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Safety Evaluation of Lipid Nanoparticle–Formulated Modified mRNA in the Sprague-Dawley Rat and Cynomolgus Monkey

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Abstract

The pharmacology, pharmacokinetics, and safety of modified mRNA formulated in lipid nanoparticles (LNPs) were evaluated after repeat intravenous infusion to rats and monkeys. In both species, modified mRNA encoding the protein for human erythropoietin (hEPO) had predictable and consistent pharmacologic and toxicologic effects. Pharmacokinetic analysis conducted following the first dose showed that measured hEPO levels were maximal at 6 hours after the end of intravenous infusion and in excess of 100-fold the anticipated efficacious exposure (17.6 ng/ml) at the highest dose tested.[24] hEPO was pharmacologically active in both the rat and the monkey, as indicated by a significant increase in red blood cell mass parameters. The primary safety-related findings were caused by the exaggerated pharmacology of hEPO and included increased hematopoiesis in the liver, spleen, and bone marrow (rats) and minimal hemorrhage in the heart (monkeys). Additional primary safety-related findings in the rat included mildly increased white blood cell counts, changes in the coagulation parameters at all doses, as well as liver injury and release of interferon γ–inducible protein 10 in high-dose groups only. In the monkey, as seen with the parenteral administration of cationic LNPs, splenic necrosis and lymphocyte depletion were observed, accompanied with mild and reversible complement activation. These findings defined a well-tolerated dose level above the anticipated efficacious dose. Overall, these combined studies indicate that LNP-formulated modified mRNA can be administered by intravenous infusion in 2 toxicologically relevant test species and generate supratherapeutic levels of protein (hEPO) in vivo.

Keywords

modified mRNA, lipid nanoparticle, toxicology, pharmacokinetics, drug discovery

The promise of mRNA as a novel modality to deliver therapeutic proteins in humans is vast, as evidenced by the growth and success of recombinant human therapeutic proteins over the last 3 decades, such as recombinant human insulin for the treatment of diabetes mellitus.[17] Protein expression directed by exogenous mRNA offers many advantages over other nucleic acid–based concepts, as well as recombinant proteins. Potential advantages of mRNA over DNA-based technology include (1) no integration into the host genome thereby circumventing the risk of deleterious chromosomal changes, and (2) faster and more efficient expression with proper modifications, since mRNA therapeutics only require access to the cytoplasm. In comparison with recombinant proteins, mRNA would have lower manufacturing costs and could enable access to intracellular as well as cell membrane–bound therapeutic targets. The biggest challenges of mRNA technology are its potential for immunogenicity and its relatively poor in vivo stability. These challenges have been addressed through progress in chemistry and sequence engineering (eg, optimization of the 5′ cap, 5′-, and 3′-untranslated regions and coding sequences) and through the use of specific nucleotide modifications.[16,21,29]

Nucleotide-modified mRNA is nearly identical to naturally occurring mammalian mRNA, with the exception that certain nucleotides, normally present in mammalian mRNA, are partially or fully replaced with nucleosides, such as pyrimidine nucleosides—specifically, pseudouridine, 2-thiouridine,

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5-methyl cytosine, or N1-methyl-pseudouridine.[2,14,16,21,29] These naturally occurring pyrimidine nucleotides are present in mammalian tRNA, rRNA, and small nuclear RNAs.[20] Incorporation of these nucleotides in place of the normal pyrimidine base has been shown to minimize the indiscriminate recognition of exogenous mRNA by pathogen-associated molecular pattern receptors, such as toll-like receptors, retinoic acid–inducible gene 1, melanoma differentiation-associated protein 5, nucleotide-binding oligomerization domain-containing protein 2, and protein kinase R.[7]

Given the lability of a naked mRNA molecule, the development of mRNA therapeutics has been further hampered by the lack of appropriate formulations for delivery and potentially as a targeting mechanism to a diseased organ or tissue.[12] However, the application of lipid-based nanoparticle delivery systems, initially developed for the in vivo delivery of siRNA, has enabled systemic administration of modified mRNA.[22] Adequate delivery of mRNA with lipid nanoparticles (LNPs) has been demonstrated for mRNA-based vaccines, where intramuscular injection of low doses of mRNA formulated in either LNPs or nanoemulsion induced immune protection from influenza and respiratory syncytial virus in mice, as well as cytomegalovirus and respiratory syncytial virus in monkeys.[9] Furthermore, a single administration of modified mRNA-LNP complexes in mice by various routes resulted in high, sustained protein production.[19] Finally, Thess et al[25] reported that repeated administration of unmodified mRNA in combination with the nonliposomal polymeric delivery system (*Trans*IT) induced high systemic protein levels and strong physiologic responses in mice. These authors also noted similar observations following single-dose administration of erythropoietin (EPO)–mRNA in LNPs to pigs and monkeys.

LNPs have been reported to be clinically effective for the delivery of siRNA.[6] The LNP vehicle is currently in late-phase clinical trials of a synthetic siRNA in patients suffering from transthyretin amyloidosis and has been well tolerated in this population.[6] Therefore, considerable work has been done to understand the safety profile of systemic administration of siRNA-LNPs.[3] Here, we set out to describe, for the first time, the pharmacology and toxicologic effects of repeated administration of hEPO-mRNA in LNPs in male Sprague-Dawley rats and female cynomolgus monkeys.

Materials and Methods

Animals and Husbandry

The study plan and any amendments or procedures involving the care and use of animals in these studies were reviewed and approved by the Institutional Animal Care and Use Committee of Charles River Laboratories Preclinical Services (Montreal and Sherbrooke, Canada). During the study, the care and use of animals were conducted according to the guidelines of the US National Research Council and the Canadian Council on Ani-

Male Sprague-Dawley rats (Charles River Laboratories) were 11 to 12 weeks old and weighed between 390 and 497 g at dose initiation. Animals were group housed in polycarbonate bins and separated during designated procedures. The temperature of the animal room was kept between 19°C and 25°C, with humidity between 30% and 70%. The light cycle was 12 hours light and 12 hours dark, except during designated procedures. Animals were fed PMI Nutrition International Certified Rodent Chow No. 5CR4 (14% protein) ad libitum throughout the in-life studies, except during designated procedures. Municipal tap water treated by reverse osmosis and ultraviolet irradiation was freely available to each animal via an automatic watering system. Environmental enrichment was provided to animals per standard operating procedures of Charles River Laboratories (Montreal, Canada), except during study procedures and activities.

Female cynomolgus monkeys (Charles River Laboratories) were 1.5 to 6 years old and weighed 2.5 to 5.1 kg at the initiation of dosing. Animals were housed in stainless-steel cages and separated during designated procedures. The temperature of the animal room was kept between 20°C and 26°C, with humidity between 30% and 70%. The light cycle was 12 hours light and 12 hours dark except during designated procedures. Animals were fed PMI Nutrition International Certified Primate Chow No. 5048 (25% protein). Municipal tap water treated by reverse osmosis and ultraviolet irradiation was freely available to each animal via an automatic watering system. Psychological and environmental enrichment was provided to animals per standard operating procedures of Charles River Laboratories (Montreal, Canada) except during study procedures and activities.

Control, Test, and Reference Items

An 850-nucleotide messenger RNA was prepared by in vitro transcription from a linearized DNA template with T7 RNA Polymerase. The DNA template encoded the T7 promoter, a 5′ untranslated region, the 579-nucleotide open reading frame encoding human EPO (hEPO) mature protein with signal sequence, a 3′untranslated region, and a polyadenylated tail. The in vitro transcription was performed with the canonical nucleotides adenosine triphosphate and guanosine triphosphate and the modified nucleotides 1-methylpseudouridine triphosphate and 5-methylcytidine triphosphate. The mRNA contains a 5′ Cap 1 structure, which consisted of 7-methylguanosine linked to the 5′ nucleoside of the mRNA chain through a 5′–5′ triphosphate bridge and 2′-O-methyl group present on the first nucleotide of the mRNA.[23] The messenger RNA was purified and buffer exchanged into low ionic strength buffer for formulation.[18] The final mRNA had a calculated molecular weight of 277 786 Da.

The mRNA-loaded LNPs were generated via stepwise ethanol dilution, with an approach adapted from previously demonstrated methods.[13,30] The LNP formulation was prepared by dissolving the lipids (6Z,9Z,28Z,31Z)-heptatriaconta-

Table 1. Experimental Design for Safety Study: Rat.[a]

Group No.	Test Material	Dose Level, mg/kg[b]	Intravenous Administration	Dose Concentration mg/ml
1	PBS	0	10-min infusion, 2×/wk	0
2	mRNA EPO	0.03	10-min infusion, 2×/wk	0.006
3	mRNA EPO	0.1	10-min infusion, 2×/wk	0.02
4	mRNA EPO	0.3	10-min infusion, 2×/wk	0.06
5	mRNA EPO	0.3	10-min infusion, 1×/wk	0.06
6	Empty LNP	0.3	10-min infusion, 2×/wk	0.06

Abbreviations: EPO, erythropoietin; LNP, lipid nanoparticle; PBS, phosphate-buffered saline.
[a]No. of males per group, $n = 24$. Dose volume per group, 5 ml/kg. Dose rate per group, 30 ml/kg/h.
[b]Dose levels in terms of mRNA content. For group No. 6, the dose level is listed in terms of the same amount of lipid:mRNA ratio (by weight).

Table 2. Experimental Design for Safety Study: Monkey.[a]

Group No.	Test Material	Dose Level (mg/kg)[b]	Intravenous Administration	Dose Concentration (mg/mL)
1	PBS	0	60-min infusion (2×/wk)	0
2	mRNA EPO	0.03	60-min infusion (2×/wk)	0.006
3	mRNA EPO	0.1	60-min infusion (2×/wk)	0.02
4	mRNA EPO	0.3	60-min infusion (2×/wk)	0.06
5	mRNA EPO	0.3	60-min infusion (1×/wk)	0.06
6	Empty LNP	0.3	60-min infusion (2×/wk)	0.06

Abbreviations: EPO, erythropoietin; LNP, lipid nanoparticle; PBS, phosphate-buffered saline.
[a]No. of females per group, $n = 3$. Dose volume per group, 5 ml/kg. Dose rate per group, 5 ml/kg/h.
[b]Dose levels in terms of mRNA content. For group No. 6, the dose level is listed in terms of the same amount of lipid to mRNA ratio (by weight).

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and 1,2-dimyristoyl-rac-glycerol, methoxypolyethylene glycol (PEG2000-DMG) in ethanol. The 4 lipids were prepared as a combined stock, with a total concentration of 12.5 mM (molar ratio of 50:10:38.5:1.5, MC3:DSPC:cholesterol: PEG2000-DMG). In brief, the solution containing lipids was mixed with an acidic aqueous buffer containing mRNA (0.18 mg/ml, pH 4.0) in a T-mixer device. The resulting LNP dispersion was diluted and subsequently purified and concentrated by tangential flow filtration. The formulation was filtered through a clarification filter (0.8/0.2 μm nominal). Prior to storage, the formulation was additionally filtered through 2 in-line sterile filters (0.2 μm) and aseptically filled into sterilized vials, stoppered, and capped. Empty LNPs were generated with a similar approach, whereby mRNA was excluded from the process.

The final LNP lipid concentration was determined with an ultraperformance liquid chromatography system with online charged aerosol detection. The total concentrations of lipids in the final mRNA-LNPs and empty LNPs were 22.0 and 13.1 mg/ml, respectively. The final mRNA content in hEPO LNPs was quantified by ultraviolet analysis, resulting in an mRNA concentration of 1.2 mg/ml. Measured lipid and mRNA concentration values enabled dilution with phosphate-buffered saline (PBS) to target levels for dosing (Tables 1, 2). Particle hydrodynamic diameters were determined by dynamic light scattering. Resulting diameters for mRNA and empty LNPs were 81 nm (0.08 polydispersity index) and 61 nm (0.10 polydispersity index), respectively. Total mRNA encapsulation was quantified with the Ribogreen assay (ThermoFisher Scientific). The final value for hEPO-mRNA in LNP encapsulation was 97%. Additional information for the control, test, and reference items is provided in Supplemental Table 1.

Male Rat Study Design

Only male rats were used for this study, as there was no expected sex-specific differences in metabolism, distribution, administered over the course of 2 weeks in a 10-minute intravenous (IV) infusion via a caudal vein at a dose level, dose volume, and frequency listed in Table 1. Dose levels for each study were based on previous pharmacology data demonstrating production of efficacious levels of hEPO in the rat and cynomolgus monkey at doses $\leq$0.03 mg/kg of mRNA. Based on pharmacokinetic (PK) data indicating predictable increases in protein expression with dose, the mid- and high doses for these studies were selected to achieve significant multiples of the efficacious dose level. Since PK behavior and physiologic consequences are well defined for EPO therapy, we employed a similar approach in our study of hEPO-mRNA in LNPs.[15,16,25] Each infused dose was administered with a temporary indwelling catheter inserted in a caudal vein connected to an injection set and infusion pump. The animals were temporarily restrained for the dose administration and not sedated. The dose volume for each animal was based on the most recent body weight measurement. The first day of dosing was designated as day 1. Six males per group were used for toxicity assessment, 12 males per group for immunology assessment, and 6 males per group for PK / pharmacodynamic (PD) assessment. The following end points were evaluated: clinical signs (including observations of the infusion sites), body weights, food consumption, PK/PD, clinical pathology (hematology, coagulation, and clinical chemistry), macro- and microscopic examination of tissues, and immunotoxicology markers: histamine, interleukin 6 (IL-6), interferon γ–induced protein 10 (IP-10), tumor necrosis factor α (TNF-α), interferon α (IFN-α), and complement (C3).

Blood samples were collected from nonfasted animals and analyzed for hematology on day 9 and from fasted animals for hematology, coagulation, and clinical chemistry on day 16 (at necropsy). For PD (hEPO) or PK (hEPO-mRNA), blood samples were collected and processed to plasma prestudy and at 2, 6, 24, and 48 hours after the end of injection/infusion on days 1 and 15. After processing, the plasma samples were stored in a freezer set to maintain −80°C until analyzed. For cytokines (ie, IL-6, IP-10, TNF-α), histamine, and complement (C3) analysis,

6, and 24 hours after the end of injection/infusion on days 1 and 15 in K_3EDTA tubes and processed to plasma or serum (no anticoagulant) for IFN-α analysis.

Female Monkey Study Design

Female monkeys were used for this study, as there was no expected sex-specific differences in metabolism, distribution, or toxicity. The negative control, test, and reference items were administered over the course of 2 weeks in a 60-minute IV infusion via an appropriate peripheral vein (eg, saphenous or brachial) at the dose level, dose volume, and frequency listed in Table 2. The dose volume for each animal was based on the most recent body weight measurement. The animals were temporarily restrained (on a sling or a chair) for the dose administration and not sedated. Each infused dose was administered with a temporary indwelling catheter inserted in a peripheral vein connected to an injection set and infusion pump. The first day of dosing was designated as day 1. The end points in this study included clinical signs (including observation of the infusion sites), body weights, food consumption, PK/PD, clinical pathology (hematology, coagulation, and serum chemistry), macro- and microscopic examination of tissues, and selected cytokines (interleukin 1 β [IL-1β], IL-6, TNF-α, and IP-10) and complement (C3a and C5b-9).

Blood samples were collected from overnight-fasted animals for hematology, coagulation, and clinical chemistry parameters at predose (baseline) and on day 16. Additionally, blood was analyzed on day 8 for hematology parameters only. For PK/PD assessments, blood samples were collected and processed to plasma at the following time points: predose; 2, 6, 24, and 48 hours after the first dose; and 6 hours after subsequent dosing occasions. After processing, the plasma samples were stored in a freezer set to −80°C until analyzed. Blood samples were collected in K_3EDTA tubes and processed to plasma for analysis of cytokines (ie, IL-1β, IL-6, TNF-α) and complement (ie, C3a and C5b-9) or to serum for analysis of IFN-α and IP-10 at the following time points for all groups: predose; at 2, 6, and 24 hours after the end of infusion on day 1; and at 2, 6, and 24 hours after the end of infusion on day 15. Additionally, for complement analysis only, blood samples were collected 2, 6, and 24 hours after the end of infusion on day 4 (groups 1–4 and 6).

Clinical Pathology

Hematology parameters were measured with Bayer Advia 120 Automated Hematology Analyzer (Siemens Healthcare). Standard coagulation parameters were measured on a START 4 Compact Stago Analyzer (Diagnostica Stago). Standard clinical chemistry parameters were measured with Modular Analy-

Histamine, Cytokine and Complement Levels

Histamine levels in the rat plasma were determined with the Histamine EIA Kit (IM-2015; Immunotech). Serum levels of IFNα were determined with the Rat IFNα ELISA Kit (KT-60242; Kamiya Biomedical Company) and the Human IFNα Multi-subtype ELISA Kit (41105-1 or 41105-2; PBL Biomedical Laboratories). IL-6, IP-10, and TNFα in rat plasma were determined with the Rat Cytokine/Chemokine Magnetic Panel Kit (RECYMAG-65K; Millipore). IL1β, IL-6, and TNFα in monkey plasma were determined with the Non-Human Primate Cytokine/Chemokine Magnetic Panel Kit (PRCYTOMAG-40K; Millipore). IP-10 in the monkey serum was determined with the Monkey IP-10 Singleplex Magnetic Kit (LHB0001; Invitrogen). C3 levels in the rat plasma were determined with the Rat C3 ELISA Kit (GWB-A8B8AF; Genway). C3a levels in the monkey plasma were determined with the Human C3a EIA Kit (A031; Quidel). C5b-9 levels in the monkey plasma were determined with the Human C5b-9 ELISA Kit (558315; BD Bioscience).

Histopathology

Representative samples of the following tissues from all animals were preserved in 10% neutral buffered formalin: bone marrow (sternum), heart, infusion site (last dose), kidney, liver, lung, spleen, and thymus. Tissues were embedded in paraffin, sectioned, mounted on glass slides, and stained with hematoxylin and eosin. The histopathologic evaluation was internally peer reviewed.

hEPO bDNA

The bioanalysis of plasma samples for quantification of hEPO-mRNA levels was conducted at AxoLabs according to the bDNA method for mRNA detection developed by QuantiGene (Affymetrix).[26] Briefly, plasma samples were directly diluted in lysis buffer. On each bDNA plate, including a customized assay-specific set of probes, a dilution curve was pipetted with spiked standards into untreated plasma. Signal amplification was carried out with oligonucleotides bound to the enzyme alkaline phosphatase. The calculated amount in picograms was normalized to the amount of plasma in the lysate and to the amount of lysate applied to the plate. Since measurements in the PBS-treated control group were within the background level range, cross-reactivity of hEPO-mRNA to rat or monkey EPO mRNA was considered negligible.

hEPO ELISA

hEPO levels were measured with a human EPO Sandwich ELISA Kit (01630; Stemcell Technologies). For this assay, the lower and upper limits of quantitation were 12 and 800 pg/ml, respectively. Since predose measurements were within the background level range, cross-reactivity of hEPO to rat or

Data Analysis and Reporting

The toxicokinetic parameters of human modified hEPO-mRNA and its expressed protein in plasma were calculated with a noncompartmental approach in WinNonlin Phoenix 64, version 6.3 (Pharsight). Dose-normalized maximum serum concentration (C_{max}/dose) and area under the curve (AUC/dose) were determined by dividing the respective parameters by dose and calculated by either WinNonlin or Excel. The mean, standard deviation, and percentage coefficient of variation of the toxicokinetic parameters were calculated in WinNonlin. All reported values were rounded to either 3 significant figures or 1 decimal place (time to reach maximum serum concentration [T_{max}], half-life [$t_{1/2}$]).

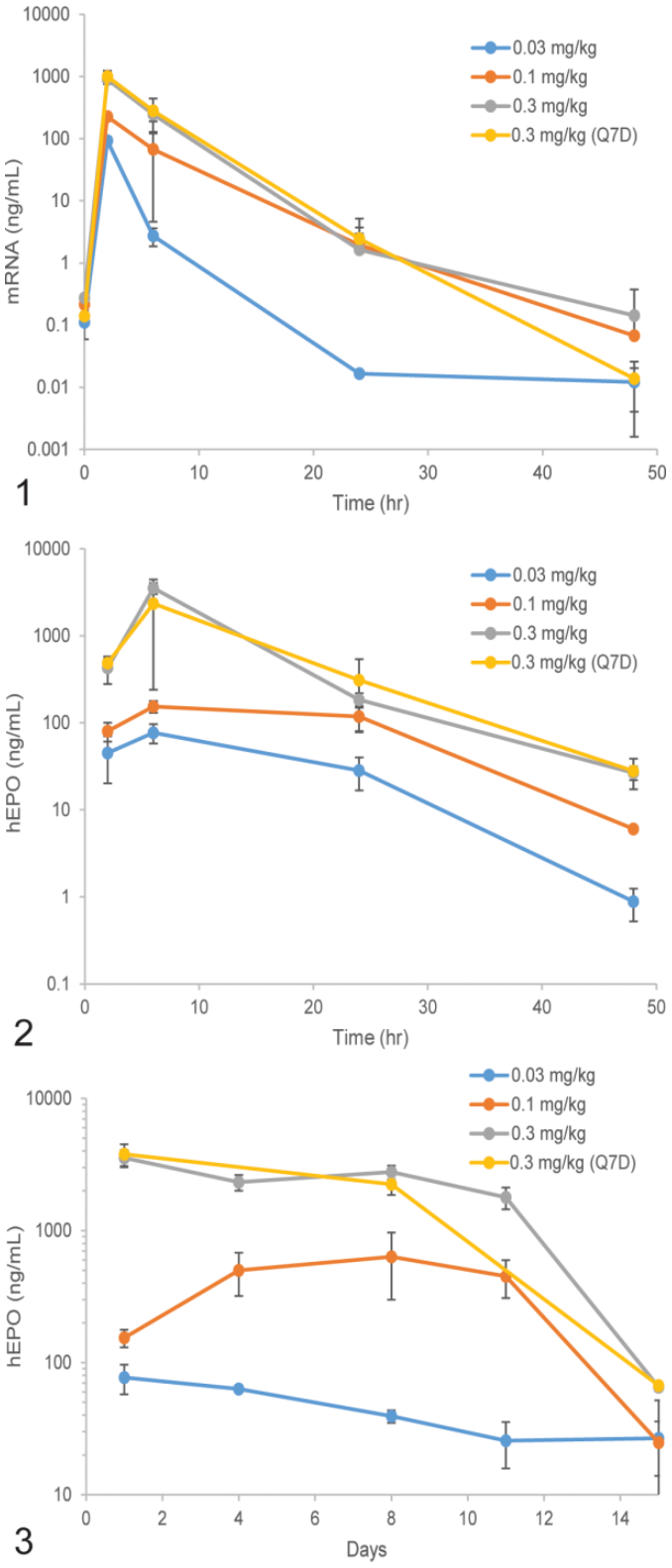
Results

Administration of hEPO-mRNA in LNP Results in Detection of Significant Serum hEPO Levels and Corresponding PD Effects in the Rat and Monkey

Toxicokinetic analysis in the rat revealed that hEPO-mRNA had a moderate half-life (2.9–5.7 hours) and low clearance (49.0–97.2 ml/h/kg; Fig. 1, Table 3). The C_{max}/dose values were consistent among the 4 dose groups, ranging from 2270 to 3320 ng/ml/mg/kg (Table 3). Measured hEPO levels were maximal approximately 6 hours after the 10-minute infusion (Fig. 2, Table 4). The AUC values (for hEPO-mRNA and hEPO) increased in more than a dose-proportional manner, between 0.03 and 0.3 mg/kg (Table 4). Plasma samples collected at 6 hours after each dose indicated that hEPO levels were constant at C_{max} at all dose levels until day 15, when measured hEPO levels were significantly decreased in the mid- and high-dosed groups (Fig. 3). Consistent with literature data,[1] peak reticulocytosis (PD marker described later) was observed by day 9, and levels remained elevated during the 15-day period. Overall, these results indicate that plasma concentrations of hEPO were mostly consistent throughout the study and exhibited greater-than-dose-proportional increases in AUC after IV administration.

Significant increases were noted in red blood cell and associated parameters (hemoglobin, hematocrit) in all male rat groups dosed with hEPO-mRNA in LNPs as compared with the PBS group and the group dosed with empty LNPs. Interestingly, the changes in red blood cell parameters (except mean corpuscular volume) were similar across all hEPO-mRNA-dosed groups and did not seem to be dose related (Fig. 4, Suppl. Fig. 1). In addition, dose-dependent increases in platelet counts and reticulocytes were noted, particularly at the highest doses administered twice weekly (Suppl. Fig. 1). Overall, these results indicate that repeated administration of hEPO-mRNA in LNPs achieves physiologically relevant and persistent hEPO levels that result in significant changes in precursor cells and mature red blood cell count at doses as low as 0.03 mg/kg.

Like in male rats, toxicokinetic findings in female monkeys



Figures 1–3. Plasma concentration of hEPO-mRNA (ng/ml; Fig. 1) and hEPO (ng/ml; Figs. 2, 3) in rats. Graphs represent mean values ($n = 6$); error bars indicate SD. Following a 10-minute infusion, peak plasma concentrations of hEPO-mRNA appear to occur at approximately 2 hours, while peak plasma concentrations of hEPO are approximately

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