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ORIGINAL RESEARCH ARTICLE

An Integrated Approach for a Structural and Functional Evaluation of Biosimilars: Implications for Erythropoietin

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Abstract

Background Authorization to market a biosimilar product by the appropriate institutions is expected based on biosimilarity with its originator product. The analogy between the originator and its biosimilar(s) is assessed through safety, purity, and potency analyses.

Objective In this study, we proposed a useful quality control system for rapid and economic primary screening of potential biosimilar drugs. For this purpose, chemical and functional characterization of the originator rhEPO alfa and two of its biosimilars was discussed.

Methods Qualitative and quantitative analyses of the originator rhEPO alfa and its biosimilars were performed using reversed-phase high-performance liquid chromatography (RP-HPLC). The identification of proteins and the separation of isoforms were studied using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF–MS) and two-dimensional gel electrophoresis (2D-PAGE), respectively. Furthermore, the biological activity of these drugs was measured both in vitro, evaluating the TF-1 cell proliferation rate, and in vivo, using the innovative experimental animal model of the zebrafish embryos.

Results Chemical analyses showed that the quantitative concentrations of rhEPO alfa were in agreement with the labeled claims by the corresponding manufacturers. The qualitative analyses performed demonstrated that the three drugs were pure and that they had the same amino acid sequence. Chemical differences were found only at the level of isoforms containing N-glycosylation; however, functional in vitro and in vivo studies did not show any significant differences from a biosimilar point of view.

Conclusion These rapid and economic structural and functional analyses were effective in the evaluation of the biosimilarity between the originator rhEPO alfa and the biosimilars analyzed.

Key Points

The structural comparison of the originator rhEPO alfa and two of its biosimilars was assessed using already recognized techniques such as reversed-phase high-performance liquid chromatography (RP-HPLC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF–MS) for qualitative and quantitative analysis of the protein content and a two-dimensional gel electrophoresis (2D-PAGE) technique for detection of the isoforms.

The biological activity of the originator rhEPO alfa and two of its biosimilars was studied at the preclinical level using two different approaches: an in vitro study on the human TF-1 cell line and an in vivo study using the innovative experimental animal model represented by zebrafish embryos.

These studies confirmed the effective structural and functional similarity between the originator rhEPO alfa and the biosimilars analyzed.

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1 Introduction

An increasing number of the drugs available for patients are now biotechnology products, namely proteins produced in living cells using recombinant DNA techniques [1].

When the patent of a biotechnological drug expires, the possibility is open to market non-innovator versions of the product. At the present time, the patent of a number of chemical small-molecule drugs has expired and the use of bioequivalent (or 'generic') drugs is being strongly pursued worldwide by health agencies as formal clinical efficacy and safety studies are not required for the bioequivalent drug to be commercialized. This approach cannot, however, be applied to copies of biotechnology drugs, due to their complexity. Indeed, since it is very difficult to show that two protein products are identical, the term 'biosimilars' was introduced in the EU.

The 2004 EU legislation, the pioneering law in this area, established a comprehensive regulatory pathway to bring biosimilars to market [2]. The European Medicines Agency (EMA) defined biosimilar as "a biological medicinal product that contains a version of the active substance of an already authorized original biological medicinal product (reference medicinal product) in the EEA [European Economic Area]" [3]. Subsequently, the EMA Committee for Medicinal Products for Human Use (CHMP) developed detailed guidance documents to develop a biosimilar drug [2–8]. To be marketed, similarity to the reference medicinal product in terms of quality characteristics, biological activity, safety, and efficacy, based on a comprehensive biosimilarity test, need to be established.

The biosimilarity process that a biosimilar has to fulfill with respect to its reference medicinal product is very complex: it includes comprehensive analyses of the proposed biosimilar and the reference medicinal product, using sensitive and robust methods to determine not only similarities, but also potential differences in quality attributes [4]. Interestingly, it is not expected that all quality attributes of the biosimilar product will be identical to the reference medicinal product; however, when qualitative and/or quantitative differences are detected, such differences should be justified and, if relevant, they should not have impact on the clinical performance of the drug. This statement may include additional pre-clinical and/or clinical data [4]. As a matter of fact, relevant pre-clinical studies should be performed during development of the biosimilar, before initiating clinical trials. The EMA suggests a stepwise preclinical approach for the comparative evaluation: "analytical and in vitro pharmaco-toxicological studies must be conducted first and a decision then made as to the extent of what, if any, in vivo work in animal studies will be required" [5]. However, despite a stringent approval process and a significant cost advantage over the originator drugs [6], acceptance of biosimilars in the medical community continues to be low [7]. Bocquet and colleagues analyzed the global rhEPO market after 5 years from the approval and market entrance of patented EPOs [8]. They concluded that determining factors to increase the uptake of biosimilar EPOs are prescription and substitution incentives, as occurs in Germany.

At present, 13 biosimilars have been authorized in the EU, five of which are biosimilars of EPO [9]. EPO is a glycoprotein, synthesized mainly by the kidney peritubular interstitial cells and in the liver. It stimulates erythropoiesis by acting on erythroid progenitor cells [10]. Its therapeutic indications include the treatment of severe anemia caused by chronic kidney disease, chemotherapy, and AIDS. Human EPO (hEPO) was the first hematopoietic growth factor to be cloned [11] and, now, the recombinant hEPO (rhEPO) is one of the best-selling protein drugs worldwide [12].

Each clinically available rhEPO displays a similar amino acid sequence of the endogenous EPO, but they differ in their glycosylation pattern. rhEPO consists of a single 165-amino acid polypeptide chain, without Arg166 in the C-terminal (lost after post-translational modification), with three N-glycosylation sites at Asn24, Asn38, andAsn83, and one O-glycosylation site at Ser126 [13]. The glycosylation level strongly influences both the pharmacokinetic and pharmacodynamic properties of rhEPO [14–16]; indeed, due to the glycosylation sites, active rhEPO presents a carbohydrate content of about 40 % [17]. It should be taken into account that, using the biotechnology approach to synthesize rhEPO, while the polypeptide chain is genetically controlled, the oligosaccharide chains are the result of species- and tissue-dependent post-translational enzymatic reactions, giving rise to a mixture of isoforms that can differ in the recombinant protein compared to the native hormone [18]. Due to the differences in these glycosylated oligosaccharides, there are many different types of rhEPO, such as EPO alfa, beta, zeta, delta, or kappa. Due to the superimposable quaternary structure as well as the glycosylation pattern, the EPO zeta is homologous to the EPO alfa.

The first patented rhEPO [rhEPO alfa, Eprex® (EPR)] was developed by Janssen-Cilag and approved by the US Food and Drug Administration (FDA) in 1989. The patent expired in 2007 and its biosimilar products Binocrit® (BIN) and Retacrit® (RET) were authorized by the EMA and are clinically available in most European countries [19]. In the literature, the comparison between the EPO biosimilars has been performed mainly in a clinical context. Few studies, however, compare biosimilars from a structural and functional point of view, as recommended by the EMA before any in vivo study [5].

The aim of this study is to propose a rapid and reliable integrated approach for a structural and functional early screening of biosimilars. To demonstrate the usefulness of this method, two biosimilar EPOs, BIN and RET, were compared to their originator EPR [20, 21].

2 Materials and Methods

2.1 Materials

All drugs were obtained in their commercially available forms as an injection solution in prefilled syringes, as reported in Table 1. All salts and solvents for chemical analyses, as well as matrix and calibration kits for matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) [MALDI-TOF–MS] analysis, were purchased from Sigma Italia (Milan, Italy). Glycine, leucine, isoleucine, phenylalanine, glutamic acid, and threonine (European Pharmacopoeia Reference Standard) were purchased from Sigma Italia. *N*-methyl *N*-(trimethyl-sylyl) trifluoroacetamide (MSTFA) and acetonitrile (>98 % purity) for the derivatization and dilution of amino acids were purchased from Sigma Italia. For gas chromatography (GC)–mass spectrometry (MS) [GC–MS] analysis a capillary column HP-5MS (30 m × 0.25 mm inner diameter, 0.25 mm film thickness; J&W Scientific, Folsom, CA, USA) was used. Human recombinant granulocyte–macrophage–colony-stimulating factor (hrecGM-CSF), sequencing-grade bovine trypsin (trypsin), and PNGase F were purchased from Roche Italia. Sodium dodecylsulphate (SDS)–polyacrylamide gel electrophoresis (SDS-PAGE) precast gels, IPG®ReadyStrips, strips for first-dimension electrophoresis, and ReadyPrep™ 2-D Kits for clean-up were purchased from Bio-Rad (Life Technologies, Milan, Italy). ZipTips C_{18} for sample purification were purchased from Millipore (Darmstadt, Germany). The TF-1 cell line (cat. ACC-334) was purchased from Leibniz-Institut DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany); RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Sigma Aldrich (Milan, Italy). The wild-type AB zebrafish strain (cat. #1175) was purchased from the EZRC—European Zebrafish Resource Center, Institute of Toxicology and Genetics (Eggenstein-Leopoldshafen, Germany).

2.2 Qualitative and Quantitative Analyses by High-Performance Liquid Chromatography (HPLC) and Gas Chromatography–Mass Spectrometry

Reversed-phase high-performance liquid chromatograph (RP-HPLC) analyses were performed using a Dionex™ UltiMate™ 3000 Thermo Fisher Scientific S.p.A (Milan, Italy) equipped with a LPG-3400SD quaternary analytical pump, an HPG-3200BX biocompatible binary semipreparative pump, a WPS-3000SL analytical autosampler, a VWD-3100 UV–Vis detector, a TCC-3000SD thermostatted column compartment, and an AFC-3000 automatic fraction collector.

An RP-HPLC method was employed to determine rhEPO in pharmaceutical preparations. Preliminary experiments were performed to assess the reproducibility of the method used (data not shown). The specific liquid chromatographic parameters were as follows: the column was a Thermo Scientific, BioBasic-4, 250 × 4.6 mm with 5 µm particle size packing; the mobile phase composition was (A) water:trifluoroacetic acid (TFA) (100.0:0.1 v/v); and (B) acetonitrile:water:TFA (70.0:30.0:0.1 v/v/v); and the mobile phase flow rate was 1 mL/min. To establish the suitable resolution, the gradient elution was determined. The profile of elution with a mixture of solvents A and B was as follows: 0–1 min, isocratic, 50 % B; 1.1–6 min, 100–50 % B; 6.1–8 min, isocratic, 100 % B; 8.1–8.2 min, 100–50 % B; and 8.2–18 min, isocratic, 50 % B.

All analyses were performed at 30 °C; the detection wavelength was 214 nm. An analytical autosampler was used to inject 20 µL of sample.

Since a standard of comparison was not available, the qualitative analysis was carried out by collecting the main peak with an automatic fraction collector and then analyzing it by mass spectrometry. Furthermore, quantitative analysis was performed using EPR as a standard calibrator with four different dilutions (Table 2).

To validate the use of EPR as a standard, it was injected in triplicate. It is the area under peak of absorbance-time curve was recorded after integration and the concentration was calculated as ppm to obtain a standard curve

Table 1 Summary of epoetin formulations used in this study

Product	Abbreviation	Company	Batch number	Country of manufacture	Country of acquisition
Eprex® 1 mL at 40,000 IU/mL or 336 µg/mL	EPR	Janssen-Cilag	DGS5G00	The Netherlands	Italy
Binocrit® 1 ml at 40,000 IU/mL or 336 µg/mL	BIN	Sandoz	47021202	Austria	Italy
Retacrit® 1 ml at 40,000 IU/ml or 336 µg/mL	RET	Hospira	3E366G3	Germany	Italy

Table 2 Quantitative analysis performed using Eprex® as a standard calibrator with four different dilutions

Units	Eprex® dilution			
	1	2	3	4
IU/mL	40,000	20,000	8000	4000
μg/mL	336	168	67.2	33.6

(Electronic Supplementary Material Fig. S2). The concentration of each compound was subjected to regression analysis obtained by the least-squares method to calculate the calibration equation and correlation coefficient. The concentration of BIN and RET was calculated using the calibration curve and correlation coefficient obtained from EPR analysis.

Two 100 μL aliquots of solutions obtained from the fraction collector (Dionex™ UltiMate™ 3000) Thermo Fisher Scientific S.p.A (Milan, Italy) and a 100 μL aliquot of amino acid standards solution were dried. For each aliquot, 100 μL of MSTFA and 100 μL of acetonitrile was added. The mixtures were heated at 100 °C for 4 h. After centrifugation, the samples were injected in the 6890 GC system Agilent Technologies (Milan, Italy) coupled with a 7683 B Series injector and the ChemStation G1701GA version D.03.00.611 (Agilent, Waldbronn, Germany). The GC conditions were as follows: splitless injection mode (heated to 250 °C), injection port temperature 200 °C; carrier gas helium 4.6; flow rate 1.2 mL min^{-1}. The oven, held at an initial temperature of 100 °C for 1 min, was then heated to 290 °C at 35 °C min^{-1} and held for 3 min, before heating to 310 °C at 40 °C min^{-1}. The 5975 mass spectrometric detector (Agilent Technologies) was operated in the electron ionization (EI) mode using Scan Ion Monitoring (range mass 40–450 *m*/*z*). GC–MS interface was set at 300 °C, MS EI source at 230 °C, and MS quadrupole at 150 °C [22].

2.3 Protein Analysis by Sodium Dodecylsulphate-Polyacrylamide Gel Electrophoresis and Western Blot

To verify the protein content, 10 μL (=3.36 μg rhEPO) of each drug was denaturized at 95 °C for 5 min, loaded on 10 % SDS-PAGE precast gel, and run for 90 min at 150 V. Proteins were visualized with silver staining, according to Shevchenko et al. [23]. Furthermore, Western blot analysis was performed to assess the quality of EPO molecules. Briefly, after SDS-PAGE gel separation, proteins were transferred onto a nitrous cellulose membrane and incubated overnight with a mouse monoclonal antibody anti-hEPO (R&D System, Minneapolis, MN, USA; work dilution 1:1000) at 4 °C, and then with a fluorescent anti-mouse secondary antibody (LI-COR Biosciences, Milan, Italy; work dilution 1:1500) for 2 h at room temperature (rt). The protein detection was performed using Odyssey (LI-COR Biotechnology, Lincoln, NE, USA).

2.4 Enzymatic Digestion with PNGase F

Aliquots (10 μg) of each rhEPO were dried by speed-vac and dissolved in 12 μL of sodium phosphate buffer (NaH_2PO_4) (pH 7.5; 50 mM). To obtain a complete deglycosylation, drugs were denatured and reduced with 1 μL of 1 % SDS (w/v) and 1 μL dithiothreitol (DTT) (100 mM) in NaH_2PO_4 (pH 7.5; 50 mM) at 95 °C for 5 min and then cooled for 5 min at rt. In order to avoid the inactivation of enzyme, 1 % SDS (w/v), 2 μL of NaH_2PO_4 (pH 7.5; 50 mM), and 2 μL of 5 % nonylphenoxypolyethoxylethanol (NP40) (w/v) were added to the samples. Two units of PNGase F were added to the denatured proteins and deglycosylation was performed at 37 °C for 3 h. The reaction was stopped by heating the samples at 75 °C for 5 min. To verify that complete deglycosylation of proteins was achieved, the undigested drugs, used as control, and PNGase F digests were loaded on 10 % SDS-PAGE precast gel and run at 150 V for around 1 h. Gels were rinsed with deionized water, fixed for 1 h in an aqueous solution with 50 % methanol and 7 % acetic acid, and finally incubated in Coomassie™ Blue solution overnight. Before the acquisition of the image, the gel was washed three times with a de-staining solution [methanol:water:acetic acid (50:40:10, v/v/v)] to eliminate the staining background.

2.5 Enzymatic Digestion with Trypsin

The whole procedure was carried out in a laminar flow sterilized hood, whilst wearing powder-free gloves, in order to reduce keratin contamination.

Gels were washed with 150 μL of water and then dehydrated with 150 μL of acetonitrile twice. Cysteine residues were reduced by incubating gels with DTT (10 mM) in ammonium bicarbonate (NH_4HCO_3 0.1 M; pH 8.5) for 30 min at 56 °C. Gels were then washed three times with acetonitrile, and derivatized by treatment with iodoacetamide (IAA) (55 mM) in NH_4HCO_3 (0.1 M) for 20 min at rt in the dark. Gels were washed three times with NH_4HCO_3 (0.1 M), dehydrated three times with acetonitrile, and finally dried in a SpeedVac™ centrifuge.

The digestion buffer was added to gels and incubated for 1 h at 4 °C. The digestion buffer consisted of trypsin 12.5 ng/μL in NH_4HCO_3 (50 mM) with $CaCl_2$ (5 mM) [24]. Once the gel plugs had become swollen with absorbed digestion buffer, the excess buffer was removed and replaced with the same buffer without trypsin. Digestion

was performed overnight at 37 °C, shaking the tubes in a Thermo-Shaker apparatus (BioSan, Riga, Latvia).

After collecting the digest supernatant, two peptide extractions were performed to increase peptide recovery, by incubating the gel plugs twice with 60 and 40 μL of acetonitrile:formic acid (95:5; v/v) at 37 °C for 15 min. Recovered peptides were pooled and concentrated by evaporating the final volume of the extracts to complete dryness in a vacuum centrifuge, and re-dissolving them in the digest supernatant previously collected [24].

Before mass spectrometry analyses, peptide digests were desalted and concentrated by using ZipTips C_{18}, according to the manufacturer's protocol.

Purified peptides were eluted directly in a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile:water:TFA (70.0:30.0:0.1, v/v/v), used as matrix for the MALDI-TOF–MS analyses.

2.6 Matrix-Assisted Laser Desorption/Ionization (MALDI) Time-of-Flight (TOF)/TOF Mass Spectrometry (MS) [MALDI-TOF/TOF–MS] Analyses

Samples (proteins and peptides) were dissolved in 0.1 % TFA and mixed with the corresponding matrix solution. 1 μL of these preparations was applied to the MALDI plate, and allowed to dry at rt.

A solution of sinapic acid (SA) (10 mg/mL) in acetonitrile:water:TFA (50.0:50.0:0.1, v/v/v) was chosen for protein analyses and a solution of CHCA (20 mg/mL) in acetonitrile:water:TFA (70.0:30.0:0.1, v/v/v) was chosen for peptide analyses. Experiments were carried out on an AB Sciex 5800 MALDI-TOF/TOF–MS, equipped with a nitrogen laser ($k = 337$ nm). Samples were measured both in linear mode, providing information on the total number of different structures, and in reflector mode, for identification of molecular formulas based on precise mass measurements. For peptides and proteins, a ProteoMass™ Peptide and Protein MALDI-MS Calibration Kit was used to calibrate.

Recorded data were processed with freeware Mascot Software utilizing the Swissprot database.

2.7 Analyses of Isoforms by Two-Dimensional Gel Electrophoresis

The different glycoform patterns of originator and biosimilar rhEPO, before and after PNGase F digestion, were investigated by two-dimensional gel electrophoresis (2D-PAGE). Since the isoelectric point of glycosylated (before digestion) and deglycosylated (after digestion) protein changed, two different protocols were applied for the first dimension separation:

- Method A: approximately 6 μg of each glycosylated drug was applied to a 70 mm pH 3–6 IPG®ReadyStrip. The strips were then actively rehydrated in the protein isoelectric focusing (IEF) cell at 50 V for 12 h. The IEF was performed in increasing voltages as follows: 300 V for 30 min, a linear gradient to 1000 V for 30 min, then another linear gradient to 5000 V for 1.5 h, and finally 40,000 V/h.
- Method B: approximately 10 μg of each PNGase F digests were purified from salts and detergent that could interfere with 2D-PAGE, using ReadyPrep™ 2-D Cleanup Kit according to the manufacturer's protocol. Drugs were dissolved in the appropriate buffer and loaded on a 70 mm pH 3–10 IPG®ReadyStrip. The strips were then actively rehydrated in the protein IEF cell at 50 V for 12 h. The IEF was performed in increasing voltages as follows: 300 V for 30 min, a linear gradient to 1000 V for 30 min, then another linear gradient to 5000 V for 1 h and 20 min, and finally 50,000 V/h.

For the second dimension, the IPG® ReadyStrips were equilibrated for 15 min with Tris–HCl (pH 6.8; 50 mM) containing urea (6 M), 1 % (w/v) SDS, 30 % (v/v) glycerol, and 0.5 % (w/v) DTT, and then re-equilibrated for 15 min in the same buffer containing 4.5 % (w/v) IAA instead of DTT. Linear gradient precast criterion TGX™ (Tris–Glycine eXtended) gels Any kD™ were used to perform second-dimension electrophoresis at 200 V for 65 min. After the 2D-PAGE, gels were analyzed by silver staining, according to Shevchenko et al. [23].

2.8 Cell Culture

TF-1 cells were maintained in RPMI 1640 medium supplemented with 10 % FBS and 5 ng/mL hrecGM-CSF. For the proliferation assay, cells were collected and washed twice in PBS to eliminate hrecGM-CSF; cells were then seeded at a density of 10^5 cells/well in a 24-well cell culture plate and grown for 72 h at 37 °C and 5 % carbon dioxide in the presence or absence of increasing concentrations (0.03–10 IU/mL) of originator and biosimilar rhEPO. At the end of treatment, cells were collected, centrifuged, re-suspended in 1 mL of PBS, and counted either by hematocytometer or by flow cytometer (Becton–Dickinson FACS calibur). A non-linear fit dose–response curve was used to calculate each concentration producing 50 % of maximum effect (EC_{50}), using the GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Then, to compare the fitted midpoints (log EC_{50}) of the three curves statistically, the F test (p value <0.05) was performed.

2.9 Fish Maintenance and Egg Collection

All zebrafish embryos were handled according to relevant national and international guidelines. Current Italian rules do not require approval for research on zebrafish embryos.

A breeding stock of healthy adult wild-type AB zebrafish strain was used for egg production. Fish were maintained at 28 °C on a constant 14 h light/10 h dark cycle, under standard laboratory conditions as described in the literature [25]. Immediately after spawning, fertilized eggs were harvested, washed, and placed in 10 cm Ø Petri dishes in fish water. The developing embryos were incubated at 28 °C and maintained in 0.003 % (w/v) 1-phenyl-2-thiourea to prevent pigmentation.

2.10 Erythrocytes Quantification

Originator and biosimilar rhEPO stock solutions were diluted to the final concentration of 24 IU/mL in 0.05 % (w/v) phenol red solution. At 48 h post-fertilization (hpf) of development 4 nL of each dilution was injected into the common cardinal vein of zebrafish dechorionated embryos [26]. 0.05 % (w/v) phenol red solution without drugs was used as negative control. Embryos were incubated at 28 °C for 2–4 h after injection and then used for erythrocytes quantification. Each experiment was repeated three times.

Groups of 25 embryos for each injected compound were fixed in 4 % (w/v) paraformaldehyde in PBS overnight at 4 °C. O-Dianisidine staining was performed as described in literature [27] to detect hemoglobin in red blood cells. Erythrocytes quantification was performed using ImageJ 1.45 s image analysis software.

Hemoglobin quantification was also performed on the total embryo extract by using a modified cyanomethemoglobin method [28]. Twenty embryos for each injected compound were anesthetized in tricaine and placed in a tube with 800 µL of Drabkin's solution. Samples were sonicated and centrifuged at maximum speed for 20 min. Absorbance of the supernatant was measured at 540 nm and it was linearly correlated with the hemoglobin concentration.

Quantifications are expressed as a mean ± standard deviation of independent experiments. Statistical analyses were made using GraphPad Prism software. Analysis of variance (one-way ANOVA) followed by a Dunnett's test was performed to identify statistically significant differences among the different groups, considering a p value <0.05 as the threshold for a significant difference.

2.11 Macrophages and Granulocytes Quantification

Originator and biosimilar rhEPO stock solutions were diluted to the final concentration of 24 IU/mL in 0.05 % (w/v) phenol red solution. At 72 hpf of development 1 nL of each dilution was injected into the otic cavity of zebrafish dechorionated embryos. As a negative control, 0.05 % (w/v) phenol red solution without the pharmaceutical compounds was used. *Escherichia coli* JM109 bacteria in 0.05 % (w/v) phenol red solution were used as positive control [29]. Embryos were incubated at 28 °C for 2 h after injection and then fixed in 4 % (w/v) paraformaldehyde in PBS overnight at 4 °C. Forty embryos for each injected compound were used to perform whole-mount in situ hybridization (WISH), according to Thisse protocol [30]. *lplastin* and *pu1* were used as probes to detect macrophages and neutrophils. Embryos were mounted in agarose-coated dishes and images were taken with a Leica MZ16F stereomicroscope equipped with DFC 480 digital camera and LAS Leica Imaging software (Leica, Wetzlar, Germany). Leukocytes quantification was performed using ImageJ 1.45 s image analysis software.

Quantifications are expressed as a mean ± standard deviation of three independent experiments. Statistical analyses were made using GraphPad Prism software. One-way ANOVA followed by a Dunnett's test was performed to identify statistically significant differences among the different groups, considering a p value <0.05 as the threshold for a significant difference.

3 Results and Discussion

3.1 Qualitative Analysis of Whole Proteins by HPLC and MALDI-TOF–MS

HPLC has been used for the qualitative and quantitative evaluation of a number of drugs over the last 30 years. It is coupled with the conventional UV detectors and is used for the analysis of drugs present in the final products or in the body fluids of individuals. Its prevalent application is due to the possibility of separating different compounds from a mixture of other ingredients, such as the pharmaceutical preparations or degradation products.

On this basis, EPR and its biosimilar products BIN and RET were analyzed using HPLC–UV. This experimental procedure allowed us to demonstrate that rhEPO in the originator EPR and in its biosimilar products BIN and RET could be identified by only one peak, with retention times of 7.053, 7.098, and 7.038 min, respectively (Fig. 1). Interestingly, as shown in Fig. 1, while chromatograms of EPR and BIN were characterized by a single peak, multiple peaks could be detected in the chromatogram of RET. Indeed, in addition to the major peak corresponding to the rhEPO, other peaks were eluted at 3.052 and 3.225 min, close to the solvent front. The extra peaks of RET were

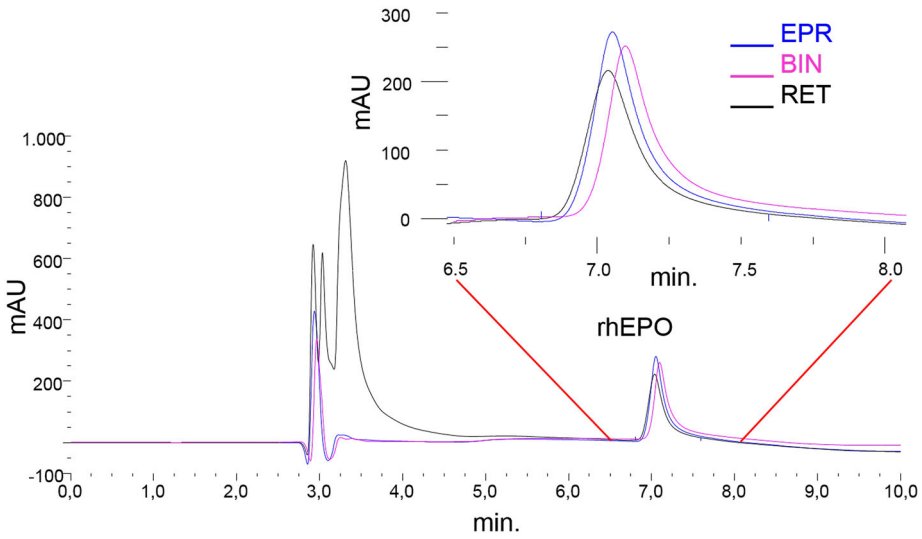

Fig. 1 Overlay of the three chromatograms obtained by qualitative analysis of Eprex® (*blue*), Binocrit® (*magenta*), and Retacrit® (*black*), showing the presence of the rhEPO peak (retention time 7.053, 7.098 and 7.038 min, respectively). *BIN* Binocrit®, *EPR* Eprex®, *RET* Retacrit®

Table 3 Composition of Eprex®, Binocrit®, and Retacrit® as declared by respective manufacturers and reported on corresponding leaflet

	EPR	BIN	RET
Drug	rhEPO alfa 336 µg	rhEPO alfa 336 µg	rhEPO zeta 336 µg
Excipients	Polysorbate 80	Polysorbate 80	Polysorbate 20
	Sodium phosphate monobasic dihydrate	Sodium phosphate monobasic dihydrate	Sodium phosphate monobasic dihydrate
	Sodium phosphate dibasic dihydrate	Sodium phosphate dibasic dihydrate	Sodium phosphate dibasic dihydrate
	Sodium chloride	Sodium chloride	Sodium chloride
	Water	Water	Water
	Glycine	Glycine	Glycine
			Leucine
			IsoLeucine
			Threonine
			Glutamic acid
			Phenylalanine
			Calcium chloride dihydrate

BIN Binocrit®, *EPO* rhEPO, *EPR* Eprex®, *RET* Retacrit®

recovered by a fraction collector and analyzed in GC–MS (Electronic Supplementary Material Fig. S1). These peaks were attributed to amino acids used as excipients in RET pharmaceutical formulation and were not present in the other two drugs (Table 3).

In order to confirm the similarity to rhEPO, the common peak of all three drugs, with a retention time approximately equal to 7 min, was recovered using a fraction collector and analyzed by mass spectrometry MALDI-TOF (Fig. 2).

A MALDI-TOF–MS procedure was then applied to investigate these drugs because the soft ionization procedures allow the measurement of proteins smaller than 100 kDa, and thus the MALDI-TOF–MS is able to reveal the exact structure of the amino acids sequence through the analysis of peptide sequences obtained after enzymatic digestion.

The mass spectra of each drug were similar. They were characterized by three peaks with an *m*/*z* value about of 14,000, 28,000, and 55,000, respectively. As reported in the literature [31], the principal peak at an *m*/*z* value of approximately 28,000 represents the single charge of rhEPO. The *m*/*z* value of about 14,000 could thus represent the double charge of rhEPO, while the *m*/*z* value of about 55,000 could represent the single charge of the rhEPO dimer. These analyses confirmed that the peaks eluted by HPLC with a retention time approximately equal to 7 were consistent with rhEPO.

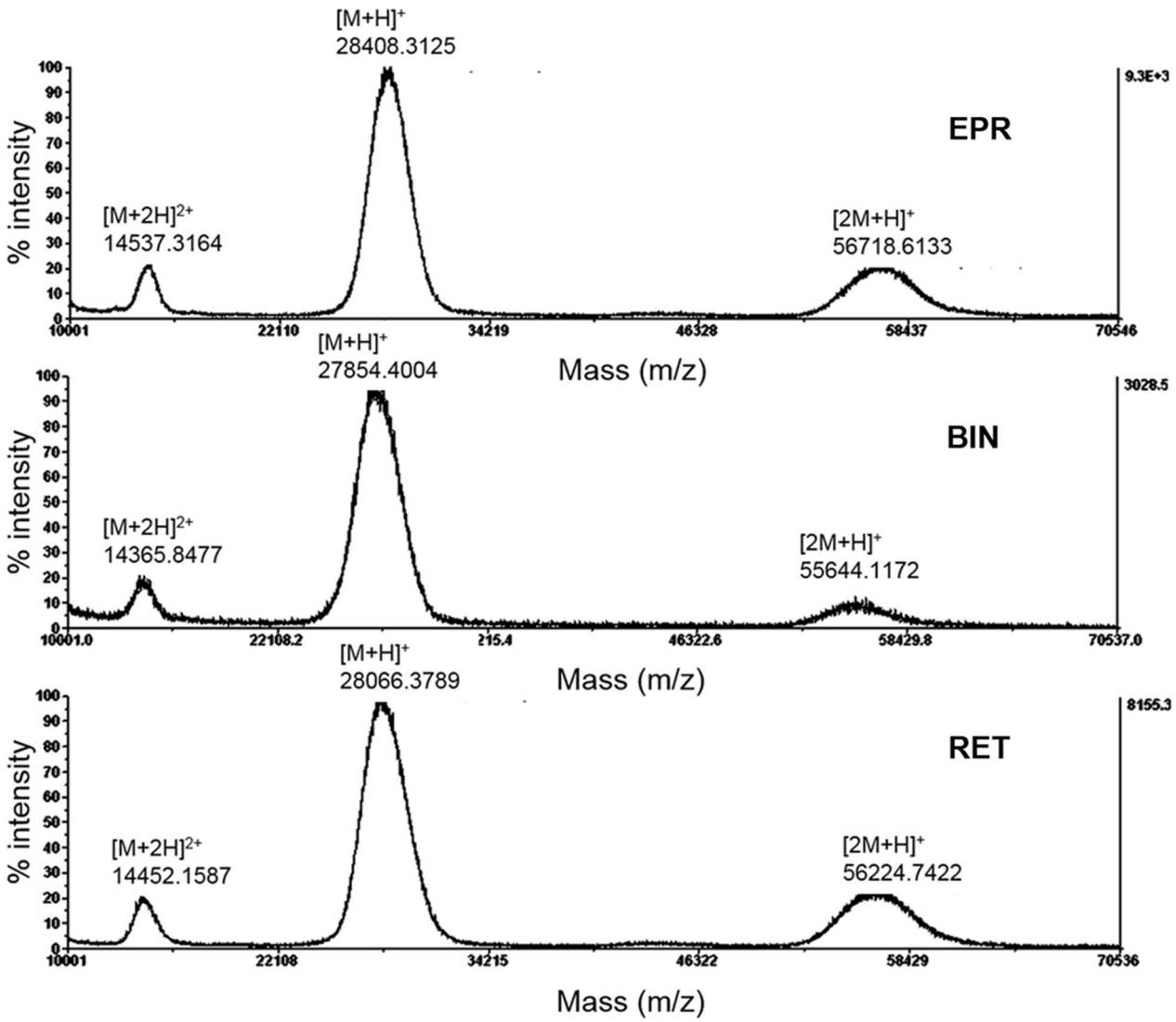

Fig. 2 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF–MS) positive ion spectra of Eprex®, Binocrit®, and Retacrit®. 1 μL of each recombinant human rhEPO solution, corresponding to 336 ng of protein content, was mixed with 1 μL of sinapic acid and analyzed with a laser at 337 nm. *BIN* Binocrit®, *EPR* Eprex®, *RET* Retacrit®

The qualitative analysis we conducted provided information about the purity of the sample under analysis but it was not able to provide information on the amount of rhEPO present. Therefore, a quantitative analysis was also carried out to compare the concentrations of rhEPO in the different pharmaceutical preparations.

3.2 Quantitative Analysis of Whole Proteins by HPLC

Before conducting the quantitative analysis of different pharmaceutical preparations, a calibration curve was performed (Electronic Supplementary Material Fig. S2). The originator EPR was used as a standard, since there were no commercially available standards for rhEPO. The linearity was studied at between 33.6 and 336 ppm. The method proved to be linear with an R^2 of 0.9994. The quantitative analyses of BIN and RET were then conducted and the results showed that the BIN and RET concentrations were, respectively, 336.032 ± 0.032 and 335.996 ± 0.026 ppm (Electronic Supplementary Material Fig. S3). Results obtained for each pharmaceutical preparation were in agreement with the claims on the label by the respective manufacturers.

3.3 Protein Analysis

To evaluate the protein content present in the final product, an SDS-PAGE was also performed (Electronic Supplementary Material Fig. S4). Results showed the presence of

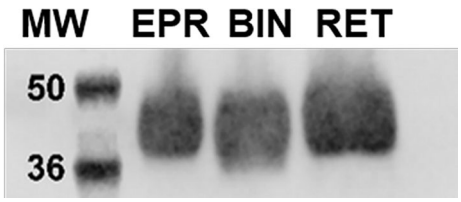

Fig. 3 Representative immunoblot of Eprex®, Binocrit®, and Retacrit® obtained using an anti-human rhEPO antibody. In the sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE) precast gel, 10 µL of each recombinant human rhEPO solution was loaded, corresponding to 3.36 µg of protein content. The Western blot protein analysis revealed a single band in all three of the comparative drugs with an apparent molecular weight of 36 kDa. *BIN* Binocrit®, *EPR* Eprex®, *MW* molecular weight, *RET* Retacrit®

a single diffuse band, due to the presence of glycoforms, with an apparent molecular weight of 36 kDa [32]. This result confirmed that, as already indicated by the HPLC analyses, there were no other protein impurities in the composition of each final product. Furthermore, Western blot analysis with a specific monoclonal anti-hEPO antibody confirmed that the band viewed by SDS-PAGE was effectively rhEPO (Fig. 3).

3.3.1 Structural Analyses of Drugs

To better characterize the chemical structure of rhEPO, the originator and biosimilar drugs were also studied with a proteomic approach. Samples were separated by SDS-PAGE (see Electronic Supplementary Material Fig. S4), digested by trypsin, and analyzed by MALDI-TOF/TOF mass spectrometry. The results are reported in Table 4, which shows that 15 identical peptides were present in each drug. The data were then compared to the peptides obtained from in silico digestion of EPO alfa, based on peptide mass [33] (Table 4—MH^+ calculated column). The small peptides consisting of only one or two amino acids were lost during sample purification or suppressed due to interference by matrix ions in the low *m*/*z* range. Fifteen peptides were found to be identical to the peptides obtained from in silico digestion except for two peptides containing N-glycosylation (Table 4—peptides 48–72 and 104–124), which have not been determined after tryptic digestion. This could be due to the fact that, as reported in the literature, the MALDI ionization of peptides cannot occur in the presence of the N-linked carbohydrates [31, 34–36]. In order to clarify this point and to complete the analysis starting from whole proteins, the carbohydrate chains were cleaved from the peptides by treatment with PNGase F [37]. Before purifying the sample by SDS-PAGE (Fig. 4), an aliquot (1 µL) of the reaction mixture was analyzed by MALDI-TOF–MS in linear mode (Fig. 5). Figure 5 shows that the principal peak at an *m*/*z* value of approximately 18,900 represented the single charge of rhEPO without N-glycosylation. The *m*/*z* value of about 9500 represented the double charge of relative rhEPO and the *m*/*z* value of about 37,800 represented the single charge of relative rhEPO dimer.

Gels obtained by SDS-PAGE (Fig. 4) were subsequently submitted to tryptic digestion for peptides identification. The digested samples were then analyzed by mass spectrometry MALDI-TOF/TOF to verify the presence of the missing peptides (Table 5). Table 5 shows that, after both digestions, the two peptides (48–72 and 104–124) were determined and that both were present in all three drugs and coincided with the peptides obtained from in silico digestion [33].

These results allowed us to conclude that the originator EPR and biosimilars BIN and RET are endowed with the same peptide sequence, which is coincident with the amino acid sequence of EPO alfa.

3.3.2 Isoforms Detection

The 2D-PAGE technique was then applied to investigate the possible presence of isoforms in the three pharmaceutical preparations. In the literature, capillary electrophoresis was used to discover chemical differences between several isoform of these drugs [38]. Here, these isoforms were discovered and separated by 2D-PAGE (METHOD_A) [32, 39]. Results obtained with this approach clearly demonstrated that, although the peptide sequence was the same between the three drugs, differences could be observed (Fig. 6a, c, e). Comparing the three gels, the trend of BIN was different from RET and EPR; indeed, there were differences in the molecular weight of BIN that did not appear in the other two drugs. To better investigate if the variation in the molecular weight was due to the presence of different sugar chains in N-glycosylation, a 2D-PAGE (METHOD_B) was performed after PNGase F digestion of the three drugs. From this analysis (Fig. 6b, d, f), different isoforms but with the same molecular weight were obtained in each pharmaceutical preparation. Therefore, it could be concluded that differences in the molecular weight previously reported for BIN were due to the presence of different carbohydrate chains compared to EPR and RET.

3.4 Functional Analyses

To investigate whether or not differences, observed from a structural point of view, could have an impact on rhEPO

Table 4 Matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF–MS)-positive ion spectrum of Eprex®, Binocrit®, and Retacrit® after trypsin digestion compared with in silico analysis (MH^+ calculated)

Peptide	Sequence	MH^+ $(m/z)^a$ calculated[b]	MH^+ $(m/z)^a$ observed		
			EPR	BIN	RET
28–31	APPR	440.2616	440.2793	440.2691	440.2606
32–37	LICDSR (Cys_CAM: 34)	763.3767	763.4197	763.4238	763.4025
38–41	VLER	516.314	516.343	516.3317	516.3189
42–47	YLLEAK	736.4239	736.4591	736.4719	736.4506
48–72	EAENITTGCAEHCSLNENITVPDTK (Cys_CAM: 56, 60)	2803.2509[c]	nd	nd	nd
73–79	VNFYAWK	927.4723	927.517	927.5312	927.5057
73–80	VNFYAWKR (1 missed cleavage)	1083.5734	1083.556	1083.5669	1083.5288
80–103	RMEVGQQAVEVWQGLALLSEAVLR (1 missed cleavage)	2683	2682.544	2682.5193	2682.4392
81–103	MEVGQQAVEVWQGLALLSEAVLR	2526.3384	2526.431	2526.4316	2526.3394
104–124	GQALLVNSSQPWEPLQLHVDK	2359.2404[d]	nd	nd	nd
125–130	AVSGLR	602.362	602.3907	602.3914	602.377
125–137	AVSGLRSLTTLLR (1 missed cleavage)	1386.8427	1387.936	1387.9481	1387.8857
131–137	SLTTLLR	803.4985	803.5432	803.5517	803.5271
144–158	EAISPPDAA**S**AAPLR	1465.7645	1465.838	1465.8389	1465.8013
144–158	EAISPPDAA**S**AAPLR + GalNAc + Galattosio	1830.9526	1830.978	1830.9824	1830.9257
159–166	TITADTFR	924.4785	924.5321	924.5418	924.5117
178–180	LFR	435.2714	435.2868	435.2772	435.2696
171–181	VYSNFLRGKLK (2 missed cleavage)	1324.7735	1324.736	1324.7417	1324.6876
171–177	VYSNFLR	898.4781	898.5297	898.5392	898.5113
178–181	GKLK (1 missed cleavage)	445.3133	445.0568	445.0473	445.0364
182–189	LYTGEACR (Cys_CAM: 188)	969.4458	969.4949	969.5083	969.4836

BIN Binocrit®, *EPR* Eprex®, *nd* not detected, *PNGase F* peptide-N4-(acetyl-b-glucosaminyl)-asparagine amidase F, *RET* Retacrit®

[a] All values are monoisotopic masses

[b] Theoretical protonated molecule ions of peptides based on peptide mass [33]

[c] Plus 2 Da after peptide-N4-(acetyl-b-glucosaminyl)-asparagine amidase-catalyzed deglycosylation [34, 35]

[d] Plus 1 Da after peptide-N4-(acetyl-b-glucosaminyl)-asparagine amidase-catalyzed deglycosylation [34, 35]

biological activity, preclinical functional analyses in the model, both in vitro and in vivo, were conducted, using innovative experimental approaches.

3.4.1 In Vitro Proliferation Assay

The in vitro functional analysis was conducted using the experimental model of the TF-1 cell line, derived from a patient diagnosed with erythroleukemia. This cell line is growth-factor dependent [40, 41] and responds to several hematopoietic growth factors, including EPO, granulocyte–macrophage–colony-stimulating factor, Interferon-γ, interleukin (IL)-3, IL-4, IL-5, IL-6, IL-13, leukemia inhibitor factor (LIF), nerve growth factor, stem cell factor, and tumor necrosis factor-α [42].

TF-1 cells were treated with the originator and biosimilar rhEPO at different concentrations ranging from 0 to 10 IU/mL for 72 h. The length of treatment was chosen on the basis of published data [43].

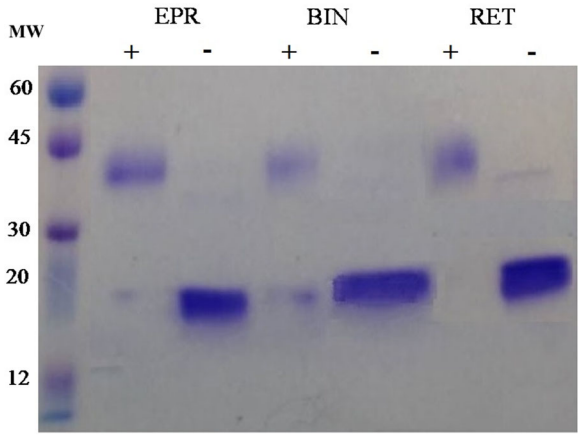

Fig. 4 Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE) purification of Eprex®, Binocrit®, and Retacrit® before (+) and after (−) PNGase F digestion. *BIN* Binocrit®, *EPR* Eprex®, *MW* molecule weight, *RET* Retacrit®

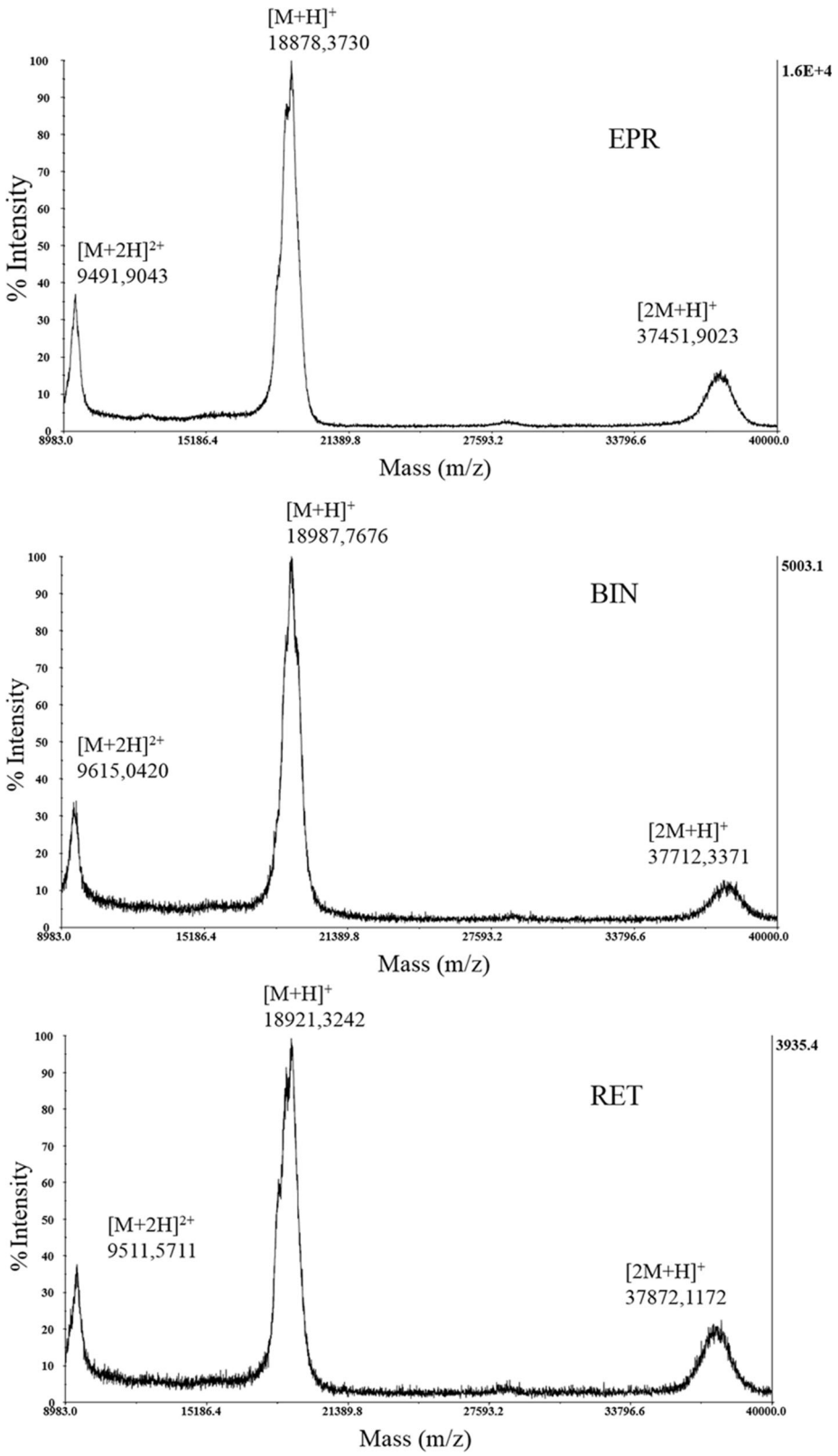

Fig. 5 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF–MS) positive ion spectra of Eprex®, Binocrit®, and Retacrit®. 1 μL of each recombinant human rhEPO solution, after PNGase F digestion, was mixed with 1 μL of sinapic acid and analyzed with a laser at 337 nm

As reported in Fig. 7, exposure of TF-1 cells to increasing concentrations of EPR, BIN, or RET induced a stimulation of the cell proliferation rate. Comparison of the concentration–response curves between drugs did not show any significant differences ($p = 0.6748$; $F = 0.3941$); indeed, the EC_{50} values were 0.22 (95 % CI

Table 5 Matrix-assisted laser desorption/ionization time-of-flight/-time-of-flight mass spectrometry (MALDI-TOF/TOF–MS) analyses of the peptides containing N-glycosylation of Eprex®, Binocrit®, and Retacrit® before and after PNGase F digestion and after trypsin digestions compared with in silico analysis (MH^+ calculated)

Peptide	Sequence	MH^+ (m/z)[a] calculated[b]	MH^+ (m/z)[a] observed					
			EPR_W	EPR_D	BIN_W	BIN_D	RET_W	RET_D
48–72	EAENITTGCAEHCSLNENITVPDTK (Cys_CAM: 56, 60)	2803.2509[c]	nd	2805.4985	nd	2805.4768	nd	2805.4790
104–124	GQALLVNSSQPWEPLQLHVDK	2359.2404[d]	nd	2360.4358	nd	2360.4250	nd	2360.4326

BIN Binocrit®, *D* after PNGase F digestion, *EPR* Eprex®, *nd* not detected, *PNGase F* peptide-N4-(acetyl-b-glucosaminyl)-asparagine amidase F, *RET* Retacrit®, *W* before PNGase F digestion

[a] All values are monoisotopic masses

[b] Theoretical protonated molecule ions of peptides based on peptide mass [33]

[c] Plus 2 Da after PNGase F-catalyzed deglycosylation [34, 35]

[d] Plus 1 Da after PNGase F-catalyzed deglycosylation [34, 35]

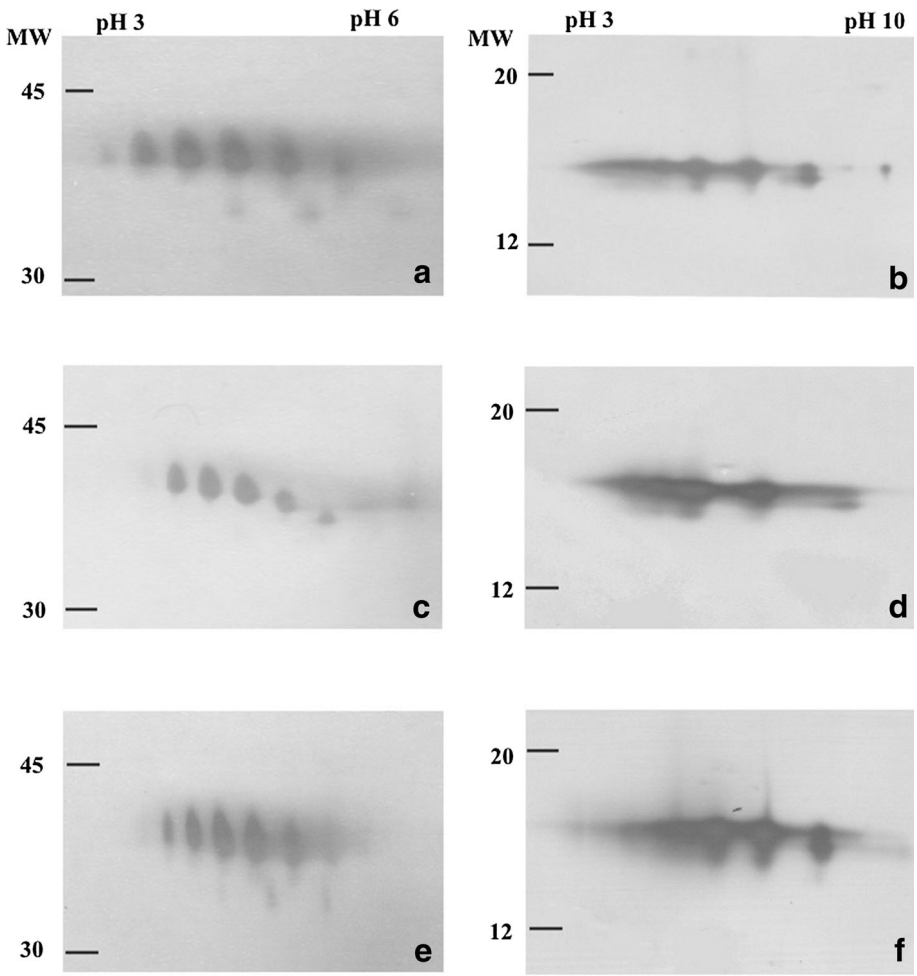

Fig. 6 Two-dimensional gel electrophoresis (2D-PAGE) of before EPR, BIN, and RET (**a**, **c** and **e**, respectively) and after (**b**, **d** and **f**, respectively) peptide-N4-(acetyl-b-glucosaminyl)-asparagine amidase F (PNGase F) digestion. EPR Eprex®, BIN Binocrit®, RET Retacrit®, *MW* molecular weight

0.09462–0.5304), 0.19 (95 % CI 0.1069–0.3355), and 0.30 IU/mL (95 % CI 0.1592–0.5781) for EPR, BIN, and RET, respectively. Time-course experiments up to 120 h, at the respective EC_{50} values, demonstrated that the reduction of the viability reached its maximum at 72 h and did not change afterwards (data not shown).

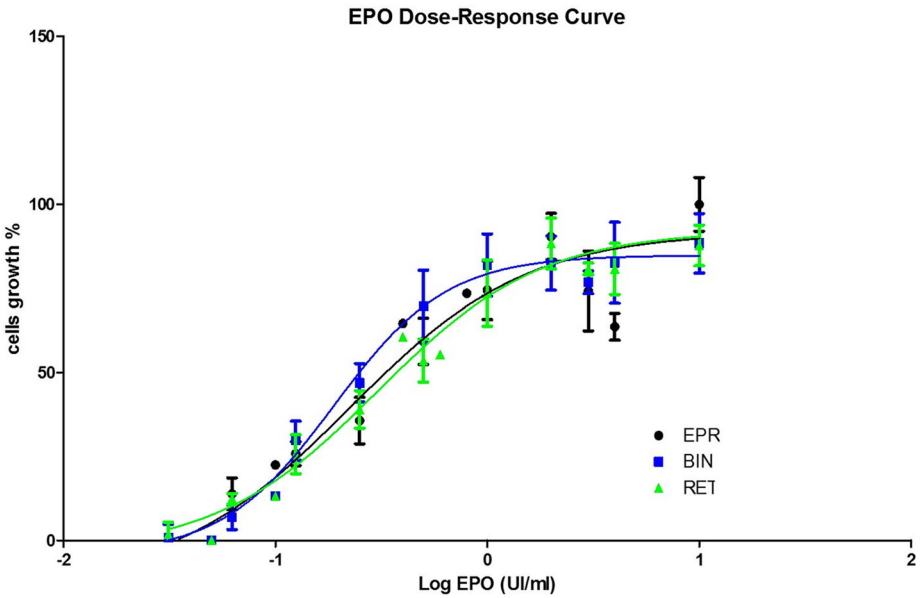

Fig. 7 Sigmoidal dose response curve of three different rhEPOs; EPREX® (*black*), Binocrit® (*blue*), and Retacrit® (*green*). *BIN* Binocrit®, *EPO* rhEPO, *EPR* Eprex®, *RET* Retacrit®

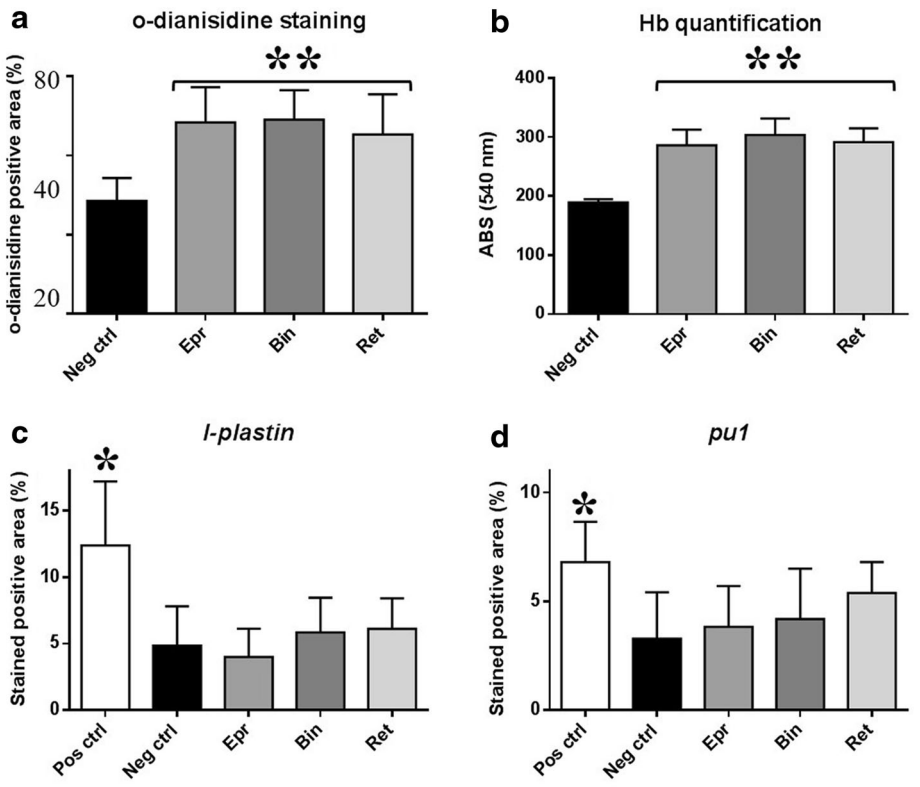

Fig. 8 Eprex®, Binocrit®, and Retacrit® injection effects on 48 and 72 hpf zebrafish embryos. Negative controls were injected with 0.05% phenol red solution. Positive controls were *E. coli* JM109 bacteria. Hemoglobin (HB) content was quantified by **a** O-dianisidine staining and **b** modified Drabkin protocol. Macrophages and neutrophils quantification was performed by whole-mount in situ hybridization with **c** *l-plastin* and **d** *pu1* probes. Data are the mean ± standard deviation of 3 experiments. *Epr* Eprex ®, *Bin* Binocrit®, *Ret* Retacrit®, *ctrl* control

3.4.2 In Vivo Hematopoiesis Stimulation

Increasing evidence suggests that zebrafish (Danio Rerio) are becoming a very interesting animal model in different fields, including pharmacology, as they represent a less expensive and more manageable model organism to conduct functional analyses than standard animal models [44]. As a matter of fact, zebrafish are widely used to study in vivo vertebrate hematopoiesis [45]. Erythrocytes, together with macrophages and granulocytes, are the first blood cells to enter the bloodstream at around 24 hpf [46]. As the main function of EPO is the proliferation of early

erythroblastoid cells, zebrafish embryos may thus represent a suitable animal model to study the effects of exogenous administered EPO in vivo.

EPR, BIN, and RET, at a 24 IU/mL concentration, were injected into the common cardinal vein of healthy zebrafish embryos at 48 hpf. Groups of 20–25 embryos coming from the same batch of fertilized eggs were used for each experimental point. Embryos were incubated at 28 °C for 2–4 h after injection to let the drugs act. Preliminary experiments have been conducted, injecting different rhEPO doses (2–48 IU/mL), to establish the most efficient treatment (data not shown). o-Dianisidine staining was performed to detect red blood cells. Figure 8 shows the percentage of o-dianisidine-positive area, proportional to the amount of red blood cells, measured in the trunk and in the tail of each embryo. Embryos treated with all the three compounds showed a statistically significant increase of o-dianisidine-positive area compared with the negative controls. Indeed, embryos injected with EPR, BIN, and RET showed a 1.69-, 1.72-, and 1.58-fold increase of erythrocytes content, respectively, when compared with the negative controls.

A modified Drabkin protocol was used to quantify the amount of hemoglobin in total embryo extracts. Figure 8 showed the hemoglobin absorbance of groups of 20 embryos for each experimental point. The measured absorbance was proportional to hemoglobin content, and it was significantly increased in embryos treated with EPO (EPR 1.51-, BIN 1.60-, and RET 1.54-fold increase) when compared with the negative controls.

The results showed that EPR, BIN, and RET were able to interact with zebrafish EPO receptor, leading to an increase of hemoglobin content, proportional to the number of circulating erythrocytes. Effects on hematopoiesis were not significantly different between the originator and biosimilar rhEPO.

Finally, to verify if samples could induce inflammation, a WISH, using *lplastin* and *pu1*as probes to detect leukocytes, was performed. EPR, BIN, and RET were injected into the optic capsule of healthy zebrafish embryos at 72 hpf. It is known that leukocytes are normally absent in this anatomical region [28]. Injected embryos were incubated at 28 °C for 2 h after injection to let the drugs act and then WISH was performed. As shown in Fig. 8, both macrophages and neutrophils were attracted to the injection site by the presence of *E. coli* bacteria (positive control), while EPR, BIN, and RET didn't show any chemoattractive properties. The leukocytes signal intensity in positive controls was 2.56- and 2.07-fold higher than negative controls for *lplastin* and *pu1* probes, respectively. In contrast, the signal quantification in embryos treated with all three compounds was comparable with that of the negative controls.

4 Conclusion

The results presented here suggest an innovative and fast approach for a comparison of a biotechnology drug with its respective biosimilars. Briefly, the structural comparison has been assessed by some already recognized techniques such as HPLC and MALDI-TOF–MS for qualitative and quantitative analysis of the protein content and a 2D-PAGE technique for isoforms detection. The biological activity of these drugs was studied at the preclinical level using two different approaches: an in vitro study on the human cell line and an in vivo study using the innovative experimental animal model represented by zebrafish embryos.

The robustness and reliability of this combined approach has thus been validated with an early screening of biosimilarity between the originator rhEPO EPR and its commercially available biosimilars BIN and RET. Briefly, chemical analysis showed that the quantitative concentrations of rhEPO were in agreement with the label claims made by the corresponding manufacturers. Furthermore, the qualitative analysis performed by HPLC demonstrated that the three drugs had a single main peak, with the only difference being in the drug RET, in which two other peaks were found; however, these were attributed to the different composition of the excipients. Moreover, with the MALDI-TOF/TOF–MS we demonstrated that all three drugs had the same amino acid sequence. The chemical differences were found only at the level of isoforms containing N-glycosylation; however, functional in vitro and in vivo studies did not show any differences and confirmed the similarity of BIN and RET to their originator EPR.

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Compliance with Ethical Standards

Conflict of interest All authors, Alessandra Gianoncelli, Sara A. Bonini, Michela Bertuzzi, Michela Guarienti, Sara Vezzoli, Rajesh Kumar, Andrea Delbarba, Andrea Mastinu, Sandra Sigala, Pierfranco Spano, Luca Pani, Sergio Pecorelli, and Maurizio Memo, did not receive funds related to the subject of this manuscript and they declare that they have no conflicts of interest.

Author contributions All authors made substantial contributions to the study development and manuscript writing. All authors were involved in experimental design and planning, structural and functional experiments, and results interpretation and discussion and final manuscript revision. All authors read and approved the final manuscript. Maurizio Memo is the guarantor for the overall content.

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