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REPORT NO. 1012588

REGULATORY DOCUMENT

TITLE:	RO0503821-000: In vivo stability and tissue localization of RO0503821 after single (IV or SC) or multiple (IV) dose administration to rats (Study Nos. D01017 and D02001)
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1. GLOSSARY OF ABBREVIATIONS

ADME	Absorption, Distribution, Metabolism and Excretion
Anti-rhEPO antibody	Rabbit polyclonal antibody against recombinant human Epoetin beta
di-PEG-epoetin beta	Epoetin beta conjugated with two 30 kDa PEG groups, a by-product of RO0503821 synthesis
ELISA	Enzyme-Linked Immunosorbent Assay
rhEPO or EPO	recombinant Human Epoetin beta (RO2053859)
HRP	horseradish peroxidase
IHC	immunohistochemistry
IV	intravenous
MOPS	(3-[N-morpholino] propanesulfonic acid) buffer
NCDS	Non-Clinical Drug Safety
PEG	polyethylene glycol
mPEG	30 kDa methoxy-PEG Butanoic acid
SC	subcutaneous
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

2. SYNOPSIS OF REGULATORY DOCUMENT_REPORT NO_1012588

COMPANY: Hoffmann-La Roche, Inc. NAME OF FINISHED MEDICAL PRODUCT: NAME OF ACTIVE INGREDIENT: _RO0503821-000-001	SUMMARY TABLE REFERRING TO PART …. OF THE DOSSIER: Volume: Page: Ref:	FOR NATIONAL AUTHORITY USE ONLY:
INDICATION	Anemia	
GLP	No	
DRUG/TITLE OF THE STUDY	RO0503821-000: In vivo stability and tissue localization of RO0503821 after single (IV or SC) or multiple (IV) dose administration to rats (Study Nos. D01017 and D02001)	
MAIN AUTHOR	DK Buckman	
INVESTIGATOR(S) STUDY DATES	D01017: Buckman, Cotler, Fulton D02001: Buckman, Cotler, Fulton and Tang D01017: 03/19/01-03/20/01 (in life) D02001: 02/19/02-2/26/02 (in life)	
INSTITUTION	Hoffmann-La Roche, Inc. Nutley, NJ	
STUDY OBJECTIVES	In vivo metabolism/distribution/excretion	
STUDY DESIGN	D01017: Parallel Groups D02001: Parallel Groups	
STUDY POPULATIONS: SPECIES/NO/SEX/BODY WEIGHT	D01017: Crl(LE) Rat/4/Male/175-318 grams D02001: Crl(LE) Rat/28/Male/~250-300 grams	
DOSE / ROUTE / REGIMEN / DURATION	D01017: 6-11 mg/kg/IV or SC/single dose/30 min-24 hrs (groups III & IV) D01017: 6-11 mg/kg/IV or SC/single dose/30 min or 24 hr (groups I & II) D02001: 8 mg/kg/IV/single dose/30 min-24 hrs D02001: 8 mg/kg/IV/multiple doses (every 3 days)/30 min-7 days	
FORMULATION LOT No.	D01017: RO0503821= HC-31848-270 D02001: RO0503821= HC-33511-172	
ANALYTICAL METHODS /Ref.	ELISA, SDS-PAGE, Western Blot, Immunohistochemistry	
BIOLOGICAL SAMPLES	Serum, Urine, Bone Marrow, Sagittal Whole Body Sections	
SENSITIVITY OF ASSAY	SDS-PAGE: ~50 ng; Western Blot: ~10 ng;	
AVERAGE INTER-ASSAY PRECISION (CV)	Not known	
DATA RETRIEVAL	Laboratory Notebook No.32716	

3. SUMMARY

The major objective of the studies in this report, was to evaluate the *in vivo* stability of RO0503821, a methoxy polyethylene glycol (PEG) conjugated recombinant human Epoetin beta (rhEPO). For study D01017, the first of two studies, rats received either a single subcutaneous (SC) or a single intravenous (IV) dose of RO0503821. Selected tissues, serum, bone marrow aspirates, urine and whole body tissue sections from these rats were examined at 30 minutes and 24 hours post dose for the presence of intact RO0503821 and/or evidence of its degradation. Similarly, the second study, D02001, monitored the stability of RO0503821 in serum and urine after single or multiple IV administrations for a period of one week. The results from these two studies suggest that RO0503821 remained intact while in the serum. RO0503821 was excreted in the urine along with an additional band of PEG related material that migrated as the 30 kDa PEG standard. Anti-PEG immunohistochemistry (IHC) of bone marrow smears from treated animals (study D01017) demonstrated that an immunoreactive PEG group had reached and bound to a specific population of bone marrow cells. Analysis by electrophoresis and iodine staining confirmed the presence of RO0503821 within bone marrow aspirate at 30 minutes after IV dosing, indicating that the molecule had reached its target tissue. When whole body tissue sections from an IV RO0503821 treated rat were immunostained with an anti-rhEPO antibody, the antibody bound predominately to liver and spleen tissues. In conclusion, both studies found only RO0503821 in rat serum samples following IV or SC administration. At 30 min post IV dosing, RO0503821 was present in bone marrow aspirates and had localized mainly to the liver. RO0503821 was excreted in the urine as a 60 kDa molecule that migrated and stained the same as RO0503821, and a 30 kDa PEG entity, which migrated and stained as the mPEG reference standards.

4. INTRODUCTION

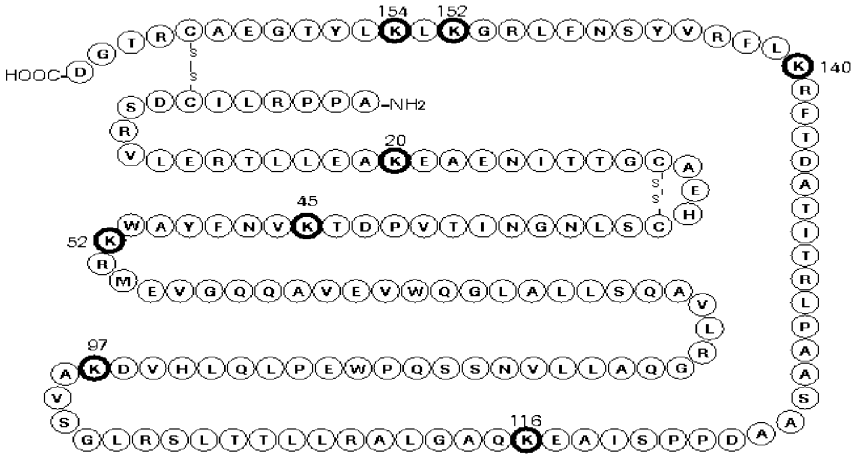
4.1 Background and Objectives

RO0503821 is a methoxy polyethylene glycol (PEG) conjugated recombinant human Epoetin beta (rhEPO) being developed for the treatment of anemia of various origins. The major objective of the present studies was to evaluate the *in vivo* stability of RO0503821. *In vivo* stability analysis of proteins conjugated with large PEG molecules is challenging due to: (a) the lack of a sensitive high resolution assay for the detection of polyethylene glycol in tissue matrices, and (b) the failure (up to this time) to find a method for labeling either the PEG or the protein components of RO0503821 that would result in a molecule that remained both bioactivity and reasonable stability.

In order to perform *in vivo* stability and tissue localization studies for RO0503821, very high doses (3-8 mg/kg) of RO0503821 were selected to overcome the detection limits of the methods to be applied. After determining that male rats could tolerate mg levels of RO0503821 for at least eight days, the following studies were initiated. The first study, D01017, examined the *in vivo* stability of RO0503821 in serum, urine and bone marrow aspirates after a single IV or SC dose. Distribution to the bone marrow and to other body tissues was also examined. A second study, D02001, investigated the time course of the stability of RO0503821 in serum and urine after either a single or multiple IV doses. All studies were performed in the Department of Non-Clinical Drug Safety (NCDS), Hoffmann-La Roche Inc., Nutley, NJ.

5. CHEMICAL STRUCTURES AND NAMES

5.1 Amino Acid Sequence and Primary Structure of Recombinant Human Epoetin beta



5.2 RO0503821 Synthesis

RO0503821 is composed of Epoetin beta, a 30 kDa glycoprotein, and a 30 kDa linear methoxy polyethylene glycol (mPEG) molecule via covalent linkage. The potential pegylation sites (K=Lysine) are circled in bold (Section 5.1). At least 90% of RO0503821 is mono-pegylated at either the N-terminus or lysine52. Most of the remaining drug substance consists of Epoetin beta conjugated with two (di-PEG-EPO) or more (oligo-PEG-EPO) PEG molecules

5.3 Lot Numbers

Study	Group ID	Test Article	Lot Number
D01017	Groups I - III	2 mg/mL RO0503821-000	HC-31848-270
D02001	Group B	6.7 mg/mL mPEG	HC-33511-175
	Groups C - J	4 mg/mL RO0503821-000	HC-33511-172
	Group A	Vehicle	HC-33511-169

6. MATERIALS AND METHODS

6.1 Reagents and Supplies

All chemicals were of the highest grade available. Whenever possible, materials and supplies that would contact with RO0503821 containing fluids were both sterile and composed of polypropylene.

6.2 Study Design

Study D01017 used four Crl:(LE)BR male rats purchased from Charles River Inc., Raleigh, NC, weighing 175-318 grams. Two received IV injections of RO0503821 at approximately 6 mg/kg (rats 2 and 3), one (rat 1) received a subcutaneous (SC) injection at 11 mg/kg, and one (rat 4) received no injection as detailed in the table below. Urine samples from rats 2-4 were collected in chilled polypropylene tubes for 24 hours post dose. Serum and bone marrow samples were collected from rat 1 at 30 minutes post dose and for rats 2-4 the samples were collected at 24 hours post dose.

Study D02001 used twenty-eight Crl:(LE)BR male rats purchased from Charles River Inc., Raleigh, NC. The animals were approximately 9 weeks old and weights ranged from 290-344 grams at the initiation of RO0503821 treatment. The first set of 16 animals (2 rats/group for groups A & B and 3 rats/group for groups C-F) was selected for the single dose arm of the study. The second set of 12 animals (groups G-J, 3 rats/group) was used in the multiple dose arm. Animals were dosed on day 1 and samples collected at each of the indicated sample collection time points (Section 6.6).

The information about animal and group assignment for both studies is detailed in the following table:

D01017: Single High Dose IV and SC Study			
Dose regimen	**Group ID**	**Animal ID**	**Sample Collection**
RO0503821 6.3 mg/kg IV	I	1	Serum, Bone Marrow 30 min post dose
RO0503821 6.7 mg/kg IV	II	2	Serum, Urine, Bone Marrow 24 hr post dose
RO0503821 11.4 mg/kg SC	III	3	Serum, Urine, Bone Marrow 24 hr post dose
Control (no injection)	VI	4	Serum, Urine, Bone Marrow at 24 hr
D02001: Single Dose Phase			
Dose regimen	**Group ID**	**Animal ID**	**Sample Collection**
Placebo Control (IV vehicle injection)	A	1-2	Serum and Urine 24 hr post dose
mPEG 6.7 mg/kg IV (PEG Control)	B	3-4	Serum 30 min post dose
RO0503821 8 mg/kg IV	C	5-7	Serum 30 min post dose
	D	8-10	Serum 3 hr post dose
	E	11-13	Serum 8 hr post dose
	F	14-16	Serum and Urine 24 hr post dose
D02001: Multiple Dose Phase			
Dose regimen	**Group ID**	**Animal ID**	**Sample Collection**
RO0503821 8 mg/kg IV On days 1, 4 and 7	G	17-19	Serum 30 min post final dose
	H	20-22	Serum 3 hr post final dose
	I	23-25	Serum 8 hr post final dose
	J	26-28[a]	Urine daily 24 hrs/day Serum 24 hr after final dose

[a] Animal 28 was found dead at 24 hr prior to the 3rd treatment. Rat 25 was used as replacement, leaving only two animals, 23 and 24, in Group I.

6.3 Husbandry and Diet

Each animal was housed individually in either a stainless steel cage or plastic metabolism cage for urine collection from animals in groups II, III, A, F and J. Each cage had an identification label showing compound number, study number, dose level, and animal number. Room temperature and relative humidity were maintained at approximately 72°F ± 4°F and 50% ± 20% respectively. An approximate 12-hour light/dark cycle (4:30 am to 4:30 pm) was maintained. Approximately 225 grams of Purina Certified Rodent Chow #5002 (meal) were provided to each animal on daily basis. The rats were allowed to eat and drink *ad libitum*.

6.4 Test and Control Articles

The compositions of the dosing solutions for both studies are listed in the table below:

Composition of Dosing Solutions				
Ingredients	**Quantity/mL**			
Study Number	D01017	D02001		
Lot Number	HC-31848-270	HC-33511-169	HC-33511-172	HC-33511-175
RO0503821/000	2 mg	-	4 mg	-
mPEG Butanoic Acid	-	-	-	6.7 mg
Sodium phosphate, Monobasic	-	1.38 mg	1.38 mg	1.38 mg
Potassium phosphate, Monobasic	0.49 mg	-	-	-
Sodium phosphate heptahydrate, dibasic	1.72 mg	-	-	-
Sodium sulfate (40 mM)	-	5.68 mg	5.68 mg	5.68 mg
Sodium Chloride	7.73 mg	-	-	-
Mannitol	-	30 mg	30 mg	30 mg
Poloxamer 188*	-	0.1 mg	0.1 mg	0.1 mg
L-methionine (10 mM)	-	1.49 mg	1.49 mg	1.49 mg
Sodium hydroxide 1N	q.s. pH 7.0± 0.1	q.s. pH 6.2 ± 0.1	q.s. pH 6.2 ± 0.1	q.s. pH 6.2 ± 0.1
HCl 10% v/v	q.s. pH 7.0± 0.1	q.s. pH 6.2 ± 0.1	q.s. pH 6.2 ± 0.1	q.s. pH 6.2 ± 0.1
Water for injection	q.s. to 1 mL	q.s. to 1 mL	q.s. to 1 mL	q.s. to 1 mL

*previously known as pluronic F68

6.5 Assay of Test and Control Articles

The Department of Pharmaceutical and Analytical Research and Development (PARD) formulated the dosing solutions. The vehicle contained either 6.7 mg/mL mPEG (Lot RC01102120-645), 2 mg/mL RO0503821/000 (Lot G003.02E) or 4 mg/mL of RO0503821/000 (Lot 10041273-G001.03E). The vehicle (HC-33511-169), or 4 mg/mL drug product solutions (HC-33511-172) were assayed and found to be satisfactory (note book reference 34082-122/SAM). The mPEG dosing solution (HC-33511-175) was formulated using the vehicle solution mentioned above. The stability of the vehicle lot HC-33511-169 has been established previously.

6.6 Experimental Conduct

Jugular vein cannulation was performed one day before the study under isofluothane anesthesia. On the experimental day, the test compounds (RO0503821, vehicle, or mPEG) were administered to the animals via the cannula at the volume of 2 mL/kg intravenously or as 1 mL subcutaneously. For multiple dose groups (G – J) the animals were dosed with RO0503821 once every third day for up to three treatments. Retro-orbital-sinus blood samples were collected under CO_2/O_2 anesthesia according to the

experimental design and the animals subsequently euthanized by CO_2 asphyxiation. Blood samples were placed in sterile polypropylene tubes and allowed to clot on ice. Clotted blood samples were centrifuged at 4°C, serum transferred to appropriately labeled individual vials and stored at -70°C. Urine samples from animals in study D01017, and from animals in groups A, F and J of study D02001, were collected over each 24 hr period into sterile polypropylene tubes surrounded by dry ice and stored at -70°C for further analysis.

For the purpose of bone marrow sample collection, both rear limbs of the animals (study D01017) were removed immediately after euthanasia at the designated times. The limbs were rinsed with ice-cold 70% ethyl alcohol, then, the femurs were removed. One femur per rat was placed in 10% formalin for sectioning and immunohistochemical (IHC) analysis. Bone marrow cells from the second femur were harvested according to the method in Mishell and Shiigi [6]. Briefly, bone marrow was flushed from the femur with ice cold PBS into a cold, sterile polypropylene tube and stored on ice or at 4°C for further analysis.

6.7 Methodology for SDS-PAGE

The reference standards used for sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were RO0503821, Epoetin beta and the PEG reagent. The concentrations of the reference standards for RO0503821 and Epoetin beta were based on the amount of Epoetin beta protein in the reagents and were calculated using an A_{280} value of 1.25 for a 1 mg/mL solution of Epoetin beta protein. The concentration of the PEG reagent reference standard was calculated as weight of dry powder in µg per mL H_2O. Frozen experimental samples were thawed on ice or at 4°C. Serum samples were diluted with ice cold PBS (1 part serum to 9 parts PBS). Then SDS-PAGE sample loading buffer was mixed with a 10 µL aliquot of diluted sample. Samples and reference standards were loaded on an Invitrogen (Invitrogen, CA) 1.0 mm 4-20% Bis-Tris gel and the gel was run using 3-[N-morpholino] propane-sulfonic acid (MOPS) running buffer.

6.8 Detection Methods

After SDS-PAGE, which separated the sample proteins according to MW, the gels were stained for the presence of PEG, total glycoprotein, total protein or transferred to membranes for the immunodetection of Epoetin beta. PEG-containing molecules were detected in the gels directly after SDS-PAGE using an iodine stain according to Kurfürst [5] and imaged immediately. The same gel was then stained using the NOVEX® Colloidal Blue Kit Protocol for NuPAGE® Bis-Tris Gels (Invitrogen, CA) to visualize total protein and/or stained with Bio-Rad Glycoprotein Staining Kit to detect glycosylated proteins. Western blots were made from unstained gels or from iodine stained gels after several additional washes (Section 6.8). Stained gels and Western blots were documented by scanning or CCD camera imaging. Selected images were analyzed by scanning densitometry using the reference standards for the standard curves.

6.9 Methodology for Western Blot

Gel proteins were transferred to nitrocellulose membranes using the NOVEX® XCell II blotting apparatus. An overnight transfer was applied to obtain a more efficient transfer of the pegylated proteins. Since the smaller Epoetin beta passed through the first

membrane during the overnight transfer, a back-up membrane was employed for all transfers. After the overnight transfer, the membranes were allowed to dry thoroughly before being placed in blocking buffer. A polyclonal rabbit anti-rhEPO antibody (pAb<EPO>rabbit-IgG(IS), Lot 1/2000 Wessner, supplied by Dr. Wolfgang Hoesel, Hoffmann-La Roche, Inc) which recognizes both RO0503821 and Epoetin beta, but not rat EPO, was used for Epoetin beta protein detection. The secondary antibody was goat anti-rabbit IgG (H+L) peroxidase conjugate (Boehringer Mannheim Corp, IN, #1814141). SuperSignal® West Dura Extended Duration Substrate (Pierce, #34075) was used for chemiluminescent detection of Epoetin beta protein on the blots. The results were recorded with a CCD camera system (NightOwl, Berthold). Images as presented have been electronically balanced for clarity.

6.10 Methodologies for Immunohistochemistry

Two immunohistochemical procedures were used to identify the tissue distribution of RO0503821. For the first procedure, bone marrow smears from the marrow in the formalin fixed femurs (study D01017, Section 6.6) were immunostained as follows for the PEG conjugate. The smears were fixed in 4% paraformaldehyde for 15 minutes then immersed in Target Retrieval Solution (Dako Ltd, Carpinteria, CA) and heated to 94ºC in a steamer (Black & Decker) for 10 minutes. To quench endogenous peroxidase activity, smears were placed in 3% H_2O_2 in Methanol for 10 min. To block non-specific tissue-bindings sites, smears were placed in 10% normal goat serum in Ultra-V (Lab Vision, Fremont, CA) at room temperature for 10 minutes. Smears were then incubated for 1 hour at room temperature with the AGP3 antibody, a mouse monoclonal antibody against PEG [3], diluted 1:50 in Antibody Diluent (Dako Ltd, Carpinteria, CA). An anti-mouse IgM antibody was used as the secondary antibody for 30 minutes followed by the addition of horseradish peroxidase-labeled streptavidin complex for 30 minutes. Vector Nova Red (Vector Laboratories, Fremont, CA) w PEG conjugated as the final chromogen for the antigen and hematoxylin was used as the nuclear counterstain. Microscopic images were captured on a Jenoptik ProgGes C14 digital camera. An anti-mouse IgM antibody was used as the secondary antibody and the microscopic images recorded.

The second procedure employed whole body immunohistochemisty to analyze the distribution of RO0503821. The frozen bodies of the 30 minute IV animal from study D01017 and a control rat were blocked in 4% carboxymethyl cellulose and sagittal 20 µm whole body tissue sections were taken at vertical depths which revealed tissues of interest. Sections were collected onto Tesa 110 mm type 4248 transparent adhesive tape and freeze dried. The whole body sections were then immunostained for the presence of Epoetin beta using the anti-rhEPO antibody described above (Section 6.9) in a procedure similar to that used for the Western Blots (Section 6.9). The secondary antibody was an anti-rabbit antibody conjugated with an infrared, IRDye800™, fluorescent dye. The fluorescently labeled whole body tissue sections were then scanned using an Odyssey Infrared Imaging System (Odyssey, LI-COR, Lincoln, NE).

6.11 Analysis of RO0503821, PEG and Epoetin beta in Serum, Urine and Bone Marrow by SDS-PAGE.

A combination of SDS-PAGE, semi-specific staining and Western blot were used to characterize the stability of RO0503821 in body fluids. After SDS-PAGE of experimental samples, the sample-containing gels were either stained immediately with an iodine stain to detect PEG molecules, or the protein contents of the gels were transferred to a nitrocellulose (NC) membrane. Gels stained for PEG could then also be stained for total protein content or for glycoproteins. Thus, serum, urine and bone marrow samples from animals administered PEG or a pegylated-protein were first separated by molecular weight and then stained to estimate quantity and molecular weights of PEG containing molecules. Intact RO0503821 and 30 kDa PEG in the SDS-PAGE gels were identified and quantities estimated by comparison with the staining and migration distance of reference standards.

Iodine stained gels of serum samples contained large bands of iodine staining material in the region in which serum albumins would be expected to migrate. Coomassie (protein) staining demonstrated that those bands did indeed contain large quantities of protein. Such staining was not seen in serum free samples. However, if generous amounts of Epoetin beta reference standard were loaded in a lane, the Epoetin beta could also be visualized with the iodine staining. Since Epoetin beta is a glycoprotein, as are many of the serum albumins, it is suggested that serum glycoproteins interact weakly with the iodine reagent. That weaker interaction could be distinguished from true PEG staining by continued washings of the iodine stained gel. Repeated washes resulted in a much decreased intensity and a slight change in color of suspect bands, while the true PEG-containing bands retained their original color.

No Epoetin beta was found in any of the experimental samples examined by iodine staining of the gels, and no sample Epoetin beta was detected by the much more sensitive Western Blot procedure. However, to rule out the presence of small amounts of Epoetin beta in the gels that were either below the detection limits of the iodine stain and/or lost during the blotting procedure, the back-up membranes were also immunostained. In addition, two further in-gel detection methods were applied. In the first method, gels were stained specifically for glycoproteins (Pierce GelCode Glycoprotein Staining Kit). The purpose of staining for glycoproteins was two-fold: a) to confirm the presence of glycoproteins in the serum 'albumin' bands, and b) to confirm the absence of detectable Epoetin beta in the gels prior to Western Blotting. A second in-gel method, in-gel immunochemistry with chemiluminescence detection, was also used to estimate potential analyte loss during the blotting procedure.

Selected SDS-PAGE gels were also stained for total protein using Invitrogen's Simply Blue Colloidal Coomassie stain. Total protein staining allows a comparison of relative quantity and composition of most of the proteins in the gel, and a comparison of the migration distances of unknown bands with the migration distances of known reference standards. The protein stain also allows an estimation of the completeness of protein transfer to the blot membrane. Although the more concentrated Epoetin beta standards were readily detected by the Simply Blue stain, very rarely were drug protein quantities in biological samples large enough to be separated and distinguished from the naturally

occurring sample proteins by Coomassie staining alone. Definitive identifications were obtained by the combination of SDS-PAGE estimated molecular weight and specific chemical or immunochemical staining as described above.

7. RESULTS AND DISCUSSION

Unless stated otherwise, the results presented in this section are from animals that received intravenous (IV) injections of the test compound.

7.1 Analysis of Urine Samples

All urine samples from animals treated with RO0503821 contained bands that migrated and stained with iodine as did the 60 kDa reference standard RO0503821 (Figure 1, page 25). The presence of a second iodine staining band in the urine was confirmed with samples from rats that had received the larger doses of RO0503821. This second band, stained and migrated the same as the 30 kDa reference mPEG standard (Figure 2. page 26). No non-pegylated Epoetin beta was detected in any of the urine samples, including the samples from the multiply dosed rats (Figure 3. page 27).

The urinary excretion of 60 kDa RO0503821 and 30 kDa PEG by the rat was not entirely unexpected. Similar results had been reported for another 60 kDa pegylated protein, and its 40 kDa branched PEG [1]. It has been hypothesized that large pores presented in the rat kidney glomerulus may allow the passage of selected large molecules into the urine [2]. However, the unconjugated protein portions of the two pegylated molecules mentioned above were not found in any of the urine samples analyzed.

While the methods used in the present studies were not designed to detect the small amounts of endogenous EPOs which are normally excreted in urine, an amount of Epoetin beta corresponding to the amount of 30 kDa PEG found in the rat urine would have been readily detected. However, to lessen the chance that detectable quantities of urinary Epoetin beta might be lost during the analytical procedures, multiple precautions were taken to prevent loss or degradation of the protein and alternate detection methods were used.

In order to prevent degradation of excreted Epoetin beta, the urine samples were collected directly from the metabolism cages into polypropylene tubes surrounded by dry ice. Each 24 hour collection was then stored at -70°C. On the day of analysis, the samples were thawed at 4°C and kept in ice water until heated for the SDS-PAGE procedure. In addition, since highly soluble PEG and pegylated proteins have been reported to be easily lost from acrylamide gels [8], gels were processed immediately after electrophoresis.

To guard against losses during the blotting procedure, a second (back-up) membrane was placed directly behind the first membrane. The purpose of the second membrane was to catch smaller molecules like Epoetin beta, that might pass through the first membrane during the extended blotting times used to transfer the larger pegylated molecules. While the Epoetin beta reference standards did pass through the first blotting membrane and were clearly visible on back-up membranes, digital enhancement of either the first (Figure 3, page 27) or the back-up membrane (Figure 3 Cont., page 28) failed to reveal detectable amounts of Epoetin beta in the urine samples. In contrast to the smaller Epoetin beta molecule and the purportedly highly soluble pegylated proteins, RO0503821

and the di-pegylated molecule were difficult to transfer from the gels to the membranes. Despite overnight transfers, a large percent of each pegylated molecule consistently remained behind in the gel.

When the urine samples from multiply dosed rats (D02001) were analyzed, bands that migrated and stained as did the reference standard 30 kDa PEG were clearly visible (Figure 2, page 26). In addition, samples from the multiply dosed rats tended to contain greater amounts of the 30 kDa PEG than of RO0503821 (Figure 4, page 29). An additional band which migrated between the RO0503821 band and the 30 kDa PEG band (arrow in Figure 2, page 26) was observed in some of the iodine stained gels. Although that band was also stained by the anti-rhEPO antibody (arrow in Figures 2 and 3, page 27) the exact nature of the band is unclear.

The gel images shown in Figures 4, 5 and 6 (page.29) all contain the same standards and daily 24 hour urine samples from the multiple dose animals. In Figure 4 (page.29), the iodine stained gel, the ratio of the amount of 30 kDa PEG to the amount of RO0503821 excreted in the urine appeared to increase as time and dosing progressed. The unidentified molecule migrating between the RO0503821 and the 30 kDa PEG bands is best seen in lane 10 (page.29). An in-gel chemiluminescent immunochemistry procedure confirmed the presence in that band of immunoreactive Epoetin beta as shown in Figure 6. (page.31). The lower bands in lanes 9 & 10 show the strongest immunoreactive response to the anti-rhEPO antibody.

The in-gel immunochemistry also confirmed the absence of detectable levels of immunoreactive material the same molecular weight as the Epoetin beta reference standard, although Epoetin beta, if present in the urine at levels comparable to the amounts of 30 kDa PEG found in the samples, would have been easily detectable.

To further rule out the possibility that low levels of Epoetin beta might have originally been present in the gels, but had passed through both membranes into the transfer buffer during the blotting procedures, a gel was stained before the transferring. As seen in Figure 5 (page.30), the glycoprotein stain had a greater sensitivity towards the reference standard Epoetin beta than the in-gel immunochemistry procedure used in Figure 6. However, no Epoetin beta could be detected above the background staining in the lanes containing the urine samples from the RO0503821 treated rats.

7.2 Analysis of Serum Samples

The analysis of serum samples by SDS-PAGE required a ten-fold dilution of the sample in PBS to reduce interference from the serum albumins. When diluted samples from either single dose or multi-dose rats were separated by SDS-PAGE, only intact RO0503821 was seen (Figures 1 and 7, page.25 and page.32 , respectively). Excluding the temporary iodine staining of abundant serum glycoproteins, seen at ~51 kDa, the only other PEG staining material found was the one that migrated with RO0503821 or its di-pegylated counterpart. Analysis by Western blot with chemiluminescent detection of Epoetin beta immunoreactive material failed to find any Epoetin beta in the samples other than the band migrating as RO0503821 (Figure 8, page.33). Digital enhancement (over-exposure) of the membrane images failed to reveal any additional Epoetin beta immunostaining on either the first membranes or on the back-up membranes. Since no

RO0503821 degradation products could be detected in any of the serum samples analyzed, and only 30 kDa PEG, but no Epoetin beta, was found in the urine, it is possible that the protein portion of RO0503821 was digested in the kidney and the remaining PEG was excreted without further catabolism.

When mPEG was intravenously administered to rats, only single iodine stained band, migrated as the 30 kDa PEG reference standard, was found in the 30 minute serum samples. The 30 kDa PEG band in the serum samples was as sharp as the corresponding band in the reference standard lane. The quantity of material in the sample band appeared to represent a quantity equivalent to the theoretical dose at the given body volume of serum. There was no visible evidence of degradation of the injected PEG.

A semi-quantitative comparison of the distribution of RO0503821 in serum and urine samples from IV or SC treated animals is shown in Table 1 (page.37). The amounts of RO0503821 in serum and urine samples from the Group I, II and III animals were determined by SDS-PAGE and scanning densitometry. From those values the percent of the original dose present in the serum at 30 minutes or at 24 hours post dose and the percent excreted over a 24 hour period were estimated. At 30 minutes, most of the IV dose was found in the serum. After 24 hours, approximately three fourths of the IV dose appeared to be removed from circulation. However, less than 2% had been excreted in the urine. In contrast, only 17% of the SC dose was found in the serum at 24 hours after treatment and only 0.3% of the dose had been excreted in the urine. These results reflected the anticipated differences in rates of RO0503821 distribution between IV and SC administrations [4][7].

7.3 Analysis of Bone Marrow Aspirates

The supernatants from bone marrow cell aspirates, collected 30 minutes post IV dose from the femur of an RO0503821 treated rat (study D01017) and from an untreated control rat, were analyzed by SDS-PAGE. Iodine staining revealed a single band, that migrated and stained as RO0503821, in the bone marrow cell supernatant from the treated rat. There was no comparable band in the bone marrow cell supernatant from the control rat (Figure 9, page.34). This finding clearly demonstrates that detectable levels of IV administered RO0503821 reached their target sites as an intact material.

7.4 Localization Patterns in Rat Bone Marrow

Bone marrow samples taken 30 minutes or 24 hours after treatment from the femurs of rats treated with IV RO0503821 contained anti-PEG immunoreactive material (Figure 10, page.35). At 30 minutes the PEG immunostaining appeared to be concentrated at the periphery of specific cells. By 24 hours, much of the PEG staining seemed to appear internal to the cell membrane and some stained cells had migrated into a bone marrow blood vessel. The identification of PEG in the bone marrow is supported by the results shown in Figure 9 (page.34), which demonstrate that RO0503821 had reached the bone marrow cell matrix by 30 minutes post IV dosing. Thus, it was concluded that the PEG immunoreactive material detected in the bone marrow sections was the same material as the iodine stained band in the bone marrow cell aspirate that migrated as RO0503821 during SDS-PAGE.

7.5 Localization Patterns in Rat Whole Body Sections

Fluorescence immunohistochemistry (IHC) of sagittal whole body sections from a control and an RO0503821 treated rat demonstrated immunoreactive material only in the treated animal. Thirty minutes after IV administration, the antigen was found primarily in the liver and, to a lesser extent, the spleen of the treated rat. These results are shown in Figure 11 (page 36).

The images of the two whole body sections have been digitally enhanced to show the location of the fluorescent tissues within the whole body sections. This view is possible because of the natural (*auto)fluorescence* of many animal tissues, and may be further enhanced by generalized non-specific binding of the secondary (fluorescent-tagged) antibody. The *autofluorescence* of many endogenous biological molecules makes the use of most fluorescent labels for whole body IHC difficult or impossible. However, since fewer biomolecules are excited by or emit in the near IR wavelengths, it is possible to detect the fluorescence of an IR800™ labeled antibody above most background *autofluorescence*.

If the background fluorescence in Figure 11 were eliminated, for example, by digitally decreasing the brightness and contrast of the image, only the brightest fluorescence of the liver, and the tissue posterior to the stomach, would be visible. While the liver fluorescence was specific for RO0503821, the fluorescence image was also appeared in the absence of the anti-rhEPO antibody at the tissue posterior to the stomach [7]. Thus, it is conclude that this fluorescence was the result of non-specific anti-body binding. In summary, these results demonstrated a specific localization of Epoetin beta immunofluorescence to the liver and spleen 24 hours after an IV injection of RO0503821. Studies using radio-labeled RO0503821, administered to rats in single or multiple doses, traced a much wider distribution of the drug at later time points [4][7].

8. SUMMARY AND CONCLUSIONS

The results of studies D01017 and D02001 suggested that:

- RO0503821 remained intact in the serum.
- RO0503821 had reached the bone marrow matrix 30 minutes after IV administration.
- Thirty minutes after IV administration of RO0503821, Epoetin beta-immunoreactive material was found primarily in the liver.
- Both RO0503821 and a 30 kDa PEG were excreted in the urine.
- Although several different analytical approaches were utilized, no unconjugated human Epoetin beta was detected in any serum, urine or bone marrow sample examined.
- No evidence indicated any *in vivo* catabolism of RO0503821 prior to its passage through the kidneys.

In conclusion, the results of these studies suggested that 60 kDa RO0503821 remains intact in the serum. It reached bone marrow target as a 60 kDa molecule and was excreted in the urine as the intact 60 kDa molecule or a 30 kDa PEG molecule.

9. DATA RETRIEVAL

Roche notebook No. 32716.

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11. SIGNATURE PAGE

DOCUMENT: **Regulatory Document No. 1012588**

TITLE: RO0503821-000: In vivo stability and tissue localization of RO0503821 after single (IV or SC) or multiple (IV) dose administration to rats (Study Nos. D01017 and D02001)

Authors:

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Study Director — Date

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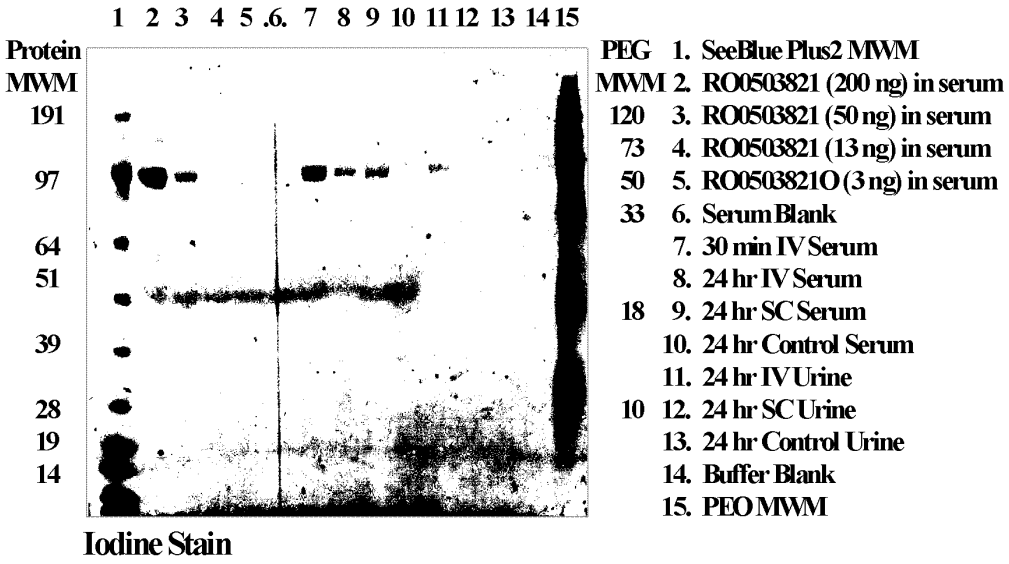
Jian-Ping Tang, PhD
Study Coordinator — Date

Figure 1 SDS-PAGE Gel Analysis of Serum and Urine Samples from Rats Administered IV or SC Doses of RO0503821 – Stained for PEG Moiety*

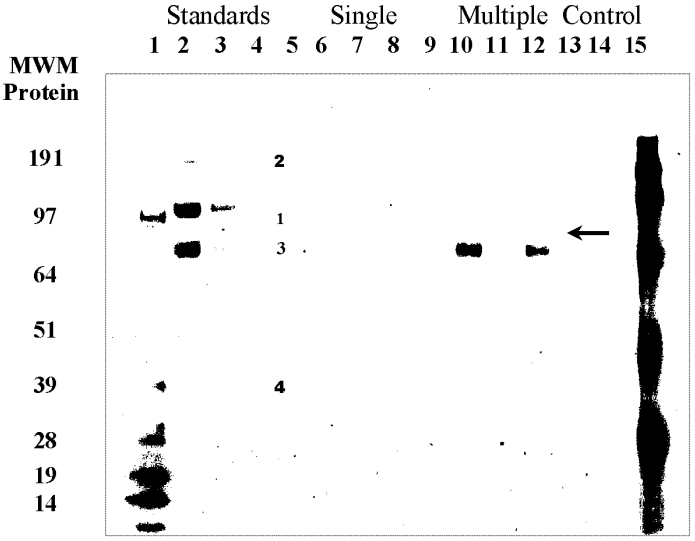


*Standards are, from top of lane down: 90 kDa Di-pegylated epoetin beta at ~190 kDa (a by-product of RO0503821 synthesis), 60 kDa RO0503821 at ~97 kDa, and 30 kDa Epoetin beta (which is not usually visible with an iodine stain) at ~39 kDa. The amount of each standard per lane is given as A280 protein. The molecular weight markers in lane 1 are SeeBlue™ Plus Pre-Stained Protein MWM from Invitrogen. In lane 15 are polyethylene oxide (PEO) MWM for PEG.

Iodine stained SDS-PAGE gel containing serum and urine samples from rats administered 6-11 mg/kg (2 mg/rat) RO0503821 (Study D01017). Iodine stains polyethylene glycols a reddish-brown. The orange-tan staining at MWM 51 kDa in lanes 2-10 is from serum glycoproteins. This staining of the serum glycoproteins fades upon additional washing. Note that the lanes containing urine samples, lanes 11-13, do not show similar staining.

The RO0503821 and epoetin beta standards were added to diluted serum (1 part serum with 9 parts PBS). Experimental serum samples were also diluted 1:9 with PBS before electrophoresis. Urine samples were not diluted.

Figure 2 **SDS-PAGE Gel Analysis of Urine Samples from Rats Administered Single or Multiple Doses of RO0503821 – Stained for PEG Moiety**



MWM PEO	
	1. Pre-stained MWM
120	2. 200 ng *EPO Stands**
73	3. 67 ng *EPO Stands*
50	4. 22 ng *EPO Stands*
	5. *Stands Blank*
35	6. Single Dose Rat F14
	7. Single Dose Rat F15
	8. Single Dose Rat F16
	9. Magic Mark MWM
18	10. Multi Dose Rat J25
	11. Multi Dose Rat J26
	12. Multi Dose Rat J27
10	13. Rat A1 Control
	14. Rat A2 Control
	15. PEO MWM

PEG in Urine (Iodine Stain)

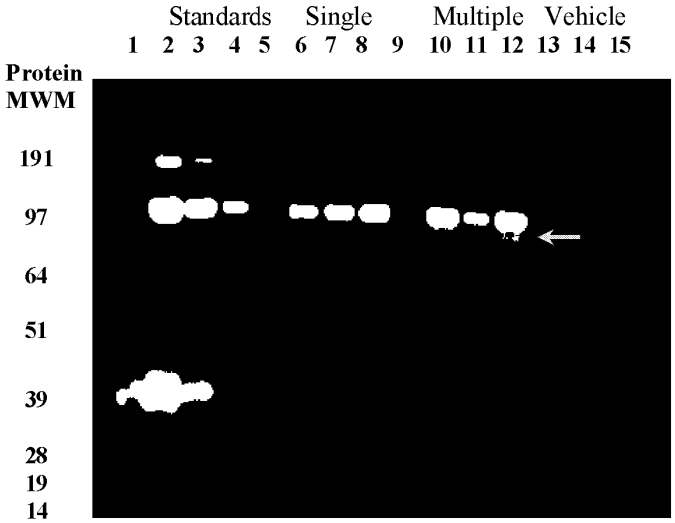
*Standards are, from top of lane down: [2]90 kDa Di-pegylated epoetin beta (a by-product of RO0503821 synthesis) at ~190 kDa; [1]60 kDa RO0503821 at ~97 kDa, [3]30 kDa PEG reagent at ~64 kDa, and [4]30 kDa Epoetin beta, which is not usually visible with the iodine stain, at ~39 kDa. The amount of each standard per lane is given as A280 protein or, as ng weight for the PEG reagent (see NOTE below). The molecular weight markers in lane 1 are SeeBlue™ Plus Pre-Stained Protein MWM from Invitrogen. In lane 15 are polyethylene oxide (PEO) MWM for PEG.

NOTE: The ng amount of Reference Standard PEG reagent in a standards lane is equivalent to the ng amount of Epoetin in the same standards lane. (The molar ratio of 30 kDa PEG to 30 kDa Epoetin beta in ***mono-pegylated*** RO0503821 is approximately one, therefore the amount of PEG reagent equivalent to that in a given amount of RO0503821 is approximately equal to the A280 protein ng weight of the RO0503821.)

Iodine stained SDS-PAGE gel containing rat urine samples from study D02001. Rats 14-16 (group F) received one 8 mg/kg IV dose of RO0503821 and their urine was collected on dry ice for 24 hours following the dose. Rats 25-27 (group J) each received 8 mg/kg IV doses on days 1, 4 and 7, for a total of 3 doses. The group J urine samples analyzed in the above gel were collected on dry ice for 24 hours following the final dose. All samples were stored at -70°C until analysis.

The **arrow** points to the location of the band of the unidentified component mentioned in Section 7.1.

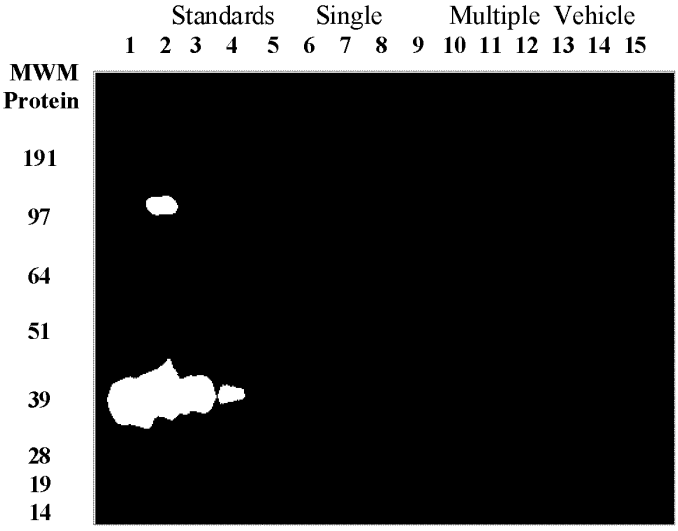
Figure 3 **Western Blot Analysis of Urine Samples from RO0503821 Treated Rats – Presence of Epoetin beta Immunoreactive Molecules in the Urine**



PEO MWM
120
73
50
35
18
10

1. Pre-stained MWM
2. 200 ng *Standards**
3. 67 ng *Standards*
4. 22 ng *Standards*
5. *Standards* Blank
6. Rat F14-24 hrs post Single Dose
7. Rat F15-24 hrs post Single Dose
8. Rat F16-24 hrs post Single Dose
9. Blank
10. Rat J25-24 hrs post §Multi Doses
11. Rat J26-24 hrs post Multi Doses
12. Rat J27-24 hrs post Multi Doses
13. Rat A1-24 hrs post Vehicle
14. Rat A2-24 hrs post Vehicle
15. Polyethylene oxide MWM

A. EPO in Urine: Western Blot (Chemiluminescence detection)



MWM PEO
120
73
50
35
18
10

1. Pre-stained MWM
2. 200 ng *Stands**
3. 67 ng *Stands*
4. 22 ng Stands
5. *Stands* Blank
6. Rat F14-24 hrs post Single Dose
7. Rat F15-24 hrs post Single Dose
8. Rat F16-24 hrs post Single Dose
9. Blank
10. Rat J25-24 hrs post §Multi Doses
11. Rat J26-24 hrs post Multi Doses
12. Rat J27-24 hrs post Multi Doses
13. Rat A1-24 hrs post Vehicle
14. Rat A2-24 hrs pose Vehicle
15. Polyethylene oxide MWM

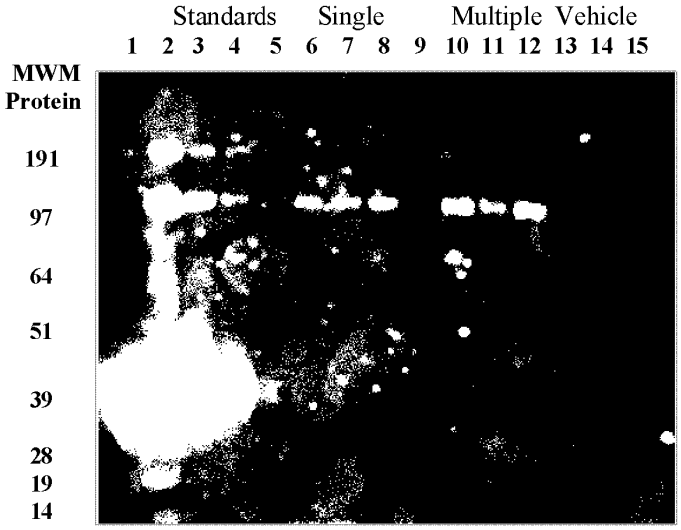
B. ***Backup Membrane*** showing the immunoreactive EPO standards that passed through first membrane (Chemiluminescence – see next page for an enhanced exposure of this membrane)

*Standards are, from top of lane down: [2]90 kDa Di-pegylated epoetin beta (a by-product of RO0503821 synthesis) at ~190 kDa; [1]60 kDa RO0503821 at ~97 kDa, [3]30 kDa PEG reagent at ~64 kDa, and [4]30 kDa Epoetin beta. (For additional description, see next page.)

§See Figure 2 for description of dosing and sample collection.

The arrow in panel A points to a band of an unidentified component found in the urine, as mentioned in Section 7.1.

Figure 3 Western Blot Analysis of Urine Samples from RO0503821 Treated Rats – Presence of Epoetin beta Immunoreactive Molecules in the Urine (Cont.)

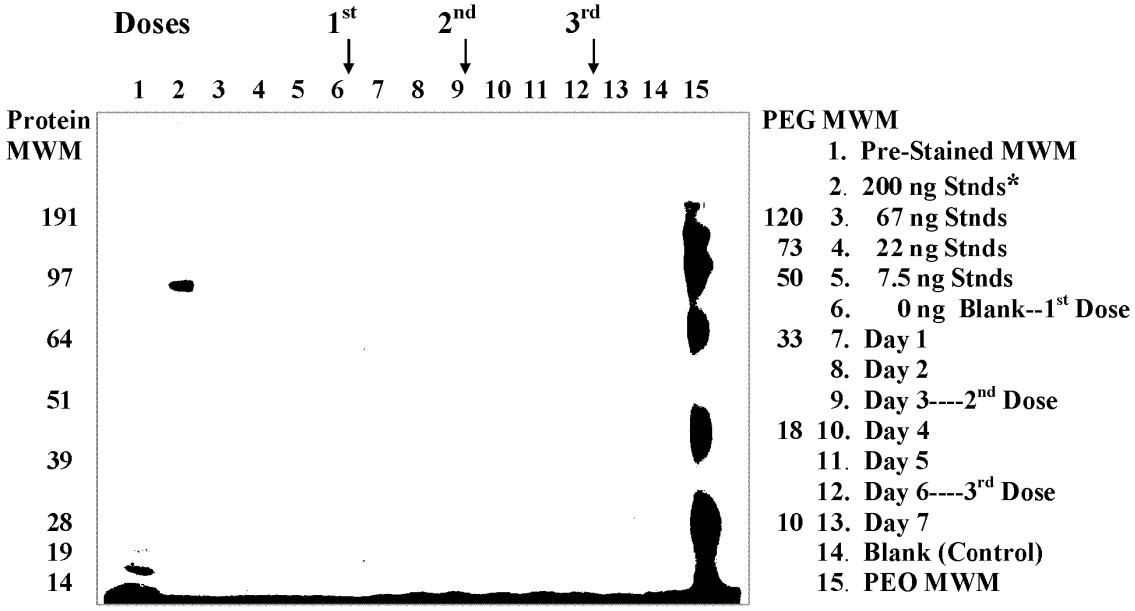


C. ***Backup Membrane*** showing the immunoreactive EPO standard that passed through first membrane (Chemiluminescence – Overexpose of B. from previous page)

*Standards are, from top of lane down: [(2)]90 kDa Di-pegylated epoetin beta (a by-product of RO0503821 synthesis) at ~190 kDa; [(1)]60 kDa RO0503821 at ~97 kDa, [(3)]30 kDa PEG reagent at ~64 kDa, and [(4)]30 kDa Epoetin beta.. The amount of each standard per lane is given as A280 protein or, as ng weight for the PEG reagent (see NOTE for Figure 2.). The molecular weight markers in lane 1 are SeeBlue™ Plus Pre-Stained Protein MWM from Invitrogen. In lane 15 are polyethylene oxide (PEO) MWM for PEG.

§Western Blot from SDS-PAGE gel containing rat urine samples from study D02001. Rats 14-16 (group F) received one 8 mg/kg IV dose of RO0503821 and their urine was collected on dry ice for 24 hours following the dose. Rats 25-27 (group J) received 8 mg/kg IV doses on days 1, 4 and 7, for a total of 3 doses. The group J urine samples analyzed in the above gel were collected on dry ice for 24 hours following the final dose. All samples were stored at -70°C until analysis.

Figure 4 **SDS-PAGE Gel Analysis of Daily Urine Samples from Rats after Multiple IV Doses of RO0503821 – Stained for PEG Moiety**

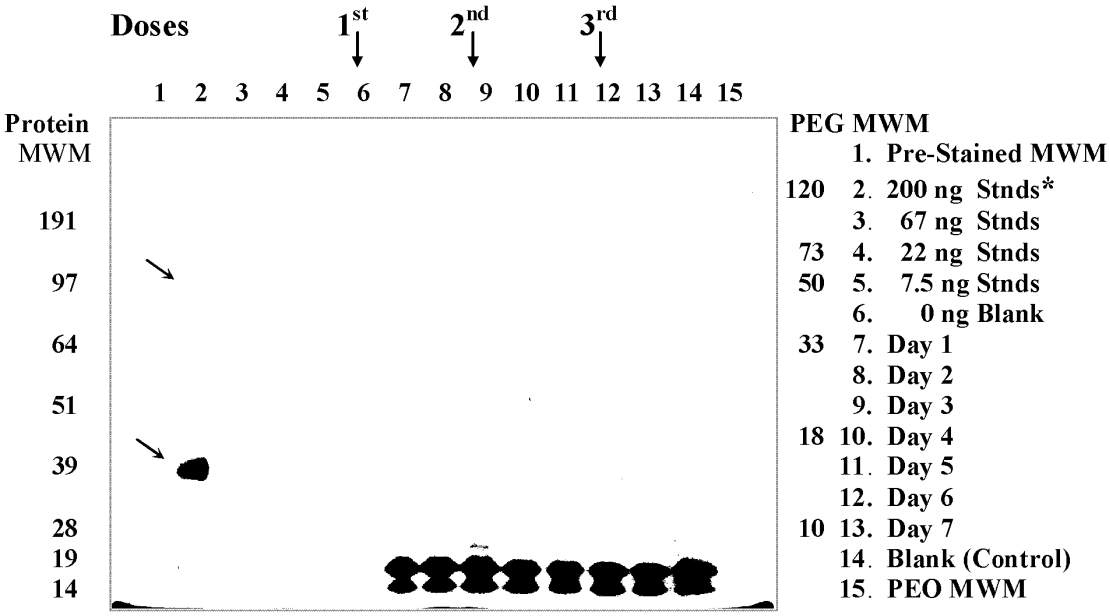


PEG in Urine (Iodine Stained Gel)

*Standards are, from top of lane down: 90 kDa-Di-pegylated Epoetin beta (a by-product of RO0503821 synthesis) at ~190 kDa; 62 kDa RO0503821 at ~97 kDa, 32 kDa PEG reagent at ~64 kDa, and 30 kDa Epoetin beta (not usually visible with iodine stain) at ~39 kDa. The amount of each standard per lane is given as A280 protein or as ng weight for the PEG reagent (see NOTE of Figure 2.). The molecular weight markers in lane 1 are SeeBlue™ Plus Pre-Stained Protein MWM from Invitrogen. In lane 15 are polyethylene oxide (PEO) MWM for PEG.

Rats 25-27 (group J, study D02001) received 8 mg/kg of RO0503821 on days 1, 4 and 7, for a total of three doses. Twenty-four hour urine samples were collected on dry ice daily from each animal and stored at -70°C. Urine aliquots from each animal/day were pooled and 10 uL of the pooled samples were analyzed by SDS-PAGE and stained for the presence of polyethylene glycol (PEG). (**Note:** Urine samples were contaminated with varying amounts of rat chow, therefore, normalization for urinary protein was not attempted.)

Figure 5 **SDS-PAGE Gel Analysis of Daily Urine Samples from Rats after Multiple IV Doses of RO0503821 – Stained for Glycoprotein**

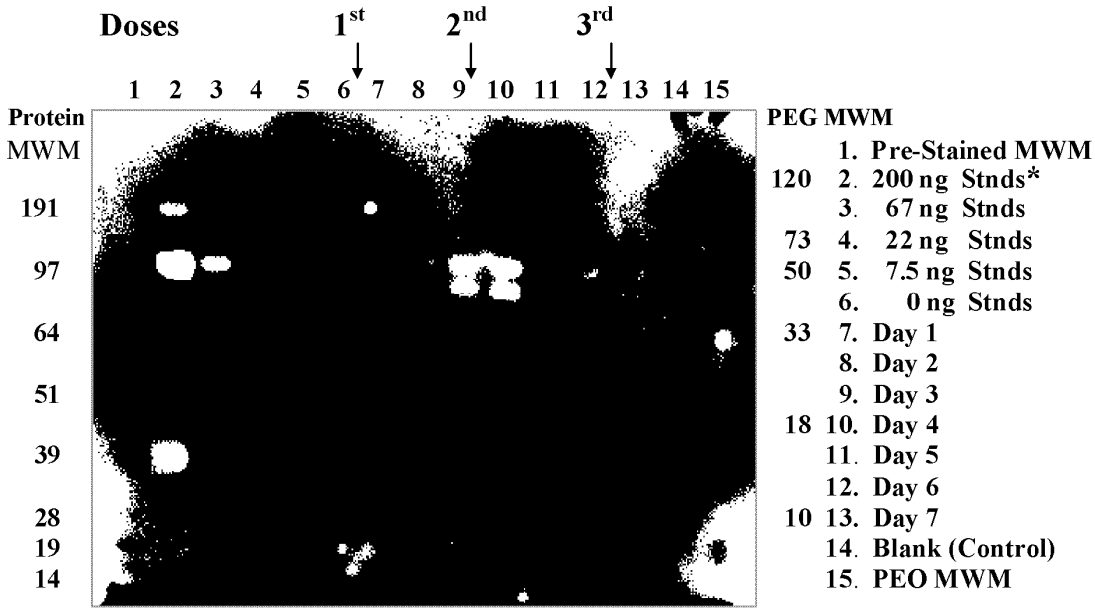


Glycoproteins in Urine (Glycoprotein Stained Gel)

*Standards are, from top of lane down: 90 kDa Di-pegylated epoetin beta (a by-product of RO0503821 synthesis) at ~190 kDa, 60 kDa RO0503821 at ~97 kDa, 30 kDa PEG reagent at ~64 kDa, and 30 kDa epoetin beta at ~39 kDa. The quantity of each standard per lane is given as A280 protein, or as ng weight for the PEG reagent (see NOTE Figure 2.). The molecular weight markers in lane 1 are SeeBlue™ Plus Pre-Stained Protein MWM from Invitrogen. In lane 15 are polyethylene oxide (PEO) MWM for PEG.

This gel, which is stained for the presence of glycoproteins, is a duplicate of the Figure 4 gel. The arrow at ~97 kDa points to the RO0503821 standard, which is a glycoprotein, and the arrow at ~39 kDa points to the glycosylated Epoetin beta standard. Observe the heavy staining of the glycosylated Epoetin beta standards. The 22 ng Epoetin beta standard in lane 4 is easily seen in this image. The 7.5 ng of Epoetin beta standard could be seen as well in the freshly stained gel. However, while several bands in the urine sample lanes were well stained, no glycosylated Epoetin beta bands could be distinguished above the background staining in any of the urine samples.

Figure 6 **SDS-PAGE Gel Analysis of Daily Urine Samples from Rats after Multiple IV Doses of RO0503821 – Immunostained for Epoetin beta**

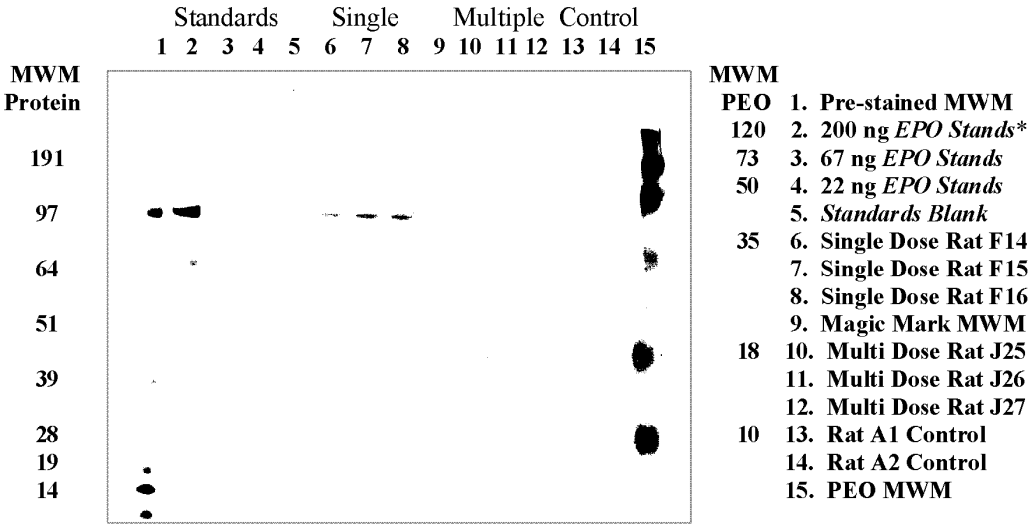


Immunoreactive EPO in Urine (Anti-EPO in-gel Chemiluminescent Immunostaining)

**Standards are, from top of lane down: 90 kDa di-pegylated epoitin beta (a by-product of RO0503821 synthesis) at ~190 kDa; 60 kDa RO0503821 at ~97 kDa, 30 kDa PEG reagent at ~64 kDa, and 30 kDa epoetin beta at ~39 kDa. The amount of each standard per lane is given as A280 protein, or as ng weight for the PEG reagent (see NOTE Figure 2.). The molecular weight markers in lane 1 are SeeBlue™ Plus Pre-Stained Protein MWM from Invitrogen. In lane 15 are polyethylene oxide (PEO) MWM for PEG.

Rats 25-27 (group J, study D02001) received 8 mg/kg doses of RO0503821 on days 1, 4 and 7, for a total of three doses. Twenty-four hour urine samples from each animal were collected daily on dry ice and stored at -70°C. For the above SDS-PAGE analysis, urine aliquots from each animal/day were pooled and 10 µL aliquots of the pooled samples were loaded on the gel. After electrophoresis, the gel was stained for the presence of immunoreactive epoetin beta by incubating the gel in the antibody against epoetin beta. The secondary antibody was a horseradish peroxidase (HRP) conjugated goat anti-rabbit antibody and was detected by Chemiluminescence. The unidentified band detected by iodine staining in the urine samples shown in Figures 2 and 3, which migrated at a molecular weight between RO0503821 (~97 kDa) and the PEG reagent (~64 kDa), is also seen in the above gel. The ratio of the unknown band to the intact RO0503821 appears much higher in this in-gel analysis than the ratios seen after transfer to a membrane. This difference may be a consequence of losses during transfer out of the gel and binding to the membrane and may indicate higher levels of that molecule than indicated by Western Blot. However, no urinary epoetin beta was detected by this in-gel method.

Figure 7 SDS-PAGE Gel Analysis of Serum Samples from RO0503821 Treated Rats

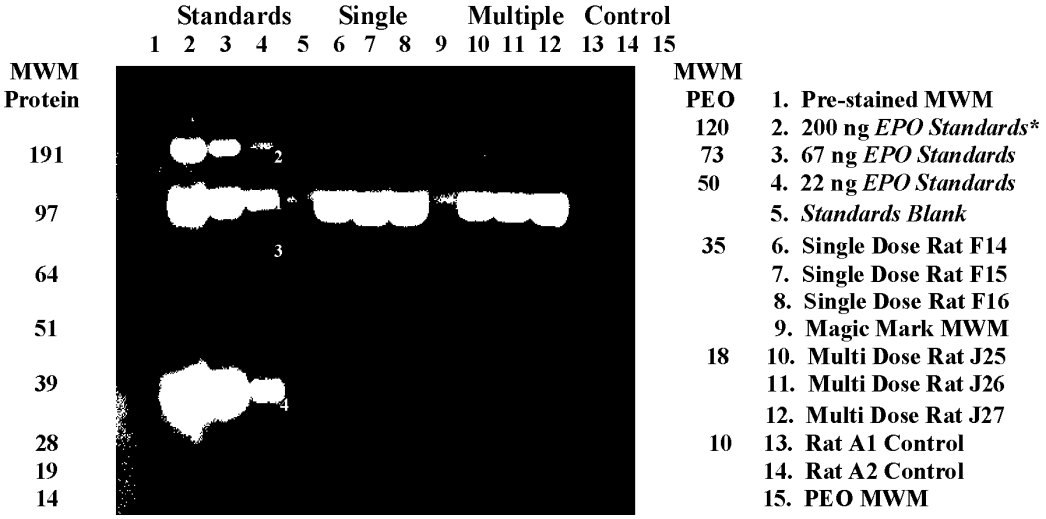


PEG in Serum (Iodine Stained Gel)

*Standards are, from top of lane down: 90 kDa di-pegylated epoetin beta (a by-product of RO0503821 synthesis) at ~190 kDa; 60 kDa RO0503821 at ~97 kDa, 30 kDa PEG reagent at ~64 kDa, and 30 kDa epoetin beta (not usually visible with iodine stain) at ~39 kDa. The amount of each standard per lane is given as A280 protein, or as ng weight for the PEG reagent (see NOTE Figure 2.). The molecular weight markers in lane 1 are SeeBlue™ Plus Pre-Stained Protein MWM from Invitrogen. In lane 15 are polyethylene oxide (PEO) MWM for PEG.

Iodine stained SDS-PAGE gel containing serum samples from the same study D02001 rats shown in Figures 2 and 3. The rats 14-16 (group F) had received one 8 mg/kg IV dose of RO0503821 and a serum sample was collected at 24 hrs post dose. Rats 25-27 (group J) received 8 mg/kg IV doses on days 1, 4 and 7, for a total of three doses. Group J serum samples were collected 24 hours following the final dose. Notice the absence of any iodine staining below the RO0503821 bands in the serum samples indicating no significant amounts of RO0503821 degradation products.

Figure 8 Western Blot Analysis of Serum Samples from RO0503821 Treated Rats – Immunostained for Epoetin beta

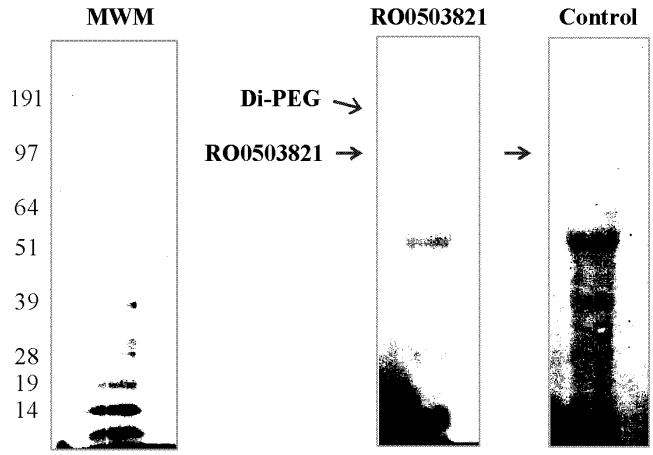


Serum Epoetin (ImmunoChemiluminescence Blot)

*Standards are, from top of lane down: [2]90 kDa-di-pegylated epoetin beta (a by-product of RO0503821 synthesis) at ~190 kDa; [1]60 kDa RO0503821 at ~97 kDa, [3]30 kDa PEG reagent at ~64 kDa, and [4]30 kDa epoetin beta at ~39 kDa. The amount of each standard per lane is given as A_{280} protein, or as ng weight for the PEG reagent (see NOTE for Figure 2.). The molecular weight markers in lane 1 are SeeBlue™ Plus Pre-Stained Protein MWM from Invitrogen. Lane 15 contains polyethylene oxide (PEO) MWM for PEG.

Western Blot of a duplicate to the Figure 7 SDS-PAGE gel. Both gels contained samples from the same study rats as shown in Figures 2 and 3. Rats 14-16 (group F) had received one 8 mg/kg IV dose of RO0503821 and a serum sample was collected 24 hrs after the single dose. Rats 25-27 (group J) received three IV doses of 8 mg/kg each on days 1, 4 and 7. Group J serum samples were collected 24 hours following the final dose. Notice the absence of any Epoetin beta chemiluminescence below the RO0503821 bands in the serum samples, indicating the absence of significant amounts of RO0503821 degradation.

Figure 9 SDS-PAGE Gel Analysis of Bone Marrow Aspirates from RO0503821 Treated and Control Rats.

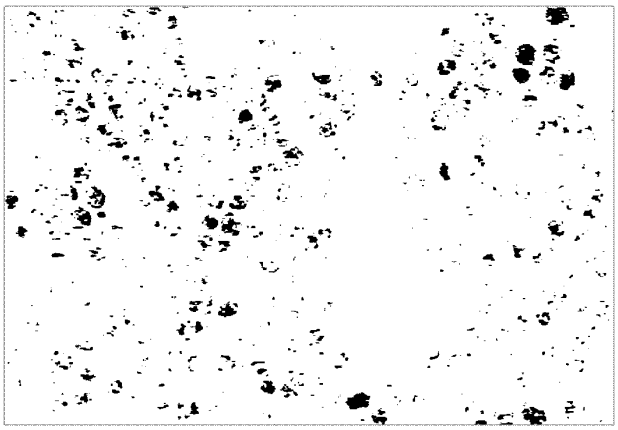


Bone marrow cell supernatant from an RO0503821 treated rat and an untreated rat (control).

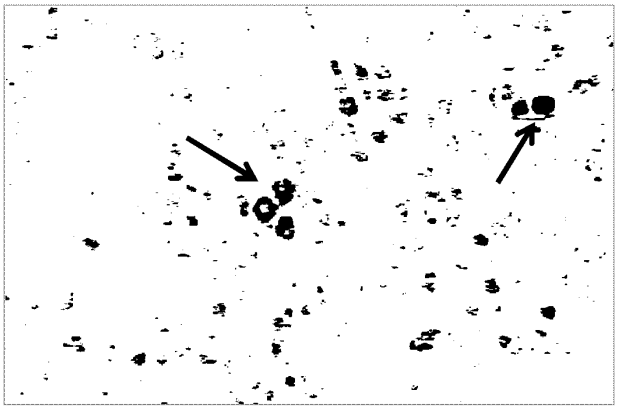
Molecular Weight Markers are from Introgen (SeeBlue™ Plus2 Pre-Stained Standards). See Figure 7. for correlation of the migration of the markers and the RO0503821 Standards.

A bone marrow (BM) cell suspension was obtained 30 minutes post dose as described in Section 6. Cells were removed by centrifugation and the remaining supernatants concentrated using a 10 kDa MWCO spin filter. Both the mono-pegylated and the di-pegylated forms of intact Epoetin beta can be seen in the cell supernatant from the RO0503821 treated rat. There is no band at the same MW in the aspirate from the control animal. The remaining iodine stained bands can be seen in both the treated and the control BM supernatants and are most likely non drug-related glycosylated proteins.

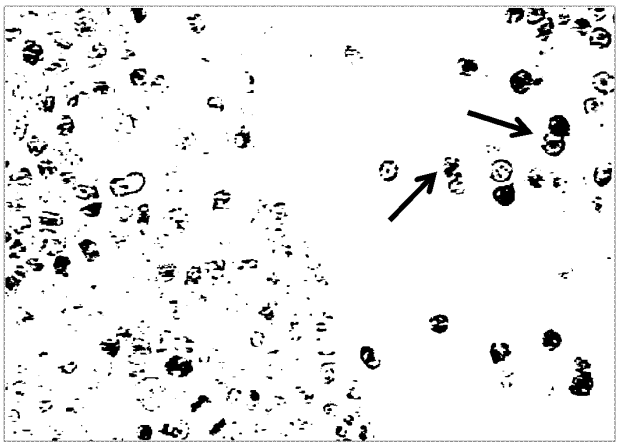
Figure 10 Immunostaining of Polyethylene Glycol (PEG Moiety) in Bone Marrow Tissue from Rats Treated with RO0503821



Rat 4: 24 hr Control



Rat 1: 30 min IV 6.3 mg/kg



Rat 2: 24 hr IV 6.7 mg/kg

Fixed bone marrow tissues from the femurs of control and RO0503821 treated rats. The doses were ~2 mg/kg, IV, and the tissue samples were taken at 30 minutes and at 24 hours as described in Section 6.

The slides were immunostained for the PEG moiety of RO0503821 using an anti-PEG antibody. The presence of PEG is indicated by a deep purple color (arrows in center and bottom pannels).

Control Bone Marrow

No staining was found in the tissue from the control rat (top).

30 Min. Post Dose

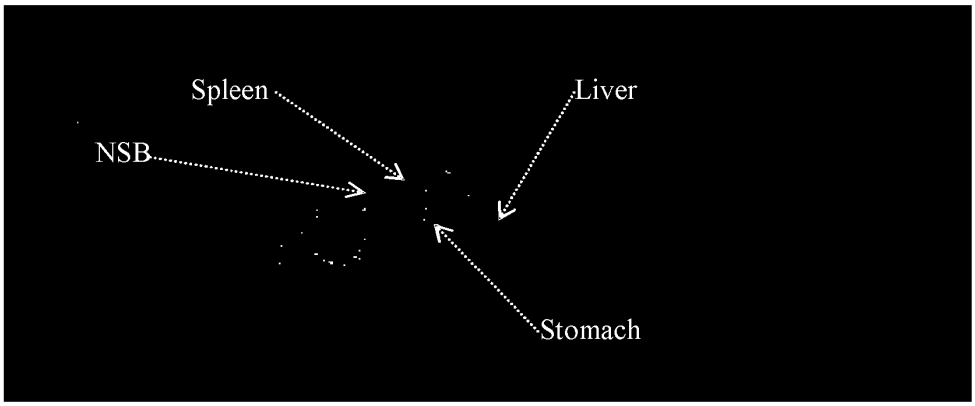
At 30 min. post dose, heavy staining appears at the periphery of specific cells (center panel).

24 Hr. Post Dose

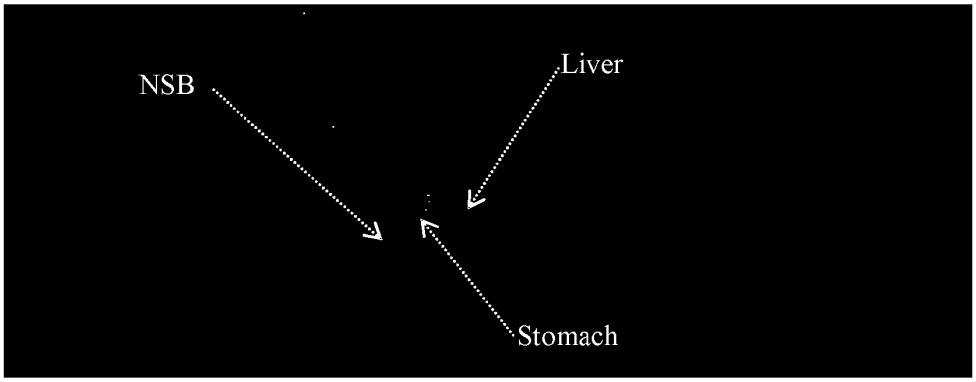
Twenty-four hours later, stained cells had moved out of the marrow and into the blood stream. Some of the staining appeared inside the cell membrane (bottom panel).

The localization of PEG immuno-reactive material in the bone marrow correlates with the presence of RO0503821 in the bone marrow aspirates analyzed by SDS-PAGE (Figure 9). Together, these results indicated that RO0503821 reached and bound to cells within the target tissue.

Figure 11 Fluorescence Immunohistochemistry of Epoetin beta in Sagittal Whole Body Sections from RO0503821 Treated and Untreated Rats



Distribution of rhEPO in rat tissues (IRDye800™ immunohistochemistry). Sagittal whole body section 30 minutes after IV administration of RO0503821.



Anti-EPO immunochemistry of whole body tissue section from an untreated rat

Tissue sections from an IV RO0503821 treated rat (Top) and from an un-treated rat (Bottom) were immunostained using the same procedure for both. The antibody used to detect epoetin beta immunoreactive material in the sections was specific for human epoetin beta. The anti-EPO antibody was detected with a goat anti-rabbit antibody labeled with a fluorescent dye (IR800Dye). The fluorescence in the sections was imaged at 800 nm (shown as green) and 700 nm (shown as red) with an IR scanner. At 30 minutes post dose most of the EPO (green fluorescence) is seen in the liver, and some possibly in the spleen, of the treated rat (Top). The red color in the digestive tract is rat chow autofluorescence from the 700 nm laser.

***NSB** (non-specific binding) caused by IgG binding proteins in the indicated tissue. Images were digitally enhanced, which increased background (auto)fluorescence throughout all tissues, to show the relative positions of tissues within the body (see discussion in Section 7.5).