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Design of Homogeneous, MonoPEGylated Erythropoietin Analogs With Preserved In Vitro Bioactivity

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Abstract

Objective—Erythropoietin (Epo) bioactivity is significantly reduced by modification of lysine residues with amine-reactive reagents, which are the most commonly used reagents for attaching polyethylene glycols (PEGs) to proteins to improve protein half-life in vivo. The aims of this study were to determine whether Epo bioactivity can be preserved by targeting attachment of maleimide-PEGs to engineered cysteine analogs of Epo, and to determine whether the PEGylated Epo cysteine analogs have improved pharmacokinetic properties in vivo.

Materials and Methods—Thirty-four Epo cysteine analogs were constructed by site-directed mutagenesis and expressed as secreted proteins in baculovirus-infected insect cells. Following purification, monoPEGylated derivatives of 12 cysteine analogs were prepared using 20 kDa-maleimide-PEGs. In vitro biological activities of the proteins were measured in an Epo-dependent cell proliferation assay. Plasma levels of insect cell-expressed wild type Epo (BV Epo) and a PEGylated Epo cysteine analog were quantitated by ELISA following intravenous administration to rats.

Results—Biological activities of 17 purified Epo cysteine analogs and 10 purified PEGylated Epo cysteine analogs were comparable to that of BV Epo in the in vitro bioassay. The only PEGylated cysteine analogs that displayed consistently reduced in vitro bioactivities were substitutions for lysine residues, PEG-K45C and PEG-K154C. The PEGylated Epo cysteine analog had a slower initial distribution phase and a longer terminal half-life than BV Epo in rats, but the majority of both proteins were cleared rapidly from the circulation.

Conclusions—Targeted attachment of maleimide-PEGs to engineered Epo cysteine analogs permits rational design of monoPEGylated Epo analogs with minimal loss of in vitro biological activity. Insect cell-expressed EPO proteins are cleared rapidly from the circulation in rats, possibly due to improper glycosylation. Site-specific PEGylation appears to improve the pharmacokinetic properties of Epo.

Introduction

Erythropoietin (Epo) is a 35-39 kDa glycoprotein that acts on immature erythrocytes to stimulate their proliferation and differentiation into mature red blood cells. Recombinant Epo is used clinically to treat anemia resulting from chronic kidney disease, chemotherapy and

complications from AIDS therapies [1]. Epo contains three N-linked glycosylation sites (N24, N38 and N83) and one O-linked glycosylation site (S126), all of which are normally glycosylated in vivo. Glycosylation is not required for in vitro biological activity of Epo, and in fact, seems to interfere with the binding of Epo to its receptor [2]. In contrast, the circulating half-life and in vivo effectiveness of Epo is highly dependent upon the protein's glycosylation pattern, in particular, the extent of sialylation of terminal galactose residues, which is thought to prevent uptake and metabolism of Epo by hepatic cells [3-5].

Recombinant, mammalian cell-expressed Epo has a half-life of 4-13 h following intravenous administration in humans, which necessitates frequent administration, generally 2- to 3-times per week, for optimum therapeutic benefits [6]. There is considerable interest in the development of longer acting Epo products that can be administered less frequently, but with comparable or superior efficacy compared to current first generation EPO products. A hyperglycosylated Epo analog, ARANESP®, was created by making 5 amino acid substitutions that add 2 additional N-linked glycosylation sites [5,7]. ARANESP® has a 2- to 3-fold longer half-life than Epo, which permits once per week dosing in the clinical setting [5,7]. Covalent modification of proteins with polyethylene glycol (PEG) is an alternative technology that has proven useful for extending the circulating half-lives of therapeutic proteins [8-11]. PEGylated proteins often have 5-fold or greater improved half-lives relative to the unmodified protein [8-11]. The most commonly used method for PEGylating proteins attaches PEG to amine groups in proteins, typically at lysine residues and/or at the N-terminal amino acid. A limitation of this approach for Epo is the fact that Epo contains 8 lysine residues in addition to the N-terminal amino acid and the PEG moiety can attach to any or all of these amino acids, resulting in a heterogeneous product. Certain of these lysine residues, e.g., K20, K45, K97 and K152, are located in regions of Epo known to be critical for receptor binding [12-16]. The importance of these regions is confirmed by the finding that extensive biotinylation or carbamylation of lysine residues reduces Epo's in vitro biological activity on hematopoietic cells by greater than 100-fold [17,18]. In this report we describe the engineering of Epo analogs that contain an added cysteine residue, which permits targeted attachment of maleimide-PEGs to predetermined sites in the protein. We demonstrate the utility of this approach for designing monoPEGylated Epo analogs that retain complete or near complete in vitro biological activity.

Materials and methods

Construction of Epo cysteine analogs

A cDNA encoding Epo was amplified from total RNA isolated from the human Hep3B cell line (American Type Culture Collection, Rockville, MD, USA) using the reverse transcriptase – polymerase chain reaction (RT-PCR) method [19,20]. The cDNA was modified for expression of Epo in insect cells (referred to as BV Epo) by adding the DNA sequence CAAA immediately upstream of the initiator ATG to enhance translation [21]. In addition, DNA encoding the 8 amino acid FLAG epitope (DYKDDDDK), preceded by a 7 amino acid flexible linker (SGGSGGS) was added immediately following DNA encoding the carboxy-terminal amino acid of Epo, R166. These modifications were made via PCR. cDNAs encoding Epo cysteine analogs were constructed by site-directed PCR-based mutagenesis [22,23] of the BV EPO gene and cloned as *Bam* HI – *Eco* RI fragments in plasmid pBlueBac4.5 (Invitrogen Corporation, Carlsbad, CA, USA). Correct gene sequences were confirmed.

Insect cell expression of BV Epo and Epo cysteine analogs

pBlueBac4.5 plasmid DNAs encoding BV Epo and Epo cysteine analogs were cotransfected with linearized Bac-N-Blue™ baculovirus DNA into insect Sf 9 cells (Invitrogen Corporation) using standard procedures. Individual positive plaques were used to inoculate 2.5×10^6 Sf 9

cells in a T25 flask containing 5 mL of Grace's insect cell media supplemented with 10% fetal bovine serum (FBS). Supernatants from the infected cells were assayed by Western Blot for Epo expression using a rabbit anti-human Epo antibody (R&D Systems, Inc., Minneapolis, MN, USA). Alkaline phosphatase conjugated goat anti-rabbit IgG (Pierce Chemical Company, Rockford, IL, USA) was used as the secondary antibody. Western blots were developed using NBT[unk]BCIP color development substrate (Promega Corporation, Madison, WI, USA). Serial dilutions of the infected cell supernatants were screened for Epo bioactivity in the UT7/Epo cell proliferation assay described below. Concentrations of the Epo proteins in the serial dilutions were measured using human Epo ELISA kits (R&D Systems). Viral stocks of positive clones were amplified and used to infect 500 mL cultures of Sf 9 or High Five™ insect cells in spinner or shake flasks. Culture supernatants containing Epo cysteine analogs were adjusted to 2mM cystine immediately after harvesting.

Purification of BV Epo and Epo cysteine analogs

BV Epo and Epo cysteine analogs were purified by affinity chromatography using anti-FLAG M2 Affinity Gel resin (Sigma-Aldrich, Inc., St. Louis, MO, USA). Bound proteins were eluted with 0.1M glycine pH 3.0, 0.05% Tween 20, 10% glycerol and neutralized to pH 8. Fractions containing the Epo analogs were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), pooled and stored at -80°C . In some cases Epo cysteine analog monomers were purified from dimers by size-exclusion column chromatography (Superdex 75 or 200 10/30 columns, Amersham Biosciences Corporation) using a column buffer of 50 mM sodium phosphate (or 50 mM Tris) pH 7.5, 150 mM NaCl, 10% glycerol.

PEGylation of Epo cysteine analogs

BV Epo and Epo cysteine analogs were incubated for 2 h at room temperature in a pH 8.0 solution containing a 15- to 20-fold molar excess of TCEP [Tris (2-carboxyethyl) phosphine]-HCl, Pierce Chemical Company], a 15- to 20-fold molar excess of 5 kDa- or 20 kDa- maleimide PEG (Nektar, Inc., Huntsville, AL, USA) and 0.01% Tween 20. An additional 5- to 10-fold molar excess of TCEP and maleimide-PEG reagent were then added to the reaction and the mixture incubated for another 1 h. Each PEGylation reaction was terminated by diluting the mixture 10-fold with 20 mM MES (2-morpholinoethanesulfonic acid) pH 5.0, 0.01% Tween 20, 10% glycerol and loading the mixture onto a 1 ml HiTrap S-Sepharose column (Amersham Biosciences Corporation) pre-equilibrated with the same buffer. Bound proteins were step eluted with a 20 mM MES (or 20 mM NaOAc) pH 5.0, 0.01% Tween 20, 10% (or 20%) glycerol, 500 mM NaCl. Fractions containing the PEG-Epo analogs were pooled and applied to a Superdex 200 10/30 column (Amersham Biosciences Corporation) equilibrated with 50 mM sodium phosphate pH 7.0, 150 mM NaCl, 10% glycerol. Fractions containing the PEGylated Epo analogs were pooled, diluted 10-fold with 20 mM MES or NaOAc pH 5.0, 0.01% Tween 20, 10% (or 20%) glycerol and step eluted off a 1 ml HiTrap S-Sepharose column as described above. Fractions containing the PEGylated Epo analogs were pooled and stored at -80°C .

In vitro bioassay

The human UT7/Epo cell line [12,24] was maintained in Iscove's Modified Delbecco's Media (IMDM) supplemented with 10% FBS, 50 units/mL penicillin, 50 $\mu\text{g/mL}$ streptomycin, 2 mM glutamine and 1 unit/mL recombinant Epo (CHO (Chinese Hamster Ovary) cell-expressed; R&D Systems, Inc.) or BV Epo. For bioassays, the cells were washed and resuspended at a concentration of 1×10^5 cells/mL in phenol red-free IMDM media containing 10% FBS, 50 units/mL penicillin, 50 $\mu\text{g/mL}$ streptomycin and 2 mM glutamine (assay media). Fifty μL (5×10^3 cells) of the cell suspension were aliquotted per test well of a flat bottom 96 well tissue culture plate. Serial 3-fold dilutions of the protein samples were prepared in assay media. Fifty

μL of the diluted protein samples were added to the test wells and the plates incubated at 37°C in a humidified 5% CO_2 tissue culture incubator. Protein samples were assayed in triplicate wells. After 3 days, 20 μL of CellTiter 96 Aqueous One Solution Reagent (Promega Corporation) was added to each well and the plates incubated at 37°C in the tissue culture incubator for 1–4 h. Absorbance of the wells, which is proportional to cell number, was read at 490 nm using a microplate reader. Control wells contained media but no cells.

Animal experiments

Experiments were performed with the approval of Premier Laboratory's Institutional Animal Care and Use Committee. Groups of three male Sprague Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA), weighing 340–370 g each, were used for this study. A pre-dose sample was drawn from each rat one day prior to injection of the proteins. Animals received a single intravenous injection (lateral tail vein) of BV Epo or 20 kDa-PEG T26C, each at a dose of 100 μg protein/kg. At 0.25, 1.5, 4, 10, 24, 48, 72 and 96 h post-injection, blood samples (0.4 mL) were drawn from the rats into EDTA-coated tubes, centrifuged and the plasma samples stored at -80°C . Serial dilutions of plasma samples from one animal in each test group were analyzed in the in vitro bioassay to identify dilutions that fell within the linear range of the ELISA (2.5 to 200 mIU Epo equivalents/mL). Duplicate samples of appropriate dilutions of plasma samples from all rats were then analyzed using human Epo ELISA kits (R & D Systems, Inc.). Pharmacokinetic parameters were analyzed using non-compartmental methods and the WinNonlin software package (Pharsight, Inc, Mountain View, CA, USA).

Biochemical methods

Concentrations of the affinity-purified proteins and PEGylated proteins were determined using a Bradford dye binding assay kit (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as the standard. Protein samples were analyzed on precast polyacrylamide gels (Invitrogen Corporation) and stained with Coomassie Blue. Protein samples were reduced using 2-mercaptoethanol (1–5% V/V). Endotoxin levels of the proteins were measured using a chromogenic Limulus Amebocyte Lysate kit (QCL-1000, BioWhittaker, Walkersville, MD, USA).

Results

Human Epo was expressed as a secreted protein in baculovirus-infected insect Sf 9 cells using the human Epo signal sequence. The 8 amino acid FLAG epitope (DYKDDDDK), preceded by a 7 amino acid linker (SGGSGGS) was added to the carboxy-terminus of Epo to facilitate purification of the protein by affinity chromatography. The purified protein, referred to as BV Epo, migrates with an apparent mass of 26.3 kDa under non-reducing conditions (Fig. 1). BV Epo stimulated proliferation of human UT7/Epo cells in vitro, with a mean EC_{50} (protein concentration that stimulates 50% of maximal stimulation) of 0.31 ng/mL, similar to the mean EC_{50} measured for a CHO-cell derived Epo standard in this assay (0.46 ng/mL; Table 1). These data indicated that the peptide linker and FLAG epitope did not adversely affect Epo in vitro bioactivity.

Construction, purification and in vitro bioactivities of Epo cysteine analogs

Epo has a compact globular structure, comprising four amphipathic helices joined by loops [12,14]. The four α helices are termed A–D beginning from the N-terminus of the protein and are shown in bold-faced type in Fig. 2. The loop regions are referred to by the helices they join; e.g., the A–B loop joins helices A and B. We constructed 33 Epo cysteine substitution analogs, primarily in the presumed non-helical regions of the BV Epo protein. We also constructed a cysteine analog (*167C) that adds a cysteine immediately following the carboxy-

terminal R166 residue. Locations of the Epo cysteine analogs in the Epo primary sequence are shown in Fig. 2.

DNAs encoding the Epo cysteine analogs were transfected into baculovirus-infected Sf9 insect cells and supernatants from positive plaques screened in the UT7/Epo cell proliferation assay to identify biologically active cysteine analogs. A supernatant from insect cells expressing BV Epo was included as a control. Concentrations of the Epo proteins in the insect cell supernatants were quantitated by Western blot and by ELISA. The data (not shown) indicated that all of cysteine analogs were biologically active and had specific activities similar (within 3-fold) to that of BV Epo. A control supernatant from a plaque expressing an unrelated gene showed no activity in the UT7/Epo cell assay.

To confirm this finding and to control for potential differences in sensitivities of the Epo cysteine analogs in the ELISAs and Western blots, we purified representative Epo cysteine analogs, determined their protein concentrations using a Bradford dye binding assay, and remeasured their activities in the in vitro bioassay. The Epo cysteine analogs were expressed using baculovirus-infected Sf9 and High Five™ insect cells and purified by affinity column chromatography as described for BV Epo, with the exception that 2 mM cystine was added to the baculovirus supernatants prior to purification. Addition of cystine to the baculovirus supernatants improved recoveries of the Epo cysteine analogs, presumably by forming a mixed disulfide with the free cysteine residue in the analogs and stabilizing the proteins during purification. Most of the purified Epo cysteine analogs, including the S126C analog that eliminates the O-glycosylation site, co-migrated with BV Epo under non-reducing SDS-PAGE conditions, consistent with the proteins being monomeric (Fig. 1). In contrast, Epo analogs containing cysteine substitutions at amino acids that comprise the N-linked glycosylation recognition sites (N24C, T26C, N38C, T40C, N83C and S85C proteins) migrated with apparent molecular masses of 25.1 kDa, slightly smaller than BV Epo, and consistent with the proteins being underglycosylated (Fig. 1). The S85C protein migrated as a doublet under both reducing and non-reducing conditions; one band of the doublet co-migrated with the major band of Epo analogs such as N24C that lack one of the N-linked glycosylation sites, while the other band co-migrated with BV Epo. These data suggest that N83 (or another amino acid) may be partially glycosylated in the S85C analog. Certain of the purified cysteine analogs such as S126C and K154C tended to form disulfide-linked dimers (apparent molecular mass of 55 kDa by non-reducing SDS-PAGE) during the purification process. Monomeric forms of these analogs could be purified away from dimers by size-exclusion chromatography.

Bioactivities of the purified Epo cysteine analogs were measured in the UT7/Epo cell proliferation assay using concentrations of the proteins determined using a Bradford dye binding assay. The bioassay results are summarized in Table 1. Most of the Epo cysteine analogs had EC₅₀s that were comparable to (within 2-fold of) the EC₅₀ of BV Epo measured on the same day (Table 1). Only the N38C, T40C and K154C proteins had in vitro bioactivities that were consistently reduced (2- to 4-fold) relative to the BV Epo control protein. Dose response curves for representative cysteine analogs are shown in Fig. 3.

Preparation and in vitro bioactivities of PEGylated Epo cysteine analogs

We reacted the purified Epo cysteine analogs with 5 kDa- and 20 kDa-maleimide PEGs using the partial reducing conditions described in **Materials and methods**. Partial reduction of the cysteine analogs was required to disrupt the mixed disulfide (formed between the added cysteine residue and cystine added during the purification process) and make the added cysteine residue available to react with the PEG reagent. The partial reducing conditions used in the PEGylation reactions did not appear to disrupt Epo's native disulfide bonds, as evidenced by the failure of BV Epo to react with the PEG reagent under similar conditions (see below). Most Epo cysteine analogs PEGylated efficiently (25 - 80 % conversion to the PEGylated species)

under these conditions and yielded only monoPEGylated proteins. SDS-PAGE analysis of a representative PEGylation experiment for the T26C analog using a 5 kDa-PEG is shown in Fig. 4. The T26C analog PEGylated (indicated by a shift of the protein to a higher molecular weight) only in the presence of both the PEG reagent and a reducing agent (Fig. 4, lane 6). No PEGylation of the T26C protein was observed when the reducing agent (TCEP) was absent from the reaction mixture (Fig. 4, lane 5). Similar results were obtained with a 20 kDa-PEG (data not shown). A 5 kDa-PEG was used in the experiment shown in Figure 4 because the excess unreacted 20 kDa-PEG in the PEGylation reaction mixtures distorts the SDS gel and interferes with migration and visualization of the PEGylated and unPEGylated proteins. Unreacted 5 kDa-PEG migrates at a different position in the gel and does not interfere with migration or visualization of the proteins. BV Epo did not PEGylate under identical conditions (Fig. 4, lane 3), strongly suggesting that the PEG molecule is attached to the free cysteine introduced into the cysteine analogs. The K154C and S126C analogs formed significant amounts of disulfide-linked dimers during the PEGylation reactions.

The PEGylated Epo cysteine analogs were purified from unreacted protein, PEG reagent and protein dimers by size-exclusion chromatography and concentrated by ion-exchange chromatography. Non-reducing SDS-PAGE analysis of representative purified Epo cysteine analogs modified with a 20 kDa-maleimide PEG is shown in Fig. 5. Mean EC_{50} s for most of the PEG-Epo cysteine analogs were similar to mean EC_{50} values determined for the corresponding non-PEGylated Epo cysteine analogs and BV EPO in the UT7/Epo cell proliferation assay (Table 1). Concentrations of the proteins used in the bioassays were determined using the Bradford dye binding assay. The only PEG-Epo analogs that showed consistently reduced in vitro bioactivities were PEG-K45C, which was reduced 3-fold in activity, and PEG-K154C, which was reduced 13-fold in activity (Table 1). Dose-response curves for representative PEG-Epo cysteine analogs are shown in Fig. 3.

Pharmacokinetic studies in rats

Circulating half-lives of the 20 kDa-PEG-T26C analog and BV Epo were measured following intravenous administration to rats at a dose of 100 μ g protein/kg (Fig. 6). Three rats were used per protein and each rat received ~ 35 μ g of protein, which corresponds to 6.65×10^6 mIU Epo, assuming a conversion factor of 1.9×10^5 mIU/ μ g Epo. Plasma levels of the proteins were quantitated by ELISA. Selected plasma samples (0-24h post injection) from one rat in each group also were analyzed in the UT7/Epo in vitro bioassay to estimate plasma concentrations of the proteins and to determine whether the proteins were biologically active. There was an excellent correlation between the in vitro bioassay results and the ELISA results (data not shown). BV Epo was eliminated rapidly from the rats. At the first time point analyzed (15 min post-injection) we recovered only 1 % of the amount of BV EPO administered to the animals (recovery of 5.9×10^3 mIU/mL versus a theoretical maximum recovery of 543×10^3 mIU/mL, which was calculated by dividing the total amount of BV Epo administered, 6.65×10^6 mIU, by the plasma volume of a 350g rat, which was estimated to be 12.2 mL or 3.5% of body weight). The BV Epo remaining in circulation had a terminal half-life of 1.7 h (measured from 1.5–10 h post-injection) and was undetectable in plasma 24 h post-injection. The 20kDa-PEG-T26C protein had a slower, but still rapid, early elimination phase and a slower terminal elimination half-life than BV Epo. At 15 min post-injection plasma levels of PEG-T26C (202×10^3 mIU/mL) were 34-fold higher than plasma levels of BV Epo. However, more than 90% of the PEG-T26C protein was eliminated from the circulation during the first 4 h post-injection. The 20 kDa-PEG-T26C protein remaining in circulation had a terminal half-life of 12.9 h (measured from 10 - 96 h post-injection) and was detectable in plasma for up to 96h post-injection.

Discussion

Our data indicate that a large number (> 30) of amino acid residues in Epo can be changed to cysteine without substantially affecting in vitro biological activity of the protein. In addition, we found that it is possible to covalently modify many of these Epo cysteine analogs with a large 20 kDa-maleimide PEG and that at least 10 of the PEGylated proteins retain complete or near complete in vitro biological activity. Since only a subset of the cysteine analogs constructed were purified for PEGylation studies, it is likely additional fully active PEGylated cysteine analogs can be prepared from the cysteine analogs we did not purify. The high percentage of fully active cysteine analogs identified in our study probably is a result of our focusing mutagenesis efforts away from known receptor binding sites in Epo, which have been localized to helices A, C and D and the C-terminal end of the A-B loop [12,13,15]. Previous substitution and deletion mutagenesis studies suggested that the non-helical regions targeted in our study, the region preceding helix A, the N-terminal end of the A-B loop, the B-C loop, the C-D loop, and the region following helix D, are largely nonessential for in vitro biological activity of Epo [12,13,15]. Our finding that large polymers can be attached to many amino acids in these regions without appreciable effects on in vitro bioactivities of the proteins supports this conclusion. Kochendoerfer et al. [25] previously reported that a synthetic Epo analog containing E89C and E117C substitutions possessed wild type Epo in vitro bioactivity; our findings are in agreement with their conclusion. More than half of the amino acids changed to cysteine residues in our study are absolutely conserved in Epo proteins from man, monkey, sheep, pig, cat and dog [26]. Apparently, the high degree of amino acid conservation in these regions does not reflect physiological importance of the conserved amino acids, at least with regard to Epo in vitro bioactivity on UT7/Epo cells.

Epo in vitro bioactivity on hematopoietic cells is severely reduced, and in some cases eliminated, by modification of lysine residues with amine-reactive reagents [17,18]. Mutagenesis studies revealed that K20, K45, K97 and K152 are important for in vitro bioactivity and/or structural stability of Epo [13,15]. The only two PEGylated cysteine analogs identified in our study that possessed obviously reduced in vitro bioactivities were modifications of lysine residues, PEG-K45C and PEG-K154C. The PEG moiety appears to be responsible for the modestly (3-fold) reduced bioactivity of PEG-K45C, because the unmodified K45C protein displayed wild type in vitro bioactivity. K45, which is located in the A-B loop, is believed to constitute part of the high affinity receptor binding site in Epo [16]. Given the presumed importance of K45 in receptor binding, it is surprising that attaching a large PEG at this position resulted in only a minor impairment in in vitro bioactivity. Previous mutagenesis studies disagreed on the importance of K154 for Epo in vitro bioactivity. Bittorf et al. [27] reported that a K154A analog possessed only 9% of the in vitro bioactivity of Epo, whereas Wen et al. [28] reported that a K154A analog was more active than wild type Epo in in vitro bioassays. Elliot et al. [15] reported that K154R and K154S analogs possessed wild type in vitro bioactivity. We found that the K154C protein possessed mildly reduced in vitro bioactivity (2- to 3-fold), whereas the PEGylated K154C protein displayed severely reduced (13-fold) in vitro bioactivity. Certain amino acids near K154 in helix D, in particular G151 and K152, form a hydrophobic surface that is postulated to constitute part of the high affinity Epo receptor binding site [16]. Substitution of G151 or K152 with alanine residues reduces Epo in vitro bioactivity greater than 10-fold [13,15,28], similar to the reduction in bioactivity we observed with PEG-K154C. Attachment of a large, hydrophilic PEG molecule to amino acid 154 in Epo may disrupt the critical hydrophobic interaction involving G151 and/or K152, and/or sterically prevent key receptor contacts from forming.

We used insect cells rather than mammalian cells for production and screening studies because insect cells allowed us to readily produce and purify milligram quantities of the Epo cysteine analogs and PEGylated cysteine analogs for accurate quantitation of the proteins for bioactivity

measurements. Insect cell-expressed and mammalian cell-expressed Epo proteins have similar EC₅₀s in in vitro bioassays; thus these studies allowed us to rapidly identify promising Epo cysteine analogs for future expression studies in mammalian cells. A drawback of the use of insect cells for our studies was the finding that insect cell-expressed Epo (BV Epo) displays quite different pharmacokinetic properties than mammalian cell-derived Epo in rats. Most notable was the apparent rapid elimination of BV Epo following intravenous administration to rats (Fig. 6). Approximately 99% of the BV Epo protein was eliminated from the circulation within 15 min of administration. In contrast, only 10-40% of mammalian cell-expressed Epo typically is eliminated during the first few hours following intravenous administration to rats [4, and our unpublished results]. BV Epo also had a shorter terminal half-life (1.7 h) than mammalian cell-expressed Epo [3-4 h; 4,5]. Epo expressed in mammalian cells is extensively glycosylated with highly branched, complex sugars and the commercial form of the protein is selected for high sialic acid content to maximize its half-life [5]. In contrast, insect cells attach only simple sugars to proteins and do not appear to add terminal sialic acids to N-linked sugars [29]. These glycosylation differences may account, at least in part, for the rapid clearance of BV Epo in rats. The pharmacokinetic properties of BV Epo were similar to those reported for asialylated, mammalian cell-expressed Epo [3,4]. Asialylated mammalian cell-derived Epo had an early distribution half-life of 2- 5 min and a terminal half-life of 1.5 h in rats [3,4]. Only 4% of the administered protein was recovered 30 min following post-injection, similar to our result with BV Epo (1% recovery at 15 min post-injection).

Attaching a single 20 kDa-PEG to an insect cell-expressed Epo cysteine analog, T26C, significantly altered the protein's pharmacokinetic profile in rats. Both the initial distribution phase and the terminal half-life were slowed. However, greater than 90% of the PEGylated protein was still eliminated from the circulation within 4 h post injection. The terminal half-life of the PEG-T26C protein remaining in the circulation was improved 7- to 8-fold relative to BV Epo (12.9 h versus 1.7 h). This is a much greater half-life improvement than the 2.5-fold half-life improvement obtained by engineering 2 extra glycosylation sites into Epo [5,7]. Whether a similar relative improvement in half-life can be obtained by site-specific PEGylation of a properly glycosylated mammalian cell-derived Epo analog remains to be determined. Other researchers reported that modification of mammalian cell-derived Epo with multiple 5 kDa-amine-reactive PEGs increased the protein's half-life approximately 9-fold following intravenous administration to rats [30]. The significant glycosylation and pharmacokinetic differences between Epo proteins expressed in insect and mammalian cells precluded meaningful in vivo efficacy comparisons between proteins expressed using these different host systems.

In conclusion, these studies demonstrate the feasibility of using site-specific PEGylation to design Epo analogs with minimal loss of in vitro bioactivity. By targeting attachment of PEG to non-essential sites in Epo using site-specific PEGylation it is possible to avoid the significant loss of in vitro bioactivity associated with random modification of lysine residues in Epo. It should be possible to use a similar approach to create designer Epo cysteine analogs modified with other types of polymers and functional groups. Antigenicity of these modified Epo cysteine analogs, which is a potential serious complication of any recombinant Epo preparation [31], remains to be tested.

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REFERENCES

1. Markham A, Bryson HM. Epoetin alpha. A review of its pharmacodynamic and pharmacokinetic properties and therapeutic use in nonrenal applications. *Drugs* 1995;49:232–254. [PubMed: 7729331]
2. Yamaguchi K, Akai K, Kawanishi G, Ueda M, Masida S, Sasaki R. Effects of site-directed removal of N-glycosylation sites in human erythropoietin on its production and biological properties. *J Biol Chem* 1991;266:20434–20439. [PubMed: 1657925]
3. Fukuda MN, Sasaki H, Lopez L, Fukuda M. Survival of recombinant erythropoietin in the circulation: the role of carbohydrates. *Blood* 1989;73:84–89. [PubMed: 2910371]
4. Spivak JL, Hogans BB. The in vivo metabolism of recombinant human erythropoietin in the rat. *Blood* 1989;73:90–99. [PubMed: 2462945]
5. Egrie JC, Browne JK. Development and characterization of novel erythropoiesis stimulating protein (NESP). *Brit J Cancer* 2001;84(Supplement 1):3–10. [PubMed: 11308268]
6. Flaherty KK. Clinical pharmacology of recombinant human erythropoietin. *Pharmacotherapy* 1990;10:9S–14S. [PubMed: 2345710]
7. Egrie JC, Dwyer E, Browne JK, Hitz A, Lykos MA. Darbepoetin alpha has a longer circulating half-life and greater in vivo potency than recombinant human erythropoietin. *Exp Hematol* 2003;31:290–299. [PubMed: 12691916]
8. Abuchowski A, Kazo GM, Verhoest CR, et al. Cancer therapy with chemically modified enzymes. I. Antitumor properties of polyethylene glycol-asparaginase conjugates. *Cancer Biochem Biophys* 1984;7:175–186. [PubMed: 6467175]
9. Molineux G, Kinstler O, Briddell B, et al. A new form of filgrastim with sustained duration in vivo and enhanced ability to mobilize PBPC in both mice and humans. *Exp Hematol* 1999;27:1724–1734. [PubMed: 10641590]
10. Wang Y-S, Youngster S, Bausch J, Zhang R, McNemar C, Wyss DF. Identification of the major positional isomer of PEGylated interferon alpha-2b. *Biochemistry* 2000;39:10634–10640.
11. Bailon P, Palleroni A, Schaffer CA, et al. Rational design of a potent, long-acting form of interferon: a 40 kDa branched polyethylene glycol-conjugated interferon α 2a for the treatment of hepatitis C. *Bioconjugate Chem* 2001;12:195–202.
12. Boissel J-P, Lee W-R, Presnell SR, Cohen FE, Bunn HF. Erythropoietin structure-function relationships. Mutant proteins that test a model of tertiary structure. *J Biol Chem* 1993;268:15983–15993. [PubMed: 8340419]
13. Matthews DJ, Topping RS, Cass RT, Giebel LB. A sequential dimerization mechanism for erythropoietin receptor activation. *Proc Natl Acad Sci USA* 1996;93:9471–9476. [PubMed: 8790354]1996
14. Cheetham JC, Smith DM, Aoki KH, et al. NMR structure of human erythropoietin and a comparison to its receptor bound form. *Nature Structural Biology* 1998;5:861–866.
15. Elliott S, Lorenzini T, Chang D, Barzilay J, Delorme E. Mapping the active site of recombinant human erythropoietin. *Blood* 1997;89:493–502. [PubMed: 9002951]
16. Syed RS, Reid SW, Cuiwei, et al. Efficiency of signaling through cytokine receptors critically depends on receptor orientation. *Nature* 1998;395:511–516. [PubMed: 9774108]
17. Wojchowski DM, Caslake L. Biotinylated recombinant human erythropoietins: bioactivity and utility as receptor ligand. *Blood* 1989;74:952–958. [PubMed: 2546635]
18. Leist M, Ghezzi P, Grasso G, et al. Derivatives of erythropoietin that are tissue protective but not erythropoietic. *Science* 2004;305:239–242. [PubMed: 15247477]
19. Kawasaki, ES. Amplification of RNA. In: Innis, MA.; Gelfand, DH.; Sninsky, JJ.; White, TJ., editors. *PCR Protocols: A Guide to Methods and Applications*. Academic Press; San Diego, CA: 1990. p. 21–27.
20. Wang GL, Semenza GL. Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. *Blood* 1993;82:3610–3615. [PubMed: 8260699]
21. Ranjan A, Hasnain SE. Influence of codon usage and translation initiation codon context in the AcNPV-based expression system: computer analysis using homologous and heterologous genes. *Virus Genes* 1995;9:149–153. [PubMed: 7537424]

22. Higuchi, R. Recombinant PCR. In: Innis, MA.; Gelfand, DH.; Sninsky, JJ.; White, TJ., editors. PCR Protocols: A Guide to Methods and Applications. Academic Press; San Diego, CA: 1990. p. 177-183.
23. Horton, RM. In Vitro Recombination and mutagenesis of DNA. SOIn together tailor- made genes. In: White, BA., editor. Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications. Humana Press; Totawa, NJ: 1993. p. 251-226.
24. Komatsu M, Nakauchi H, Miwa A, et al. Establishment and characterization of a human leukemic cell line with megakaryotic features: dependency on granulocyte-macrophage colony-stimulating factor, interleukin-3, or erythropoietin for growth and survival. *Cancer Res* 1991;51:341–348. [PubMed: 1824823]
25. Kochendoerfer GG, Chen SY, Mao F, et al. Design and chemical synthesis of a homogeneous polymer-modified erythropoiesis protein. *Science* 2003;299:884–887. [PubMed: 12574628]
26. Wen D, Boissel J-PR, Tracy TE, et al. Erythropoietin structure-function relationships: high degree of sequence homology among mammals. *Blood* 1993;82:1507–1516. [PubMed: 8364201]
27. Bittorf T, Jaster R, Brock J. Structural and functional characterization of recombinant human erythropoietin analogues. *FEBS Lett* 1993;366:133–136. [PubMed: 8262196]
28. Wen D, Boissel J-P, Showers M, Ruch BC, Bunn HF. Erythropoietin structure-function relationships. Identification of functionally important domains. *J Biol Chem* 1994;269:22839–22846. [PubMed: 8077236]
29. Tomiya N, Narang S, Lee YC, Betenbaugh MJ. Comparing N-glycan processing in mammalian cell lines to native and engineered lepidopteran insect cell lines. *Glycoconj J* 2004;21:343–360. [PubMed: 15514482]
30. Jolling K, Ruixo JJP, Hemeryck A, Piotrovskij V, Greway T. Population pharmacokinetic analysis of Pegylated erythropoietin in rats. *J Phar Sci* 2004;93:3027–3038.
31. Casadevall N. Pure red cell aplasia and anti-erythropoietin antibodies in patients treated with epoetin. *Nephrol Dial Transplant* 2003;18(Supplement 8):viii37–41. [PubMed: 14608000]

Figure 1. Non-Reducing SDS-PAGE of aliquots of purified BV Epo and representative Epo cysteine analogs. Proteins were purified by ant-FLAG M2 affinity column chromatography. MW indicates molecular weight markers.

Figure 2.

Amino acid sequence and secondary structure features of human Epo (adapted from [14]). Amino acids that comprise alpha helices A-D are indicated in bold. Amino acids that were changed to cysteine residues are underlined. An asterisk (*) denotes the *167C analog, which contains a cysteine residue added after R166.

Figure 3.

Dose response curves for affinity-purified BV Epo, Epo cysteine analogs and PEGylated Epo cysteine analogs for stimulating proliferation of human UT7/Epo cells. Data are means of triplicate wells $\pm$ SD from representative experiments. Proteins shown in the same panel were assayed on the same day. Protein concentrations were measured using a Bradford dye binding assay. Absorbance values are proportional to cell number.

Figure 4.

Specificity of PEG reaction. BV Epo and the T26C analog were incubated with a 5 kDa-maleimide PEG and the reducing agent TCEP using the reaction conditions described in **Materials and methods**. The PEG reactions were analyzed by reducing SDS-PAGE on 14% Tris Glycine gels. Positions of Epo monomers and PEG-T26C are marked. Lane 1, BV Epo; lane 2, BV Epo + PEG; lane 3, BV Epo + PEG + TCEP; lane 4, T26C; lane 5, T26C + PEG; lane 6, T26C + PEG + TCEP. MW indicates molecular weight markers.

Figure 5. Non-Reducing SDS-PAGE of purified monoPEGylated Epo cysteine analogs and BV Epo. Epo cysteine analogs were modified with a 20 kDa-maleimide PEG. MW indicates molecular weight markers.

Figure 6.

Changes in plasma protein levels following intravenous administration of BV Epo and 20 kDa-PEG-T26C to rats. Protein levels were measured using human Epo ELISA kits. Data are means $\pm$ SD for 3 rats per group.

Table 1

In vitro bioactivities of purified BV Epo, Epo cysteine analogs and PEGylated Epo cysteine analogs in UT7/Epo cell proliferation assay.

Protein	UnPEGylated Protein		20 kDa-PEG-Protein	
	EC ₅₀ [*] (ng/mL)	Mut/WT EC ₅₀ ratio [§]	EC ₅₀ [*] (ng/mL)	Mut/WT EC ₅₀ ratio [§]
BV Epo	0.31 ± 0.19			
Epo WT CHO ^{**}	0.46 ± 0.17			
A1C	0.21 ± 0.04	0.75	0.37 ± 0.05	1.61
P3C	0.41 ± 0.13	1.37	0.24 ± 0.08	1.26
N24C	0.76 ± 0.16	1.65		
T26C	0.17 ± 0.03	0.77	0.22 ± 0.04	1.00
N38C	1.05 ± 0.35	2.28		
T40C	0.95 ± 0.48	3.65		
K45C	0.30 ± 0.06	1.00	0.84 ± 0.04	2.80
N83C	0.50 ± 0.09	1.92		
S85C	0.72 ± 0.19	1.57		
E89C	0.39 ± 0.11	1.20	0.31 ± 0.08	0.94
Q115C	0.39 ± 0.05	0.86	0.23 ± 0.03	0.51
E117C	0.33 ± 0.12	1.10	0.23 ± 0.05	0.79
S126C	0.74 ± 0.09	0.90	0.48 ± 0.09	0.59
A128C	0.27 ± 0.05	0.90	0.23 ± 0.05	0.77
K154C	1.10 ± 0.26	2.44	5.80 ± 0.97	12.90
T163C	0.23 ± 0.07	0.79	0.37 ± 0.09	1.30
I67C [*]	0.35 ± 0.16	1.46	0.31 ± 0.09	1.29

* Means ± standard deviations of at least 3 assays for each protein. Concentrations of the purified BV Epo, Epo cysteine analogs and PEGylated Epo cysteine analogs were measured using a Bradford dye binding assay. Concentration of the recombinant CHO Epo protein was provided by the supplier (R & D Systems, Inc.)

§ Mutant EC₅₀/BV Epo EC₅₀ in assays performed on the same days.

** Recombinant wild type Epo expressed in CHO cells (R&D Systems, Inc.).