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Differential pharmacokinetic analysis of *in vivo* erythropoietin receptor interaction with erythropoietin and continuous erythropoietin receptor activator in sheep

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Abstract

The two erythropoiesis stimulating agents (ESAs), short acting recombinant human erythropoietin (EPO) and long acting continuous erythropoietin receptor activator (CERA), have been hypothesized to share an *in vivo* elimination pathway that involves binding to erythropoietin receptor (EPOR) and subsequent internalization. A physiologically based recirculation model and a pharmacokinetic tracer interaction methodology (TIM) were used to compare the *in vivo* interaction kinetics with EPOR between the two ESAs in adult sheep. Animals treated with EPO experienced a greater EPOR up-regulation than those treated with CERA, as evidenced by an eightfold-higher initial EPOR normalized production rate constant, k_{syn}/R_0 , versus a twofold-larger EPOR degradation rate constant, k_{deg} . In agreement with *in vitro* studies, EPO had a lower *in vivo* equilibrium dissociation constant from EPOR than CERA ($K_D = 6$ versus 88.4 pmol/l, respectively, $p < 0.01$). The internalization and/or degradation of the EPO–EPOR complex was faster than that of the CERA–EPOR complex ($k_{\text{int}} = 24$ versus 2.41 h^{−1}, respectively, $p < 0.01$). The adopted model enables a mechanism-based explanation for CERA's slower elimination and greater erythropoietic activity *in vivo*. As predicted by the model, the slower elimination of CERA is due to: (1) less EPOR up-regulation induced by CERA administration; (2) slower binding of CERA to EPOR; and (3) reduced internalization and/or degradation rate of surface-bound CERA. Slower CERA/EPOR complex elimination explains the greater *in vivo* erythropoiesis reported for CERA, despite its lower affinity to EPOR. A sensitivity analysis showed that the model parameters were reliably estimated using the TIM methodology.

Keywords

erythropoietin; erythropoiesis stimulating agent (ESA); CERA; pharmacokinetics; receptor; physiological model; receptor binding; recirculation model

Introduction

Erythropoietin (EPO) is a 34 kD glycoprotein hormone that regulates erythropoiesis. It binds to cell surface receptors (EPOR) on bone marrow erythroid progenitor cells (burst-forming unit-erythroids and colony-forming unit-erythroids), promoting their proliferation and differentiation into mature red blood cells (RBC) and protecting them from apoptosis [1,2]. Binding of EPO to the cell surface induces dimerization of two EPOR molecules, which, in

turn, initiates the Janus kinase intracellular signal transduction required for erythropoiesis [3]. Simultaneously, activation of EPOR by EPO binding promotes degradation of the formed complex via two pathways [4]: (1) proteolysis by proteosomes at the cell surface; and (2) internalization followed by targeting to lysosomes. On a macroscopic level, receptor-mediated elimination is responsible for the nonlinear clearance of EPO that is observed in various animal species [5–8], including humans [9].

Continuous erythropoietin receptor activator (CERA) is a novel erythropoiesis stimulating agent (ESA). Unlike EPO, CERA contains methoxy polyethylene glycol chains linked to amino groups via amide bonds, resulting in a molecule weighing approximately 60 kD [10]. Studies in humans have shown that CERA has a long half-life (approximately 130 h) [11] and is able to correct anemia and maintain a stable hemoglobin level for extended dosing intervals. These features allow the direct conversion of patients with chronic kidney disease from shorter frequency (three times a week to one time every second week) EPO treatment to once a month CERA administration [12,13].

Preclinical studies in animal models demonstrated that CERA has slower systemic clearance, longer elimination half-life and higher *in vivo* erythropoietic activity than EPO [14,15]. The differences in pharmacokinetic (PK) and pharmacodynamic (PD) properties of EPO and CERA have been hypothesized to be attributed to a unique interaction between CERA and EPOR, which involves a slower association, but a slightly faster dissociation, than those observed between EPOR and EPO. However, this hypothesis was based solely on *in vitro* binding studies that reported 50 to 100-fold lower affinity of CERA for EPOR binding sites [16].

In order to elucidate the mechanism behind the significantly slower elimination and prolonged activity of CERA *in vivo*, the interactions between EPOR and both EPO and CERA were studied in adult sheep using a sensitive PK methodology, the tracer interaction method (TIM) [17], and a receptor-based physiological recirculation model. The model incorporates parameters for drug-receptor binding, receptor turnover and drug-receptor complex endocytosis. The modeling is done within a physiological modeling framework that considers heterogeneous tissues connected through a recirculatory vascular network. A sensitivity analysis was performed to investigate the reliability of the parameters estimation.

Materials and Methods

Animals

All animal care and experimental procedures were approved by the University of Iowa Institutional Animal Care and Use Committee and adhere to the ‘Principles of Laboratory Animal Care’ (NIH publication 85–23, revised in 1985). Seven healthy young adult sheep, 2–4 months old, 24.9 ± 3.5 kg (mean $\pm$ SD), were selected for the EPO experiments, and five sheep, 2–4 months old, 28.4 ± 2.44 kg, were used for the CERA experiments. The animals were housed in an indoor, light- and temperature-controlled environment, with *ad libitum* access to feed and water. Prior to study initiation, jugular venous catheters were aseptically placed under pentobarbital anesthesia. Intravenous ampicillin (1 g) was administered daily for 3 days following catheter placement. The long-term infusion of CERA required in the TIM experiments was done with a portable infusion pump (Pegasus infusion pump, Instech/Solomon Laboratories Plymouth Meeting, PA, USA) mounted on a specially designed sheep jacket.

Study protocol

A detailed description of the theory and principles of the TIM was published previously [17]. In the current study, each animal underwent a single TIM PK experiment. A single i.v.

bolus dose of ^{125}I -labeled drug (465 mU/kg for EPO and 50 mU/kg for CERA) was initially administered, then immediately followed by a constant rate infusion of the tracer (117 mU/h/kg for EPO and 1.6 mU/h/kg for CERA). An i.v. bolus injection of the non-tracer (94.8 U/kg for EPO and 104 U/kg for CERA) was administered when the plasma level of the tracer approached steady state (i.e. at approximately 4 h for EPO and 20 h for CERA). Blood samples (0.5 ml per sample) were collected subsequent to the tracer and the non-tracer administrations for determination of plasma EPO and plasma CERA levels. For the tracer, about 38 samples were collected for EPO from 0 to 14 h, while about 50 samples were collected for CERA from 0 to 85 h. For the non-tracer, 24 samples were collected for EPO from 4 to 14 h, while 30 samples were collected for CERA from 20 to 85 h. The longer sampling period for CERA was required due to the slower elimination of CERA. To minimize hemoglobin and red cell loss due to frequent blood sampling, blood was centrifuged, the plasma removed and the red cells re-infused.

Assay

Radiolabeled drug—Radiolabeled EPO (^{125}I -EPO) and radiolabeled CERA (^{125}I -CERA) were prepared by iodination of 1 μg of the drug with 1 mCi of ^{125}I Na (Perkin Elmer, Billerica, MA, USA) using 8 μg chloramine-T (Sigma-Aldrich, St Louis, MO, USA) in a 10 s reaction that was terminated with 24 μg sodium metabisulfite (Sigma-Aldrich, St Louis, MO, USA). This reaction mixture was desalted on a Sephadex 25 column (PD10, GE Healthcare Biosciences Corp., Piscataway, NJ, USA) and stored at 4 °C before final purification on a size separation column (Sephadex g100, GE Healthcare Biosciences Corp.). The radiolabeled drug was measured in plasma samples or infusion solution by non-specific protein precipitation followed by separation by centrifugation. Specifically 1 ml duplicate aliquots of plasma sample or infusion solution were added to 12 $\times$ 75 glass test tubes, 1 ml 10% w/v trichloro-acetic acid (TCA) is added, the precipitated proteins were pelleted by a 30 min centrifugation, the supernatant was decanted and the pellet was counted for radioactivity.

Unlabeled drug—Plasma EPO concentrations were measured in triplicate using a double-antibody radioimmunoassay (RIA) procedure (lower limit of quantitation 1 mU/ml) [18]. All samples from the same animal were measured in the same assay to reduce variability.

CERA was provided as 5.9 mg of protein/ml solution (Lot No. R78238600, Hoffmann-LaRoche Inc.), and was stored at $-70\text{ }^{\circ}\text{C}$. This stock was used to prepare working stocks (in 50 mM sodium phosphate with 0.02% sodium azide and 5% BSA, pH 7.4) at a concentration of 0.14 mg of protein/ml. Preparation and analysis of the unknown CERA plasma, standard curve, non-specific binding and zero standard samples was identical to the unlabeled EPO determination. The unknown CERA concentrations were determined using the EPO standard curve between the EPO EC_{80} and EC_{20} as mU EPO equivalents/ml. The EPO standard curve was used instead of CERA standard curve for convenience and our extensive experience with the EPO RIA. The use of the EPO standard curve to measure CERA was validated by performing 1:2 dilutions of the CERA stock solution until the response was between the EC_{80} and EC_{20} on the EPO standard curve. The dilution corrected responses were determined and had a CV of 8.3% across the linear range of the EPO standard curve, demonstrating a 1-to-1 relationship between the determined mU EPO equivalents/ml and the ng/ml of CERA within this range. These validations resulted in a CERA conversion constant of 71300 mU/ μg of protein ($n = 13$). All unknown CERA samples were measured in duplicate or triplicate and diluted between the EC_{80} and EC_{20} on the EPO standard curve, which corresponds to a linear CERA range of 65.5–647 pg of protein/ml.

Pharmacokinetic analysis

A previously introduced receptor-based recirculation PK model [19] was adopted to analyse the kinetics of EPO and CERA using tracer data and the TIM methodology [17]. Briefly, the model consists of three tissues, heart-lung segment (HLS), non-target mediated disposition (NTM) and target mediated disposition (TM), interconnected by arteries and veins (Figure1). The cardiac output is given by Q , and P_i is the fraction of blood flow to i th tissue for $i = \text{TM or NTM}$ ($P_{\text{TM}} + P_{\text{NTM}} = 1$). Drug disposition in the HLS and NTM tissues was assumed to be linear. Therefore, the output rates from both tissues ($f_{\text{out}}^i(t)$, for $i = \text{HLS or NTM}$) are described by the following convolution equations (* denotes convolution) that relate the input and output rates for a given tissue to a unit impulse response function ($UIR(t)$):

$$f_{\text{out}}^{\text{HLS}}(t) = [D_{\text{iv}}\delta(t) + \text{Inf}(t) + f_{\text{out}}^{\text{NTM}}(t) + f_{\text{out}}^{\text{TM}}(t)] * UIR(t) \quad (1)$$

$$f_{\text{out}}^{\text{NTM}}(t) = P_{\text{NTM}} f_{\text{out}}^{\text{HLS}}(t) * UIR(t) \quad (2)$$

where, $D_{\text{iv}}\delta(t)$ and $\text{Inf}(t)$ are i.v. bolus dose and constant rate infusion of the tracer, respectively.

Assuming both HLS and NTM tissues are non-extracting, the $UIR(t)$ function reduces to a transit time density function ($g(t)$) that was nonparametrically described as a biexponential function:

$$UIR(t) = g(t) = \lambda \alpha \exp(-\alpha t) + (1-\lambda)\beta \exp(-\beta t), \quad 0 \leq \lambda \leq 1 \quad (3)$$

Nonlinear drug disposition through TM tissues was considered by a receptor-based model (Figure 2) that follows the generalized target mediated drug disposition model [20] and its quasi-equilibrium approximation [21]. Accordingly, the interaction between drug input concentration ($C_{\text{in}}^{\text{TM}}(t)$) and free EPOR in the extracellular space (R), occurs with a second-order rate constant (k_{on}), to form a Drug-EPOR complex (R^*), which dissociates at a first-order rate constant (k_{off}), or is internalized into the intracellular space with a first-order rate constant (k_{int}), and then subsequently degraded by lysosomes. The EPOR is assumed to be produced at a zero-order rate constant (k_{syn}) and degraded at a first-order rate constant (k_{deg}) (Figure 2).

Quasi-equilibrium conditions, where binding and de-binding of the drug with receptor are much faster than the other processes of the system, result in:

$$\frac{RC_{\text{in}}^{\text{TM}}(t)}{R^*} = \frac{(R_{\text{tot}} - R^*)C_{\text{in}}^{\text{TM}}(t)}{R^*} = K_D \quad (4)$$

where, R_{tot} is total EPOR, ($R_{\text{tot}} = R + R^*$), K_D is drug/EPOR equilibrium dissociation constant ($K_D = k_{\text{off}}/k_{\text{on}}$).

Rearranging Equation (4) for R^* and normalizing the resulting equation by initial total EPOR (R_0) gives:

$$R^*/R_0 = \frac{R_{\text{tot}}/R_0 C_{\text{in}}^{\text{TM}}(t)}{K_D + C_{\text{in}}^{\text{TM}}(t)} \quad (5)$$

The changes in R and R^* were added to obtain the change in R_{tot} and the resulting equation was normalized by R_0 :

$$\frac{dR_{\text{tot}}}{dt}/R_0 = k_{\text{syn}}/R_0 - k_{\text{deg}}R_{\text{tot}}/R_0 + (k_{\text{deg}} - k_{\text{int}})R^*/R_0, \quad R_{\text{tot}}(0)/R_0 = 1 \quad (6)$$

The normalization in Equations (5) and (6) is necessary because R_0 cannot be estimated from drug plasma concentrations in this study.

To establish a link between the receptor-based model in TM tissues and the rest of the recirculation model, a receptor-dependent extraction function, $E_{\text{TM}}(t)$, was defined as:

$$E_{\text{TM}}(t) = \frac{R^*/R_0}{C_{\text{in}}^{\text{TM}}(t)}, \quad 0 \leq E_{\text{TM}}(t) \leq 1 \quad (7)$$

where, $C_{\text{in}}^{\text{TM}}(t)$ is given by:

$$C_{\text{in}}^{\text{TM}}(t) = P_{\text{TM}} f_{\text{out}}^{\text{HLS}}(t) / P_{\text{TM}} Q = f_{\text{out}}^{\text{HLS}} / Q \quad (8)$$

Assuming that distribution equilibrium is achieved rapidly between the tissues and the emerging venous blood the output rate from TM tissues is given by:

$$f_{\text{out}}^{\text{TM}}(t) = P_{\text{TM}} (1 - E_{\text{TM}}(t)) f_{\text{out}}^{\text{HLS}}(t) \quad (9)$$

Finally, the total plasma tracer concentration that is measured in the venous blood ($C_{\text{out}}^{\text{tot}}(t)$) is given by:

$$C_{\text{out}}^{\text{tot}}(t) = (f_{\text{out}}^{\text{NTM}}(t) + f_{\text{out}}^{\text{TM}}(t)) / Q \quad (10)$$

Equations (1) to (3) were conveniently converted to the following equivalent equations (Eq. 11–16). The latter six equations are simpler to deal with numerically and computationally because they include first-order differential equations and avoid the convolution operator.

$$dy_1/dt = -\alpha y_1 + \lambda \alpha (Inf(t) + f_{\text{out}}^{\text{NTM}}(t) + f_{\text{out}}^{\text{TM}}(t)), y_1(0) = D_{\text{iv}} \lambda \alpha \quad (11)$$

$$dy_2/dt = -\beta y_2 + (1 - \lambda) \beta (Inf(t) + f_{\text{out}}^{\text{NTM}}(t) + f_{\text{out}}^{\text{TM}}(t)), y_2(0) = D_{\text{iv}} (1 - \lambda) \beta \quad (12)$$

$$f_{\text{out}}^{\text{HLS}}(t) = y_1 + y_2 \quad (13)$$

$$dy_3/dt = -\alpha y_3 + P_{\text{NTM}} \lambda \alpha f_{\text{out}}^{\text{HLS}}(t), y_3(0) = 0 \quad (14)$$

$$dy_4/dt = -\beta y_4 + P_{\text{NTM}} (1 - \lambda) \beta f_{\text{out}}^{\text{HLS}}(t), y_4(0) = 0 \quad (15)$$

$$f_{\text{out}}^{\text{NTM}}(t) = y_3 + y_4 \quad (16)$$

Equation (5) was modified to account for the TIM procedure:

$$R^*/R_0 = \begin{cases} \frac{R_{\text{tot}}/R_0 C_{\text{in}}^{\text{TM}}(t)}{K_D + C_{\text{in}}^{\text{TM}}(t)} & \text{for } 0 < t \leq t_{\text{cold}} \\ \frac{R_{\text{tot}}/R_0 C_{\text{in}}^{\text{TM}}(t)}{K_D + C_{\text{cold}}^{\text{TM}}(t)} & \text{for } t > t_{\text{cold}} \end{cases} \quad (17)$$

where, $c_{\text{cold}}(t)$ is plasma non-tracer concentrations represented as a cross-validation cubic spline [22] when fitting the model to tracer data and t_{cold} is time of injection of the non-tracer.

To improve the estimation of the parameters, the sheep physiological parameters, Q and P_{TM} , were fixed to values drawn from literature [23], with $Q = 7122.7$ ml/h/kg, and P_{TM} was assumed to be equal to the fraction of blood flow to the bone marrow (0.0597).

The numerical solution for Equation (10) was individually fitted to sheep tracer data for the pre- ($t < t_{\text{cold}}$) and post- ($t > t_{\text{cold}}$) unlabeled drug administration phases using WINFUNFIT, an interactive Microsoft Windows program evolved from the general non-linear regression program FUNFIT [24]. The ratio of maximum unlabeled drug concentration to equilibrium dissociation constant, K_D , was calculated and taken as a nonlinearity index (NLI). Statistical comparisons of mean values for the TIM receptor-related PK parameters (Table 1) between EPO and CERA were done using a one-tailed Student's t -test with correction for non-equal variance, if needed, after testing by an F-ratio test. Values of p of 0.05 and 0.01 were taken as the levels of significance for the type I null hypotheses error. All statistical tests were done using SAS/STAT (SAS Institute Inc., Cary, NC).

WINFUNFIT was used to simulate bound EPOR (R^*)-to-initial EPOR (R_0) concentration ratio profiles in EPO and CERA treatment groups. The simulations were performed using the mean parameter estimates of both drugs, and the physiological parameters of a sheep animal weighing 30 kg. The input was assumed to be an i.v. bolus dose of 100 pmol/kg.

Sensitivity analysis

A sensitivity analysis was implemented to evaluate the influence of each model parameter on the predicted plasma concentration–time profile. The model parameters investigated included the transit time density function parameters; α , β , λ , and receptor-related parameters; k_{syn}/R_0 , k_{deg} , K_D and k_{int} .

Sensitivity function ($S(t)$) was obtained using the partial derivatives of predicted plasma concentration–time profile with respect to model parameters [25,26]. The $S(t)$ was calculated using the central difference method:

$$S(t) = \frac{\partial C(\theta_i, t)}{\partial \theta_i} \cong \frac{C(\theta_i + \Delta\theta_i, t) - C(\theta_i - \Delta\theta_i, t)}{2\Delta\theta_i}$$

where $C(\theta_i, t)$ is the total plasma tracer concentration predicted at the i th parameter θ_i and time t . $\Delta\theta_i$ is the change in parameter θ_i (5% was used for all parameters). While parameter θ_i was investigated, all other parameters were held constant. The log-normalized $S(t)$:

$$NS(t) \frac{\partial \ln C(\theta_i, t)}{\partial \ln \theta_i} \cong S(t) \cdot \frac{\theta_i}{C(\theta_i, t)}$$

determines the relative change in $C(\theta_i, t)$ caused by a small relative change in θ_i [25,26]. A $NS(t)$ that is near zero indicates that the corresponding θ_i has only little influence on the plasma concentration response. A θ_i that has $NS(t)$ further away from zero, in the positive or the negative side, is associated with a greater influence on $C(\theta_i, t)$. Clewell *et al.* [25] has defined cut-off values for high, medium and low normalized sensitivity values to be: >0.5 , $0.2-0.5$ and <0.2 , respectively. A negative $NS(t)$ indicates a decrease in $C(\theta_i, t)$ as a result of an increase in θ_i , and vice versa. A positive $NS(t)$ occurs when both θ_i and $C(\theta_i, t)$ increase, or decrease together.

All simulations were performed using WIN-FUNFIT [24] at the experimental conditions for CERA, including time range, tracer bolus dose, tracer infusion rate and time of non-tracer bolus input. The nominal parameter values were the mean values of CERA parameter estimates.

Results

Pharmacokinetic analysis

The elimination patterns of both EPO (Figure 3) and CERA (Figure 4) were nonlinear, as evidenced by the significant perturbation in plasma tracer level (square symbols) caused by the injection of the non-tracer bolus (triangle symbols). This pronounced phenomenon was observed consistently in all animals. The plasma tracer concentrations were adequately described by the receptor-based recirculation model, as indicated by mean correlation coefficients of 0.984 and 0.993 for the fits of EPO and CERA data, respectively. This conclusion was supported by strong correlations between predicted and observed plasma tracer concentrations in both EPO and CERA treatment populations (Figure 5).

Receptor-related parameters obtained from the model fittings are summarized in Table 1. The inter-individual coefficients of variation (CV) of all parameter estimates were less than 33%, suggesting small variability between subjects. EPO and CERA had significantly different parameter values ($p < 0.05$). The most extreme difference was observed for the equilibrium dissociation constant (K_D), where the mean value was 15-fold larger for CERA than for EPO. The nonlinearity index (NLI), the initial total EPOR-normalized production rate constant (k_{syn}/R_0), and the internalization rate constant of the drug/EPOR complex (k_{int}), were approximately 7.5-, 8- and 10-fold higher for EPO than for CERA, respectively. The EPOR degradation rate constant (k_{deg}) was the most similar for the two compounds with a mean value that was about 1.5-fold larger for EPO than for CERA. The EPOR underwent faster elimination in the presence of EPO than in the presence of CERA, as indicated by mean half-life values of both degradation ($t_{1/2}(k_{deg})$) and internalization ($t_{1/2}(k_{int})$) processes that were 1.5- and 10-fold longer for CERA, respectively.

The time course of receptor occupancy in EPO and CERA treatment groups is shown in Figure 6. The bound EPOR (R^*)-to-initial EPOR (R_0) concentration ratio profiles for both drugs showed a rapid initial increase, followed by a gradual decline to the initial level. Although the EPOR occupation level was much higher for EPO than CERA, as indicated by the initial rise to 50 versus 28, the EPOR activation interval was longer for CERA, as indicated by the secondary slower decrease. CERA's lower EPOR occupancy but prolonged signaling compared with EPO (Figure 6) is consistent with its significantly larger K_D , and smaller k_{int} values, respectively (Table 1).

Sensitivity analysis

Figure 7 shows the time-dependent sensitivity of the venous blood tracer concentrations predicted by the model to both the transit-time density function and receptor-related parameters. All sensitivity values ranged from -1.2 to $+1.2$. All parameters exhibited medium (0.2 to 0.5) to high (>0.5) absolute relative sensitivities at given time ranges, indicating that those parameters were reliably estimated with the implemented experimental design.

Sensitivity values of the transit time density function parameters peaked prior to non-tracer administration with minor perturbations that occurred during or after the non-tracer input. Those perturbations fell rapidly to zero and remained there almost to the end of the experiment (Figure 7A). On the other hand, sensitivities of receptor-related parameters increased gradually with time and peaked during or after the non-tracer perturbation (Figure 7B). After non-tracer input, the overall model output was much more sensitive to changes in receptor-related parameters than to changes in density function parameters, indicating that the drug–receptor interaction was the most important factor determining the shape of the tracer concentration–time curve after perturbation.

Maximum sensitivity of parameter β was larger than that of parameters α and λ ($+1$ versus $+0.3$, and -0.3 , respectively). Maximum sensitivity occurred at approximately zero hour for parameter β and at approximately 8 h for parameter α (Figure 7A). Thus, β indicates a fast initial distribution phase from highly perfused tissues, while α reflects a slow terminal distribution from poorly perfused tissues.

Among receptor-related parameters, k_{int} showed the lowest sensitivity (maximum sensitivity: $+0.5$ versus -1.2 , $+1.2$, $+1.2$ for k_{syn}/R_0 , k_{deg} and K_D , respectively). Although K_D , k_{deg} and k_{syn}/R_0 are important in determining system response between approximately zero and 10 h after the non-tracer input, it seems that only k_{deg} and k_{syn}/R_0 can influence the response at later times (i.e. from 10 h after non-tracer administration until the end of the experiment). Unlike K_D , the sensitivity of which peaks at a non-tracer input time and vanishes afterwards, the sensitivity of k_{int} peaks 40 h after the non-tracer input.

Discussion

The present study hypothesizes that EPOR-expressing cells play a major role in the elimination of ESAs from the body and that this process is initiated by binding of the ESA to surface EPOR, followed by internalization and degradation by lysosomes. Thus, changes in receptor homeostasis due to EPO or CERA administration, as well as receptor binding and endocytosis properties of both hormones, should explain their different rates of metabolism. In this work these processes were indirectly quantified using the TIM, which is an experimental procedure that specifically deals with the competition between labeled and unlabeled forms of a drug on receptor binding sites enabling the kinetic mechanism of the binding to be elucidated.

Receptor-based in vivo elimination of EPO and CERA

Our previous studies provided significant evidence that EPOR-mediated pathways play a major role in the *in vivo* elimination of EPO and CERA. Busulfan-induced bone marrow ablation resulted in pronounced reduction of EPO clearance in adult sheep [27,28]. The same phenomenon was observed for CERA as a consequence of busulfan administration to adult sheep [19]. Furthermore, in phlebotomized sheep, a significant correlation was found between increases in EPOR mRNA and EPO clearance [29].

The kidneys and liver are not considered important in the *in vivo* elimination of EPO [30]. Likewise, given the lack of differences in CERA PK properties among healthy humans, chronic kidney disease patients and patients with impaired hepatic function, it appears that these organs are also unimportant in CERA elimination [11,31,32]. The long-term stability of EPO in whole blood at room temperature indicates no significant biotransformation taking place in the circulation [33].

EPOR turnover

For a fixed volume of target-mediated tissues, the free EPOR concentration (R) is proportional to the EPOR mass and therefore determines the capacity of the receptor-mediated elimination process. The larger the R , the greater the rate of elimination via the EPOR route. Assuming negligible variations in pre-administration receptor concentration (R_0) between animals in the EPO and CERA treatment groups, the observed difference in k_{syn}/R_0 is mainly due to a difference in k_{syn} . The k_{syn}/R_0 ratio was eightfold larger, while k_{deg} was only about twofold larger, for EPO than for CERA ($p < 0.01$) (Table 1). This finding suggests that animals injected with EPO have experienced a greater up-regulation in EPOR than those injected with CERA. In a study analysing the effect of EPO on regulation of its receptor expression in sheep, it was found that EPOR mRNA was up-regulated as a result of increased endogenous EPO levels after phlebotomy induced anemia [29]. An *in vitro* study using murine cell lines reported an increase in the number of EPOR per cell after 6 days of incubation with EPO, without altering EPOR binding affinity [34]. In a different study, erythroleukemic cells cultured in the presence of EPO had a higher number of EPOR than cytokine stimulated cells [35]. EPOR up-regulation was also reported in mice cerebral vascular endothelial cells after treatment with EPO [36].

EPOR binding with EPO and CERA

The equilibrium dissociation constant (K_D , the ratio of the dissociation and association rate constants) was significantly higher for CERA ($p < 0.01$, Table 1), indicating that it has a slower association rate and/or a faster dissociation rate than EPO. Analysis of equilibration curves in a comparative *in vitro* binding study for EPO and CERA with soluble recombinant EPOR and EPOR expressing cultured human cells indicated that the difference in affinity is driven mainly by slower association [16].

The faster occupation of EPOR by EPO molecules suggests that, for a given time interval, production of the EPO/EPOR complex is higher than production of the CERA/EPOR complex. The faster binding between EPO and EPOR on the cell surface can explain the significantly higher k_{syn}/R_0 in the EPO treatment group, where rapid disappearance of free EPOR from the medium stimulates the synthesis of more receptors by a positive feedback control mechanism.

Disposition nonlinearity of EPO and CERA

According to Equation (5), the degree of nonlinearity is determined by K_D and $C_{\text{in}}^{\text{TM}}(t)$, the input concentration to target-mediated tissues. When K_D and $C_{\text{in}}^{\text{TM}}(t)$ have comparable values, the drug metabolic rate depends nonlinearly on drug concentration. A very large K_D value or a very small $C_{\text{in}}^{\text{TM}}(t)$ results in linear, first-order kinetics. The maximum degree of nonlinearity is expected at the highest $C_{\text{in}}^{\text{TM}}(t)$ observed at the highest observed plasma concentration. The nonlinearity index was much greater than 1 (Table 1), which indicates a significant degree of nonlinearity for both drugs. The nonlinearity of EPO was greater, in accordance with its larger nonlinearity index ($p < 0.01$).

Endocytosis and metabolism of surface-bound EPO and CERA

Slow internalization of the drug/receptor complex to the intracellular space reduces drug elimination by increasing the probability of drug dissociation into the medium; slow degradation in lysosomes counteracts elimination by increasing the likelihood that the complex will be re-secreted, intact, to the cell surface. The parameter k_{int} lumps the processes of internalization and degradation together. Thus, the results of the current analysis cannot elucidate which process drives the smaller k_{int} value observed for CERA (Table 1). Panchapakesan *et al.* hypothesized that the very slow association but fast dissociation between CERA and EPOR results in a brief binding period that is barely long enough to permit internalization [37]. Our finding that the difference between EPO and CERA k_{int} values agree well with the differences in K_D values (10 versus 15-fold, respectively) supports the hypothesis of Panchapakesan *et al.* and suggests that internalization of surface-bound EPOR is the rate-limiting step in post-binding events.

Although EPOR expressed in cultured Ba/F and UT-7/EPO cells was bound approximately five times more quickly by EPO than by darbepoetin alpha, both drugs were internalized at the same rate and there were no differences in their fractions degraded or re-secreted after internalization [38]. Contrary to these patterns, it was found that EPO and CERA had different k_{int} values, which suggests that the metabolic fate of the CERA-EPOR complex is unique.

In vivo erythropoiesis of EPO and CERA

It has been proposed that ESAs function via the same mechanism [39]: they interact directly with EPOR on the cell surface and induce a conformational change in the receptor, which triggers the intracellular Janus kinase signal transduction required for the production of RBCs [3]. In this setup, the bound receptor concentration can serve as a PD marker of the pharmacological effect of ESAs with K_D and k_{int} values being considered as qualitative measures for receptor occupancy and receptor activation duration, respectively. The fact that CERA has a higher K_D value (i.e. lower occupancy) suggests that the greater *in vivo* erythropoietic activity reported for CERA compared with EPO [14] can be attributed to the slower internalization and/or degradation of CERA-EPOR complex (i.e. smaller k_{int} value) which results in prolonged signaling *in vivo*, as shown in the initial EPOR normalized bound receptors profiles (Figure 6). Our finding agrees with the hypothesis that the magnitude of increase in RBC concentration is primarily controlled by the length of exposure to an ESA, rather than by the number of occupied receptors [38–40]. Increased EPOR occupancy increases the rate of erythropoiesis, not by increasing the rate of cell division, but by increasing the recruitment and differentiation of more erythroid progenitor cells. However, once all available progenitors are actively dividing, the occupation of further receptors becomes trivial [39]. This hypothesis is supported by the fact that maximum erythropoiesis is elicited even when only a minor fraction of EPORs is activated [41,42].

Conclusion

The adopted receptor-based recirculation model accounts for: (1) drug-receptor binding, (2) receptor turnover, and (3) drug/receptor complex endocytosis and lysosomal degradation enabling an in-depth mechanistic analysis of the PK/PD of EPO and CERA in adult sheep. As predicted by the model, the slower elimination of CERA can be explained in terms of the following mechanisms: (1) less EPOR up-regulation induced by CERA administration; (2) slower binding of CERA to EPOR; and (3) reduced internalization and/or degradation rate of surface-bound CERA. Slower CERA/EPOR complex elimination explains the greater *in vivo* erythropoiesis observed for CERA [14] despite its lower affinity to its receptor. A

comprehensive sensitivity analysis showed that the model parameters were reliably estimated from the TIM experiments.

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Appendix A. List of non-standard abbreviations in text

*	Convolution operator
α, β	Rate parameters of transit time density function
$C_{in}^{TM}(t)$	Input concentration to TM tissues
$C_{out}^{TM}(t)$	Output concentration from TM tissues
$C_{out}^{tot}(t)$	Total output concentration
$C_{cold}(t)$	Unlabeled drug plasma concentrations
$C(\theta_i, t)$	Model output calculated at time t and parameter value θ
CV%	Coefficient of variation

CERA	Continuous erythropoietin receptor activator
D_{iv}	i.v. bolus dose
$\delta(t)$	Dirac delta function
$E_{TM}(t)$	Receptor-dependent extraction function
EPO	Erythropoietin
EPOR	Erythropoietin receptors
ESA	Erythropoiesis stimulating agent
$f_{out}^i(t)$	Output rate from i th tissue for $i = \text{HLS, TM or NTM}$
$g(t)$	Transit time density function
HLS	Heart-lung segment
$Inf(t)$	Constant rate infusion input function
$^{125}\text{I-CERA}$	Radiolabeled CERA
$^{125}\text{I-EPO}$	Radiolabeled EPO
i.v.	Intravenous
K_D	Drug/receptor equilibrium dissociation constant
k_{deg}	First-order receptor degradation rate constant
k_{int}	First-order drug/receptor internalization rate constant
k_{off}	First-order drug/receptor dissociation rate constant
k_{on}	Second-order drug/receptor association rate constant
k_{syn}	Zero-order receptor synthesis rate constant
λ	Mixing parameter of transit time density function
NLI	Nonlinearity index
NTM	Non-target mediated

P_i	Fraction of blood flow to i th tissue for $i = \text{TM or NTM}$
PD	Pharmacodynamics
PK	Pharmacokinetics
Q	Cardiac output
RBC	Red blood cell
RIA	Radioimmunoassay
R	Free receptor concentration
R^*	Bound receptor concentration
R_{tot}	Total receptor concentration
R_0	Initial receptor concentration
$S(t)$	Sensitivity function
$NS(t)$	Normalized sensitivity function
θ	Model parameter vector
t_{cold}	Time of injection of unlabeled drug
TIM	Tracer interaction method
TM	Target mediated
TMD	Target mediated disposition
$UIR(t)$	Unit impulse response function
y	Dummy variables

Figure 1.
Scheme of the recirculation model adopted to compare EPO and CERA PK

Figure 2.
Scheme of the receptor-based model adopted to compare EPO and CERA PK in target mediated disposition tissues

Figure 3. Representative fit of the receptor-based recirculation model to the ^{125}I -EPO TIM tracer data (squares) in adult sheep. The non-tracer EPO data (triangles) are fitted to a general cross validation cubic spline function

Figure 4.

Representative fit of the receptor-based recirculation model to the ^{125}I -CERA TIM tracer data (squares) in adult sheep. The non-tracer CERA data (triangles) are fitted to a general cross validation cubic spline function

Figure 5.
Predicted versus observed plasma tracer concentrations in EPO and CERA treatment populations

Figure 6.
Simulated bound EPOR-to-initial EPOR concentration ratio profiles in EPO and CERA treatment groups

Figure 7. Normalized sensitivity function for CERA transit time density function parameters (A) and receptor-related parameters (B) using TIM plasma tracer concentrations as the model output

Table 1

Receptor-related pharmacokinetic parameters of EPO and CERA obtained by fitting a receptor-based recirculation model to TIM data from adult sheep

Parameter (units)	Definition	EPO (<i>n</i> = 7)	CERA (<i>n</i> = 5)	<i>p</i>
		Mean (CV%)	Mean (CV%)	
k_{syn}/R_0 (1/h)	Initial EPOR normalized synthesis rate constant	1240 (17)	164.3 (31.3)	<0.01
k_{deg} (1/h)	EPOR degradation rate constant	65.6 (19.6)	42.5 (32.4)	<0.01
$t_{1/2}(k_{\text{deg}})^*$ (min)	EPOR degradation half-life	0.656 (19.9)	1.06 (29)	<0.05
K_D (pmol)	Drug/EPOR equilibrium dissociation constant	6.0 (29.6)	88.4 (18.3)	<0.01
<i>NLI</i>	Nonlinearity index	106 (31.1)	14.8 (20.2)	<0.01
k_{int} (1/h)	Drug/EPOR internalization rate constant	24.0 (2.2)	2.41 (17.7)	<0.01
$t_{1/2}(k_{\text{int}})^*$ (min)	Drug/EPOR internalization half-life	1.72 (2.2)	17.7 (16.1)	<0.01

CV%, coefficient of variation.

* Half-lives were calculated as: $\ln(2)/\text{corresponding rate constant}$.