

## Differential Effects of Long-lived Erythropoietin Receptor Agonists in Rats

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### 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- $\alpha$  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 kD 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 proteasome 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- $\alpha$ , 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 *in vitro* activity in UT7<sub>EPO</sub> cells) and pharmacokinetic behavior (terminal  $t_{1/2}$  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- $\alpha$  was obtained from Ortho Biotech (Raritan, NJ) and darbepoetin- $\alpha$  (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.

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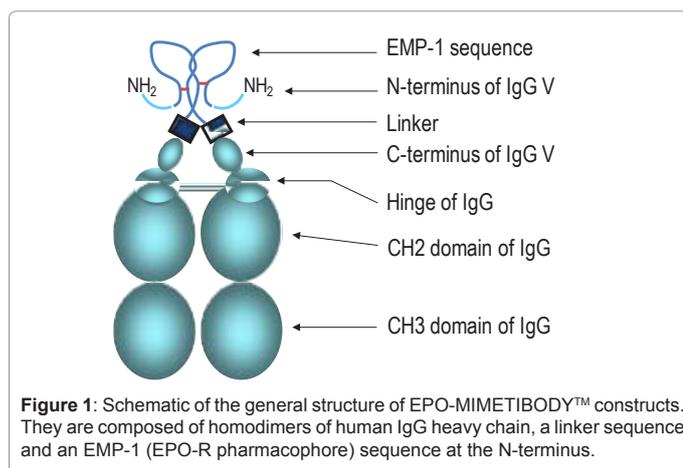
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## UT7 Assay

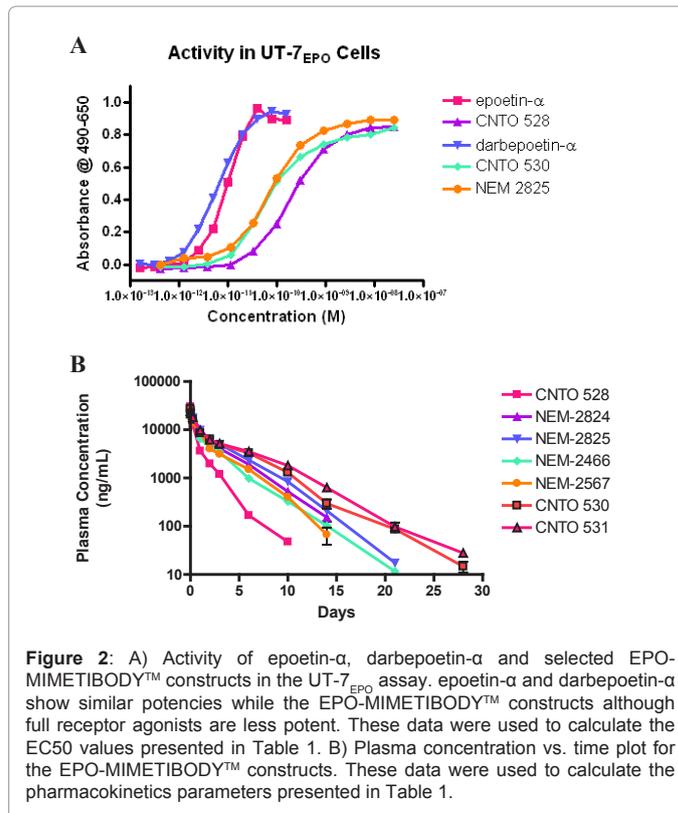
Bioactivity of EPO-R agonists was determined using a UT-7<sub>EPO</sub> cell proliferation assay. UT-7<sub>EPO</sub> cells are an EPO dependent subline of UT-7, a human megakaryoblastic leukemia cells [19]. Cells were washed thrice in PBS with final resuspension in Iscove's modified Dulbecco's medium supplemented with 2mM L-glutamine and 5% fetal bovine serum (I5Q) 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 I5Q. Cells were distributed at 30,000 cells per well in duplicate in a 96-well plate, EPO-R agonists were added and the plate incubated in 5% CO<sub>2</sub> at 37°C. After 48 h, 20 μL of MTS reagent (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, 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 (EC<sub>50</sub>).



Test Article	Fc	Mol Wt	EC <sub>50</sub> in UT-7 <sub>EPO</sub> Assay	Terminal t <sub>1/2</sub> (hr)	Cl (mL/day/kg)
Epoetin-α	NA	34 kDa	9.55 x 10 <sup>-12</sup>	2.5	425
Darbepoetin-α	NA	37 kDa	6.31 x 10 <sup>-12</sup>	6.9	115
CNTO 528	IgG1	62 kDa	2.08 x 10 <sup>-10</sup>	37.0	56.81
NEM-2824	IgG1ala-ala	58 kDa	8.00 x 10 <sup>-11</sup>	50.4	23.08
NEM-2825	IgG1ala-ala	58 kDa	7.69 x 10 <sup>-11</sup>	48.0	20.32
NEM-2466	IgG4ala-alaS>P	58 kDa	9.36 x 10 <sup>-11</sup>	53.3	30.01
NEM-2467	IgG4ala-alaS>P	58 kDa	1.01 x 10 <sup>-10</sup>	45.8	28.93
CNTO 530	IgG4ala-alaS>P	58 kDa	6.60 x 10 <sup>-11</sup>	72.7	19.00
CNTO 531	IgG4ala-alaS>P	58 kDa	8.27 x 10 <sup>-11</sup>	71.5	16.78

\*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.



**Figure 2:** A) Activity of epoetin-α, darbepoetin-α and selected EPO-MIMETIBODY™ constructs in the UT-7<sub>EPO</sub> 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 EC<sub>50</sub> 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 CO<sub>2</sub> and a target volume of 300 μL 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

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:

$$RBC(t_N) = ((RBC(t_{N-1}) + RTC(t_N)) \cdot SF)$$

where

$RBC(t_N)$  = Total RBC count on Day N

$RTC(t_N)$  = Reticulocyte count on Day N

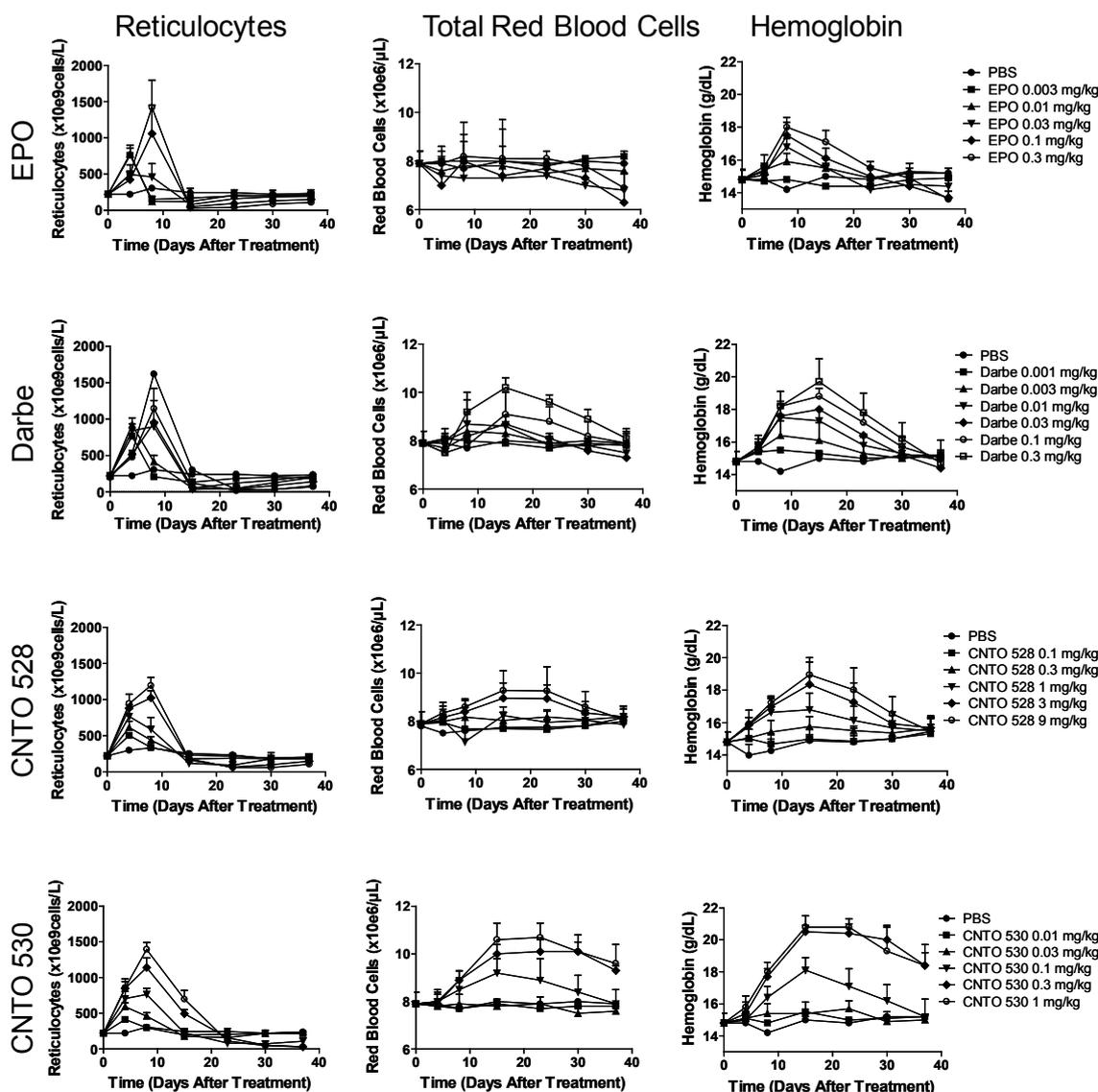
SF = surviving fraction in PBS treated rats

And,  $RBC(t_0)$  = Average RBC count in PBS treated rats on Day 4

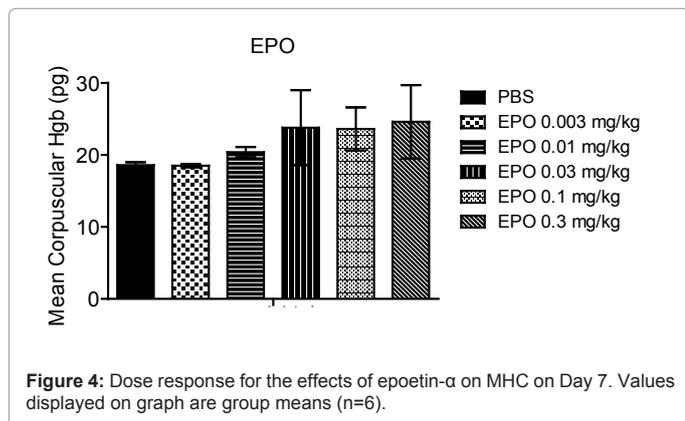
To test the hypothesis that the differential effects on conversion of RTC to RBC had practical significance, the area under the change in hemoglobin (Hgb) vs. time curves (Hgb AUC) was calculated. Hgb  $AUC_{(1-37)}$  was calculated for the change in Hgb between Days 1 and 37 (when the mean Hgb values for epoetin- $\alpha$  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:

$$Hgb\ AUC_{(1-37)} = a \cdot \log(\text{dose}) + b$$

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- $\alpha$ , darbepoetin- $\alpha$  and the EPO-MIMETIBODY™ constructs on RTC, RBC (not corrected for reticulocyte count) and Hgb. Values displayed on graph are group means (n=6).

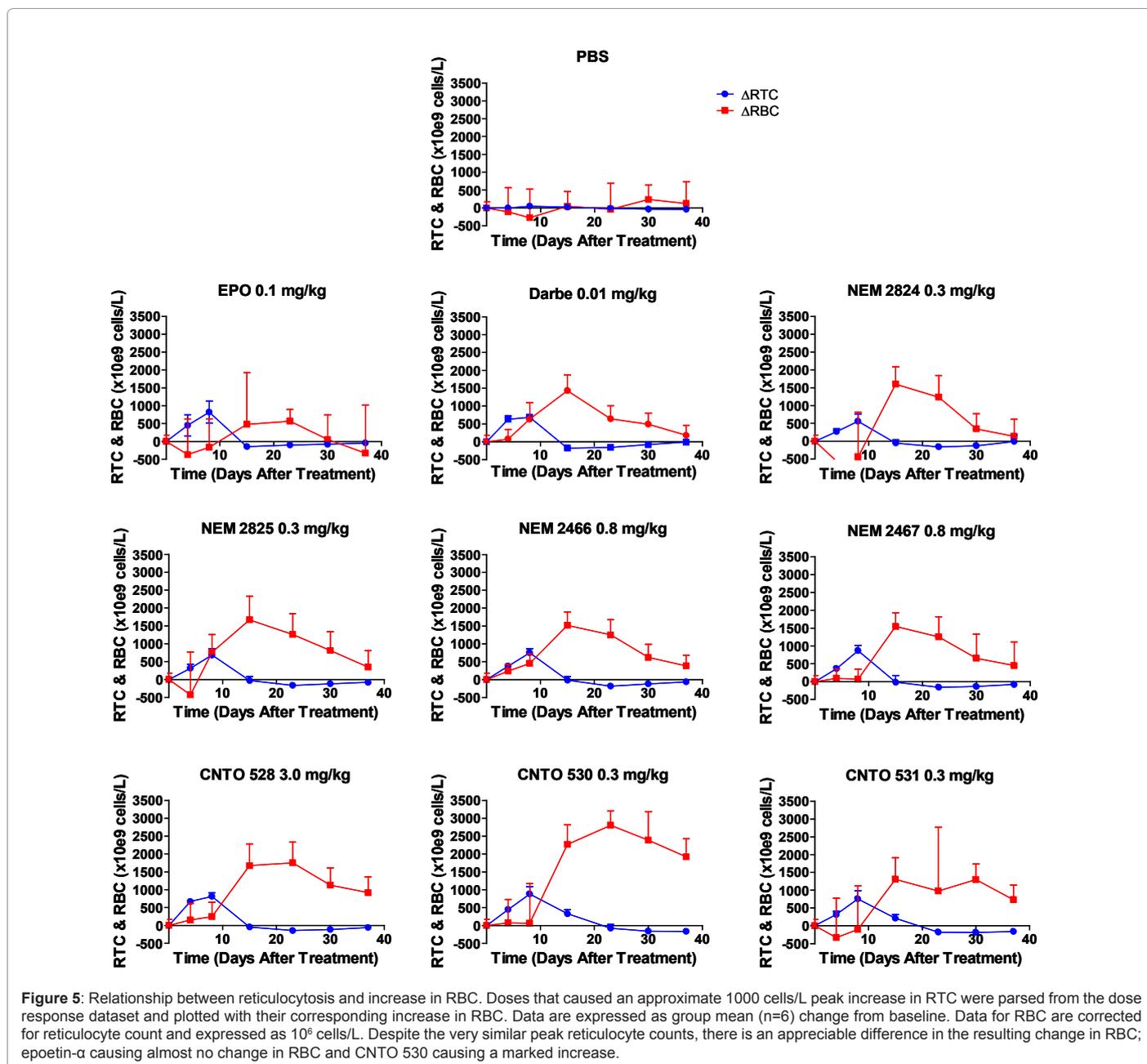


curves and the clearance of the EPO-R agonists were evaluated using the Spearman Rank Order correlation. P values less than 0.05 were accepted as significant.

## Results

The ability of the epoetin- $\alpha$ , darbepoetin- $\alpha$  and the EPO-MIMETIBODY™ constructs to activate EPO-R was studied in UT-7<sub>EPO</sub> cells. Representative data are shown in Figure 2a and the EC<sub>50</sub> values are presented in Table 1. All the EPO-MIMETIBODY™ constructs supported proliferation of UT-7<sub>EPO</sub> cells, albeit at as much as ~100 fold lower potency compared to epoetin- $\alpha$  and darbepoetin- $\alpha$ .

The results of the pharmacokinetic analysis of the EPO-MIMETIBODY™ constructs are shown in Figure 2b and the terminal t<sub>1/2</sub> and systemic clearance in Table 1. The EPO-MIMETIBODY™

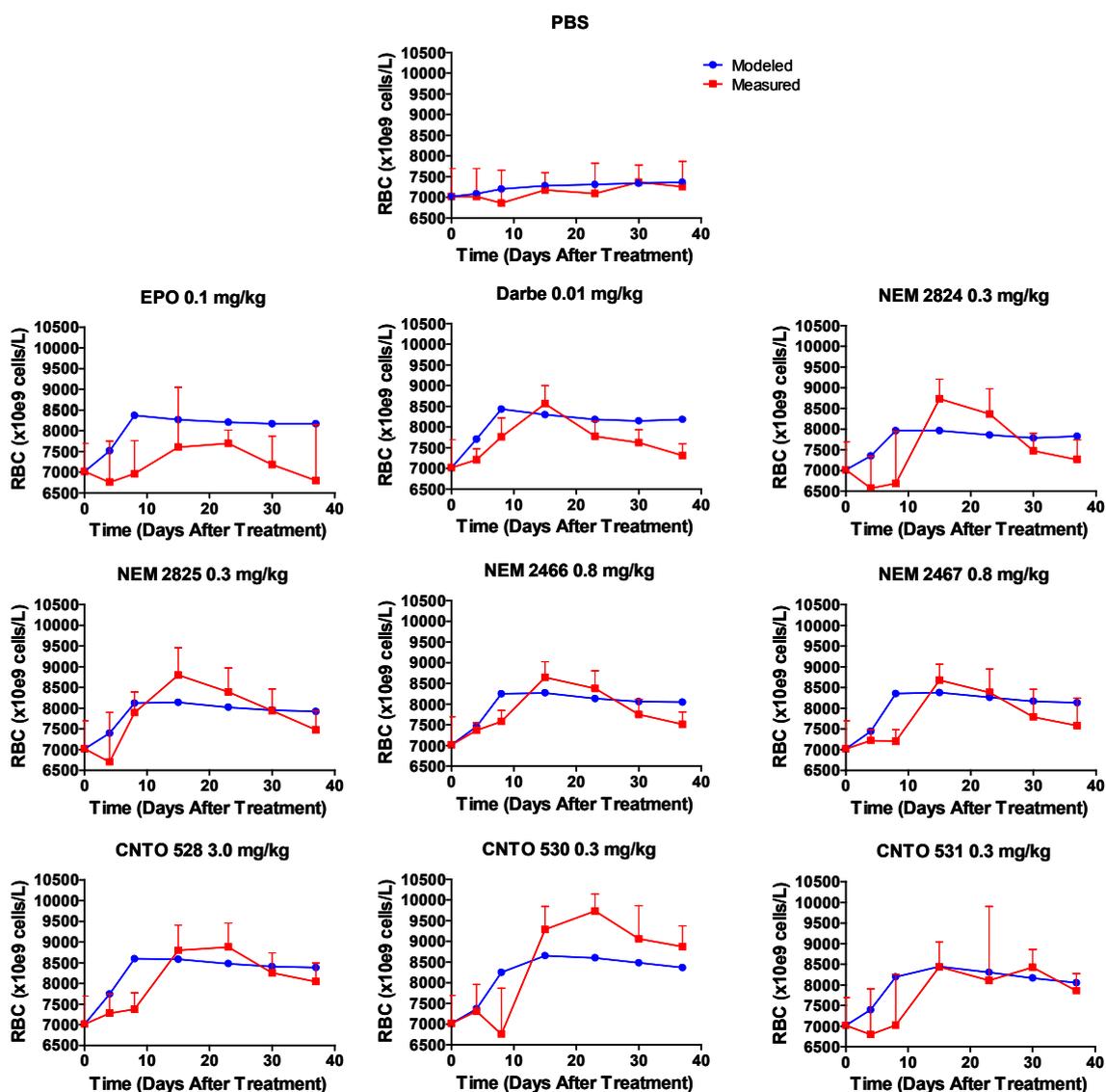


constructs showed as much as 10 fold longer  $t_{1/2}$  and lower clearance than epoetin- $\alpha$ .

To study the pharmacodynamic effects of epoetin- $\alpha$ , darbepoetin- $\alpha$  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- $\alpha$ , darbepoetin- $\alpha$ , 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- $\alpha$ , 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- $\alpha$  treated rats. The peak MHC on Day 7 is illustrated for epoetin- $\alpha$  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- $\alpha$ , 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- and darbepoetin- $\alpha$  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- $\alpha$  and darbepoetin- $\alpha$  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<sub>(1-37)</sub> for the complete dose response dataset plotted as a function of log<sub>10</sub> 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<sub>EPO</sub> cells are as follows: Epoetin- $\alpha$ , Darbepoetin- $\alpha$ , NEM-2466, CNTO 531, NEM-2824, NEM-2825, CNTO 530, CNTO 528 and NEM-2467. Interestingly, for in vivo effects on hemoglobin AUC<sub>(1-37)</sub> the order changes to CNTO 531, CNTO 530, NEM-2467, NEM-2466, NEM-2825, NEM-2824, CNTO 528, Darbepoetin- $\alpha$  and Epoetin- $\alpha$ .

## 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- $\alpha$  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<sub>(1-37)</sub> 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<sub>(1-37)</sub> and potency in the UT-7<sub>EPO</sub> assay. Taken together, these data suggest that maintaining the blood levels of the EPO-R agonist over the time required for maturation of RTC 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 <sup>125</sup>I-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 <sup>125</sup>I-EPO. More recently, Mihov et al. [27] were able to demonstrate low-level specific binding of <sup>125</sup>I-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 <sup>125</sup>I-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

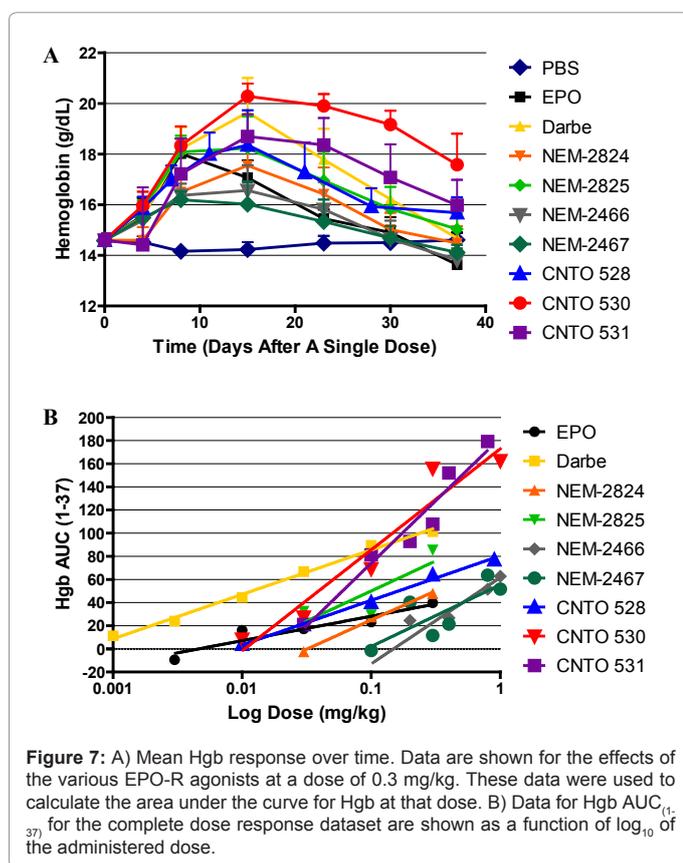


Figure 7: A) Mean Hgb response over time. Data are shown for the effects of the various EPO-R agonists at a dose of 0.3 mg/kg. These data were used to calculate the area under the curve for Hgb at that dose. B) Data for Hgb AUC<sub>(1-37)</sub> for the complete dose response dataset are shown as a function of log<sub>10</sub> of the administered dose.

Test Article	Cl mL/day/kg	a	b	R <sup>2</sup>
Epoetin- $\alpha$	507.84	9.2	49	0.89
Darbepoetin- $\alpha$	163.20	16.7	124	0.99
CNTO 528	56.81	16.7	80	0.99
NEM-2824	23.08	21.9	75	0.99
NEM-2825	20.32	22.6	102	0.68
NEM-2466	30.01	32.3	62	0.92
NEM-2467	28.93	23.1	55	0.68
CNTO 530	19.00	37.9	173	0.93
CNTO 531	16.78	47.0	182	0.95

Table 2: Hgb AUC<sub>(1-37)</sub> Regression Analysis (Hgb AUC<sub>(1-37)</sub> = a\*log(dose)+b).

who showed EPO can influence of a variety of physiologic functions in RTC and RBC: inhibition of  $\text{Ca}^{2+}$ -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 proteasome/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 a 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 (PLSCR1) [43] that is activated by increased cytosolic free  $\text{Ca}^{2+}$  to redistribute PS between the leaflets in proteoliposomes. Of interest, the  $\text{Ca}^{2+}$  ionophore A23187 has been shown to mediate exposure of PS on RBC [44], EPO has been shown to inhibit  $\text{Ca}^{2+}$ -permeable cation channels, thus decreasing cytosolic free  $\text{Ca}^{2+}$  [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.

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## References

1. Chen K, Liu J, Heck S, Chasis JA, An X, Mohandas N. (2009) Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. *Proc Natl Acad Sci U S A* 106: 17413-17418.

2. Koury MJ, Sawyer ST, Brandt SJ (2002) New insights into erythropoiesis. *Current Opinion in Hematology* 9: 93-100.
3. Fried W (2009) Erythropoietin and erythropoiesis. *Experimental Hematology* 37: 1007-1015.
4. Wen D, Boissel JP, Tracy TE, Gruninger RH, Mulcahy LS, et al. (1993) Erythropoietin structure-function relationships: high degree of sequence homology among mammals. *Blood* 82: 1507-1516.
5. Wojchowski DM, Sathyanarayana P, Dev A (2010) Erythropoietin receptor response circuits. *Curr Opin Hematol* 17: 169-176.
6. Pearson PL, Smith TP, Sonstegard TS, Klemcke HG, Christenson RK, et al. (2000) Porcine erythropoietin receptor: molecular cloning and expression in embryonic and fetal liver. *Domest Anim Endocrinol* 19: 25-38.
7. Lacombe C, Mayeux P (1999) The molecular biology of erythropoietin. *Nephrology Dialysis and Transplantation* 14: 22-28.
8. Livnah O, Johnson D, Stura E, Farrell F, Barbone F, et al. (1998) An antagonist peptide-EPO receptor complex suggests that receptor dimerization is not sufficient for activation. *Nat Struct Biol* 5: 993-1004.
9. Ratajczak J, Majka M, Kijowski J, Baj M, Pan Z, et al. (2001) Biological significance of MAPK, AKT and JAK-STAT protein activation by various erythropoietic factors in normal human early erythroid cells. *Br J Haematol* 115: 195-204.
10. Verdier F, Walrafen P, Hubert N, Chretien S, Gisselbrecht S, et al. (2000) Proteasomes regulate the duration of erythropoietin receptor activation by controlling down-regulation of cell surface receptors. *J Biol Chem* 275: 18375-18381.
11. Grossi A, Vannucchi AM, Bacci P, Caporale R, Cappelli G, et al. (1998) Erythropoietin upregulates the expression of its own receptor in TF-1 cell line. *Leuk Res* 22: 145-151.
12. Testa U (2004) Apoptotic mechanisms in the control of erythropoiesis. *Leukemia* 18: 1176-1199.
13. Bugelski PJ, Capocasale RJ, Makropoulos D, Marshall D, Fisher PW, et al. (2008) CNTO 530: molecular pharmacology in human UT-7EPO cells and pharmacokinetics and pharmacodynamics in mice. *J Biotechnol* 134: 171-180.
14. Bugelski PJ, Nesspor T, Volk A, O'Brien J, Makropoulos D, et al. (2008) Pharmacodynamics of recombinant human erythropoietin in murine bone marrow. *Pharm Res* 25: 369-378.
15. Alfrey CP, Rice L, Udden MM, Driscoll TB (1997) Neocytolysis: physiological down-regulator of red-cell mass. *Lancet* 349: 1389-1390.
16. Achuthanandam R, Makropoulos D, Johns L, Volk A, Brosnan K, et al. (2011) Pharmacodynamics of CNTO 530 and darbepoetin- $\alpha$  in human TNF- $\alpha$  transgenic mice, a murine model of anemia of chronic disease. *Pharmacology Pharmacy* 2: 63-76.
17. Johnson D, Farrell F, Barbone F, McMahon F, Tullai J, et al. (1998) Identification of a 13 amino acid peptide mimetic of erythropoietin and description of amino acids critical for the mimetic activity of EMP1. *Biochemistry* 37: 3699-3710.
18. Bouman-Thio E, Franson K, Miller B, Getsy J, Cohen A, et al. (2008) A phase I, single and fractionated, ascending-dose study evaluating the safety, pharmacokinetics, pharmacodynamics, and immunogenicity of an erythropoietin mimetic antibody fusion protein (CNTO 528) in healthy male subjects. *J Clin Pharmacol* 48: 1197-1207.
19. Komatsu N, Yamamoto M, Fujita H, Miwa A, Hatake K, et al. (1993) Establishment and characterization of an erythropoietin-dependent subline, UT-7/Epo, derived from human leukemia cell line, UT-7. *Blood* 82: 456-464.
20. Kliwinski C, Makropoulos D, Kwok D, Volk A, Foster K, et al. (2010) Pharmacokinetics and pharmacodynamics of an EPO-mimetic fusion protein in a model of chronic renal insufficiency anemia. *Open Hematology Journal* 4: 17-20.
21. Landschulz KT, Noyes AN, Rogers O, Boyer SH (1989) Erythropoietin receptors on murine erythroid colony-forming units: natural history. *Blood* 73: 1476-1486.
22. Fraser JK, Lin FK, Berridge MV (1988) Expression of high affinity receptors for erythropoietin on human bone marrow cells and on the human erythroleukemic cell line, HEL. *Exp Hematol* 16: 836-842.

23. Broudy VC, Lin N, Brice M, Nakamoto B, Papayannopoulou T (1991) Erythropoietin receptor characteristics on primary human erythroid cells. *Blood* 77: 2583-2590.
24. Baciú I, Ivanof L, Pavel T, Marina C, Zirbo M, et al. (1985) Erythropoietin binding to the red cell membranes. *Physiologie* 22: 227-231.
25. Akahane K, Tojo A, Fukamachi H, Kitamura T, Saito T, et al. (1989) Binding of iodinated erythropoietin to rat bone marrow cells under normal and anemic conditions. *Exp Hematol* 17: 177-182.
26. Wickrema A, Krantz SB, Winkelmann JC, Bondurant MC (1992) Differentiation and erythropoietin receptor gene expression in human erythroid progenitor cells. *Blood* 80: 1940-1949.
27. Mihov D, Vogel J, Gassmann M, Bogdanova A (2009) Erythropoietin activates nitric oxide synthase in murine erythrocytes. *Am J Physiol Cell Physiol* 297: C378-388.
28. Myssina S, Huber SM, Birka C, Lang PA, Lang KS, et al. (2003) Inhibition of erythrocyte cation channels by erythropoietin. *J Am Soc Nephrol* 14: 2750-2757.
29. Chakraborty M, Ghosal J, Biswas T, Datta AG (1986) Effect of erythropoietin on the different ATPases and acetylcholinesterase of rat RBC membrane. *Biochem Med Metab Biol* 36: 231-238.
30. Lawrence WD, Davis PJ, Blas SD (1987) Action of erythropoietin in vitro on rabbit reticulocyte membrane Ca<sup>2+</sup>-ATPase activity. *J Clin Invest* 80: 586-589.
31. Ghosal J, Chakraborty M, Biswas T, Ganguly CK, Datta AG (1987) Effect of erythropoietin on the glucose transport of rat erythrocytes and bone marrow cells. *Biochem Med Metab Biol* 38: 134-141.
32. Chakraborty M, Ghosal J, Biswas T, Datta AG (1988) Effect of erythropoietin on membrane lipid peroxidation, superoxide dismutase, catalase, and glutathione peroxidase of rat RBC. *Biochem Med Metab Biol* 40: 8-18.
33. Lang F, Gulbins E, Lang PA, Zappulla D, Foller M (2010) Ceramide in suicidal death of erythrocytes. *Cell Physiol Biochem* 26: 21-28.
34. Koury M, Koury S, Kopsombut P, Bondurant M (2005) In vitro maturation of nascent reticulocytes to erythrocytes. *Blood* 105: 2168-2174.
35. Brines M (2010) The therapeutic potential of erythropoiesis-stimulating agents for tissue protection: a tale of two receptors. *Blood Purif* 29: 86-92.
36. Chong ZZ, Kang J-Q, Maiese K (2002) Hematopoietic factor erythropoietin fosters neuroprotection through novel signal transduction cascades. *J Cereb Blood Flow Metab* 22:503-514.
37. Leventis PA, Grinstein S (2010) The distribution and function of phosphatidylserine in cellular membranes. *Annu Rev Biophys* 39: 407-427.
38. Bevers EM, Williamson PL (2010) Phospholipid scramblase: an update. *FEBS Lett* 584: 2724-2730.
39. Sahu SK, Gummadi SN, Manoj N, Aradhyam GK (2007) Phospholipid scramblases: an overview. *Arch Biochem Biophys* 462: 103-114.
40. Wu Y, Tibrewal N, Birge RB (2006) Phosphatidylserine recognition by phagocytes: a view to a kill. *Trends Cell Biol* 16: 189-197.
41. Bosman GJCGM, Willekens FLA, Werre JM (2005) Erythrocyte aging: a more than superficial resemblance to apoptosis? *Cell Physiol Biochem* 16: 1-8.
42. Kuypers FA, de Jong K (2004) The role of phosphatidylserine in recognition and removal of erythrocytes. *Cell Mol Biol (Noisy-le-grand)* 50: 147-158.
43. Basse F, Stout J, Sims PJ, Weidmer T (1996) Isolation of an erythrocyte membrane protein that mediates Ca<sup>2+</sup>-dependent transbilayer movement of phospholipid. *J Biol Chem* 271: 17205-17210.
44. Balasubramanian K, Mirnikjoo B, Schroit AJ (2007) Regulated externalization of phosphatidylserine at the cell surface: implications for apoptosis. *J Biol Chem* 282: 18357-18364.
45. Föller M, Kasinathan RS, Koka S, Huber SM, Schuler B, et al. (2007) Enhanced susceptibility to suicidal death of erythrocytes from transgenic mice overexpressing erythropoietin. *Am J Physiol Regul Integr Comp Physiol*. 293: R1127-1134.
46. Egrie JC, Dwyer E, Browne JK, Hitz A, Lykos MA (2003) Darbepoetin alfa has a longer circulating half-life and greater in vivo potency than recombinant human erythropoietin. *Exp Hematol* 31: 290-299.