Effects of Pegylation and Immune Complex Formation on the Pharmacokinetics and Biodistribution of Recombinant Interleukin 10 in Mice

Hamsell M. Alvarez, On-Yee So, SuChun Hsieh, Natasha Shinsky-Bjorde, Huipeng Ma, Yaoli Song, Yinuo Pang, Melinda Marian, and Enrique Escandón

anti-human IL-10 12G8 monoclonal antibody; mαmIL-10, mouse anti-mouse IL-10 11D8 monoclonal antibody.
DMD #42531

Abstract

Interleukin 10 (IL-10) is a potent cytokine homodimer with multiple immunoregulatory functions. Here, we have characterized the effects of pegylation and formation of hIL-10/hαhIL-10 (humanized anti-hIL-10) immune complexes in the pharmacokinetics (PK), biodistribution and biotransformation of IL-10 in mice. In order to assess the fate of native, pegylated and antibody bound IL-10; we implemented an analytical set of fluorescent-emission-linked assays (FELA). Plasma size exclusion chromatography analysis indicated that fluoro-labeled native and pegylated murine IL-10 (mIL-10 and PEG-mIL-10) are stable in the circulation. Pegylation of IL-10 resulted in a 21-fold increased exposure, 2.7-fold increase in half-life and a 20-fold reduction in clearance. Kidney is the major organ of disposition for both native and pegylated mIL-10 with renal uptake directly related to systemic clearance. Fluorescent signal in the kidneys reached tissue-to-blood ratios up to 150 and 20 for native and PEG-mIL10, respectively. hIL-10/hαhIL-10 immune complexes are detectable in the circulation without evidence of unbound or degraded hIL-10. The exposure of hIL-10 present in immune complexes vs. hIL-10 alone, increased from 0.53 to 11.28 µg*day/ml, with a half-life of 1.16 days and a 23-fold reduction in clearance. Unlike hIL-10 alone, antibody bound hIL-10 was targeted mainly to the liver with minimal renal distribution. In addition, we found an 11-fold reduction (from 9.9 to 113 nM) in binding to the Neonatal Fc Receptor (FcRn) when the hαhIL10 antibody is conjugated to hIL-10. The potential changes in FcRn binding in vivo and increased liver uptake may explain the unique PK properties of hIL-10/hαhIL-10 immune complexes.
Introduction

Interleukin 10 is a T helper 2-type cytokine with pleiotropic immunoregulatory functions (Mocellin et al., 2004) which include inhibitory activities that limit myeloid tissue destructive inflammation, and immunostimulatory actions that support antigen-specific effector functions (Pestka et al., 2004).

Human IL-10 exists in solution as a non-covalent homodimer, composed of two aglycosylated polypeptide chains (Vieira et al., 1991). The murine IL-10 consists of 157 amino acids, with approximately 73% overall sequence identity to the human homolog. On the basis of its high helical content, it has been proposed that IL-10 is a member of a large family of helical cytokines and growth factors (Kotenko, 2002). IL-10 mediates its anti-inflammatory actions upon specific binding to a tetrameric cell surface receptor formed by two IL-10R1 and two IL-10R2 polypeptide chains (Moore et al., 2001), ultimately resulting in transcription of STAT3-responsive genes, such as SOCS-3 (Ito et al., 1999).

The therapeutic effect of IL-10 has been investigated clinically for inflammatory indications, with phase 2 or phase 3 studies completed for Crohn’s disease, rheumatoid arthritis and psoriasis (Fedorak et al., 2000; Braat et al., 2006; Keystone et al., 1998; Asadullah et al., 1998; Asadullah et al., 1999). More recently, a pegylated version of IL-10 has been considered for oncology indications based primarily on its ability to drive CD8 T cell activation and recruitment into the tumor resulting in strong anti-tumor activity in several pre-clinical models (Gérard et al., 1996) (J. B. Mumm, J. Emmerich, X. Zhnag, L Wu, S. Mauze, S. Blaisdell, J. Dai, C. Sheppard, K. Hong, C. Cutler, S. Turner, D. LaFace, M. Kleinschek, M. Judo, G. Ayanoglu, J. Langowski, D. Gu, B. Paporello, Y. Pang, E. Murphy, V. Sriram, S. Naravula, B. Desai, M. Marian, S. Medicherla, T. McClanahan, W. Seghezzi, S. Cannon-Carlson, A. Beebe, and M. Oft, manuscript in preparation). To date, there are no published information concerning the distribution and stability of exogenously administered recombinant IL-10. In order to gain a
better understanding of the pharmacological potential of pegylated vs. native IL-10 as a therapeutic candidate, we have assessed their disposition in rodents. These studies demonstrate that both pegylation of IL-10 and immune complex formation resulted in clear alterations in the systemic exposure and disposition of IL-10. However, pegylation of mIL-10 mainly reduced renal uptake consistent with an increase in the molecular weight of PEG-mIL10; while immune complex formation resulted in a major switch in organ uptake, changing native IL-10 route of elimination from renal to hepatic disposition.
**Materials and Methods**

**Fluorescent Labeling.** DyLight-549, 649 or 680 labeling kits (Thermo Scientific, Rockford, IL) were used to conjugate an N-hydroxysuccinimide ester fluorescence dye (Ex 550/Em 568 nm, Ex 646/Em 674 nm, and Ex 692/Em 712 nm, respectively) to the experimental proteins. Before labeling, protein (mIL-10, PEG-mIL-10 and hIL-10) or antibodies (hαhIL-10 12G8, patent # US7662379) were buffer-exchanged to 50 mM sodium borate (Thermo Scientific, Walthman, MA) plus 75 mM NaCl buffer pH 8.5 using a 10 kDa molecular weight cut-off Slide-A-Lizer dialysis cassette (Thermo Scientific, Rockford, IL). Each reaction mixture contained 1.2 mg protein at a final volume 0.5 mL. The reaction was initiated by combining each protein with the dye and continued for 1 hour at room temperature (RT) protected from light. Unconjugated dye was removed using a purification resin (Thermo Scientific, Walthman, MA) packed into spin columns. When necessary, samples were further concentrated by Amicon ultra centrifugation filter devices with a 10 kDa cut-off membrane (Millipore, Billerica, MA). The labeled materials were filtered using a 0.22 µm Durapore PVDF membrane (Millipore, Billerica, MA). A NanoDrop apparatus (Thermo Scientific, Walthman, MA) was used to characterize the labeled reagents for determination of protein concentration and degree of labeling (DOL) as dye to protein, mole to mole ratio. Integrity of the labeled proteins and antibodies were also assessed by SEC-HPLC, binding ELISA (data not shown), and in vitro activity determinations (data not shown). When large amount of labeled regents were needed, the original procedures were scaled up to allow the efficient labeling of up to 50 mg of protein/reaction.

**SEC-HPLC Analysis.** Samples were applied onto a BioSep-SEC-S 300 column equipped with a Security Guard cartridge (Phenomenex, Torrance, CA) and separated using an Agilent 1200 HPLC system equipped with an integrated UV (Agilent Technologies Inc., Santa Clara, CA) and fluorescent detector (Hamamatsu Corporation, Bridgewater, NJ).
The size exclusion procedure was a 15 minutes isocratic run with Dulbecco’s Phosphate-Buffered Saline (DPBS) buffer (Mediatech, Manassas, VA) as mobile phase at a flow rate of 1 mL per minute at ambient temperature. The effluent was monitored optically at 280 nm and by total fluorescent intensity at excitation/emission of 550/568 or 646/674 nm, respectively. The percentage of peak area was calculated by dividing the area of the main intact protein peaks by the total fluorescent area of each chromatographic profile. Data collection and analysis were done using the Agilent ChemStation software V2 (Agilent Technologies Inc., Santa Clara, CA) and EXCEL 2003 SP-1 (Microsoft Corporation, Seattle, WA), respectively. Molecular weight markers (Bio-Rad, Hercules, CA) were run before and after the analysis for assessment of column performance.

Bioactivity Proliferation Assay. Among all the proteins and antibodies labeled in our studies, the bioactivities of 649-labeled mIL-10 and PEG-mIL-10 were assessed in a $\gamma^{14}$C-thymidine incorporation assay measuring the proliferation of MC9 cells that expresses mIL-10R$\alpha$ and mIL-10R$\beta$ receptors. Healthy cells are plated at 7500 cells/well density in 96-well Cytostar-T scintillating microplates (GE Healthcare, Piscataway, NJ) and exposed to serial dilutions of mIL-10 or PEG-mIL-10 from 100ng/ml plus 10ng/ml of mIL-4 (R&D Systems, Minneapolis, MN), and 2% $\gamma^{14}$C-labeled thymidine (GE Healthcare, Piscataway, NJ) in assay medium consisting of RPMI-1640, 10% heat-inactivated FBS, 50 μM 2-mercaptoethanol, 2 mM L-Glutamine, 100 IU/ml Penicillin, and 100 μg/ml Streptomycin. The assay culture plates are incubated for 24 hours at 37°C, 5% CO$_2$. Proliferation is represented as accumulated incorporation of radiolabel and is assayed using scintillation proximity and measured on a TopCount Microplate Liquid Scintillation Counter (Packard Bioscience, Meriden, CT). Proliferation is plotted against IL-10
concentration and the EC$_{50}$ value for bioactivity is determined using non-linear regression (curve fit) of sigmoidal dose-response in GraphPad Prism 4 (Graphad Software Inc, La Jolla, CA).

**FcRn Expression and Purification.** The extracellular domain of murine FcRn α-chain (including a Gly-His$_6$ and a human glutathione S-transferase tags) and β$_2$-microglobulin genes were subcloned into pTT5 mammalian cell expression vectors for transfection into the Epstein-Barr virus antigen (EBNA1)-transformed human embryonic kidney cell line 293 (293E) using linear polyethylenimine (PEI). Supernatants were collected 72 hr after transfection and the soluble mFcRn.His$_6$.GST/β$_2$m protein was purified and characterized as previously described (Shields et al., 2001).

**FcRn Binding ELISA.** A polyclonal rabbit αGST antibody (Invitrogen, Carlsbad CA) was coated on ELISA plates (Nunc-Immuno Plate with MaxiSorp; Nunc International, Rochester, NY) overnight at 4°C. After blocking, murine FcRn-His$_6$-GST was bound to ELISA plates. Serial 3-fold dilutions of recombinant proteins were added to the plate at pH 6.0. Captured antibodies or antibody complexes were detected with peroxidase-conjugated AffiniPure F(ab')$_2$ fragment donkey α-human (H+L) secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) using TMB (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as substrate. Absorbance measured at 450 nm minus 650 nm was read on a Spectramax Plus 384 plate reader (Molecular Devices) and plotted against the logarithm of the various antibodies concentration. Control plates were incubated in identical conditions in plates without murine FcRn-His$_6$-GST coating and values where subtracted as background. Data were fitted and analyzed using a sigmoidal dose-response (variable slope) nonlinear regression fit using GraphPad Prism 4 (Graphad Software Inc, La Jolla, CA). The absorbance at the midpoint of the
titration curves and its corresponding concentration were determined (EC \textsubscript{50}) and reported as an approximate dissociation constant (K\textsubscript{d}).

**In Vivo Study Design and Dosing.** Animals used for the in vivo study were BALB/c AnNCrl\textsuperscript{®} and CD-1 female mice from Charles River Laboratories (Wilmington, MA), approximately 8-16 weeks of age and body weights of 23 to 27 g. All animals were dosed i.v. (tail vein) or s.c. (intrascapular area) with unlabeled and fluoroprobe labeled test materials. Dosing solutions (0.250-0.70 µL) were prepared using body weight averages of each group. Animals placed on alfalfa-free diet were fed with Teklad TD.97184 (Harlan, Indianapolis, IN) for at least 5 days prior to dosing and until study termination. Table 1 summarizes the animal study design. All studies were approved by the Institutional Animal Care and Use Committee and were performed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care.

**Pharmacokinetic Analysis.** The PK and Biodistribution of mIL-10, hIL-10, PEG-mIL10, h\textalpha\textsubscript{h}IL-10 and hIL-10 in complex with \textalpha\textsubscript{m}IL-10 antibodies (h\textalpha\textsubscript{h}IL-10 & m\textalpha\textsubscript{m}IL-10) were characterized following single i.v. or s.c. bolus administration in normal mice (Table 1). Their PK properties were compared and evaluated.

Concentrations of test materials in serum samples and dosing solution retainers were determined by an Electrochemiluminescence Assay (ECL) for mIL-10, and Enzyme-Linked Immunosorbent Assays (ELISA) for PEG-mIL-10 and h\textalpha\textsubscript{h}IL-10 12G8; while plasma samples were analyzed by Fluorescence Emission-linked assay (FELA) for hIL-10, h\textalpha\textsubscript{h}IL-10 12G8 and immune complexes. The lower limits of quantification were: 21 pg/mL for mIL-10, 100 pg/mL for PEG-mIL-10 (ELISA), approximate 5.0 ng/mL for all 649-labeled experimental proteins.
Concentration-time data for mIL-10 and PEG-mIL-10 were analyzed using noncompartmental methods. PK parameters for mIL-10 and PEG-mIL-10 were estimated or calculated using WinNonlin Model 200 for s.c. administration or Model 201 for i.v. administration (WinNonlin, Version 5.2.1; Pharsight, Sunnyvale, CA). Values of $r^2$ or $r^2$-adjusted ≥ 0.80 (where $r$ is the correlation coefficient) were required for acceptance of Lambda z ($\lambda_z$) estimates in noncompartmental analyses. ELISA and FELA data for hohIL-10 12G8 were modeled using a two-compartment PK model (WinNonlin, Version 5.2.1, Model 7; Pharsight, Sunnyvale, CA), and PK parameters were calculated using a noncompartmental Model 201 (i.v.) using WinNonlin (Pharsight, Sunnyvale, CA). PK parameters of FELA concentration-time data for hIL-10 alone (i.v. or s.c) or in immune complexes with hohIL-10 12G8 or mamIL-10 11D8 were calculated using an appropriate noncompartmental model as described before. Concentration data for all proteins and antibodies was plotted using GraphPad Prism 4 (Graphad Software Inc, La Jolla, CA) and presented with standard deviation for all proteins and antibodies with exception of hIL-10 (s.c.) and hIL-10 (s.c.) dosed 5 minutes after administration of mamIL-10 (i.v.).

**Blood and Tissue Collection.** Animals were anesthetized with carbon dioxide prior to euthanasia. Blood (0.5 ml) was collected via cardiac puncture in EDTA-K$_2$ Capiject plasma separator tubes (Terumo, Somerset, NJ) and kept at 4°C. Plasma was stored at -80°C. Blood samples (0.1 ml) were collected into polypropylene vials (Corning, Corning, NY). Urine and organ samples were collected terminally. For lysate preparation, organ samples were weighed and placed on dry ice. Partially frozen samples were minced and homogenized using a MiniBeadbeater 96 device (Cole-Parmer, Vernon Hills, IL). One hundred miligrams of wet tissue per 1 mL (1:10 dilution) of DPBS lysis buffer containing 1% Triton X-100 (MP Biomedical, Solon, OH) and Halt protease inhibitor single-use cocktail (Pierce, Rockford, IL) were homogenized.
DMD #42531

using 1 mm zirconia/silica beads. The tissue slurry was centrifuged (10000 rpm for 6 min, 4°C) and the tissue lysate supernatants collected and stored at -80°C until analysis.

Whole-Body Computer Tomography (CT)-fusion fluorescence imaging analysis. An OPTIX CT-fusion fluorescence apparatus (ART, Montreal, QC) was used to determine the disposition of 680-labeled native and PEG-IL10 in live mice following subcutaneous administration. Animals were shaved under isoflurane anesthesia, and pre-scanned prior to dosing. Mice were injected i.v. with 0.3 - 0.5 mL of Fenestra VC or LC contrast agent 2 - 4 hours prior to scanning.

Analysis of Total Fluorescence Intensity by Fluorescence Plate Reader. Total fluorescence intensity was measured using a Modulus TM microplate multimode reader (Turner BioSystems, Sunnyvale, CA) equipped with a fluorescence optical kit filter with excitation/emission wavelengths of 625/660-720 nm, respectively. Individual calibration curves were prepared for all the tissues to be analyzed resulting in three main concentration vs. fluorescent intensity slopes represented by whole blood, plasma, and liver. Aliquots were transferred to low fluorescent background 96-well polystyrene assay plates (Corning, Corning, NY). A set of corresponding blank tissue lysates was used for background correction. A linear function was fitted to the data using regression. Concentrations were calculated as micro gram equivalents per gram of wet tissue. Tissue to blood ratios, and percent of injected dose were calculated based on the drug concentration in tested tissues and total administered dose per animal respectively. Data was calculated using EXCEL 2003 SP-1 (Microsoft Corporation, Seattle, WA) or GraphPad Prism 4 (Graphpad Software Inc, La Jolla, CA).

Immunoprecipitation and SDS-PAGE Fluorescence Scanning Analysis. Aliquots of
selected tissue lysates were thawed and centrifuged for 10 min at 2000 x g. Supernatants from the hIL-10 treated group were immunoprecipitated with 100 µg of mαmIL-10 11D8 and incubated for 60 min at 4°C, while the immune complexes group was not subjected to incubation with an antibody. Protein-A sepharose (Sigma-Aldrich, St. Louis, MO) in PBS slurry was added to the samples that were previously passed through a ½ cc Lo-dose U-100 insulin syringe and incubated overnight at 4°C with gentle agitation. The Protein-A sepharose pellets were washed four times with ice cold lysis buffer. After centrifugation, the supernatants were aspirated and the pellets were resuspended in 60 µl of SDS-PAGE sample buffer. Samples were incubated at 70°C for 5 min. Samples (10 µl) were run on a 4 -12% Bis-Tris gel (Invitrogen, Carlsbad CA) and analyzed by fluorescence scanning using a Typhoon™ apparatus (GE HealthCare, Piscataway, NJ). BenchMark Fluorescence Protein Standard (Invitrogen, Carlsbad CA) were used for molecular weight assessment of the experimental bands. Images were analyzed using the ImageQuant Total Lab software (GE HealthCare, Piscataway, NJ).

**Immunohistochemistry.** Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded 5-µm-thick tissue sections using a mouse anti-DyLight-649 monoclonal antibody (BC15.12A3.G4 IgG1/k). Sections were deparaffinized, quenched with 3% hydrogen peroxide, and heat retrieved with citrate buffer (Dako Corporation, Carpinteria CA) using a decloaking chamber (Biocare Medical, Concord, CA). Slides were then mounted in an autostainer (Dako Corporation, Carpinteria, CA), incubated with mouse anti-DyLight-649 primary antibody, treated with rabbit anti-FITC secondary antibody (Invitrogen, Carlsbad, CA), and incubated with rabbit envision-plus (Dako Corporation, Carpinteria, CA). Slides were developed with DAB-Plus (Dako Corporation, Carpinteria, CA), counterstained in modified Mayer’s hematoxylin and blued in 0.3% ammonia water.
Multichannel Fluorescence Whole-Slide Scanning. Scanning fluorescence analysis was performed on 5-µm-thick, 4% paraformaldehyde-fixed frozen tissue sections (kidney and liver) mounted in medium containing DAPI (ProLong Gold anti-fade reagent with DAPI, Invitrogen, Carlsbad, CA). All slides were scanned using an Aperio Scan Scope FL (Aperio Technologies, Vista, CA) at Ex 550/Em 568 nm for DyLight-549, and Ex 646/Em 674 nm for DyLight-649 experimental proteins. Images were analyzed using ImageScope software and Spectrum Plus (Aperio Technologies).
Results

Characterization of Labeled mIL-10, PEG-mIL-10, hIL-10 and hαhIL-10. In order to follow the fate of exogenously administered experimental proteins in complex biological matrices both in vitro and in vivo; test materials were chemically labeled with the fluorescent dye (DyLight-649). In specific studies, DyLight-549 for co-localization studies and DyLight-680 for whole in vivo imaging were also used. The purity, integrity and biological activity of the fluorescence labeled protein therapeutics were characterized by several methods.

SEC-HPLC analysis of labeled and unlabeled native mIL-10 and PEG-mIL-10 measured at 280 UV and 649 nm fluorescent respectively are identical (Fig. 1) with no evidence of aggregate formation, depegylation, monomer release or degradation products resulting from the labeling procedure with identical recovery results for 649- and 680-labeled materials (data not shown). mIL-10 homodimer runs as a single peak with an approximate molecular weight of 35 kDa (Fig. 1A and 1D), while PEG-mIL-10 is depicted by two major peaks of 158 and 95 kDa representing a Di- and Mono- pegylated mIL-10 moieties respectively (Fig. 1B and 1E). mIL-10 has been pegylated at the N-terminus with a 5 kDa linear aldehyde activated PEG through a reductive alkylation using sodium cyanoborohydride as a reductant. The degree of labeling (DOL) for native mIL-10 and PEG-mIL-10 ranged between 1.2 and 5 (dye to protein molar ratio). The recovery of the labeled materials measured by a capture ELISA was 100% compared to the unlabeled control. Analysis of biological activity was also measured in a proliferation assay. EC$_{50}$ values for unlabeled and labeled native mIL-10 were 0.543 and 0.449 ng/ml and for unlabeled and labeled PEG-mIL-10 were 1 and 1.9 respectively, indicating that the labeling protocol did not have a major impact in the bioactivity of the test materials (data not shown). SEC-HPLC analysis of unlabeled and labeled hαhIL-10 12G8 antibody (Fig. 1C and 1F) demonstrated no changes in the chromatographic profile due to the labeling procedure. There are no chromatographic differences between native human and murine IL-10. The expected
molecular weight of approximate 150 kDa for hαhIL-10 12G8 was confirmed by size-exclusion chromatography coupled with multiple angle light scattering (SEC-MALCS) (data not shown). The average degree of labeling (DOL) for hIL-10 and hαhIL-10 was 1.5 and 3.7 moles of dye per moles of protein, respectively and the recovery ranged from 72 to 83%.

Characterization of hIL-10 immune complexes. Figure 2 shows the SEC-HPLC fluorescent profiles of 649-labeled hIL-10 in preformed immune complexes with hαhIL-10 12G8 or mαmIL-10 11D8 monoclonal antibodies (Fig. 2A and 2B, respectively) following incubation in freshly prepared neat EDTA-K2 murine plasma at different antibody:hIL-10 molar ratios. Labeled hIL-10 alone was identified in plasma by a single peak with an apparent molecular weight of approximately 35 kDa. In contrast, three main HMW complexes were identified in the presence of hαhIL-10 12G8. The approximate molecular weights were 223 kDa, 348 kDa and 528 kDa (peaks I, II and III respectively) (Fig. 2A). The sizes of these immune complexes are consistent with antigen/antibody interactions containing two hIL-10 molecules bound to one and two antibodies respectively (expected MW of 225 kDa and 375 kDa for peak I and II), and three ligands plus three antibodies (expected MW 563 kDa for peak III). The molecular weight of hIL-10 with hαhIL-10 12G8 immune complexes was confirmed by size-exclusion chromatography coupled with multiple angle light scattering (SEC-MALCS) (data not shown). Once formed, these immune complexes were very stable over time and largely unaffected by dilution (data not shown). hIL-10 in the presence of the murine monoclonal mαmIL-10 11D8 (Fig. 2B), formed only one major HMW complex with an approximate molecular weight of 222 kDa, consistent with the interaction of two ligands bound to one antibody (expected MW of 225 kDa). This mαmIL-10 monoclonal antibody also binds hIL-10 with high affinity.
Pharmacokinetics of mIL-10, PEG-mIL-10 and hαhIL-10 after a single i.v. or s.c. Administration. The mIL-10 and PEG-mIL-10 serum concentration-time profiles are presented in Figure 3A (unlabeled samples). A noncompartmental model was conducted and generated PK parameters are summarized in Table 2. The pegylation of mIL-10 reduced the clearance by 20-fold (from 4584 mL/day/kg to 220.80 mL/day/kg) and increased t½ by 2.7-fold (0.098 days to 0.26 days) as compared to the native protein when it is administered intravenously. A similar effect was observed when PEG-mIL-10 was dosed subcutaneously. The volume of distribution decreased 1.5-fold for the pegylated protein (from 95.40 mL/kg to 62.40 mL/kg), suggesting that the PEG moiety reduces the ability of the mIL-10 to distribute outside of the central vasculature. Accordingly, pegylation of mIL-10 following i.v. or s.c. administration resulted in a significant increase in systemic exposure as compare to the wild-type protein (from 0.65 and 0.27 µg*day/ml to 13.5 and 6.03 µg*day/ml, respectively). Figure 3B shows the concentration time profile of labeled and fluor-labeled hαhIL-10 monoclonal antibody 12G8 following i.v. dosing in mice. A two-compartmental PK model provided a good fit to the observed data, while a non-compartmental analysis was used to generate the PK parameters (Table 3). Our results demonstrated that both bioanalytical methods (FELA vs. ELISA) provide similar profiles and PK parameters for the labeled and unlabeled hαhIL-10 antibody 12G8. hαhIL-10 12G8 has a good systemic exposure with clearance and terminal t½ of a typical humanized monoclonal antibody in rodents (Kim et al., 1999).

Pharmacokinetics of hIL-10 immune complexes. Figure 4 shows the plasma concentration-time profiles of 649-labeled-hIL-10 alone or in preformed immune complexes with 12G8 (antibody to ligand molar ratio of 2.0) following i.v. administration. The concentration-time profile of immune complexes formed in vivo using mαmIL-10 11D8 monoclonal antibody is also shown. The murine analog antibody was given i.v. 5 minutes before a s.c. injection of 649-hIL-10.
DMD #42531

(antibody to ligand molar ratio of 0.8). In both cases, the levels of hIL-10 present in immune complexes were calculated using FELA. The s.c. PK profile of hIL-10 alone was also calculated.

A noncompartmental model was used to generate the PK parameters (Table 4). Binding to the humanized antibody in preformed complexes reduces hIL-10 clearance by 23-fold (5655.89 mL/day/kg to 243.34 mL/day/kg) and increases t1/2 by 29-fold respectively (from 0.04 days to 1.16 days). Complexation of hIL-10 in vivo to the murine monoclonal antibody 11D8 (antibody i.v., and ligand s.c., dosed sequentially), also increased the t1/2 of hIL-10 but to a lesser degree (from 0.54 days to 1.16 days).

**Binding of hαhIL-10 to murine FcRn.** In order to ascertain the role of binding to FcRn in the changes in half-life and clearance (Presta, 2008) of hαhIL-10 antibody when bound to the ligand, we characterized the FcRn binding properties in vitro of hαhIL-10 12G8 alone or as hIL-10/hαhIL-10 immune complexes. Bevacizumab (Avastin), a humanized αVEGF monoclonal antibody (Ferrara et al, 2004) was used as a control for immune complexes specificity in the presence of excess hIL-10. The approximate Kd values are shown in Table 5. Similar mFcRn binding parameters were observed between hαhIL-10 and Bevacizumab (approximate Kd of 9.9 nM and 7.1 nM respectively) in the absence of hIL-10. These FcRn binding values are comparable to those previously reported for a humanized anti-TNFα monoclonal antibody binding to murine FcRn (Kd = 12.1 nM) (Deng et al, 2010). Importantly, the presence of hIL-10 causes a 10-fold decreased in the binding of hαhIL10 (Kd = 113 nM) but not Bevacizumab to mFcRn (Kd = 7.8 nM) demonstrating a profound effect in FcRn-antibody interactions when bound to the ligand.

**Characterization of mIL-10, PEG-mIL-10, hαhIL-10 and hIL-10/hαhIL-10 Immune Complexes stability and interactions in vivo.** Sequential HPLC fluorescent analysis from
plasma collected over a 2 days period following s.c. dosing of 3 mg/kg 649-labeled mIL-10 and PEG-mIL-10 are presented in Figure 5A and 5B. Native mIL-10 peaks at 1hr and it is only detectable up to 2 hours post dosing. At any time point, detectable mIL-10 was mostly composed of the intact 35 kDa homodimer with only low levels of free dye and HMW complexes present systemically. PEG-mIL-10 represented by two chromatographic peaks (Mono vs. Di-PEG homodimer) also remained fully intact in the circulation and it was detectable up to 48 hours after s.c. administration. SEC-HPLC analysis also demonstrated that the absorption and elimination profiles of PEG-mIL10 are size dependent. The relative amounts of each peak reverse over time in the circulation. At earlier time points (up to 2 hours) Mono-PEG mIL-10 is the dominant peak while at six hours Di-PEG mIL-10 form has become predominant and by one day is the only peak present in plasma. At the later time points there were low levels of HMW complexes, with no evidence of catabolic species, de-pegylation or significant fluoroprobe release. The relative amounts of Di- vs. Mono-PEG forms present over time in vivo are shown in Table 6. Urine samples from animals dosed with mIL-10 or PEG-mIL-10 contained large amounts of fluorescent signal composed only of low molecular weight moieties or free fluorescent dye (< 1.4 kDa), consistent with renal elimination (data not shown).

Figure 5C shows the HPLC plasma profiles of 649-labeled-hαhIL-10 antibody 12G8 following i.v. administration of 3 mg/kg as a single major peak of approximate 150 kDa MW representing unmodified IgG up to 28 days after dosing. Only low levels of free dye or HMW complex formation were observed. Similarly, plasma HPLC analysis of preformed hIL-10/hαhIL10 immune complexes at a 1 to 2 molar ratio following dosed i.v. (Fig. 5D) indicated that fluoro-labeled hIL-10 remains bound to the antibody at every time point investigated, indicative of strong systemic stability in these conditions. Only immune complexes and no free hIL-10 were detectable in the circulation up to 3 days after dosing. The size and relative amounts of immune complexes present in plasma are identical to those characterized in the
dosing solutions as shown in Figure 2. There was no evidence of catabolites or degradation products including free dye, unbound or monomeric hIL-10 in the circulation. Only low molecular weight (approximate 3.5 kDa) catabolites were identified in urine (data not shown). The formation and stability in vivo of immune complexes was also demonstrated in mice dosed i.v. with the mαmIL-10 antibody analogue 11D8, followed by the s.c. administration of labeled hIL-10 (data not shown). These complexes in vivo were undistinguishable in size to those characterized previously in vitro with the mαmIL10 antibody (Fig. 2).

**Analysis of mIL-10, PEG-mIL-10 and hαhIL-10 distribution in tissues.** Total fluorescent profiles (µg-Eq/g of tissue) of labeled mIL-10 and PEG-mIL-10 after s.c. administration were measured over time in organs, whole blood and plasma. The highest levels of total fluorescent signal after native or PEG-IL10 dosing (Fig. 6A and 6B, respectively) were detected in the kidneys (131 µg-Eq/g for mIL-10 vs. 36 µg-Eq/g for PEG-mIL-10 at 6 hours postdose). The native mIL-10 tissue-to-blood ratios over time (Fig. 7A) were considerably higher than 1 in several organs, indicative of potential tissue uptake and accumulation. Kidney-to-blood ratios were exceptionally high (approximate 150) indicating a major role for this organ in the disposition and clearance of mIL-10. In comparison, tissue-to-blood ratios for PEG-mIL-10 (Fig. 7B), with exception of the kidneys (approximate 20) ranged around 1-2 in most organs at any given time with the exception of colon and mesenteric lymph nodes 2 days after dosing (10 and 9, respectively). Supplemental material (Supplemental Table 1 and 2) shows disposition of mIL-10 and PEG-mIL-10 described as % of injected dose per gram of tissue. The organ with the highest percentage of injected dose for both tested materials were the kidneys at 6 hr post dose, 87% for mIL-10 and 24% for PEG-mIL-10. These data indicate that the kidneys play and important role in the clearance and disposition of both native and pegylated IL-10.
Concentration profiles and tissue-to-blood ratios of 649-labeled hαhIL-10 antibody 12G8 following i.v. administrations are presented in Figures 8A and 8B. No evidence of organ uptake was observed. The tissue-to-blood ratios over time were lower than 1 in all analyzed organs, indicative of a negative tissue uptake or accumulation.

**In vivo CT-fusion fluorescent imaging of native and pegylated mIL-10 in normal mice.** Whole animal in vivo fluorescence imaging studies where conducted to complement the biodistribution analysis of native vs. pegylated mIL-10. Figure 9 shows the strong kidney localization of 680-labeled native and pegylated mIL-10, 2 hours following a s.c. dose of 3 mg/kg. Consistent with the previous quantitative analysis (Fig. 6) there is a stronger fluorescent signal in the kidneys of animals injected with the native mIL-10.

**Analysis of hIL-10 immune complexes distribution in tissues.** Total fluorescent profiles (µg-Eq/g of tissue) of labeled hIL-10 and hIL-10 bound to the hαhIL-10 antibody 12G8 following i.v. administration of preformed immune complexes were measured over time in various organs. The highest level of signal after hIL-10 administration alone (Fig. 10A) was detected in the kidney (125 µg/g at 1 hr postdose). Liver and spleen disposition of hIL-10 was also observed at lower levels (4.5 µg/g and 0.89 µg/g at 1 hr, respectively). In contrast, in the presence of hαhIL-10 antibody, the uptake of this cytokine to the kidney was dramatically reduced (2.7 µg/g at 1 hr), while the disposition to the liver and spleen of the immune complexes increased to a peak of 15 µg/g and 3.3 µg/g at 1 hr, respectively. Kidney-to-liver ratios for hIL-10 alone were close to 30 at 1 hr while in the presence of the antibody the highest hIL-10 kidney-to-liver ratio was below 1 (Fig. 10B). Liver-to-kidney ratios for hIL-10 alone or in the presence of the antibody are shown in Figure 10C. Overall, these results demonstrate that the presence of the antibody changes the disposition of hIL-10 mainly from the kidney to the liver. Similar data were found.
when the murine monoclonal antibody 11D8 was dosed i.v. 5 minutes before a s.c. administration of labeled hIL-10 (Fig. 11A to C). Therefore, complexation of IL-10 in vivo prevents kidney localization and promotes liver uptake.

To determine the relative degree of 649-labeled hIL-10 processing in vivo, tissue lysates and biomatrices were subjected to immunoprecipitation and SDS-PAGE fluorescent analysis (Fig. 12). One major band of approximately 18 kDa is present in whole blood, plasma and spleens of mice dosed with hIL-10 alone or in the presence of circulating mαmIL-10 antibody up to 2 hours postdosing. The presence of an additional band of slightly higher MW represents reduced hIL-10 forms (data not shown). The relative band intensities for labeled hIL-10 alone or in the presence of mαmIL-10 antibody in liver and kidney reflect the quantitative analysis.

Characterization of 649-labeled hIL-10 organ disposition by immunohistochemistry and fluorescent microscopy. Figure 13 is an immunohistochemistry analysis of liver, kidney and spleen tissues 2 hours after s.c. administration of 649-labeled hIL-10 alone (20 mg/kg) or 5 minutes after an i.v. dose (60 mg/kg) of unlabeled mαmIL-10 11D8 antibody. Mice dosed with hIL-10 alone show a strong signal in a subset of proximal convoluted renal tubes and along a portion of parietal epithelium in Bowman’s capsule and to lesser degree endothelial cells in the glomeruli (Fig. 13A). hIL-10 kidney staining is dramatically reduced in mice pre-dosed with the mαmIL-10 antibody (Fig. 13D). Liver and spleen tissues show only moderate staining for hIL-10 alone (Fig. 13B and C), however in the presence of the mαmIL-10 antibody, both organs display a noticeable increase in hIL-10 staining mostly in the sinusoidal lining cells of the liver and to a lesser degree, cellular components of the red pulp in the spleen (Fig. 13E and F). These immunohistochemistry results confirmed that the presence of the antibody changes the biodistribution of hIL-10 mainly from the kidney to the liver.
A dual fluorescence microscopy analysis was conducted in order to further characterize the disposition of hIL-10 and hIL-10 in immune complexes in vivo. Normal CD-1 mice were dosed i.v. with 649-hIL-10 alone (Fig. 14A to C) or with preformed immune complexes using an additional non-overlapping fluorescent dye (DyLight-549) to label the hαhIL-10 antibody (Fig. 14D to I). hIL-10 alone is distributed in punctuate compartments and concentrated in the proximal convoluted renal tubes (Fig. 14A and C). In contrast, minimal hIL-10 fluorescence signal was observed in kidney tissues 1 hr postdose of preformed immune complexes (Fig. 14D and F). The bottom panel demonstrates that both hIL-10 and antibody signal colocalize in the liver indicative of the presence of immune complexes in this organ.

The presence of hIL-10 immune complexes in organs was confirmed by SEC-HPLC fluorescent analysis (Fig. 15). Chromatographic profiles of liver, kidney and spleen tissue lysates following 649-hIL-10 i.v. dosing in preformed immune complexes demonstrate the presence of these complexes in vivo and the absence of free hIL-10. The estimated sizes of these immune complexes (340 kDa and 525 kDa) are identical as the ones characterized in the dosing solution. The presence of degradation products of lower molecular weight and large amounts of free dye in the liver but not systemically, is indicative of active catabolism in this organ.
Discussion

The anti-inflammatory cytokine interleukin-10 has been previously evaluated clinically for the treatment of Crohn’s disease, rheumatoid arthritis and psoriasis. More recently a pegylated form of IL-10 has been considered for oncology indications based on its strong anti-tumor activity in several preclinical models. To date, there is limited information concerning the in vivo distribution of recombinant native or pegylated IL-10.

In order to characterize the fate of these experimental proteins in vivo, our biodistribution studies were conducted using an innovative, fully integrated analytical set of fluorescent emission-linked assays (FELA). Similar to isotope tagging, FELA is a direct measurement of signal intensity without the need of additional reagents or steps in the analysis and therefore minimizes potential biomatrix mediated interferences. In contrast to $^{125}$I-labeling, see Vugmeyster et al., 2010 for review, we have implemented effective fluorescence modification procedures up to 50 mg of protein/reaction allowing the use of fully labeled experimental proteins in pharmacological studies requiring mg/kg doses. Comparative analysis with ELISA based methods validated this approach as a valuable alternative platform assay for PK studies.

We have shown that pegylation of mIL-10 improves its PK properties as previously described for other pegylated proteins including Interferon α2a, α2b and G-CSF (Veronese and Pasut, 2005). Compared to regular mIL-10, PEG-mIL-10 has a higher systemic exposure, slower clearance and longer $t_{1/2}$. Likewise, hIL-10 bound to a hαhIL-10 antibody also has increased $t_{1/2}$ and reduced clearance. Similar effects in the PK properties of other antibody-bound biologics have been observed for rhVEGF-αVEGF and IgE-αIgE immune complexes (Hsei et al., 2002; Fox et al., 1996). Although little is know about the precise mechanisms regulating the PK, elimination properties and possible biological functions of soluble small immune complexes like these in vivo, it is expected that potential changes in FcRn recycling, may play an important role (Qiao et al., 2008; Liu et al., 2011). Therefore we evaluated the
DMD #42531

binding to recombinant mFcRn of hαhIL-10 12G8 antibody alone or as an immune complex in the presence of excess hIL-10. Noticeably, the affinity of hαhIL-10-in-complex is 11-fold lower than the antibody alone. A similar decrease in apparent Kd values from 1.2 to 3-fold has been reported for anti-TNFα antibodies and TNFα-receptor Fc fusion constructs (Adalimumab, Infliximab and Etanercept) when bound to TNFα (Suzuki et al., 2010). Our results demonstrate that hIL-10/hαhIL-10 immune complexes are clear faster than free hαhIL-10 (243.34 vs. 8.48 ml/day/kg) and this correlates with a weaker FcRn interaction. In contrast, the clearance values for hIL-10 when in immune complexes are between those for hIL-10 and antibody alone (5655 vs. 8.48 ml/day/kg respectively) following a 3 mg/kg i.v. dose. The fact that hIL-10 in immune complexes clears significantly slower than free hIL-10, in part explains the general systemic increase in ligand levels and related changes in other PK properties of endogenous targets following the administration of neutralizing monoclonal antibodies, as previously observed for different soluble targets including IgE, TNFα and VEGF (Schoenhoff et al., 1995; Charles et al., 1999; Gordon et al., 2001). The importance of the Fab domain in potentially modulating FcRn binding has been recently indicated by Wang et al., 2011. In rodents, clearance of large cross linking immune complexes occurs mainly by binding to Fcγ receptors present in the reticuloendothelial system (RES) (Gessner et al., 1998; Tabrizi et al., 2010). The precise role of Fcγ receptors in degradation and clearance of soluble small (< 600 kDa MW) non-cross linking immune complexes, typical of those produced by monoclonal therapeutic antibodies in vivo, has not been properly characterized to date.

The dramatic and reproducible changes in PK properties of mIL-10 after pegylation or immune complex formation provided us with an in vivo model to better characterize potential biodistribution changes responsible for the altered PK parameters of native mIL-10 and to gain valuable insight on the fate of non-cross linking immune complexes in vivo. Fluoroprobe labeled native and pegylated mIL-10 are stable in mouse plasma up to 2 and 24 hours respectively after
DMD #42531

s.c. administration. SEC-HPLC analysis of PEG-mIL-10 in vivo revealed that the relative amounts of Mono- vs. Di-PEG mIL-10 forms changes over time in the circulation. At earlier time points there is a higher percentage of the smaller Mono-PEG form but by 6 hours after dosing there is a shift in the relative amounts and the larger Di-PEG form is the prevalent one. These changes in Mono-PEG to Di-PEG mIL-10 levels in the absence of noticeable catabolism, may represent the size-dependent differences in rates of absorption from the subcutaneous injection site into the blood compartment and the rates at which each pegylated form are cleared systemically. Only small amounts of degradation products or oligomer complexes were detected in plasma samples, indicative of the stability and longer systemic exposure of PEG-mIL10.

Kidney is the major organ of accumulation and catabolism of native and PEG-mIL10 in mice. Disposition to the kidneys by PEG-mIL10 was significantly decreased; however kidney was still the major organ of clearance for both molecules. Renal disposition was also confirmed by in vivo whole-body CT-fusion fluorescence imaging. The decreased PEG-mIL-10 kidney uptake was correlated to the slower clearance and enhanced systemic exposure. In agreement with our results, it has been shown that renal uptake is the major route of non-specific disposition for recombinant cytokines with high isoelectric points such as IL-11, Apo2L and TNFα (Takagi et al., 1995; Takagi et al., 1997; Xiang et al., 2004; Miyazaki et al., 1988) or relative small molecular weight such as insulin (Rabkin et al., 1984; Maack, et al., 1979). Interestingly, the biodistribution pattern of labeled hIL-10 present in immune complexes indicated that the liver instead of the kidney has become the major route of clearance and disposition for antibody bound IL-10.

Histological analysis of kidney sections from mice treated with hIL-10 display a very strong signal associated with renal proximal tubular epithelial cells, the Bowman's capsule and to a lesser degree glomeruli’s endothelial cells. hIL-10 alone staining in the kidneys is consistent with an active process of glomerular filtration, protein degradation, and resorption of metabolites.
by the proximal convoluted tubular epithelium as we have previously shown for recombinant
Apo2L (Xiang et al., 2004). In contrast hIL-10 bound to m\alpha mIL-10 in vivo, showed a dramatic
reduction in renal staining with a concomitant staining of hepatic sinusoidal tracks. A dual
fluorescence microscopy analysis demonstrated that both hohIL-10 antibody and hIL-10
colocalize in the liver, suggesting that indeed mostly hIL-10/hohIL-10 immune complexes were
present in this organ. The characterization of the cells or receptors responsible for these
interactions needs additional studies, but it has been previously demonstrated that larger,
polyclonal immune complexes are taken up and degraded by Kupffer and liver sinusoidal
endothelial cells probably through FcγR binding (Johansson et al., 1996; Johansson et al.,
2000; Johansson et al., 2002; Kosugi et al., 1992) in particular FcγRIIb2 (Ali-Mousavi et al.,
2007). Interestingly, Fox et al. (1996) did not find evidence of increased liver uptake in monkeys
dosed with an \alpha IgE monoclonal antibody, suggesting that hepatic disposition of immune
complexes is a process affected by multiple variables likely to include their relative size and
configuration, systemic levels, and the nature of the antigen among others (Mannik, 1980). In
this study, we have shown evidence that the size of the hIL-10/hohIL-10 immune complexes is
not altered upon organ uptake and confirms that labeled hIL-10 remained in the immune
complex configuration, with only trace amounts of free hIL-10 present in tissues.

In summary, we have used FELA for direct determination of stability and drug
quantitation in the circulation and tissues. We have combined whole live imaging with direct
fluorescent microscopy and immunohistochemistry analysis in organs using anti-dye antibodies.
We have shown that protein pegylation as well as immune complex formation resulted in
dramatic changes in the PK of hIL-10, both causing an increase in systemic exposure and
reduced clearance. However, pegylation of mIL-10 mainly reduced renal uptake consistent with
an increase in the molecular weight of PEG-mIL10. In contrast, immune complex formation
DMD #42531 resulted in a major switch in organ uptake, changing native hIL-10 major organ of clearance and route of elimination from renal to hepatic disposition.
DMD #42531

Acknowledgments

We acknowledge the contributions of Dr. Robert Pierce, Patricia A. Bourne, Dr. Ying Yu, Scott Turner and Doug Hodges at the Department of Pathology for the immunohistochemistry and fluorescence scanning analysis; Dr. Laurence Fayadat-Dilman and Fahimeh Raoufi at the Department of Protein Chemistry for providing the hIL-10, PEG-hIL-10, hαhIL-10 12G8 and mαmIL-10 11D8 and SEC-MALS analysis; Dr. Diane Hollenbaugh at the Department of Antibody Engineering for production of a mouse anti-DyLight-649 antibody; Daniel Gorman, Salvatore Santino and Mehrdad Mosferati at the Department of Molecular Biology for the mFcRn cloning and expression; Dr. Wolfgang Seghezzi at the Department of Bioanalytics for performing ELISA and ECL assays for mIL-10, PEG-mIL-10 and hαhIL-10 12G8; and Dr. Mohammad Tabrizi, Dr. Leo Tseng, Dr. Amy Beebe, and Dr. Leonard Presta for valuable discussions.
DMD #42531

Authorship Contributions

Participated in research design: Alvarez and Escandón.

Conducted experiments: Alvarez, So, Hsieh, Shinsky-Bjorde and Ma.

Contributed new reagents or analytic tools: Alvarez, So and Escandón.

Performed data analysis: Alvarez, Song, Pang and Marian.

Wrote or contributed to the writing of the manuscript: Alvarez and Escandón.
DMD #42531

References


DMD #42531


human colon carcinoma COLO205 tumor-bearing nude mice. *Drug Metab Dispos* **32**: 1230-1238.
Legend for Figures

Fig. 1. In vitro characterization of mIL-10, PEG-mIL-10 and hαhIL-10 antibody 12G8. SEC-HPLC profile analysis of native mIL-10 (A), PEG-mIL-10 (B) and hαhIL-10 (C); and 649-fluorescent labeled mIL-10 (D), PEG-mIL-10 (E) and hαhIL-10 (F). Black arrows indicate the position of proteins. All samples were run on a BioSep-SEC-S 3000 Phenomenex column at 1mL/minute elution rate.

Fig. 2. In vitro characterization of hIL-10/αIL-10 immune complexes incubated in plasma. SEC-HPLC profile analysis of 649-fluorescent labeled hIL-10 incubated in the presence of unlabeled hαhIL-10 12G8 (A) or mαmIL-10 11D8 (B). Molar ratios of hαhIL-10/hIL-10 or mαmIL-10/hIL-10 are shown in the legends. Black arrows indicate the position of proteins and immune complexes. All samples were run on a BioSep-SEC-S 3000 Phenomenex column at 1mL/minute elution rate.

Fig. 3. Concentration versus time profiles following administration of 3 mg/kg mIL-10, PEG-mIL-10 and hαhIL-10 12G8. A, Serum concentrations of mice following i.v. and s.c. bolus administration of mIL-10 were determined by ELISA at 5, 15, 30 min, 1, 2, 3, 4, 8, 10, 16 and 24 hr; and at 15, 30 min, 1, 2, 3, 4, 8, 10, 16, 24 and 30 hr, respectively. Serum concentration following i.v. and s.c. bolus administration of PEG-mIL-10 were determined by ELISA at 5, 15, 30 min, 1, 2, 3, 4, 8, 10, 16, 24 and 48 hr; and at 15, 30 min, 1, 2, 3, 4, 8, 10, 16, 24 and 48 hr, respectively. Serum concentration following i.v. and s.c. bolus administration of hαhIL-10 were determined by ELISA at 5, 15, 30 min, 1, 2, 3, 4, 8, 10, 16, 24 and 48 hr; and at 15, 30 min, 1, 2, 3, 4, 8, 10, 16, 24 and 48 hr, respectively. B, hαhIL-10 plasma concentrations following i.v. bolus administration were determined by FELA at 5, 30 min, 1, 3, 6, 16 hr, 1, 2, 4, 5, 7, 10, 14, 21 and 28 days; while serum concentrations were determined by ELISA at 30 min, 1, 3, 6, 10, 16 hr, 1, 2, 3, 5, 7, 10, 14, 21 and 28 days. The results represent the average of three mice per time point. Standard deviation is shown by the bars.
Fig. 4. Concentration versus time profiles following administration of hIL-10 + αIL-10. Plasma concentrations following i.v. bolus administration in rodents of hIL-10 alone (3 mg/kg) or in preformed immune complexes with hαhIL-10 12G8 (3 mg/kg + 25 mg/kg, respectively) were determined by FELA at 5, 15, 30 min, 1, 2, 4, and 8 hr for hIL-10, and at 5, 15, 30 min, 1, 2, 4, 8, 16 hr, 1, 2 and 3 days for hαhIL-10 immune complexes, respectively. The results represent the average of three mice per time point. Standard deviation is shown by the bars. Plasma concentrations following a s.c. administered hIL-10 (20 mg/kg) alone or 5 minutes after an i.v. administration of mαmIL-10 11D8 (20 mg/kg + 60 mg/kg, respectively) were determined by FELA at 1, 2, 4, 8, 16 hr, 1 and 2 days for hIL-10, and at 1, 2, 4, 8 hr, 1, 2 and 4 days for mαmIL-10 immune complexes, respectively. The results represent the average of two mice per time point.

Fig. 5. Plasma fluorescent SEC-HPLC profiles of 649-labeled mIL-10 (A), PEG-mIL-10 (B), hαhIL-10 12G8 (C) and hIL-10/hαhIL-10 12G8 immune complexes (D) after administration in rodents. Plasma samples were collected at 0.25, 0.5, 1, 2, 6 hr, 1 and 2 days for mIL-10 (3 mg/kg); and 0.5, 1, 2, 6 hr, 1 and 2 days for PEG-mIL-10 (3 mg/kg) following s.c. administration. For hαhIL-10 treated mice (3 mg/kg), plasma samples were collected at 0.08, 0.5, 1, 3, 6, 16 hr, 1, 2, 4, 5, 7, 10, 14, 21 and 28 days, and at 5, 30 min, 1, 2, 4 hrs, 1, 2 and 3 days following i.v. administration of hIL-10/hαhIL-10 (3 mg/kg + 25 mg/kg, respectively). Undiluted aliquots were run on a BioSep-SEC-S 3000 Phenomenex column as described before. Black arrows indicate the position of the main fluorescent peaks. Only one set of time points are shown.
DMD #42531

Fig. 6. Microgram-equivalents of 649-labeled mIL-10 and PEG-mIL-10 in tissues. Tissue concentrations in liver, heart, lung, spleen, thymus, colon, muscle, brain, lymph nodes, plasma, whole blood and kidney were measured at 0.5, 1, 2, 6 hr, 1 and 2 days after s.c. dosing with mIL-10 (A) and PEG-mIL-10 (B). Organs and biomatrices were collected and process for microplate quantitative analysis. Values are expressed as microgram-equivalent per gram of wet tissue. The results represent the average of two mice per time point.

Fig. 7. Tissue-to-blood ratios of 649-labeled mIL-10 and PEG-mIL-10 following s.c. administration in mice. Liver, heart, lung, spleen, thymus, colon, muscle, brain, lymph nodes, plasma, whole blood and kidney were measured at the indicated time points. Organs and biomatrices were collected and process for microplate quantitative analysis. Ratios above 1 are indicative of tissue uptake. The results represent the average of two mice per time point.

Fig. 8. Microgram-equivalents and tissue-to-blood ratios of hαhIL-10 12G8. Tissue concentrations (A) and tissue-to-blood ratios (B) in liver, kidney, heart, lung, spleen, colon, ileum, brain, lymph nodes, urine, plasma and whole blood were measured at 5 min, 1, 2, 6 hr, 1, 2, 4, 7 and 14 days after i.v. dosing with hαhIL-10. Organs and biomatrices were collected and process for microplate quantitative analysis. Values are expressed as microgram-equivalent per gram of wet tissue. Ratios above 1 are indicative of tissue uptake. The results represent the average of two mice per time point.

Fig. 9. Whole-body in vivo imaging (anterior-posterior view) of mIL-10 and PEG-mIL-10 dosed mice using computer tomography (CT)-fusion fluorescence analysis. CT scan and fluorescence analysis of BALB/c mice 2 hours after s.c. injection (3 mg/kg) with 680-labeled mIL-10 (A, B)
Fig. 10. Biodistribution of hIL-10 and hIL-10/hαhIL-10 12G8 in immune complexes following i.v. administration. Microgram-equivalents (A), kidney-to-liver ratios (B), and liver-to-kidney ratios (C) of 649-labeled hIL-10 alone (3 mg/kg) or in preformed immune complexes with hαhIL-10 12G8 (25 mg/kg) at 5, 30 min, 1, 2, 4, 24 and 48 hr. Values are expressed as microgram-equivalent per gram of wet tissue. The results represent the average of two mice per time point.

Fig. 11. Biodistribution of hIL-10 and hIL-10/mαmIL-10 11D8 in immune complexes following s.c. administration. Microgram-equivalents (A), kidney-to-liver ratios (B), and liver-to-kidney ratios (C) of s.c. administered 649-hIL-10 alone (20 mg/kg) or 5 minutes after an IV administration of mαmIL-10 11D8 (20 mg/kg + 60 mg/kg, respectively) at 30 min, 1, 2, 6, 16, 24 and 48 hr. Values are expressed as microgram-equivalent per gram of wet tissue. The results represent the average of two mice per time point.

Fig. 12. SDS-PAGE fluorescence analysis of plasma and tissue lysate samples. SDS-PAGE of immunoprecipitated tissue samples collected after s.c. dosing of 649-hIL-10 alone [-] (20 mg/kg) or 5 minutes after an i.v. administration of mαmIL-10 11D8 [+] (20 mg/kg + 60 mg/kg, respectively). Black arrows indicate the molecular weight of hIL-10 (monomer, ~18 kDa). Organs and biomatrices were collected at 0.5, 1, 2, 6 and 24 hrs after dosing.

Fig. 13. Anti-649 immunohistochemistry (IHC) analysis of mice tissue samples after 2 hr treatment with hIL-10 and hIL-10/mαmIL-10 11D8 immune complexes. Tissue samples were collected after s.c. dosing of 649-hIL-10 alone (20 mg/kg) (1, 2 and 3) or 5 minutes after an i.v.
administration of mαmIL-10 11D8 (20 mg/kg + 60 mg/kg, respectively) (4, 5 and 6). Scale bars indicate 100 µm (original magnification x20).

Fig. 14. Dual fluorescence microscopy analysis following i.v. dosing of hIL-10 alone on in preformed immune complexes with hαhIL-10 12G8. Frozen liver and kidney sections (5 µm) were analyzed 1 hr after i.v. administration of 649-hIL-10 (5 mg/kg) alone (1, 2 and 3) or in preformed immune complexes with 549-hαhIL-10 12G8 (5 mg/kg + 25 mg/kg, respectively) (4 to 9). Red and green signals represent 649 and 549 fluorescent signals individually. Yellow represents the overlay of both fluorescent channels indicative of colocalization. Scale bars indicate 100 µm (original magnification x20).

Fig. 15. SEC-HPLC characterization of tissue lysates and plasma of mice i.v. dosed with 649-hIL-10 in preformed immune complexes with hαhIL-10 12G8. Tissue samples were collected at 1 hr and lysates were prepared as previously described. Plasma HPLC profiles of hIL-10 and hαhIL-10 12G8 are included for comparison. Black arrows indicate the position of the main fluorescent peaks. Only one set of time points are shown.


**TABLE 1**

Study design of s.c. and i.v. single dosing of IL-10 and αIL-10 in BALB/C or CD-1 female mice

<table>
<thead>
<tr>
<th>Protein or Antibody</th>
<th>Route</th>
<th>Dose</th>
<th>Observation period (day)</th>
<th>Mice</th>
<th>PK analysis</th>
<th>TD analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>mIL-10</td>
<td>i.v.</td>
<td>3.0</td>
<td>1</td>
<td>BALB/c</td>
<td>ECL</td>
<td>___</td>
</tr>
<tr>
<td>PEG-mIL-10</td>
<td>i.v.</td>
<td>3.0</td>
<td>2</td>
<td>BALB/c</td>
<td>ELISA</td>
<td>___</td>
</tr>
<tr>
<td>native &amp; 649-labeled mIL-10</td>
<td>s.c.</td>
<td>3.0</td>
<td>1.25</td>
<td>BALB/c</td>
<td>ECL</td>
<td>FELA</td>
</tr>
<tr>
<td>native &amp; 649-labeled PEG-mIL-10</td>
<td>s.c.</td>
<td>3.0</td>
<td>2</td>
<td>BALB/c</td>
<td>ELISA</td>
<td>FELA</td>
</tr>
<tr>
<td>native &amp; 649-labeled hahIL-10 12G8</td>
<td>i.v.</td>
<td>3.0</td>
<td>28</td>
<td>CD-1</td>
<td>ELISA</td>
<td>FELA</td>
</tr>
<tr>
<td>649-hahIL-10 12G8</td>
<td>i.v.</td>
<td>3.0</td>
<td>28</td>
<td>CD-1</td>
<td>FELA</td>
<td>FELA</td>
</tr>
<tr>
<td>649-hIL-10</td>
<td>i.v.</td>
<td>3.0</td>
<td>3</td>
<td>CD-1</td>
<td>FELA</td>
<td>FELA</td>
</tr>
<tr>
<td>649-hIL-10 + hahIL-10 12G8</td>
<td>i.v.</td>
<td>L: 3.0, mAb: 25.0</td>
<td>3</td>
<td>CD-1</td>
<td>FELA</td>
<td>FELA</td>
</tr>
<tr>
<td>649-hIL-10</td>
<td>s.c.</td>
<td>20.0</td>
<td>2</td>
<td>BALB/c</td>
<td>FELA</td>
<td>FELA</td>
</tr>
<tr>
<td>649-hIL-10 + mIL-10 11D8</td>
<td>L: s.c., mAb: i.v.</td>
<td>L: 20.0, mAb: 60.0</td>
<td>4</td>
<td>BALB/c</td>
<td>FELA</td>
<td>FELA</td>
</tr>
<tr>
<td>649-hIL-10 + 549-hahIL-10 12G8</td>
<td>i.v.</td>
<td>L: 5, mAb: 25.0</td>
<td>0.04</td>
<td>CD-1</td>
<td>___</td>
<td>FELA&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>In vivo experiment was only performed for fluorescence scanning analysis.
TABLE 2
Pharmacokinetic Parameters for mL-10 and PEG-mL-10

Parameters were calculated using a non-compartmental Model for i.v. and s.c. Administration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>mL-10</th>
<th>mL-10</th>
<th>PEG-mL-10</th>
<th>PEG-mL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg) &amp; Route</td>
<td>3.0, i.v.</td>
<td>3.0, s.c.</td>
<td>3.0, i.v.</td>
<td>3.0, s.c.</td>
</tr>
<tr>
<td>Clearance (mL/day/kg)</td>
<td>4584.0</td>
<td>---</td>
<td>220.80</td>
<td>---</td>
</tr>
<tr>
<td>Vss (mL/kg)</td>
<td>95.40</td>
<td>---</td>
<td>62.40</td>
<td>---</td>
</tr>
<tr>
<td>AUC last (µg*day/mL)</td>
<td>0.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.58&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; terminal (day)</td>
<td>0.098</td>
<td>0.16</td>
<td>0.26</td>
<td>0.29</td>
</tr>
<tr>
<td>Tmax (day)</td>
<td>---</td>
<td>0.04</td>
<td>---</td>
<td>0.42</td>
</tr>
<tr>
<td>Cmax (µg/ml)</td>
<td>---</td>
<td>1.96</td>
<td>---</td>
<td>8.60</td>
</tr>
</tbody>
</table>

<sup>a</sup>AUC last calculated up to 24 hr, <sup>b</sup>AUC last calculated up to 30 hr, and <sup>c</sup>AUC last calculated up to 48 hr.
# TABLE 3

Pharmacokinetic Parameters for hαhIL-10

Parameters were calculated using a non-compartmental Model for i.v. administration.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>hαhIL-10 12G8 (FELA)</th>
<th>hαhIL-10 12G8 (ELISA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Clearance (mL/day/kg)</td>
<td>8.48</td>
<td>8.25</td>
</tr>
<tr>
<td>Vss (mL/kg)</td>
<td>158.26</td>
<td>135.32</td>
</tr>
<tr>
<td>AUC last (µg*day/mL)</td>
<td>272.12</td>
<td>292.75</td>
</tr>
<tr>
<td>t₁/₂ terminal (day)</td>
<td>13.66</td>
<td>11.89</td>
</tr>
</tbody>
</table>
TABLE 4

Pharmacokinetic Parameters for hαhIL-10/hIL-10 and mαmIL-10/hIL-10 immune complex

Parameters were calculated using a non-compartmental Model for i.v. and s.c. Administration.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>hIL-10/hαhIL-10 (12G8 (FELA))a</th>
<th>hIL-10 (FELA)b</th>
<th>hIL-10/mαmIL-10 (11D8 (FELA))b</th>
<th>hIL-10 (FELA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg) &amp; Route</td>
<td>hαhIL-10: 25, i.v. &amp; 3.0, i.v.</td>
<td>mαmIL-10: 60, i.v. &amp; hIL-10: 20, s.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clearance</td>
<td>243.34</td>
<td>5655.89</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Vss (mL/kg)</td>
<td>221.46</td>
<td>75.27</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>AUC last (µg*day/mL)</td>
<td>11.28</td>
<td>0.53</td>
<td>4.88</td>
<td>1.29</td>
</tr>
<tr>
<td>t1/2 terminal (day)</td>
<td>1.16</td>
<td>0.04</td>
<td>1.16</td>
<td>0.54</td>
</tr>
<tr>
<td>Tmax (day)</td>
<td>---</td>
<td>---</td>
<td>0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Cmax (µg/ml)</td>
<td>---</td>
<td>---</td>
<td>11.80</td>
<td>8.02</td>
</tr>
</tbody>
</table>

aCalculated PK parameters up to 3 days for hαhIL-10/hIL-10, while only up to 8 hours for hIL-10.

bCalculated PK parameters only up to 48 hours, due that at 96 hours the AUC_%Extrap_pred > 25%.
### TABLE 5

Monoclonal antibody binding affinity to murine FcRn at pH 6.0

Approximate $K_d$ values (nM) indicated data determined from kinetic analysis of two independent binding experiments

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>control</th>
<th>pre-incubate with hIL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>hαhIL-10</td>
<td>9.9</td>
<td>113</td>
</tr>
<tr>
<td>hαVEGF</td>
<td>7.1</td>
<td>7.8</td>
</tr>
</tbody>
</table>
TABLE 6

Relative amounts of Di- vs. Mono-forms present in total PEG-mIL-10 over time following s.c. dosing in mice measured by SEC-HPLC analysis.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>% of Di-PEG mIL-10</th>
<th>% of Mono-PEG-mIL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>33.8</td>
<td>66</td>
</tr>
<tr>
<td>2</td>
<td>39.7</td>
<td>60</td>
</tr>
<tr>
<td>6</td>
<td>61.8</td>
<td>38</td>
</tr>
<tr>
<td>24</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
**FIGURE 2**

A. Fluorescence vs. Retention Time (Rt, min) for different concentrations of ligand (0.5, 1.5, 2.5, and 4) in blank plasma. Key points:
- Rt = 6.54 min
- Rt = 7.22 min
- Rt = 7.78 min
- Rt = 9.40 min

B. Fluorescence vs. Retention Time (Rt, min) for different concentrations of ligand (0.15, 0.25, 0.5, 1.5, and 2.5) in blank plasma. Key points:
- Rt = 7.70 min
- Rt = 9.34 min
- Rt = 11.05 min

Legend:
- blank plasma
- only ligand
- 0.5
- 1.5
- 2.5
- 4
FIGURE 3

A

Serum mIL-10, PEGmIL-10 (µg/mL)

Time (day)

mIL10 (i.v.)
PmG mIL10 (i.v.)
mIL10 (s.c.)
PEG mIL10 (s.c.)

B

Plasma 649-αhIL-10 (µgs/mL)

Time (day)

αhIL-10 (i.v.) - FELA
αhIL-10 (i.v.) - ELISA
FIGURE 4

Plasma 649-hIL-10 (μg/ml) over time (days) for different treatments:
- hIL-10 (i.v.)
- hIL-10/hαhIL-10 immune complexes (i.v.)
- hIL-10 (s.c.)
- hIL-10 (s.c.) after mαmIL-10 (i.v.)
FIGURE 5

Panel A: Plot showing retention time (Rt) vs. fluorescence for blank plasma and mIL-10. mIL-10 has a retention time (Rt) of 9.47 min.

Panel B: Plot showing retention time (Rt) vs. fluorescence for blank plasma and Di-PEG mIL-10. Di-PEG mIL-10 has a retention time (Rt) of 8.52 min.

Panel C: Plot showing retention time (Rt) vs. fluorescence for blank plasma and hGhIL-10. hGhIL-10 has a retention time (Rt) of 8.58 min.

Panel D: Plot showing retention time (Rt) vs. fluorescence for blank plasma and other samples. III has a retention time (Rt) of 7.37 min, II has a retention time (Rt) of 7.88 min, and I has a retention time (Rt) of 6.74 min.
FIGURE 6

A

Liver
Kidney
Heart
Lung
Spleen
Colon
Muscle
Brain
Thymus
Lymph Nodes
Plasma
WB

649-miIL-10 Tissue Concentration
(μg/g Tissue)

Time (hr)

B

Liver
Kidney
Heart
Lung
Spleen
Colon
Muscle
Brain
Thymus
Lymph Nodes
Plasma
WB

649-PEGmIL-10 Tissue Concentration
(μg/g Tissue)

Time (hr)
FIGURE 9

CT scan

fluorescence

mIL-10

PEG-mIL-10
FIGURE 10

A

649-hIL-10 Tissue Concentration (mg/g Tissue)

- Liver hIL-10/hαIL-10
- Liver hIL-10 alone
- Kidney hIL-10/hαIL-10
- Kidney hIL-10 alone
- Spleen hIL-10/hαIL-10
- Spleen hIL-10 alone
- Plasma hIL-10/hαIL-10
- Plasma hIL-10 alone

Time (hrs)

B

Kidney:Liver Ratio

- 5 min
- 30 min
- 1 hr
- 2 hr
- 4 hr
- 1 day
- 2 day

C

Liver:Kidney Ratio

- 5 min
- 30 min
- 1 hr
- 2 hr
- 4 hr
- 1 day
- 2 day

Treatment

This article has not been copyedited and formatted. The final version may differ from this version.
FIGURE 12

- Plasma
- Blood
- Liver
- Kidney
- Spleen

18 kDa bands at 0.5, 1, 2, 6, and 24 hours.
FIGURE 15

Retention Time (Rt, min)

Fluorescence $\lambda = 649$ nm (LU)

- Liver
- Kidney
- Spleen
- Plasma
- Free DL-649 dye
- hαhIL-10
- hIL-10
- hxIL-10

$Rt = 6.50$ min
$Rt = 7.15$ min
$Rt = 8.58$ min
$Rt = 9.40$ min
$Rt = 11.10$ min
Supplemental Material

Effects of Pegylation and Immune Complex Formation on the Pharmacokinetics and Biodistribution of Recombinant Interleukin 10 in mice

Hamsell M. Alvarez, On-Yee So, SuChun Hsieh, Natasha Shinsky-Bjorde, Huiping Ma, Yaoli Song, Yinuo Pang, Melinda Marian, and Enrique Escandón


Address reprint requests to: Enrique Escandón, Ph.D., 901 S. California Avenue, Palo Alto, CA 94304-1104. Phone: (650) 496-6564, FAX: (650) 496-1200, Email: enrique.escandon@merck.com.
SUPPLEMENTAL TABLE 1

mIL10 % of injected dose per gram of tissue following SC administration in normal mice.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Liver</th>
<th>Kidney</th>
<th>Heart</th>
<th>Lung</th>
<th>Spleen</th>
<th>Colon</th>
<th>Ileum</th>
<th>Muscle</th>
<th>Brain</th>
<th>Skin</th>
<th>Thymus</th>
<th>L. Nodes</th>
<th>WB</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.60</td>
<td>5.4</td>
<td>0.32</td>
<td>0.04</td>
<td>0.04</td>
<td>0.08</td>
<td>0.59</td>
<td>0.07</td>
<td>0.10</td>
<td>65.8</td>
<td>0.19</td>
<td>0.14</td>
<td>0.64</td>
<td>1.16</td>
</tr>
<tr>
<td>0.5</td>
<td>1.47</td>
<td>11.2</td>
<td>0.35</td>
<td>0.03</td>
<td>0.38</td>
<td>0.00</td>
<td>0.14</td>
<td>0.09</td>
<td>0.08</td>
<td>142.7</td>
<td>2.51</td>
<td>0.05</td>
<td>0.75</td>
<td>1.50</td>
</tr>
<tr>
<td>1</td>
<td>2.98</td>
<td>51.2</td>
<td>0.59</td>
<td>0.43</td>
<td>1.16</td>
<td>0.32</td>
<td>0.76</td>
<td>0.19</td>
<td>0.09</td>
<td>61.7</td>
<td>0.37</td>
<td>0.59</td>
<td>1.12</td>
<td>2.09</td>
</tr>
<tr>
<td>2</td>
<td>3.77</td>
<td>62.5</td>
<td>0.80</td>
<td>0.50</td>
<td>1.72</td>
<td>0.63</td>
<td>1.35</td>
<td>0.35</td>
<td>0.10</td>
<td>69.7</td>
<td>0.42</td>
<td>0.74</td>
<td>0.57</td>
<td>1.14</td>
</tr>
<tr>
<td>6</td>
<td>5.31</td>
<td>87.5</td>
<td>0.93</td>
<td>0.48</td>
<td>1.97</td>
<td>3.28</td>
<td>6.87</td>
<td>0.20</td>
<td>0.12</td>
<td>62.1</td>
<td>0.30</td>
<td>0.52</td>
<td>0.62</td>
<td>0.67</td>
</tr>
<tr>
<td>24</td>
<td>1.49</td>
<td>19.9</td>
<td>0.21</td>
<td>0.00</td>
<td>0.22</td>
<td>0.43</td>
<td>0.27</td>
<td>0.00</td>
<td>0.03</td>
<td>20.4</td>
<td>0.04</td>
<td>0.22</td>
<td>0.15</td>
<td>0.18</td>
</tr>
<tr>
<td>48</td>
<td>1.05</td>
<td>6.7</td>
<td>0.11</td>
<td>0.00</td>
<td>0.27</td>
<td>0.12</td>
<td>0.10</td>
<td>0.01</td>
<td>0.13</td>
<td>13.8</td>
<td>0.04</td>
<td>0.04</td>
<td>0.06</td>
<td>0.06</td>
</tr>
</tbody>
</table>
**SUPPLEMENTAL TABLE 2**

PEG-mIL-10 % of injected dose per gram of tissue following SC administration in normal mice.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Liver</th>
<th>Kidney</th>
<th>Heart</th>
<th>Lung</th>
<th>Spleen</th>
<th>Colon</th>
<th>Muscle</th>
<th>Brain</th>
<th>Skin</th>
<th>Thymus</th>
<th>L. Nodes</th>
<th>WB</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.00</td>
<td>0.00</td>
<td>0.17</td>
<td>0.01</td>
<td>0.00</td>
<td>0.11</td>
<td>0.26</td>
<td>112</td>
<td>0.36</td>
<td>0.70</td>
<td>0.42</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.04</td>
<td>0.90</td>
<td>0.36</td>
<td>0.20</td>
<td>0.04</td>
<td>0.16</td>
<td>0.06</td>
<td>137</td>
<td>0.38</td>
<td>0.78</td>
<td>0.90</td>
<td>2.14</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.11</td>
<td>8.80</td>
<td>1.23</td>
<td>1.76</td>
<td>1.45</td>
<td>0.00</td>
<td>0.24</td>
<td>99.2</td>
<td>1.46</td>
<td>1.02</td>
<td>3.42</td>
<td>7.50</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2.47</td>
<td>24.0</td>
<td>2.27</td>
<td>4.09</td>
<td>2.10</td>
<td>3.48</td>
<td>0.55</td>
<td>48.1</td>
<td>1.26</td>
<td>1.83</td>
<td>4.70</td>
<td>8.75</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>2.20</td>
<td>19.0</td>
<td>1.28</td>
<td>2.14</td>
<td>1.14</td>
<td>2.67</td>
<td>0.53</td>
<td>19.4</td>
<td>0.95</td>
<td>1.30</td>
<td>1.66</td>
<td>3.60</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>0.43</td>
<td>4.23</td>
<td>0.34</td>
<td>0.52</td>
<td>0.20</td>
<td>2.02</td>
<td>0.11</td>
<td>10.9</td>
<td>0.47</td>
<td>1.83</td>
<td>0.20</td>
<td>0.52</td>
<td></td>
</tr>
</tbody>
</table>