics of rhLIF following s.c. administration was confounded by the nonlinear disposition, flip-flop kinetics, and poorly defined absorption mechanisms. Initially, a wide range of absorption models, including single zero- and first-order absorption processes and combinations of zero- and first-order input occurring in parallel or sequentially, with or without lag times, were evaluated for their ability to characterize the absorption of rhLIF. The absorption process was best described by first-order input into a delay compartment and subsequent first-order transfer to the systemic circulation. This model likely reflects the slow transport of the relatively large-sized rhLIF through the interstitium, a process that may further be hindered by the interaction of highly basic rhLIF (pI 9.15) with negatively charged interstitial glycosaminoglycans. The slow rate of absorption of subcutaneously administered proteins has also been attributed to uptake into and transport through the lymphatic system (Radvanski et al., 1998). Several studies have quantitatively demonstrated that the lymphatics are the primary absorption pathway for proteins with a molecular weight above 16 kDa (Supersaxo et al., 1990; Charman et al., 2000; McLennan et al., 2003); therefore, it is expected that this pathway would contribute to the absorption of subcutaneously administered rhLIF (19.71 kDa). The data in the current study are not sufficiently rich to delineate the relative contributions of interstitial transfer and lymphatic transport to the absorption kinetics of rhLIF; however, studies investigating the rate and extent of rhLIF lymphatic transport are currently underway. These studies will provide a greater understanding of the underlying absorption mechanisms for this protein.

In the s.c. model, the estimate for $k_{a1}$ was fixed to be equal for all three dose levels; however, the rate constant for the transfer of rhLIF from the absorption delay compartment to the systemic circulation ($k_{ad}$) was best modeled as a different value for each of the s.c. doses to capture the apparent differences in the time to reach $C_{max}$. Although definitive evidence is lacking, a possible explanation for the quicker absorption with the higher rhLIF doses is that the higher protein concentration in the formulation (note that injection volumes were constant for all s.c. dose levels) increased the oncotic pressure in the interstitium, promoting interstitial transport and/or lymphatic filling.

In summary, the current study is the first to comprehensively examine the dose proportionality of rhLIF pharmacokinetics and has demonstrated that the pharmacokinetics of rhLIF in sheep are complex and significantly nonlinear following both i.v. and s.c. administration. The nonlinear disposition was successfully characterized using a model that featured specific binding of rhLIF to high-affinity, low-capacity cell surface receptors, followed by internalization and degradation of the rhLIF-receptor complex. The apparent time course of receptor occupancy predicted by the model could potentially be used as a basis for future pharmacodynamic models of rhLIF pharmacological effects. More generally, the results further support the relevance of this pharmacokinetic model for cytokines and protein drugs, where the specific site of action also acts as a clearance pathway.

References


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