Activation of the erythropoietin receptor by multivalent molecules

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Activation of the Erythropoietin Receptor by Multivalent Molecules

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A la mémoire de Keith

Ton expérience,
Ton inépuisable savoir,
Ta passion des sciences,
Ton rire communicatif,
Ton parfait anglais,

Resteront dans mes souvenirs à jamais.

Merci

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Résumé en français

Activation du récepteur à l'érythropoietine par des molécules multivalentes

L'érythropoïétine (EPO) est une hormone de 34 kDa qui régule la production et la différentiation des globules rouges. Elle interagit avec le récepteur à l'EPO (EPOR) afin de transmettre à la cellule l'information nécessaire à sa survie et à sa croissance. L'EPO recombinante est utilisée en clinique pour le traitement de l'anémie et plusieurs protéines dérivées ont été synthétisés afin d'augmenter l'efficacité du traitement. En 1996, un peptide de 20 acide aminés, EMP pour "EPO mimetic peptide", dont la séquence n'a rien de semblable avec celle de l'EPO à démontré la capacité d'activer l'EPOR en interagissant sur le même site que l'EPO. Sa faible activité initiale a pu être amplifiée jusqu'à 1000 fois en reliant les extrémités de 2 peptides, soit par un long polymère ou par un court segment peptidique. Un autre peptide, ERP pour "EPOR-derived peptide", possède lui aussi la capacité d'activer le récepteur de l'EPO, mais en interagissant avec un différent site sur le récepteur. Il a démontré une grande synergie avec l'EPO humaine lors d'expériences sur des souris.

Les travaux effectués durant ma thèse ont porté sur la recherche de nouveaux composés multimériques ayant une action sur le récepteur de l'EPO. Plusieurs stratégies ont été étudiées impliquant la synthèse chimique de dimères et tétramères de peptides, ainsi que la synthèse chimique d'un dimère d'EPO.

Une première approche décrit la stratégie et la synthèse chimique d'un dimère d'EPO. La séquence de 165 acides aminés a été divisée en 4 fragments, chacun synthétisé séparément, et qui seront ensuite reliés par liguation chimique native (Native chemical ligation). Suite à l'attachement de 3 fragments ensemble, le projet à dû être abandonné à cause d'importants problèmes de précipitation.

Une deuxième approche décrit ensuite la synthèse et la caractérisation biologique de peptides hétéro-dimères composés d'un EMP et d'un ERP. La synergie d'ERP avec EPO laissait augurer d'un grand potentiel sur l'activité d'un dimère, étant donné que chaque molécule interagit avec un site différent sur l'EPOR. Contrairement à ce que l'on attendait, l'activité d'EMP n'a pas été augmentée par l'ajout d'une molécule ERP, étant même diminuée dans

certains cas. Le fait qu'ERP n'avait qu'une très faible activité sur notre lignée cellulaire, qui est différentes que celle utilisée dans la publication originale, est certainement la cause de la faible activité de nos dimères.

Une troisième approche décrit la caractérisation chimique et biologique des dimères et tétramères d'EMP, reliés par des chaînes de polymères. Une première série de dimères a été purifiée afin d'étudier l'influence de la longueur du lien ("linker") ainsi que du site d'attachement du linker sur les peptides (extrémité N- ou C-terminale) sur l'activité des peptides. Des tests de prolifération cellulaire ont démontré la très faible influence de ces modifications sur l'activité des dimères. La poursuite de ce travail à démontré que pour des tétramères d'EMP synthétisés en reliant deux courts dimères par une très longue chaîne PEG (poly-ethylène glycole), la longueur du linker, le site d'attachement sur le peptide ainsi que la chimie utilisée pour relier les peptides influençaient l'activité des tétramères. Il a pu être démontré que la présence d'un groupe aromatique à l'extrémité des linkers était défavorable sur l'activité des molécules et que le remplacement de ce groupe aromatique par une chaîne linéaire augmentait l'activité des molécules. Le plus actif des tétramères égale presque l'activité de l'EPO humaine, représentant le peptide le plus actif sur l'EPOR jamais décrit jusqu'ici. Une hypothèse pour l'augmentation de l'activité des tétramères synthétisés avec de très long linkers est la possibilité d'interagir avec plus d'un complexe EPOR à la fois.

Finalement, la synthèse d'un dimère d'EMP par pont disulfure, a démontré la possibilité d'activer l'EPOR par une molécule rigide. Cette molécule a une activité légèrement inférieure aux dimères d'EMP flexibles, mais les perspectives d'optimisation structurale via l'allongement de la liaison entre les 2 peptides laissent entrevoir de belles perspectives quant à la découverte d'une molécule peptidique ayant une activité supérieure à l'EPO humaine.

La caractérisation de toutes ces molécules a contribué à la compréhension de l'interaction entre EMP et le récepteur à l'EPO, ouvrant d'intéressantes perspectives pour la synthèse de nouvelles molécules stimulant la formation des globules rouges.

General introduction

At the time when I started my Thesis in summer 2005, recombinant erythropoietin (EPO) was in clinical use for more than 15 years for the treatment of anaemia. An EPO biosimilar (or erythropoiesis stimulating agents (ESAs)) had already been approved by the FDA in 2001, possessing an increased circulation half-life in human plasma thanks to additional glycans (Darbepoetin alfa, Aranesp®, Amgen). Its major concurrent on the market today, CERA (Continuous EPO receptor activator, Mircera®, Roche) was in clinical trials and got approved in Europe in 2007. Other EPO derivatives with increased biological properties had been tested, for example EPO dimers synthesized by recombinant and chemical means or a synthetic EPO with branched polyamide polymers instead of the natural glycans (SEP), but none of the latter molecules went beyond Phase I.

Apart from the EPO analogues approved as drugs, many researches had been undertaken to find small molecule peptides mimicking the action of the endogenous hormone. Erythropoietin mimetic peptide (EMP), a short 20 amino acid peptide which sequence is not related to EPO, was shown to be a potent activator of EPO receptor (EPOR). Dimerization of EMP considerably increased its activity compared to monomers, one pegylated EMP dimer just finishing a Phase 1 clinical trial in summer 2005 (Hematide®, Affymax) . At the time I am writing this document, a Phase 3 clinical trial is in progress. Another short peptide, EPOR-derived peptide (ERP) was shown to induce signalisation by interacting with a different site on EPOR. This peptide, although requiring much higher doses than EMP or EPO to induce a signal, displayed synergic action with EPO.

The search for molecules activating the EPOR dates from the late 1970s, but the interest for ESA was still very present in 2005. Moreover, multimerization of peptides to increase their

biological activity, as observed with EPO and EMP dimers, was a promising strategy that was rapidly expanding. Several models describing the thermodynamic parameters governing multivalent interactions had been published and medical applications employing that strategy started to emerge.

Based on preliminary results obtained with EMP dimers synthesized in the group of Professor Keith Rose, I started my thesis work by repeating the synthesis of these molecules and rapidly got attracted by the synthesis of new multimeric EPOR agonists. Projects using any of the three molecules known to activate EPOR signalling were undertaken (EPO, EMP and ERP), always involving solid phase peptide synthesis and multivalency. My work mainly focused on EMP multimers, for which several parameters like the linker length, the peptide linkage site, the linker stiffness and the valency were tested. Altogether, more than 60 molecules have been synthesized, characterized and tested for biological activity by cell proliferation assay.

After an introductory part that describes multivalency and the biology of erythropoietin, the different projects that have explored the activation of the EPOR by multivalent agonists are presented.

Part I:

Description of multivalency and available tools for the synthesis of new multivalent molecules

Introduction

Multivalency refers to the interaction between connected ligands and connected receptors (Figure 1) [1]. Much higher binding affinities can be achieved by multivalent interactions compared to corresponding monovalent interactions. This phenomenon, which is termed binding avidity, is driven by both enthalpy and entropy. In biology, many processes are regulated by multivalent interactions which confer selectivity and sensitivity that could be not achieved via monovalent interactions.

The potential of multivalency has been harnessed in the synthesis of new medicines, in drug delivery systems, in imaging reagents and in vaccine generation. As a result of developments in engineering approaches, the use of multivalency has rapidly expanded in recent decades. These advances offer a range of promising strategies for the generation of new bioactive compounds. In this review, we discuss the thermodynamic principles related to multivalent interactions and highlight recent advances made in the field.

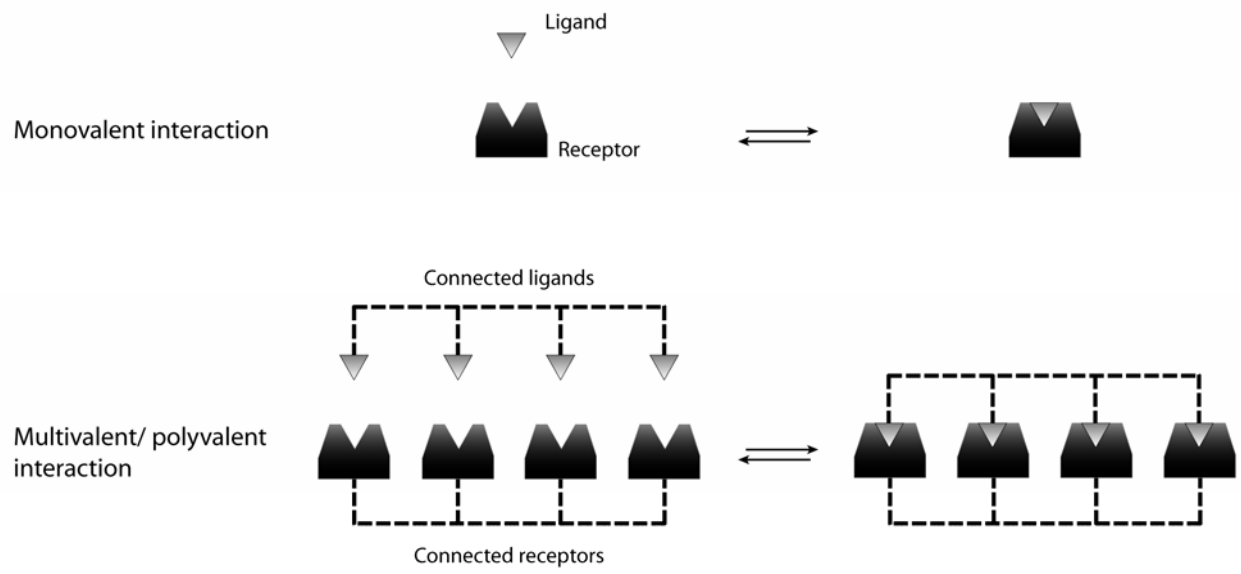

Figure 1: Monovalent and multivalent interactions

Nomenclature

The two components interacting during a molecular association are often referred to as "ligands" and "receptors", however the biological meaning of the term "receptor" can be misleading. Thus we chose to refer to the two components as "ligand" and "target" (Figure 2). The binding elements of multivalent ligands will be referred to as "ligand units"; these are connected to each other by a scaffold. The binding elements of polyvalent targets will be referred to as "target units".

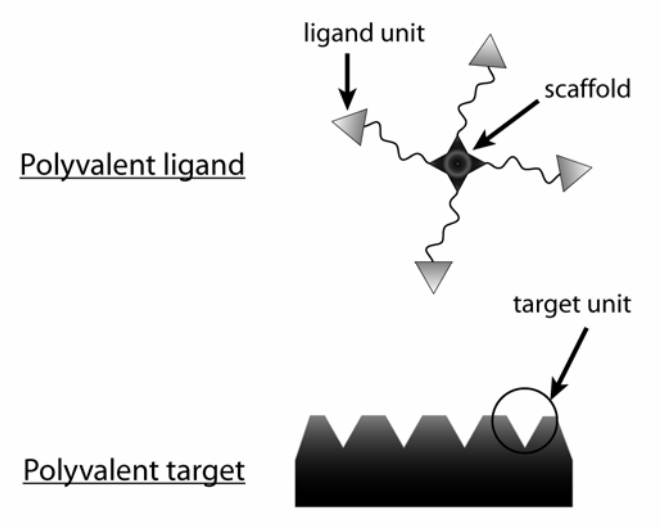

Figure 2: Nomenclature used for multivalent ligands and multivalent targets

Thermodynamics of ligand-receptor interactions

A molecular association is driven by free energy. The total energy difference between the initial and final state of an interaction is characterised by the change in Gibbs free energy, ΔG. The relationship between Gibbs free energy and the equilibrium constant (K_{equ}) is shown in Equation 1.

$$\alpha[A] + \beta[B] \leftrightarrow \gamma[C] + \delta[D]$$

Equation 1
$$\Delta G^\circ = -RT \ln(K_{equ}) = -RT \ln\left(\frac{[C]^\gamma [D]^\delta}{[A]^\alpha [B]^\beta}\right)$$

R is the gas constant (R = 8.314472 J mol^{-1} K^{-1}) and T is the absolute temperature (K).

Equation 2
$$\Delta G = \Delta H - T\Delta S$$

The free energy change of a binding interaction is determined by changes in enthalpy (ΔH) and entropy (ΔS), as shown in Equation 2. How enthalpy and entropy contribute to binding energy, and how they exert their influence on multivalent interactions, is described in the following section.

Enthalpy

In the case of molecular association, the enthalpy change (ΔH) results from the formation and breaking of non-covalent forces: hydrogen bonds, Van der Waals bonds and electrostatic interactions [2]. Enthalpically favourable non-covalent interactions have ΔH values <0 and are exothermic. A stable interaction between a ligand and a target implies that the forces created when the ligand binds to the target are stronger than the sum of forces that have to be broken between each partners and their environment (generally the solvent) [3-5]. In multivalent interactions (n identical ligand units interacting with n target units), the total enthalpy change upon binding is often considered as the sum of the enthalpies of each monovalent interaction ($\Delta H_{poly} = n\Delta H_{mono}$) [1, 6-9]. This approximation is based on two

assumptions; firstly, that the properties of a given "ligand-unit-target-unit" interaction is not influenced by the binding of its neighbours (non-cooperative binding); and secondly, that no interactions between the scaffold and the target, or between individual ligand units occur [7, 10].

Entropy

Entropy is the measure of the disorder of a system. The second law of thermodynamics states that the entropy of an isolated system will tend to increase over time, approaching a maximum value at equilibrium. In the case of molecular interactions, it relates to the freedom of movement, the concentration and the conformation of the interacting molecules. Entropy can be divided into contributions stemming from hydration, rotational, translational and conformational entropies, with the latter three contributions generally unfavourable upon complex formation [1]. An accurate definition of the four entropic contributions and their implications for polyvalent interactions is described below.

Hydration entropy

Hydration entropy refers to the energy gained by the release of ordered water molecules from the surface of a molecule into its environment [11]. This phenomenon, involving hydrophobic protein surfaces, is a positive force that is essential for protein folding and greatly contributes to the energy of binding [12-14]. For polyvalent interactions, the hydration entropy of each ligand unit-target-unit interaction is assumed to be identical whether the units are free or connected. As for enthalpy changes, this implies that no interaction between the scaffold and the receptor, or between the individual ligand units occur.

Rotational and translational entropies

Rotational and translational entropies refer to the freedom of ligands and targets to move and rotate through space. When a ligand associates with a target, entropic losses arise from the reduction in translational and rotational freedom that can be attributed to the loss of six entropy degrees of freedom (three rotational and three translational, one each along each axis) [12]. When the ligand binds to a membrane-associated target, it will lose almost all of its rotational and translational freedom, resulting in large entropy costs [15]. In the case of

polyvalent interactions, the connection of ligand units to a scaffold can have major effects on rotational and translational entropies [1]. Binding of the first ligand unit to the target can be comparable to monovalent interaction (see Figure 3A step 2, "inter-molecular" binding). Once the first ligand-unit is bound to the target, the freedom of movement of the unbounded ligand units is reduced (Figure 3A, step 2).

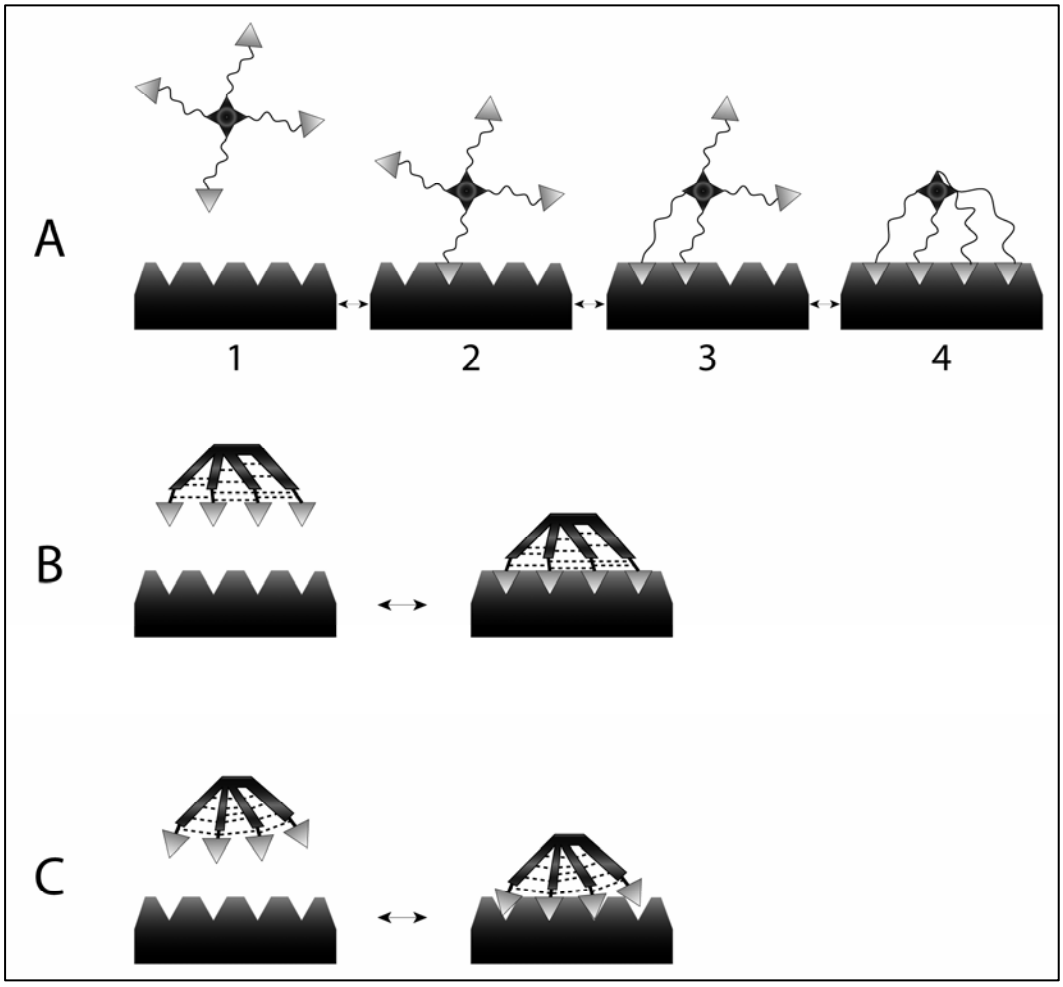

Figure 3: Interaction of a tetrameric ligand with a tetrameric target. **A:** Polyvalent interaction between a flexible ligand and a rigid target. Step 2 represents the "inter-molecular" binding event. Steps 3 and 4 are the subsequent "intra-molecular" binding events, favoured by the binding of the first ligand unit to the target. **B:** Polyvalent interaction of a rigid ligand with a rigid target. The conformation of the ligand closely matches the target conformation. this lowers the conformational entropy cost of binding of the tetrameric ligand almost to that of a monovalent interaction. **C:** Polyvalent interaction between a rigid ligand and a rigid target. The conformation of the ligand does not correspond to the target structure. Therefore, enthalpy of interaction will be lower compared to the sum of monovalent interactions.

This space confinement favours the subsequent binding of the ligand units by lowering their rotational and translational entropy cost of binding (Figure 3A steps 3 and 4, "intra-molecular binding"). This reduction in entropy costs of binding is one of the major factors underlying the enhanced binding energies achieved by multivalent interactions compared to the component monovalent interactions.

Conformational entropy

Conformational entropy is associated with structural rearrangement of molecules involved in an interaction, and is directly related to their flexibility. In the case of molecular association, binding of a flexible ligand to a rigid target structure imposes rigidification of the ligand, resulting in conformational entropy loss. To reduce this conformational entropy loss, scientists have designed ligands constrained into conformations that match those required to activate the targets [16, 17].

Appropriately constrained peptides can show significantly increased affinity compared to unconstrained peptides [18, 19]. However, inappropriately constrained ligands will lose binding affinity for the target because the incompatibility of the structures will prevent the formation of stabilizing forces, reducing the enthalpy of interaction. Conformational entropy also affects polyvalent interactions via the stiffness of the scaffold [1]. In the case of ligand units connected via a flexible scaffold, the freedom of movement after the first binding event is lower, resulting in reduced conformational entropy (Figure 3A steps 3 and 4). The use of rigid scaffolds to hold the ligand units in a conformation closely fitting the target structure considerably reduces the conformational entropy loss upon binding compared to flexible ligands, leading to significant gains in binding energy (Figure 3B). As is the case for inappropriately constrained monovalent ligands, rigid polyvalent molecules presenting the ligand units in a conformation that does not corresponds to the target structure will have a reduced affinity resulting from lower enthalpy of association (Figure 3C).

Multivalent interactions in biology

In biology, many processes involve multivalent interactions. Compared to monomeric interactions, the main advantage of multivalency is the increased affinity that can be achieved by adding up weak interactions. Regulation of signalling, either by dimerization of receptors or through the modulation of receptor density on cell membrane are other examples of mechanisms exploiting multivalency. A description of biological reactions involving multivalency will be discussed in the next section [1].

Multivalent interactions in immunity

A well known example involving multivalent ligands is the recognition of antigens by immunoglobulins [20]. As well as possessing many equivalent antigen recognition sites (from two for IgD, to ten for IgM), antibodies have a different binding site on the Fc portion [9]. The equivalent ligand units mostly interact with receptors present at the surface of invading pathogens, like viruses or bacteria. There, polyvalency is used to increase affinity in order to compensate unspecific interactions (Figure 4) [21-23]. In addition, multivalency contributes to the regulation of the signal triggered by the Fc portions, as simultaneous presentation of many Fc portions is required to transduce a signal that a single antibody cannot generate [24]. The immune system exploits three aspects of multivalency to fight against pathogens: the increased affinity by multiple similar interactions, the recruitment of specific cell types by two different binding sites (hetero-bifunctionality) and the regulation of signal transduction by density-specific interactions.

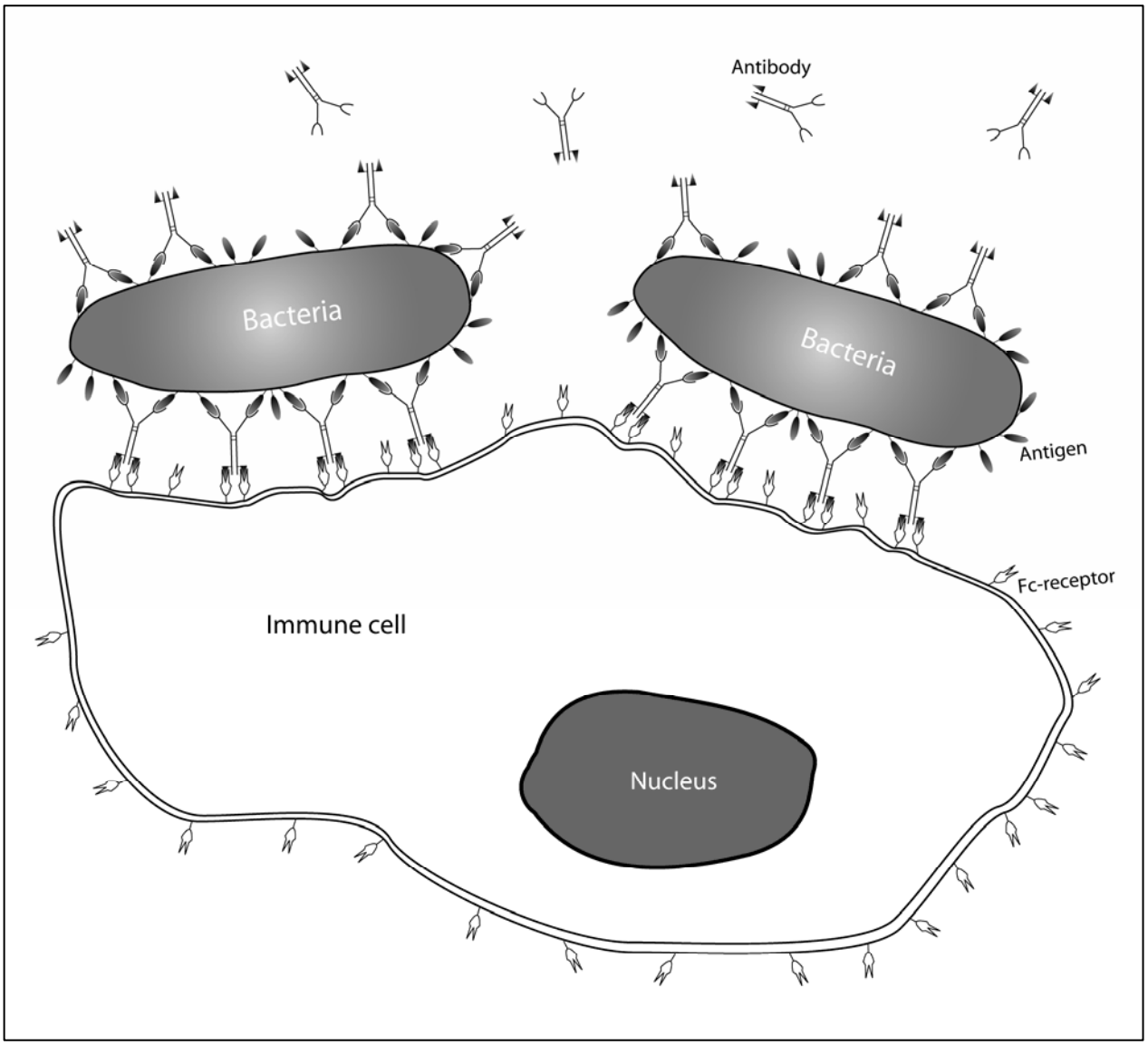

Figure 4: Implication of multivalency in immunity. The first step in bacteria recognition involves the interaction of divalent antibodies with antigens present at the surface of the pathogens. Then, the Fc region of the antibodies interact with Fc-receptors at the surface of immune cells, for which multiple simultaneous interactions are required to trigger a signal.

Multivalent interactions at cell surface

The transmission of signals from the extracellular to the intracellular environment and vice versa is controlled by molecules present at the cell surface. For example, in inflammation, cell surface selectins interact with glycoproteins at the surface of leukocytes in the blood flow and initiate their adhesion to the endothelium [25]. The strength of interactions between the cells depends on the valency of interactions [26]. Cell-cell recognition and cell migration are also

controlled by multivalency via the regulation of adhesion strength [27-30]. Finally, pathogens use similar mechanisms to adhere and invade host cells [31]. Viruses and bacteria express many copies of receptor ligand mimicks at their surface to create firm adhesion with the target cell and, in some cases, to initiate their internalization [22, 31-39].

Multivalent interactions in signal transduction

The coordination of activating molecules via multivalency is essential for the polymerization of actin, which is involved in the control of cell motility [40, 41]. The control of receptor density on the cell surface is another example of a regulation mechanism involving multivalency [42]. Through the control of receptor expression, a cell can modulate its sensitivity to extracellular signals, for example via the formation of clusters [43].

Dimerization of receptors and modulation of their selectivity by the formation of homo- or hetero-dimers are critical events in the activation of signalling cascades [44-47]. The JAK-STAT (Janus Kinase- Signal transducers and activators of transcription) signalling pathway is a good example of a system that requires association for activation [48-51]. Formation of homo- or hetero- dimers between several members of the JAK and STAT families dictate selectivity toward ligand recognition and allow many functions to be regulated specifically. The ability of these kinases to make different combinations between members of the same family allows a large variety of processes to be regulated by the same molecules.

Multivalent ligands have been used to study the importance of multivalency in signal transduction [52-54]. Via the control of the valency, the orientation and the density of the ligand units, a good understanding of the mechanism of activation of a selected receptor can be obtained.

Medical applications of polyvalent molecules

The possibility to modulate biological activity of molecules by connecting them to a central scaffold has found many applications in the medical environment. Multivalency has been employed in the development of drugs, to increase the affinity of molecules for polyvalent targets. In the case of peptide vaccines, the simultaneous presentation of multiple antigens has shown to increases immunogenicity, playing the role of an adjuvant. Other medical applications using multivalent effects involve tumor targeting, drug delivery and imaging. These aspects will be discussed in the next section [55-57].

Multivalent drugs

Because some drug targets possess multiple binding sites, polyvalent drugs have been developed to increase the activity of monovalent molecules [58]. As discussed previously, viruses and bacteria contain high density of proteins at their surface. Polyvalent inhibitors against viruses and bacteria have been prepared by attaching many copies of ligand units to a flexible polymer, simultaneously interacting with many antigens exposed at the pathogen surface [22, 32, 59, 60]. A large number of studies report on the synthesis of Shiga-like toxins polyvalent inhibitors [61-63]. Based on crystal structure, optimisation of the inhibitor conformation leads to a decavalent molecule with potency more than a million-fold higher than the monovalent ligand [64, 65]. Many polyvalent proteins have been targeted by polyvalent inhibitors, some displaying potent *in vivo* inhibition activity [66-75]. The mechanisms of inhibition are attributed to two factors: competitive binding of toxins and steric stabilization [22, 32]. Polyvalent agonists have also been created with much higher activity than monomeric molecules. Short peptides dimers targeting the erythropoietin receptor have been identified and are currently in phase 3 for the treatment of anaemia (Hematide) [76-80].

Multivalency in drug delivery and imaging

Once a potent ligand has been selected to become a drug, the molecule has to be directed to the site of action and must avoid side-reactions with non-specific receptors. To help the

transport, formulations have been developed that optimize the stability and availability of the drugs. Multivalent structures have found new applications as drug delivery devices, resulting from the higher affinity and specificity obtainable by multivalency [81-85]. A drug can be targeted to a specific site by combining two types of ligand units on the same molecule, one acting as a drug and the other directing the molecule. Globular molecules that have been derivatized to expose many copies of targeting molecules at their surface can carry drugs in their cavity and release them on the action site [83, 86-90]. Another application of polyvalent molecules is in biomedical imaging [91, 92]. Globular polyvalent ligands encapsulating luminescent molecules in their cavity can be functionalized to specifically target a cell subtype, allowing localisation of the desired cell population by positron emission tomography of fluorescence techniques [93-98]. Moore et al. developed a multifunctional nanoparticle tool that combines targeting, imaging and gene delivery [99]. Using fluorescence microscopy, they followed the entrance, release and action of a siRNA in tumor cells.

Multivalent presentation of antigens for vaccine production

The use of peptides to generate a protective immune response has found wide application for the generation of vaccines. Because single antigens do not trigger antibody response, the use of adjuvents is generally employed. It was found that the simultaneous presentation of densely packed antigens could generate an immune response, thus replacing the action of the adjuvent. These new molecules, termed multiple antigenic peptides (MAP), where first described by J. Tam in 1988 and have since found many applications [100]. MAPs have been used to generate vaccines against malaria and HIV, measles, influenza and hepatitis [101-108]. Such molecules offer advantages in the controlled valency and homogeneity of the constructs compared to peptide mixtures

Scaffolds for multivalent ligands

Many scaffolds are available to connect ligands units, playing a crucial role in the conformation and function of the multivalent ligand. Scaffolds used in polyvalent ligands can be divided in two main categories: proteic and non-proteic scaffolds. Each type offers a large choice of structures that vary in flexibility, size, valency, homogeneity and encapsulation feasibility. Protein scaffolds start from known protein complexes that are derivatized to bear ligand units at their surface. Non-proteic scaffolds are generally prepared to fit with a desired conformation, using different chemical entities to obtain the desired characteristics. The choice of scaffold will mainly depend on the target and the application. In this section, a large panel of scaffolds will be described, which have been used for the synthesis of polyvalent ligands (Figure 5).

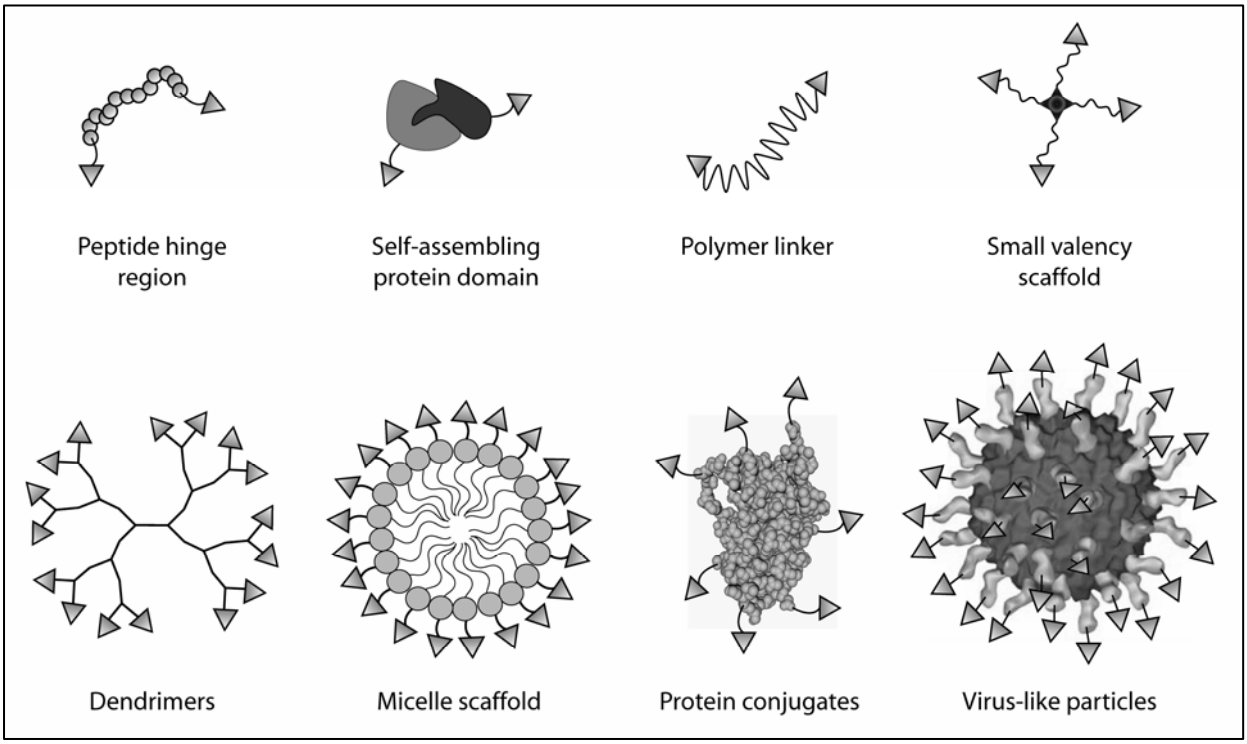

Figure 5: Scaffolds for multivalent molecules

Protein scaffolds

Protein Conjugates

Large proteins can be used as carriers for ligand units attached at their surface, the most widely used being bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) [109, 110]. The ligand units can be attached at surface exposed lysine or cysteine residues, which are targeted by chemoselective linkage units. Such polyvalent molecules offer advantages of solubility and high valency, their synthesis being facilitated by the availability of carrier proteins and derivatization reagents. Unfortunately, these constructs often lack homogeneity because of the limited control of the valency and orientation of the ligand units [54].

Peptide hinge region

In the situation where the ligand units can be synthesized by recombinant techniques, an easy way to link them is to modify the primary structure in order to obtain one molecule composed of the two ligand units linked by a polypeptide hinge region. The sequence of the peptide linker is crucial for controlling flexibility and protease resistance. An important detail with such linkage is that only attachment from the N-terminus to the C-terminus is possible, positions that can be involved in biological function. This strategy was successfully applied to the synthesis of recombinant erythropoietin (EPO) dimers, where the proteins were linked by a 9- or 17-amino acid linker [111, 112]. Peptide hinge regions can also be employed to connect ligand units to a central scaffold [113].

Self-assembling protein domains

The synthesis of antibodies and modified antibodies was revolutionized by advances in recombinant expression techniques [114-116]. Synthesis of single chain variable fragments (scFv) coupled to several multimerization domains allowed the synthesis of molecules with lower mass together with higher valency and specificity than natural proteins [117-120]. Other ligands have also been multimerized with self-assembling protein domains, some example are described below.

Biotin-streptavidin interaction as tetramerization scaffold

The strong interaction between biotin and avidin and the tetramerization domain of avidin have been widely exploited in biochemistry [121-123]. Avidin and its bacterial equivalent streptavidin are composed of four identical subunits that associate to form a stable tetramer. Each subunit possesses an independent biotin binding site [124]. The subunits were fused to antibodies by recombinant techniques, to form tetrameric complexes that still contain free biotin binding sites [125]. The latter can be used for additional interactions with biotinylated molecules, thus forming bifunctional ligands [126]. Interaction of biotinylated ligand units with fluorescently labelled streptavidins forms tetrameric ligands that are useful markers for *in vivo* localization of targets [127]. Production of tetramers based on a streptavidin scaffold is relatively straightforward given the commercial availability of streptavidins and several biotinylation reagents. One characteristic of the streptavidin scaffold is its relatively large mass (53 kDa).

Coiled coil superstructures

A coiled coil is a structural motif that consists of up to seven α-helices assembled in a supercoil structure [128-130]. Mutagenesis offers the possibility to modulate the orientation and the number of associating helices, giving rise to many possible conformations [131-134]. Additional cysteines can be incorporated at the extremity of the helices to stabilize the complex [113, 135].

These multimerization domains were used for the synthesis of dimers, trimers and tetramers linked to scFv or to integrin binding domains [136]. A homogeneous pentameric ligand based on the coiled-coil region of the cartilage matrix was obtained that exhibited a 10^5 increase in affinity compared to monomeric ligands [137]. These examples show the great flexibility in the synthesis of controlled coiled coil multimeric assemblies with the advantage of making a complex of relatively low molecular weight that self-assembles *in vivo* [135].

Protein p53 tetramerization domain

The transcription factor p53 possesses a tetramerization domain that is essential for activity, which was exploited to create synthetic ligand tetramers [138, 139]. Association of polycationic import sequences or of anti-tumor scFv with this p53 tetramerization domain

significantly increased their biological activity [140, 141] [119]. A combined multimerization and PEGylation strategy further enhanced the *in vivo* biological activity of such polymeric scFv [142, 143]. As in the case of coiled coil motifs, the tetramerization domain of the p53 protein offers a stable platform for the presentation of ligand units with the advantages of being small (31 amino acids) and not toxic [141].

Virus-like particles

Recently, the use of self assembling capsid proteins to display protein epitopes was developed for vaccine application [144-146]. The great advantage of these carrier proteins is that they can present a high number of ligand units at their surface with a great density, thus eliciting an immune response without the need for adjuvants. Generally, virus-like particles (VLPs) are modified to carry an epitope region on the terminus of the self-assembling proteins [147, 148]. For steric reasons, cross-linking strategies were developed to allow the attachment of large molecular weight proteins, further exploited to attach different proteins on a single capsid [149, 150]. VLPs from several virus types have been used (hepatitis B, tobacco mosaic virus and cowpea mosaic virus), possessing specific characteristics of high valency, homogeneity and ease of synthesis with proteins self-assembling *in vivo* [151-153].

Escherichia coli verotoxin oligomerization domain

Apart from virus capsid proteins, toxins were also adapted to serve as scaffolds. *Escherichia coli* verotoxin 1 (VT1) is a pentameric toxin that interacts with host cells through protein-glycolipid interactions [154]. The 40 kDa self-associating domain was used as a scaffold for the synthesis of pentameric ligands [155, 156]. Neutralising antibodies against VT1 have been synthesised by coupling scFvs to VT1 pentamerization domain [157]. This strategy of using the target as a scaffold for inhibitor synthesis proved successful with blocking of up to 90% of the cytotoxicity *in vitro* [62]. An interesting feature of this association domain is that the C- and N- termini of the subunits are exposed on opposite sides of the molecule [158, 159]. Thus, by fusing ligand units on each side of the subunits, bispecific decavalent molecules can be constructed [160]. This scaffold adapted from toxin offers excellent thermostability and protease resistance.

Other self-assembling protein domains

In addition to the well-characterized protein association domains, some particular structures have found applications in the synthesis of multivalent molecules. One example is the dock and lock method that was developed recently [161]. This method takes advantage of the specific interaction between the regulatory subunits and the anchoring domain of two proteins. By connecting Fab portions to each subunit, the isolation of bispecific trimeric and hexameric molecules was possible. A known structure that was also derivatized for the synthesis of protein trimers and hexamers is collagen [162].

Dendrimers

Dendrimers are branched synthetic polymers possessing structure like trees which are often used as scaffolds for polyvalent molecules. They are homogeneous with a globular shape and can be derivatized to expose reactive sites at their surface. Their stepwise synthesis allows the control of the flexibility, density, solubility, valency and size of the polyvalent ligand [82, 101, 163]. These molecules have found many applications in drug delivery, biomedical imaging, tumor targeting and vaccine development [83-85, 90, 164-167]. Dendrimers can also act as catalysts [168]. Strategies for the encapsulation of molecules inside the core of the dendrimer have recently been developed, suggesting different approaches to deal with the release of the trapped molecule [86-89]. The great flexibility of synthesis which combines different cores, several arm lengths and an increasing number of chemical "hooks" capable of reacting with specific sites on ligands offers numerous possibilities for the tuning of dendritic constructs. Compared to self-assembling scaffolds like VLPs or micelles that present the ligand units in a similar way, dendrimers have the advantage of having covalent linkages, with a more stable polymeric structure.

Small valency scaffolds

Many chemical entities can be used as a scaffold once they possess more than one reactive centre. Benzene derivatives and other functionalized small molecules such as porphyrins, adamantine and azetidinone have been used for the synthesis of multivalent ligands [169-172]. Calixarene-based scaffold offers the possibility to encapsulate guest species [173].

Natural products have also been adapted with the intention of avoiding immune responses when administred *in vivo*. Polyvalent ligands with carbohydrate moieties, diketopiperazine or DNA have been synthesized, forming multi-functional molecules [65, 174-176]. The use of hydrogen bond linkage has also been investigated to obtain pseudo-dimeric ligands [177]. Molecules capable of connecting proteins *in vivo*, chemical inducers of dimerization (CID), have been used in the exploration of receptor binding surfaces [54, 178]. The valency, orientation, flexibility, length, encapsulation properties and size of the scaffolds can easily be tuned with respect to the target. With their small size and resistant covalent structures, small molecules can adopt almost any conformation and are thus a excellent tool for the synthesis of polyvalent structures.

Polymer scaffolds

Probably the most common polymer scaffolds used today are based on Poly(ethylene glycol) (PEG) chains [179]. PEG polymers are composed of a mixture of different oligomer sizes in broadly or narrowly defined molecular weight ranges. The great advantages of these molecules include their high levels of flexibility, water solubility, their commercial availability and the fact that they are non-immunogenic. The main drawback is their heterogeneity. Linear and branched polymers have been used as scaffolds for polyvalent ligands. An general application of PEG in drug development is for the increase of protein mass to enhance their circulation half time *in vivo* (pegylation) [143]. Successful studies to measure the distance spanning receptor binding sites have been performed with PEG linkers of different sizes [179]. Other polymers have been used for the synthesis of multivalent ligands, bearing peptides on the repeating units or at the extremity of the molecule [22, 43, 180-182]. To avoid the problem of inhomogeneity of polymers, a technique was developed to synthesise monodisperse polyamide linkers with similar physical properties to the PEGs [183]. These linkers have been used to test the influence of linker length on the activity of erythropoietin mimetic peptide dimers and tetramers [78, 79].

Lipid scaffolds

In solution, lipids form spherical aggregates with hydrophilic heads in contact with the water, sequestrating hydrophobic tails. Two types of aggregates can form: liposomes, which have a

bilayer membrane, and micelles, composed of a monolayer membrane. Depending on the structure of the lipids, aggregates can adopt different characteristics of size, shape and fluidity within the layer. Functionalized lipids can be used to obtain liposomes and micelles with ligand units at their surface. Inhibitors of influenza virus attachment and of anthrax toxin have been obtained with derivatized liposomes [60, 69]. Because of the propensity of micelles to disaggregate in specific conditions, these molecules have been used as drug delivery systems [184-186]. Given the fluidity of amphiphilic molecules within the lipid layer and the ability of these structures to fuse with cell membranes, they are not appropriate as scaffolds for multivalent ligands.

Linkage chemistries

Two strategies can be employed for the synthesis of peptide multimers: the divergent and convergent approaches [101]. The divergent strategy starts from the synthesis of the scaffold followed by the stepwise addition of ligand units. This strategy does not require any chemoselective linkage since the whole molecule is synthesized step-by-step from a single starting molecule [100]. The convergent strategy is a two step process where the scaffold and the ligand units are synthesized in parallel and then connected. In biology, attachment reactions are catalyzed by enzymes that orient the two reactive groups, facilitating bond formation. Because enzymes are very selective toward ligand structure, chemists have developed chemoselective ligation strategies to link molecules. To connect ligand units to a scaffold, the chemoselective reactions should occur ideally in mild conditions, avoiding cross-reactivity with amino acids. Such reactions were first developed for peptide synthesis, and have been used successfully for the synthesis of multivalent ligands [101, 181, 187-191].

Disulfide bonds

A strategy that is exploited in nature to make protein conjugates is the creation of disulfide bonds. Two thiol groups present on cysteine side chains can be oxidized to form a disulfide bridge. This covalent interaction is specific and thus offers the requirements for strong attachment, and because cysteine is a relatively rare amino acid in nature (natural abundance of 1.5%), it is a reasonable strategy for chemoselective attachment. One advantage of this reaction is that cysteine is a natural amino acid that can be introduced by mutagenesis. Disulfide bond linkage has a major attribute: the link is cleaved in reducing conditions, being an advantage or a desadvantage depending on the application Use of disulfide bonds for the synthesis of polyvalent molecules was applied to MAPs and to the formation of stable membrane receptor dimers [44, 192-194].

Thiol addition reactions

Many activated alkenes such as maleimides, vinyl sulfones and acrylates selectively react with the thiol group of cysteine to form stable covalent bonds under mild conditions [181].

Side reactions with amines can occur, although they can be minimized by controlling the reaction conditions [195]. Because many maleimide reagents are commercially available, this strategy has found many applications in the synthesis of multivalent molecules [69, 151, 153]. Another reaction specific to cysteine is radical thiol alkylation. Alkylation of cysteine with labelled tags was employed extensively for the identification of proteins and peptides using isotope-coded affinity tags [196]. As observed with activated alkenes, radical alkylation suffers from side reactions with amino groups.

Chemoselective reactions involving natural amino acids

The coupling reaction between an activated carboxylic acid and a primary amine is well known in peptide chemistry and offers a direct and efficient method for conjugating ligand units to a scaffold. Lysine side chains and α-amino groups of peptides can be targeted by activatetd carboxylic acid. The reactions are performed in mild conditions, in the absence of catalysts and with very high yields. One limitation of this strategy is that only a single amino group can be exposed by the ligand or the target. Homo- and hetero-bifunctional linkers functionalized with amino reactive and/or thiol reactive moieties are commercially available and have been widely employed [76, 179, 197]. Native chemical ligation was developed to form an amide bond between two unprotected peptides [198]. This selective reaction between an N-terminal cysteine and a thioester is performed in mild conditions. Although that reaction was primarily developed for peptide fragments coupling, it has also been used for the attachment of ligand units to a dendrimer scaffold [199]. Amino terminal cysteines, serine and threonine specifically react with aldehydes to form pseudo-proline linkages, a strategy that was used for the conjugation of unprotected fragments [200].

Other chemoselective reactions

Previously described chemoselective reactions always involved natural amino acid. Because the presence of more than one reactive amino acid on a ligand will form inhomogeneous constructs, chemoselective reactions between chemical moieties not present in natural amino acids were developed. Commonly used chemoselective reactions are the oxime and hydrazone

linkages because they can be performed in mild conditions and form a stable linkage *in vivo* without side reaction [201-205]. Oxime ligation, which is the reaction between an aldehyde and an aminooxy moiety, has found many applications in the synthesis of multivalent molecules and in the production of multiple antigen peptides [78, 79, 103]. One advantage of these techniques involving aldehyde moieties is that the aldehyde can be obtained by oxidizing an N-terminal serine residue, thus not necessitating a non natural amino acid [206]. Other chemoselective reactions are convenient for the conjugation of ligand units to a scaffold, although these were use to a lesser extent than the previously cited reactions because of the synthesis conditions. These involve the Staudinger ligation [207-209], click chemistry reactions [210, 211], and carbon-carbon forming ligations catalyzed by palladium like the Heck, Sonogashira and Suzuki reactions [212-216].

Biotin/avidin interaction

Among the chemoselective reactions used to couple ligand units to a scaffold, the biotin-avidin interaction does not form covalent linkages. This very tight interaction was used for the attachment of ligand units to a preformed scaffold [217].

Conclusion

Multivalency is a rapidly growing field that has found many applications. Thermodynamically, the advantage of multivalency over monovalency is explained by the additivity of binding enthalpies and by the gains in rotational and translational entropy for the intra-molecular binding events. Using rigid scaffolds can greatly reduce the conformational entropy losses upon binding compared to flexible ligands, providing the ligand conformation closely fits with the target structure. Biological interactions have highlighted the functions that can be modulated by multivalency. Not only the strength of interaction can be increased by combining many low affinity associations, but also specificity and the rate of a reaction can be controlled through polyvalent associations. The different combinations of proteins to form hetero-dimer complexes offer the possibility to create new biological entities without the synthesis of new molecules. The use of multivalency in drug design is rapidly expanding, especially toward polyvalent targets. Multivalent molecules are already commercially available for medical imaging and as a vaccine, where polyvalency replaces the use of adjuvants. A very broad variety of scaffolds are available that possess different characteristics in size, shape, valency, stability and flexibility. From large self-assembling protein domains to small organic molecules, the large choice of molecules increases the possibilities to obtain a ligand whose structure closely fits with the target conformation. For multivalent ligands, the scaffold is the central element that will determine the importance of the entropy contribution. A wide choice of chemoselective reactions is available to connect ligand units to a preformed scaffold. Depending on the ligand unit, a specific amino acid can be targeted or a chemical handle can be introduced to specifically react with the scaffold without affecting the rest of the ligand unit.

This review highlights numerous applications of multivalency and gives promising directions for the synthesis of active multivalent molecules. Further studies of protein complexes in cells will certainly identify new assembling domains that could be used as scaffolds for the synthesis of multivalent ligands. The substantial increases in affinity provided by this technique will likely find new applications in the pharmaceutical industry. The increasing number of polyvalent molecules that have been described has enlarged the basic knowledge in that field, and can be expected contribute to the discovery of new active molecules.

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Part II:

Erythropoietin and erythropoiesis stimulating agents

Introduction

Erythropoietin (EPO) is a glycoprotein hormone that regulates the differentiation and proliferation of red blood cells (RBCs). It is produced in the kidney and is regulated by oxygen level in the blood. Interaction with its cognate receptor, EPO receptor (EPOR), activates a signalling cascade that leads to the transcription of specific genes that control erythropoiesis.

The first evidence of a substance regulating haematopoiesis dates from 1906, when Carnot and De Flandre demonstrated the relation between oxygen levels and RBC production [1]. That is only in 1948 that the term "erythropoietin" was introduced, after Bondsdorff and Jalavisto proved that this substance was specific to RBCs [2]. Later, EPO production was shown to be situated in the kidney under the control of hypoxia [3, 4]. Isolation of the hormone in urine of anaemic patients was achieved in 1977 and the gene sequence was determined 8 years later [5-7]. The first clinical trials with recombinant EPO dates from 1987 for the treatment of uraemic patients [8]. Since then, many studies have been performed to understand the structure, function and regulation of erythropoietin. Recombinant EPO and biosimilars have been synthesized for the treatment of anaemia and recently, erythropoietin was also shown to possess neuroprotective and cardioprotective potentials. Although the American Food and Drug Administration (FDA) has warned about the risks associated with EPO treatment following cancer chemotherapy, the therapeutic potential of EPO remains very large.

Biology of erythropoietin

Structure, synthesis and clearance of erythropoietin

Erythropoietin is a glycoprotein hormone of 165 amino acids possessing four cysteines forming two disulfide bridges [9]. The gene product has 193 amino acids and cleavage of the leader sequence of 27 amino acids as well as the carboxy-terminal arginine gives the active hormone [10]. The 34000 Dalton protein possesses three N-linked carbohydrate moieties at Asn 24, 38 and 83 and one O-linked carbohydrate moiety at Ser 126 that compose approximately 40% of the molecular weight. Glycans are mainly responsible for protein stability, but also participate in the biosynthesis and secretion of the hormone [11-15].

The hormone is primarily produced in the kidney, with low level of expression being detected in the liver, kidney, lung, spleen, brain and testes [16-20]. In the foetus, EPO is produced in the liver, beginning to switch at the age of 40 days [21]. The level of oxygen in the blood regulates the production of EPO through hypoxia responsible elements that are conserved for many hypoxia-inducible genes [3, 22-24]. Under hypoxic conditions, hypoxia-inducible factor-1 (HIF-1) is activated by dimerization and interacts with a DNA enhancer region to activate EPO gene transcription. When oxygen is present, the HIF-1α subunit is hydroxylated by prolyl-hydroxylase, leading to its rapid degradation.

In adults, EPO has a plasma half-life between 2 and 13 h and a concentration in the picomolar range. The clearance of the hormone has not been extensively studied, but it seems that degradation is coupled with EPO receptor internalization [25]. After interaction of the hormone with its receptor, the complex is rapidly internalized. After dissociation, approximately 60% of internalized EPO is resecreted while the remaining 40% are degraded [26].

Erythropoietin in erythropoiesis

Erythropoiesis

Erythrocytes compose approximately half of the blood cell population and are the final product of multiple differentiation steps starting from a multipotent haematopoietic stem cell (HSC) (Figure 6) [27]. Because erythrocytes have a limited lifespan of about 120 days, continual renewal of the red cell population is essential. The multipotent HSC, present in the bone marrow and in fetal liver, can develop into all lineages of blood cells, depending on the environment and regulation by transcriptional factors. These cells rapidly self-renew and can differentiate into progenitor cells which are committed to more specific lineages. Erythroid progenitors include burst-forming unit erythroids (BFU-E), which then differentiate into colony-forming unit erythroid (CFU-E) [28]. At this stage, erythroid commited cells become more and more sensitive to EPO and in contrast possess limited self-renewal capacity. Further differentiation into erythroid precursors, erythroblasts, gives rise to cells with morphological characteristics and accumulation of haemoglobin in the cytoplasm. Maturation of erythroid precursors into reticulocytes will form the earliest erythroid anuclear cells. This immature RBC finally leaves the bone marrow and pass into the bloodstream to become a mature erythrocyte.

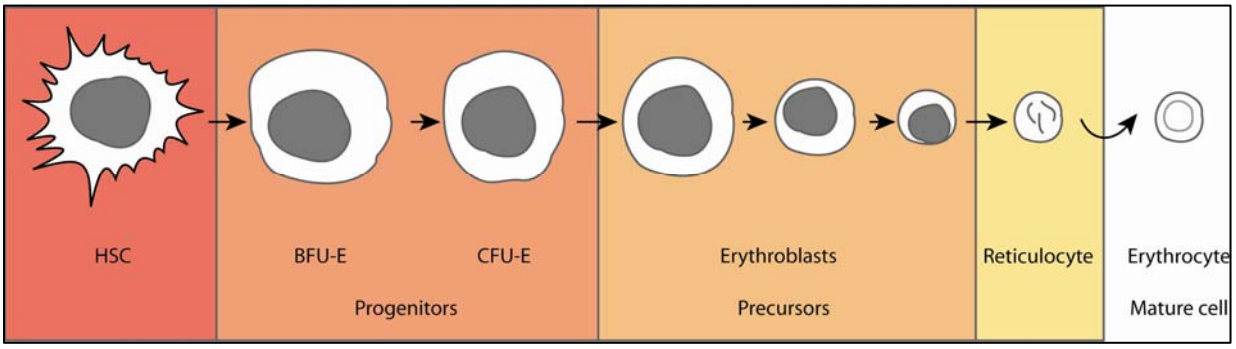

Figure 6: Major differentiation steps of a Haematopoietic Stem Cell to become an erythrocyte

Regulation of erythropoiesis

The stepwise maturation of a multipotent stem cell into a mature erythrocyte is regulated by several cytokines and transcription factors. These elements exert either positive or negative

control on the expression of all erythroid genes and can either act individually or simultaneously. Because erythropoiesis takes place in distinct anatomic sites, the local microenvironments provide specific regulatory factors that ensure proper development of erythrocytes.

Transcription factors bind to DNA or interact with proteins to modulate their activity [29]. Regulation elements are active throughough erythropoiesis, depending on the cell type and differentiation stage. One of the main regulator, zinc-finger transcription factor GATA-1, has been shown to interact with many proteins like FOG-1, EKLF, SP1, CBP/p300 and PU.1 [29].

Through the control of survival, proliferation, differentiation, commitment and maturation, hematopoietic cytokines are essential to the production of RBCs [30]. These cytokines, which can either be lineage specific or act on several haematopoietic cells, are generally effective at different stages of the maturation. Among the cytokines involved in the regulation of erythropoiesis (like interleukins, granulocyte-macrophage colony-stimulating factor (GM-CSF) and thrombopoietin), EPO is most specific and most involved [28, 31-34]. Immature progenitor BFU-Es are only slightly sensitive to EPO, CFU-Es and more mature cells becoming increasingly more sensitive to the cytokine through the differentiation process. Mainly acting as an anti-apopoptic agent, EPO synergistically acts with other cytokines to regulate the maturation and proliferation of RBCs.

Erythropoietin and anaemia

Anaemia is defined as a deficiency in RBCs. It is the result either of a reduction in the production of RBCs or an increase in the loss of erythrocytes, the main symptom being unusual tiredness. Anaemia can be minor, moderate or life threatening and can be caused by several deregulations [27]. The most common anaemia is caused by iron deficiency, but many types are related to endogenous EPO secretion. Inadequate EPO secretion is observed in many diseases, mainly related to renal failure, and these anaemias can either be treated by blood transfusions or by injection of exogenous EPO and other erythropoietin stimulating agents (ESA). EPO was first isolated from urine before the protein could be synthesized by recombinant means and used for the treatment of patients suffering from chronic renal diseases, decreasing the need for blood transfustions with associated risks of contamination [7, 8, 35]. The first recombinant hormones, epoetins alfa and beta, were synthesized in

Chinese hamster ovary cells and required 3-times weekly injections to maintain hematocrit level at normal values [36, 37]. Modified EPO were synthesized with prolonged plasma half life, thus reducing the frequency of injections [38-45]. These synthetic agents stimulating erythropoiesis will be discussed in more details below. Most EPO derivatives have been used in the treatment of anaemias resulting from renal failure [35, 46-48], but recently these compounds have been tested for the treatment of anaemias resulting from cancer chemotherapy [49-52]. Despite the success of these compounds, the American Food and Drug Administration (FDA) has recently warned about the use of ESAs for the treatment of cancer associated anaemias [53-58]. In some studies, use of ESA seemed to encourage tumour growth and the effect on survival was worse than placebo [59-61].

Non-heamatopoietic actions of EPO

In parallel with its well established role in erythrocyte maturation, EPO also possesses nonhaematopoietic biological functions. Localization of EPO receptor (EPOR) expression in nonhaematopoietic tissues has recently provided new therapeutic potential for EPO. The first evidence of action on non erythroid cells was demonstrated on endothelial cells where EPO promoted the proliferation, migration and activation of angiogenic factors related to diabetic retinopathy [62-67].

Tissue protection by inhibition of apoptotic signals is currently the most studied effect of EPO as a cytokine [61, 68, 69]. Multiple studies indicate that this protective effect does not cross-react with its erythropoietic effect because it is mediated by different receptors [70]. A carbamoylated EPO derivative was shown to possess tissue protective properties but lacks erythropoietic activity, thus being a potent therapeutic for the treatment of tissue damage [71]. Neuroprotection has also been reported for recombinant human (rh)EPO, the cytokine crossing the blood brain barrier and improving neuron survival [68, 72-77]. Protective effects have been observed in animal models exhibiting cerebral ischemia, spinal compression and sciatic nerve crush [71]. Several preclinical tests have also proven the protection of cardiac myocytes following ischemia/reperfusion [78, 79]. These protective effects observed in nonhaematopoietic tissues involve the activation of anti apoptotic signals by the MAP kinase and phosphoinositide-3 kinase/ Akt pathways [79-82].

The erythropoietin receptor (EPOR)

The first evidence of a membrane target for EPO was demonstrated in erythroid cells in the 1970s, rapidly followed by the detection of low-level of EPOR expressions in other tissues [20, 62, 83-88]. Because of the very low concentration of EPO in human samples, it was only after the hormone was cloned and expressed recombinantly that it became possible to study its receptor in more detail [6, 7]. Determination of the structure of the extracellular portion of the receptor led to a more detailed characterization of the elements involved in ligand binding and signalling [89-91].

Structure of the EPOR

The EPOR is a glycosylated and phosphorylated transmembrane receptor. The murine EPOR gene, which was first cloned in 1989, codes for a 507 amino acid product [92]. Mature human EPOR is 484 residues in length, resultinf from the cleavage of a 24 amino acids signal peptide. Mass measurement of EPOR by gel electrophoresis revealed several bands between 60 and 66 kDa, probably depending on different glycosylation and ubiquitination patterns [93, 94]. The EPOR is a member of the class I cytokine receptor superfamily, carrying the characteristic conserved cysteine residues and WSXWS motif on its N-terminal extracellular domain, a single transmembrane segment and a cytosolic domain lacking catalytic activity [95-97]. The 225 amino acid extracellular domain is responsible of the interaction with EPO, and structural studies have demonstrated that one ligand binds to two receptors chains [89, 90]. Controversial studies have identified two separate receptor-binding sites, one high affinity site with a K_D in the picomolar range and one low affinity site with a K_D in the nanomolar range, but other groups only identified a single binding region [98-100]. The 236 amino acid cytoplasmic domain is divided in two parts, the Box-1 membrane proximal region, which is necessary for interaction with JAK2 kinase and the Box-2 region, which is involved in receptor trafficking [101-104].

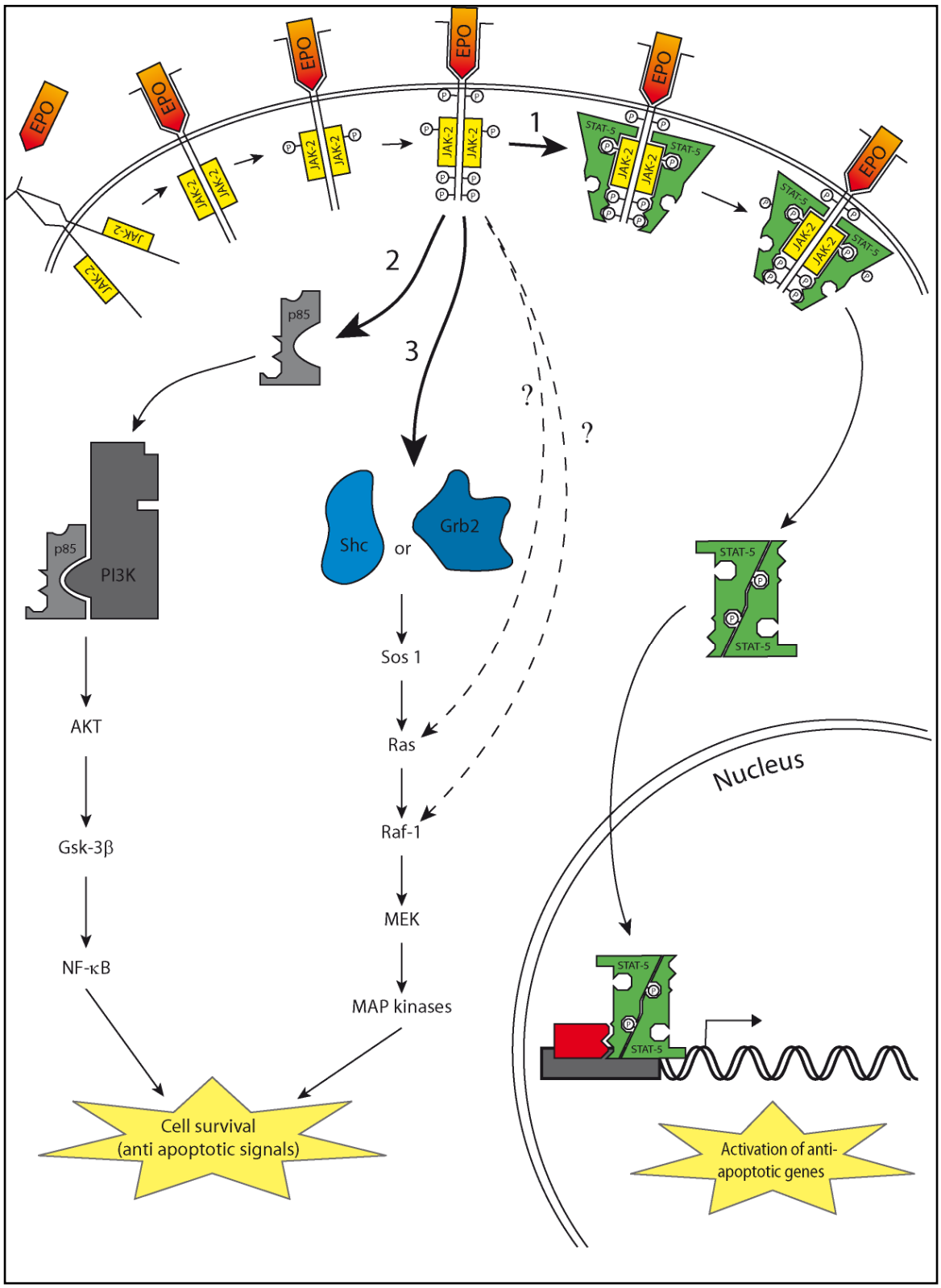

Figure 7: Activation of signalling pathways by the EPOR

Localization and trafficking of the EPOR

Erythropoietin receptors are mainly located on erythroid cells, progenitors CFU-Es expressing up to 1000 EPOR/ cell [105-107]. Other cell tissues also express low amounts of EPOR that participate in nonhaematopoietic functions [20, 62, 83-86]. Recently, EPOR have been detected in tumour cells, but their function remains incompletely understood [108-113]. Transcription of the EPOR gene is constitutive, but hypoxia and anaemia have been shown to upregulate expression in the brain [114, 115]. Following activation by EPO, the EPOR is rapidly down regulated and internalized by mechanisms involving the proteasome and lysosomes, maintaining short duration of intracellular signalling [116, 117]. After ligand stimulation, the receptor is ubiquitinated and allows the recruitement of the proteasome, which degrades the C-terminal part and inactivates the signal. In parallel, internalization of the receptor-ligand complex to the lysosomes for degradation ensures limited signalling duration.

Signalling via EPOR

Binding of erythropoietin to its receptor dimer induces a conformational change that affects the cytosolic domain of EPOR, leading to the activation of several signalling cascades (Figure 7). The first step in the activation of EPOR is the reorientation of the two cytosolic subunits upon binding of the ligand, activating the associated Janus Kinases 2 (Jak2) by trans phosphorylation [90, 91, 118]. Activated Jak2 then phosphorylates tyrosine residues of the EPOR cytosolic domain, creating docking sites for the recruitment of proteins containing Sarc homology 2 (SH2) domains [101, 119-122]. The major pathways involved in haematopoiesis involve activation of Signal Transducer and activator of transcription 5 (STAT5), Mitogen Activated Protein (MAP) kinases and Phosphoinositide 3-kinase (PI3K) [61, 113].

The active STAT5 proteins homodimerize in the cytoplasm, translocate to the nucleus and initiate the transcription of specific genes [123-127]. The JAK2-STAT5 signalling pathway activates anti apoptotic genes that are necessary for the survival of erythroid progenitors and precursors [128].

The MAP kinase pathway appears essential for erythropoiesis, mediating mitogenic, differentiation, haemoglobinization and anti apoptotic signals. Mechanisms of activation in

erythropoiesis via Raf, RAS and other signalling proteins is complex and still incompletely understood (see picture 2). Different proteins have been shown to be recruited to the plasma membrane by activated EPOR in different cell lines and activation of upstream (Shc, Grb2) and downstream (Ras, Raf-1) signalling molecules of the pathway has been observed [86, 129-132]

The PI3K pathway is activated by recruitment of the p85 regulatory subunit at phosphotyrosine docking sites [133-136]. Downstream activation of protein kinase B (PKB/AKT), glycogen synthase kinase-3β (Gsk-3β) and nuclear factor-κB (NF-κB) will contribute to the survival of haematopoietic cells [137].

Erythropoietin receptor stimulating agents

Since its first application in the clinic 20 years ago, recombinant human erythropoietin (rhEPO) has been extensively used for the treatment of anaemia. A number of strategies have since been developed to increase the activity of EPO, with the aim to limit the frequency of injections. The first commercialized molecules were epoetin alfa and beta, followed approximately 10 years later by modified proteins with prolonged plasma half life. Peptide derivatives and small molecule agonists have also been tested, one peptide being currently in clinical trial (Hematide). These studies underline the major pharmaceutical interest of EPO and EPOR for the treatment of anaemia and more recently for its tissue protective effects. The different molecules that activate EPOR signalling will be described in the next section [47, 48, 138-141].

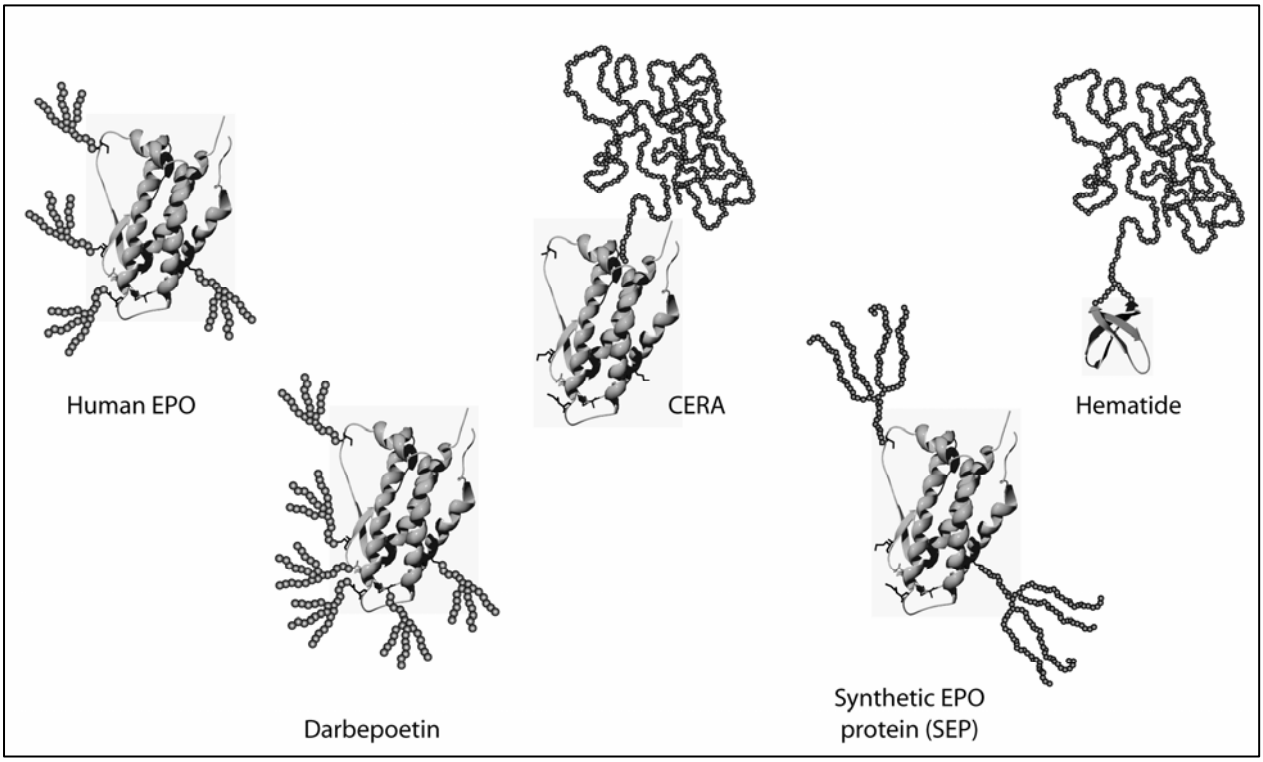

Figure 8: Schematic view of EPO stimulating agents. Human EPO possess 4 carbohydrate moieties that are essential for its stability. Darbepoetin is a hyperglycosylated EPO derivative that has two additional N-glycans. Continuous EPOR activator (CERA) is a pegylated EPO of about 70 kDa. The SEP moleculewas synthesized by solid phase peptide synthesis and possess two branched polymers that replace the function of natural glycans. Hematide is a peptide dimer that was pegylated to possess prolonged duration of action *in vivo.*

Epoetins

The first EPO biosimilars epoetin alfa and beta possess the same amino acid sequence as the endogenous hormone but differ slightly in their glycosylation pattern [7, 36, 37, 142]. Both are expressed in Chinese hamster ovary cells (CHO) and were the first Erythropoiesis Stimulating Agents (ESA) to be licenced for the treatment of chronic kidney disease (Figure 8). Because the patents recently expired, new EPO biosimilars are emerging in Europe, some being currently on the market. Epoetin omega, commercialized in Asia and Central America, is a rhEPO produced in baby hamster kidney cells (BHK) that possesses a different N-glycosylation pattern [143-145]. Although structural differences exist, this molecule possesses similar pharmacokinetic and pharmacodynamic profile to rhEPO. Epoetin delta, first marketed in 2007, is another rhEPO product that was expressed in human fibrosarcoma cell cultures, thus having very similar glycosylation pattern than the endogenous hormone [146-150]. However, the similarity with the native human EPO has proven no clear advantage yet. Erythropoietin was even produced in plant cells, exhibiting a distinct glycosylation pattern than rhEPO but with enhanced receptor affinity and potent protection of kidney epithelial cells [151]. All these epoetins possess the same 165 amino acid sequence but differ in their glycosylation pattern. No clear in vivo efficacy differences were observed between the epoetins, all molecules being administered for identical therapeutic reasons. Although these molecules have been extensively used for more than 20 years without major complains, two undesired side effects have been reported. The first drawback is the development of antibodies against epoetins that cross react with endogenous EPO, leading to pure red cell aplasia[56, 152]. The second effect relates to the survival of patients treated with ESA following cancer chemotherapy, where elevated hematocrit was linked with higher mortality [55].

Darbepoetin

Darbepoetin alfa is a hyperglycosylated version of EPO that exhibits an increased serum half life [38-40, 153, 154]. Mutation of the original EPO sequence at 5 positions were introduced that added two Asn residues at positions 30 and 80, resulting in the expression of a molecule with 5 N-linked glycans compared to 3 for endogenous EPO. The other three mutations (His32Thr, Pro87Val and Pro90Thr) were performed to ensured proper expression, refolding

and glycosylation of the modified EPO. The net effect on the protein is an increase in carbohydrate content of about 10%, prolonging its elimination half-life after intravenous injection to 25.3 hours compared to 8.5 hours for epoetin alfa [26, 42]. Although darbepoetin is more potent *in vivo* than rhEPO, it has a 4-fold lower affinity for EPOR. Thus, the decrease in affinity for EPOR created by two additional sialic acid chains is compensated by prolonged circulation half time that increases its biological activity. The consequence is a lower frequency of injection compared to rhEPO, with patients receiving two to four times monthly injections. Since its approval in 2001, darbepoetin has gained many market shares compared to epoetin alfa and beta [55].

Continuous EPO Receptor Activator (CERA)

With the objective of increasing molecular size to obtain a molecule with better *in vivo* stability, a pegylated epoetin beta was synthesized (Continuous erythropoietin receptor activator (CERA)) [43, 155-157]. Addition of a 30 kDa methoxy-polyethyleneglycol polymer chain by formation of an amide linkage either at the α-N terminal amino group or at ε-amino groups of Lysine-45 or Lysine 52 created a molecule of about 60 kDa. With a size doubled compared to endogenous EPO, CERA has a considerably prolonged half-life in man of about 130 h and can be administred once or twice monthly to control hematocrit in patients suffering from chronic renal failure [44, 45]. Studies of the interaction with EPOR have demonstrated that binding of CERA was much slower than rhEPO (on-rate), but that dissociation (off-rate) was faster. Such kinetic behaviour permits longer signalling action and sustained activation of EPOR, the molecule dissociating from the receptor before internalization [138]. The molecule was first approved in Europe in summer 2007 and directly competes for market shares with darbepoetin alfa.

Synthetic Erythropoietin Protein (SEP)

A totally synthetic EPO molecule was created by solid phase peptide synthesis, the sequence being divided in four fragments that were coupled using native chemical ligation [158, 159]. Incorporation of two ketone moieties by mutagenesis and levulinic acid coupling on ε-amino groups of two additional Lysine residues (Lys^{24} and Lys^{126}) allowed the attachment of two branched precision polymers by oxime ligation [160, 161]. The homogeneous 51 kDa EPO

derivative had haematopoietic activity similar to rhEPO with a prolonged circulation half-life similar to darbepoetin and CERA. Studies on SEP were abandoned because the molecule failed in Phase I (personal communication).

Erythropoietin fusion proteins

Another way to increase the mass is to create EPO fusion proteins either by making EPO multimers or by fusing EPO with other proteins. Erythropoietin dimers have been synthesized recombinantly with a 17- or 9- amino acid linker [162, 163]. One dimer exhibited a significant increase in RBC production after 7 days in mice at doses to which EPO monomer was ineffective. An inactive EPO mutant, R103A, recovered activity upon dimerization with a poly glycine amino acid linker [164]. Erythropoietin chemical dimers and trimers have also been synthesized and shown to have prolonged action *in vivo* compared to EPO monomers [165]. For the synthesis, one set of EPO molecules was modified to carry additional sulfhydryl groups while maleimido groups were introduced into another set. Reaction of the two molecules resulted in a mixture of dimers and trimers linked by covalent bonds. Formation of EPO homo dimers and trimers proved successful in stimulating erythropoiesis, but none of these big cytokines has advanced to clinical trials.

Fusion of EPO with other proteins was also tested to improve its biological activity. A hybrid molecule of EPO fused to the Fc region of human IgG was synthesized with the idea to improve its circulation half time both acting on the mass and on the trafficking of EPO [166, 167]. Rearrangement of the cysteine pattern improved stability of the fusion protein, increasing its effect on RBC production [168]. Recently, fusion of the carboxyl-terminal peptide of human chorionic gonadotropin β-Subunit with EPO created a molecule that retains the same *in vitro* properties as rhEPO, which is not the case for darbepoetin alfa [169]. Moreover, this fusion molecule has an increased circulation half-time and maintains haematocrit in mice with once weekly injections at doses where rhEPO is ineffective.

To increase its erythropoietic activity, EPO was fused to other cytokines involved in erythropoiesis using recombinant technology. An interleukin-3/EPO fusion protein maintained the biological activity of the separate molecules but did not show any beneficial effect compared to a mixture of the two cytokines [170]. A hybrid molecule composed of EPO and GM-CSF was shown to be potent erythropoiesis stimulating agent in cynomolgus

monkeys, but studies had to stop because of immunogenic responses inducing severe anaemia [171-173]. A hetero-dimeric fusion of EPO and thrombopoietin was created for the treatment of thrombocytopenia, but the effect on erythropoiesis was not further studied [174].

Erythropoietin Mimetic Peptide (EMP)

In 1996, a small 20 amino acid peptide discovered by phage display was shown to be capable of activating the EPOR *in vitro* and *in vivo* in a similar manner to rhEPO [175]. Erythropoietin Mimetic Peptide 1 (EMP1), which possesses two cysteines that form a disulfide bridge and does not have any sequence similarity with rhEPO. The X-ray structure of the peptide in complex with the EPOR revealed that the peptide activates the receptor using the same mechanism as EPO, but that 2 peptides occupy the binding site used by one EPO hormone [176]. This finding inspired scientists to create EMP dimers, either using a short Lysine-linker to the C-terminus or a longer PEG chain to couple the two N-terminal amino groups [177, 178]. The most potent PEG dimer was up to 1000-times more potent at stimulating proliferation of an EPO-dependent cell line compared to its monovalent peptide, with an EC50 values of about 100 pM [179]. Compared to rhEPO, the potency is still approximately one order of magnitude lower. Alanine scanning and truncation of EMP1 identified a 13 amino acid sequence that is sufficient for erythropoietic activity [180].

Hematide

Based on the studies of EMPs, a new erythropoietin stimulating agent was developed that could avoid generation of antibodies cross reacting with endogenous EPO, thus limiting the risk of pure red cell aplasia [56, 152, 181]. This synthetic molecule, named Hematide, is a pegylated molecule composed of two peptidic chains linked at the C-terminus by a lysine linker. The sequence is similar to EMPs and the molecule was shown correct anaemia associated with chronic kidney disease and cancer [182]. The measured circulation half life in monkeys is between 14 to 60 h and the potency to stimulate growth of EPO-dependent cells is approximately 10 times lower than rhEPO, similar to a non-pegylated EMP dimer. Once a month injections seems effective at maintaining hematocrit levels [48, 183]. This molecule is described as easier and cheaper to produce than recombinant derivatives because it dose not necessitate use of cell lines. Hematide is currently is phase III clinical trials.

Erythropoietin Receptor-derived Peptide (ERP)

During the search for small peptide agonists, a molecule capable of activating the erythropoietin receptor through an alternative binding site to that used by EPO and EMP was discovered [184, 185]. The Erythropoietin Receptor-derived Peptide (ERP) has a sequence that corresponds to the EPOR amino acids 194-216, was demonstrated to interact with that region. The peptide can activate EPOR signalling but at micromolar concentrations compared to picomolar concentrations for rhEPO. Combined activation of signalling by EPO and ERP was shown to be strongly synergistic, with phosphorylation of JAK5 being observed at concentrations where the hormone and peptide alone could not induce any signal. This molecule was not further developed, probably because of the high doses required.

Nonpeptide molecules

Development of small molecule agonists of the EPOR has been investigated with the hope to obtain an orally available drug. Screening of small molecule libraries has identified molecules competing with EPO to bind EPOR [22, 186, 187]. Some molecules were combined to form oligomers that improved EPOR activation, but still at concentrations even higher than monomeric EMP1. These studies showed that mimicking EPO with orally available small molecule is possible but that considerable optimization to increase activity will be necessary.

Novel strategies to stimulate erythropoiesis

In addition to the stimulation of erythropoiesis by EPO or its derivatives, other targets have been identified that could potentially be used to treat anaemia. Hypoxia inducible factor-1α (HIF-1α) is the major regulator of EPO expression through oxygen sensing. Expression of HIF-1α is inactivated by prolyl hydroxylase in normoxic conditions, leading to its degradation [23]. Orally available inhibitors of prolyl hydroxylase, recently referred as HIF stabilizers, have been shown to stimulate erythropoiesis, opening the way to new treatment of anaemia [188]. Unfortunately, many genes are regulated by HIF elements and the lack of specificity of these molecules could cause severe adverse effects, the most alarming being their angiogenic properties [189]. Another negative transcription factor, GATA-2, was targeted by inhibitors that lead to increased EPO production in mouse [190]. Apart from transcription factors, inhibition of protein acting on the down-regulation mechanism of EPOR signalling was investigated. Haemopoietic cell phosphatase (HCP/SHP-1) is an enzyme that negatively regulates JAK2 by phosphorylation. Inhibition of its kinase activity by a small molecule resulted in dose-dependent erythropoiesis stimulation [191]. Because maintaining a constant EPO concentration seems essential to successful treatments, gene therapy is regarded as an attractive option . Moreover, the site of delivery does not seem matter as EPO can circulate to reach its target. Several strategies have been tested to deliver EPO gene, the biggest issue being the control of expression [192-196]. No tests in human have been undertaken so far.

Conclusion, perspectives

The glycoprotein hormone erythropoietin is of major therapeutic interest, especially for the treatment of anaemia. The protein controls the proliferation and differentiation of erythroid cells through activation of anti apoptotic signals involving JAK/STAT, MAP kinases and PI3 kinases signalling pathways. Since the first usage of rhEPO 20 years ago to treat patients suffering from renal diseases, many investigations have been undertaken to obtain superactive EPO biosimilars that will diminish the administration frequency of the drug. Hyperglycosylated and pegylated rhEPO have proven very effective in treating anaemia by once- or twice-monthly injections, and the next step in drug research is the development of orally available molecules. Erythropoietin has reduced the need for blood transfusions in cases of anaemia, with dosage of the drug that has proven essential in the treatment of anaemia resulting from cancer chemotherapy. Because of the concomitant findings that tumour cells bear EPOR and that EPO has angiogenic properties additionally to its anti apoptotic effect, fear has arise that EPO could facilitate tumour progression. Such effects have not been demonstrated so far. In the search for orally available drugs, alternative activation of erythropoiesis by acting on EPO transcription elements is a promising strategy, although the control of specificity has proven difficult. Acting on downstream signalling elements of EPOR signalling is another strategy that has only recently been proposed, as well as modifying the trafficking of EPOR after activation by ligand binding. Good perspectives for the treatment of anaemia exists, but the replacement of a very potent molecule (EPO is active at picomolar concentrations) that has already proven very successful during 20 years will not be straitforward.

Erythropoietin has recently shown to induce anti apoptotic signals in non haematopoietic tissues, thus opening new perspectives for the use of EPO or biosimilars in tissue protection. Effects in neurons, in the kidney and in cardiac cells have been demonstrated and new tissues will certainly be identified as new targets. Use of EPO as tissue protective agent suffers from specificity concerns because of its role in erythropoiesis, but derivatives like carbamoylated EPO that is cardioprotective but lacks erythropoietic activity, are maybe opening a new way in the therapeutic usage of erythropoiesis stimulating agents. Thus, albeit EPO and ESA have been extensively studied and used in the last 20 years to treat anaemia, their therapeutic

potential is still expanding and the next challenge is the development of potent EPO derivatives with specific non haematopoietic activities.

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Chapter 1

Synthesis of a synthetic erythropoietin dimer

1.1Abstract

Since its identification as the major regulator of red blood cells production, erythropoietin (EPO) has been extensively used for the treatment of anaemia. In the search for more potent hormones, EPO derivatives carrying either additional glycans or additional PEG polymer have been shown to display increased biological activity *in vivo* compared to EPO (EPO). Several recombinant EPO dimers and trimers have been described, which exhibit increased *in vivo* potency, as well as a totally synthetic EPO protein (SEP). In the present study, we explored the possibility of synthesizing a synthetic EPO dimer by using a combination of solid phase peptide synthesis, native chemical ligation and dimerization via PEG polymers. That molecule was expected to possess increased biological activity compared to the native hormone by benefiting from increased affinity thanks to multimerization and from reduced clearance *in vivo* thanks to its increased mass. However, following precipitation problems during one of the synthesis steps, the project proved to be intractable. Comparison of the strategies applied for the synthesis of our dimer and for SEP provides a potential explanation of the difficulties encountered. New strategies applicable to successfully complete the synthesis are presented.

1.2 Introduction

Erythropoietin (EPO) is a glycoprotein hormone of 165 amino acids that controls the differentiation and proliferation of red blood cells [1]. The protein is highly glycosylated with approximately 40% of its mass made up of carbohydrates, these glycans possessing important functions in the biosynthesis, the secretion and the *in vivo* biological activity of the hormone, but not affecting its *in vitro* potency [2, 3]. To exert its action, one EPO hormone interacts with a preformed EPO receptor (EPOR) dimer, modifying the orientation of the cytosolic region upon binding (Figure 1-1) [4, 5]. This interaction stimulates the activation of several signaling cascades, leading to the transcription of specific genes involved in cell maturation and survival [6-8].

Recombinant EPO has been used for more than 20 years in the treatment of anaemia. To increase its activity, large moieties have been attached to the native hormone, like additional glycans of PEG chains, which prolong its duration of action *in vivo* [9, 10]. These molecules are currently in clinical use for the treatment of anaemia. Other strategies have been tested to increase the size of EPO to obtain molecules with better biological activity. Recombinant erythropoietin dimers have been produced by expression of a gene coding for two EPO proteins linked via a peptide hinge region of 9 to 17 amino acids linker.

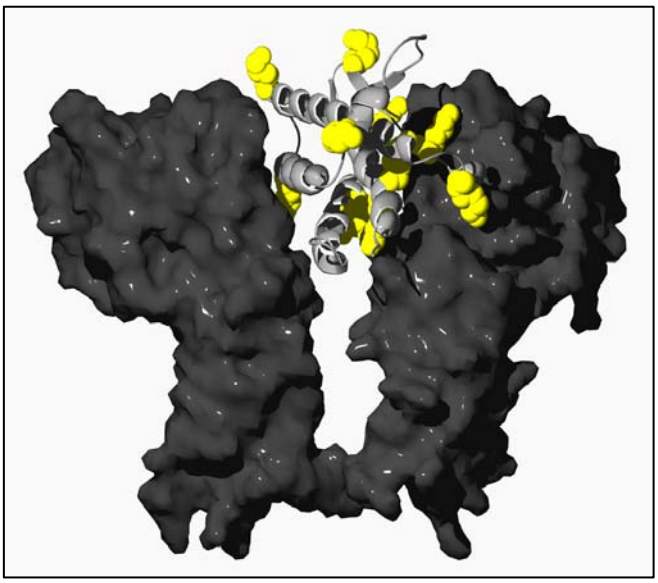

Figure 1-1: Structure of EPO in complex with EPOR. Side chains of the Lys residues of EPO are shown with bowls

These dimers exhibited 6-times enhanced biological activity both *in vitro* and *in vivo* compared to monomer, whereas use of a shorter linker of 2 to 7 amino acids led to decreased activity [11-13]. Chemical attachment of recombinant human EPO (rhEPO) modified at lysine residues resulted in the formation of dimers and higher order oligomers with enhanced *in vivo* activity [14]. Although these rhEPO multimers exhibited prolonged circulation half time in rabbits, they were very inhomogeneous because the linkage strategy was not specific enough. Any of the surface-exposed lysine residues of EPO could be involved in the association (Figure 1-1), ending up in a mixture of aggregates connected via different sites. A totally synthetic EPO protein (SEP) was also described, for which the four carbohydrate moieties of the native protein were replaced by two branched polymers [15, 16]. For synthesis of that molecule, the EPO sequence was divided in four fragments that where coupled using native chemical ligation (NCL), the attachment of the polymers employing another chemoselective reaction: oxime ligation [17, 18].

We hypothesized that a homogeneous synthetic EPO dimer linked via a large polyethylene glycol (PEG) polymer would combine increased biological activity *in vitro* because of dimerization and prolonged duration of action *in vivo* thanks to its substantially increased mass. We developed a strategy based on SEP for the synthesis of a synthetic EPO dimer, using NCL and oxime chemistry for linker attachment. We report here an attempt for the synthesis of a synthetic EPO dimer, which could not be completed owing to solubility problems. Studies of the protocols used for SEP synthesis compared to our approach provide a potential explanation of the role played by the branched polymer moiety of SEP on ligation product solubility.

1.3 Material and methods

1.3.1 Material

Peptide synthesis grade DMF and DIEA were purchased from Biosolve. DCM, NMM, N-methylpyrrolidinone (NMP), diethyl ether, DMSO, trifluoroethanol (TFE), carbonyldiimidazole (CDI), thiophenol, TFMSA, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and DIC, were purchased from Fluka, Switzerland. TFA was from Halocarbon, New Jersey. Acetonitrile CHROMASOLV® gradient grade for HPLC was from Sigma-Aldrich. HBTU was from Iris Biotech GmbH Germany) and HATU was from GL Biochem (Shanghai) Ltd. HOBt was from NovaBiochem (Switzerland) and amino acids were from AnaSpec (San Jose) or from NovaBiochem. Standard EPO is EPREX (epoetin alpha) from Janssen-Cilag AG. IMDM (with l-glutamine and 25mM HEPES), penicillin, streptomycin were from Invitrogen. MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide was from Sigma, 2-propanol and 1.25N HCl/ isopropanol was from Fluka.

1.3.2 Analytical High Pressure Liquid Chromatography (HPLC)

Analytical reverse-phase HPLC was performed at 0.6ml/min on Waters equipment using a Macherey-Nagel C_8 column (4 x 250 mm 300Å 5µm particle size). Solvent A was 0.1% TFA in HPLC grade water. Solvent B was 90% acetonitrile with 0.1% TFA. Elution was done with a 40min linear gradient 0-80%B.

1.3.3 Preparative High Pressure Liquid Chromatography (HPLC)

Preparative reverse-phased HPLC was performed at 15ml/min on Waters equipment using a Vydac C_8 column (22 x 250 mm 300Å 10-15µm particle size). Elution with previously described solvents A and B peptides was done with an appropriate linear gradient, usually 1%/min. UV monitoring was at 214nm. Peaks were collected manually and the product was recovered by lyophilization.

1.3.4 Mass Spectrometry

MALDI-TOF mass spectrometry was performed in linear mode using sinapinic acid as matrix on a Voyager-DE STR (Applied Biosystems) equipped with delayed extraction. External calibration was performed using porcine insulin (Novo Nordisk).

1.3.5 Solid Phase Synthesis of the peptide fragments

Solid phase peptide synthesis [19] was performed on a modified ABI 433A machine using Boc chemistry and *in situ* neutralization as previously described [20]. Peptides were prepared on a 0.2 mmol scale; fragment 4 using Boc-Arg-PAM and fragments 1, 2 and 3 using thioester resin of the type Boc-X-S-CH2-CO-Leu-PAM-Gly-RAPP, X representing the C-terminal amino acid of the fragment. Boc-amino acids were protected by the following groups: Arg(Tos), Asn(Xan), Asp(OcHx), Cys (Mob), Glu(OcHx), His(Dnp), Lys(Z(2Cl)), Ser(Bzl), Thr(Bzl), Trp(For), Tyr(Z(2Br)). On fragment 4, Lys126 was introduced as Boc-Lys(Fmoc) to allow orthogonal deprotection on resin of the ε-amine with piperidine treatment. Then, levulinic acid was coupled on the Lys126 ε-amine as a symmetrical anhydride after activation with DIC. Four mmol Levulinic cid were dissolved in DCM and 2 mmol DIC were added. After 20 min, DCM was evaporated and the symmetrical anhydride dissolved in DMF for on-resin coupling. Reaction time was 45 min, followed by Kaiser test to confirm completion of the coupling. The N-terminal Cys of fragments 2 and 3 were introduced as Boc-L-Thiazolidine ("Thio-Proline", NeoMPS, France) to avoid cyclization with the C-terminal thioester. Acid cleavage was done with HF containing 5% p-cresol for 60 minutes at 0°C. Peptides were precipitated and then washed with cold diethyl ether. Crude peptide was exposed to high vacuum overnight then purified by preparative reversed phase HPLC and lyophilization. Sequences of the fragments are presented in Table 1-1.

1.3.6 Native Chemical Ligation (NCL)

Ligation 1: 10mg (1.81µmol) of fragment 4 are dissolved in ligation buffer (6 M Guanidine-HCl / 0.2 M phosphate / 50 mM L-methionine / pH 7.5) at 5 mM final concentration.

Fragment 3 (2.16 µmol, 1.2 excess) previously dissolved in ligation buffer is incorporated to the solution. Thiophenol is added as a catalyst (1% v/v) and the reaction is performed over night at room temperature under stirring. Trp(CHO) and His(Dnp) protecting groups are removed by addition of 2-mercaptoethanol at a final concentration of 20 % (v/v). Hydrazine is added dropwise to bring the pH to 9 and the solution is agitated 60 min at 37°C. Reaction mixture is then adjusted to pH 4-4.5 by addition of acetic acid. Conversion of the ThioProline into cysteine is done by adding 1 volumle of a 1M methyl-hydroxylamine / M Guanidine-HCl / pH 3.5 solution. The pH is adjusted to 3.5 and the solution stirred for 2 hours at 37°C. Addition of 10 molar excess of TCEP for 2 hours is necessary before dilution and purification by RP-HPLC. Ligation 2 and 3 are performed as the first ligation with alkylation of cysteines after ligation 2.

1:	APPRL I**C**DSR ^{11}VLERY LLEAK ^{21}EAENI TTG**C**A ^{31}EH
2:	**C$_{(Tp)}$**SL NENIT ^{41}VPDTK VNFYA ^{51}WKRME VGQQA ^{61}VEVWQ GLALL ^{71}SEAVL RGQAL ^{81}LVNSS QPW
3:	C$_{(Tp)}$P ^{91}LQLHV DKAVS ^{101}GLRSL TTLLR ^{111}ALGAQ K
4:	CAIS ^{121}PPDAA K$_{(levul)}$AAPL ^{131}RTITA DTFRK ^{141}LFRVY SNFLR ^{151}GKLKL YTGEA ^{161}**C**$_{(Acm)}$RTGD R

Table 1-1: Sequence of the 4 fragments used to synthesize an synthetic EPO dimer. C(Tp) is for ThioProline and K(levul) is for attachment of levulinate to the ε-amino group of Lys. Highlighted is grey are the glutamic acid and glutamine residues that could be use

1.3.7 Alkylation of cysteines 89 and 117 with bromoacetic acid

Complex of fragments 2+3+4 with free Cys89 and Cys117 and Acm protected Cys161 is dissolved in TFE at 1mM, and diluted with 10 volumes 6M Gn-HCl-300mM phosphate (pH 7.9). Bromoacetic acid is added at 25-fold excess (relative to free thiol groups). After 1 hour stirring, purification of the complex, carboxymethylated at Cys89 and Cys117, is done by RP-HPLC.

1.3.8 Reversibility of the oxime ligation

To test if oxime formed during conversion of the thiazolidine (ThioProline) into cysteine was reversible, fragment four carrying an oxime between Lys16 levulinate and methyl-hydroxylamine was incubated with aminooxy acetic acid. Reaction was performed with 10 and 20 equivalent of aminooxy acetid acid over the fragment for 2 hours.

1.4 Results

1.4.1 Synthesis of the fragments

The starting point for the synthesis of a synthetic EPO dimer was to synthesize a non glycosylated EPO modified at one residue to allow linker attachment (see Figure 1-2). This 166 amino acid sequence is too long to be synthesized in one step by SPPS, so the sequence was divided in four fragments having of 28 to 56 residues, according to the protocol described for SEP synthesis [15]. Ligation of the fragments and linker attachment necessitated two distinct steps, thus orthogonal chemoselective strategies were required. We decided to use the same strategies employed for the synthesis of SEP: NCL for fragment ligation and oxime ligation for linker attachment. Because NCL requires fragments with N-terminal cysteines, the division of the whole sequence in fragments was chosen to avoid excessive mutations. Similarly as for SEP, mutations to cysteine were introduced at positions Glu^{89} and Glu^{117}, and Ser^{126} was mutated to lysine for introduction of a levulinate on the ε-lysine amino group by orthogonal protection scheme. The four fragments were synthesized by automated SPPS and purified by RP-HPLC. To be able to use NCL, fragments 1, 2 and 3 possessed C-terminal thioester moieties and fragments 2, 3 and 4 had an N-terminal cysteine. In contrast with SEP which employed acetamidomethyl (Acm) protecting group, initial cysteines of fragments 2 and 3 where introduced as L-thiazolidine ("thioproline") to avoid fragment cyclization, which could be transformed to cysteine after

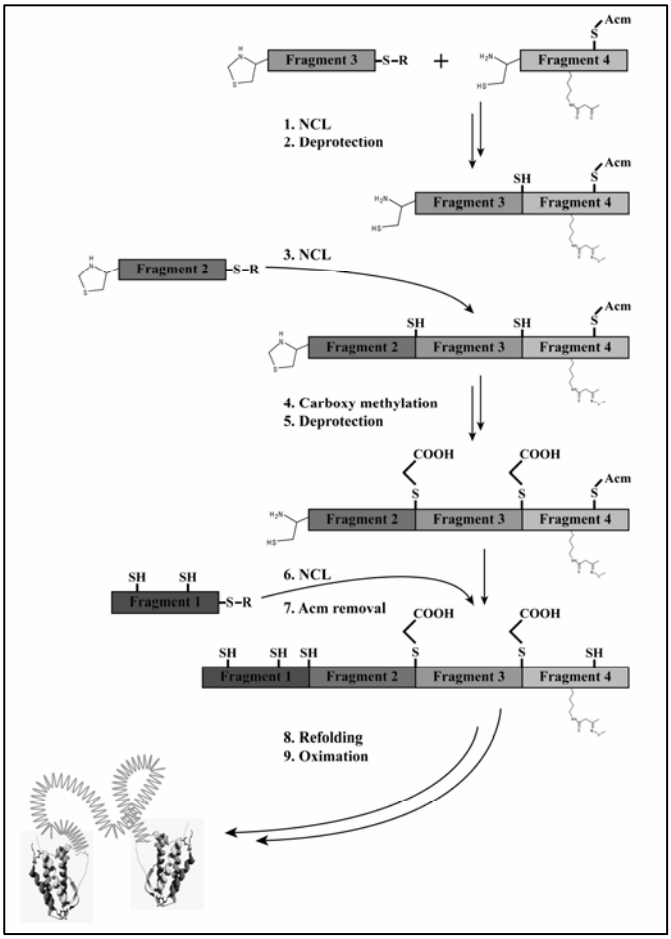

Figure 1-2: EPO chemical dimer synthesis strategy

ligation to allow the next ligation step. Purity was assessed by analytical RP-HPLC and MALDI-TOF mass spectrometry (see Figure 1-3).

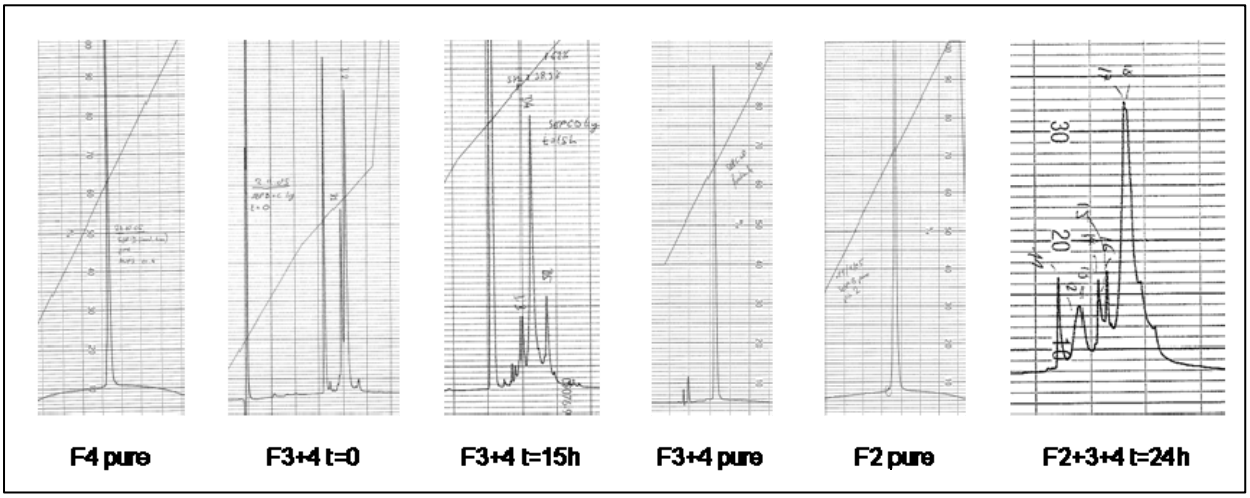

Figure 1-3: HPLC chromatograms of pure fragments and ligation steps. Abbreviation "F" is for fragment. HPLC chromatogram of the ligation of fragments 2 + 3 + 4 indicates two closely eluting peaks, but none of these two corresponds to the expected mass of the complex.

1.4.2 Reversible oxime ligation

For dimerization of EPO using a homo-bifunctional aminooxy polymer linker, a ketone group had to be introduced at the surface of the protein. Amino acid Ser^{126} is present on top of the EPO-EPOR complex and does not participate in receptor binding, as shown in Figure 1-4, and thus was mutated to lysine for further integration of a levulinate moiety. Although oximes formed with pyruvate are more stable, levulinate moiety was chosen because it forms reversible oxime bonds [16]. Deprotection of thioproline residues to obtain cysteines requires methoxamine, which reacts with levulinate to form oximes. For linker attachment, the oxime formed with methoxamine must be replaced with the aminooxy polymer. A small scale test was performed to inverse a methoxamine-oxime with 10 and 20 aminooxyacetic acid that proved successful (see Figure 1-4).

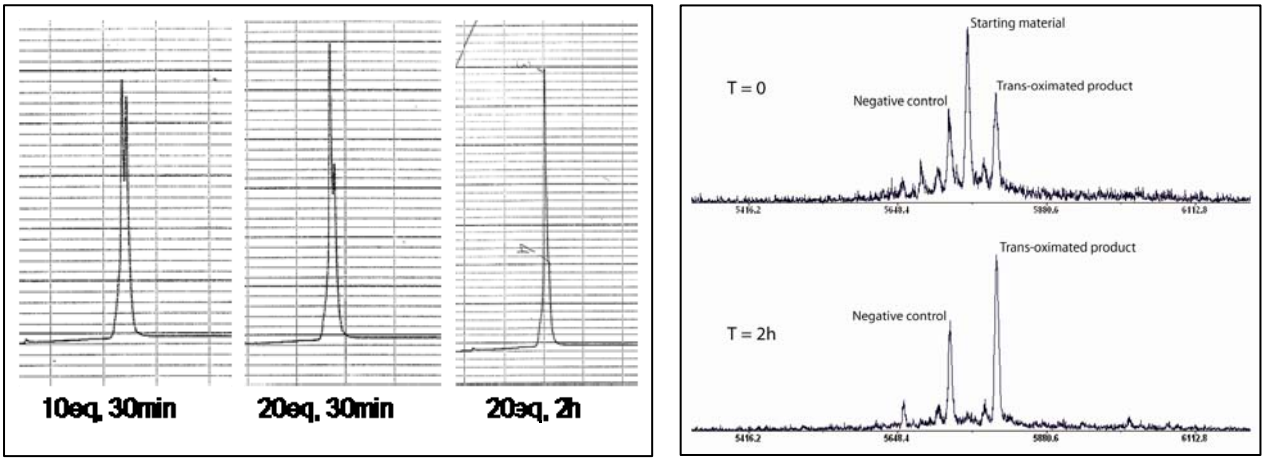

Figure 1-4: HPLC traces and MALDI-TOF MS spectra of the transoximation reaction of methyl-hydroxylamine oxime with aminooxyacetic acid in excess.

1.4.3 Ligation of the fragments using native chemical ligation

Ligation of fragments 3 with fragment 4 proceeded as expected and the ligation product was readily purified by RP-HPLC. Significant precipitation was observed when this product was ligated to fragment 2, with the desired product that could not be isolated (Figure 1-3). To introduce charges onto the ligation product that would facilitate solubilisation, three additional lysine residues where coupled on to the N-terminus of fragment 2. This did not resolve the precipitation and purification problems. Thus, the planned synthesis could not be continued and instead we sought to use modeling simulation to find an explanation as to why such an insoluble precipitate was formed at this step.

1.5 Discussion and perspectives

Based on the description of a synthetic EPO protein (SEP), we have attempted to create a homogeneous EPO chemical dimer. After successful synthesis of the four peptide fragments, ligation to obtain the entire protein was not achieved. Ligation of fragments 3 with fragment 4 proceeded as expected, but further attachment of fragment 2 yielded an insoluble mixture from which the desired product could not be isolated. This result was surprising because our molecule was so similar to the protein described by Kochendoerfer et al, the division of EPO sequence in four fragments being identical for both syntheses [15, 16]. In comparison to our strategy, which implies ligation of all the fragments before refolding and dimerization, SEP synthesis introduced a polymer moiety of approximately 16 kDa to fragment 4 before the ligation steps with fragments 3 and 2 (Figure 1-2). A model of the surfaces of each ligation product predicts that fragment 3 and 4 may form a loop, into which fragment 2 would have to pass to adopt the folded structure (Figure 1-5). Performing the ligation step on a fragment lacking the large hydrophilic group may have been the cause of the precipitation problem.

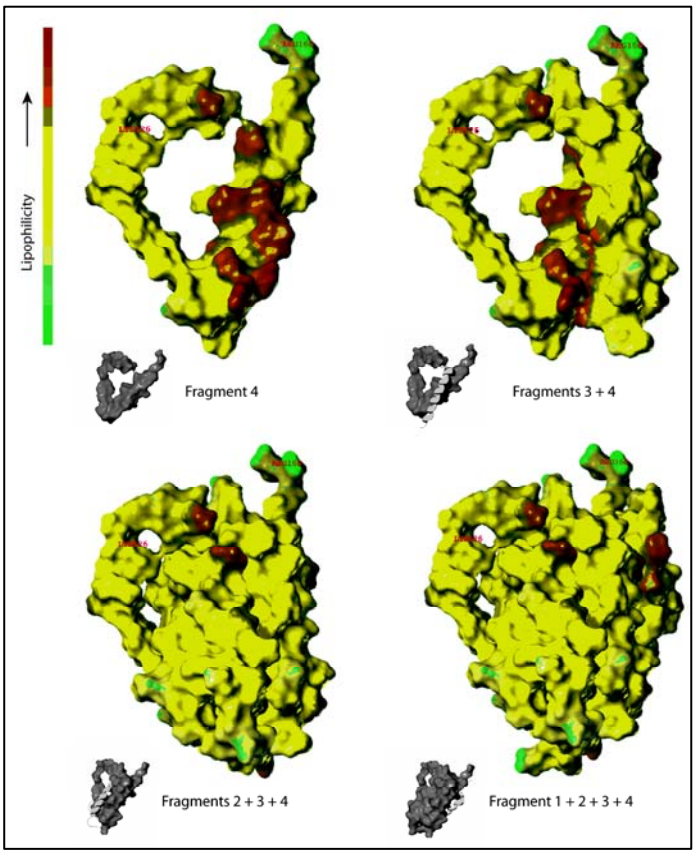

Figure 1-5: Model of the stepwise ligation of fragments 4 to fragment 1 to form the native EPO structure. Lipophilic regions of each ligation products are shown in brown. Small structures indicate the additional fragment in light grey with their secondary structure. The position of the Lys126 residue in fragment 4 in indicated in red.

1.5.1 Proposition of new synthesis strategies

An alternative would be to attach a large polymer moiety at the mutated Lys^{126}, similarly as what was done for SEP, and to exchange it with a bifunctional linker after synthesis completion. The synthesis of monomeric synthetic EPO protein being already described, an additional reversible oxime ligation step would give the synthetic EPO dimer. This inversion has been tested at small scale, so if sufficient amount of bis-aminooxy PEG linker is available, the desired reaction should occur.

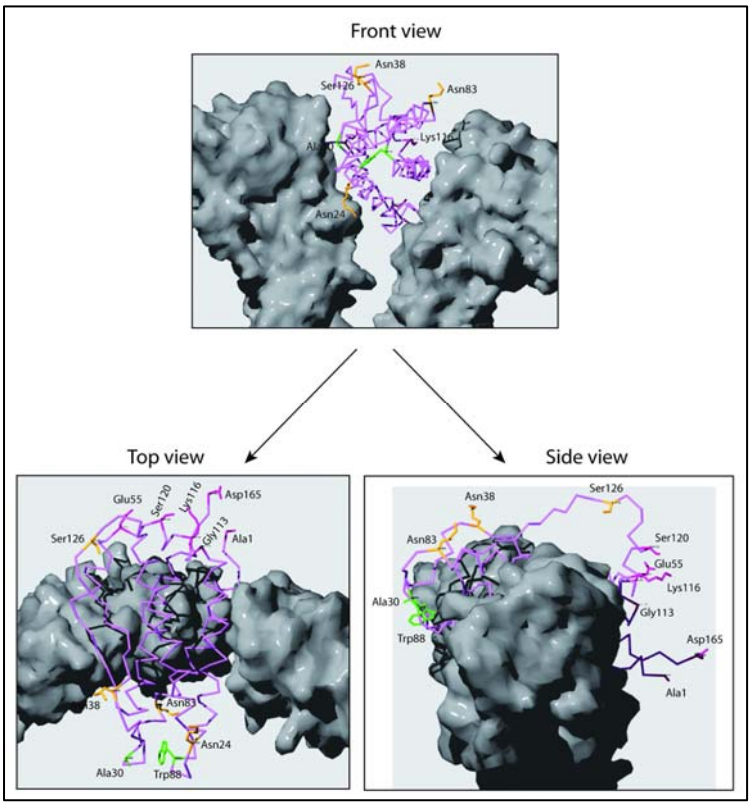

Figure 1-6: Carbon alpha trace of native EPO with natural glycosylation sites (orange), mutated residues carrying glycans in darbepoetin (green) and potential sites of linker attachment (dark pink)

With the same objective to obtain EPO dimer but by combining recombinant and chemical strategies, expression of a recombinant EPO mutant possessing an additional cysteine would provide a free thiol moiety that can selectively be targeted by thiol reactive coumpounds [21]. Residues that should tolerate mutagenesis have to be at the surface of the hormone and neither interferes with the structure nor participate in receptor interactions. One possibility would be

to mutate one of the residues that was used for introducing additional sialic acids in darbepoetin alfa, Ala^{30} or Trp^{88} [22]. Other residues situated on the opposite side of EPO could also be targeted for polymer attachment, as seen in Figure 1-6. Reaction of thiol-containing rEPO with a bis-maleimido PEG linker should form homogeneous EPO dimers. One problem that could be faced with this strategy is the formation of dimers by disulfide bond, but optimization of the reaction conditions for PEG dimerization will ensure formation of the expected product.

1.6 Conclusion

The synthesis of a synthetic EPO dimer based on the previously described SEP has proven intractable for solubility reasons. The similarity between the two strategies suggests that the absence of a large hydrophilic moiety on the initial fragment may be the cause of the solubility problems. The work described in this report proved that synthesis of large proteins combining solid phase peptide chemistry and native chemical ligation is not a routine task. Although chemical synthesis of peptides and proteins allows incorporation of non natural amino acids and polymer moieties, recombinant techniques still seems more adapted to the purification of proteins larger than 100 amino acids. The division of EPO protein in 4 fragments demonstrated that isolated portions of protein do not behave similarly as when part of a folded structure, whith this aspect that can lead to significant synthesis troubles.

1.7 References

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Chapter 2

Hetero-dimeric peptide agonist of the erythropoietin receptor

2.1 Abstract

In addition to its natural ligand, the erythropoietin (EPO) receptor has been shown to be activated by peptides that engage two distinct binding sites. The EPO mimetic peptides (EMP), which are cyclic peptides of about 20 amino acids with no sequence similarity with EPO, activate the EPO receptor (EPOR) by engaging the same binding site as EPO, although at a much lower potency. The erythropoietin receptor-derived peptide (ERP) is another 20 amino acid peptide that activates EPOR via interaction with a different site on the receptor. ERP has very low *in vitro* and *in vivo* potency, but when administered in combination with low doses of EPO, a synergistic effect was observed. Since EMP dimers with significantly increased biological activity over monovalent EMP have been reported, we anticipated that hetero-dimers composed of one EMP and one ERP molecule linked by a flexible linker could represent potent EPOR agonists. Based on the X-ray structure of the EPOR in complex with EMP, we designed and synthesized a series of hetero-dimeric peptides featuring different linker lengths and attachment sites on the EMP moiety. *In vitro* assays of EPOR-dependent cell proliferation indicated that the peptide dimers had lower potency than monomeric EMP, and did not confirm the previously reported synergy observed for ERP with EPO. Several hypotheses can explain this result, including the assay format, steric hindrance, conformation of the linker in solution and the modification of ERP cysteine residue. Novel synthesis approaches are discussed to improve the activity of the described molecules.

2.2 Introduction

By regulating survival and proliferation of erythroid progenitor cells, erythropoietin (EPO) controls the production and maturation of red blood cells [1]. Erythropoietin interacts with its receptor present at cell membrane to stimulate intracellular signaling events. The EPO receptor (EPOR) is a member of the class 1 cytokine receptor superfamily which contains conserved features: an extracellular domain responsible of ligand binding and dimerization, a single transmembrane segment and a cytosolic region that lacks enzymatic activity [2]. Receptor activation by EPO modifies the orientation of the two cytosolic subunits, activating the associated Janus Kinases 2 (JAK2) that further activate other signalling molecules, leading to the transcription of anti-apoptotic genes [3-7].

Several molecules have been developed that can also stimulate erythropoiesis via the EPOR. Modified EPO proteins with increased molecular weight displayed potent *in vivo* activity, with some molecules in clinical use for the treatment of anaemia [8, 9]. A peptide derivative was described that competes with EPO for receptor binding that has no sequence similarity with the native hormone. This 20 amino acid EPO mimetic peptide (EMP) stimulates erythropoiesis *in vitro* and *in vivo*, although at much lower potency than recombinant human EPO (rhEPO) [10, 11]. Dimerization of EMP, either with a PEG linker joining the two N-termini or with a short lysine-linker connecting the C-termini, resulted in up to 1000-fold increase in cell proliferation potency compared to monomer [12, 13].

Another peptide was discovered that can activate the EPOR in absence of EPO via an alternative binding site (Figure 2-2, Figure 2-1) [14]. This peptide has a sequence corresponding to amino acids 194-216 of the EPOR, a region involved in receptor dimerization and activation. Assumed to bind to its homologue sequence on EPOR, this EPO receptor-derived peptide (ERP) has been reported to stimulate the JAK/STAT signalling pathway *in vitro* and *in vivo* at high nanomolar concentrations [14, 15]. Moreover, ERP has been reported to exhibit synergistic activity with EPO. The mechanism of EPOR activation by ERP is still unclear, but it has been suggested that partial inhibition of EPOR internalization was involved.

We anticipated that a heterodimeric peptide composed of EMP and ERP joined by a flexible linker might increase the activity of EMP owing to a combination of multivalency and synergistic effects. In this report, we describe the *in vitro* biological characterization of previously described homogeneous ERP-EMP heterodimers linked by polyamide linkers [16, 17]. Linkers of two different sizes were employed, connecting the N-terminus of ERP to either the N- or the C-terminus of EMP. Synthesis of the peptides and of the linker was done by solid phase peptide synthesis, and oxime ligation was employed for the attachment of unprotected peptides to the linker [18]. Cell proliferation assays with an EPO-dependent cell line showed that the dimeric molecules had lower potency than EMP alone, the previously described synergy with EPO not being observed with EMP. Several hypotheses are discussed to explain this result.

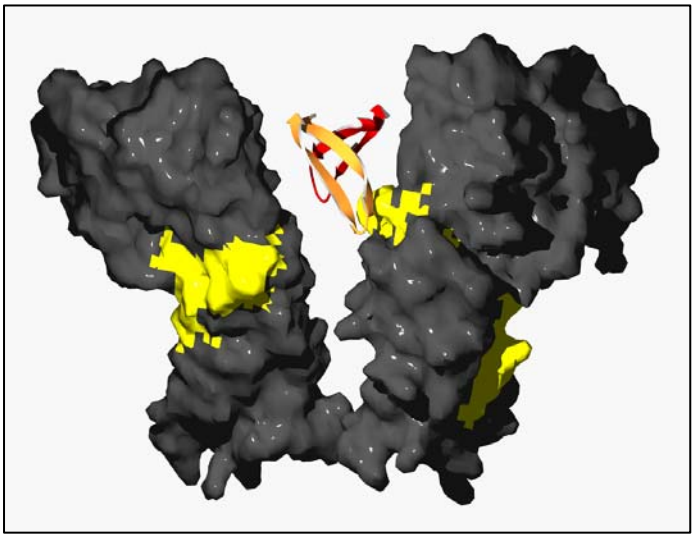

Figure 2-2: EPOR structure in complex with two EMPs. Highlighted on EPOR surface is the ERP binding region

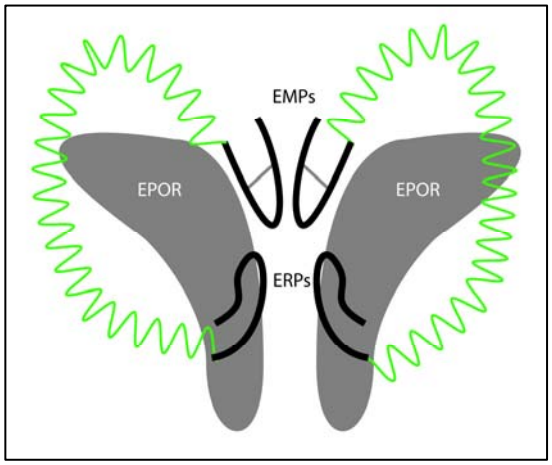

Figure 2-1: Schematic view of EPOR in complex with hetero-dimeric EMP-ERP peptide. The linker has to go over the receptor to allow the two peptides to bind with their different receptor binding sites.

2.3 Material and methods

2.3.1 Material

EMP and ERP monomers and hetero-dimers as described in Vadas and Rose [16]. Peptide synthesis grade DMF and DIEA was purchased from Biosolve. DCM, NMM, N-methylpyrrolidinone (NMP), diethyl ether, DMSO, carbonyldiimidazole (CDI), TFMSA, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and DIC, were purchased from Fluka, Switzerland. TFA was from Halocarbon, New Jersey. Acetonitrile CHROMASOLV® gradient grade for HPLC was from Sigma-Aldrich. HBTU was from Iris Biotech GmbH Germany) and HATU was from GL Biochem (Shanghai) Ltd. HOBt was from NovaBiochem (Switzerland) and amino acids were from AnaSpec (San Jose) or from NovaBiochem

2.3.2 Synthesis of heterodimers

Synthesis of EMP-ERP heterodimers was performed as previously described in Vadas and Rose (Figure 2-3) [16]. Briefly, ERP was synthesized by solid phase peptide synthesis on MBHA resin, using Boc/Bzl chemistry. After coupling of the last amino acid, successive addition of succinic anhydride, CDI and 4,7,10-trioxa-1,13-tridecanediamine (commercial PEG diamine from Fluka) added a polyamide moiety on the N-terminus of ERP. Two linker lengths where synthesized, with 5 and 6 diacid-diamine additions. Boc-Ser(Bzl) was couple to the last amine after HBTU activation. Resin cleavage was done with HF containing 5% p-cresol for 60 minutes at 0°C. Peptides were precipitated and washed with cold diethyl ether before lyophilization. After reverse-phase high performance liquid chromatography (RP-HPLC) purification, the terminal serine was oxidized with sodium periodate. The molecule was dissolved in 50mM Imidazole buffer pH 7 at a final Ser concentration of 200µM. In the presence of 50 molar excess of methionine, each serine was oxidized with 4 equivalent of sodium periodate. After 5min, 1000 equivalent (over periodate) of ethylene glycol were added and the solution brought to pH 4-5 with acetic acid before purification by RP-HPLC. Pure glyoxylyl-polyamide-ERP where finally reacted with aminooxyacetal-derivatized EMP (AoA-EMP) to form oxime bond. Peptides were dissolved in acetate buffer at pH 4.6 with 50%

acetonitrile with 1.2 excess of AoA-EMP over polyamide ERP. The mixture was stirred for 15h at room temperature before final purification by RP-HPLC

2.3.3 Cell proliferation assay

Cell proliferation assays were performed as previously described in Vadas et al. [19]. Serial dilutions of molecules were prepared in 96-well plates at 50µl/well in cell culture buffer (Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS, 50 units/mL penicillin, 50 µg/ml streptomycin, 2 mM glutamine). 5000 UT-7/EPO cells deprived in EPO were added in each well and the plates were incubated at 37°C in a humidified 5% CO2 tissue culture incubator. Proliferation was measured after 4 days using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) [20]. Absorbance of the wells was measured at 570 nm and data were analyzed using GraphPad Prism software to calculate EC_{50} values.

ERP: QRVEI LEGRT ECVLS NLRGR TRY

EMP: GGL**Y**A **C**HM**GP** M**TW**V**C** Q**P**LRG

Figure 2-3: Sequences of ERP and EMP. The only Cys residue of ERP is in the middle of the sequence. Cys 6 and Cys15 of EMP form a disulfide bond that cyclise the peptide. Highlighted in bold character are the amino acids essential for EMP activity.

2.4 Results of *in vitro* assays

Potency of the constructs was tested by cell proliferation assay using an EPO dependent cell line [21]. A diagram summarizing the pEC50 values obtained for each compound is shown in Figure 2-4. Hetero-dimers all have lower potency compared to monomeric EMP, the shortest dimers being slightly more potent than the longer ones. As observed with previously described EMP homo-dimers [19], the linkage attachment site on EMP had no influence on the activity of the hetero-dimers. Even at the highest possible concentrations (above 30 µM, where solubility of the peptide was not complete) ERP induced a level of proliferation that was barely detectable (Figure 2-5). In an experiment to verify the previously reported synergy between ERP and EPO, increasing concentrations of ERP were added to cells together with 8 pM of EPO, (a concentration providing approximately 5% of the maximum proliferative signal). Under these conditions, no synergistic effect was detectable at any ERP concentration used

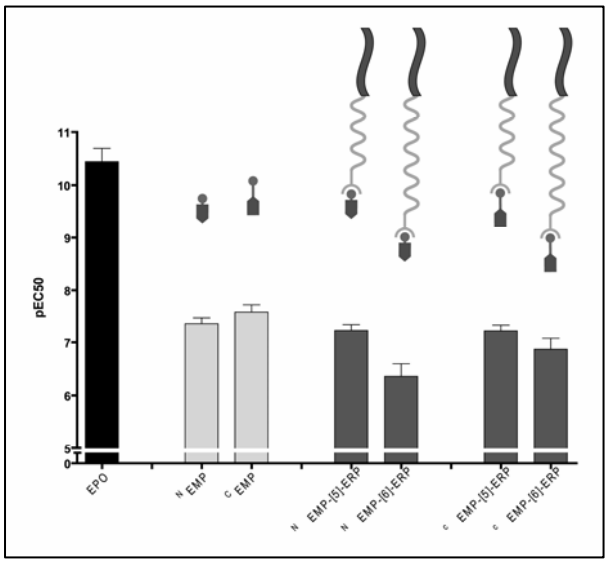

Figure 2-4: Graph of pEC50 values for monomers and ERP-EMP heterodimers. No activity was detected for monomeric ERP. Abbreviations: ^{N}EMP and ^{C}EMP refer to the linkage attachment site on EMP, respectively N- and C-termini. [X] refers to the number of "PEG-succ" units of the linker (see Vadas et al (2007)).

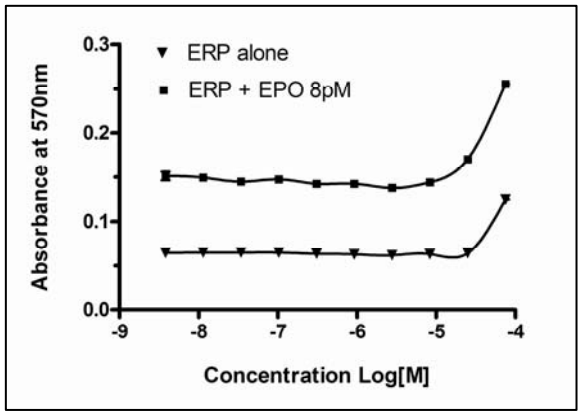

Figure 2-5: Comparison of cell proliferation assays with ERP alone or in presence of 8 pM EPO. We observe that ERP alone sustain little cell proliferation at concentration above 30 µM, and that in the presence of EPO, a minor effect is observed at concentration slightly lower (8 µM).

2.5 Discussion and perspectives

The objective of the present study was to increase the activity of EMP by connecting it to ERP via a flexible linker. Based on the observations of (i) synergy between ERP and EPO [14] and (ii) the significantly increased potency over EMP homodimers over EMP monomers [12, 13], we anticipated that an EMP-ERP dimer would show improved potency over the corresponding monomers.

To test this hypothesis, we designed and synthesized EMP-ERP hetero-dimers connected via polyamide linkers of 145 Å and 175 Å (estimated length of stretched polymer), the linkers being 3- to 5-fold longer than the distance separating the peptides when connected to their respective binding sites on EPOR according to the available structural model. Cell proliferation assay results indicated that hetero-dimers have lower potency than the most active monomeric component, EMP. Of note with respect with the low activity of hetero-dimers seen in this study is that (i) ERP has an extremely weak activity in our assay and (ii) we did not observe any synergy with EPO. Previous measurement of ERP potency by cell proliferation assays were performed with the TF-1 cell line (bone marrow, erythroleukemia, human, ATCC CRL-2003) [14], even then reporting activity at micromolar concentrations. We used a different cell line for our assay (UT-7/EPO), which was validate by previous experiments on EMP derivatives and by measuring EPO activity [19, 21]. Moreover, the latter cell line has been recently employed to test novel erythropoiesis stimulating agent [22]. The different cell line employed for the *in vitro* bioassays may explain the very low agonist activity of the ERP monomers and dimers tested in this study.

2.6 Conclusion

This study has highlighted the difficulties to increase the activity of a peptide by fusing it with another molecule having a comparable function, but that uses a different mode of action. The activity of molecules can be different depending on the assay format, some results not being observed by using other models. The synergy produced by ERP with EPO as described in the article of Naranda is very promising, providing the results can be reproduced. The continuation of that project would require a precise characterization of how ERP stimulates erythropoiesis, and what is the exact function of the interaction between ERP and EPOR. Solving the X-ray structure of the complex would certainly bring new information relevant to that question.

2.7 References

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Chapter 3

Multivalency – a way to enhance binding avidities and bioactivity – preliminary applications to EPO

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3.1 Abstract

Multi-valency has advantages over mono-valency for binding interactions and even for activity. In particular, avidity is higher since the off-rate of a multivalent species is much slower than that of a monomer. This is particularly profitable for ligands binding receptors that require dimerization for activity, like the receptor of erythropoietin (EPOR). Peptides that mimic the action of erythropoietin (EPO) have been described with no sequence similarity with the human hormone: EPO mimetic peptide (EMP) and EPO receptor peptide (ERP). These two peptides have similar activity but interact through different sites on EPOR. Here we describe the construction of several new synthetic homo- and hetero-dimers based on EMP ERP sequences. To link the monomeric molecules together, several monodisperse polyamide linkers of different lengths were synthesized with dialdehyde functionalities. The chemoselective oxime chemistry was used to obtain homogeneous constructs. Certain chemical incompatibilities were dealt with via a protection approach. The oximes are stable under normal conditions and so lend themselves to biological testing.

3.2 Introduction

Natural polyvalent molecules exist of which IgM with its ten binding sites is probably the most well-known example. Multivalency has been approached from a theoretical point of view on many occasions, including in a recent article describing the thermodynamic parameters[1]. Experimentally, the pentameric recombinant "peptabody" [2] showed 10^5 times tighter binding than the constituent monomeric peptides and was proved to be biologically active. Besides proteins, multivalent molecules may be non-peptidic organic chemicals, nucleotides [3], antibiotics [4] etc. Hetero- and homo-dimeric synthetic peptides have been described, as well as molecules with higher multiplicity, by many groups including our own.

Synthetic or recombinant dimers can bind to and activate those receptors which require dimerization to activate. Examples of synthetic dimers which bind to and activate receptors include the peptide mimetic of thrombopoietin [5] and a series of erythropoietin mimetic peptides [5] [6]. Erythropoietin (EPO) is a 166 amino acid protein which regulates the production of red blood cells [7]. The recombinant protein (rhEPO) is used clinically for the treatment of anaemia [8], and a hyperglycosylated analogue that has a prolonged duration of action can also be administered [9]. Other EPO analogues were designed by pegylation for having prolonged in vivo activity [10, 11]. The EPO receptor (EPOR) is a good example of a receptor which requires dimerization for activity [12]. The receptor may be activated not only by EPO, but by monomeric and dimeric Erythropoietin Mimetic Peptides (EMPs): 13-20 amino acid peptides having no sequence similarity with EPO that were discovered by phage display[5]. Erythropoietin Receptor Peptide (ERP), a 23-residue peptide with the sequence identical to a site on the EPO receptor is also active on EPO-responsive cells but involves another mode of action [13]. EPO itself has been dimerized in an attempt to improve activity, either by recombinant techniques (with a 17 residue linker, [14]) or chemically [14-16]. The aim of such studies is to find a molecule which has improved pharmacological properties and that is cheaper to produce than rhEPO as a therapeutic for the treatment of chronic anaemia.

Several dimers of erythropoietin mimetic peptides (EMP) have been described [6] which are more active than the corresponding monomers although considerably less active than the

recombinant protein. While the distance between the monomer units in synthetic dimers may sometimes be quite short [5], in other cases a linking moiety of considerable length is required for receptor activation [3, 17]. As exemplified by studies on other multimeric molecules, the distance between monomeric peptides is crucial for activity [3, 18]. We have previously described a simple, stepwise, solid-phase procedure for the synthesis of linking moieties that have a defined structure in spite of their great length [17]. These flexible linkers, produced by coupling commercially available diacids and diamines in a controlled manner, have a repeat unit $–[NH\text{-}Y\text{-}NHCO\text{-}X\text{-}CO]_n–$, where the value of n depends on the number of coupling cycles performed. The combination X = $-CH_2CH_2-$, Y = $-CH_2CH_2CH_2-(OCH_2CH_2)_3-CH_2-$ has particularly favourable solubility properties and is resistant to proteolytic digestion. In the present study, we describe the synthesis of several new EMP and ERP constructs. By synthesizing the polyamide linkers, we were able to design linkers with different lengths that were monodisperse, in comparison with commercially available PEGs. To attach the peptides on each side of the linkers, we decided to use the chemoselective oxime chemistry that requires very mild conditions and that is robust and biocompatible.

Combining the advantages of defined linker length and monodispersity with specificity of the oxime chemistry, we synthesized several homogeneous EMP homo-dimers and even EMP-ERP hetero-dimers.

3.3 Material and methods

3.3.1 Material

Peptide synthesis grade DMF and DIEA was purchased from Biosolve. DCM, NMM, N-methylpyrrolidinone (NMP), diethyl ether, DMSO, carbonyldiimidazole (CDI), TFMSA, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and DIC, were purchased from Fluka, Switzerland. TFA was from Halocarbon, New Jersey. Acetonitrile CHROMASOLV® gradient grade for HPLC was from Sigma-Aldrich. HBTU was from Iris Biotech GmbH Germany) and HATU was from GL Biochem (Shanghai) Ltd. HOBt was from NovaBiochem (Switzerland) and amino acids were from AnaSpec (San Jose) or from NovaBiochem

3.3.2 Analytical High Pressure Liquid Chromatography (HPLC)

Analytical reverse-phase HPLC was performed at 0.6ml/min on Waters equipment using a Macherey-Nagel C_8 column (4 x 250 mm 300Å 5µm particle size). Solvent A was 0.1% TFA in HPLC grade water. Solvent B was 90% acetonitrile with 0.1% TFA. Elution was done with a 40min linear gradient 0-80%B.

3.3.3 Preparative High Pressure Liquid Chromatography (HPLC)

Preparative reverse-phased HPLC was performed at 15ml/min on Waters equipment using a Vydac C_8 column (22 x 250 mm 300Å 10-15µm particle size). Elution with previously described solvents A and B Peptides was done with an appropriate linear gradient, usually 1%/min. UV monitoring was at 214nm. Peaks were collected manually and the product was recovered by lyophilization.

3.3.4 Mass Spectrometry

MALDI-TOF mass spectrometry was performed in linear mode using sinapinic acid as matrix on a Voyager-DE STR (Applied Biosystems) equipped with delayed extraction. External calibration was performed using porcine insulin (Novo Nordisk).

3.3.5 Solid Phase Peptide Synthesis

Solid phase peptide synthesis [19] was performed on a modified ABI 433A machine using Boc chemistry and *in situ* neutralization as previously described [20]. Peptides were prepared on a 0.2 mmol scale on MBHA cross linked with 1% DVB resin (0.9mmol/g, Senn Chemicals, Switzerland). Boc-amino acids were protected by the following groups: Arg(Tos), Asn(Xan), Asp(OcHx), Cys (Mob), Glu(OcHx), His(Dnp), Lys(Z(2Cl)), Ser(Bzl), Thr(Bzl), Trp(For), Tyr(Z(2Br)). After chain elongation, a Boc-aminooxyacetyl group (Boc-AoA) was manually coupled as its *N*-hydroxysuccinimide ester (Boc-AoA-OSu) used in 1.2 molar excess in DMSO with NMM as base[21]. Certain protecting groups were removed prior to acid cleavage (Dnp with 20% 2-mercaptoethanol and 10% DIEA; formyl with 20% piperidine; Boc with neat TFA) with HF containing 5% p-cresol for 60 minutes at 0°C. Peptides were precipitated and then washed with cold diethyl ether. Crude peptide was exposed to high vacuum overnight then purified by preparative reversed phase HPLC and the purified product was lyophilized. EMP sequences after resin cleavage were AoA-GGLYACHMGPMTWVCQPLRG-amide for the *N*-terminally modified peptide (referred to as AoA-EMP), and GGLYACHMGPMTWVCQPLRGK(AoA)-amide for the *C*-terminally modified peptide (referred to as EMPK-AoA). For the synthesis of the *C*-terminally modified EMP, Boc-Lys(Fmoc) was used to initiate the synthesis. Deprotection of the Fmoc group was performed with 20% piperidine in DMF after synthesis completion and Boc-AoA-OSu was coupled manually with 1.2 equivalents. The ERP peptide had the following sequence QRVEILEGRTECVLSNLRGRTRY. Five “PEG-succ” units were manually coupled to the *N*-terminus of the ERP and finally a Boc-Ser(Bzl)-OH was added (see details below).

3.3.6 Disulfide bond formation of EMP

20mg of peptide were dissolved in 20ml water and the pH adjusted to 7 with 1% ammonia solution. 245µl of a 3% H_2O_2 solution was added and the mixture left to react for 30min. After acidification with acetic acid, the solution was directly injected onto preparative RP-HPLC for purification.

3.3.7 Synthesis of a PEG-polyamide linker

PEG-like dialdehyde linkers of various lengths were synthesized as described in [17]. Briefly, 0.3mmol of Sasrin resin (1.02mmol/g, 200-400 mesh, Bachem Switzerland) was acylated with succinic anhydride, 4mmol in 8 ml of DMF containing 0.5 M of DMAP to which 0.4 ml of DIEA was added. Free carboxyl groups were activated with CDI for 30min. The activated carboxyl group was then aminolysed with 4,7,10-trioxa-1,13-tridecanediamine (commercial PEG diamine from Fluka) in the presence of HOBt for 60min in DMF [17]. The growing molecule was again acylated with succinic anhydride, 4mmol in 8 ml of DMF containing 0.5 M HOBt to which 0.4 ml of DIEA was added. The polyamide chain was further grown by successive activation, aminolysis and acylation steps. Each "acylation-activation-aminolysis" cycle will add a "PEG-succ" unit (-$NHCH_2(CH_2CH_2O)_3CH_2CH_2CH_2NH$-$COCH_2CH_2CO$-) to the resin. After addition of the desired number of units, Boc-Ser(Bzl)-OH was coupled to the free amine. For this, 1.2mmol (4eq) of Boc-L-Ser(Bzl) was activated with 1.2mmol (4eq) HBTU dissolved in DMF. 2mmol (8eq) of DIEA were added and the solution reacted for 40min. Ninhydrin test (Fluka) was performed to verify the acylation coupling had succeeded[22]. If the test was positive, the coupling was repeated for another 40min. Products were cleaved from the resin with 1% TFA in DCM and neutralized in pyridine/methanol (9/1) before being precipitated with cold diethyl ether. HPLC preparative purification was employed. The linker was then coupled [17] to the commercial PEG diamine: 4 equivalent of linker with 4 equivalent of HATU and 8 equivalent DIEA were preactivated for 5 min before reaction with 1 equivalent of the commercially available diamine. After 4 hour incubation, the product was isolated by RP-HPLC. The symmetrical linker with the formula Boc-Ser(Bzl)-[PEG-succ]$_{4,6,8}$-PEG-[PEG-succ]$_{4,6,8}$-Ser(Bzl)-Boc was obtained. The Boc and Bzl protecting groups were removed by dissolving 10mg of linker in 300µl TFA for 4min followed by

addition of 30µl of TFMSA for 25min. TFA was evaporated with air and the product precipitated and washed with cold ether, and dried in a dessicator under high vacuum.

3.3.8 Serine oxidation to a glyoxylyl function

The N-terminal serine is oxidized with periodate to a glyoxylyl function as described in [23, 24]. The molecule is dissolved in 50mM Imidazole buffer pH 7 at a final Ser concentration of 200µM. In the presence of 50 molar excess of methionine, each serine is oxidized with 4 equivalent of sodium periodate. After 5min, 1000 equivalent (over periodate) of ethylene glycol are added and the solution is brought to pH 4-5 with acetic acid. The solution is then injected on a RP-HPLC for purification.

3.3.9 Alkylation of the cysteine of ERP

10mg of peptide were dissolved in 2.3ml of 100mM phosphate buffer pH8. 6.5 mg of TCEP were added and the solution was incubated for 30min at 37°C. Then 8.5mg of iodoacetamide were added and incubated for 90min at 37°C. The solution was acidified before injection on RP-HPLC for purification.

3.3.10 Oximation of the dialdehyde linker with aminooxy EMP derivatives

200µl of a 14mM aldehyde solution (HPLC fraction concentrated) was quickly added to 200µl (1.5 excess over aldehyde groups) of a 20mM solution of aminooxy-peptide (dissolved in 10mM acetate buffer, pH 4.6 with 50% acetonitrile) [21]. The reaction was stirred for 15h at room temperature and purified by RP-HPLC as previously described.

3.4 Results and discussion

3.4.1 Synthesis of EMP homo-dimers

The EPO dimer analogues were assembled using the highly specific oxime reaction between an aldehyde and an aminooxy compound [21]. Based on previously described strategy [17], we decided to synthesize EMP dimers of precise length, with the aminooxy monomeric peptides attached to the linker either by their N- or C-terminus (Figure 3-3). By designing monodisperse linkers of different lengths, we synthesized a panel of new compounds expected to be agonists of EPOR. Peptides were synthesized using Boc chemistry on MBHA resin and have an identical sequence, excepted that an additional Boc-Lys(Fmoc) was introduced at the C-terminal EMP derivative (EMPK-AoA) to attach the aminooxy group. Following chain extension, selective deprotection by piperidine was followed by AoA-OSu coupling on resin and allowed us to obtain the desired peptide. After resin cleavage and HPLC purification, the peptides containing two cysteines were oxidized with hydrogen peroxide to the disulfide. This rather strong oxidation method was preferred to air oxidation, since the latter was not efficient enough.

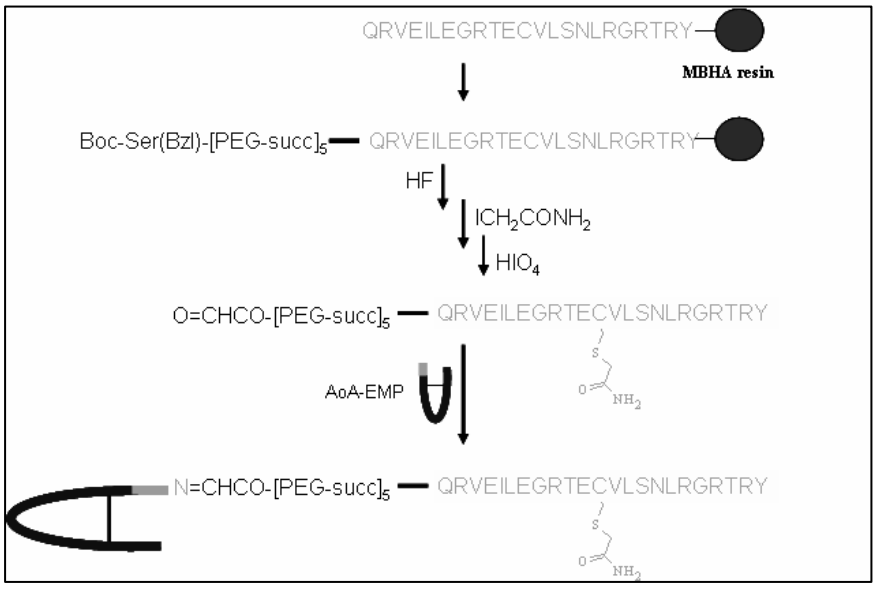

Figure 3-1: Strategy for the synthesis of N-EMP-ERP heterodimer. ERP was synthesized by SPPS on MBHA resin and five "PEG-succ" units were added on-resin to the chain. After acid cleavage, Cys protection with iodoacetamide and Ser oxidation to glyoxylyl function, the ERP construct with an aldehyde

After purification, both peptides gave a single peak on analytical RP-HPLC and possessed the expected masses (Table 3-1, Figure 3-2). The PEG-like linkers were synthesized on hydroxyl resin following the protocol described in [17]. This allowed us to prepare monodisperse linkers of desired lengths and with a chosen functionality on both ends. We decided to synthesize three different linker lengths composed of various number of "PEG-succ" groups. After successive and controlled addition of a selected number of "PEG" and succ" units, a Boc-protected serine residue was coupled for later transformation to an aldehyde functionality. To avoid the presence of linkers with missing PEG-succ units, we used large excesses of reagents. These Boc-Ser(Bzl)-[PEG-succ]4,6,8-OH polyamide fragments were then used to acylate both amino groups of the PEG diamine to obtain symmetrical linkers composed of either 8, 12 or 16 PEG-succ units (referred as short, medium and long). After deprotection, terminal serine residues were oxidized with periodate to obtain the dialdehyde linker [23, 25]. We found important not to dry the dialdehydes since they became less soluble and less reactive.

Molecule	Calculated mass (Da)	Measured mass by MS (Da)	difference
AoA-EMP (monomer)	2247.65	2247.48	0.17
EMPK-AoA (monomer)	2375.82	2377.24	1.42
N-EMP short dimer	7210.60	7209.80	0.8
N-EMP medium dimer	8420.10	8420.47	0.37
N-EMP long dimer	9629.52	9629.50	0.02
C-EMPK-AoA short dimer	7466.90	7466.94	0.04
C-EMPK-AoA medium dimer	8676.40	8676.79	0.39
C-EMPK-AoA long dimer	9885.80	9886.71	0.91
N-EMP-ERP hetero dimer	6603.75	6603.79	0.04
C-EMPK-AoA-ERP hetero dimer	6731.92	6731.94	0.02

Table 3-1: List of constructs with calculated and measured masses

Finally, the purified aminooxy peptides (either N- or C-terminal derivative) were reacted with the dialdehyde linkers to form oxime dimers in mild conditions (pH 7 in Imidazole buffer). A 1.5 fold excess of peptide over the aldehyde groups was used. This chemoselective reaction allowed formation of exclusively N- or C-terminally linked dimers of precise length (N-EMP

dimer or C-EMPK dimer), without any side reaction between the linker and peptide side chains, in contrast to general acylation which may react with either α or ε amino groups [6, 21]. All constructs gave a single peak on RP-HPLC and possessed the expected masses (Table 3-1, Figure 3-2). Traces of linker missing a "PEG-succ" unit were observable by MS, but these were insignificant compared to the inhomogeneity of the commercially available PEGs [17]. MALDI analysis of monomeric AoA EMP derivatives showed an additional mass with 164 Da adduct that could not be identified. Since the pure EMP dimer molecules had one single mass, no further purification was performed. Biological activity of the different compounds is currently under investigation.

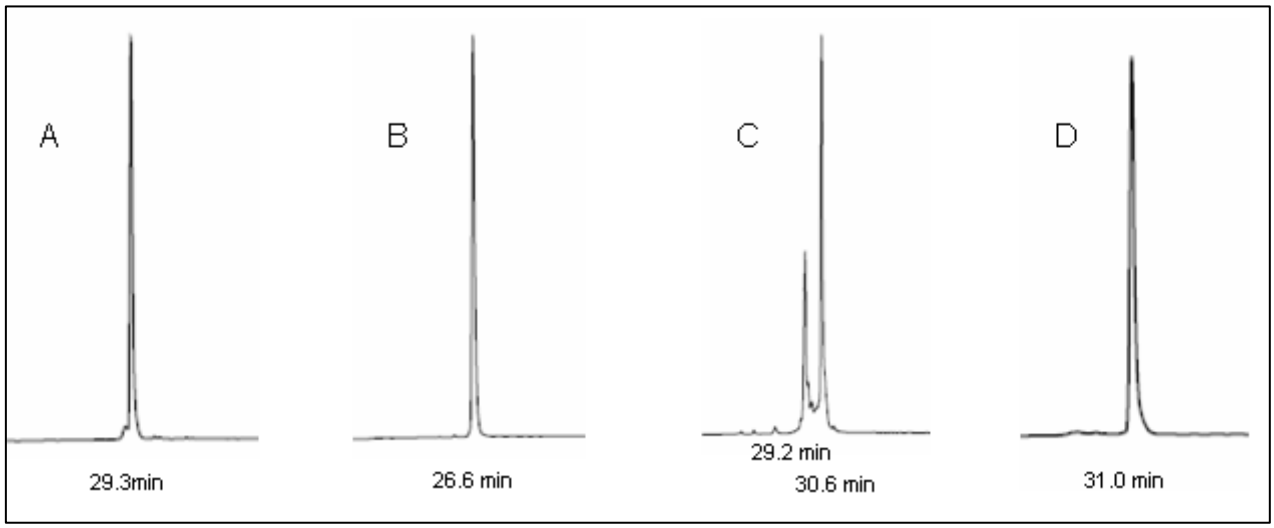

Figure 3-2: HPLC of N-EMP long dimer synthesis. Pure AoA-EMP (A) was reacted with pure long dialdehyde (B) by oxime chemistry. After 60min reaction (C), two species were observed and the expected product was purified by HPLC (D).

3.4.2 Synthesis of ERP-EMP hetero-dimers

A new type of EPO analogue, a hetero-dimer, was synthesized in an attempt to profit from multivalency (Figure 3-1). Besides EMP, the peptide ERP was synthesized and used. ERP was found to activate EPOR by interacting with a site distant from the hormone binding site [13, 26], whereas EMP was shown to bind EPOR at the same site as EPO [27]. Moreover,

ERP and EPO were shown to have synergic action in vitro [13]. Hence, a heterodimer EMP-ERP is expected to bind more strongly to EPOR than an EMP dimer or an ERP dimer.

For this heterodimeric construct, the idea was to synthesize a hetero-dimer composed of one ERP and one EMP linked by a flexible linker. The synthesis strategy was different than for the EMP homo-dimers, although a similar monodisperse polyamide linker was used: for the heterodimer we synthesized the ERP construct by SPPS and added on-resin five "PEG-succ" units on its N-terminus. By finally coupling a serine residue, we obtained pure Ser-[PEG-succ]5-ERP after resin cleavage and HPLC purification. As they were many impurities in the crude material, we tried coupling pre-purified Boc-Ser(Bzl)-[PEG-succ]6-OH on-resin to chemically synthesized ERP. Using PyBOP as coupling agent with 3 equivalents of DIEA, the expected product was synthesized but unfortunately over-acylation was observed. Solution-phase coupling was also attempted but no reaction occured. This was probably due to the remaining water content of the linker, even after 15 hours of exposure to high vacuum on the lyophilizer. To oxidize the terminal serine to glyoxylyl, we first needed to protect the free cysteine of the ERP to avoid sulfonic acid formation. This was performed by alkylating the molecule with 20 equivalents of iodoacetamide. Alkylation was a reasonable approach since ERP was reported to be active under reducing conditions, showing that a preformed disulphide was probably not necessary for activity [26].

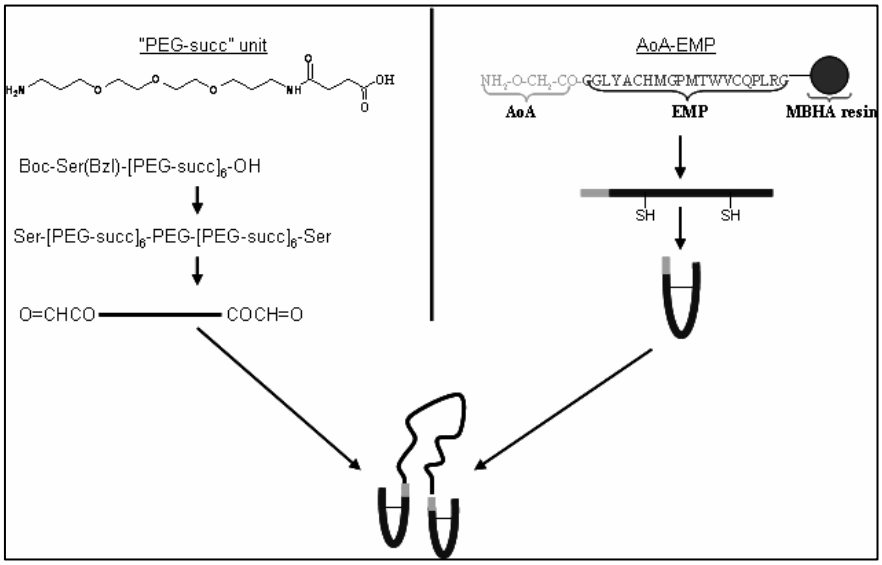

Figure 3-3: Strategy for N-EMP medium dimer synthesis. On one side the dialdehyde linker is synthesized by successive addition of "PEG-succ" units, alkylation of the carboxylic linker parts with a diamine and finally serine oxidation to glyoxylyl function. On the other side, AoA-EMP is synthesized by SPPS and the disulfide bond formed after resin cleavage. Finally, both molecules are reacted together to form the N-terminal EMP dimer by oxime ligation.

The serine was then oxidized with periodate to obtain the necessary aldehyde for oxime ligation. Reaction with pure AoA-EMP or EMPK-AoA, gave the expected hetero-dimer products (N-EMP-ERP and C-EMPK-ERP hetero dimers). Analytical RP-HPLC was one single peak and MS data were as expected (Table 3-1, Figure 3-4).

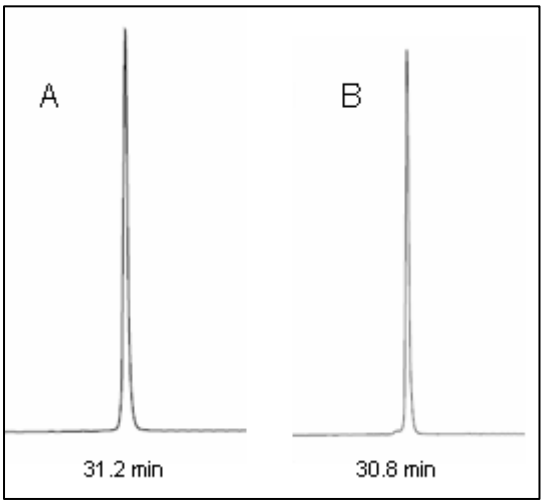

Figure 3-4: HPLC of N-EMP-ERP (A) and C-EMPK-ERP (B) constructs

3.5 Conclusions

New synthetic EPO analogues dimers have been synthesized following a protocol that allows great flexibility in linker length and attachment point. Starting from known EMP sequences that were discovered by phage display [5], and from ERP (a peptide active on EPOR through an alternative binding site) new multivalent constructs were designed. Using monodisperse polyamide linkers of desired lengths, monomeric peptides [6] were separated by chosen distances. Attaching the peptides to the linkers by oxime bonds allowed the synthesis of homogenous constructs compared to previous EPO analogues [6, 15], with the possibility to attach the peptides through their N- or C-terminus. With a choice of linkers of different lengths and EMPs with functional aminooxy group at different position, the possibilities for new compounds are large. The synthesis strategy used in this paper for the synthesis of multivalent compounds is applicable to any active peptide for a corresponding receptor. The oxime bond is compatible with human use [28] and stable under physiological conditions [29], so the molecules lend themselves to biological tests.

The hetero-dimeric constructs also combine the advantages of monodispersity of the linker with construct homogeneity and opens a new approach for EPOR targeting. By targeting the receptor on two distinct sites with one single molecule, moreover with active peptides that have synergic activity, the improvement for biological activity is promising.

3.6 Acknowledgements

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Chapter 4

Characterization of new multimeric erythropoietin receptor (EPOR) agonists

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4.1 Abstract

In addition to its natural ligand, the receptor for erythropoietin can be activated by small peptides known as Erythropoietin Mimetic Peptides (EMPs). Although EMPs are less potent than the natural ligand, EMP dimers, consisting of two EMPs joined via a linker, have been shown to exhibit significantly improved activity compared to the corresponding monomers, with potency approaching that of the native hormone. In this study, we used a panel of novel EMP dimers to explore the effects of linker length and EMP attachment site on potency. The EC50 values obtained in an EPO-dependent proliferation assay indicated that, as has been shown with similar molecules, EMP dimerization can lead to increases in potency of more than two orders of magnitude. We found that both C-terminal and N-terminal attachment of the linker to EMP was tolerated, and that, with the exception of the shortest linker, all of the linker lengths tested provided a similar increase in potency. In follow-up work devised to explore the potential benefit of contacting additional cell surface EPO receptors, we designed a tetrameric template consisting of lysine based dimers joined via commercial PEG linkers of various molecular weights. Evaluation of the resulting molecules indicated a clear effect of PEG linker size on activity: while the 'dimer of dimer' with the shortest linker exhibited 10-fold lower potency than the corresponding dimer, the longest tetramer increased potency by 5-fold. We discuss the implications of these results for the further development of EMP multimers.

4.2 Introduction

Through its effects on the specificity and affinity of binding interactions, multivalency plays an important role in biology [1, 2]. Peptide scientists have taken advantage of the increased potency available through multimerization by synthesizing multimeric molecules that present an ordered array of two or more ligands [3-6]. Dimers can be readily synthesized using a bifunctional PEG linker or a lysine residue as spacer [7, 8]. For synthetic multimers to obtain maximum benefit from branching effects, two key criteria must be met: first, attachment of the linker must not interfere with the activity of the peptide, and second, the linker must be of a length and rigidity appropriate for a given target [8]. Dimeric ligands have particularly favourable applications for receptors that are active as dimers [7, 9, 10].

Erythropoietin (EPO), a glycoprotein of 34 kDa, regulates the proliferation and differentiation of red blood cells by activation of its receptor, EPOR, through dimerization. EPO is used to treat anaemia due to renal failure, and during the last decade, a number of studies have been carried out with the goal of obtaining molecules more active than the native hormone [11]. The main strategy involved increasing the size of the protein in order to prolong its circulating life time in the body, using either hyperglycolysation [12], the introduction of PEG chains [13], or by the addition of polyamide polymers on a synthetic EPO analogue [14]. Creating EPO dimers by recombinant techniques also led to a molecule with increased potency compared to monomeric EPO [15]. In 1996, Wrighton et al. discovered EMP (Erythropoietin Mimetic Peptide), a 20-residue peptide that activates EPOR [16]. While the potency of EMP is lower than that of EPO, improvement of its potency by up to three orders of magnitude has been achieved using dimerization strategies, either via the amino-termini using a PEG linker [17], or via the C-termini with a lysine-based linker [10]. Although the most active EMP dimers are still less potent than native EPO, they remain promising candidates for drug development, and one EMP dimer has entered clinical trials [18].

The present article describes the synthesis and biological characterization of a series of new EMP multimers, using an EPO-dependent cell proliferation assay [19]. A group of dimers, linked via monodisperse PEG-based polyamide linkers of various sizes, were used to study the effect of linker length and EMP attachment site on activity [20]. A second set of

tetrameric molecules was then used to explore the potential benefit of bridging two EPOR dimer pairs. We discuss the implications of the results obtained for further development of EMP multimers.

4.3 Material and methods

4.3.1 Materials

EMP monomers and dimers as described in Vadas and Rose [20]. Peptide synthesis grade DMF and DIEA were purchased from Biosolve. DCM, NMM, N-methylpyrrolidinone (NMP), diethyl ether, DMSO, carbonyldiimidazole (CDI), TFMSA, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and DIC, were purchased from Fluka, Switzerland. TFA was from Halocarbon, New Jersey. Acetonitrile CHROMASOLV® gradient grade for HPLC was from Sigma-Aldrich. HBTU was from Iris Biotech GmbH Germany) and HATU was from GL Biochem (Shanghai) Ltd. HOBt was from NovaBiochem (Switzerland) and amino acids were from AnaSpec (San Jose) or from NovaBiochem. Standard EPO is EPREX (epoetin alpha) from Janssen-Cilag AG. IMDM (with l-glutamine and 25mM HEPES), penicillin, streptomycin were from Invitrogen. MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide was from Sigma, 2-propanol and 1.25N HCl/ isopropanol was from Fluka.

4.3.2 Solid Phase Peptide Synthesis

Solid phase peptide synthesis was performed on a modified ABI 433A machine using Boc chemistry and in situ neutralization as previously described [21, 22]. Peptides were prepared on a 0.2 mmol scale on MBHA cross linked with 1% DVB resin (0.9 mmol/g, Senn Chemicals, Switzerland). Boc-amino acids were protected by the following groups: Arg(Tos), Asn(Xan), Asp(OcHx), Cys (Mob), Glu(OcHx), His(Dnp), Lys(Z(2Cl)), Ser(Bzl), Thr(Bzl), Trp(For), Tyr(Z(2Br)). After chain elongation, a Boc-aminooxyacetyl group (Boc-AoA) was manually coupled as its N-hydroxysuccinimide ester (Boc-AoA-OSu) used in 1.2 molar excess in DMSO with NMM as base. Certain protecting groups were removed prior to acid cleavage (Dnp with 20% 2-mercaptoethanol and 10% DIEA; formyl with 20% piperidine; Boc with neat TFA) with HF containing 5% p-cresol for 60 minutes at 0°C. Peptides were precipitated and washed with cold diethyl ether. Crude peptide was exposed to high vacuum overnight then purified by preparative reversed phase HPLC and the purified product was

lyophilized. EMP sequences are referred in Figure 4-1. For the synthesis of the C-EMP, Boc-Lys(Fmoc)-OH was used to initiate the synthesis. Deprotection of the Fmoc group was performed with 20% piperidine in DMF after synthesis completion and Boc-AoA-OSu was coupled manually with 1.2 equivalents.

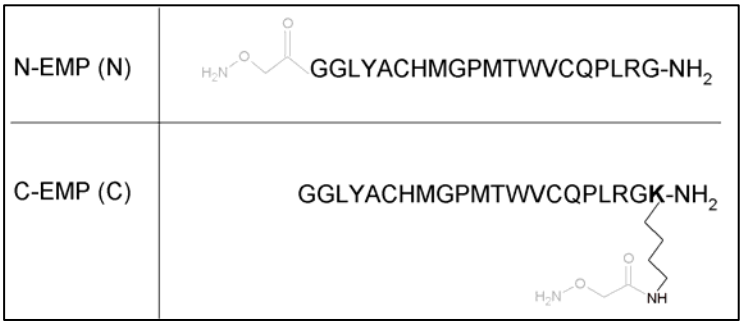

Figure 4-1: Structure of N-EMP and C-EMP peptides

4.3.3 Synthesis of a PEG-polyamide linker for dimers

PEG-like dialdehyde linkers of various lengths were synthesized as described in Vadas & Rose [20, 23]. Briefly, 0.3 mmol of Sasrin resin (1.02 mmol/g, 200-400 mesh, Bachem Switzerland) was acylated with succinic anhydride, 4 mmol in 8 ml of DMF containing 0.5 M of DMAP to which 0.4 ml of DIEA was added. Free carboxyl groups were activated with CDI for 30 min. The activated carboxyl group was then aminolysed with 4,7,10-trioxa-1,13-tridecanediamine (commercial PEG diamine from Fluka) in the presence of HOBt for 60min in DMF [23]. The growing molecule was again acylated with succinic anhydride, 4 mmol in 8 ml of DMF containing 0.5 M HOBt to which 0.4 ml of DIEA was added. The polyamide chain was further grown by successive activation, aminolysis and acylation steps. Each "acylation-activation-aminolysis" cycle adds a "PEG-succ" unit (-NHCH2(CH2CH2O)3CH2CH2CH2NH-COCH2CH2CO-) to the resin. After addition of the desired number of units, Boc-Ser(Bzl)-OH was coupled to the free amine. For this, 1.2 mmol (4eq) of Boc-L-Ser(Bzl) was activated with 1.2 mmol (4eq) HBTU dissolved in DMF. 2 mmol (8eq) of DIEA were added and the solution reacted for 40 min. Ninhydrin test (Fluka) was performed to verify the acylation coupling had succeeded [24]. If the test was positive, the coupling was repeated for another 40 min. Products were cleaved from the resin with 1%

TFA in DCM and neutralized in pyridine/methanol (9/1) before being precipitated with cold diethyl ether. HPLC preparative purification was employed. The linker was then coupled to the commercial PEG diamine: 4 equivalent of linker with 4 equivalent of HATU and 8 equivalent DIEA were preactivated for 5 min before reaction with 1 equivalent of the commercially available diamine. After 4 hour incubation, the product was isolated by RP-HPLC. The symmetrical linker with the formula Boc-Ser(Bzl)-[PEG-succ]4,6,8-PEG-[PEG-succ]4,6,8-Ser(Bzl)-Boc was obtained. The Boc and Bzl protecting groups were removed by dissolving 10mg of linker in 300 µl TFA for 4 min followed by addition of 30 µl of TFMSA for 25 min. TFA was evaporated with air and the product precipitated and washed with cold ether, and dried in a dessicator under high vacuum.

4.3.4 Serine oxidation to a glyoxylyl function

The N-terminal serine was oxidized with periodate to a glyoxylyl function as described in [25, 26]. The molecules were dissolved in 50 mM Imidazole buffer pH 7 at a final Ser concentration of 200 µM. In the presence of 50 molar excess of methionine, each serine was oxidized with 4 equivalents of sodium periodate. After 5 min, 1000 equivalent (over periodate) of ethylene glycol were added and the solution brought to pH 4-5 with acetic acid. The solution was then injected on a RP-HPLC for purification.

4.3.5 Synthesis of PEG tetraaldehyde linker

Four tetraaldehyde PEG linkers were synthesized based on the same protocol. Starting from 1 µmol of PEG diamine (3,4 kDa, 6 kDa, 10 kDa, 20 kDa), both amino groups were acylated with Boc-Lys(Boc)-OH. Eight µmol of amino acid were activated with 7.6 µmol HATU with 12µmol DIEA. After 40min reaction, the product was precipited and washed 3 times in cold ether. Neat TFA was added for 3 min to remove Boc protecting groups. After TFA evaporation, the product was precipitated and washed with ice cold ether. The four amino groups were acylated with 16µmol of 4-formyl-benzoicacid, activated with 15.2 µmol HATU in the presence of 25 µmol DIEA. The crude product was precipitated, washed with ether and dried under vacuum. Non purified linker was oximated with aminooxyacetyl peptides as described below. Structure of tetraaldehyde linker is presented in Figure 4-2.

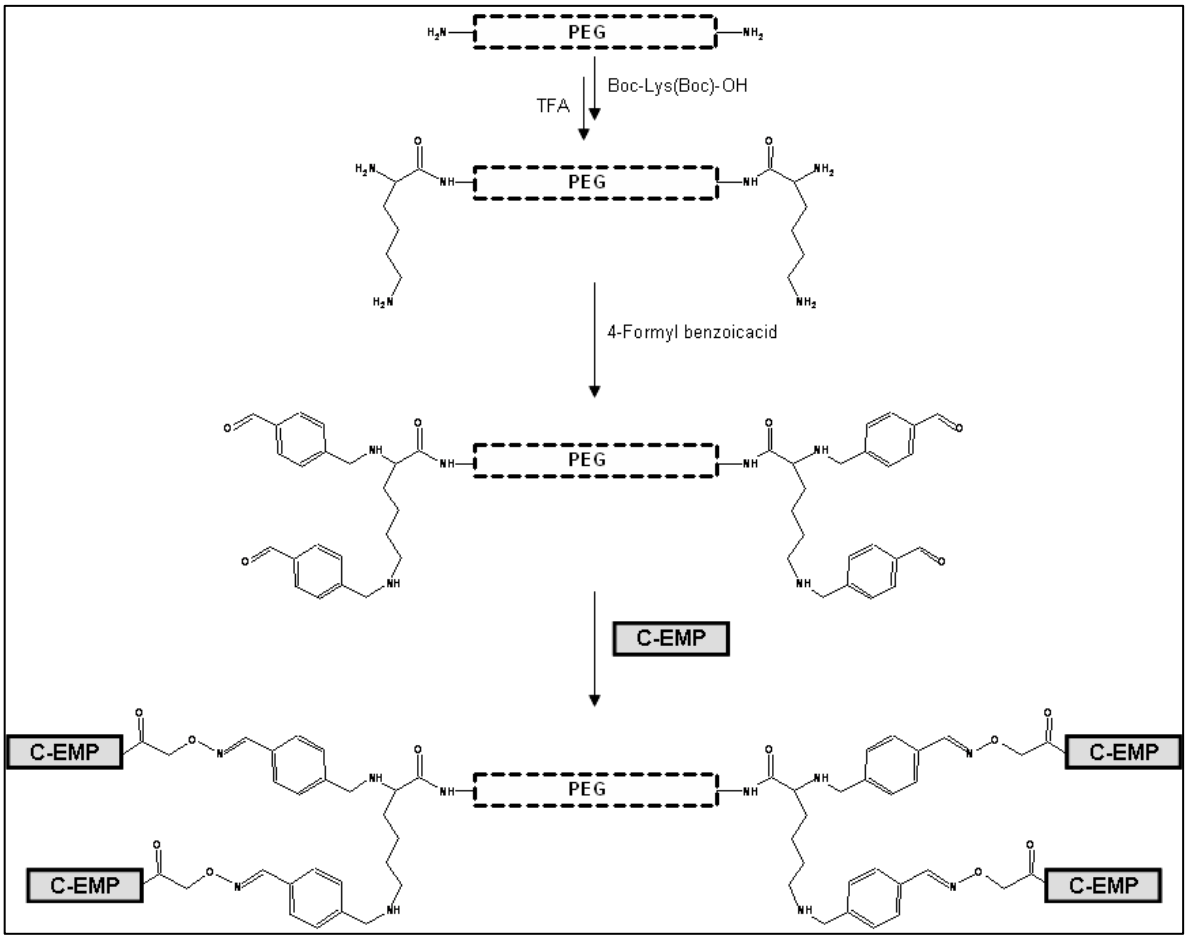

Figure 4-2: Synthesis of a C-EMP tetramer starting from commercial PEG diamine

4.3.6 Synthesis of control PEG-EMP dimer (short dimer with a polymeric chain)

The lysine based control molecule was synthesized on-resin following the same protocol as the synthesis of PEG-based polyamide linkers, except for the last steps. On 0.03 mmol SASRIN, six "acylation-activation-aminolysis" cycles were performed as described above. After the 6th aminolysis step, Fmoc-Lys(Fmoc)-OH was coupled. 0.12 mmol Fmoc-Lys(Fmoc)-OH were activated with 0.114 mmol HBTU in the presence of 0.2 mmol DIEA. After 60 min, resin was washed with DMF and Fmoc protecting groups were removed with 20% piperidine/DMF. Coupling of 0.3 mmol of 4-formyl-benzoicacid preactivated with 0.28 mmol HATU in the presence of 0.6 mmol DIEA was done for 60 min. Product was cleaved and neutralized as described above. Crude product was precipitated and washed in cold ether

prior to lyophilization. No HPLC purification was done prior to oximation with aminooxyacetyl peptides.

4.3.7 Oximation of aldehyde linkers with aminooxyacetyl EMP derivatives

200 µl of a 10 mM aldehyde solution (HPLC fraction concentrated) was quickly added to 150 µl (1.5 excess over aldehyde groups) of a 20 mM solution of aminooxyacetyl-peptide (dissolved in 10 mM acetate buffer, pH 4.6 with 50% acetonitrile) 20. Reaction was stirred for 15 h at room temperature and purified by RP-HPLC.

4.3.8 Cell proliferation assays

The human EPO-dependent UT-7/EPO cell line [19] was maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS, 50 units/mL penicillin, 50 µg/ml streptomycin, 2 mM glutamine and 1 U/mL EPO (Eprex). For bioassays, cells were washed 3 times and resuspended in media deprived of EPO at 10^5 cells/mL. Serial dilutions of agonists were prepared in triplicate in flat-bottom 96-well plate, 50 µl/well. Five thousand cells were added in each well and the plates incubated at 37°C in a humidified 5% CO2 tissue culture incubator. Proliferation was assessed after 4 days using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) [27]. Absorbance of the wells was measured at 570 nm and data were analyzed using GraphPad Prism software to calculate EC_{50} values.

4.4 Results

4.4.1 Biological activity of EMP dimers

A series of previously synthesized EMP dimers [20], comprising a group in which linkage was via the N-termini and a group in which linkage was via the C-termini (N- and C-EMP dimers, see Figure 4-1) was tested for their capacity to stimulate proliferation of an EPO-dependent cell line [19]. Each series had dimers varying in linker size: 250 Å for short dimers (Nsd, Csd); 370Å for medium dimers (Nmd, Cmd) and 480 Å for long dimers (Nld, Cld). Lengths of linkers are estimated values for fully extended linkers.

Compound abbreviation	Linker length (extended linker)	Calculated Mw [Da]	Mean pEC50 values ± standard deviation	Mean EC50 [pM]	Fold increase in potency
EPO	N/A	34000	10.55 ± 0.18	28	-
N-EMP	N/A	2247.7	7.36 ± 0.11	43284	-
Nsd	250 Å	7210.6	9.88 ± 0.11	131	330
Nmd	370 Å	8420.1	9.89 ± 0.15	129	335
Nld	480 Å	9629.5	10.01 ± 0.09	97	447
C-EMP	N/A	2375.8	9.73 ± 0.08	25949	-
C-PEG	monomer with PEG	4321.2	6.94 ± 0.08	115226	0.23
Cvsd	8 Å	4848.3	8.05 ± 0.08	8972	3
Csd	250 Å	7466.9	9.73 ± 0.26	186	140
Cmd	370 Å	8676.4	9.81 ± 0.17	154	169
Cld	480 Å	9885.8	9.57 ± 0.14	271	96

Table 4-1: Mean pE50 (± Standard Deviation) and mean EC50 values of N-EMP and C-EMP dimers are presented with the gain in potency compared to monomers. Abbreviations: C, C-EMP; N, N-EMP; Vsd, very short dimer; sd, short dimer; md, medium dimer; ld, long dimer; C-PEG, C-EMP monomer bound to a PEG polymer

All of the dimers exhibited similar potency, with EC_{50} values of approximately 120 pM for the N-EMP dimers and approximately 200 pM for the C-EMP dimers (Table 4-1). These potencies represented improvements of approximately 2 orders of magnitude with respect to the corresponding monomers (45 nM for N-EMP and 30 nM for C-EMP monomers), but still less active than native EPO (30 pM). A C-EMP dimer featuring a very short linker (Cvsd, 10 Å linker length) showed potency similar to monomer and a pegylated derivative of the C-EMP monomer (C-PEG) had a potency that decreased 4 fold with respect to C-EMP.

The increased potency we observed with the dimers was similar to that previously observed for EMP dimers linked through their N-termini via commercial PEG and a C-terminal lysine-based linker. Our results indicate that dimerization involving a range of linker lengths from 250 to 500 Å lead to a similar improvement in potency, and only when the linker length becomes as short as 10 Å is potency reduced to the level of the monomer.

4.4.2 Synthesis of EMP tetramers

In an attempt to explore the potential benefit of bridging two EPOR dimer pairs, we next chose to link two lysine-based EMP dimers together via a longer PEG linker, thereby creating a tetrameric template (Figure 4-2). Synthesis involved the reaction of tetraaldehyde linkers with previously described aminooxyacetyl EMP peptides to obtain final product by chemoselective oxime ligation [22]. Starting from commercially available PEG diamines of either 3.4, 6, 10 or 20 kDa, Boc-Lys(Boc)-OH was coupled and deprotected with TFA to obtain tetraamine linkers. The aldehyde function necessary to react with aminooxyacetyl peptides was reported to form more stable oximes with aromatic aldehyde than with linear ones (Dawson, P, APS symposium 07, Montreal). Thus, instead of coupling a serine residue and oxidizing it to the glyoxylyl, we preferred to acylate the extremities of the linker with 4-formyl-benzaldehyde. This strategy proved to be an improvement over the serine oxidation approach because of the reduction in the number of steps in the synthesis. Synthesis of the control molecule (C2-PEG), a lysine-based dimer attached to a PEG chain, was done on solid phase. Tetraaldehyde and the dialdehyde linkers were reacted with C-EMP and final products were purified by RP-HPLC. Characterization of the products revealed single HPLC peaks rather broad for each construct, typical of molecule made with commercial PEG chains. MALDI-TOF MS spectra displayed characteristic ranges of masses, with centroid mass

corresponding to the expected m/z ratio for each construct (Table 4-2). A typical HPLC chromatogram and MS spectra obtained with our tetramers is exemplified in Figure 4-3, with a C-EMP tetramer prepared from a 3.4 kDa PEG linker (C2-P3.4-C2).

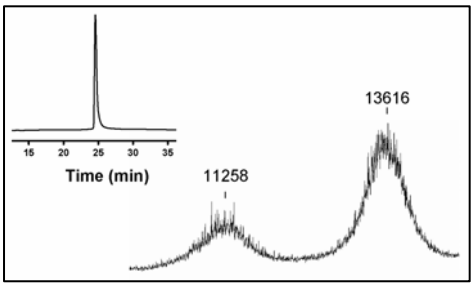

Figure 4-3: HPLC chromatogram and MALDI-TOF MS spectra of C-EMP tetramer with 3.4 kDa linker (C2-P3.4-C2).The expected m/z ratio for the tetrameric molecule is observed (13616). The additional peaks around 11258 Da are usual oxime cleavage generated by the laser of the MALDI-TOF analyzer.

4.4.3 Biological activity of EMP tetramers

The EMP tetramers, as well as the constituent C-EMP lysine-based dimer (C2-PEG), were tested for their activity in our EPO-dependent cell proliferation assay (Table 4-2, Figure 4-4). While the C-EMP control dimer that represents a half tetramer does show improved potency with respect to the C-EMP monomer, its potency is about 5-fold lower than some of the other dimers featuring longer polymer-based linkers (e.g. Cld, Csd in Table 4-1).

Compound abbreviation	Linker length (extended linker)	Calculated Mw [Da]	Observed mass [Da]	N	Mean pEC50 values ± standard deviation	Mean EC50 [pM]	Fold increase in potency
EPO	N/A	34000	-	29	10.55 ± 0.18	28	-
C-EMP	N/A	2376	2377	8	9.73 ± 0.08	25949	-
C2-PEG	Control dimer, "half-tetramer"	6938	6939	7	9.18 ± 0.43	663	-
C2-P3.4-C2	340 Å	13613	13631	7	7.98 ± 0.17	10516	0.063
C2-P6-C2	600 Å	16213	16630	7	9.14 ± 0.31	722	1
C2-P10-C2	1000 Å	20779	21033	6	9.41 ± 0.31	386	2
C2-P20-C2	2000 Å	30213	32631	6	9.74 ± 0.26	182	4

Table 4-2: Calculated and measured masses of C-EMP tetrmers, mean pEC50 (± standard deviation) and mean EC50 values, withthe gain in potency compared to control molecules are presented. N is the number of experiments performed in triplicate. Abbreviations: C2-PEG refers to control molecule, a C-EMP lysine-based dimer attached to a PEG polymer. C2-Px-C2 refers to C-EMP lysine-based tetramers separated by a PEG of x kDa.

Notably, there was a clear relationship between the size of the large PEG linker used to join the dimers and the potency of the tetrameric construct. The tetramer featuring the shortest linker (3.4 KDa) showed a 10-fold loss in potency with respect to the control dimer, the tetramers featuring medium sized linkers (6 KDa and 10 KDa) showed potency equivalent to the control dimer, and the tetramer made with the longest linker, 20 kDa, displayed an increase in potency of about 5-fold (205 pM and 929 pM respectively).

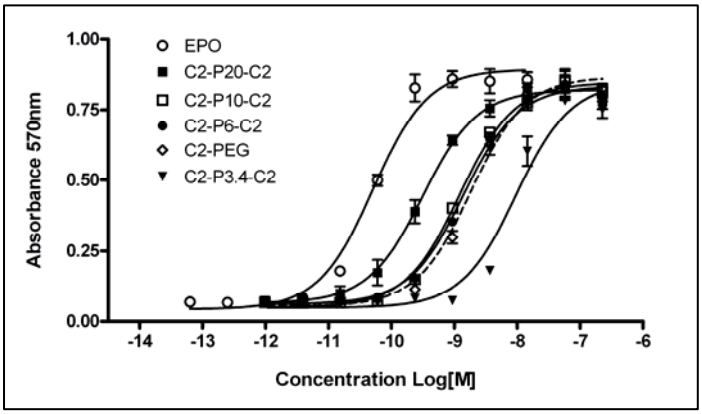

Figure 4-4: Cell proliferation assay for tetrameric compounds. Data present mean values for triplicate measurement ± SEM. The dose-response curve for the control lysine-based dimer is indicated with a dotted line.

4.5 Discussion

In this study, we describe the synthesis and in vitro biological characterization of new erythropoietin receptor (EPOR) multimeric agonists based on erythropoietin mimetic peptide (EMP).

4.5.1 Activity of EMP dimers

First, we used an EPO proliferation assay to evaluate a panel of EMP dimers [20], in which three different linker lengths were used and in which the attachment site of the linker was either on the N- or C-terminus of EMP. In agreement with previously published work concerning structurally similar molecules [10, 17], we observed that dimerization of EMP leads to significantly increased potency (Table 4-1), irrespective of whether linkage is via either the C- or the N-termini. We did not see any trend between chain length and potency for dimers with linker lengths between 250 Å and 500 Å (theoretical size of fully extended linkers), suggesting that linker sizes in this range are compatible with simultaneous interactions with both receptors in a cell surface EPOR dimer. The relatively broad tolerance towards linker length seen in the dimers probably relates to the conformational flexibility of the linkers used; linkers of each size are capable of adopting conformations that provide optimal spacing of the EMP units. An EMP dimer with a too short linker, exemplified by Cvsd (10 Å linker), shows potency no higher than that of the corresponding monomer, suggesting that it lacks the necessary length and/or flexibility to allow interaction with two adjacent receptors. These observations led us to conclude that once the linker reaches a threshold length that allows two EMP peptides to interact simultaneously with the two components of an EPO receptor dimer, potency is increased by approximately 2 orders of magnitude, whereas when the linker is below the threshold length, simultaneous interaction with the two components of the receptor dimer is not possible, and potency does not exceed that of the EMP monomers.

4.5.2 Synthesis of EMP tetramers

In an attempt to explore the potential benefit of bridging two EPOR dimer pairs, we next worked on a series of EMP multimers based on a tetrameric template, featuring EMP dimers linked through their C-termini via a branched lysine structure, itself linked to an identical dimer via a PEG chain of variable size (Figure 4-2). The PEG sizes used were between 3.4 kDa and 20 kDa, giving fully extended chain lengths between 340 Å and 2000 Å. Synthesis based on this template, which involved oximation of an aminooxy peptide with benzaldehyde linkers, led to the isolation of products apparent as single HPLC peaks and with the expected range of masses corresponding to the PEG mixtures used. Inhomogeneity of commercial PEG linkers is associated with rather broad peaks on HPLC chromatograms (Figure 4-3).

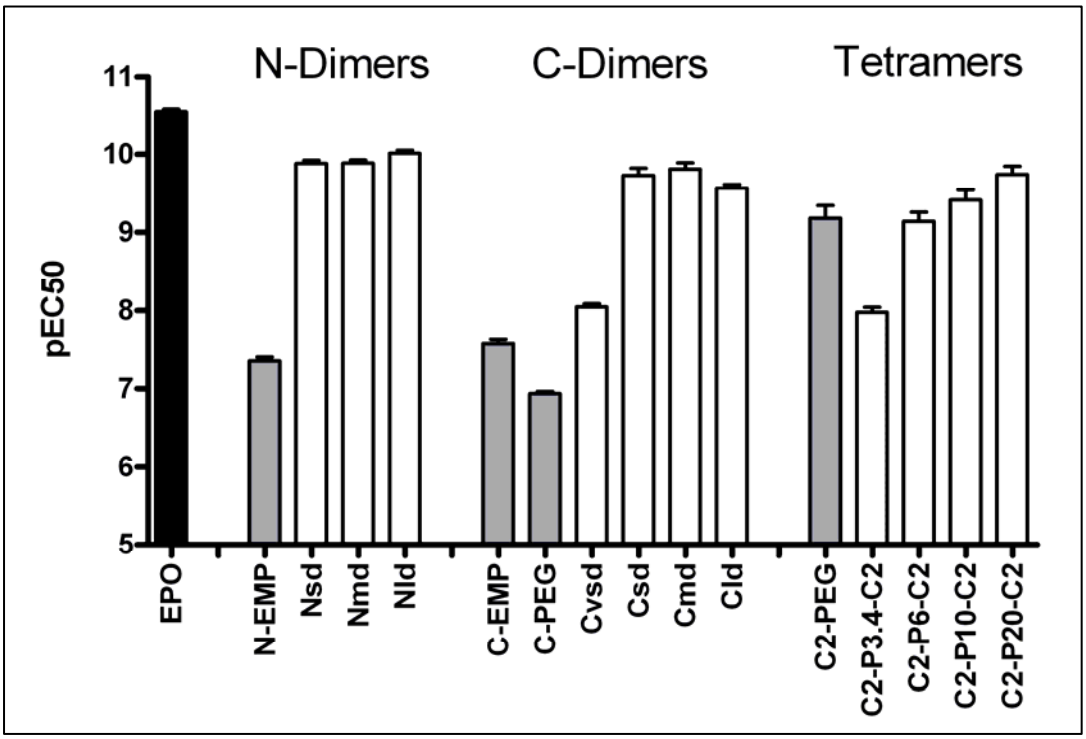

Figure 4-5: Comparison of pEC50 values of all described EMP multimers

4.5.3 Evaluation of EMP tetramers

The biological activity of the tetramers was tested in our EPO proliferation assay, using the corresponding PEG-EMP-dimer (C2-PEG,) as a control (Figure 4-3 and Figure 4-4). We noted that while the C2-PEG dimer shows significantly increased potency with respect to the corresponding EMP monomer, its potency is not as high as that of some of the other polymer-linked dimers (Figure 4-5). Among possible explanations for this result are (i) the linker that we used lacks the necessary size and/or flexibility, (ii) attachment of the PEG moiety affects interaction with the EPOR or (iii) benzaldehyde oximes offer less plasticity than glyoxylyl structures.

Across the group of tetramers, we noted a major effect on potency related to the size of the PEG linker used. While the smallest linker used showed a clear reduction in potency compared to the PEG-EMP-dimer, linkers of intermediate size showed comparable activity, and the longest linker used (20 KDa) showed a 5-fold increase in potency. These results indicate that it may be possible to enhance potency by contacting additional EPOR dimers on the cell surface, but that relatively long linkers are required.

4.6 Conclusions

We have used a new panel of EMP dimers to confirm previous observations that the potency of EMP can be significantly enhanced through dimerization via either the N- or the C-termini, using linkers of length ≥ 250 Å. Use of a very short linker (10 Å) reduced potency to the level of an EMP monomer, suggesting that in order to show increased potency, EMP dimers must feature linkers longer than a threshold length (between 10 Å and 250 Å) which allows simultaneous interaction with both components of an EPO receptor dimer.

Our preliminary studies of a group of EMP tetramers provide some hope that additional gains in potency could be reached via the use of templates which would enable more than one EPOR dimer to be contacted simultaneously on the cell surface. Further work should help determine whether more complex EMP multimers such as tetramers have the capacity to generate more potent EPOR agonists.

4.7 Acknowedgements

We thank Dr. Nikolett Mihala for the fruitfull discussions on the project and Dr. Jean Vizzavona for the advice on peptide synthesis. Chemical and analytical facilities used in this work were supported by the Swiss National Science Foundation, the Ernst & Lucie Schmidheiny Foundation, Société Académique of Geneva and the Faculty of Medicine of the University of Geneva.

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Chapter 5

Erythropoietin mimetic peptide tetramers with comparable potency as native hormone

5.1 Abstract

It was previously reported that a 20-amino acid erythropoietin mimetic peptide (EMP) is capable of activating the erythropoietin (EPO) receptor, although at high concentrations, and that dimerization of EMP, either via its N-terminus or through its C-terminus, considerably enhanced its potency. Only minor differences in potency were observed for the two types of dimerization strategies, and further experiments also demonstrated that linker length had a very small influence on potency. Study of a series of tetramers, prepared by attaching a pair of EMP dimers via large PEG polymers, revealed a correlation between PEG linker length and activity. Preliminary results also indicated that significant differences in activity were observed between tetramers prepared with peptides linked via the N-terminus and those linked via the C-terminus (Figure 5-1). After confirmation of this result, we supposed that the chemistry employed to connect the peptides to the linker could influence the activity of the tetramers. This proved right, the activity of tetramers prepared with linear linkers being up to 10 fold higher than tetramers prepared with aromatic linkers. Finally, to test if the correlation between PEG linker length and activity observed with tetramers also applied to EMP dimers, a series of dimers employing the same PEG linkers as for the tetramers was prepared (Figure 5-1). A comparable relation between potency and linker length was observed, supporting the previous model which allocates the gain in activity of the longest molecules to the connection of more than one EPO receptor cluster by the same ligand.

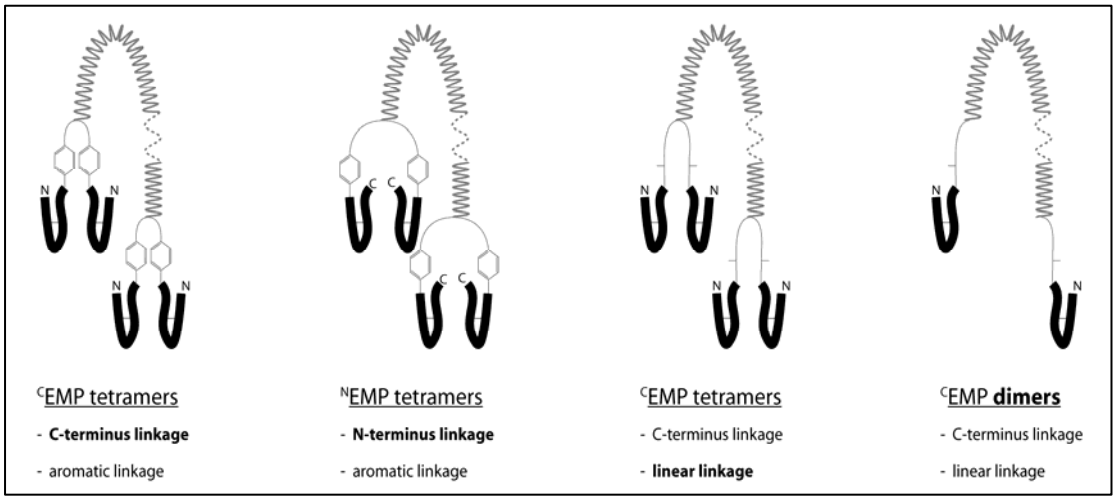

Figure 5-1: Illustration of the different series EMP multimers. Each series was prepared with four linker lengths ranging from 340 Å to 2000 Å (stretched polymers). The linkage site on EMP, the linkage chemistry and the difference between dimerization and tetramerization were tested.

5.2 Introduction

The erythropoietin (EPO) receptor is member of the class I cytokine receptor superfamily that requires dimerization to trigger signalling events [1]. In 1996, a small 20 amino acid peptide was discovered by phage display that activates the EPO receptor (EPOR), although at concentrations much higher than the native hormone [2]. Structural studies have shown that this EPO mimetic peptide (EMP) competes with native EPO for EPOR binding and that two peptides mimic the action of a single EPO hormone [3]. Covalent dimerization of EMP greatly enhanced potency, with the most active compound being a dimer where the peptides have been linked through their C-termini with a 3.4 kDa PEG polymer [4, 5]. That EMP dimer is 1000-fold more potent than the corresponding monomer, as tested in an *in vitro* cell proliferation assay. These examples demonstrate the capacity of multivalency to enhance EMP activity, particularly because the results were obtained without structural optimization.

Where linker length and peptide attachment site showed little influence on the potency of EMP dimers, study of EMP tetramers prepared by connecting two dimers with large PEG polymers revealed a correlation between activity and linker length (Figure 5-2) [6, 7]. The tetramer featuring the longest polymer linkage had a 50 times higher potency than the shortest tetramer, which was equal to that of the component EMP dimers. Preliminary results indicated that attachment of the peptides via their N-termini (^{N}EMP) or their C-termini (^{C}EMP) also influenced the potency of EMP tetramers, with the ^{N}EMP tetramers showing significantly lower potency than the corresponding ^{C}EMP tetramers (unpublished work).

The main difference between the previously described homogeneous EMP dimers, for which linker length and peptide linkage site have only minor influence on activity, and the EMP tetramers, is the chemistry employed to connect the peptide to the linker [6, 7]. Linkers used for peptide dimers possess linear aldehydes at their extremity, whereas tetrameric linkers have aromatic moieties. These different linker structures could also affect the activity of EMP multimers.

In order to perform a more detailed study on the effect of peptide linkage site and linkage chemistry on activity, new sets of EMP tetramers were synthesized and tested for biological activity.

The first series of tetramers validated our preliminary observation that linker attachment site on peptide can influence activity. A second series of ^{C}EMP tetramers was then synthesized and tested, in which the aromatic aldehyde on the linker was replaced by a linear moiety. Finally, to verify if the trend in activity observed with long tetramers also applied to long dimers, a new series of EMP dimers was synthesized using the same PEG linkers employed than for the tetramers, which are much longer than the one used in the previously described EMP dimers [6].

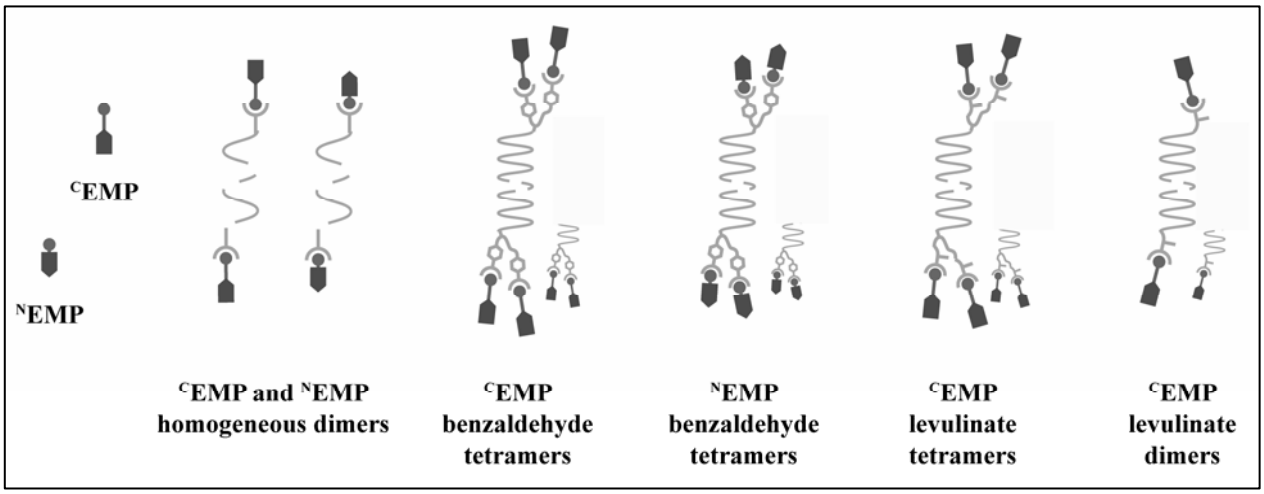

Figure 5-2: Schematic view of EMP multimers. The longer distance separating ^{C}EMP to linker attachment moiety compared to ^{N}EMP is well visible for monomeric peptides. Homogeneous dimers refer to previously described molecules possessing polyamide linkers between 250 and 500 Å (lengths of stretched polymers). All tetramers have been synthesized with PEG linkers spanning distances between 340 and 2000 Å (stretched chains). Control molecules for each series of PEG multimer are presented at smaller scale.

5.3 Material and methods

5.3.1 Material

EMP monomers, dimers and benzaldehyde-oxime tetramers as described in Vadas and Rose [6, 7]. Peptide synthesis grade DMF and DIEA were purchased from Biosolve. DCM, NMM, N-methylpyrrolidinone (NMP), diethyl ether, DMSO, carbonyldiimidazole (CDI), TFMSA, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and DIC, were purchased from Fluka, Switzerland. TFA was from Halocarbon, New Jersey. Acetonitrile CHROMASOLV® gradient grade for HPLC was from Sigma-Aldrich. HBTU was from Iris Biotech GmbH Germany) and HATU was from GL Biochem (Shanghai) Ltd. HOBt was from NovaBiochem (Switzerland) and amino acids were from AnaSpec (San Jose) or from NovaBiochem. Standard EPO is EPREX (epoetin alpha) from Janssen-Cilag AG. IMDM (with l-glutamine and 25mM HEPES), penicillin, streptomycin were from Invitrogen. MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide was from Sigma, 2-propanol and 1.25N HCl/ isopropanol was from Fluka.

5.3.2 Solid Phase Peptide Synthesis

Solid phase peptide synthesis was performed on a modified ABI 433A machine using Boc chemistry and in situ neutralization as previously described [6-9]. Peptides were prepared on a 0.2 mmol scale on MBHA cross linked with 1% DVB resin (0.9 mmol/g, Senn Chemicals, Switzerland). Boc-amino acids were protected by the following groups: Arg(Tos), Asn(Xan), Asp(OcHx), Cys (Mob), Glu(OcHx), His(Dnp), Lys(Z(2Cl)), Ser(Bzl), Thr(Bzl), Trp(For), Tyr(Z(2Br)). After chain elongation, a Boc-aminooxyacetyl group (Boc-AoA) was manually coupled as its N-hydroxysuccinimide ester (Boc-AoA-OSu) used in 1.2 molar excess in DMSO with NMM as base. Certain protecting groups were removed prior to acid cleavage (Dnp with 20% 2-mercaptoethanol and 10% DIEA; formyl with 20% piperidine; Boc with neat TFA) with HF containing 5% p-cresol for 60 minutes at 0°C. Peptides were precipitated and washed with cold diethyl ether. Crude peptide was exposed to high vacuum overnight then purified by preparative reversed phase HPLC and the purified product was lyophilized.

For the synthesis of the C-EMP, Boc-Lys(Fmoc)-OH was used to initiate the synthesis. Deprotection of the Fmoc group was performed with 20% piperidine in DMF after synthesis completion and Boc-AoA-OSu was coupled manually with 1.2 equivalents.

5.3.3 Synthesis of PEG aldehyde linkers

Tetraaldehyde PEG linkers were synthesized from commercial PEG diamines similarly as in Vadas et al [6]. Starting from 1 µmol of PEG diamine (3,4 kDa, 6 kDa, 10 kDa, 20 kDa), both amino groups were acylated with Boc-Lys(Boc)-OH. Eight µmol of amino acid were activated with 7.6 µmol HATU with 12 µmol DIEA. After 40 min reaction, the product was precipited and washed 3 times in cold ether. Neat TFA was added for 3 min to remove Boc protecting groups. After TFA evaporation, the product was precipitated and washed with ice cold ether. The four amino groups were acylated with 100 µmol of levulinic acid, previously activated as a symmetrical anhydride. 100 µmol of levulinic acid where dissolved in DCM in the presence of 50 µmole of dicyclohexyl carbodiimide (DCC). After 20 min stirring at 0°C, DCM was removed by evaporation and the activated levulinic anhydride was reacted with the tetraamine in DCM in presence of 11 µl N-methylmorpholine (NMM). The crude product was precipitated, washed with ether and dried under vacuum. Non purified linker was oximated with aminooxyacetyl peptides as described below.

The lysine based control molecules where synthesized starting from methoxy-PEG-NH_2 to obtain short dialdehydes linked to long PEG polymers. Methoxy-PEGs of 2 kDa, 10 kDa and 20 kDa where employed and the same strategy of acylation-deprotection steps followed for tetraaldehyde linkers was followed.

The long dialdehyde linkers were prepared by coupling either benzaldehyde or levulinic acid to PEG diamines, following the same coupling protocol described for the synthesis of tetraaldehyde linkers.

5.3.4 Oximation of aldehyde linkers with aminooxyacetyl EMP derivatives

200 µl of a 10 mM aldehyde solution (HPLC fraction concentrated) was quickly added to 150 µl (1.5 excess over aldehyde groups) of a 20 mM solution of aminooxyacetyl-peptide

(dissolved in 10 mM acetate buffer, pH 4.6 with 50% acetonitrile) 20. Reaction was stirred for 15 h at room temperature and purified by RP-HPLC.

5.3.5 Cell proliferation assays

The human EPO-dependent UT-7/EPO cell line [10] was maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS, 50 units/mL penicillin, 50 µg/ml streptomycin, 2 mM glutamine and 1 U/mL EPO (Eprex). For bioassays, cells were washed 3 times and resuspended in media deprived of EPO at 10^5 cells/mL. Serial dilutions of agonists were prepared in triplicate in flat-bottom 96-well plate, 50 µl/well. Five thousand cells were added in each well and the plates incubated at 37°C in a humidified 5% CO2 tissue culture incubator. Proliferation was assessed after 4 days using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) [11]. Absorbance of the wells was measured at 570 nm and data were analyzed using GraphPad Prism software to calculate EC_{50} values.

5.4 Results

5.4.1 Synthesis of EMP multimers

To test the influence of linkage attachment site, the same tetra benzaldehyde linkers that were employed for the synthesis of ^{C}EMP tetramers and for the corresponding control molecule were reacted with aminooxy-functionalized ^{N}EMP monomers [6, 7]. Purification by RP-HPLC displayed the same profile for the two types of molecules, only differing in their masses. The second series of ^{C}EMP tetramers used the same peptides as for the previous molecules but the aromatic moiety at the extremity of the linkers was replaced by levulinate, a linear ketone known to form stable oximes with AoA peptides [12]. Levulinic acid coupling to tetraamino linkers or to the control diamino linker was achieved by activating levulinic acid through formation of symmetrical anhydride with DCC. A third series of molecules, ^{C}EMP dimers, was prepared by directly coupling activated levulinic anhydride to PEG diamines of 3.4, 6, 10 and 20 kDa.

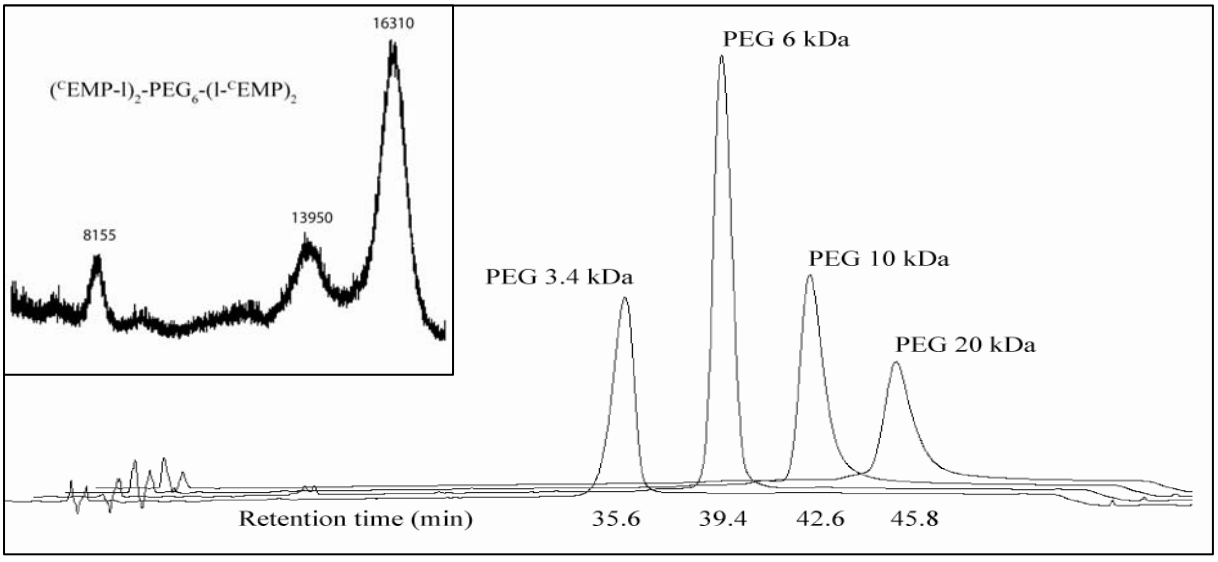

Figure 5-3: Superposition of HPLC traces corresponding to ^{C}EMP tetramers with benzaldehyde chemistry having four different PEG linkers. The peaks are large, typical of PEG constructs, and the purity of each molecule is above 90%. On top-left, MALDI-TOF MS spectra of a ^{C}EMP tetramer prepared with a 6000 Da PEG derivatized with four levulinates is presented. The expected profile is observed, the highest peak corresponding to the final product, the peak at 13950 resulting of one oxime cleavage by the laser and the peak at 8155 being the final product bis-charged. The large peaks are typical of molecules containing inhomogeneous PEG polymers. Similar profiles are observed for tetramers prepared with any linker chemistry.

Oxime reaction with the aminooxy-derivatized ^{C}EMP yielded the desired dimers. The purity of each EMP multimer was assessed by analytical RP-HPLC and MALDI-TOF MS. HPLC chromatograms displayed single broad peaks typical of PEG constructs and MS characterization displayed the expected m/z ratios with centroid mass corresponding to calculated masses. (Figure 5-3).

5.4.2 Influence of peptide linkage site on activity

The influence of linker attachment site on the peptides, either via the N-terminus or the C-terminus, was verified by measuring the cell proliferative activity of ^{N}EMP and ^{C}EMP tetramers prepared with identical linkers. Previously described ^{C}EMP tetramers displayed a potency that was related to linker length, the longest molecule being 50 fold more potent than the shortest tetramer [6]. (Figure 5-4, Table 5-1). In contrast, activity of all ^{N}EMP tetramers was much lower, and the length of the linker had no influence on activity. Compared to previously described EMP dimers, which activity is not affected by the peptide linkage site, the tetramers appear to be very sensitive to the linker attachment site. We suspected that this difference between dimers and tetramers emanated from the linkage chemistry employed, the tetramers possessing linkers with aromatic moieties (benzaldehyde) whereas the dimers were prepared with linear linkers (Figure 5-1 and Figure 5-2).

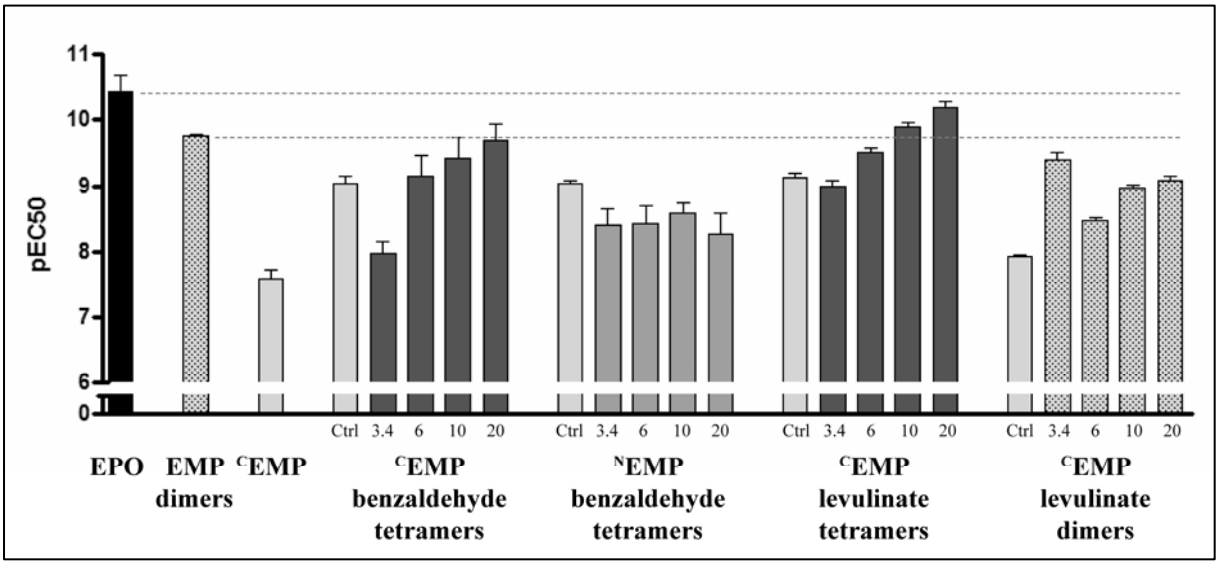

Figure 5-4: Graph of pEC50 values and standard deviation for EMP multimers with their controls. "EMP dimers" is a mean value of all previously described ^{N}EMP and ^{C}EMP homogeneous dimers. "Ctrl" is for control molecule of the series and the numbers below the graph indicate the mass of the PEG linkers in kDa.

Aromatic structures are known to form pi-pi interactions (stacking) that could impose constraints to the tetramers, lowering their activity.

	Length of stretched linker (Å)	EC50 [nM]	Activity vs control	Activity vs shortest tetramer	Activity vs EPO	Nb of assays
EPO	-	0.039			1	54
Homogeneous flexible dimers	255 - 482	0.18			0.217	61
^{C}**EMP** tetramers with	control	0.93	1		0.042	2
benzaldehyde chemistry	340	10.52	0.1	1	0.004	7
	600	0.72	1.3	14.6	0.054	7
	1000	0.39	2.4	27.3	0.101	6
	2000	0.2	4.6	51.6	0.192	8
^{N}**EMP** tetramers with	control	0.91	1		0.043	3
benzaldehyde chemistry	340	4	0.2	1	0.010	7
	600	3.9	0.2	1.0	0.010	8
	1000	2.6	0.3	1.5	0.015	7
	2000	5.6	0.2	0.7	0.007	7
^{C}**EMP** tetramers with	control	0.76	1		0.052	2
levulinate chemistry	340	1	0.7	1	0.038	2
	600	0.3	2.5	3.4	0.128	2
	1000	0.13	5.9	8.1	0.303	4
	2000	0.064	11.8	16.3	0.625	4
^{C}**EMP** dimers with	control	11.9	1		0.003	2
levulinate chemistry	340	0.4	30.0	1	0.099	4
	600	3.5	3.4	0.1	0.011	2
	1000	1.1	10.7	0.4	0.035	2
	2000	0.83	14.3	0.5	0.047	2

Table 5-1: Results of cell proliferation assays of EMP tetramers and dimers prepared with PEG polymers.

5.4.3 Influence of linkage chemistry on EMP tetramers activity

A series of ^{C}EMP tetramers possessing linear linkers were compared to the first series of ^{C}EMP tetramers prepared with aromatic linkers to test the influence of linkage chemistry on activity. Similar ^{N}EMP tetramers with linear linkers were not synthesized because of time and

resources limitations. Cell proliferation assays indicated that the potency of tetramers with linear linkers was 3 times higher than aromatic tetramers, the trend inactivity related to linker length being observed for the two series of tetramers. The longest linear tetramer almost equals the activity of native hormone (EC50 of 64 pM versus 39 pM for EPO, Table 5-1), making that molecule the most active peptide identified so far at stimulating cell proliferation of an EPO dependent cell line [4, 6].

5.4.4 Activity of EMP dimers linked with large PEG polymers

To investigate if the trend in activity related to linker length that is observed with tetramers also applies to long dimers, a series of EMP dimers prepared with the same PEG polymers as the one used for the tetramers was tested (Figure 5-1). Previous series of EMP dimers prepared with homogeneous linkers ranging from 250 Å to 500 Å did not show any effect on activity [6], but because the PEG linkers used for the tetramers were up to 2000 Å long, we expected that the activity of the dimers would be increased by using longer linkers. Cell proliferation assays indicated that the activity of the long PEG dimers was related to linker length, but that the shortest dimer was the most active compound (Figure 5-4, Table 5-1). The shortest PEG dimer, which length is in the range of the previously described homogeneous EMP dimers (340 Å), possess an activity comparable with the latter dimers (Table 5-1) [6]. The PEG dimers with linkers ranging from 600 Å to 2000 Å are less active than the shortest molecule; however, they display a correlation between linker length and activity that is comparable with the results observed with tetramers, the longest dimer being 4-times more potent than the short tetramer. This experiment confirmed that activity of EMP multimers can be enhanced by using long enough linkers.

5.5 Discussion and perspectives

Previous results indicated that the length of the linker and the peptide linkage sites had no effect on the activity of EMP dimers, but that tetramers prepared with the same peptides were sensitive to these modifications [6]. In this study, we have demonstrated that additionally to the influence of linker length, the peptide linkage site and the linkage chemistry affected the activity of EMP tetramers. Attachment of EMP via its N-terminus of via its C-terminus

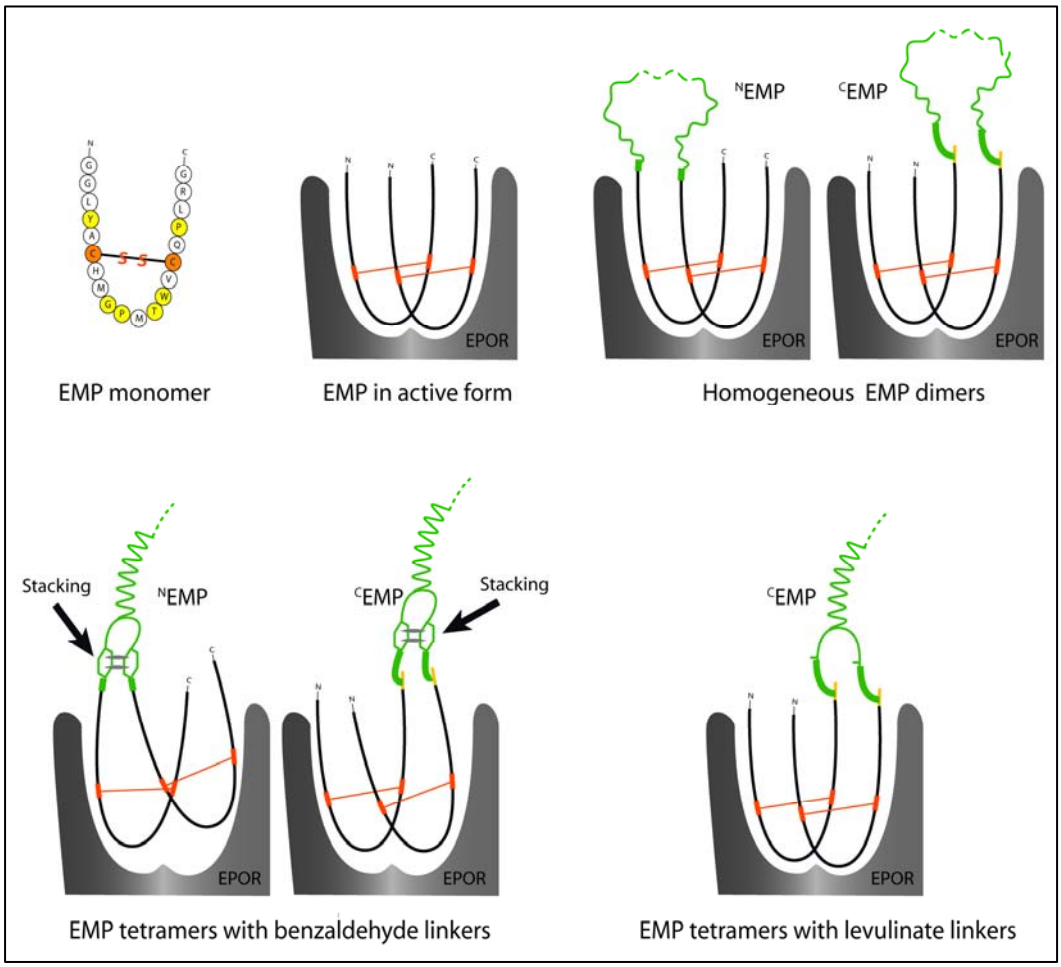

Figure 5-5: Schematic view of EMP in contact with EPO receptor (EPOR). The residues highlighted in yellow of EMP monomer are essential for peptide activity. The distance separating the two N-termini and C-termini is almost equal and probably explains why homogeneous ^{N}EMP and ^{C}EMP dimers have almost identical potency. Visible for the tetramers prepared with benzaldehyde linkers is the stacking of the two aromatic moieties (indicated with arrows), imposing unfavourable constraints especially to ^{N}EMP tetramers because the distance from the extremity of the peptide to the linker is shorter than for ^{C}EMP tetramers. Replacement of benzaldehyde with levulinate allows more plasticity to the peptides, which can better accommodate within the receptor binding pocked.

proved to have significant effect on activity, ^{C}EMP tetramers being much more active than ^{N}EMP tetramers. We believe that the difference in activity between ^{N}EMP and ^{C}EMP tetramers can be allocated to the stacking of the aromatic moieties on the linkers, and to the distance separating the peptides to the linker. As seen in Figure 5-2 and Figure 5-5, the distance between the peptide extremity and the linker is slightly longer for ^{C}EMP than for ^{N}EMP [7]. The rigidity imposed by the pi-pi interactions is supposed to lower the potency of the peptide multimers, with ^{C}EMP tetramers that can better accommodate this effect thanks to longer linkage (Figure 5-5). This assumption is confirmed by the increased activity of ^{C}EMP tetramers prepared with linear linkers compared to aromatic tetramers (Table 5-1). The longest linear EMP tetramer is the most active erythropoiesis stimulating agent tested by cell proliferation assay.

The higher activity of EMP tetramers possessing long PEG linkers compared to shorter ones was previously assigned to their capacity of contacting additional EPO receptor on the cell surface [6]. In the present study, cell proliferation assay results of EMP dimers prepared with the same long PEG linkers as the one employed for EMP tetramers also displayed a correlation between activity and linker length, supporting the hypothesis that more than one EPO receptor complex can be contacted by a single molecule, provided is has a long enough linker (Figure 5-6). In the situation where only one receptor complex can be reached a ligand dimer, we expect that longer linkers will provoke the activity to decrease, until it reaches the activity of the monomer [13]. The opposite situation is observed with our EMP multimers, which may reflect the activation of more than one EPOR by a single ligand.

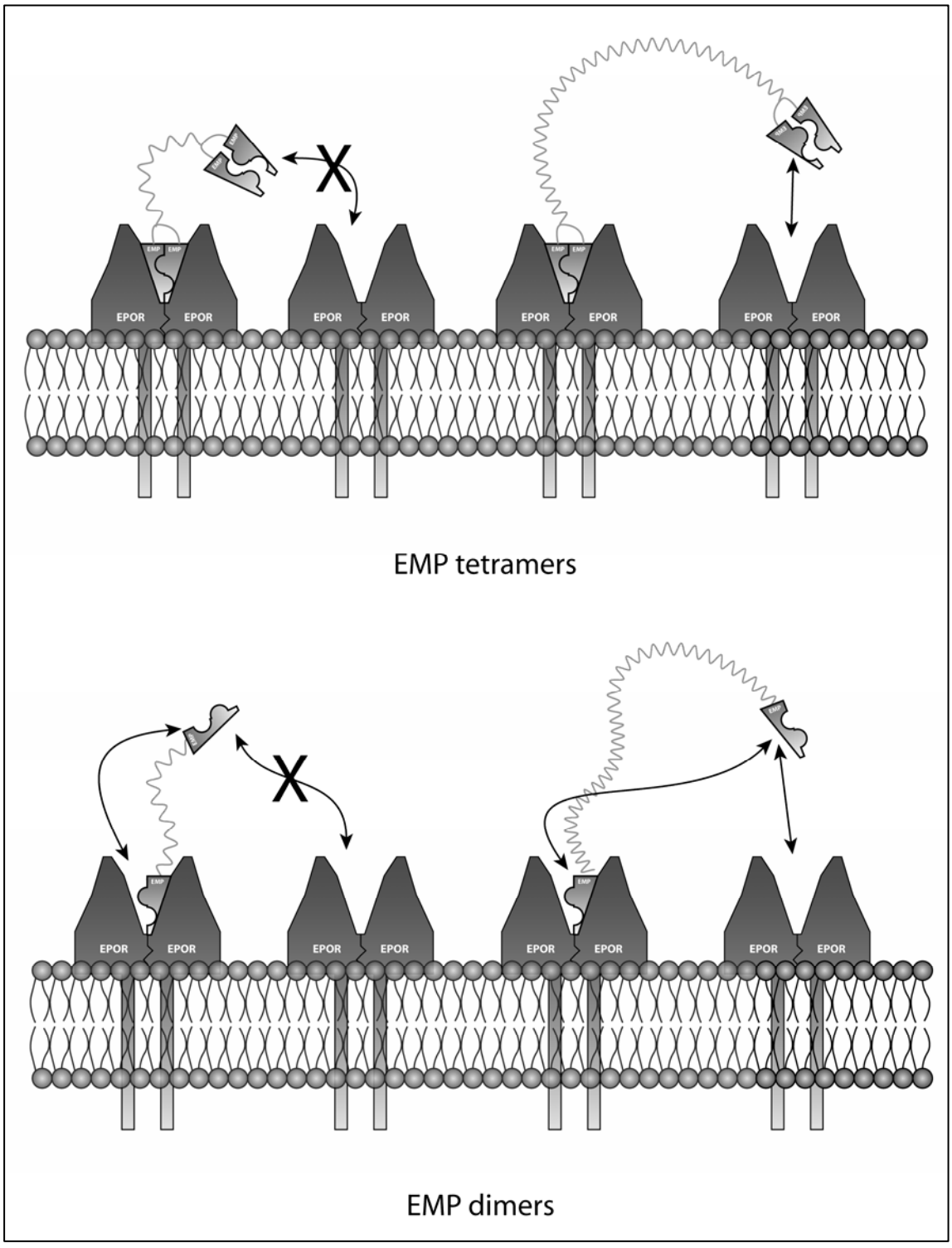

Figure 5-6: Schematic representation of the possibility for long EMP multimers to contact additional EPO receptor (EPOR) complexes at the cell surface. For EMP tetramers, one short dimer activates one EPOR complex and long enough tetramers have the possibility to activate a second receptor complex. For a short EMP dimer, once the first peptide has bound to a receptor, the second connected ligand can only target the same EPOR complex. For long EMP dimers, following the first binding event, the unbounded peptide has two possibilities for contacting a receptor, either targeting the same receptor complex or a different one. This characteristic is supposed to account for the higher potencies of long EMP multimers compared to shorter

5.6 Conclusion

In conclusion, we have demonstrated the influence of linker length, linkage site and linkage chemistry on the activity of EMP tetramers. The presence of an aromatic moiety at the extremity of the linkers proved particularly unfavourable, especially for ^{N}EMP multimers which peptides are very close to the linker extremity. By optimization of the linker structure, we have identified a molecule which is the most potent peptide at stimulating erythropoiesis *in vitro*, almost reaching the activity of native EPO. We have also shown that, as for EMP tetramers which display an enhanced activity related to linker length, the same phenomenon applied to EMP dimers prepared with the same long PEG linkers, supporting the hypothesis that more than one EPO receptor complex can be contacted at the cell membrane by a single molecule. These results confirm the capacity of multivalency for increasing peptide activity, and may open new perspectives if the hypothesis that more than one complex can be targeted by a single molecule is confirmed. With its high affinity for EPOR and its large mass (35 kDa), we expect that the longest levulinate tetramer also exhibit potent *in vivo* activity.

5.7 References

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Chapter 6

Synthesis of a rigid erythropoietin mimetic peptide dimer

6.1 Abstract

Erythropoietin mimetic peptide (EMP), a 20 amino acid peptide which sequence is not related to native EPO, was shown to activate the erythropoietin (EPO) receptor via the same receptor binding site as EPO. Structure of the receptor in complex with EMP indicates that two peptides occupy the receptor binding site used by one EPO hormone. Potency of EMP is much lower than that of native EPO, but dimerization of the peptide with flexible linkers results in molecules with up to 1000-fold increase in cell proliferation potency. Previous studies performed in our laboratory on a series of EMP dimers indicated that peptide linkage via the N- or C-termini using flexible linkers between 250 Å and 500 Å resulted in molecules with very similar potency. Since major affinity increase can be achieved by rigid molecules compared to component flexible ligands thanks to conformational entropy factors, we anticipated that a rigid EMP dimer constrained in the conformation required for receptor activation would exhibit a higher potency than the EMP flexible dimers. Based on the X-ray structure of two EMPs in complex with the EPO receptor (EPOR), we identified residues of the two EMP chains distant of 6-7 Å, corresponding closely to the length of a disulfide bond. A rigid EMP dimer synthesized by linking two peptides via disulfide bond exhibited a major increase in potency compared to EMP monomers, but was still 5-times less potent than the flexible dimers. This result indicates that further optimisation is required to obtain a constrained molecule which structure closely matches with the conformation required to activate the EPOR. New synthesis strategies to increase the biological activity of the described molecule are discussed.

6.2 Introduction

Erythropoietin (EPO) is a glycoprotein hormone of 34 kDa that controls the differentiation and proliferation of red blood cells [1, 2]. Erythropoietin interacts with its cognate receptor to activate several signalling cascades, including the JAK2/STAT5, the Mitogen Activated Protein (MAP) kinase and Phosphoinositide 3 kinase (PI3K) signalling pathways [3-6]. Several molecules have been developed that can stimulate erythropoiesis via activation of the EPOR. A peptide derivative was described that competes with EPO for receptor binding, although it has no sequence similarity with the native hormone [7, 8]. This 20 amino acid EPO mimetic peptide (EMP) stimulates erythropoiesis *in vitro* and *in vivo*, although at much higher concentrations than recombinant human hormone [9, 10]. The X-ray structure of the peptide in complex with EPOR showed that two peptides occupy the receptor binding site to

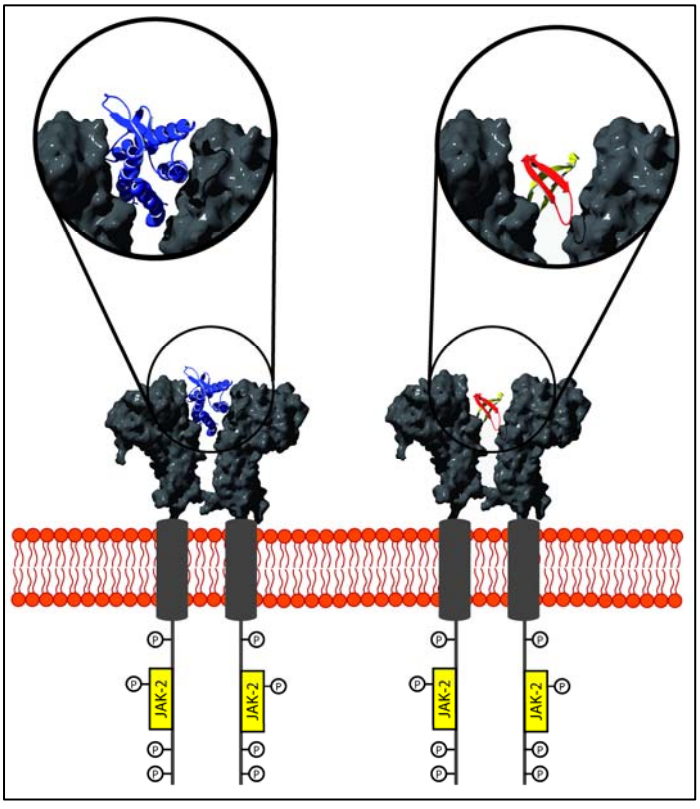

Figure 6-1: Structural comparison of EPO and EMP in complex with EPOR. One hormone activates the receptor, whereas two peptides are present in the receptor binding site, their extremities pointing away from the receptor.

mimic the action of one EPO molecule (Figure 6-1) [10]. The two cyclic peptides have their extremities pointing at the same direction, away from the receptor. Dimerization of EMP, either with a short lysine-linker connecting the C-termini or with a polymer linker joining the two N-termini, resulted in up to 1000-fold increase in cell proliferation potency compared to monomers [11, 12]. These examples show that multivalency is an effective strategy to increase EMP activity, although no conformational optimization was performed [13]. Previous studies have demonstrated that dimerization of peptides with flexible linkers greatly increase their affinity for a target compared to their monovalent constituents [12, 14].

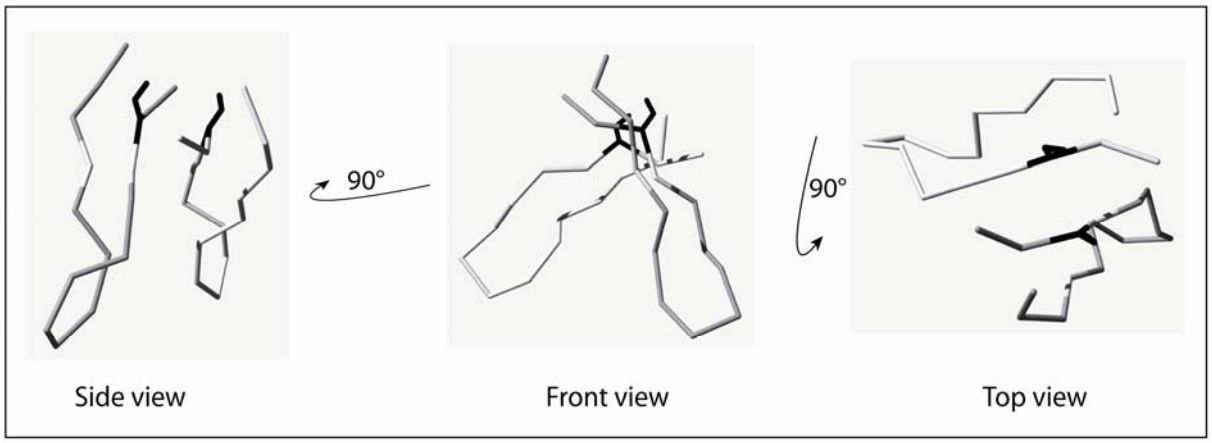

Figure 6-2: Carbon alpha trace of EMP in active conformation, when connected to the EPOR. Highlighted in black is the Ala5 residues that have been mutated to cysteine for the dimerization strategy.

Thermodynamically, this increase in affinity can be explained by considerations of enthalpy and entropy. The enthalpy of binding for multivalent compounds corresponds closely to the sum of each monovalent contribution, largely favouring affinity of multivalent interactions [15]. When flexible linkers are used, the entropy contributions for polyvalent interactions compared to monovalent interactions is mainly concerned with reduction of rotational and translational entropies. Binding of the first peptide carry the same entropy lost as monovalent binding, but because the second ligand is then maintained in close proximity to its partner by the linker, its rotational and translational entropy lost upon binding will be reduced compared to monovalent binding. The entropy cost of binding for flexible molecules can further be reduced if they are constrained in their active conformation, thanks to conformational entropy considerations [16]. Thus, multimerization and rigidification of peptides can result in

tremendous increases in biological activity, if the rigidified ligand is properly aligned with the receptor binding sites.

For the EMP-EPOR interaction, we anticipated that a rigid EMP dimer constrained in the conformation required for receptor engagement would considerably enhance its activity compared to flexible EMP dimers. Based on the X-ray structure of EMP bound to EPOR, we synthesized a rigid EMP dimer by connecting the Alanine5 residues of both EMPs through disulfide bridge (Figure 6-2 and Figure 6-3). *In vitro* biological characterization by cell proliferation assays showed that the rigid dimer had a much increased potency compared to monomers, although not reaching the potency of flexible dimers. Activity of the rigid EMP dimer and possible synthesis to increase its activity are discussed.

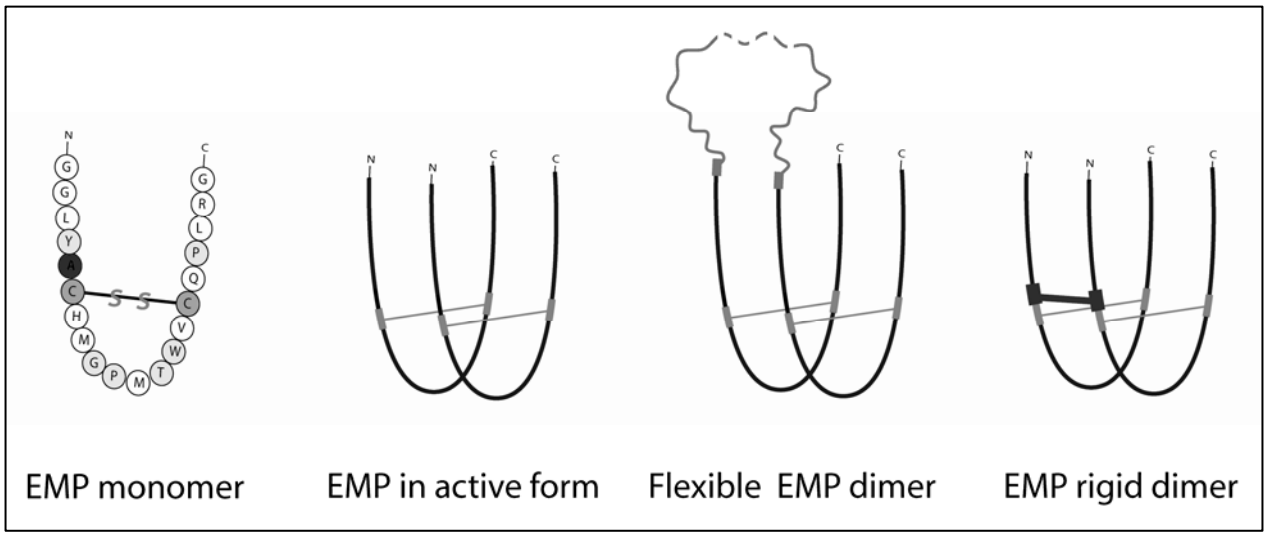

Figure 6-3: Schematic view of EMP in its active conformation. The intra-molecular disulfide bonds between Cys 6 and Cys15 is indicated with a thin line. Dimerization with a flexible linker via the extremity of the peptides and rigidification by an inter-molecular disulfide linkage are presented.

6.3 Material and methods

6.3.1 Material

Peptide synthesis grade DMF and DIEA were purchased from Biosolve. DCM, NMM, N-methylpyrrolidinone (NMP), diethyl ether, DMSO, carbonyldiimidazole (CDI), TFMSA, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and DIC, were purchased from Fluka, Switzerland. TFA was from Halocarbon, New Jersey. Acetonitrile CHROMASOLV® gradient grade for HPLC was from Sigma-Aldrich. HBTU was from Iris Biotech GmbH Germany) and HATU was from GL Biochem (Shanghai) Ltd. HOBt was from NovaBiochem (Switzerland) and amino acids were from AnaSpec (San Jose) or from NovaBiochem. Standard EPO is EPREX (epoetin alpha) from Janssen-Cilag AG. IMDM (with l-glutamine and 25mM HEPES), penicillin, streptomycin were from Invitrogen. MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide was from Sigma, 2-propanol and 1.25N HCl/ isopropanol was from Fluka.

6.3.2 Modeling

Computer simulation was performed with the software Sybyl in collaboration with Professor L. Scapozza. The Ala5 of both EMPs in the EPOR binding sites were mutated to Cys and a inter-molecular disulfide bridge was created. Cycles of energy minimization were performed to test the influence of the modification on the conformation of the peptides in the receptor binding site.

6.3.3 Peptide synthesis

Solid phase peptide synthesis was performed on a modified ABI 433A machine using Boc chemistry and in situ neutralization as previously described [17]. Peptides were prepared on a 0.2 mmol scale on MBHA cross linked with 1% DVB resin (0.9 mmol/g, Senn Chemicals, Switzerland). Boc-amino acids were protected by the following groups: Arg(Tos), Asn(Xan), Asp(OcHx), Cys (Mob), Glu(OcHx), His(Dnp), Lys(Z(2Cl)), Ser(Bzl), Thr(Bzl), Trp(For),

Tyr(Z(2Br)). The Cys5 residue was orthogonally protected with HF-resistant acetamidomethyl (Acm) protecting group and the peptide was acylated after chain elongation with 20 equ of actetic anhydride for 20 min in DMF. Certain protecting groups were removed prior to acid cleavage (Dnp with 20% 2-mercaptoethanol and 10% DIEA; formyl with 20% piperidine; Boc with neat TFA) with HF containing 5% p-cresol for 60 minutes at 0°C. Peptides were precipitated and washed with cold diethyl ether, before exposition to high vacuum overnight. After purification of the crude peptide with RP-HPLC, the monomeric peptide had the following sequence: Ac-GGLYC(Acm)CHMGPMTWVCQPLRG-NH_2. Cyclization by disulfide bond formation was performed with 25 equ hydrogen peroxide as previously described [18]. Dimerization of two peptides by inter-molecular disulfide bridge was performed by Cys5 deprotection and oxidation in a single step using iodine. Four mg of Acm-protected peptide were dissolved in 400 µl of a 50% acetic acid solution. After addition of 44 µl of a 1 M HCl solution, 10 equivalents (per Acm) of iodine were incorporated and the solution stirred for 30 min (Iodine was dissolved at 0.1 M concentration in acetic acid). The reaction was quenched by addition of 10 equ. (per Acm) of a 1 M sodium thiosulfate solution. The final product was purified by RP-HPLC and characterization was done by analytical RP-HPLC and MALDI-TOF MS.

6.3.4 Cell proliferation assays

The human EPO-dependent UT-7/EPO cell line [19] was maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS, 50 units/mL penicillin, 50 µg/ml streptomycin, 2 mM glutamine and 1 U/mL EPO (Eprex). For bioassays, cells were washed 3 times and resuspended in media deprived of EPO at 10^5 cells/mL. Serial dilutions of agonists were prepared in triplicate in flat-bottom 96-well plate, 50 µl/well. Five thousand cells were added in each well and the plates incubated at 37°C in a humidified 5% CO2 tissue culture incubator. Proliferation was assessed after 4 days using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) [20]. Absorbance of the wells was measured at 570 nm and data were analyzed using GraphPad Prism software to calculate EC50 values.

6.4 Results

6.4.1 Modeling

To test the possibility of linking two EMPs with an inter-molecular disulfide bond, we performed computer simulations based on the X-ray structure of EMP bound to the extracellular portion of EPOR [10]. The alanine 5 residues were found to be distant of 6 to 7 Å, slightly longer than the distance spanned by a disulfide bridge (around 4 Å, Figure 6-2). Alanines were mutated to cysteine and energy minimization simulations were performed to predict the influence of the linkage on peptides conformation. Some conformation modifications to the backbone and certain side chains occur, but the overall structure of the peptides in the dimer remains similar to the conformation of the non-connected EMPs. These minor changes in the structure were expected to have minimal effects on peptide activity.

6.4.2 Synthesis of rigid EMP dimer using orthogonal cysteine protection scheme

The synthesis of EMP dimer linked by disulfide bond involved only a single mutation of Ala5 to Cys. The synthesis was done by automated solid phase synthesis with Boc/Bzl chemistry. To ensure correct formation of intra-molecular disulfide bond, cysteine 5 was protected with an acetamidomethyl (Acm) protecting group that is resistant to hydrogen fluoride cleavage. Folding of EMP using standard protocol with hydrogen peroxide was possible, and a single step was necessary to remove Acm protecting group and oxidize the Cys5 using iodine.

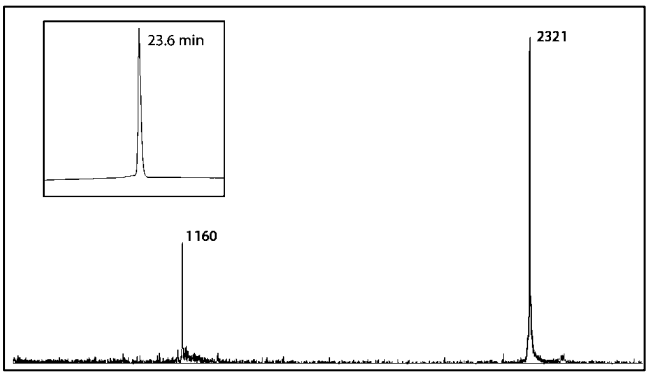

Figure 6-4: HPLC chromatogram and MALDI-TOF MS spectra of rigid EMP dimer

Purification of the dimer was done by RP-HPLC and characterization of the final product was tested by MALDI-TOF mass spectrometry and analytical RP-HPLC. The expected mass was obtained and purity of the construct was above 90% as measured by HPLC (Figure 6-4).

6.4.3 Biological characterization

To test the biological activity of the rigid dimer, we performed cell proliferation assays with an EPO-dependent cell line [19]. Serial dilutions of compounds where prepared in 96 well plates in triplicate, and 5000 UT-7/EPO cells deprived in EPO where added in each well. Cells where then incubated for four days and cell survival was measured with a colorimetric assay (MTT) [20]. Determination of the concentration at which half-maximal proliferation was achieved (EC50) was used to compare the potency of the compounds. The presence of an Acm protecting group on Cys5 altered the activity of monomeric EMP, with the protected monomer exhibiting a 10 fold reduction in potency compared to the free peptide (Figure 6-5, Table 6-1). Dimerization via a disulfide linkage greatly enhanced the potency of EMP, the rigid dimer exhibiting a 250-fold increase compared to the Acm-protected peptide, and a 25-fold increase over free EMP. This potency was nonetheless 6-fold lower than that of the best flexible dimers [21].

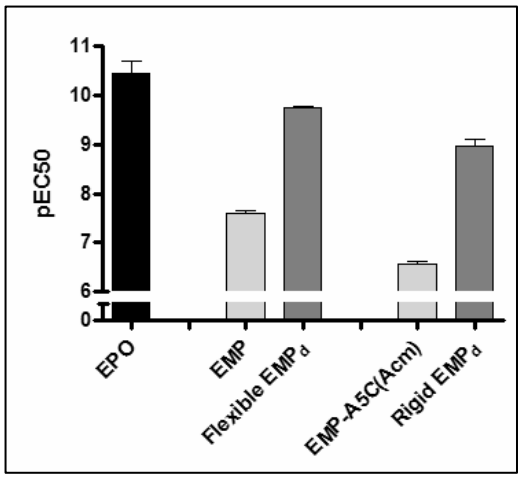

Figure 6-5: pEC50 values obtained by cell proliferation assay. Difference between monomers and dimers are comparable, flexible EMP dimer being more potent than rigid EMP dimer.

	EC50 [nM]	Activity vs EPO	Nb of assays
EPO	0.039	1	54
EMP	25.95	0.0015	8
Flexible EMP dimers	0.18	0.217	61
EMP-A5C(Acm)	285	0.00014	9
Rigid EMP dimer	1.13	0.035	12

Table 6-1: Activity of the rigid and flexible EMP dimers with their controls. EMP-A5C(Acm) is for monomeric EMP with Ala 5 mutated to Cys, the cysteine being protected with acetamidomethyl.

6.5 Discussion and perspectives

In the present study, we have shown that dimerization of EMP via disulfide bridge increased its activity, almost reaching the activity of flexible dimers. Modelling simulations had predicted that only minor changes in the conformation of the EMPs when engaged with the EPOR would result from their attachment via disulfide linkage. Biological characterization confirmed that rigidification of EMP dimers was successful at increasing activity compared to monomers, although the potency of flexible dimers could not be reached. The X-ray structure of EMP in complex with EPOR indicates that the two peptides are tightly packed within the receptor binding site. As a consequence, minor structural changes to the ligand could have major effects on activity.

An explanation for the lower potency of the rigid EMP dimer compared to the flexible ones is that the disulfide linkage employed constrains the dimer in a conformation that is not optimal for receptor engagement because the linker is too short. Appropriately constrained peptides can show significantly increased affinity compared to unconstrained peptides thanks to conformational entropy factors [16, 22, 23]. However, this large gain in affinity can be offset by enthalpy losses if the peptides are constrained in an inappropriate conformation. Our rigid dimer certainly has more favourable conformational entropy of binding compared to the flexible linkers, but because it is inappropriately constrained, its activity is reduced for enthalpy reasons.

The significant increase in activity of the rigid EMP dimer compared to monomer indicates that the rigidification strategy was reasonable, but because the potency is not as high as for the flexible dimers, structural optimization is required. To further increase the potency of the rigid EMP dimer, slightly longer linkages than a disulfide bond should be tested. Replacement of the cysteine by homo cysteine is one possibility that would expand the linker length of two carbon-carbon bonds. Other possibilities include the use of other chemoselective reactions like the oxime or hydrazone ligations [24, 25].

If replacement of the disulfide bond linkage proves successful at increasing peptide potency, additional constraints to the dimer could be introduced to further increase its activity thanks to

conformational entropy gains. New linkages could be created at the extremity of the peptides, because these positions have already shown to tolerate modifications [21]. New modelling simulations could validate that proposition and may suggest alternative possibilities for rigidification of EMP dimers in the conformation required for EPOR activation.

6.6 Conclusion

In conclusion, we have described a new strategy to activate the erythropoietin receptor by small peptide agonists. Rigidification of EMP dimer via disulfide bond formed a molecule with slightly lower potency than flexible EMP dimers, but still 25-fold more potent than monomeric EMP. Visualization of the X-ray structure of the peptide in complex with the EPOR indicates that a linkage slightly longer than a disulfide bond would fit better with the conformation observed for the peptides when engaged with the EPOR. Synthesis of such a molecule could result in a peptide dimer with much higher potency than the flexible dimer, possessing similar enthalpy but much increased entropy contribution upon binding.

6.7 References

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General conclusions

Main issues of each project

In the present document, four different strategies to activate the erythropoietin receptor by multivalent molecules have been investigated, each involving solid phase peptide synthesis.

The objective of the first project was the synthesis of a homogeneous EPO chemical dimer by ligation of four fragments using native chemical ligation (chapter 1). After successful synthesis of the fragments, completion of the project proved to be intractable because of major solubility problems during the ligation steps. Comparison with the synthesis strategy for a previously described synthetic EPO protein suggests that the solubility problems could be solved by the incorporation of a large hydrophilic moiety on to one of the fragments...

The second project explores the possible increase of activity that can be achieved by connecting two peptides that engage the EPOR via two different receptor binding sites (chapter 2). Based on the reported activity of the ERP peptide, the reported synergy between ERP and EPO, and the relative proximity of the two binding sites on the EPOR, polyvalent molecules separated by flexible linkers of different lengths were designed and synthesized. All hetero-dimers displayed lower potency than monomeric EMP, with ERP itself showing very low activity and no synergy with EPO. The most plausible explanation for these results is the use of a different cell line in our proliferation assay compared to the original publication that reported activity of ERP.

In follow-up study based on earlier work with EMP dimers conducted in the laboratory prior to the start of my thesis project, a new series of EMP dimers with several linker lengths and two different peptide linkage sites was designed and synthesized (chapters 3 and 4). *In vitro* assays confirmed the capacity of multivalency to increasing potency of the peptide monomers,

and indicated that peptide linkage site and linker lengths had minor influence on peptide dimer activity, provided that sufficiently long linkers were employed. Following these promising results, EMP multimers became the main focus of my work.

With the objective of further increasing the activity of the EMP dimers, a series of EMP tetramers was designed and synthesized by connecting two EMP dimers with large PEG polymers (chapters 4 and 5). The biological activity of the EMP tetramers was shown to be affected by the linkage chemistry, with the presence of an aromatic moiety on the linker reducing activity of tetramers compared to that of corresponding tetramers featuring linear linkers. The most potent molecule almost equals the potency of native EPO in a cell proliferation assay. Additionally, an unexpected correlation between PEG linker length and activity was observed, with tetramers featuring longer linkers showing greater potency than tetramers featuring shorter linkers. This may reflect the capacity of large EMP multimers to contact more than one EPOR complex at the cell surface.

Finally, an attempt was made to improve the potency of EMP dimers by reducing the conformational entropy loss upon receptor binding (chapter 6). Based on the X-ray structure of EMP in complex with EPOR, an EMP dimer rigidified by a disulfide bond linkage was designed and synthesized. Cell proliferation assays confirmed that, as expected the dimer showed increased potency over the monomer components, but did not match the activity of some of the other flexible dimers produced in the project, indicating that further optimization will be required to maximize enthalpy and entropy gains.

Comparison with previously described erythropoiesis stimulating agents

The work performed during my thesis has explored five strategies to activate the EPOR, four of them involving EMP multimers. Compared to the previously described erythropoiesis stimulating agents (ESAs), my investigations have identified a molecule with a better cell proliferation potency than previously described peptide-based molecules. The only peptide-based therapeutic described for the treatment of anaemia (Hematide, currently in Phase 3 clinical trials) has a lower potency for EPOR than my best tetramer (levulinate tetramers) as determined in comparable proliferation assays. Since one of the advantages of the EPO biosimilars compared to native EPO is their prolonged circulation half-life in human plasma, *in vivo* tests of my molecule would be necessary to compare them with the previously

described ESAs. In this case I would expect that the longest levulinate tetramer would exhibit similar or superior activity *in vivo* compared to Hematide, its in vitro potency being superior, and its long PEG linker providing a large molecular radius.

The rigidification of EMP dimers is a new promising strategy for increasing peptide activity that may lead to the identification of a molecule with higher potency than native EPO, providing the dimer structure can be optimized to closely fit with the structure required to engage the EPOR.

Structure-activity relationship

All EMP multimers that have been characterized in this work have contributed to better understand the mode of activation of the EPOR by small peptides.

The comparable potency of flexible EMP dimers featuring different linker lengths and different attachment sites have demonstrated the relative tolerance of EPOR to accommodate with EMPs modified at their extremity. Studies on the rigid EMP dimer indicate that the conformation of the two peptides in the receptor binding pocket is essential for activity, with a relatively small distortion of the two EMP chain structures imposed by use of the linker having significant impact on activity. The high potency of EMP tetramers prepared with long polymer linkers has provided a new direction in the search for active ligands. If the hypothesis that these molecules can contact more than one EPOR dimer on the cell membrane is confirmed, this strategy could be applied to any peptide or small molecule ligand targeting cell surface receptors.

Future perspectives

With the hope to identify a novel ESA with higher potency than the described molecules, the strategies applied to the synthesis of a rigid EMP dimer and to the synthesis of long EMP tetramers could be combined. I believe that optimisation of the rigid EMP dimer could succeed in the development of a molecule with better potency than seen with the EMP flexible dimers. Connection of two rigid dimers with a large PEG polymer to allow the molecule to contact additional EPOR, as for tetramers, could further increase potency.