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A comparative pharmacokinetic and pharmacodynamic study of two novel Cuban PEGylated rHuEPO *versus* MIRCERA® and ior®EPOCIM

[Estudio comparativo farmacocinético y farmacodinámico de dos nuevas rHuEPO PEGiladas cubanas *versus* ior®EPOCIM y MIRCERA®]

Gledys Reynaldo[1*], Leyanis Rodríguez[1], Roberto Menéndez[2], Joaquín Solazábal[3], Daniel Amaro[3], María de los A. Becquer[4], Yamila Colom[5], Haydee Gil[5], Juan C. Polo[1], Gilberto Castañeda[6], Braulio Jiménez-Vélez[7], Jorge Duconge[8], Eduardo M. Fernández-Sánchez[1,4]

[1]Department of Pharmacy, Institute of Pharmacy & Foods, University of Havana, 222 St. and 23 Av. La Coronela, La Lisa, 13600, Havana, Cuba.
[2]Center of Neurosciences of Cuba, 190 St between 25 and 27, Cubanacan, Playa, 11600, Havana, Cuba.
[3]Center of Molecular Immunology, 216 St. and 15 St. Atabey, Playa. 11600, Havana, Cuba.
[4]Center for Research and Biological Evaluation, Institute of Pharmacy & Foods, University of Havana, 222 St. and 23 Av. La Coronela, La Lisa, 13600, Havana, Cuba.
[5]National Institute of Oncology and Radiobiology (INOR), 29 St. and F, Vedado, Plaza de la Revolución, 10400, Havana, Cuba.
[6]Department of Pharmacology, CINVESTAV-IPN, Av. IPN No. 2508, Col. San Pedro Zacatenco, 07360, Delegación Gustavo A Madero, Mexico City, Mexico.
[7]University of Puerto Rico Medical Sciences Campus, Department of Biochemistry, School of Medicine, PO Box 365067, 00936-5067, San Juan, Puerto Rico.
[8]University of Puerto Rico Medical Sciences Campus, Department of Pharmaceutical Sciences, School of Pharmacy, PO Box 365067, 00936-5067, San Juan, Puerto Rico.
*E-mail: gledysrf@ifal.uh.cu

Abstract

Context: The recombinant human erythropoietin (rHuEPO) stimulates the erythropoiesis process. Because this glycoprotein has a short half-life, it needs to be administrated two to three times a week. One of the technics to solve this issue is the PEGgilation.

Aims: To evaluate the pharmacokinetics (PK) and pharmacodynamics of two new branched PEGylated erythropoietins (i.e., an asymmetric 32 kDa-PEG_2-rHuEPO and a symmetric 40 kDa-PEG_2-rHuEPO molecule) compared to non-PEGylated ior®EPOCIM and MIRCERA®.

Methods: Serum concentrations of both PEGylated and non-PEGylated erythropoietins were measured at various time points in order to determine PK parameters using non-compartmental analysis approach. The reticulocyte (%), erythrocyte count and hemoglobin levels were ascertained in order to compare the effect of these molecules after administrating a single intravenous dose (10 µg/kg) of each product in male New Zealand rabbits.

Results: Both branched PEGylated erythropoietin forms exhibited half-lives that were significantly longer than ior®EPOCIM ($p<0.05$), but not statistically different to MIRCERA®. The mean elimination half-life increased from 4 h (ior®EPOCIM) to 131 h for the 32 kDa-PEG2-rHuEPO and 119 h for the 40 kDa-PEG2-rHuEPO. Conversely, MIRCERA® exhibits a half-life of 64 h. Both PEGylated erythropoietin products significantly enhanced the stimulating effect on reticulocytes and erythrocytes formation, as well as on hemoglobin levels, when compared to ior®EPOCIM treatment up to 42 days post-dose.

Conclusions: The PEGylation strategy employed in this study is an effective method to modify the pharmacokinetics and pharmacodynamics of rHuEPO molecule achieving higher half-lives and, therefore, longer *in vivo* bioactivity. Both of the branched PEGylated-EPO forms tested are promising candidates for human testing.

Keywords: erythropoietin; non-compartmental analysis; PEGylated EPO; pharmacodynamics; pharmacokinetics; reticulocytes.

Resumen

Contexto: La eritropoyetina humana recombinante (rHuEPO) estimula la formación de eritrocitos en la medula ósea. Esta glicoproteína terapéutica presenta rápida eliminación en el organismo. Una de las estrategias tecnológicas para resolver esta problemática es la PEGgilación.

Objetivos: Evaluar la farmacocinética y la farmacodinámica de dos nuevas eritropoyetinas PEGiladas ramificadas (una asimétrica de 32 kDa-PEG_2-rHuEPO y otra simétrica de 40 kDa-PEG_2-rHuEPO) en comparación con ior®EPOCIM y el producto de referencia MIRCERA®.

Métodos: Se midieron las concentraciones séricas de eritropoyetina PEGilada y no PEGilada, a diferentes tiempos, para determinar los parámetros farmacocinéticos usando el método de análisis no compartimental. Se determinaron los % de reticulocitos, los niveles de eritrocitos y hemoglobina para comparar el efecto de estas moléculas después de administrar a dosis única 10 µg/kg por vía intravenosa en conejos Nueva Zelanda machos.

Resultados: Las eritropoyetinas PEGiladas ramificadas presentaron semividas significativamente superiores a ior®EPOCIM ($p<0,05$), pero no fueron estadísticamente diferentes a MIRCERA®. El $t_{1/2}$ aumentó de 4 h (ior®EPOCIM) a 131 h para la 32 kDa-PEG2-rHuEPO y 119 h para la 40 kDa-PEG2-rHuEPO, respectivamente. Ambas eritropoyetinas PEGiladas mejoraron significativamente el efecto estimulante sobre la formación de reticulocitos y eritrocitos, así como los niveles de hemoglobina, en comparación con ior®EPOCIM hasta 42 días después de la dosis.

Conclusiones: La estrategia de PEGilación, empleada en este estudio, es un método efectivo para modificar la farmacocinética y farmacodinamia de moléculas de eritropoyetinas. Esta tecnología permitió aumentar la semivida de estas moléculas, así como prolongar su bioactividad *in vivo*. Ambas formas ramificadas de rHuEPO PEGiladas son candidatos prometedores para su uso clínico.

Palabras Clave: análisis no compartimental; eritropoyetina; eritropoyetina PEGilada; farmacocinética; farmacodinámica; reticulocitos.

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Abbreviations:

AUC: area under the disposition curve; **AUEC:** area under the effect curve; **CIM:** Center of Molecular Immunology; **CIGB:** Center of Genetic Engineering and Biotechnology; **CL:** Clearance; **EPO:** erythropoietin; **ESAs:** erythropoiesis-stimulating agents; **HGB:** hemoglobin; **MRT:** mean residence time; **NCA:** non compartmental analysis; **NZ:** New Zealand; **PK:** pharmacokinetics; **PD:** pharmacodynamics; **PEG:** polyethylene glycol; **RBC:** red blood cell or erythrocyte; **RET:** reticulocytes; **Rmax:** maximum response; **rHuEPO:** recombinant human erythropoietin; **T(Rmax)**: time to reach the maximum response; $\mathbf{t_{1/2}}$: terminal half-life; **Vss:** volume of steady-state distribution.

INTRODUCTION

Biotechnological products have opened a new era in medicine and are presently an active research area (Kinch, 2014). Erythropoietin (EPO) is a glycoproteic hormone of approximately 30.4 kDa produced mainly by the endothelial cells of the peritubular capillaries in the renal cortex. EPO is released under hypoxic conditions, being the main physiological regulator of the maturation and differentiation processes of precursor erythrocyte cells in the bone narrow (Jelkmann, 2013).

Production of recombinant human erythropoietin (rHuEPO) through biotechnology is carried out by isolation of the human gene and its expression in Chinese hamster ovary (CHO) cells and other cell types (Jelkmann, 2013; Ostrowski and Heinrich, 2018). The (rHuEPO) obtaining has facilitated its use in therapeutics for the treatment of anemia associated with chronic renal insufficiency, cancer chemotherapy, HIV infection, among other diseases (Thilaka and Kumar, 2016).

The use of erythropoiesis stimulating agents has reduced morbidity, hospitalization and mortality, as well as increasing patient quality of life, though they may also increase the incidence of thromboembolism (Biggar and Kim, 2017). However, rHuEPO exhibits some limitations such as its short half-life. Hence, it must be administrated at short dosing intervals to ultimately achieve the target hemoglobin level (Sinclair, 2013). Several strategies have been developed to overcome this drawback, such as the covalent linkage with polyethylene glycol (PEG). PEGylation has been well established as an efficient method for peptide stabilization, reduction of renal excretion, prolong the systemic circulation of proteins, decreased toxicity and immunogenicity (Fishburn, 2008; Dozier and Distefano, 2015).

In the international market, there is an innovative PEGylated EPO product available commercialized known by the brand name MIRCERA® produced by Hoffmann-LaRoche. This product consists of an attached linear methoxy-polyethylene glycol chain of 30 kDa, which considerably increased its half-life allowing a prolonged dosing interval of up to one month with regard to rHuEPO (Saglimbene et al., 2017).

Notwithstanding their efficacy and safety, access to these innovative biotechnological products are restricted for low-income countries due to its high cost. Hence, production of biosimilars, but less costly, products appears as a suitable strategy to increase the access to such medicines (Mikhail and Farouk, 2013).

The ior®EPOCIM is a rHuEPO alpha that was registered in 1998 by the CECMED (Cuban National Regulatory Agency) under different codes such as 0995, 0996 and B-04-020-B03. It is available in 1-mL bulbs for SC or IV injections at strengths of 2000, 4000 and 10,000 IU, respectively. The ior®EPOCIM is considered one of the top biotech product among those produced at the CIM facilities. At present, ior®EPOCIM is routinely in use within the Cuban Healthcare System to treat anemia in patients with chronic renal disease, chemotherapy- or zidovudine-induced anemia in cancer or AIDS patients, respectively. This product exhibits similar properties to Epoetin alpha, which is marketed as Eprex® by Janssen Inc. (Pucaj et al., 2014). However, one of the limitations is its short half-life. In this sense, with the objective of improving its PK and PD characteristics two new candidates of PEGylated rHuEPO were developed: i.e., 32 kDa-PEG_2-rHuEPO and 40 kDa-PEG_2-rHuEPO (Páez et al., 2016). The invention provides for a conjugate of rHuEPO with two branched polymerized structures and their corresponding pharmaceutical ingredients. One of these products is an asymmetric arrangement of two mono-methoxy-polyethylene glycol (mPEG) structural motifs of 20 and 12 kDa each; whereas, the other is a symmetrical arrangement of two identical mPEG units of 20 kDa.

During the development or modification of any product, PK and PD studies are required in relevant species before using it in human. Therefore, the present work investigates the PK and PD properties of these two new branched PEGylated-rHuEPO products and compares them with ior®EPOCIM and the innovative PEGylated MIRCERA®, using New Zealand rabbits.

MATERIAL AND METHODS

Experimental material

Experimental formulations of 32 kDa-PEG_2-rHuEPO and 40 kDa-PEG_2-rHuEPO were developed by the Center Immunology Molecular and the Center of Genetic Engineering and Biotechnology, Havana, Cuba. Each formulation was prepared in homogenous solution and packaged in sterile ampoules at a concentration of 100 µg/mL. However commercial formulation of rHuEPO (ior®EPOCIM, nominal concentration 10,000 IU/mL) was obtained from CIM (CIMAB S.A., Atabey Playa, Cuba). Commercial MIRCERA® (Roche, Basel, Switzerland) in pre-charged syringes of 100 µg/0.3 mL.

Experimental animals

Male New Zealand (NZ) rabbits of 1.5 to 2.3 kg (PK study) and 1.6 to 2.0 kg (PD study) of weight were selected. Rabbits were obtained from the National Center of Laboratory Animal Production (CENPALAB, Havana, Cuba). The health and quality of all animals was certified. Rabbits were placed in individual cages under controlled conditions. Temperature was 19.5 ± 2°C, humidity was 71% and the light/dark cycle was 12h/12h. The register code of protocol EPOPEG/PK-PD/2012 was approved by The Ethics Committee for Animals Protection at the Center for Research and Biological Evaluation and the National Institute of Oncology and Radiobiology, both from Havana Cuba. Besides, all procedures, were conducted in compliance with the Caring for Animals for Better Science Directive of the European Union (European Commission, 2010). For both the PK and PD studies, four groups of five animals each were formed. In each study, a single dose of 10 µg/kg of each product was administrated per experimental group. Animals were kept in fasting condition overnight before drug administration, but food and water were given *ad libitum* 2 h later.

Pharmacokinetic study

A single intravenous (IV) bolus dose of 10 µg/kg of 32 kDa-PEG_2-rHuEPO, 40 kDa-PEG_2-rHuEPO, ior®EPOCIM or MIRCERA® was injected into the left marginal ear vein. Blood samples (1 mL) were drawn from the right marginal ear vein at 0, 0.5, 1, 3, 6, 8, 16, 24, 36, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h after administration. Blood was placed in Eppendorf tubes and let stand for 1 h. Serum was then obtained by centrifugation at 3000 rpm during 15 min and kept frozen at -70°C. EPO serum samples were analyzed using a commercial EPO ELISA kit (Roche diagnostics GmbH, Germany). The rHuEPO calibrations curves were constructed in the range of 0 to 200 mIU/mL. The lower limit of quantification for EPO was 0.24 mIU/mL.

Pharmacodynamics

A single intravenous bolus dose of 10 µg/kg of 32 kDa-PEG_2-rHuEPO, 40 kDa-PEG_2-rHuEPO, ior®-EPOCIM or MIRCERA® was injected into the left marginal ear vein. Blood samples were drawn at 0, 72, 168, 240, 504, 864, 1008 h post-dose and the following hematological variables were measured: reticulocyte count, erythrocyte count and hemoglobin levels. One mL blood was placed in Eppendorf tubes containing 10 µL EDTA (10%) as anticoagulant.

Hemoglobin and erythrocyte determinations were performed by a PENTRA 120 automatic hematologic analyzer (Horiba, Montpellier, France). Reticulocytes were counted manually, according to the INOR laboratory standards and literature reports (Bain et al., 2012). All experiments were carried out at the same time of the day to avoid circadian variations. Reticulocytes were counted the same day of blood samples were obtained.

Statistical analysis

The individual concentration versus time profiles obtained were analyzed by the non-compartmental analysis (NCA) using a combined linear/log linear trapezoidal rule approach. Data are expressed as mean ± standard error or mean (SEM). Pharmacokinetic and pharmacodynamic parameters were calculated using the Phoenix® WinNonlin® 7.0 software (Pharsight Corp. Certara, USA). An exploratory analysis was performed to verify data requirements for normality and randomness using goodness of fit (Shapiro-Wilks test) and the runs test, respectively. A Levene's test was used for testing homoscedasticity. Pharmacokinetic and pharmacodynamic parameters were statistically

analyzed using the one-way analysis of variance (ANOVA) and Dunnett *post hoc* to test differences between groups. Comparison between basal and final level of reticulocytes, hemoglobin and erythrocytes was done by paired t test. A significance level of 5% was considered for these purposes. Statistical analyses were performed using the IBMSPSS Statistics version 22.0 for Windows.

RESULTS

Pharmacokinetics

Fig. 1 depicts the corresponding mean ± SEM serum drug concentrations *versus* time profiles (i.e., raw data) of 32 kDa-PEG$_2$-rHuEPO, 40 kDa-PEG$_2$-rHuEPO, ior®EPOCIM and MIRCERA® after single IV bolus doses of 10 µg/kg given to each experimental group. As expected, the plasma concentration values declined exponentially in all the four experimental groups, following the typical disposition pattern for IV bolus administrations. Besides, an up-and-down pattern during the exponential decay of biological products was also observed. This up-and-down pattern has been previously described by others after single IV injection of recombinant human biological products (Okabe et al., 1990; Duconge et al., 2005). The systemic disposition profile of Ior®EPOCIM showed a biphasic exponential decline until 48 h post-dose; whereas, the PEGylated derivatives followed similar pharmacokinetic profiles but with more prolonged elimination phases that last until after 240 h post-dose. Pharmacokinetic parameters (mean ± SEM) are summarized in Table 1. The pharmacokinetic parameters $t_{1/2}$, MRT and AUC of the PEGylated derivatives were significantly higher than that of the non-PEGylated molecule. The volumes of distribution at steady-state (Vss) fluctuated between 356 and 614 mL/kg (Table 1), which are larger than reported by others for a PEGylated EPO (Maleki et al., 2011). These values denote a broad distribution of the EPO molecules beyond the vascular compartment into the extracellular fluid as the blood volume in rabbits is reportedly to range from 44 to 70 mL/kg (Suckow et al., 2012). This probably is a direct consequence of high binding to EPO receptor on progenitor cells in bone marrow. No significant differences were found for the Vss values between the studied molecules. However, the clearance values of the PEGylated molecules shown a slower rate than that reported for ior®EPOCIM. Moreover, no significant differences were found between the two branched molecules with regard to MIRCERA®. On the other hand, the branched PEG molecules showed high SEM values of PK parameters with regard to the commercial molecules. This can be a consequence of the remarkable intra- and inter-animal variability associated with a higher intrinsic heterogeneity of the molecules. There are some literature reports in support of this argument (Macdougall et al., 2006; Cao et al., 2014; Wadhwa et al., 2015).

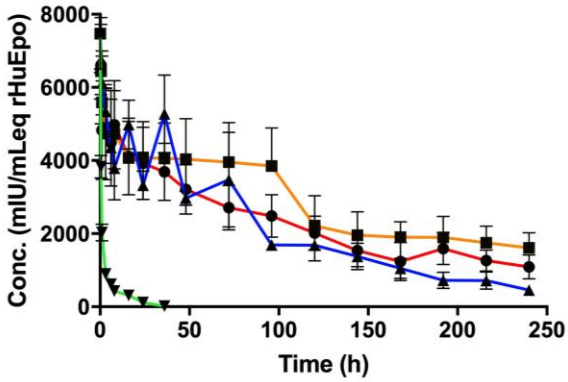

Figure 1. Mean serum concentration *versus* time profiles of erythropoietins (●) 32 kDa-PEG$_2$-rHuEPO, (■) 40 kDa-PEG$_2$-rHuEPO, (▼) ior®EPOCIM and (▲) MIRCERA® after administration of single IV bolus doses of 10 µg/kg to male New Zealand rabbits.

Each point represents the mean ± standard error (SEM) in four (MIRCERA®) or in five rabbits per group.

In vivo biological activity

A significant increase in reticulocytes (%) was observed in this study (see Fig. 2A). Significant departures from baseline were observed after dosing that persist up to day 10th. Maximal induction of reticulocytes (%) oscillated between 7 and 10 days post-treatment. This effect starts to diminish from day 10 onward, returning to basal values at 21 days. As can be seen in Fig. 2A, the PEGylated variants showed a larger effect of induction on reticulocyte with regard to ior®EPOCIM, with the 40 kDa PEG2-rHuEPO compound displaying the highest % of response at day 10 and MIRCERA® the lesser at the same time among the PEGylated products.

As observed in Fig. 2B-C, PEGylated compounds also gradually increased the erythrocytes and hemoglobin concentrations until after 21-36 days post-treatment when peaks were achieved. Afterward, decreases of these responses until day 42 were observed but without returning to basal levels. On the other hand, ior®EPOCIM achieved its maximal effects at day 21 for both RBC and HGB and then decreased continuously to return to basal values at day 42.

Table 1. Pharmacokinetic parameters of 32 kDa-PEG$_2$-rHuEPO, 40 kDa-PEG$_2$-rHuEPO, ior®EPOCIM and MIRCERA® after administration of single IV bolus doses of 10 µg/kg to male New Zealand rabbits.

PK parameters	EPOCIM®	MIRCERA®	32 kDa-PEG2-rHuEPO	40 kDa-PEG2-rHuEPO
$t_{1/2}$ (h)	3.91 ± 0.45	64.05 ± 6.57	131.03 ± 38.74**	119.21 ± 18.55*
MRT (h)	6.50 ± 0.85	92.54 ± 9.50	194.33 ± 53.76**	185.21 ± 22.55**
$[AUC]_{0-\infty}$ (mIU·h/mL)	15672.60 ± 1051.36	510316.62 ± 96239.61	545150.34 ± 118800.88**	747280.02 ± 193814.85**
Cl (mL/h/kg)	64.16 ± 3.97	4.04 ± 0.79**	4.20 ± 2.04**	2.47 ± 0.51**
Vss (mL/kg)	407.63 ± 43.50	355.98 ± 43.52	614.39 ± 175.70	468.67 ± 127.93

Data are expressed as mean ± SEM of five rabbits per group. *P<0.05; **p<0.01. Comparison *versus* ior®EPOCIM (ANOVA followed by Dunnett).

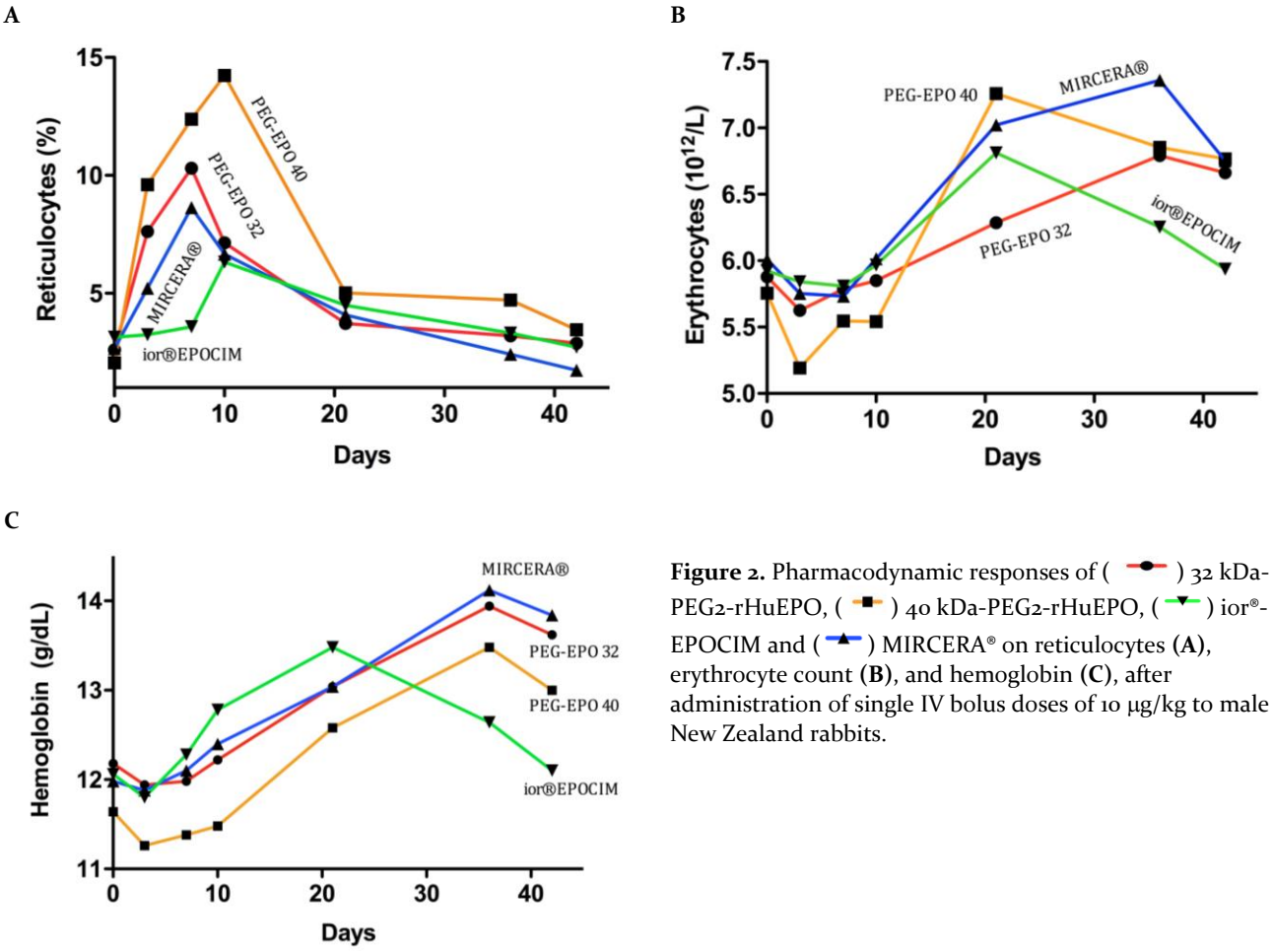

Figure 2. Pharmacodynamic responses of ( ) 32 kDa-PEG2-rHuEPO, ( ) 40 kDa-PEG2-rHuEPO, ( ) ior®-EPOCIM and ( ) MIRCERA® on reticulocytes **(A)**, erythrocyte count **(B)**, and hemoglobin **(C)**, after administration of single IV bolus doses of 10 µg/kg to male New Zealand rabbits.

The mean peak values (Rmax) of reticulocytes, erythrocytes and HGB for both ior®EPOCIM and the PEGylated molecules were significantly higher than to basal values (Table 2).

However, the single dose administration of each PEGylated derivative resulted in significant increases of the mean values of RBC and HBG, which persisted longer and were, therefore, higher than that of the ior®EPOCIM treatment at the end of the experimental period. Statistically significant differences were found in the comparison of the maximum induction of reticulocytes (%, Rmax) among the products (one-way ANOVAs, as presented in Table 3), denoting not only a superiority of the PEGylated products over ior®EPOCIM but also of the branched PEGylated molecules over MIRCERA®. Regarding the AUEC of % RET, differences were observed between the branched PEGylated products and ior®EPOCIM, but not between MIRCERA® and ior®EPOCIM. Likewise, a significant difference was detected between MIRCERA® and the 40 kDa-PEG_2-rHuEPO, but not with regard to the 32 kDa-PEG_2-rHuEPO. Finally, the T(Rmax) values were found to be statistically different among the groups, except for the comparison between ior®EPOCIM and 40 kDa-PEG_2-rHuEPO as well as between MIRCERA® and 32 kDa-PEG_2-rHuEPO. Similar to what was observed in the pharmacokinetic study, there were no statistical differences between the two branched molecules and MIRCERA® in the PD parameters of the other two tested biomarkers (i.e., between MIRCERA® and 40 kDa-PEG_2-rHuEPO for the RBC response and between MIRCERA® and both 32 and 40 kDa-PEG_2-rHuEPOs for the HGB response). Nonetheless, a significant difference was indeed found between MIRCERA® and 40 kDa-PEG_2-rHuEPO for the reticulocyte (%) response.

Table 2. Erythropoiesis response (i.e., % RET, levels of RBC and HGB) before and after the administration of PEGylated and non-PEGylated derivatives of rHuEPO in NZ rabbits.

Products	Baseline (Mean ± SEM)	Rmax (Mean ± SEM)
	RET (%)	
EPOCIM®	3.14 ± 0.24	6.32 ± 0.53*
MIRCERA®	2.66 ± 0.13	8.64 ± 0.13*
32 kDa-PEG2-rHuEPO	2.60 ± 0.24	10.56 ± 0.73*
40 kDa-PEG2-rHuEPO	2.06 ± 0.19	14.24 ± 0.33*
	RBC (x 10^{12}/L)	
EPOCIM®	5.92 ± 0.13	6.81 ± 0.09*
MIRCERA®	6.01 ± 0.29	7.36 ± 0.05*
32 kDa-PEG2-rHuEPO	5.88 ± 0.12	6.86 ± 0.08*
40 kDa-PEG2-rHuEPO	5.76 ± 0.16	7.29 ± 0.10*
	HGB (g/dL)	
EPOCIM®	12.06 ± 0.06	13.48 ± 0.26*
MIRCERA®	11.98 ± 0.58	14.12 ± 0.14*
32 kDa-PEG2-rHuEPO	12.18 ± 0.10	14.08 ± 0.26*
40 kDa-PEG2-rHuEPO	11.64 ± 0.38	13.58 ± 0.20*

Statistical analysis for the comparison of the corresponding responses at To (baseline) and T(Rmax) were performed using a Student's t-test for paired samples. *Significant differences between any two group). Significance level was set at 5%.

RET: reticulocytes; RBC: red blood cell or erythrocytes; HGB: hemoglobin.

Table 3. Pharmacodynamic parameters after a single intravenous administration of 10 ug/kg to healthy rabbits.

PD parameters	EPOCIM®	MIRCERA®	32 kDa-PEG2-rHuEPO	40 kDa-PEG2-rHuEPO
	RET (%)			
AUEC (h)	174.34 ± 9.37	182.79 ± 4.32	207.20 ± 3.63*¶	304.90 ± 13.72*+
T(Rmax) (h)	10.00 ± 0.0	7.00 ± 0.0*	7.60 ± 0.60*¶	10.00 ± 0.0+
Rmax (mL/h)	6.32 ± 0.53	8.64 ± 0.12*	10.56 ± 0.73*	14.24 ± 0.33*
	RBC (x 10^{12}/L)			
AUEC (h)	263.48 ± 2.73	280.14 ± 3.69*	262.73 ± 4.21+	271.64 ± 4.73
T(Rmax) (h)	21.00 ± 0.0	36.00 ± 0.0*	38.40 ± 1.47*¶	25.20 ± 4.20+
Rmax (mL/h)	6.81 ± 0.10	7.36 ± 0.05*	6.86 ± 0.08+¶	7.29 ± 0.10*
	HGB (g/dL)			
AUEC (h)	536.09 ± 8.08	548.00 ± 15.11	544.28 ± 4.80	521.14 ± 7.01
T(Rmax) (h)	21.00 ± 0.00	36.00 ± 0.00 *	38.40 ± 1.47*	34.20 ± 3.50*
Rmax (mL/h)	13.48 ± 0.26	14.12 ± 0.14	14.08 ± 0.26	13.58 ± 0.20

Data are expressed as mean ± SEM of five rabbits per group. Statistical analysis for the comparison of the corresponding PD parameters were performed using one-way ANOVAs followed by *post hoc* Dunnett's Multiple Comparison Test. *Significant differences between ior®EPOCIM and the rest of formulations. +Significant differences between MIRCERA® and the branched PEGylated derivatives. ¶Significant difference between the two branched PEGylated derivatives. Significance level was set at 5%.

DISCUSSION

The pharmacokinetic results in this study were consistent with previous reports of PEGylated and non-PEGylated rHuEPO products (Maleki et al, 2011). PEGylation can indeed modify the product affinity to cellular receptors, decrease the enzymatic degradation, increase physical and thermal stabilities as well as solubility, leading to a reduced clearance and, therefore, a longer half-life (Dozier and Distefano, 2015). Such effects are mainly produced by the modification of several physicochemical properties including conformational changes, steric hindrance, changes in electrostatic fixation and hydrophobicity (Fishburn, 2008). The majority of these changes could also be explained by an expansion in the hydrodynamic ratio of the protein-PEG conjugate. It is possible because of the PEG structure that is able to coordinate numerous molecules of water (Gokarn et al., 2012). According to Harris, each unit of oxiethyleneglycol is capable of coordinating 3-5 molecules of water, increasing the hydrodynamic volume in 5-10 folds the original molecular mass (Harris et al., 2001). Thus, it is reasonable to observe a significant PK differences between the PEGylated compounds and the non-PEGylated molecule. This research shows that the elimination half-life time and the clearance of the newly PEG-rHuEPO are obviously longer than that of the parental rHuEPO, giving them potential to be clinically used and reducing the administration frequency. However, PEGylation strongly decrease the binding of PEGylated EPO to its receptor, which results in significant loss of activity (Fishburn, 2008). This effect may cause a decrease in their clinical efficacy. Nevertheless, there is a non-inferiority of both the PEGylated products over ior®EPOCIM and these branched PEGylated molecules over MIRCERA®, as determined by the PD parameters AUEC and Rmax (Table 3). The PEGylation of these branched products does not interfere with the mechanism of action proposed for rHuEPO. Indeed, with the exception of the % RET response (where the PEGylated products seemed to be superior over ior®EPOCIM, as presented in Table 3), the results suggest that the PEGylated and non-PEGylated molecules have quite similar erythropoietic effects but differences in their duration are observed ($p < 0.001$). These results are consistent with those obtained by other studies comparing the *in vivo* biological activity of native EPO and monoPEGylated EPO in normocitemic

mice (Páez et al., 2016). On the other hand, it is observed that the erythropoietic response for these PEGylated derivatives shows a very similar behavior to MIRCERA®. This fact gives reliability to the method of PEGylation and increases its potential for clinical use. However, a study of the effects of these new derivatives on erythropoiesis in animal models of disease-states and after multiple dosing are necessary to confirm this hypothesis.

The fundamental biological activity of the EPO is the production of red blood cells (i.e., erythropoiesis) not only under normal but also in hypoxic conditions. Erythropoiesis occurs as a result of a narrowly controlled cell proliferation and differentiation pathway whose end result is the production of erythrocytes. In consequence, the *in vivo* biological activity of the PEGylated derivatives and ior®EPOCIM was evaluated by studying reticulocytes, erythrocytes and hemoglobin levels within 42 days after the administration a single dose in healthy rabbits. It should be noted that the reticulocytes, hemoglobin and erythrocytes measures after the administration of the PEGylated derivatives were above those observed with the non-PEGylated EPO in samples taken over the entire study period. These results may be due to the fact that increases in erythropoiesis (Elliot et al., 2008) are more favored by the presence of sustained concentrations of EPO over time than by high concentrations of short duration. Accordingly, it seems that the improvement in the pharmacokinetic parameters (i.e., as a result of the PEGylation-induced changes in the original molecule) led to a greater biological activity with respect to ior®EPOCIM.

The significant increase in RET (%) was observed short after the first dose of rHuEPO in this study (see Fig. 2A), similar to those reported earlier by others (Maleki et al., 2011; Pucaj et al., 2014). Since the increase in %RET resulted from a direct effect of erythropoietin on erythroid progenitors at the bone marrow, this response biomarker is considered as an indicator of positive outcome in the PD characterization of the studied products.

The effects of erythropoietin on marrow erythroid progenitor cells are well known (Jelkmann, 2013; Ostrowski and Heinrich, 2018). Previous studies indicated that multiple doses of rHuEPO administered to normal iron-replete subjects stimulated erythropoiesis and raised circulating HGB by an increase in RBC. As expected, increases in RBC and HGB are the direct consequence of increased numbers of circulating reticulocytes and of the synthesis of HGB under the influence of rHuEPO. In the present study with NZ rabbits, neither RBC nor HGB increases proportionately after a single dose, in spite of a significant increase in the % reticulocytes (Flaharty et al., 1990; Major et al., 1994). Both the RBC count and the HGB levels were found to be kept relatively unaltered after a slight increment up to day 10th. More important, only slightly or no significant differences were in general found among the three PEGylated products (i.e., MIRCERA® versus either 32 kDa-PEG2-rHuEPO or 40 kDa-PEG2-rHuEPO).

In a previous report by Souillard et al. (1996), a significant increase in % reticulocytes was observed two days after the first dose of rHuEpo in treated subjects; with up to 250% of increase with respect to day 0. However, the maximum increases were only 36 and 4.6% for RBC and HGB, respectively (Souillard et al., 1996). These findings suggest that not all the reticulocytes formed as a consequence of a direct effect from rHuEPO are ultimately maturated into RBC, which might be explained by either the hypothesis of a negative-feedback loop of RBC production or a delayed (lagged time) response of the biological system to increase RBC count.

As the maturation of erythroid cells requires 5 to 9 days under normal physiological conditions, the increase of RBC after the administration of PEGylated rHuEPO products is not immediate (i.e., time-dependent PD behavior). In this work, a significant increase in RET (%) was seen at 7-10 days after single IV administrations of the PEGylated products into the rabbits, which could be a consequence of the direct action of these rHuEPO molecules on the expulsion of reticulocytes (Major et al., 1994). Subsequently, the numbers of reticulocytes remained elevated for the following next days after dosing. A negative-feedback loop of RBC production –further to high HGB values– could explain the observed lesser changes in both RBC and HGB levels, as previously described by others (Koury and Bondurant, 1992).

On the other hand, the rate of erythropoiesis change (about 4-fold) is smaller than the EPO con-

centrations changes (about 1000-fold) (Eschbach et al., 1987). Thus, the magnitude of increase in RBC concentration is primarily controlled by the length of time EPO concentrations are maintained and not by the EPO concentration level per se. Moreover, it should keep in mind that the experimental model used in this study is a "healthy" animal (NZ rabbits) and, therefore, their RBC levels are expected to be normal. Under such an experimental condition, no further maturation of reticulocytes to RBC are required and hence a feedback mechanism is triggered by the change of RBC levels with respect to baseline so that the RBC count remain ultimately unaltered. Further studies in animal models at disease-state are warranted in order to validate the hypothesis. Such studies should also be designed to comparatively assess PK/PD responses of rHuEPO at steady-state condition, after multiple dosing (i.e., control *vs*. disease and single *vs.* multiple doses comparisons).

In this study, a single IV bolus dose seemed to be insufficient to maintain the necessary length of exposure time to produce the magnitude of increase in RBC concentrations, as depicted in Fig. 2B. These results are in good agreement with the fact that hematopoietic activity of rHuEPO is not immediate, as the differentiation and proliferation processes of the precursor cells in the bone marrow may take several days (Nandakumar et al., 2016). This effect starts to diminish from day 10 onward, returning to basal values at 21 days. It should be noted that the production of reticulocytes is the rate-limiting step in the erythropoiesis pathway.

CONCLUSIONS

The PEGylation strategy employed in this study is an effective method to modify the pharmacokinetics and pharmacodynamics of rHuEPO molecule achieving longer half-lives and, therefore, significantly higher *in vivo* bioactivity. This enables the development of novel long-acting erythropoiesis-stimulating agents with improved physicochemical and biological properties.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

Bain BJ, Bates I, Laffan M, Lewis SM (2012) Dacie and Lewis Practical Haematology, London, UK: 11th edn. Churchill Livingstone Elsevier.

Biggar P, Kim GH (2017) Treatment of renal anemia: Erythropoiesis stimulating agents and beyond. Kidney Res Clin Pract 36: 209–223.

Cao X, Chen Z, Yu Z, Ge Y, Zeng X (2014) Pharmacokinetics of PEGylated recombinant human erythropoietin in rats. J Anal Methods Chem 2014: 1–6.

Dozier JK, Distefano MD (2015) Site-specific PEGylation of therapeutic proteins. Int J Mol Sci 16(10): 25831–25864.

Duconge J, Rodríguez-Vera L, Valenzuela C, Álvarez D, Ramírez O, de la Luz-Hernández KR, Rabeza-Legon EY, Casaco A, Fernandez-Sanchez E (2005) Pharmacokinetic comparison of two recombinant human granulocyte colony-stimulating factor after subcutaneous administration in rabbits. Eur J Pharm Biopharm 61: 142–148.

Elliot S, Pham E, Macdougall IC (2008) Erythropoietins: A common mechanism of action. Exp Hematol (Charlottesv) 36: 1573–1584.

Eschbach JW, Egrie JC, Downing MR, Browne JK, Adamson JW (1987) Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. Results of a combined phase I and II clinical trial. N Engl J Med 316: 73–78.

European Commission (2010) Caring for animals aiming for better science of the European Union. Directive 2010/63/EU on protection of animals used for scientific purposes. http://ec.europa.eu/environment/chemicals/lab_animals/pdf/guidance/inspections/en.pdf [Accessed on November 1, 2017].

Fishburn CS (2008) The pharmacology of PEGylation: Balancing PD with PK to generate novel therapeutics. J Pharm Sci 97(10): 4167–4183.

Flaharty KK, Caro J, Erslev A, Whalen JJ, Morris EM, Bjornsson TD, Vlasses PH (1990) Pharmacokinetics and erythropoietin response to human recombinant erythropoietin in healthy men. Clin Pharmacol Ther 47: 557–564.

Gokarn YR, Matthew McLean, Laue TM (2012) Effect of PEGylation on protein hydrodynamics. Mol Pharm 9: 762–773.

Harris JM, Martin NE, Modi M (2001) PEGylation: a novel process for modifying pharmacokinetics. Clin Pharmacokinet 40(7): 539–551.

Jelkmann W (2013) Physiology and pharmacology of erythropoietin. Transfus Med Hemother 40: 302–309.

Kinch MS (2014) The rise (and decline?) of biotechnology. Drug Discovery Today 19(11): 1686–1690.

Koury MJ, Bondurant MC (1992) The molecular mechanism of erythropoietin action. Eur J Biochem 210: 649–663.

Macdougall IC, Robson R, Opatrna S, Liogier X, Pannier A, Jordan P, Dougherty FC and Reigner B (2006) Pharmacokinetics and pharmacodynamics of intravenous and subcutaneous continuous erythropoietin receptor activator (C.E.R.A.) in patients with chronic kidney disease. Clin J Am Soc Nephrol 1: 1211–1215.

Major A, Bauer C, Breymann C, Huch A, Huch R (1994) RH-erythropoietin stimulates immature reticulocyte release in man. Br J Haematol 87: 605–608.

Maleki A, Rouholamini A, Roohvand F, Shafiee A, Khanahmad H, Faghihi H, Hedayati MH, Tajerzadeh H (2011) Evaluation of bioactivity and pharmacokinetic characteristics of PEGylated *P. pastoris*-expressed erythropoietin. Drug Delivery 18(8): 570–577.

Mikhail A, Farouk M (2013) Epoetin biosimilars in Europe: five years on. Adv Ther 30(1):28-40.

Nandakumar SK, Ulirsch JC and Sankaran VG (2016) Advances in understanding erythropoiesis: evolving perspectives. British journal of haematology 173(2): 206–218.

Okabe M, Asano M, Kuga T, Komatsu Y, Yamasaki M, Yokoo Y, Itoh S, Morimoto M, Oka T (1990) *In vitro* and *in vivo* hematopoietic effect of mutant human granulocyte colony stimulating factor. Blood 75(9): 1788–1793.

Ostrowski D, Heinrich R (2018) Alternative erythropoietin receptors in the nervous system. J Clin Med 7: 24.

Páez R, Amaro DE, Castro FR, Hernández Y, Ruiz GA (2016) Conjugate comprising erythropoietin and a branched polymer structure. US Patent No. 2016/0317674A1.

Pucaj K, Riddle K, Taylor S, Ledon N, Golger G (2014) Safety and biosimilarity of ior®EPOCIM compared with Eprex based on toxicologic, pharmacodynamic, and pharmacokinetic studies in the Sprague–Dawley rat. J Pharm Sci 103: 3432–3441.

Saglimbene VM, Palmer SC, Ruospo M, Natale P, Craig JC, Strippoli GF (2017) Continuous erythropoiesis receptor activator (CERA) for the anaemia of chronic kidney disease. Cochrane Database Syst Rev 8: CD009904.

Sinclair AM (2013) Erythropoiesis stimulating agents: approaches to modulate activity. Biologics 7: 161–174.

Souillard A, Audran M, Bressolle F, Gareau R, Duvallet A, Chanal JL (1996) Pharmacokinetics and pharmacodynamics of recombinant human erythropoietin in athletes. Blood sampling and doping control. Br J Clin Pharmacol 42: 355–364.

Suckow MA, Schroeder V, Douglas FA (2012) The Laboratory Rabbit, 2nd edn., Boca Raton, London New York: CRC Press Taylor & Francis Group.

Thilaka GK, Kumar SV (2016) A review on pharmacological use of recombinant human erythropoietin in renal and nonrenal anemia and other potential applications in clinical practice. Apollo Medicine 13(2): 80–85.

Wadhwa M, Bird C, Dougall T, Rigsby P, Bristow A, Thorpe R and participants of the study (2015) Establishment of the first international standard for PEGylated granulocyte colony stimulating factor (PEG-G-CSF): Report of an international collaborative study. J Immunol Methods 416: 17–28.

Author contribution:

Contribution	Reynaldo G	Rodríguez L	Menéndez R	Solazabal J	Amaro D	Becquer MA	Colom Y	Gil H	Polo JC	Castañeda G	Jiménez-Vélez B	Duconge J	Fernández-Sánchez EM
Concepts or ideas	X				X								X
Design	X												X
Definition of intellectual content	X												X
Literature search	X												X
Experimental studies	X			X		X	X	X					
Data acquisition	X	X		X		X	X	X					
Data analysis	X	X	X	X					X	X	X	X	X
Statistical analysis	X		X						X				
Manuscript preparation	X	X	X	X	X	X	X	X	X	X	X	X	X
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