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A dimeric peptide with erythropoiesis stimulating activity uniquely affects erythropoietin receptor ligation and cell surface expression

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Abstract

Erythropoiesis stimulating agents (ESAs) recently have been developed that exert long acting anti-anemia effects, but via poorly understood mechanisms. For one such ESA, peginesatide, analyses reveal unique EPOR binding properties for this synthetic EPOR agonist. As compared to rHu-EPO and darbepoietin, peginesatide exhibited a slow on-rate, but sustained EPOR residency and resistant displacement. In EPO-dependent human erythroid progenitor UT7epo cells, culture in peginesatide also unexpectedly up-modulated endogenous cell surface EPOR levels with parallel apparent relative increases in full-length EPOR-68K levels. These unique properties are suggested to contribute to the durable activity of this (and perhaps additional) dimeric peptide hematopoietic growth factor receptor agonists.

Introduction

For select therapeutic hematopoietic growth factors (HGFs), recombinant protein production together with clinical utility have been relatively tractable to achieve. As one example, rHu-GSF (and recently developed biosimilars) can be produced in bacterial systems, purified with recovery of high in vivo activity, and used with excellent success to treat neutropenia and to mobilize bone marrow CD34^{pos} progenitor cells for transplantation (Aapro, *et al* 2011, Publicover, *et al* 2013, Welte 2014). Other HGFs, in contrast, have been substantially

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more challenging to develop including Thrombopoietin (TPO) and Erythropoietin (EPO). rHu-TPO while effective in stimulating megakaryocyte and platelet production, proved in clinical trials to induce TPO- reactive antibody formation (Basser 2002). Inventive efforts to develop TPO mimetic factors as receptor agonists nonetheless have yielded useful anti-thrombocytopenia agents. These are in the form of the dimeric peptide and humanized Ig heavy chain fusion protein romiplostim (Chalmers and Tarantino 2015), and the small molecule MPL dimerizing and activating agent eltrombopag (Pathak, *et al* 2013) each of which exert clinically important anti-thrombocytopenia activities (Balduini and Noris 2016).

For EPO, the production of highly active, uniformly glycosylated rHu-EPO (and biosimilars) involves expression in engineered mammalian producer cells, non-trivial purification procedures, and associated elevated costs (Rainville, *et al* 2015). Recently, a fully synthetic dimeric peptide EPO mimetic, peginesatide, was developed and FDA- approved as a promising long-acting synthetic EPOR agonist (Fishbane, *et al* 2013). Upon broadened use, this mimetic (or perhaps a formulation component)(Weaver, *et al* 2015) unfortunately proved to evoke allergenic side-effects in certain first-time recipients, with severe anaphylactic reactions infrequently experienced (Bennett, *et al* 2014). While this hinders momentum towards the development of EPO mimetics, it remains valuable to consider how certain mimetics are able to exert sustained erythropoiesis stimulating activity upon single dosing (Fishbane, *et al* 2013, Sathyanarayana, *et al* 2009) – up to one month for peginesatide. Here, we report first on unique EPOR binding properties of peginesatide. Additionally, we describe an unexpected action mechanism for this dimeric EPO mimetic involving a substantial up-modulation of EPOR levels in erythroid progenitor cells. This is in contrast to a model that emphasizes ligand-independent EPOR trafficking (Becker, *et al* 2010), and points to novel EPOR interaction properties that in part may underlie the durable activity of this (and perhaps related) dimeric growth factor mimetics.

Materials and Methods

Characterization of ligand EPOR binding properties

Ligand binding analyses utilized surface plasmon resonance (Biacore™ platform), and an immobilized recombinant hEPOR-Fc construct. Via standard amino coupling methods, an ECD-EPOR-Fc construct was immobilized on CM4 sensor chips (Biacore T100, GE Healthcare) at two surface densities (surface-1, 675 RU; surface-2, 225 RU). For each surface, protein and peptide sample binding properties were analyzed (with replicates) at 3-fold serial dilutions. rHu-EPO was from OrthoBiotech (Epoetin-alfa). For response data from each surface, global fits were determined by a 1:1 interaction model (Scrubber2, BioLogic Software PTY Ltd.). Rate constants were then determined.

Cell culture, and estimation of functional dose equivalences for peginesatide and rHu-EPO

UT7epo cells and Baf3-hEPOR cells were cultured as per Singh et al (Singh, *et al* 2012), and Boehm et al (Boehm, *et al* 2014). Towards establishing dose equivalencies for peginesatide and rHu-EPO, cells were plated (1.5×10^5 cells/mL) and cultured in either peginesatide or rHu-EPO at varied concentrations. Ligand-dependent proliferation levels

were then determined (at day 2.5 of culture). In experiments with extended time-courses, media plus ligand were replaced daily to control for possible differential ligand depletion.

Flow cytometry, and western blotting

Assays of cell surface EPOR expression were performed using antibody EC-c38.5 as described previously (Singh, *et al* 2012). Briefly, cells in antibody binding buffer (ABB) were incubated for 10 minutes at 4°C with 0.3µg/mL Fc blocker (Stem Cell Technologies, cat #01470) and then with hIgG (Jackson ImmunoResearch, cat #009-000-003) (1µg per 0.2mL assay). Subsequently, cells were collected and incubated at 4°C with anti-hEPOR antibody EC-c38.5 at 1.0 µg per assay for 1 hour at 4°C. Antibody- EPOR complexes then were detected using Alexafluor-647 goat anti-rabbit IgG (0.02 µg/assay) (Invitrogen, cat #A21245) (30 minutes at 4°C). Negative controls included non-immune rabbit IgG, and second antibody only. Western blotting was as detailed previously (Singh, *et al* 2012) with anti-hEPOR antibody IC-c1.1, a rabbit monoclonal antibody to pY344-hEPOR (Singh, *et al* 2012), and anti- GAPDH (Cell Signaling Technologies).

Results and Discussion

To gain initial insight into peginesatide's interactions with the hEPOR, Biacore analyses were performed for ligand binding to an EPOR extracellular domain- IgFc construct (Figure 1A) (for details, please see Supplementary Methods). For comparison, binding properties of rHu-EPO, Darbepoetin, and unconjugated dimeric peptide (not coupled to PEG) also were analyzed. For each, ligand binding properties were determined at varied densities of EPOR immobilization (Figure 1B). Peginesatide proved to exhibit an attenuated association rate constant, but once bound, additionally exhibited an attenuated dissociation rate constant (an order-of-magnitude less than for rHu-EPO), together with uniquely highly stable binding to the EPOR (Figure 1C). Specifically, EPOR residence time for peginesatide was estimated to be 17.1 fold greater than that of rHu-EPO. In addition, a 12.4 fold greater concentration of competing rHu-EPO was required for IC-50 displacement of EPOR- ligated peginesatide as compared to rHu-EPO. Thus, once ligation is achieved, binding of peginesatide to the EPOR is uniquely sustained.

Recently, we developed and characterized highly specific monoclonal rabbit antibodies to the hEPOR, including those useful for flow cytometry and western blotting (Singh, *et al* 2012). This provided unique opportunities to examine possible effects of peginesatide on EPOR expression levels. For these experiments, we first assessed ESA- dependent UT7epo cell growth profiles in order to determine matched biological dose equivalency for peginesatide and rHu-EPO (Figure 2A). This also was determined (with highly comparable outcomes) in murine BaF3 cells stably expressing the hEPOR (Figure 2A, right panel). Subsequent analyses used matched doses of rHu-EPO and peginesatide at 1.5U/mL vs 2.1 nM, and at 2.0 U/mL vs 2.8nM, to investigate possible ligand-dependent effects on endogenous EPOR expression. As studied in human EPO-dependent erythroid progenitor cells, when endogenous EPOR expression was assessed (by flow cytometry), notably elevated cell surface EPOR levels were observed for erythroid progenitor UT7epo cells propagated in peginesatide as compared directly to cells propagated in rHu-EPO (Figure 2B

and Supplemental Figure S-1). This was reflected (with clear statistical significance) in both median and mean fluorescence intensities.

These studies next were extended (again using varied peginesatide and rHu-EPO dosing) to include western blot time-course analyses of EPOR expression. Peginesatide as the EPOR ligand proved to give rise to elevated levels of full-length EPOR-68K receptor forms (Figure 3). By comparison, no such increases were observed for a lower molecular weight EPOR-36K form which has been implicated to be an EPOR turnover product (Singh, *et al* 2012). Here it is noted that an uncommon EPOR-T isoform (~35.8 K molecular weight) is not endogenously expressed in UT-7 derived cell lines (Shimizu, *et al* 1999). In quantitative RT-PCR analyses, no differential effects of peginesatide vs rHu-EPO on *EPOR* transcript levels were observed (data not shown), indicating effects at post-transcriptional levels.

The present observations concerning unexpected effects of a durable EPO mimetic on EPOR binding and expression have several potentially significant implications. First, peginesatide, (and possibly other long-acting ESAs) may exert sustained ESA effects in part by promoting enhanced cell surface EPOR expression levels. One contributing mechanism could relate to peginesatide's presently characterized attenuated on-rate, and prolonged time required to achieve EPOR engagement. This consequently may lead to a compensatory up-modulation of cell surface EPOR expression within targeted erythroid progenitor cells. Second, the molecular specifics of how dimeric peptides are scaffolded within ESAs may be an additional contributing factor. In particular, peginesatide's scaffold component is an anionic PEG polymer that might exert charge repulsion, steric hinderance and/or plasma membrane effects as factors which could alter EPOR trafficking. Such possible ESA action mechanisms would also be consistent with observed trends towards apparent lessened processing of the EPOR to lower molecular weight forms when peginesatide is the EPOR ligand.

Overall, we show that a long-acting synthetic dimeric peptide ESA binds in an initially attenuated but ultimately persistent fashion to the hEPOR. This furthermore occurs in association with heightened endogenous EPOR expression as studied in an EPO-dependent human erythroid progenitor cell model (Goupille, *et al* 2012, Singh, *et al* 2012). In certain individuals, peginesatide unfortunately can also somehow stimulate allergenic reactivity (Bennett, *et al* 2014). This can include rare fatal anaphylaxis, and this ESA therefore can not presently be considered for therapeutic use. Recently, this side effect has been suggested to perhaps relate to effects of the production and/or formulation of peginesatide on mast cells (Weaver, *et al* 2015), but additional in-depth studies of such side effects clearly are needed to resolve this essential matter. This, however, does not discount the potential high-merit scientific insight gained for deciphering how certain ESAs exert sustained anti-anemia effects beyond those explained by their modestly higher pharmacokinetic half-lives (Fishbane, *et al* 2013, Sathyanarayana, *et al* 2009). This (in the authors' opinions) includes the notion to continue to consider the development of safe and effective synthetic dimeric peptide EPO mimetics, and/or small hEPOR dimerizing agents.

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- Engineered homodimeric EPOR (and TPOR) agonists can exert sustained bioactivity.
- For the EPOR ligand peginesatide, unique binding properties presently are defined.
- Peginesatide also heightens cell surface EPOR expression.
- These properties may contribute to peginesatide's durable in vivo activity.

Figure 1. Unique EPOR binding properties of the dimeric peptide EPO mimetic, peginesatide.
A] Schematics are outlined for the human EPOR (as a dimer co-assembled with JAK2), rHu-EPO and peginesatide. B] EPOR binding properties of rHu-EPO, Darbepoietin, EPO mimetic peptide dimer and peginesatide: The hEPOR (EC domain, Ig Fc fusion) was immobilized on Biacore chips at two different densities (left, right panels). Ligands were then introduced (at the concentrations indicated) and for the time courses shown, binding was assayed. C] Summary ligand-hEPOR binding properties including data for association rate constants, dissociation rate constants, EPOR residence time, and competitive displacement IC₅₀'s of ligands by rHu-EPO.

Figure 2. Up-modulation of cell surface hEPOR expression by Peginesatide

A] Estimation of matched dosing for peginesatide and rHu-EPO in UT7epo, and Baf3-hEPOR cells: Data illustrate ligand- dependent UT7epo cell growth (means $\pm$ SE, $n=4$) for the three indicated sets of rHu-EPO and peginesatide doses. UT7epo data, left 3 sub-panels; Baf3-hEPOR data, right panel. **B]** As an EPOR agonist, peginesatide heightens cell surface EPOR expression: UT7epo cells were cultured in the indicated matched doses of rHu-EPO or peginesatide. At days 4 and 8 of culture, cell surface EPOR expression levels were assayed via flow cytometry. **C]** Summary data also are shown for replicate analyses (at each day) for median and mean fluorescence intensities (mean values $\pm$ SE, $n=3$).

Figure 3. Peginesatide-dependent increases in levels of full-length EPOR

UT7epo cells were propagated in either rHu-EPO or peginesatide at the doses indicated. Cell lysates were then prepared and analyzed via western blotting for EPOR expression levels, and EPOR molecular weight species (68K, 40K, 36K).