Differential Effects of Long-lived Erythropoietin Receptor Agonists in Rats

Peter J. Bugelski, Dorie Makropoulos*, Tracey Spinka-Doms, Ed Eirikis, Amy Volk, Qun Jiao, Chichi Huang

Biologics Toxicology Centocor R&D, Inc., 145 King of Prussia Road Radnor, PA 19087, USA

Abstract

Erythropoietin (EPO) regulates proliferation and differentiation of erythroid precursor cells into erythrocytes. Here, we report on experiments designed to study how the pharmacokinetic profiles of EPO receptor agonists, ranging from the short-lived epoetin-α to the long-lived EPO-MIMETIBODY™ constructs CNTO 530 and CNTO 531, influence the pharmacodynamic response in rats. Rats received a single dose of an EPO-R agonist and the effects on reticulocytes, red blood cells and hemoglobin were measured over time. The increase in red blood cells and hemoglobin were negatively correlated with clearance. At doses that cause a similar effect on reticulocytes, very long-lived EPO-R agonists caused prolonged production of red blood cells. In conclusion, we have shown that very long-lived EPO-R agonists cause prolonged production of red blood cells and increase in hemoglobin that is independent of their in vitro potency or the peak release of reticulocytes. These data suggest that EPO may be a survival factor for reticulocytes.

Keywords: Hematology; Erythropoiesis; Structure

Introduction

Erythropoiesis is the process of forming red blood cells (RBC) from multipotent stem cells. Production of a mature RBC is the culmination of a complex and tightly regulated process initiated in the central sinus beds of medullary marrow and completed in circulation [1,2]. Erythropoietin (EPO) plays a central role in erythropoiesis [2,3]. EPO is a ~30 kDa glycoprotein whose amino acid sequence is highly conserved among mammals (91% identity between monkey and human, ~80% identity between rodents and human [4]). It is well established that erythropoietin (EPO) is a growth and survival factor for the early stages of erythropoiesis. EPO acts by binding and activating EPO receptors (EPO-R) on the surface of populations of erythroid precursor cells [5]. Like that of EPO, the amino acid sequence of EPO-R is also highly conserved [6]. EPO-R exists constitutively in association with Janus 2 kinase (Jak2) [7]. Binding of EPO-R by EPO or EMP1 results in activation of Jak2, and phosphorylation of Jak2, EPO-R, Shc, and Stat5 [8]. This in turn results in the activation of three distinct signaling pathways: MAPK, Stat5, and AKT [9]. In addition to mediating activation of cell signaling, binding of EPO-R by EPO also results in internalization of EPO-R. This results in down-regulation of receptor expression on the cell surface as internalized receptors are believed to be degraded by the endosome-lysosome or proteosome pathways [10]. EPO-R are replenished on the cell surface from a pre-existing intracellular pool and, as has been shown in an EPO-dependent cell line, long term exposure to EPO can result in up-regulation of its own receptor [11].

EPO functions as a growth factor and is also believed to act as a survival factor for erythroid precursors, inhibiting apoptosis in early precursors in vitro, particularly erythroid burst forming units (BFU-e) and colony forming units (CFU-e) [12]. Recent in vivo work has also shown that EPO and novel EPO-R agonists can influence apoptosis in later stage erythroid precursors in the bone marrow [13,14] and the precipitous drop in RBC following withdrawal of EPO [15] suggests that EPO can influence the survival of anucleate reticulocytes (RTC) and nascent RBC after these cells leave the bone marrow.

Previously, we have shown that CNTO 530 and darbepoetin-α, two long-lived EPO-R agonists have little effect on RBC life span in mice [16]. The purpose of this study was to use a number of EPO-R agonists with varying potency (in vitro activity in UT7Epo cells) and pharmacokinetic behavior (terminal t½ and clearance) to test the hypothesis that EPO acts as a survival factor for RTC. We have found that short-lived EPO-R agonists can increase RTC without increasing RBC, that longer-lived EPO-R agonists cause an increase in RBC consistent with the expected 2% daily loss of senescent RBC, and very long-lived EPO-R agonists can increase RBC to a greater extent than expected. Taken together, our findings suggest that the presence of high circulating levels of an EPO-R agonist can act as a survival factor for RTC and RBC and thus foster improved efficiency of end-stage erythropoiesis.

Materials and Methods

Epoetin-α was obtained from Ortho Biotech (Raritan, NJ) and darbepoetin-α (Aranesp®, Amgen, Inc. Thousand Oaks, CA) was purchased commercially.

EPO-MIMETIBODY™ constructs

The general structure of the EPO-MIMETIBODY™ constructs is shown schematically in Figure 1. Each construct contains two EMP-1 sequences as the pharmacophore. EMP-1 is a 20-amino acid peptide that was discovered by screening combinatorial libraries of random sequence peptides using phage display technology [17]. EMP-1 binds to EPO-R and expresses EPO-like bioactivity in both in vitro and in vivo systems [8,17]. EPO-MIMETIBODY™ constructs were expressed in mammalian cells and purified by routine methods and supplied by Centocor R&D as described previously [13,18]. Their characteristics are summarized in Table 1.

*Corresponding author: Dorie Makropoulos, Biologics Toxicology Centocor R&D, Inc., 145 King of Prussia Road Radnor, PA 19087, USA; Tel: 610-240-8542; Fax: 610-651-7363; E-mail: dmakropo@its.jnj.com

Received August 09, 2011; Accepted September 23, 2011; Published September 26, 2011


Copyright: © 2011 Bugelski PJ, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
UT7 Assay

Bioactivity of EPO-R agonists was determined using a UT-7EPO cell proliferation assay. UT-7EPO cells are an EPO dependent subline of UT-7, a human megakaryoblastic leukemia cells [19]. Cells were washed thrice in PBS with final resuspension in Iscove’s modified Dulbecco’s medium supplemented with 2mM L-glutamine and 5% fetal bovine serum (15Q) but no epoetin-α for overnight EPO starvation. After 24 hours of EPO starvation, cells were washed once in PBS, counted, and ultimately re-suspended in fresh 15Q. Cells were distributed at 30,000 cells per well in duplicate in a 96-well plate, EPO-R agonists were added along with 20 µL of MTS (Promega) per 100 µL reaction was added to each well. Readings were taken at hourly intervals, starting one hour after reagent addition. Plates were read at a wavelength of 490 nm with a reference wavelength of 650 nm subtracted. Three hour data were analyzed using GraphPad PRISM based on a sigmoidal curve fit of the data as described previously [13].

Data are reported as the concentration that caused a 50% maximal response (EC₅₀).

**Figure 1** Schematic of the general structure of EPO-MIMETIBODY™ constructs. They are composed of homodimers of human IgG heavy chain, a linker sequence: Schematic of the general structure of EPO-MIMETIBODY™ constructs.

### Table 1: Characteristics of EPO-R Agonists used in this study.

<table>
<thead>
<tr>
<th>Test Article</th>
<th>Fc</th>
<th>Mol Wt (kDa)</th>
<th>T₅₀ (hr)</th>
<th>CI (mL/day/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epoetin-α</td>
<td>NA</td>
<td>34</td>
<td>9.55 x 10⁻¹²</td>
<td>2.5</td>
</tr>
<tr>
<td>Darbepoetin-α</td>
<td>NA</td>
<td>37</td>
<td>6.31 x 10⁻¹²</td>
<td>6.9</td>
</tr>
<tr>
<td>CNTO 528</td>
<td>IgG1</td>
<td>62</td>
<td>2.08 x 10⁻¹⁰</td>
<td>37.0</td>
</tr>
<tr>
<td>NEM-2824</td>
<td>IgG1ala-ala</td>
<td>58</td>
<td>8.00 x 10⁻¹¹</td>
<td>50.4</td>
</tr>
<tr>
<td>NEM-2825</td>
<td>IgG1ala-ala</td>
<td>58</td>
<td>7.69 x 10⁻¹¹</td>
<td>48.0</td>
</tr>
<tr>
<td>NEM-2466</td>
<td>IgG4ala-ala-S+P</td>
<td>58</td>
<td>9.36 x 10⁻¹¹</td>
<td>53.3</td>
</tr>
<tr>
<td>NEM-2467</td>
<td>IgG4ala-ala-S+P</td>
<td>58</td>
<td>1.01 x 10⁻¹⁰</td>
<td>45.8</td>
</tr>
<tr>
<td>CNTO 530</td>
<td>IgG4ala-ala-S+P</td>
<td>58</td>
<td>6.60 x 10⁻¹¹</td>
<td>72.7</td>
</tr>
<tr>
<td>CNTO 531</td>
<td>IgG4ala-ala-S+P</td>
<td>58</td>
<td>8.27 x 10⁻¹¹</td>
<td>71.5</td>
</tr>
</tbody>
</table>

*Pharmacokinetic values for a single IV dose of epoetin-α and darbepoetin-α in rats are taken from Egrie et al. [46].

**Figure 2**: A) Activity of epoetin-α, darbepoetin-α and selected EPO-MIMETIBODY™ constructs in the UT-7EPO assay. Epoetin-α and darbepoetin-α show similar potencies while the EPO-MIMETIBODY™ constructs although full receptor agonists are less potent. These data were used to calculate the EC50 values presented in Table 1. B) Plasma concentration vs. time plot for the EPO-MIMETIBODY™ constructs. These data were used to calculate the pharmacokinetics parameters presented in Table 1.

### Rats

Female Sprague Dawley CD rats weighing approximately 300 grams were obtained from Charles Rivers Laboratories (Raleigh, NC). Rats were housed 2 per cage in filter topped plastic shoe-box style cages in a 12 hr light/dark cycle and fed and watered ad libitum. The rats were identified with ear tags, placed at least 1 week prior to the start of the study. Cage cards labeled with animal number, test article, treatment, study number and IACUC protocol number were affixed to the cages. All procedures were reviewed by the Centocor R&D Institutional Animal Care and Use Committee and were conducted in an AALAC approved facility.

### Pharmacokinetics

On Day 0 all rats were weighed and received a weight-adjusted, single I.V. injection of 1 mg/kg (4 mL/kg) test article. Blood samples were taken at 20 min, 60 min, 6hr, 24hr, 48hr, 3, 6, 10, 14, 21, 28 days. For sampling, animals were anesthetized with CO₂ and a target volume of 300 ul. of blood collected via retro-orbital bleed. Blood was allowed to clot and centrifuged to separate serum. Serum was stored at –80°C. Serum levels of the EPO-MIMETIBODY™ constructs were measured by ELISA using goat anti-huFc capture/goat anti-huFc detection and using anti-EMP-1 Fab capture/goat anti-huFc detection as described previously for mouse plasma [13]. Serum concentration data were used to calculate standard pharmacokinetics parameters using non-compartmental analysis. (WinNonlin version 5.1, Pharsight Corporation, Mountain View, CA) as described previously [13].

### Pharmacodynamics

Hematological parameters were evaluated from rat whole blood using an ADVIA® 120 hematology analyzer (Siemens Medical Solutions) and the plate incubated in 5% CO₂ at 37ºC. After 48 h, 20 µL of MTS cells per well in duplicate in a 96-well plate, EPO-R agonists were added to the plate incubated in 5% CO₂ at 37ºC. After 48 h, 20 µL of MTS cells were distributed at 30,000 cells per well in duplicate in a 96-well plate, EPO-R agonists were added to the plate incubated in 5% CO₂ at 37ºC. After 48 h, 20 µL of MTS cells were washed once in PBS, counted, and ultimately re-suspended in fresh 15Q.

**Pharmacokinetic values for a single IV dose of epoetin-α and darbepoetin-α in rats are taken from Egrie et al. [46].**

**Table 1**: Characteristics of EPO-R Agonists used in this study.

Page 2 of 8
Diagnostics, Tarrytown, NY) as described previously [20]. Blood samples were taken on Study Days 4, 8, 15, 23, 30, and 37. RTC counts were enumerated by multiplying % RTC by total RBC counts and total RBC counts were corrected for RTC by subtraction.

To test the hypothesis that the various EPO-R agonists have a differential effect on the efficiency of RTC maturation, a model was constructed using data from control rats and then applied to the treatment groups:

\[ \text{RTC(t)}_{\text{N}} = \left( \frac{\text{RTC(t)}_{\text{N-1}} + \text{RTC(t)}_{\text{N}}}{2} \right) \times \text{SF} \]

where

- \( \text{RTC(t)}_{\text{N}} \) = Total RTC count on Day N
- \( \text{RTC(t)}_{\text{N}} \) = Reticulocyte count on Day N
- \( \text{SF} \) = surviving fraction in PBS treated rats

And, \( \text{RBC(t)}_{\text{N}} \) = Average RBC count in PBS treated rats on Day 4

To test the hypothesis that the differential effects on conversion of RTC to RBC had practical significance, the area under the change in hemoglobin (Hgb) vs. time curves (Hgb AUC) was calculated. Hgb AUC \(_{(1-37)}\) was calculated for the change in Hgb between Days 1 and 37 (when the mean Hgb values for epoetin-α had returned to baseline) was calculated by subtracting the mean Hgb value of the control group from the treated groups at each time point, multiplying this value by the sampling interval and summing the resultant value. Data for Hgb AUC \(_{(1-37)}\) were plotted as a function of log of the administered dose (mg/kg) and fitted with the following equation:

\[ \text{Hgb AUC}_{(1-37)} = a \times \log(\text{dose}) + b \]

Statistical analysis was performed with SigmaStat v2.03 (SPSS, Inc. San Rafael, CA). Correlations between the slope of the Hgb AUC \(_{(1-37)}\)

**Figure 3:** Dose response curves for the effects of epoetin-α, darbepoetin-α and the EPO-MIMETIBODY\(^{10}\) constructs on RTC, RBC (not corrected for reticulocyte count) and Hgb. Values displayed on graph are group means (n=6).
curves and the clearance of the EPO-R agonists were evaluated using the Spearman Rank Order correlation. P values less than 0.05 were accepted as significant.

**Results**

The ability of the epoetin-α, darbepoetin-α and the EPO-MIMETIBODY™ constructs to activate EPO-R was studied in UT-7EPO cells. Representative data are shown in Figure 2a and the EC₅₀ values are presented in Table 1. All the EPO-MIMETIBODY™ constructs supported proliferation of UT-7EPO cells, albeit at as much as ~100 fold lower potency compared to epoetin-α and darbepoetin-α.

The results of the pharmacokinetic analysis of the EPO-MIMETIBODY™ constructs are shown in Figure 2b and the terminal t₁/₂ and systemic clearance in Table 1. The EPO-MIMETIBODY™
constructs showed as much as 10 fold longer $t_{1/2}$ and lower clearance than epoetin-α.

To study the pharmacodynamic effects of epoetin-α, darbepoetin-α and the EPO-MIMETIBODY™ constructs, rats received a single sc dose of each test article over a range of doses on Day 1 and serial blood samples were collected starting on Day 4. (To avoid an endogenous RTC response to blood collection, pre-dose samples were not collected and Day 4 values from the PBS treated rats were used as Day 0 values for graphing.) Representative dose response data for RTC, total RBC and Hgb for epoetin-α, darbepoetin-α, CNTO 528 and CNTO 530 are shown in Figure 3. The dose responses for other EPO-MIMETIBODY™ constructs were similar to those of CNTO 530 (Data not shown). As is evident from the figure, although all test articles caused a dose responsive increase in peak RTC, the time to peak RTC and the duration of the increase in RTC were related to the administered dose and the clearance of the EPO-R agonist. It is also evident that epoetin-α, although causing a dose responsive increase in Hgb, did not cause a commensurate dose responsive increase in total RBC. An increase in mean corpuscular Hgb (MHC) explains the increase in Hgb in the epoetin-α treated rats. The peak MHC on Day 7 is illustrated for epoetin-α in Figure 4.

From the dose response data, doses that caused the same peak increase in RTC were selected for further study. The RTC and RBC (corrected for RTC counts) responses for these doses are shown in Figure 5. The results for the test articles are arranged by efficiency of RBC production. As is evident from the figure, a similar peak RTC response was not necessarily followed by a commensurate increase in RBC. To confirm this observation, we constructed a pharmacodynamic

---

**Figure 6**: Modeling of the conversion of RTC to RBC at dose that cause a similar increase in RTC. The model is describe in Materials and Methods and assumes no loss in RTC and a 2% loss in RBC. The model closely follows the measured RBC values for the control mice and most EPO-MIMETIBODY™ constructs. In contrast, the model overestimates the measured response for epoetin-α, but underestimates the response to CNTO 530.
model that predicts RBC based on RTC. The results of the model are graphed with the measured RBC (corrected for RTC) in Figure 6. The survival fraction for RTC was determined based on the Day 4 and Day 8 RBC values for the PBS treated rats. A value of 2% loss was found. The model accurately predicted the RBC values for the PBS and CNTO 531. For epoetin-α and darbepoetin-α the model over-predicted the RBC response and the model under-predicted the RBC response for the other EPO-MIMETIBODY™ constructs. Taken as a whole, the performance of the model indicates that for epoetin-α and darbepoetin-α the efficiency of conversion of RTC to RBC was lower than in control rats and, with the exception of CNTO 531, for the EPO-MIMETIBODY™ constructs the efficiency was greater than expected. This suggests that a long terminal half-life and low clearance of an EPO-R agonist may influence the efficiency of conversion of an RTC to an RBC.

To determine if the purported increase in efficiency of converting an RTC to an RBC had a meaningful effect on the increase in Hgb, the area under the Hgb vs. time curve was calculated. Data for the effects of the various EPO-R agonists at a dose of 0.3 mg/kg on Hgb are shown in Figure 7A. These data were used to calculate the area under the curve for Hgb at that dose. Data for Hgb AUC(1-37) for the complete dose response dataset plotted as a function of log₁₀ of the administered dose are shown in Figure 7B. The data were well modeled by a log linear relationship and the constants for the regression analysis are shown in Table 2. There was a statistically significant correlation between the slope of the regression curves and the clearance of the EPO-R agonist (Coefficient of Correlation = −0.845, P < 0.001).

To summarize, the rank order from highest potency in UT-7 cells are as follows: Epoetin-α, Darbepoetin-α, NEM-2466, CNTO 531, NEM-2824, NEM-2825, CNTO 530, CNTO 528 and NEM-2467. Interestingly, for in vivo effects on hemoglobin AUC(1-37), the order changes to CNTO 531, CNTO 530, NEM-2467, NEM-2825, NEM-2824, CNTO 528, Darbepoetin-α and Epoetin-α.

**Discussion**

In these experiments, all EPO-R agonists caused a dose responsive increase in RTC and Hgb. However, at doses that caused the same peak increase in RTC widely divergent effects on RBC were seen; epoetin-α causing a negligible increase in RBC and CNTO 530 causing a 6 fold greater increase suggesting that the efficiency of maturation of RTC to RBC was different among the agonists tested. Finally, to determine if the differential effects observed on the maturation of RTC to RBC had practical implications, we examined the dose response of the various EPO-R agonists on increasing Hgb. The negative correlation between the slope of the Hgb AUC(1-37) vs. dose curves and the rate of pharmacokinetic clearance of the various EPO-R agonists demonstrated that those with slowest clearance showed the greatest efficacy. Interestingly, there was no significant correlation between Hgb AUC(1-37) and potency in the UT-7 assay. Taken together, these data suggest that maintaining the blood levels of the EPO-R agonist over the time required for maturation of RBC may be an important factor in determining efficacy.

Critical to interpreting the results of this study is the issue if RTC and RBC express EPO-R. It is well established that early erythroid precursor cells express EPO-R and that as these cells mature, expression of EPO-R decreases [21-23]. More problematic is expression of EPO-R by late stage erythroblasts, RTC and nascent RBC. In pioneering work, Baciu et al. [24] demonstrated in vitro binding of unlabeled EPO to membranes from human RTC and RBC. Akahane et al. [25] studied binding of [125I]-EPO in rat bone marrow and found low-level expression of EPO-R on polychromic but not orthochromic erythroblasts (Poly/OrthoEB). In contrast, Fraser et al. [22] showed that OrthoEB from cultured human bone marrow cells retained 30% of the initial number of EPO-R. Working with cultured Friend virus infected mouse bone marrow cells Wickrema et al. [26] found that RTC stage cells expressed 15% of the initial EPO-R mRNA content and bound 5% of the initial amount of [125I]-EPO. More recently, Mihov et al. [27] were able to demonstrate low-level specific binding of [125I]-EPO to RTC and RBC and using Scatchard analysis estimated 105 binding sites per RTC and 1-4 binding sites per mature RBC. Interestingly, although the value for EPO binding sites per RBC is very small, it is in keeping with the number of specific [125I]-EPO binding sites on RBC measured by Myssina et al. [28].

That the EPO binding sites on RTC and RBC represent expression of functional EPO-R is supported by the work of a number of groups...
who showed EPO can influence a variety of physiologic functions in RTC and RBC: inhibition of Ca\(^{2+}\)-ATPase activity in rat and rabbit reticulocyte membranes [29,30] and human RBC [28]; activation of nitric oxide synthase activity in murine RTC and RBC [27]; glucose transport in rat RBC [31] reactive oxygen metabolism and in rat RBC [32] and the work of Lang et al. [33] who have shown that EPO can inhibit eryptosis mediated in human RBC by a wide variety of insults (reviewed in Lang et al. [33]). Thus, when levels of EPO-R agonist fall, the lack of signaling by the remaining EPO-R on RTC may result in their rapid clearance via eryptosis.

The mechanism by which the lack of signaling by the small number of EPO-R expressed on RTC controls eryptosis is uncertain. EPO-R is a type I receptor that in early erythroid precursors is internalized after ligand binding via coated pits and targeted for destruction in by the proteosome/lysosome pathway [10]. However, during maturation RTC progressively lose coated pits [34] and thus may be unable to internalize and down-regulate EPO-R. Moreover, EPO can protect cardiac myocytes [35] and neuronal cells [36] from apoptosis; cell types not traditionally known to express EPO-R. Although RTC may express only a small fraction of the number of EPO-R expressed on earlier precursors, this low number of receptors may still be sufficient to influence cell behavior.

In normal cells, phosphatidylserine (PS) is preferentially distributed to the inner leaflet of the plasma membrane and loss of PS asymmetry is an early indicator of apoptosis [37]. Scramblases catalyze the bidirectional movement of phospholipids across the plasma membrane resulting in net redistribution of PS from the inner to the outer leaflet [38,39]. Increased exposure of PS at the cell surface has been shown to mediate recognition and phagocytosis by macrophages [40] and exposure of PS is believed to contribute to clearance of senescent RBC [41,42]. A 37 kDa type II membrane protein scramblase has been isolated from human RBC (PILSCR1) [43] that is activated by increased cytosolic free Ca\(^{2+}\) to redistribute PS between the leaflets in proteosomes. Of interest, the Ca\(^{2+}\) ionophore A23187 has been shown to mediate exposure of PS on RBC [44], EPO has been shown to inhibit Ca\(^{2+}\)-permeable cation channels, thus decreasing cytosolic free Ca\(^{2+}\) [28] and acute deprivation of EPO has been shown to increase PS exposure in RTC and RBC [45]. It is thus a reasonable hypothesis that lack of EPO-R signaling can lead to the activation of scramblase activity resulting in the rapid exposure of PS on RTC (and nascent RBC) and that exposure of PS subsequently leads to untimely phagocytosis and poor efficiency of end-stage erythropoiesis.

**Conclusion**

In conclusion, we have shown that very long-lived EPO-R agonists cause an unexpectedly high production of RBC and increase in Hgb that is independent of their in vitro potency or the peak release of RTC. These data suggest that EPO may be a survival factor for RTC.

**Acknowledgements**

This work was supported by Centocor R&D, Inc. The authors thank Christine McCauley, Kimberly Foster, Paul Fisher, and Thomas Nesspor for their excellent technical support. Thomas Nesspor also reviewed the manuscript. The authors would like to dedicate this research article in memory of Peter Bugelski. Peter was a true innovator and a real inspiration to have the pleasure and honor to work with. Peter will be truly and sincerely missed by us all.

**References**


