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Computational and functional analysis of biopharmaceutical drugs in zebrafish: Erythropoietin as a test model

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ABSTRACT

The zebrafish (*Danio rerio*) is a very popular vertebrate model system, especially embryos represent a valuable tool for *in vivo* pharmacological assays. This is mainly due to the zebrafish advantages when compared to other animal models.

Erythropoietin is a glycoprotein hormone that acts principally on erythroid progenitors, stimulating their survival, proliferation and differentiation. Recombinant human erythropoietin (rhEPO) has been widely used in medicine to treat anemia and it is one of the best-selling biotherapeutics worldwide. The recombinant molecule, industrially produced in CHO cells, has the same amino acid sequence of endogenous human erythropoietin, but differs in the glycosylation pattern. This may influence efficacy and safety, particularly immunogenicity, of the final product.

We employed the zebrafish embryo as a vertebrate animal model to perform *in vivo* pharmacological assays. We conducted a functional analysis of rhEPO alpha Eprex® and two biosimilars, the erythropoietin alpha Binocrit® and zeta Retacrit®.

By *in silico* analysis and 3D modeling we proved the interaction between recombinant human erythropoietin and zebrafish endogenous erythropoietin receptor. Then we treated zebrafish embryos with the 3 rhEPOs and we investigated their effect on erythrocytes production with different assays. By real time-PCR we observed the relative upregulation of *gata1* (2.4 ± 0.3 fold), embryonic α-*Hb* (1.9 ± 0.2 fold) and β-*Hb* (1.6 ± 0.1 fold) transcripts. A significant increase in Stat5 phosphorylation was also assessed in embryos treated with rhEPOs when compared with the negative controls. Live imaging in tg (*kdrl*:EGFP; *gata1*:dsred) embryos, o-dianisidine positive area quantification and cyanomethemoglobin content quantification revealed a 1.8 ± 0.3 fold increase of erythrocytes amount in embryos treated with rhEPOs when compared with the negative controls. Finally, we verified that recombinant human erythropoietins did not cause any inflammatory response in the treated embryos.

Our data showed that zebrafish embryo can be a valuable tool to study *in vivo* effects of complex pharmacological compounds, such as recombinant human glycoproteins, allowing to perform fast and reproducible pharmacological assays with excellent results.

Abbreviations: hpf, hours post fertilization; dpf, days post fertilization; CHO, Chinese hamster ovary; EPO, erythropoietin; EPOR, erythropoietin receptor; rhEPO, recombinant human erythropoietin; *hepo*, human erythropoietin gene; hEPO, human erythropoietin protein; *hEPOR*, human erythropoietin receptor gene; hEPOR, human erythropoietin receptor protein; *zepo*, zebrafish erythropoietin gene; zEpo, zebrafish erythropoietin protein; *zepor*, zebrafish erythropoietin receptor gene; zEpor, zebrafish erythropoietin receptor protein; WISH, whole mount *in situ* hybridization.

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1. Introduction

The zebrafish (*Danio rerio*) is widely used as an experimental vertebrate animal model in many fields of scientific research. For instance, because of eggs transparency, rapid *ex utero* development and easy access to experimental manipulation, zebrafish embryos represent a valuable tool for *in vivo* pharmacological assays, offering several advantages when compared to other animal models. Strong correlations have been observed between the effects of many compounds tested both on zebrafish and humans. Moreover, drug administration to embryos is simple and fast, and a large number of individuals can be analyzed at the same time [1–3].

The zebrafish is also a very useful animal model to study *in vivo* vertebrate hematopoiesis [4]. The genes and the molecular pathways involved in hematopoiesis are very well conserved between zebrafish and higher vertebrates. Moreover, morphology and function of zebrafish blood cells are comparable to their mammalian counterparts [5,6].

The first myeloid progenitors mature into primitive macrophages and granulocytes, which have the ability to migrate towards the site of a wound and to phagocytose foreign particles by 2 days post fertilization (dpf). Proerythroblasts are the first blood cell entering the circulation around 24 hpf and mature into primitive erythrocytes, that are the only circulating red blood cells till 4 dpf [5,7].

Zebrafish erythrocytes show the typical elliptical shape and, as all the other nonmammalian vertebrates, they retain their nucleus also in the mature adult stage. Erythropoiesis is finely regulated by different molecular mechanisms, such as transcription factors (*i.e.*, *scl*, *lmo* and *gata* family) or activation of signaling pathways, the most important of which is the erythropoietin pathway [8].

Erythropoietin (EPO) is a glycoprotein hormone that acts principally on erythroid progenitors, stimulating their survival, proliferation and differentiation. In mammals, EPO is produced by fetal hepatocytes during development and mainly by peritubular interstitial fibroblasts in adult kidney. EPO binding to its homodimeric receptor (EPOR) on progenitors surface, causes a conformational change in EPOR intracellular domain. This leads to JAK2 transphosphorylation and EPOR activation, which activates the downstream signaling cascade *via* STAT5, PI3K/AKT and MAPK pathways [9].

Human erythropoietin gene (*hEPO*) was cloned and characterized for the first time in 1985 [10,11]. Since then, recombinant DNA technology allowed the production of recombinant human erythropoietin (rhEPO) using mammalian Chinese hamster ovary (CHO) cells as expression system. rhEPO has been widely used to treat patients with chronic anemia caused by renal failure, but also chemotherapy-associated anemia in cancer patients, severe anemia due to antiviral therapy in AIDS patients and perioperative anemia after surgery [12].

In 2007, two decades after human erythropoietin synthesis, zebrafish erythropoietin and erythropoietin receptor genes (*zepo* and *zepor*, respectively) were cloned and biochemically characterized [13,14]. It has been shown that, despite the low identity score between human and zebrafish erythropoietin proteins (hEPO and zEpo, respectively), the most important residues are maintained among them. As well, protein sequences of human and zebrafish erythropoietin receptors (hEPOR and zEpor, respectively) share low identity score, but very well conserved functional domains [15]. The biological function of both zEpo and zEpor seems to be maintained: downregulation of *zepo* or *zepor* genes causes a dramatic decrease of hemoglobin, while overexpression of *zepo* leads to an increase in circulating erythroid cells [15].

Due to these findings, we employed the zebrafish embryo as a vertebrate animal model to study the effects of 3 commercially available rhEPOs *in vivo*. In the present paper we performed a functional analysis of Eprex® and its biosimilars Binocrit® and Retacrit® [16]. Eprex® was the first patented rhEPO approved by FDA in 1989, while the erythropoietin alpha Binocrit® and zeta Retacrit® were authorized as Eprex® biosimilars in 2007. Because of the similar amino acid sequence and the glycosylation pattern, the erythropoietin zeta Retacrit® is considered homologous of the erythropoietins alpha [17]. Bioequivalence of erythropoietin alpha and zeta was demonstrated by pharmacokinetic studies [18]. The biosimilarity between the 3 different rhEPO proteins was also demonstrated in our laboratory by a proteomic approach (submitted manuscript).

In the present study we first performed a computational analysis to predict if rhEPO could interact with zEpor. We treated zebrafish embryos with the 3 rhEPOs and we investigated their effect on erythrocytes production, analyzing changes both in quality and in quantity. Moreover, we examined whether the presence of these exogenous compounds could cause an inflammatory response in the treated embryos. In this paper, we firstly show that zebrafish embryo can be a good animal model to study *in vivo* effects of complex pharmacological compounds.

2. Material and methods

2.1. In silico *analysis*

The amino acid sequence of rhEPO published on the European Pharmacopoeia 8th edition (http://online6.edqm.eu/ep805/) was used to BLAST search the human and zebrafish Ensembl genome sequence database at http://www.ensembl.org The database supplies the more updated genome assembly, which is GRCh38 (Db version 78.38) for human, and Zv9 (Db version 78.9) for zebrafish [19]. The "tblastn" and the "blastp" algorithms allowed us to identify the human endogenous erythropoietin transcript and protein sequences, respectively. Three zebrafish splice variants of the same erythropoietin gene were found, and each of these transcripts encoded for a protein. The hEPO protein information collected in the UniProt database (http://www.uniprot.org) were used to obtain hEPOR entry, in the "Protein Interaction" section [20]. Ensembl full length sequences of human erythropoietin receptor transcript and protein were deduced and used to BLAST search the zebrafish Zv9 Ensembl genome assembly. One full length zebrafish transcript, encoding for one erythropoietin receptor protein, was identified.

A comparative analysis of gene organization between human and zebrafish erythropoietin and EPO receptor genes was carried out, employing information supplied by the Ensembl database [19].

Synteny analysis was performed using the Synteny database (http://syntenydb.uoregon.edu/synteny_db/) and the Genomicus genome browser (http://www.genomicus.biologie.ens.fr/genomicus-78.01/cgi-bin/search.pl) [21,22].

The protein sequences of rhEPO, endogenous hEPO and zEpo were employed to perform multiple sequence alignment by ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) [23]. The same analysis was carried out using hEPOR and zEpor protein sequences.

2.2. Homology modeling

To better evaluate the functional similarity between human and zebrafish erythropoietin receptors and to assess if rhEPO could recognize and bind the zebrafish erythropoietin receptor, a 3D model of zEpor was created. The zEpor protein sequence (NP_001036799.1) and PSI-BLAST were used to identify a list of 7 suitable templates from the RCSB protein data bank (http://www.rcsb.org/pdb/home/home.do) [24].

The 1EER complex, representing the extracellular portion of hEPOR, was selected as best template given its similarity with zEpor

and the inclusion of the human EPO substrate. A single chain (chain B) from this template was used in I-Tasser (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) [25] to perform homology modeling and generate the predicted structure of zEpor extracellular domain, identified from residues Y26 to T232. The N-terminal domain and the transmembrane and intracellular regions were excluded. The final model of zEpor monomer was then superimposed on the human protein structure to generate a prediction of the zEpor homo-dimer and to evaluate the placement of relevant residues involved in disulfide bonds and contact surface with hEPO. Protein structure was visualized and refined using PyMOL v1.7.0.

To better analyze the possible interaction between zEpor homodimer and hEPO, a complex between the two molecules was predicted using GRAMM-X [26], with the 3 residues F108, I164 and Y207, imposed as obligate contact amino acids in zEpor. The best predicted complex that resemble the correct hEPO orientation was then used to evaluate contact surfaces and placement of relevant residues in zEpor model.

The surface interaction between zEpor and hEPO was evaluated using SC program from CCP4 package [27,28], considering the predicted complex of hEPO ligand and zEpor structure, and an interaction space of 5 Å. The same calculation was performed on the 1EER structure considering chain B of the hEPOR and hEPO molecules.

2.3. Fish maintenance and egg collection

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

Adult zebrafish of two strains were used for egg production: the wild type AB strain and the transgenic line *casper* tg (*kdrl*:EGFP; *gata1*:ds-red), the last one a kind gift of Prof. Cotelli (Department of Biosciences, University of Milan). Fishes were maintained in the zebrafish facility at University of Brescia (Department of Molecular and Translational Medicine) under standard laboratory conditions as described, at 28 °C on a 14 h light/10 h dark cycle [29]. Fertilized eggs were collected immediately after spawning, harvested, washed and placed in fish water. The developing embryos were raised in a 28 °C incubator in 0.003% 1-phenyl-2-thiourea (Sigma–Aldrich) to prevent pigmentation.

2.4. rhEPO administration

Three commercially available rhEPOs, sold as injectable solutions in prefilled syringes (1 ml at 40,000 IU/ml), were used as stock solutions. The following batch were employed: Eprex® (Janssen-Cilag©) #DGS5G00, Binocrit® (Sandoz©) #47021202 and Retacrit® (Hospira©) #3E366G3.

Eprex®, Binocrit® and Retacrit® stock solutions were diluted to the final concentration of 48 IU/ml in 0.05% phenol red solution (Sigma–Aldrich). The 0.05% phenol red solution without any pharmaceutical compound was used as negative control. The compounds were administered in two different ways to the dechorionated embryos. For the subsequent erythrocytes visualization and quantification experiments, 4 nl of each compound was injected into the common cardinal vein of 48 hpf embryos [30]. To assess primitive myelocytes activation, 1 nl of each dilution was injected into the otic cavity of 72 hpf embryos. *Escherichia coli* JM109 bacteria in 0.05% phenol red solution were used as positive control [31].

2.5. Real time quantitative RT-PCR (Q-RT-PCR)

Total RNA was extracted from groups of 30 embryos for each injected compound using the RNAeasy kit (Qiagen) and 1 mg was transcribed into cDNA, using the M-MLV reverse transcriptase (Promega). Relative gene expression of *gata1*, α-*eHb* and β-*eHb* [32] was evaluated by Q-RT-PCR with the ViiA7 Real Time PCR System (Life Technologies), using the SYBR Green Method (Bio-Rad). Analysis was performed in triplicate, ΔCt was calculated using β-*actin* as the housekeeping gene. The experiment was repeated twice, using different groups of embryos.

2.6. Western blot

Total embryo protein extracts were obtained by homogenizing groups of 10 treated embryos in lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% Triton X-100 with a cocktail of protease and phosphatase inhibitors). For Western blot analysis 25 μg of protein extracts were loaded per lane on a 10% Mini-PROTEAN TGX Gel (Bio-Rad) and transferred to a nitrocellulose paper. The membrane was incubated with 1:200 rabbit anti-Phospho-Stat5 primary antibody (Cell Signaling) overnight at 4 °C, followed by 1:1500 IRDye 800RD or 680RD secondary antibody (LI-COR Biotechnology) for 1 h at RT. Membranes were washed and reprobed with anti-Actin primary antibody (Sigma–Aldrich). The Odyssey Infrared Imaging System (LI-COR Biotechnology) was employed for protein detection. The Western blot analysis was repeated 3 times.

2.7. Erythrocytes visualization and quantification

Treated embryos were incubated at 28 °C for 4 h after injection and then used for erythrocytes visualization and/or quantification. Each experiment was repeated 3 times.

Groups of 25 transgenic *casper* treated embryos were anesthetized in tricaine (Sigma–Aldrich) and photographed under a Leica MZ16F stereomicroscope equipped with GFP3 and G fluorescence filters, DFC 480 digital camera and LAS Leica Imaging software (Leica, Wetzlar, Germany). Red and green signals were quantified by using ImageJ 1.45 s image analysis software.

Groups of 25 wild type embryos for each injected compound were fixed in 4% paraformaldehyde overnight at 4 °C. O-Dianisidine staining was performed as described in literature to detect hemoglobin in red blood cells [13]. To visualize erythrocytes distribution, embryos were mounted in agarose-coated dishes and photographed. Images were used to quantify erythrocytes on selected region of interest by using ImageJ 1.45 s image analysis software.

Hemoglobin quantification on total embryo extract was performed by using a modified cyanomethemoglobin method [33]. Groups of 20 wild type embryos for each injected compound were anesthetized in tricaine (Sigma–Aldrich) and sonicated in 1 ml of Drabkin solution (Sigma–Aldrich). After centrifugation, absorbance of the supernatant of each sample was measured at 540 nm and it was linearly correlated with hemoglobin content.

2.8. Macrophages and granulocytes quantification

Groups of 40 treated embryos for each compound were incubated at 28 °C for 2 h after injection and then fixed in 4% paraformaldehyde in PBS overnight at 4 °C. Whole-mount *in situ* hybridization (WISH) was performed according to Thisse protocol [34]. *pu1*, *lplastin* and *mpx* were used as probes to detect leukocyte precursors, macrophages and neutrophils [7,35]. 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).

Leucocytes quantification was performed using ImageJ 1.45 s image analysis software. The experiment was repeated 3 times.

2.9. Statistical analysis

Quantifications are expressed as mean ± standard deviation of independent experiments. Statistical analysis were made using GraphPad Prism 6.01 version (GraphPad Software). Analysis of variance (one-way ANOVA) followed by Dunnett's test was performed to evaluate significant differences between the groups of data (p value < 0.05).

3. Results and discussion

In order to evaluate if zebrafish could be used as an animal model for rhEPO functional analysis, we first assessed the similarity between human and zebrafish erythropoietin receptors by computational analysis and 3D modeling techniques. Then we investigated the rhEPOs effects on erythrocytes production and their inflammatory response.

3.1. In silico *analysis and homology modeling*

In order to identify the *hEPO* gene ortholog in zebrafish, the 165 amino acids sequence of rhEPO was used as query in a BLAST search performed against the human and zebrafish Ensembl genome sequence database at http://www.ensembl.org [19]. The rhEPO sequence fully matched with the amino acids 28-192 of the human endogenous immature erythropoietin protein ENSP00000252723, corresponding to the RefSeq: NP_000790.2 on the Reference Sequence peptide database at NCBI (http://www.ncbi.nlm.nih.gov, RefSeq Release 69) [36]. The hEPO protein is encoded by the 1330 bp transcript ENST00000252723 (RefSeq: NM_000799.2), which is the product of the *hEPO* gene ENSG00000130427 located on human chromosome 7.

In zebrafish, according to literature, we found 3 splice variants of the same *zepo* gene (ENSDARG00000055163), located on zebrafish chromosome 7 [15]. The 3 *zepo* transcripts on the Ensembl database are called respectively *epo*-001 (ENSDART00000111066), *epo*-002 (ENSDART00000020288) and *epo*-201 (ENSDART00000077483). They correspond to the NCBI *zepo* isoforms L2 (RefSeq: NM_001038009), L1 (RefSeq: NM_001115128) and S (RefSeq: NM_001115127), respectively.

Both human and zebrafish genes have a very similar organization, consisting of 5 coding exons and 4 introns. The shortest *zepo* isoform 201 (or S isoform), with only four coding exons, differs from the other ones.

It is well established that a conserved co-localization of gene clusters among different species often correspond to a conserved protein function [37]. A synteny analysis was performed between the human and zebrafish chromosome 7, in the genomic regions that contain the erythropoietin gene (Fig. S1A and B). By using the Genomicus genome browser (http://www.genomicus.biologie.ens.fr/genomicus-78.01/cgi-bin/search.pl) [22] we found 5 ortholog genes in the erythropoietin syntenic region, two of which maintained the same orientation and 3 orientated in the opposite direction (Fig. S1A). The analysis was repeated by using the Synteny database (http://syntenydb.uoregon.edu/synteny_db/) [21], which allowed to evaluate a more extended genomic region. By analyzing a 100-gene window, 3 more ortholog genes associated with the erythropoietin gene were highlighted, and they maintained the same orientation on both chromosomes (Fig. S1B).

The amino acid sequences of hEPO and zEpo were employed to perform ClustalW multiple sequence alignment [23]. The 3 zebrafish splice variants of the *zepo* gene encode for 3 proteins, that are named respectively Epo-L1 (RefSeq: NP_001108600), Epo-L2 (RefSeq: NP_001033098) and Epo-S (RefSeq: NP_001108599). The 3 isoforms are identical, except for only few amino acids in the N-terminal signal peptide (Fig. S1C), that is cleaved in the 3 mature forms. The L1 isoform was used to perform protein alignment, because it is the longest isoform and the most abundant [15]. The hEPO amino acid sequence covered 99% of zEpo sequence, with 34.55% identity and 66% similarity (Fig. S1D). By using the information collected in the UniProt database (http://www.uniprot.org) [20] we identified in the human hEPO sequence (UniProt: P01588) the most important amino acids, responsible for erythropoietin post-translational modifications (Fig. S1D). The 4 cysteine Cys34, Cys56, Cys60 and Cys188 were conserved in the zEpo sequence (in position 33, 55, 59 and 181, respectively). These amino acids are essential to form two disulfide bond, necessary for the correct protein folding and function. Moreover, the O-glycosylation site in position 153 and 2 out of 3 N-glycosylation sites in position 65 and 110 were conserved in the zebrafish zEpo sequence (in position 144, 64 and 104, respectively). The glycosylation of erythropoietin is important for its correct function and half life [38,39] and these conserved residues suggest functional conservation between human and zebrafish erythropoietin.

The information supplied by the UniProt database (http://www.uniprot.org) [20] allowed us to identify, in the "Protein Interaction" section, the human full length hEPOR entry, UniProt: P19235. This was used to search the Ensembl database for hEPOR 508 amino acids full length protein sequence (ENSP00000222139) [19]. The hEPOR protein is encoded by the 2411 bp transcript ENST00000222139, which is the product of the *hEPOR* gene ENSG00000187266 located on human chromosome 19. The Ensembl human erythropoietin receptor protein and transcript sequences correspond to the NCBI database RefSeq: NP_000112 and RefSeq: NM_000121, respectively [36].

The hEPOR sequence was used to BLAST search the zebrafish Zv9 Ensembl genome assembly [19]. We identified one full length zebrafish transcript ENSDART00000064033 (RefSeq: NM_001043334) which is the product of *zepor* gene ENSDARG00000043609, located on zebrafish chromosome 3. The 1656 bp zebrafish transcript encoded for the 509aa zEpor protein ENSDARP00000064032 (RefSeq: NP_001036799).

The gene organization of both human and zebrafish erythropoietin receptor is very similar, constituted by eight coding exons and seven introns. The genomic regions that contain the erythropoietin receptor gene on human chromosome 19 and zebrafish chromosome 3 showed a high degree of synteny (Fig. S2A and B). The Genomicus genome browser underlined ten ortholog genes in the syntenic region (Fig. S2A), while by using a 100-gene window in the Synteny database we found nine more ortholog genes associated with the erythropoietin receptor gene on both human and zebrafish chromosomes (Fig. S2B) [21,22].

A ClustalW multiple sequence alignment was performed by using the amino acid sequences of hEPOR and zEpor as inputs (Fig. S2C) [23]. The human protein covered 97% of the whole zebrafish sequence, with an identity score of 27.11% and an overall similarity of 62%. The most important residues and motifs of the human hEPOR sequence, annotated in the UniProt database, were conserved also in the zebrafish zEpor sequence (Fig. S2C) [20]. In the extracellular region the four cysteine Cys52, Cys62, Cys91 and Cys107, necessary to form two disulfide bond, were conserved (respectively Cys44, Cys54, Cys83 and Cys100 in zEpor). The WSXWS motif, essential for the correct folding and function of the erythropoietin receptor, was present in both proteins (aa 233-237 in hEPOR, aa 221-225 in zEpor). Moreover, the 3 residues responsible for the ligand binding, Phe117, Met174 and Phe229 in hEPOR, were also conserved in the zEpor sequence (Phe108, Ile164 and Tyr217). The transmembrane domain had two con-

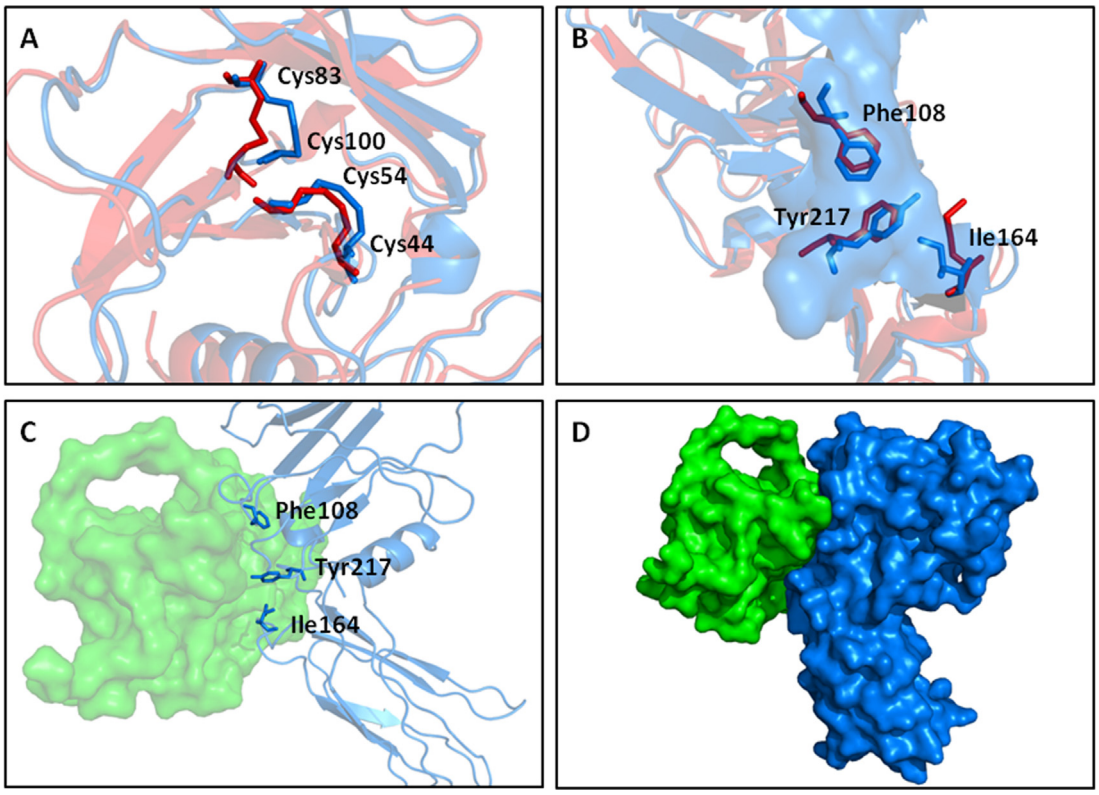

Fig. 1. Structural 3D model of zEpor extracellular domain. (A), (B) Superimposition of zEpor (in blue) on hEPOR (in red) showing: (A) the residues involved in disulfide bond formation; (B) the residues involved in EPO binding. (C), (D) Analysis of the interaction between zEpor (in blue) and hEPO (in green) showing: (C) the contact between hEPO and the three zEpor residues involved in EPO binding; (D) surface representation of the interaction between the two molecules (Sc score 0.507). Amino acid residues refers to zEpor RefSeq: NP_001036799. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

served leucine residues (Leu265-266 and Leu254-255 in human and zebrafish sequences, respectively), necessary for the correct receptor dimerization and the activation of the signaling cascade. The cytoplasmic region of both proteins contained Box1 (aa 282-290 in hEPOR, aa 271-279 in zEpor) and Box2 (aa 328-336 in hEPOR, aa 317-325 in zEpor), essential domains for the interaction and activation of JAK2. Also the ITIM motif (aa 452-457), containing two phospho-Tyr residues, was conserved. Three other phospho-tyrosine (Tyr468, Tyr485 and Tyr489), important for downstream signaling, were conserved in hEPOR and zEpor proteins.

To better evaluate the functional similarity between human and zebrafish erythropoietin receptors andto assess if rhEPO could recognize and bind the zebrafish erythropoietin receptor, a three-dimensional model of zEpor was created (Fig. S3). We used I-Tasser (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) and the structure of the extracellular portion of human EPOR (PDB: 1EER) to generate the predicted structural model of zEpor extracellular domain by homology modeling [25]. Superimposition on the human protein structure revealed that the four cysteine putatively involved in disulfide bonds in zEpor (Cys44, Cys54, Cys83 and Cys100) were placed near each other, in a configuration compatible with the bond (Fig. 1A). Analysis of the 3 conserved residues involved in EPO binding, namely Phe108, Ile164 and Tyr217, showed that they were placed in a configuration resembling those of the human corresponding Phe117, Met174 and Phe229, respectively (Fig. 1B). Moreover, the predicted contact surface of zEpor was similar to that of hEPOR (Fig. 1C and D; Fig. S3) [26]. The molecular docking of hEPO with the predicted structure of zEpor showed also an acceptable compatibility of contact surfaces (Sc score 0.507), compared to the score of the original human complex 1EER determined by crystallography (Sc score 0.71) [27,28].

3.2. Erythrocytes visualization and quantification

To perform a functional analysis of rhEPO *in vivo* we conducted several preliminary experiments in order to set the optimal concentration to be used in zebrafish embryos. We diluted Eprex® in 0.05% phenol red solution (Sigma–Aldrich) to a series of concentrations ranging from 2 to 48 IU/ml. The different Eprex® concentrations were injected into the common cardinal vein of 48 hpf embryos and they were incubated at 28 °C for up to 12 h after injection. As negative control embryos were injected with the 0.05% phenol red solution without drug. Finally we performed an o-dianisidine staining to detect red blood cells [13]. Time-course and concentration-curves demonstrated a concentration-dependent increase in the erythrocytes number, that reached its maximum 4 h after injection (data not shown). Based on the results obtained in these preliminary experiments, we used Eprex®, Binocrit® and Retacrit® at the concentration of 48 IU/ml.

To examine the *in vivo* effects of rhEPO administration on zebrafish erythropoiesis, the expression of 3 lineage-specific genes was investigated by real time Q-RT-PCR after rhEPO treatment. The analysis was performed in triplicate and the experiment was repeated twice, using different groups of embryos. Total RNA was extracted from groups of 30 embryos injected with Eprex®, Binocrit®, Retacrit® or negative controls and gene expression levels were normalized using β-*actin* as the housekeeping gene. The relative expression of *gata1* was significantly upregulated in embryos treated with rhEPOs compared to the negative controls (2.4 ± 0.3 fold increase), as well as the relative expression of α-*eHB* (1.87 ± 0.2 fold increase) and β-*eHb* (1.57 ± 0.1 fold increase). No statistically significant differences in gene expression have been found between Eprex® and its biosimilars (Fig. 2).

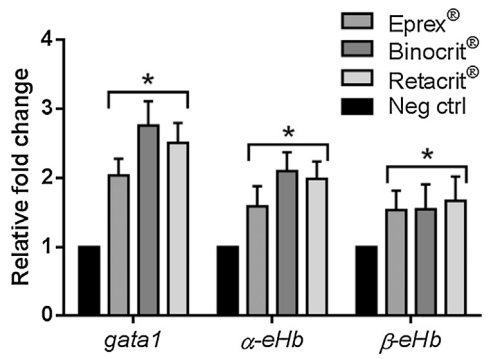

Fig. 2. Relative gene expression levels of *gata1*, *α-eHB* and *β-eHb* in embryos injected with negative control (Neg ctrl), Eprex®, Binocrit® or Retacrit®. Q-RT-PCR was performed using total RNA extracted from groups of 30 embryos for each injected compound. Data are expressed as fold change of target gene expression normalized to the internal control gene (*β-actin*). Data were analyzed according to the comparative Ct method. Asterisks indicate statistically significant fold changes of the 3 target genes expression ($p < 0.05$). The experiment was repeated twice, using different groups of embryos.

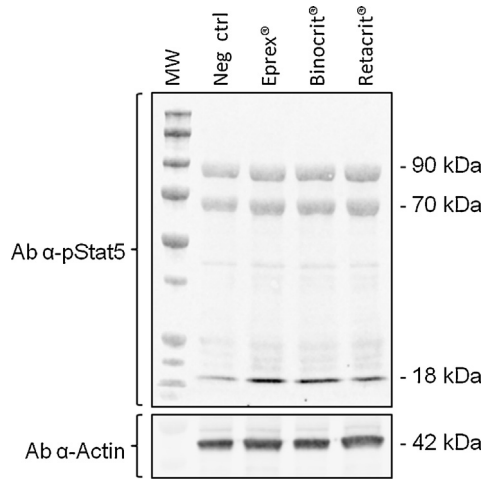

Fig. 3. Effects of Eprex®, Binocrit® and Retacrit® treatment on Stat5 phosphorylation. Western blot was performed on total protein extracts obtained from groups of 10 treated embryos. The anti-Phospho-Stat5 primary antibody revealed three isoforms of phosphorylated Stat5, at around 90, 70 and 18 kDa respectively. The anti-actin primary antibody was used as internal control. The western blot image was representative of 3 independent analysis.

To confirm that rhEPO was functionally active on zebrafish embryos, we investigated the effects of Eprex®, Binocrit® and Retacrit® injection on Jak/Stat pathway. The phosphorylation status of Stat5 protein was examined on total embryo protein extracts obtained from groups of 10 embryos for each injected compound. Indeed, rhEPO binding to zEpor is expected to cause Jak2 activation, which leads to Stat5 phosphorylation [9]. As shown in Fig. 3, the rabbit anti-Phospho-Stat5 primary antibody was able to detect basal levels of 3 different isoforms of phosphorylated Stat5 (Fig. 3, lane 1) [40]. After treatment with Eprex®, Binocrit® and Retacrit® the phosphorylation of Stat5, especially the shortest isoform, was clearly upregulated respect to the untreated negative controls (Fig. 3). Of note, to the best of our knowledge, only few papers studying phospho-Stat5 on total embryo protein extracts are available in literature. We will further investigate why the shortest isoform of Stat5 resulted the most phosphorylated one after rhEPO treatment.

The three rhEPOs were then injected in groups of 25 tg (*kdrl*:EGFP; *gata1*:ds-red) *casper* embryos, for subsequent live imaging acquisition. Double-channel fluorescence images (Fig. 4A) revealed that blood vessels (expressing EGFP) were pervious and red blood cells (expressing ds-red) were able to easily circulate in the whole embryo. Images acquired in single-red channel (Fig. 4B) highlighted an increase of fluorescence intensity in embryos injected with Eprex®, Binocrit® and Retacrit® compared to the negative controls. This effect was caused by the higher number of circulating erythrocytes in embryos treated with rhEPOs. It could be visualized both in the principal vessels (*i.e.* dorsal aorta and posterior cardinal vein) and in the smallest blood vessels (*i.e.* intersegmental vessels and caudal venous plexus) [30]. Quantification of the red and green fluorescence signal, performed using ImageJ analysis software, is reported in Fig. 4C and D as a mean of 3 independent experiments. The red signal intensity, proportional to the circulating erythrocytes, was 2.13 ± 0.09 fold higher in embryos treated with rhEPOs compared to the negative controls. No statistically significant differences were highlighted between the three biosimilars (Fig. 4C). On the contrary, the green signal intensity, produced by the endothelial cells of the blood vessels, remained constant in all the analyzed embryos (Fig. 4D).

Groups of 25 wild type embryos were fixed in 4% paraformaldehyde after injection with negative control or Eprex®, Binocrit® and Retacrit®, each group was incubated in o-dianisidine staining buffer and then photographed (Fig. 5A). Hemoglobin catalyzes the oxidation of o-dianisidine, producing a dark red-brown stain in cells containing hemoglobin [13]. Erythrocytes were quantified in the trunk and in the tail of each embryo by using ImageJ analysis software. Fig. 5B reports the results of three independent experiments: the percentage of o-dianisidine positive area is proportional to the amount of red blood cells measured in the region of interest. Embryos treated with rhEPOs showed a 1.67 ± 0.06 fold increase of erythrocytes content when compared to the negative controls. Interestingly, no differences have been found between Eprex® and its biosimilars also in the o-dianisidine staining assay.

These data were confirmed with a quantitative hemoglobin assay on total embryo extract by using a modified cyanomethemoglobin method [33]. According to the manufacturer, Drabkin's solution (Sigma–Aldrich) reacts with hemoglobin causing its oxidation to methemoglobin. In the presence of potassium cyanide, methemoglobin is converted to cyanomethemoglobin, which maximum absorbance can be measured at 540 nm. Groups of 20 wild type embryos injected with negative control solution or Eprex®, Binocrit® and Retacrit® were anesthetized in tricaine and used for the Drabkin assay. Fig. 5C shows the cyanomethemoglobin optical density (O.D.) at 540 nm of each group of treated embryos, as a mean of three independent experiments. The hemoglobin content was significantly increased (1.53 ± 0.05 fold) in embryos treated with rhEPO when compared with the negative control.

The results showed that Eprex®, Binocrit® and Retacrit® were able to interact with zebrafish epo-receptor, leading to an increase of hemoglobin content, proportional to the number of circulating erythrocytes. When compared among them Eprex®, Binocrit® and Retacrit® did not show any statistically significant differences.

3.3. Macrophages and granulocytes quantification

We then investigated whether Eprex® and its biosimilars Binocrit® and Retacrit® were able to activate primitive monocytes/macrophages and granulocytes neutrophils to induce an inflammatory state. The three rhEPOs samples, negative and positive (*E. coli* JM109 bacteria) controls were injected into the otic capsule of groups of 40 healthy zebrafish embryos at 72 hpf. It is well known that leukocytes are normally absent in that anatomical region, but external stimuli can induce their migration to the site of infection/inflammation [31]. Moreover, it has been demonstrated that at this stage monocytes/macrophages are present in higher number and have more phagocytic activity than granulocytes neu-

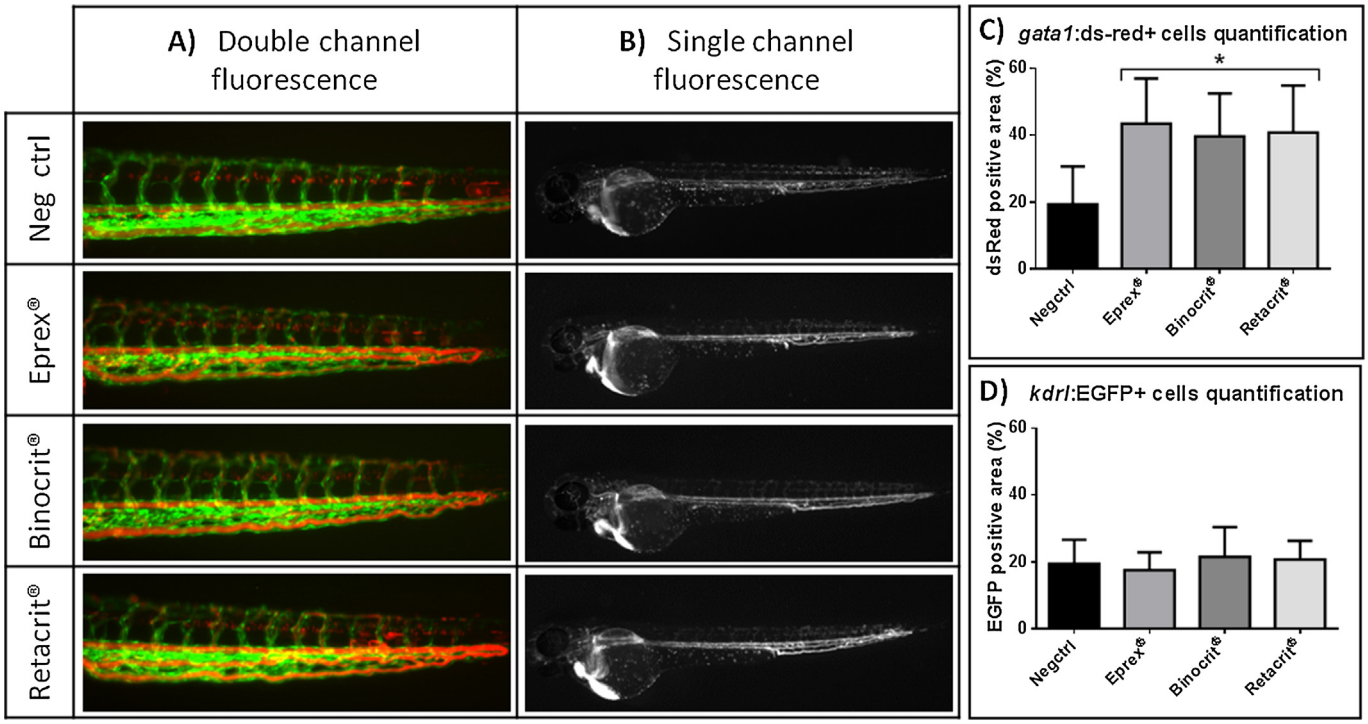

Fig. 4. Negative control (Neg ctrl), Eprex®, Binocrit® or Retacrit® injected in 48 hpf tg (*kdrl*:EGFP; *gata1*:ds-red) casper embryos and photographed after 4 h. (A) Double channel fluorescence: in green blood vessels, expressing *kdrl*:EGFP and in red circulating erythrocytes, expressing *gata1*:ds-red. Lateral views, anterior to the left, 11.5X magnification; (B) single red-channel fluorescence shows circulating erythrocytes, expressing *gata1*:ds-red. Lateral views, anterior to the left, 4X magnification. Quantification of (C) red fluorescence signal and (D) green fluorescence signal, measured with ImageJ 1.45 s image analysis software on a mean of 25 embryos for each experimental point. Asterisk indicates statistically significant increase of the positive area ($p < 0.05$), data are the mean ± S.D. of 3 independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

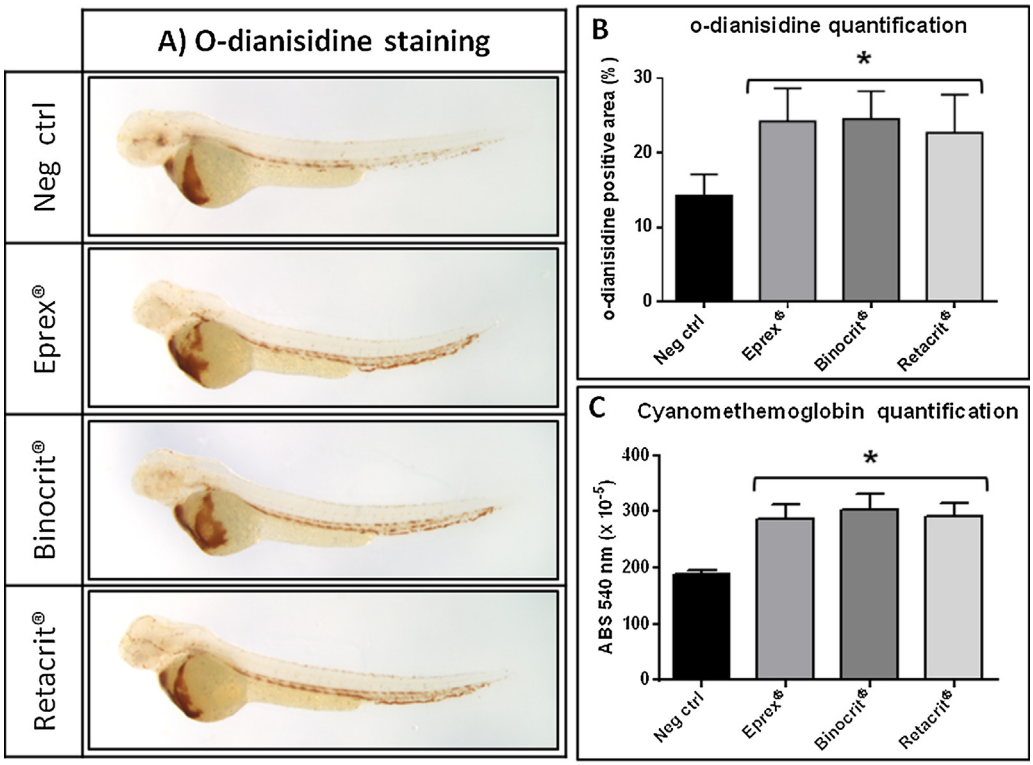

Fig. 5. O-dianisidine staining and Drabkin assay quantification of 48 hpf wild type AB embryos injected with negative control (Neg ctrl), Eprex®, Binocrit® or Retacrit®. (A) Representative images of embryos stained with o-dianisidine 4 h after treatment. Lateral views, anterior to the left, 4X magnification; (B) quantification of o-dianisidine positive area in the region of trunk and tail, measured with ImageJ 1.45 s image analysis software on a mean of 25 embryos for each experimental point. Asterisk indicates statistically significant increase of the positive area ($p < 0.05$), data are the mean ± S.D. of 3 measurements; (C) cyanomethemoglobin absorbance measured at 540 nm, proportional to the amount of red blood cells in groups of 20 embryos. Asterisk indicates statistically significant increase of absorbance ($p < 0.05$), data are the mean ± S.D. of 3 measurements.

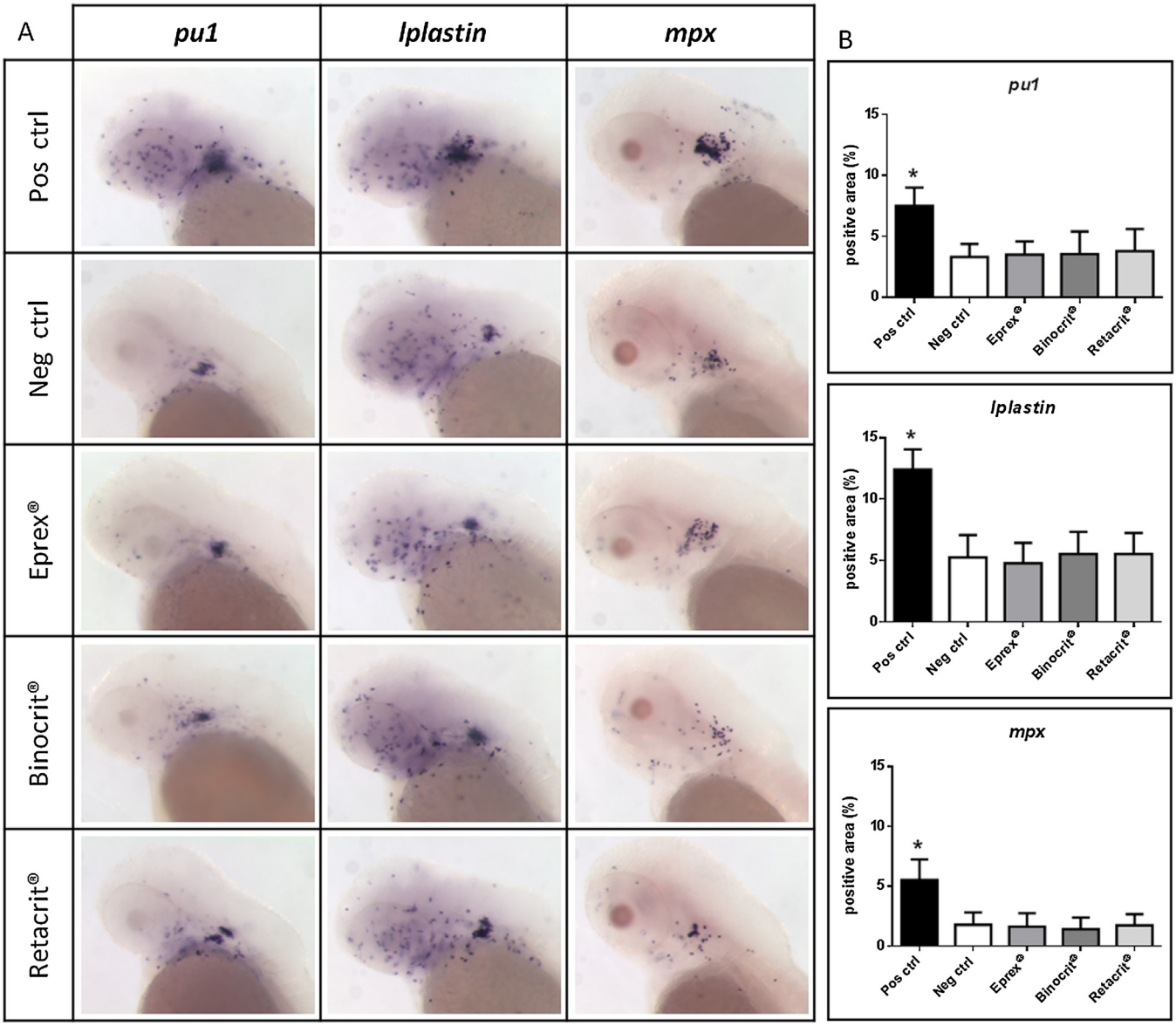

Fig. 6. *In situ* hybridization with *pu1*, *lplastin* and *mpx* probes to detect leukocytes precursors, monocytes/macrophages and granulocytes neutrophils, respectively. (A) Representative images of 72 hpf embryos injected into the otic capsule with Eprex®, Binocrit® or Retacrit® and positive or negative control (Pos ctrl and Neg ctrl, respectively). Lateral views, anterior to the left, 11.5X magnification; (B) quantification of *pu1*, *lplastin* and *mpx* positive area, measured with ImageJ 1.45 s image analysis software on a mean of 40 embryos for each experimental point. Asterisk indicates statistically significant increase of the positive area ($p < 0.05$), data are the mean ± S.D. of 3 measurements.

trophils [31]. Primitive leukocytes were detected by WISH, using *pu1*, *lplastin* and *mpx* as molecular probes. The transcription factor *pu1* is essential for early hematopoiesis, in particular for the myeloid cell development, including both macrophages and granulocytes precursor. The actin-binding protein encoded by *lplastin* is predominately expressed in monocytes/macrophages, while the enzyme myeloperoxidase, encoded by *mpx*, is tipically located in neutrophils granules [7,35].

Fig. 6 reports leukocytes response to the injection into the otic capsule of groups of 40 embryos, as the mean of three independent experiments. As expected, the amount of macrophages was about twice the number of neutrophils in the otic capsule and in the surrounding area of all the analyzed embryos. Both macrophages and neutrophils were strongly attracted to the injection site by the presence of the positive control of *E. Coli* bacteria, while the negative control had a very mild effect on leukocytes, similar to that of Eprex® and its biosimilars Binocrit® and Retacrit® (Fig. 6A). The leukocytes signal intensity in positive controls was 2.25 fold higher than negative controls for *pu1* probe, 2.35 fold higher for *lplastin* probe and 4.49 fold higher for *mpx* probe (Fig. 6B). On the contrary, the signal quantification for all the three probes in embryos treated with rhEPOs was comparable to that of the negative controls (1.01 ± 0.09 fold increase, Fig. 6B). The results showed that Eprex®, Binocrit® and Retacrit® were able to act on EPO-receptor without causing any acute pro-inflammatory effects on the zebrafish embryos.

4. Conclusions

Due to its valuable characteristics, in the last decade the zebrafish embryo has been widely used as a vertebrate model for high throughput screening of chemicals. However, more recently it has been increasingly exploited to test not only chemically synthesized drugs, but also more complex molecules (*i.e.*, biopharmaceuticals) [1–3]. In this paper, we used the zebrafish embryo to perform *in vivo* pharmacological assays. We performed a functional analysis of human recombinant erythropoietin alpha Eprex® and its biosimilars Binocrit® and Retacrit®. The recombi-

nant molecules, industrially produced in CHO cells, have the same amino acid sequence of endogenous hEPO but differ in the glycosylation pattern. This may influence efficacy and safety, particularly immunogenicity, of the final product [38,39].

Data obtained by computational analysis and 3D modeling techniques strongly suggested that rhEPO could recognize and bind the zebrafish erythropoietin receptor. The 3D model of zEpor was created by homology modeling starting from the structure of the extracellular portion of hEPOR. It confirmed the results obtained with the ClustalW multiple sequence alignment, in particular the structural conservation of the most important functional residues. The analysis of surfaces putatively involved in EPO binding revealed a high degree of similarity between zEpor and hEPOR structure, indeed the molecular docking of hEPO with the predicted structure of zEpor showed a compatibility of contact surfaces (Sc score 0.507). These data supported the hypothesis that hEPO could be recognized and bound by the zEpor.

Then it was demonstrated that Eprex® and its biosimilars were able to produce a significant increase of the number of circulating erythrocytes in treated embryos. The relative expression of *gata1*, α-*eHB* and β-*eHb* transcripts was significantly upregulated in embryos treated with rhEPOs, as well as the phosphorylation of Stat5 protein. Live imaging in tg (*kdrl*:EGFP; *gata1*:ds-red) *casper* embryos revealed that both the principal vessels and the smallest blood vessels were pervious and the concentration of circulating erythrocytes was higher in embryos treated with rhEPO respect to the negative controls. Also o-dianisidine positive area quantification and cyanomethemoglobin content quantification revealed a significant increase of erythrocytes amount in embryos treated with rhEPOs when compared with the negative controls.

Finally, this experimental animal model confirmed the lack of immunogenicity, required for the regulatory approval of biopharmaceutical drugs. Indeed embryos injected with Eprex® or its biosimilars showed an almost null effect on activation of primitive monocytes/macrophages and granulocytes neutrophils, comparable to that of negative controls.

In conclusion, our data demonstrated that zebrafish embryo could represent an new, reproducible and robust experimental vertebrate animal model to study the efficacy and the safety of complex biotherapeutics and biosimilars, such as recombinant human glycoproteins. Indeed, using the zebrafish embryo, we were able to test *in vivo* effects of rhEPO, in terms of both biological activity and safety. Zebrafish embryo demonstrated to be a valuable tool, that allows to perform fast and reproducible pharmacological assays with excellent results.

Conflict of interest

None.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2015.09.004.

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