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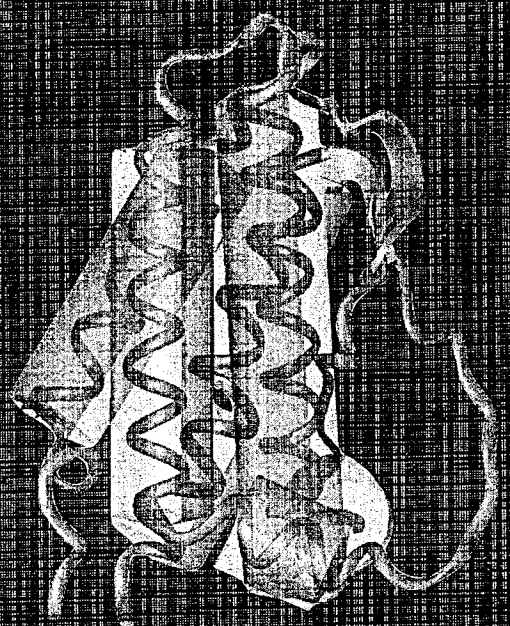
Milestones in Drug Therapy

Michael J. Parnham
Jacques Bruinvels
Series Editors

Erythropoietins and Erythropoiesis

Molecular, Cellular, Preclinical, and Clinical Biology

G. Molineux
M.A. Foote
S.G. Elliott
Editors



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G. Molineux, M.A. Foote, and S.G. Elliott (Editors)

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Erythropoietins and Erythropoiesis

Molecular, Cellular, Preclinical, and Clinical Biology

Edited by G. Molineux, M.A. Foote, and S.G. Elliott

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Erythropoietins and Erythropoiesis
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New molecules and formulations of recombinant human erythropoietin

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Introduction

Recombinant human erythropoietin (rHuEPO), is a glycoprotein hormone commonly used for the treatment of anemia associated with chronic kidney disease [1–3]. It is also indicated for the treatment of anemia associated with cancer, human immunodeficiency syndrome virus (HIV) infection, and for use in surgical situations to reduce allogeneic blood transfusion requirements. (For further information see Chapters 9, 10, 11, and 12). A number of studies have shown that rHuEPO is well tolerated and effective at ameliorating anemia, restoring energy levels, and improving patient quality of life in these indications. The clinical benefits of rHuEPO are well understood and appreciated.

The endogenous EPO protein is naturally optimized for maintenance of hemostasis in the body where the protein can be produced on demand in the amounts needed. However, that which is optimal for natural *in vivo* production may be sub-optimal in the context of clinical intervention. For example, rHuEPO must be administered frequently by injection to be efficacious. The discomfort of injections and the inconvenience to the patient and healthcare provider burdens both groups.

Approaches to enhancing a drug's properties have included new formulations and delivery systems of the existing drugs whereby the circulating concentration of the drug is maintained for extended periods of time (sustained delivery). Such improvement can be accomplished in several ways, including use of pumps or slow-release formulations. Alternatively, proteins can be modified chemically with attached polymers that confer a longer half-life (sustained duration of action). Attempts to modify the protein itself by *in vitro* mutagenesis or through protein fusions to other peptides or protein hormones have been explored. Glycoengineering has been successfully applied to rHuEPO. With this process, new carbohydrate attachment points are introduced into the protein, increasing the amount of attached carbohydrate and increasing *in vivo* activity and serum half-life without substantially lowering *in vitro* activity. The new molecule can be administered with extended dosing intervals with no loss of efficacy.

The inherent limitations of rHuEPO due to its peptidic composition or mechanism of action can potentially be bypassed entirely with new molecular entities such as small molecules or antibodies (EPO mimetics). These compounds may have advantageous biological or chemical properties not present in rHuEPO, such as an oral route of delivery, (small molecule), or structural conformations that hold little in common with EPO, thus exploiting different routes of elimination. Finally, stimulation of erythropoiesis by mechanisms different from those of rHuEPO, such as those steps upstream or downstream from receptor activation step, have been attempted. This chapter discusses new molecules being considered or developed and their limitations, if any.

Considerations of new formulations and drug entities

The purpose of any therapeutic intervention is to treat the patient without causing harm, and rHuEPO has been particularly successful in this regard. The molecule is very effective at stimulating erythropoiesis with minimal side-effects. This excellent safety profile has created a high standard against which any new erythropoiesis-stimulating molecule will be measured. Not only should a new drug entity have improved properties, but it should also match the excellent safety profile of rHuEPO. The preferred properties of any new drug or formulation include retention or increase in efficacy and lack of new or unwanted side-effects or toxicities.

One particular concern is anti-EPO antibodies [4]. (See Chapter 14 for further information.) Antibody formation to new drug entities is important not only because the drug may lose efficacy, but also because such antibodies might cross-react with endogenously produced EPO resulting in pure red cell aplasia, a very serious and severe form of anemia. Potential causes for antibody formation not only include the structure of the molecule itself, but also the breakdown products or aggregates generated during manufacturing or storage. Accordingly, new drugs should be designed and manufacturing procedures put in place to minimize this risk.

EPO molecules with altered activity

One approach to increase activity of EPO is to alter the interaction with the EPO receptor (EPOR). EPO activates erythroid precursor cells by binding and activating EPOR on the surface of erythroid progenitor cells [5]. (See Chapters 3 and 5 for further discussion.) Receptor activation occurs through homodimerization, whereby the two EPOR binding sites on a single EPO molecule crosslink two EPOR [6]. The two binding sites on rHuEPO have different affinities, high (approximately 1 nM) and low (approximately 10 μM) [7]. Initial binding is to the high-affinity binding site, followed by homodimerization of the receptor by binding to the low-affinity binding site [8].

Molecules that alter or increase affinity at either of the two sites can have increased activity.

Despite numerous attempts to identify them, no erythropoietic molecules suitable for clinical development have been reported that increase biological effect as a consequence of increased receptor affinity. The reasons are several-fold. First is the theoretical concern that antibodies, were they to develop in patients, may be targeted to the changed region of the molecule and would be neutralizing because they would interfere with the EPO:EPOR interaction. Should these antibodies cross-react with endogenous EPO, pure red cell aplasia would likely result. A second reason is that increased affinity does not always translate into increased *in vivo* activity because there are additional requirements beyond receptor binding required to effect a biologic response *in vivo*. Each receptor-binding event is transient because EPO:EPOR complexes are rapidly internalized and degraded [9, 10]. In addition, the EPOR-signaling pathway is down-modulated shortly after activation [10–12]. Thus, *in vivo* erythropoiesis requires continuous stimulation of multiple EPOR through multiple binding events. As a consequence, molecules cleared quickly have low *in vivo* activity. For example, increased *in vitro* activity and increased receptor affinity have been observed with EPO analogs that remove sialic acid from carbohydrates or remove *N*-linked carbohydrates entirely [13, 14]; however, these molecules have reduced *in vivo* activity due to a more rapid clearance [13, 15]. Increased concentrations can partially compensate for the increased clearance; however, these compounds must be administered more frequently to be fully efficacious.

Changes in rHuEPO amino acid sequence can result in increased stability. These changes can include removal of amino acids that are unstable (tryptophan [16]); or are subject to oxidation (methionine), deamidation (asparagine), or changes that confer increased conformational stability, such as those that stabilize alpha helices or connecting loops. Such molecules may be more amenable to long-term storage or suitable for formulations where more stable EPO molecules are essential, such as in slow-release formulations or automated delivery systems. Removal of proteolytic cleavage sites by *in vitro* mutagenesis can enhance *in vivo* stability. It may be possible to remove antigenic sites, thereby reducing immunogenicity.

Compounds that bind through the high-affinity site but do not dimerize because of reduced binding at the low-affinity site can function as antagonists [8]. Such molecules may have some clinical utility for treatment of EPO responsive polycythemias, and several such molecules have been described [8, 17].

Molecules with increased serum half-life

A longer duration of action can allow for reduced frequency of administration. One approach that has been successfully applied to rHuEPO is glycoengineer-

ing [18]. Glycoengineered molecules bind and activate EPOR in the same manner as rHuEPO, resulting in similar biological responses while at the same time reducing clearance and enhancing activity [19]. Other strategies to increase duration of action of EPO included chemical modifications, such as the addition of a polyethylene glycol molecule (pegylation) or gene fusions between EPO and other proteins. In these cases, the goal is to reduce clearance rate by increasing hydrodynamic size.

Glycoengineering

rHuEPO is a glycoprotein hormone consisting of approximately 40% carbohydrate [20]. The carbohydrate component consists of three *N*-linked carbohydrates attached to Asp at amino acid positions 24, 38, and 83, and an *O*-linked carbohydrate attached to Ser at amino acid position 126 [20] (Fig. 1). Unlike the invariant protein sequence, the carbohydrate is variable in structure, resulting in glycoforms with modest differences in sizes, structures, and sugar content [22, 23]. A typical *N*-linked carbohydrate made by mammalian cells is

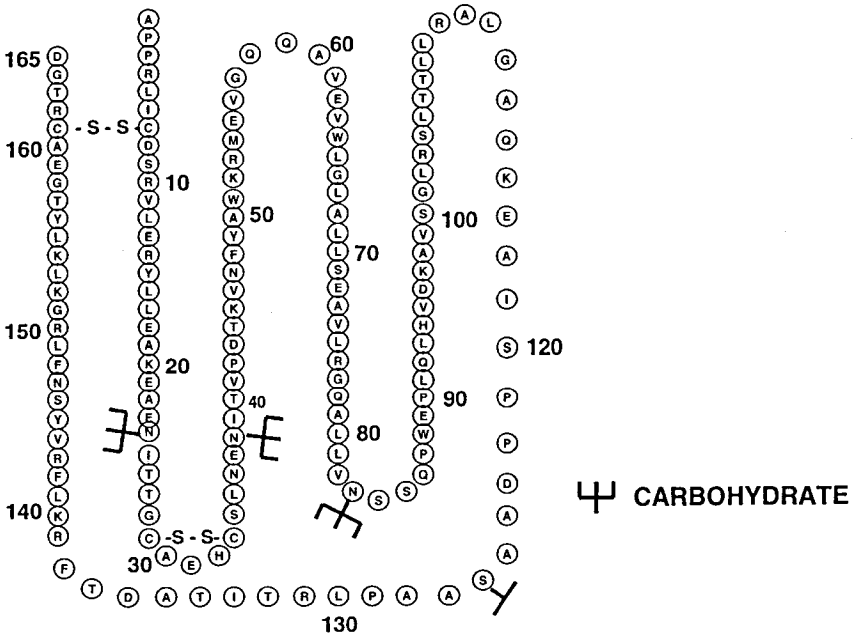

Figure 1. Amino acid sequence of human erythropoietin. Recombinant human erythropoietin (rHuEPO) is 165 amino acids in length [21]. Disulfide bonds (-S-S-) join Cys7 to Cys161 and Cys29 to Cys33. The 3 *N*-linked glycosylation attachment points are at Asn24, Asn38, and Asn83 and the *O*-linked carbohydrate is attached to Ser126. Forked structures schematically depict the attached carbohydrates.

branched with two to four arms. The end of each arm is usually capped by a sialic acid; sialic acid content exhibits microheterogeneity in the different glycoforms. The sialic acid is of importance because it is the only negatively-charged sugar on the carbohydrate. Variations in the amount of sialic acid can affect the electrostatic properties of the molecules to which it is attached.

The carbohydrate is essential for *in vivo* but not for *in vitro* biological activity [13, 24]. The sialic acid component of carbohydrate, in particular, plays a critical role in the *in vivo* biological activity of rHuEPO. Removal of sialic acid from the carbohydrate of EPO results in almost complete loss of *in vivo* activity [14, 25]. Studies on glycoforms of rHuEPO containing different sialic acid contents demonstrated a direct relationship between increased sialic acid content and increased *in vivo* activity [19]. The increased *in vivo* activity was due to an increased serum half-life of the molecule and not increased receptor affinity. The theoretical maximum number of sialic acids on rHuEPO is 14 (up to four sialic acids for each of the three *N*-linked carbohydrates and up to two sialic acids for the *O*-linked carbohydrate) [23]. It was hypothesized that *in vivo* activity may be increased beyond that observed with rHuEPO by adding new sialic acid containing *N*-linked carbohydrates. Each new *N*-linked chain could add up to four additional sialic acids.

To add more *N*-linked carbohydrate, new *N*-linked glycosylation sites were introduced into the amino acid sequence of EPO. *N*-linked carbohydrate is attached to an Asn present in the consensus sequence Asn-Xxx-Ser/Thr (where Xxx can be any amino acid except proline) [26]. This sequence is necessary but not sufficient for *N*-linked carbohydrate addition [27]. During synthesis of a glycoprotein, appropriate consensus sequences are recognized by oligotransferases in the cell, resulting in attachment of carbohydrate. This carbohydrate is subsequently modified by the action of additional intracellular enzymes. The protein is then secreted from the cell into the circulation [28].

For the purpose of potentially developing a new drug with properties superior to then available products, it became apparent that simply adding an *N*-linked consensus sequence to rHuEPO would not be sufficient. The changes needed to be introduced in such a way that the resultant molecule was efficiently glycosylated and retained activity, conformation, and stability. To increase the likelihood of success, the amino acid changes were introduced into a region of the molecule distal to the receptor-binding site to ensure that the molecule would efficiently activate EPOR. This effort was aided by structure-function studies that defined the active sites of rHuEPO and determination of amino acids important for maintenance of structure [17, 29, 30].

EPO glycosylation analogs with introduced *N*-linked glycosylation consensus sequences were constructed and tested [18]. Several candidates containing additional carbohydrate had acceptable activity and conformation characteristics. Two of these consensus sequences (Asn30-Thr32 and Val87-Asn88-Thr90) were combined to generate a new molecule with two additional *N*-linked carbohydrates. This molecule (darbepoetin alfa) had near-normal *in vitro* activity, was glycosylated efficiently, and had a similar conformation and

stability to rHuEPO. The carbohydrate content was increased from 40% to 51%, the size from approximately 30 Kd to approximately 37 Kd, and the maximum number of sialic acids was increased from 14 to 22.

Studies in mice administered darbepoetin alfa revealed that more rHuEPO was required to obtain a response similar to that of darbepoetin alfa [18, 19]. In pre-clinical studies, animals were administered rHuEPO or darbepoetin alfa at various dose intervals. The relative *in vivo* activity difference of rHuEPO and darbepoetin alfa increased as the dosing interval increased. Three-fold more rHuEPO than darbepoetin alfa was required to elicit a similar response when the drugs were administered three times per week. This difference increased to 13-fold when the molecules were administered at weekly intervals [19]. The increased *in vivo* activity and the differing potencies with changes in dose interval could be explained by an observed three-fold increase in serum half-life of darbepoetin alfa over rHuEPO [19, 31]. The observation that the serum half-life increased in proportion to the number of added carbohydrate chains indicated that the carbohydrate directly affected clearance. Testing in humans mirrored the results found in animals. The serum half-life of darbepoetin administered intravenously was increased approximately three-fold [31]. In clinical trials, patients were converted from rHuEPO administered two to three times per week to darbepoetin alfa administered once per week or from weekly rHuEPO to once-every-other-week darbepoetin alfa. The hemoglobin concentrations were successfully maintained with the less frequent dosing schedule [32, 33]. More recently, clinical results suggest that hemoglobin concentrations can be successfully maintained when darbepoetin alfa is administered at once every three to four weeks dosing intervals [34].

One theoretical concern with any alteration in a protein's amino acid sequence or structure is immunogenicity. Several characteristics of darbepoetin alfa and its methods of manufacture, minimize the potential for antibody formation. The particular amino acid substitutions in darbepoetin alfa had a minimal effect on structure and stability. The carbohydrate and sialic acid content of the material selected during the purification process was maximized for several reasons: first, the higher carbohydrate content enhances the *in vivo* activity. Secondly, carbohydrate can increase solubility and stability of proteins thereby inhibiting formation of aggregates and other byproducts [35–38]. Finally, *N*-linked carbohydrate is large relative to the peptide backbone giving the carbohydrate a "shielding" effect potentially inhibiting antibody formation. Antibody formation was monitored during clinical trials with darbepoetin alfa and in all patient samples examined, no neutralizing antibody was detected [33, 39].

Pegylation

Pegylation of proteins has been used successfully to increase serum half-life of proteins [40]. Pegylation involves chemical attachment of the polymer, polyethylene glycol (PEG), to reactive regions of proteins or carbohydrates.

Pegylated molecules have an increased hydrodynamic size because they create a "water shell" around the molecule. The attachment of PEG is thought to improve solubility and possibly reduce immunogenicity due to shielding by the conjugate. In addition, the increased hydrodynamic size can result in reduced clearance and thus increase *in vivo* activity.

Mixing activated polyethylene polymers with proteins under appropriate chemical reaction conditions produces pegylated proteins. The duration of the drug development process using this strategy is relatively short because existing starting materials can often be used for the chemical reaction. PEG is thought to be relatively inert and non-immunogenic by itself, so it is a suitable starting material for protein-conjugate therapeutics.

One issue with drugs made by solution or solid-phase chemistry can be poor specificity of conjugation in the chemical reaction or generation of undesirable by-products. Many pegylation chemistries have been tried to reduce undesirable by-products, improve the specificity and efficacy of PEG attachments, and minimize immunogenicity risk of the protein conjugate while maximizing the *in vitro* and *in vivo* activity of the resultant molecule [41]. The current chemistries typically target the reactive amino groups on Lys or the amino terminal amine. rHuEPO has eight Lys, some of which are part of the active site [17]. Therefore, some pegylated EPO molecules conjugated to Lys may have low activity because PEG may interfere with receptor binding and activation. Other pegylated EPO molecules may have low activity because attached polymer results in structural alterations that interfere with receptor interaction.

Analogs of proteins can be made to increase specificity. For example, Cys substitutions at targeted regions can allow addition of the conjugate with high specificity to the sulfhydryl group [42, 43]. Another strategy is to make pegylated EPO synthetically: During synthesis, a PEG-conjugated amino acid could be introduced instead of the unconjugated amino acid. This approach allows specific targeting of particular amino acid positions for PEG attachment, such as the glycosylation sites, and reduces heterogeneity and the potential for loss of *in vitro* activity. It is not clear that these molecules will retain the same stability, *in vivo* activity, and lack of immunogenicity as their glycosylated counterparts, however.

Fusion proteins

Several EPO gene fusion proteins have been reported, including EPO/interleukin-3 (IL-3) [44], and EPO/granulocyte-macrophage colony-stimulating factor (GM-CSF) [45]. The theory behind creation of such molecules is that they can impart to the fusion protein biologic properties of both molecules. One can imagine that co-administration of an early-acting growth factor such as rHuIL-3 with rHuEPO can increase efficacy of rHuEPO. The fusion protein being larger may impart increased activity for both partners because of reduced clearance. The fusion protein also ensures that both molecular entities

are simultaneously present. Simultaneous administration by fusing two drugs can simplify administration, especially when the two proteins have different pharmacokinetic parameters. The ability to independently adjust dosing of the fusion partners is lost, however. Furthermore, the difficulty in retaining a non-immunogenic structure has been a challenge and neither EPO/IL-3 nor EPO/GM-CSF fusion molecules have been approved for use in humans.

EPO dimer has been generated as a potential therapeutic [46, 47]. In general, the increased size can reduce clearance because of slowed transport out of the serum compartment [46]. EPO dimer may also have increased *in vitro* activity due to altered avidity to the receptor [48].

The two protein partners are typically joined by a linker peptide that included Gly, Ala, and Ser. These three amino acids are thought to result in linkers that are flexible and relatively inert, allowing independent folding of the two proteins into their appropriate three-dimensional structures. Full *in vitro* activity of both proteins in fusion proteins does not always occur [44]. The fusion proteins have been reported to have increased risk of immunogenicity [49], presumably because of altered folding or stability.

EPO mimetics

rHuEPO is currently administered by either subcutaneous or intravenous injection. Because of its large size and peptidic nature, delivery by other routes such as oral or transdermal or by inhalation can be quite challenging. The potential use of rHuEPO for treatment of neuronal trauma by promoting neuronal survival [50] is limited by its poor transport across the blood brain barrier [51]. One possible solution is a small molecule, an EPO mimetic, capable of stimulating EPOR. EPO mimetics are compounds that mimic the activity of EPO but bear no structural homology. EPO mimetics can have new biological or biophysical properties not present in EPO. Designed appropriately, such a compound has the potential to be delivered by routes that are more convenient than currently in use for rHuEPO.

Significant challenges are associated with the identification and development of a useful small molecule EPO mimetic. First, the need to be small to be delivered orally is confounded by the need to be large enough to have sufficient affinity for EPOR to be effective. The compound must have appropriate pharmacokinetic parameters so that it persists sufficiently long in the serum to be efficacious. Finally, the compound should not have unwanted side-effects due to either toxicities of the compound itself or breakdown products of it. In spite of these challenges, work has proceeded and progress has been made in attempts to identify lead compounds that may be amenable to oral delivery.

Several strategies have been used to identify EPO mimetics. The first is to screen peptide and small molecule libraries for those compounds that can stimulate erythropoiesis using *in vitro* bioassays as screens. According to this strategy, an understanding of the mechanism of activation is not necessary, and

compounds active in the assay may activate EPOR by a different mechanism than by rHuEPO. Another strategy is to identify molecules that directly bind to and agonize EPOR in a manner similar to that of rHuEPO. The latter strategy can be performed in two steps: the first step is to identify compounds that bind EPOR and the second step is to covalently link the compounds into bivalent dimers that can agonize the receptor by EPOR homodimerization.

The latter strategy takes advantage of the observation that an EPOR mutant containing an Arg129 to Cys129 mutation was constitutively active [52]. A disulfide bond formed between the Cys129 residues on the receptors resulted in homodimerization and receptor activation demonstrating that EPO was not essential for receptor activation (Fig. 2). X-ray crystallography results demonstrated that EPOR forms a 2:1 complex with EPO [57]. Each receptor uses the same region on its surface to bind to two surfaces on EPO, resulting in receptor homodimerization. Further confirmation of the homodimerization mechanism was the discovery of agonist monoclonal IgG antibodies that could homodimerize EPOR [53]. Anti-EPOR antibodies activated because they were bivalent, had two binding sites, and could simultaneously bind and cross-link two EPOR (Fig. 2). Monovalent, Fab fragments could bind but did not agonize

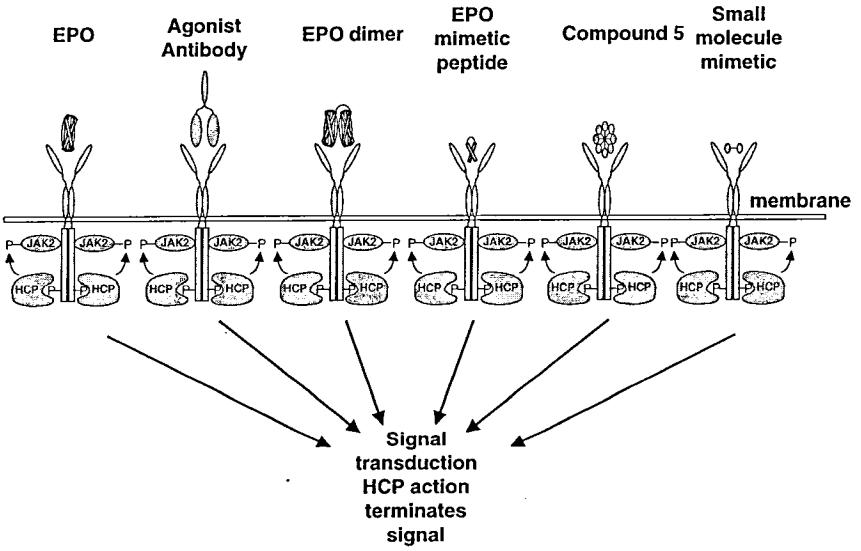

Figure 2. Mechanism of erythropoietin receptor (EPOR) activation. EPOR are homodimerized because of the two asymmetric receptor-binding sites on rHuEPO. EPO binding results in phosphorylation of EPOR, JAK-2 recruitment, and phosphorylation of JAK-2. The activation of JAK-2 results in downstream signaling events. Hematopoietic cell phosphatase (HCP) can bind the activated (phosphorylated) receptor resulting in dephosphorylation of JAK-2, thereby terminating signal transduction. The EPO mimetic compounds; agonist antibody [53], EPO dimer [46, 47], EPO mimetic peptide [54], compound 5 [55], and small-molecule mimetics [55, 56] can all homodimerize and activate EPOR in a manner similar to that of rHuEPO.

the receptor, confirming a requirement for bivalent binding. Wrighton and colleagues screened peptide phage libraries and one peptide was identified that could bind and agonize EPOR [54]. This peptide, AF11154, had no homology to EPO. It self associates into dimers to form a bivalent molecule that could homodimerize EPOR in a 2:2 mimetic:EPOR complex [58]. Additional sequence modification of this peptide resulted in EMP1, a 20-amino acid peptide with an approximate 50-fold increase in affinity over the starting peptide (Tab. 1). The affinity was increased more by covalent linkage of the peptide [59]. The activity of this peptide was still significantly lower (500-fold) than that of rHuEPO when tested in an *in vitro* bioassay. In addition, the *in vivo* activity was very low (25,000-fold less than rHuEPO). This work demonstrated, however, that a molecule smaller than rHuEPO could successfully dimerize and activate the receptor. An attempt to discover other EPO agonist peptides by another group was also successful [61]. The *in vitro* activity was not increased, however, and the size of this peptide was not decreased compared with EMP1.

One explanation for the low *in vivo* activity of mimetic peptides is their rapid clearance. One group addressed this problem by creating a fusion protein between EMP1 and a larger protein, plasminogen activator inhibitor (PAI1)[60], resulting in an increase in molecular weight from 4.8 Kd to 66 Kd. The *in vivo* activity was significantly increased (2500-fold); its *in vivo* activity, however, was still significantly less than that of rHuEPO (100-fold) and the ability to be delivered orally was compromised by the size increase.

The peptide mimetics described above are significantly larger (4.2 Kd) than the preferred size of an orally bioavailable compound (<0.6 Kd). These peptides may be used to design lead compounds of smaller size. Some small molecule agonists have been isolated based on the EMP1 structure [56]; however, their *in vitro* activities were low (Tab. 1). An independent approach was to directly screen for small-molecule EPO mimetics that could dimerize EPOR. Small molecule libraries containing compounds with two-fold symmetry were screened to find dimerizing compounds that agonize the receptor [55, 63]. This strategy did not result in discovery of agonist compounds, however. A small molecule (compound 1, approximately 5 Kd) that bound but did not agonize EPOR was discovered. Compound 1 was made active *in vitro* by oligomerizing it with a multivalent crosslinker resulting in a molecule (compound 5) containing eight compound-1 molecules joined together. Compound 5 binding to EPOR was increased somewhat (10-fold) over that of compound 1; however, the size (6.4 Kd) was greater than that required to be orally bioavailable. In addition, its *in vitro* activity was low relative to rHuEPO and the compound had toxicity. The feasibility of the small-molecule approach to discovery of small molecules that could agonize EPOR was demonstrated, however.

Further progress in development of small molecule EPO mimetics has been slow. Progress has been made with the development of small molecules that can agonize other cytokine receptors including granulocyte colony-stimulating factor receptor [64] and insulin receptor [65]. The insulin mimetic is noteworthy in that it is orally active in rodents [66]. This work demonstrates that small

Table 1. Activity of erythropoietin and mimetics

Compound	MW (Kd)	*In vitro* activity (EC50)	Binding (IC50)	*In vivo* activity (U/mg)	Relative difference in activity (molar)	Notes	Ref
rHuEPO	18,200	5–10 pM	100 pM	>100,000	1	peptide monomer	[20,53]
EMP1	4200	400 nM	200 nM	2	25,000	peptide dimer	[54, 58]
EMP dimer	4800	approx 20 nM	approx 2 nM	200	250	peptidedimer	[59]
EMP-PAI1	63,000	NA	NA	5000	100	peptide dimer	[60]
ERP	3085	100 nM	approx 1 nM	NA	NA	peptide dimer	[61]
ERB	2170	approx 3 nM	45 nM	NA	NA	peptide monomer	[62]
EPO Mab 71	150,000	200 pM	1 nM	NA	NA	peptide dimer	[53]
Compound 1	500	0	60 μM	NA	NA	nonpeptide monomer	[55, 63]
Compound 5	6400	1–5 nM	4 μM	NA	NA	nonpeptide dimer	[55]
A1B10C1	2100	1–10 nM	NA	NA	NA	nonpeptide dimer	[56]

EC, extracellular; IC, intracellular.

molecules can be identified that can activate cytokine receptors and retain properties suitable for oral delivery.

EPO mimetics without homodimerization

Difficulties with development of small molecules that activate EPOR by dimerization can be bypassed by targeting a different mechanism. One report describes a peptide that activates EPOR by binding to a domain on EPOR similar to major histocompatibility complex (MHC) peptides (Fig. 2). This 23-amino acid peptide is reported to have both *in vitro* and *in vivo* EPO activity [62]. The molecule appears to activate by binding EPOR at a region distal to its binding site (transmembrane domain), suggesting that it activates differently than does rHuEPO. The mechanism may be similar to that of the virus envelope protein, gp55, that also activates·EPOR by an interaction with the EPOR transmembrane region [67, 68]. The nature of how activation EPOR occurs by gp55 is not understood.

Another approach to mimetic discovery is to modulate steps downstream from receptor activation such as by inhibiting hematopoietic cell phosphatase (HCP) [69, 70]. HCP is an enzyme that dephosphorylates JAK-2, a kinase that is part of the EPO signal transduction cascade [5]. JAK-2 is normally activated (phosphorylated) as a consequence of EPOR activation. HCP binds to activated (phosphorylated) EPOR and then dephosphorylates JAK-2, terminating signal transduction. Truncated EPOR lack the HCP binding site, and thus HCP cannot dephosporylate JAK-2, resulting in hypersensitivity of the receptor to EPO. EPOR truncations have been described in humans whereby the affected individuals have increased hemoglobin concentrations but very low EPO concentrations due to a hypersensitive EPOR [71, 72]. These observations suggest that small molecule antagonists of HCP may result in increased EPOR activity that increases erythropoiesis in the absence of added EPO.

One concern of HCP inhibitors relates to observations associated with HCP mutations in mice. These mice (motheaten) have a defective *HCP* gene [73] and have multiple hematopoietic abnormalities, including increases in macrophages, lymphocytes, and erythrocytes. HCP is a negative regulator for several different cytokine receptors besides EPOR [69, 70, 74, 75]. Although HCP inhibitors may be effective at increasing erythroid cell number, increases in cell number of other hematopoietic cells may limit the usefulness of these compounds.

Gene therapy

Controlled delivery of *EPO* genes to humans is another promising approach for EPO therapy. Early work in this field depended on direct injection of plasmid DNA containing constitutively active *EPO* genes into the muscles of mice

[76], resulting in a measurable increase in hematocrit. Several concerns became apparent from these studies, including inefficient and variable delivery of the *EPO* gene and subsequent variations in EPO concentration. EPO expression also decreased over time. Expression systems and gene delivery methods with improved efficiency have been reported [77–80]. Current *EPO* gene therapy protocols require repeated administration of EPO genes. In addition there is concern that the therapy may be irreversible or result in altered gene expression resulting in tumorigenicity. Over-expression of *EPO* genes could result in polycythemia with little ability to correct the condition.

The efficiency and irreversibility concerns have been addressed by developing implantable capsules containing EPO-expressing cells [81]. The capsules can be removed, halting EPO delivery. A further improvement would be to construct vectors whereby EPO expression is controlled by a small molecule such as tetracycline, enabling increased EPO expression in response to oral administration of the gene activator [82]. Controlled expression of the *EPO* gene has been demonstrated in mice using tetracycline [77, 78, 83], mifepristone [84], or rapamycin [85]. Additional advances are the development of vectors where EPO expression is controlled by oxygen tension [82], or methods that target the kidney for gene transfer [86]. Despite these advances, safe and controlled EPO delivery using gene therapy methods suitable for human use remains a distant but tantalizing opportunity.

New formulations and devices

Endogenous EPO concentration is exquisitely controlled in the body by rapid changes in expression. In contrast, protein therapeutics are placed in a non-physiologic environment for extended periods of time, which in some instances may be years. Safe storage in any formulation requires that conditions and formulations be designed to minimize formation and accumulation of unnatural breakdown products or alterations in EPO structure. Inappropriate formulations that do not maintain the integrity of the product can risk exposing the patient to an abnormal form of the protein.

Formulations containing rHuEPO have been successfully developed and used safely and effectively for more than a decade. Despite the success of current formulations, change is sometimes required to keep up with regulatory or safety concerns or to allow for new technologies, such as new devices or delivery systems. Such manufacturing and formulation changes included removal of excipients such as human serum albumin or bovine-derived products.

Prolonged stimulation of erythropoiesis is one desirable property that may be addressed by new delivery systems, including devices that allow controlled release of rHuEPO over a long time. This approach necessitates that the molecule remain stable in the device for a prolonged period of time. Another approach is to introduce rHuEPO into a biodegradable matrix that degrades slowly over time (slow release) [87, 88]. This strategy requires development of

methods to immobilize rHuEPO in a matrix, such as microparticles, that breakdown at predictable rates and release the product in a controlled manner.

The use of an appropriate slow-release process has not been successful for several reasons. The main one is a requirement that the protein remain intact and unchanged during both the processing of the material and during the prolonged exposure in the body. Protein integrity is a particularly difficult requirement in biodegradable matrices because the protein is in a concentrated hydrated state at physiologic temperatures for extended periods of time. Small amounts of contaminating rHuEPO aggregates, misfolded rHuEPO, or breakdown products may compromise not only efficacy but also safety (immunogenicity). Another concern is that too rapid breakdown of the matrix may result in excessive delivery resulting in an overdose.

Conclusions

Nearly two decades have passed from the heady days when the *EPO* gene was cloned and rHuEPO was first administered to a patient. Recombinant HuEPO has proven to be a safe and efficacious molecule for EPO replacement therapy, setting the bar high for any improvements that may follow. Nonetheless there remains a desire for better erythropoietic molecules, new formulations, or more useful delivery systems. The discovery, development and regulatory approval of darbepoetin alfa shows that it is possible to improve EPO replacement therapy in a safe and effective manner. In this case, darbepoetin alfa performs the same function as rHuEPO, but has increased *in vivo* activity and reduced serum clearance, and a similar safety profile. Further progress is anticipated as new devices that can simplify the administration of these drugs are developed. Additional changes in rHuEPO or in formulations are anticipated that may need to be developed to allow effective use of these delivery devices. The future will be exciting as small-molecule EPO mimetics are discovered, though matching the safety profile of rHuEPO presents a substantial hurdle to any small-molecule program. New erythropoietic agents may be administered orally. Permanent correction of anemia may occur through gene therapy, thereby allowing additional treatment opportunities. These developments may require extensive research and testing; however, many believe that these developments are not a question of if, but when.

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Erythropoietins and Erythropoiesis
Molecular, Cellular, Preclinical, and Clinical Biology
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This volume is a one-source guide to the most current information about red blood cell formation and the action of recombinant human erythropoietins. Topics in the fields of erythropoiesis, recombinant protein discovery and production, and treatment of patients with anemia will be covered. The newest theories in erythropoiesis (receptors, signaling), manufacturing, new formulations, and clinical research are discussed.

It is of interest to researchers and clinical investigators in academia and biotechnology and pharmaceutical companies, to clinical research associates, clinical monitors, and physician investigators.

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