CHAPTER NINE

Peptide Derivatives of Erythropoietin in the Treatment of Neuroinflammation and Neurodegeneration

Ilkcan Ercan*, Kemal Ugur Tufekci*, Ezgi Karaca*, Sermin Genc*,†, Kursad Genc†,1

*International Biomedicine and Genome Institute, Dokuz Eylul University, Balcova, Izmir, Turkey
†Institute of Health Science, Dokuz Eylul University, Balcova, Izmir, Turkey
[1]Corresponding author: e-mail address: kkursadgenc@hotmail.com

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Abstract

During the past 35 years, recombinant DNA technology has allowed the production of a wide range of hematopoietic and neurotrophic growth factors including erythropoietin (EPO). These have emerged as promising protein drugs in various human diseases. Accumulated evidences have recently demonstrated the neuroprotective effect of EPO in preclinical models of acute and chronic degenerative disorders. Nevertheless, tissue protective effect of EPO could not be translated to the clinical trials because of common lethal thromboembolic events, erythropoiesis and hypertension. Although chemically modified nonerythropoietic analogs of EPO bypass these side effects, high expense, development of antidrug antibodies, and promotion of tumorigenicity are still concern especially in long-term use. As an alternative, nonerythropoietic EPO mimetic peptides can be used as candidate drugs with their high potency and selectivity, easy production, low cost, and immunogenicity properties. Recent experimental studies suggest that these peptides prevent ischemic brain injury and neuroinflammation. The results of clinical trial in patients with neuropathic pain are also promising. Herein, we summarize these studies and review advanced experimental and in silico methods in peptide drug discovery.

ABBREVIATIONS

Aβ	amyloid beta
ACTH	adrenocorticotropic hormone
ADME	absorption, distribution, metabolism, and excretion
BBB	blood–brain barrier
CCM	corneal confocal microscopy
CEPO	carbamoylated EPO
CERA	continuous erythropoietin receptor activator
CFA	complete Freund's adjuvant
cGMPs	current good manufacturing practices
CHBP	cyclic helix B peptide
CKD	chronic kidney disease
CNTF	ciliary neurotrophic factor
CSF	cerebrospinal fluid
CSF2Rβ	colony-stimulating factor 2 receptor beta
EAE	experimental autoimmune encephalomyelitis
EAN	experimental allergic neuritis
EPHB4	ephrin receptor B4
EPO	erythropoietin
EPOR	erythropoietin receptor
ERK1/2	extracellular signal-regulated kinase 1/2
ESAs	erythropoiesis-stimulating agents

FDA	the US Food and Drug Administration
fMRI	functional MRI
GEPO	glutaraldehyde EPO
GLP-1	glucagon-like peptide-1
GM-CSF	granulocyte-macrophage colony-stimulating factor
HbA1c	hemoglobin A1c
HBSP	helix B surface peptide
HSA	human serum albumin
IL-3	interleukin 3
IL-5	interleukin 5
IP	intraperitoneal
ITP	immune thrombocytopenic purpura
IV	intravenous
JAK2	janus kinase 2
KA	kainic acid
KDR	kinase domain receptor
LPS	lipopolysaccharide
MOG	myelin oligodendrocyte glycoprotein
NO	nitric oxide
NRF2	nuclear factor (erythroid-derived 2)-like 2
PEG	polyethylene glycol
PI3-K	phosphoinositide-3-kinase
PLP	proteolipid protein
PPIs	protein–protein interactions
PTMs	posttranslational modifications
QST	quantitative sensory testing
rhEPO	recombinant human protein EPO
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SC	subcutaneous
SHP-1	Src homology region 2 domain-containing phosphatase-1
STAT5	signal transducer and activator of transcription 5
TBI	traumatic brain injury
TNF-α	tumor necrosis factor-alpha
TRPV1	transient receptor potential vanilloid 1
VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor 2
βCR	β common receptor (CD131)

1. PEPTIDE DRUGS

Peptides are natural biological or artificially generated synthetic compounds consisting of two or more amino acids linked by peptide (amide) bonds. They are also considered as small proteins because they are less than

50 residues and their molecular weight is less than 10 kDa. So far, around 7000 natural peptides have been identified, which have an important role in the most biological processes such as acting like hormones, growth factors, neurotransmitters, ion channel ligands, and anti-infectives. Formation of peptides is linked with protein degradation and turnover inside cells. Although most of the degraded products are produced as a result of cellular homeostasis, they are hydrolyzed to be recycled in protein synthesis, and regulate protein phosphatases and kinases. The importance of biologically active peptides in normal and pathological cellular events provides the inspiration for pinpointing new therapeutic candidates (Qvit, Rubin, Urban, Mochly-Rosen, & Gross, 2017). Today, there are more than 60 FDA-approved peptide drugs. Nearly 140 clinical trials with peptide drugs are ongoing research, and preclinical studies are carried out with over 150 peptide molecules (Fosgerau & Hoffmann, 2015).

As therapeutic agents, peptides have unique features over proteins or small molecule drugs: They are small in size, have a higher activity, and are highly specific toward their targets. Since peptides are rapidly degraded, they do not accumulate in specific organs (kidney, liver, etc.) (Table 1). So, they are less toxic than small molecule drugs (Marqus, Pirogova, & Piva, 2017). An added benefit of using peptides in treatment is that they are less immunogenic when compared to antibodies or proteins and also have minimal drug–drug interaction (McGregor, 2008). In addition to these advantages, therapeutic peptides have also some drawbacks. They have a short half-life and low oral bioavailability, since proteolytic enzymes of the digestive system can break them down quickly. They also have difficulties in crossing the physiological

Table 1 Advantages and Disadvantages of Peptide Drugs

Advantages	Disadvantages
High activity	Low oral bioavailability
Low toxicity	Short half-life
High potency and selectivity	Low stability
Low accumulation in tissues	Low membrane permeability
Broad range of targets	Tendency for aggregation
High chemical and biological diversity	Risk of immunogenic effects
Minimal drug–drug interactions	High production cost

barriers due to their hydrophilic character. Their high conformational flexibility appears to be an advantage, though sometimes this results in a lack of selectivity and leads to their interactions with different targets (Vlieghe, Lisowski, Martinez, & Khrestchatisky, 2010).

2. STRATEGIES TO IMPROVE THE PHARMACOKINETIC PROPERTIES OF PEPTIDE DRUGS

Different strategies can be applied to improve the biological effects of peptide drugs (Table 2). Modifications of peptides during formulation such as cyclization, peptide stapling, imposing N-methylation, or using a transporter system, increasing permeability using surfactants, phospholipids, fatty acids, and nanoparticles are available approaches that can improve the absorption of peptide drugs (Di, 2015; Furman, Chiu, & Hunter, 2015). Both diffusion and convection mechanisms provide biodistribution of peptide drugs, which can be enhanced via conjugation to endogenous proteins or structural modification of peptides (Di, 2015).

The metabolism of the peptide drugs occurs in blood, liver, kidneys, and small intestine by proteolytic degradation. Degraded peptide metabolites and fragments may continue their biological actions (Otvos & Wade, 2014). Half-lives of peptide drugs may be extended as demonstrated for adrenocorticotropic hormone (ACTH), parathormone, and relaxin (Otvos & Wade, 2014). Structure modification of peptides, such as inclusion of nonnatural amino acids; conjugation to macromolecules (polyethylene glycol (PEG), XTEN or antibody Fc portion) and using protease inhibitors are effective strategies to improve stability of the peptide (Blaskovich, 2016; Furman et al., 2015; Goldflam & Ullman, 2015; Vhora, Patil, Bhatt, & Misra, 2015). Macromolecules for conjugation should be selected carefully. For example, a PEGylated erythropoietic peptide, peginesatide, was quickly withdrawn from the market due to unexplained serious adverse drug reactions (Bennett, Jacob, Hymes, Usvyat, & Maddux, 2014; Hermanson, Bennett, & Macdougall, 2016). Moreover, degradable polymers such as XTEN are replaced with PEG to enhance peptide stability. Peptides are rapidly filtered through glomeruli du to their small size and their limited tubular uptake. Renal clearance of peptide drugs could be modified by their conjugation to polymers, albumin-binding small molecules, or increasing their plasma protein binding (Diao & Meibohm, 2013).

Physicochemical properties of peptides are critical for their absorption, distribution, metabolism, and excretion (ADME) properties. Prediction of

Table 2 Strategies to Improve Poor Pharmacokinetic Properties of Peptide Drugs

Properties	Solutions	Mechanisms
Low absorption	N-methylation, cyclization, introducing intramolecular hydrogen bonds, incorporation of positively charged amino acids, repetitive arginine-containing modules, stapled peptides	Reduce hydrogen bonding potential, increase rigidity, reduce intermolecular hydrogen bonds and flexibility, enhance absorption
	Using the vitamin B12 multivitamin uptake system	Use transporter system
	Bile salts, phospholipids, FAs, glycerides, surfactants, EDTA, heparin	Enhance absorption, increase solubility and paracellular permeability
	Mildly perturbing mucosal surfaces, oral permeability enhancers, cell-penetrating peptides	Enhance transcellular permeation
	Incorporation of various sugars, binding to endogenous proteins, conjugation to nanoparticles, biotin conjugates	Enhance absorption
Insufficient biodistribution	Cyclization, C-terminal amide, N-terminal acetylation, N-terminal ring formation	Protect C and N terminus
	Conjugation	Increase membrane transport
Rapid metabolism	Replacing L-amino acids with D-amino acids, modification of amino acids (αMet-AAs, N-me-AAs, β-AAs), side chain modification, disulfide-rich peptides, asparagine deamidation/aspartic acid isomerization, acylation	Protect from proteolysis
	Conjugation to macromolecules, antibody Fc portion	Protect from proteolysis, prolong half-life
	Protease inhibitors	Protect from proteolysis
Rapid excretion	Conjugation to large polymers (PEG, PSA, XTEN, HES, PVP)	Reduce glomerular filtration, prolong half-life
	Covalent linkage to albumin-binding small molecules	
	Fusion to long-live plasma proteins	

ADME properties with in silico tools can improve drug potential of peptides. Besides, transporters and permeability enhancers could take essential role in peptide pharmacokinetic features.

3. PEPTIDE DRUG DISCOVERY

3.1 The Empirical Approach

The empirical approach is the most commonly used method to discover bioactive peptides that are obtained from food proteins. Protein extract from foods is hydrolyzed by using proteolytic enzymes or by fermentation to obtain peptide fragments (Darewicz, Borawska, Vegarud, Minkiewicz, & Iwaniak, 2014; Ha et al., 2015). Those fragments can then be fractionated based on their structural properties and can further be tested for their benefits in vitro. Furthermore, the active fractions of the peptides can be defined using liquid chromatography–tandem mass spectrometry (Garcia-Tejedor et al., 2014). Using this approach, Bidasolo et al. discovered functional peptides after digesting donkey's milk casein with pepsin and corolase enzymes (Bidasolo, Ramos, & Gomez-Ruiz, 2012). Even though this approach eases the process of novel bioactive peptide discovery in protein samples, it usually neglects the less concentrated peptides present in the media. Also, this method is costly and time consuming for screening process. Therefore, computational protein design emerges as an economical alternative to this approach.

3.2 Computational Design of Peptide Ligands for Protein Receptors

The majority of the computational design methods utilize a rational strategy while constructing the peptide of interest. In the rational strategy, availability of the receptor structure obtained either by structural biology techniques (i.e., X-ray crystallography, nuclear magnetic resonance spectroscopy, electron microscopy) or homology modeling is assumed. When the receptor structure is at hand, binding of the desired peptide can be modeled by using different algorithms. Such designs ultimately aim at blocking or enhancing specific protein–protein interactions (PPIs) (Modell, Blosser, & Arora, 2016). There is a growing interest in designing peptides with such a structure-based approach, not only because it is cost effective but also because it can provide a physics-based model on predicting the design's affinity toward its receptor (Kilburg & Gallicchio, 2016; Sable & Jois, 2015).

3.2.1 General Rules for Receptor–Peptide Binding

Peptides are flexible by nature, which allows them to refold upon binding their receptors. To compensate this entropic cost, they form weak interactions with their partners. Their flexibility and weak binding nature make the description of their complex formation very difficult (London, Raveh, & Schueler-Furman, 2013; Trellet, Melquiond, & Bonvin, 2013). To that end, a noteworthy effort has been invested in unveiling the receptor–peptide binding patterns. This effort has been embodied in gathering databases of available receptor–peptide structures [PepBind (Das, Sharma, Kumar, Krishna, & Mathur, 2013), DOMMINO (Kuang et al., 2012), peptiDB (London, Movshovitz-Attias, & Schueler-Furman, 2010), PixelDB (Frappier, Duran, & Keating, 2017), THPdb (Usmani et al., 2017)]. Statistical analyses performed on these databases have delivered a number of peptide characteristics:

- Peptides are more complex and flexible than standard medicinal compounds. They tend to establish extended interactions with their protein partners (Kilburg & Gallicchio, 2016).
- Compared to PPIs, they form a more packed interface enriched in main chain hydrogen bonding (London et al., 2013).
- Receptor–peptide interactions are driven mainly by hotspots[a] positioned on the receptor (London et al., 2013; Sable & Jois, 2015).
- Core-binding sites on peptides are mostly made of hydrophobic residues, i.e., *Leu*, *Met*, and *Phe* (Frappier et al., 2017).
- Peptide binding generally does not induce conformational change in the receptor (Vanhee et al., 2011).
- Protein–peptide interactions follow structural folds that are observed within monomeric proteins (Vanhee et al., 2009).

Current knowledge on receptor–peptide binding has shaped the content of the receptor–peptide structure prediction algorithms. In Table 3, you will find a brief collection of these prediction tools and also how they make use of the current knowledge in predicting different types of receptor–peptide binding.

3.2.2 Available Methods

Prediction of a receptor–peptide complex involves two layers of complexity: prediction of (i) the peptide's binding site on the receptor and (ii) the structure of the bound peptide on the receptor (Peterson et al., 2017). As addressing

[a] If replacement of an amino acid residue by alanine lowers the free energy of binding at least by 2 kcal/mol, then this residue is classified as a "hotspot." Frequently observed hotspot residues are Tyr, Trp, and Arg.

Table 3 Structure-Based Methods for the Prediction of Receptor–Peptide Binding

Method and Availability	Category	Input Peptide	Reference
Data-driven tools			
Rosetta FlexPepDock ab initio http://flexpepdock.furmanlab.cs.huji.ac.il	Docking	Extended backbone conformation	Raveh, London, Zimmerman, and Schueler-Furman (2011)
HADDOCK http://haddock.science.uu.nl/services/HADDOCK2.2/haddock.php	Docking	Ensemble of three canonical peptide conformations: an a-helix, extended, or polyproline II	Trellet et al. (2013)
GalaxyPepDock http://galaxy.seoklab.org/pepdock	Knowledge-based modeling (uses similar protein–peptide interactions as templates)	Peptide sequence	Lee, Heo, Lee, and Seok (2015)
PepComposer	Knowledge-based modeling (uses motifs found in monomeric proteins as backbone scaffolds)	No input is required, plausible peptide sequences that can bind to a given pocket are listed	Obarska-Kosinska, Iacoangeli, Lepore, and Tramontano (2016)
AnchorDock	Molecular dynamics (uses ANCHORSmap's output)	Peptide sequence	Ben-Shimon and Niv (2015)
Ab initio tools			
MedusaDock	Adapted molecular dynamics (with highly weighted electrostatics)	Peptide sequence	Dagliyan, Proctor, D'Auria, Ding, and Dokholyan (2011)

Continued

Table 3 Structure-Based Methods for the Prediction of Receptor–Peptide Binding—cont'd

Method and Availability	Category	Input Peptide	Reference
FoldX Suite http://foldxsuite.crg.eu	Knowledge-based modeling (uses motifs found in monomeric proteins)	Peptide sequence	Verschueren, Vanhee, Rousseau, Schymkowitz, and Serrano (2013)
PiPreD http://www.bioinsilico.org/PIPRED/	Knowledge-based modeling (uses precompiled and bespoken library of structural motifs, extracted from protein complexes)	Peptide sequence	Oliva and Fernandez-Fuentes (2015)
pepATTRACT http://bioserv.rpbs.univ-paris-diderot.fr/services/pepATTRACT	Docking	Peptide sequence	de Vries, Rey, Schindler, Zacharias, and Tuffery (2017)
IDP-LZerD http://kiharalab.org/proteindocking/idplzerd.php	Docking	Peptide sequence (can also account peptides having a sequence length >15 amino acids)	Peterson, Roy, Christoffer, Terashi, and Kihara (2017)
CABS-dock http://biocomp.chem.uw.edu.pl/CABSdock/	Docking	Peptide sequence	Kurcinski, Jamroz, Blaszczyk, Kolinski, and Kmiecik (2015)

both problems is a challenging task, the majority of the structure prediction methods necessitate knowledge of the peptide-binding site on the receptor (data-driven modeling). Binding site information can be obtained via different sorts of binding experiments (e.g., mutagenesis) or bioinformatic predictions (e.g., Pepsite (Trabuco, Lise, Petsalaki, & Russell, 2012), Peptimap (Lavi et al., 2013), ANCHORSmap (Ben-Shimon & Eisenstein, 2010)). Some other methods, on the other hand, perform a global search on the receptor (ab initio modeling) for finding the optimum binding pocket. Available data-driven and ab initio algorithms are constructed either as docking,[b] knowledge-based,[c] or molecular dynamics[d] modeling packages (Kilburg & Gallicchio, 2016; London et al., 2013; Sable & Jois, 2015). Based on the available information on the peptide under study, any of the mentioned packages can be utilized.

3.3 Phage Display

Phage display method was first defined by G. P. Smith et al. in 1985 to express cloned antigens on viral surface (Smith, 1985). It is a combinatorial technology which attracts a great deal of attention for the future of drug discovery. Other than phage display, different molecular display techniques can be also employed in peptide discovery, namely, yeast and bacterial display, ribosome display, mRNA display, CIS, and covalent antibody display (Wu, Liu, Lu, & Wu, 2016). In phage display method, coat protein of the phage is engineered to express random sequence peptides or antibodies on the surface. Afterward, the phage library is tested on desired target in terms of target-ligand binding (Hamzeh-Mivehroud, Alizadeh, Morris, Church, & Dastmalchi, 2013). This method is a robust tool in drug discovery principally for peptide drug identification (Rothe, Hosse, & Power, 2006). Phage display method enables researchers to construct libraries and rapidly isolate and identify specific protein interactions of molecular targets (Noren & Noren, 2001). Furthermore, this technique facilitates ligand identification for receptors, enzyme blocker discovery, protein/DNA–protein interactions, cDNA expression screening, antibody epitope mapping, antibody engineering, and vaccine design (Hamzeh-Mivehroud et al., 2013). Phage display has several advantages over traditional screening techniques, considering its ease of use, cost effectiveness, agility, and more importantly high-throughput screening

[b] Docking depicts plausible binding modes of the peptide on the receptor.

[c] Knowledge-based modeling builds receptor–peptide interactions by following structurally related interaction patterns deposited in structural databases.

[d] Whereas molecular dynamics look for stable contacts within a time course of the simulation.

ability of peptide and peptide variants. In phage display, after binding of phage to target, selected phages are eluted and phage DNA is sequenced for identification (Omidfar & Daneshpour, 2015). Afterward, selected ligands can be structurally analyzed to produce specifics of the receptor–ligand interaction (Omidfar & Daneshpour, 2015). Bioactive peptide screen using phage display is a powerful approach for also food-derived peptides. The newly discovered peptides using phage display technique can then be cloned and overexpressed to increase yield in hybrid technology combining solid and liquid synthesis.

3.4 Integrated Approach

As an alternative to the isolated use of empirical or computational methods, integrated approaches can be employed in bioactive peptide discovery. These approaches combine empirical and computational tools, in order to maximize the advantages of both methods in designing and testing therapeutic peptides in the functional food and health applications (Daliri, Lee, & Oh, 2017). As an example, in silico determined peptides derived from food proteins can be chemically synthesized, which can lead to the discovery of novel peptides (Udenigwe & Aluko, 2011).

3.5 Peptide Production and Manufacturing

Apart from erythropoietin (EPO)-derived peptides, there are many other peptide types that are studied preclinically and clinically. For the production of such peptides, there are alternative methods and definite determinants, like size and their nature. So, peptides can be produced by several methods, namely, chemical synthesis, recombinant expression, and in vitro translation.

Chemical peptide synthesis can be used to produce peptides less than 100 aa length. This method allows researchers to synthesize peptides composed of nonnatural or D-amino acids, pseudo-peptide bonds, and directed disulfide bonds to allow broader diversity than other peptide synthesis methods (Furman et al., 2015). Substitution of natural amino acids to unnatural ones also results in higher plasma stability as they can escape from proteases (Vlieghe et al., 2010). Furthermore, constructing synthetic combinations of peptide library that is composed of random peptides can enhance drug discovery rate and candidate selection.

Chemical peptide synthesis has been first performed by Vigneaud et al. in 1953 (Vigneaud et al., 1953), and since then, novel techniques have been developed for synthesis of the peptides in terms of coupling efficiency, diversity of amino acids, and addition of functional groups. Current advancements

in the synthetic peptide synthesis by automated systems have accelerated this process, while the yield of peptide synthesis is greatly affected by coupling efficiencies. As the length of peptide increases, poorer coupling reactions are encountered in the final yield of pure peptide (Furman et al., 2015).

In the case that pseudo-peptide bond and unnatural amino acids are not required, recombinant protein expression methods may be the best option for manufacturing. In this method a fusion protein, human serum albumin (HSA), Fc, and XTEN polymer, is attached to N- or T-termini of the protein to be manufactured (Doknic, Stojanovic, & Popovic, 2014; Kenanova et al., 2010). This system also requires a host organism to express protein, such as transgenic plants and animals, bacteria, yeast, insect cells, and mammalian cells. Furthermore, there are also some commercially available recombinant peptide therapeutics available including nesiritide, teriparatide, and salmon calcitonin (Chandrudu, Simerska, & Toth, 2013; Vlieghe et al., 2010).

Another method for protein manufacturing includes cell-free (in vitro) translation technologies. Those technologies include necessary components for translation machinery obtained from cell extracts of *Escherichia coli* S30, rabbit reticulocytes, wheat germ, *Leishmania*, *Thermus*, or human (Furman et al., 2015). This technique has gained some advantages over traditional cell-based methods (Murray & Baliga, 2013). First, this method eliminated the limitations of cell wall or membranes by adding chaperones, isomerases, or posttranslational modifier enzymes to achieve proper translation and folding. Second, it allows more efficient use of reactor volumes and eliminates cell viability limitations (Harris & Jewett, 2012).

Nevertheless, cell-free systems have been generally utilized in small-scale peptide production, resulting in batch-to-batch variability and improper folding in the case of scale-ups. Recent advancements could allow manufacturers to linearly scale up under cGMP conditions, which is transferrable to industrial scale (Uhlig et al., 2014). As an example, 100 L batch of biologically active granulocyte-macrophage colony-stimulating factor (GM-CSF), a 127-amino acid protein can give yield of 700 mg/L in 10-h period (Hipolito & Suga, 2012). So, this method can be a prominent alternative to traditional peptide generation methods. FDA's guideline specific for quality control in peptide manufacturing includes guidelines for the specifications of GMP peptides (Commission Directive, 2003).

Peptide drug preparations must be stable, sterile, and pyrogen- and particulate-free. Especially during long-term storage microbial contamination of peptide preparations results in loss of biological activity by proteolysis. Thus, preservative formulations in ingredients are edited (Bak et al., 2015;

Furman et al., 2015). Peptides must be stored in suitable containers to avoid their adsorption on surfaces. Appropriate container retains stability of the peptides during processing and storage (Bak et al., 2015).

Currently, most peptide drugs are administered by parenteral route. However, orally bioavailable peptides are more suitable for patient's convenience, compliance, and adherence. There are only a few oral peptide drugs in the market, for example, cyclosporine, desmopressin, and calcitonin (Bak et al., 2015). These drugs have also spray formulations for intranasal delivery. The disadvantage of peroral and intranasal administration is intra- and inter-individual variability of bioavailability. Oral formulations often interact with food. As an alternative, peptide drug delivery through intestinal patch system can be used instead of oral usage (Banerjee & Mitragotri, 2017). This innovative approach facilitates paracellular uptake of peptides from intestinal wall to systemic circulation.

Because unmodified peptides with short half-life are eliminated before they act, they should be provided in frequent high doses through parenteral delivery (Diao & Meibohm, 2013). For example, long-acting analog of glucagon-like peptide-1 (GLP-1) is used for the normalization of blood glucose (Deacon, 2004). Another example is depot injection form of leuprorelin that provides slow, continuous, and controlled release of the drug into the systemic circulation. Monthly intramuscular injection of leuprorelin stimulates the release of follicle-stimulating hormone and luteinizing hormone (Periti, Mazzei, & Mini, 2002).

4. ERYTHROPOIETIN

4.1 Erythropoietin and Erythropoietin Receptor

EPO is a 34-kDa glycoprotein hormone, cytokine, and hematopoietic growth factor, belonging to the type I cytokine superfamily (Broxmeyer, 2013; Genc, Koroglu, & Genc, 2004a). The primary production of EPO is in the liver during fetal development. After birth, production subsequently changes to the kidney (Souma, Suzuki, & Yamamoto, 2015). EPO binds to its receptor (EPOR) on erythroid precursor cells in bone marrow and sustains their viability, proliferation, and differentiation in normoblast (Genc, Koroglu, & Genc, 2004b). A few amount of EPO was first isolated from 2500 L of urine of aplastic anemia patients by Goldwasser and Kung (Miyake, Kung, & Goldwasser, 1977). Obtaining pure human protein was a pioneering approach for the partial identification of amino acid sequence, construction of isolated DNA probes according to tryptic fragments, and finally gene cloning

(Jacobs et al., 1985; Lin et al., 1985). Right after cloning, DNA-derived recombinant human protein EPO (rhEPO) was successfully used to treat anemia in end-stage renal disease in clinical trials, and the US Food and Drug Administration (FDA) approved to use EPO for the treatment of anemia related with renal failure, prematurity, human immunodeficiency virus infection, cancer, and chronic inflammatory disease (Fisher, 2010; Genc et al., 2004a).

EPO gene encodes a 193-amino acid sequence protein at the beginning, and after posttranslational modifications (PTMs), the remainder sequence leads to a mature protein containing 165 amino acids to which four glycans are attached (Rahbek-Nielsen et al., 1997; Ribatti, 2008). Three N-glycans and one O-glycan are making up EPO's carbohydrate part (~40% of the protein). N-glycans provide protection to EPO from proteases and prevent disruption of its receptor-binding affinity (Jelkmann, 2013).

EPOR is a 59-kDa glycoprotein and member of cytokine receptor superfamily (Jelkmann, 2011). It is encoded by the human EPOR gene, located in the short (p) arm of chromosome 19 at position 13.2 (Winkelmann, Penny, Deaven, Forget, & Jenkins, 1990), containing 8 exons that synthesize 508 residues. The first 24 amino acids code a signal peptide, which is cut for maturation. After glycosylation, phosphorylation, and ubiquitination processes, mature human EPOR protein is produced (Farrell & Lee, 2004). EPOR has three major domains: an extracellular domain, including two pairs of cysteine residues to provide binding of the ligand and also conserved motif (WSXWS); a single transmembrane domain; and an intracellular domain lacking catalytic action (Farrell & Lee, 2004). EPOR preexists on the surface of erythroid progenitors as homodimers, which experiences a conformational change upon binding of its ligand EPO. This change brings its intracellular domains into close position (Livnah et al., 1999; Remy, Wilson, & Michnick, 1999). Dimerization of EPOR subunits assembles with janus kinase 2 (JAK2) and other members of molecular signaling machinery. This results in autophosphorylation and activation of JAK2 kinases (Youssoufian, Longmore, Neumann, Yoshimura, & Lodish, 1993). Phosphorylation of tyrosine residues leads to several downstream signaling pathways. One of them is induction of several antiapoptotic proteins (Bcl-2 and Bcl-XL) by active phosphoinositide-3-kinase (PI3-K) (Broxmeyer, 2013). One of the other pathways is dimerization and translocation of signal transducer and activator of transcription 5 (STAT5) to the nucleus where proliferation and cell survival genes are transcribed with the induced STAT5. STAT5 has a role in stimulation of mitochondrial antiapoptotic protein BCL-XL as well (Sargin, Friedrichs, El-Kordi, & Ehrenreich, 2010). Activation of extracellular

signal-regulated kinase 1/2 (ERK1/2) by EPOR signaling stimulates nuclear translocation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and plays pivotal role in neuroprotective effects of EPO in SHSY-5Y cell line and primary rat cortical neurons (Genc, Egrilmez, & Genc, 2010; Zhang, Zhang, Zhao, & Sun, 2017). Inactivation of the receptor is mediated by dephosphorylation of Src homology region 2 domain-containing phosphatase-1 (SHP-1). Overall, proliferation, inhibition of apoptosis, and differentiation are the net effect of EPOR stimulation (Sargin et al., 2010).

4.2 EPOR-Associated Receptors

Recently, it has been shown that EPO binds to various receptors that mediate diverse effects of EPO in different tissues. The diversity of biological roles of same molecule is achieved by the expression of receptor type and affinity of molecules.

The β common receptor (βCR; CD131) is a member of type-1 cytokine receptor family. βCR, also known as a hemopoietin receptor, shares a common motif (WSXWS) in the extracellular part with EPO and has a long intracellular tail as well (Watowich, 2011). βCR is encoded by the human colony-stimulating factor 2 receptor beta (CSF2Rβ) gene which is located at the chromosome 22 (position 22q13.1) and involves 14 exons (Debeljak, Solar, & Sytkowski, 2014). βCR has a common beta chain subunit of the high-affinity receptor for GM-CSF, interleukin 3 (IL-3), and interleukin 5 (IL-5) (Hermanson et al., 2016). Binding of specific cytokine α receptor subunits to the βCR by disulfide bonds indicates ligand specified as observed for EPOR. The interaction of EPOR and βCR receptor was first hypothesized to play a role in hematopoiesis based on the phosphorylation of βCR in hematopoietic cells with EPO signaling. Moreover, the protection effect of EPO observed in nonhematopoietic tissues (kidney, vessels, heart, and brain) can be appear through EPOR–βCR heterodimer or EPOR–βCR–EPOR heterotrimer (Brines & Cerami, 2006). The βCR and EPOR expression has been examined in a variety of tissues including central and peripheral nervous system, heart, kidney, retina, endothelium, and muscle (Brines & Cerami, 2012). Its signaling pathway is still not well understood; however, it is anticipated that the signaling pathways activated by EPOR–βCR heterodimer are different from the signaling pathway occurred by EPOR dimerization. Consequently, the only clear fact is that βCR–EPOR heterocomplex has a role in tissue protective response (Brines & Cerami, 2012; Ghezzi & Brines, 2004).

Ephrin receptors which form the largest family of receptor tyrosine kinase (RTK) family and their ligands, the ephrins, have a role in several developmental processes, mainly in the nervous system (Debeljak et al., 2014). The protein encoded by this gene was specified as survival factor in several cancers and also binds to ephrin-B2 ligand to play an important role in vascular development (Debeljak et al., 2014). Identification of the EphB4 as an alternative EPOR has many consequences including opportunities such as anti-EphB4 approaches to clear away the stimulatory effects of EPO on tumor growth (Pradeep et al., 2015). Glioma cells exhibits higher EphB4 expression and overexpression of EphB4 leads to growth of glioma cells (Chen et al., 2013). In 2013, there was a new European patent application made for NEPOR, a novel tissue protective EPO-binding receptor complex. NEPOR is composed of EPOR heteroreceptor dimer EPOR–EPHB4, homoreceptor EPOR–EPOR, and EPHB4–EPHB and other components stemming from ephrin biology (Debeljak et al., 2014).

EPO and vascular endothelial growth factor (VEGF) have effects on the endothelium and endothelial progenitor cells to increase the number and performance properties that are dependent on nitric oxide (NO) stimulated by both EPO and VEGF (Sautina et al., 2010). VEGF has many receptors and among them, vascular endothelial growth factor receptor 2 (VEGFR2) has a serious role in EPO function. VEGFR2 is a kinase domain receptor (KDR) and its gene is located on chromosome 4 (position: 4q12), encoding 30 exons (KDR Kinase Insert Domain Receptor, 2017). VEGFR2 is stimulated to become active in neoangiogenesis in hypoxic endothelial cells and be a member of the EPOR–βCR complex which is essential for production of nitric oxide with the addition of VEGFR2 (Brines & Cerami, 2012; Kimakova, Solar, Solarova, Komel, & Debeljak, 2017). In addition, βCR also colloborates with VEGFR2 in other cellular systems and forms homo or heterotrimers (Brines & Cerami, 2012).

Unfortunately, the expression pattern of the different EPOR complexes and their activation in different cell types are not well understood yet.

4.3 Adverse Effects of Recombinant Human Erythropoietin

The erythropoietic activity of EPO has proven very beneficial for the treatment of anemia. Both biosynthetic and synthetic analogs of EPO are available in the market (Jelkmann, 2013). However, several side effects can emerge according to desired erythropoietic activity, such as pure red cell aplasia, tumor growth, and thromboembolic and hypertension complications. According to

FDA statement, when compared with the patients that did not treat with EPO, treatment with EPO analogs resulted in earlier progression or sooner death (Uversky & Redwan, 2016).

Some clarifications for the adverse effect of rhEPO in anemic cancer patients include tumor promotion via signaling involving EPO or erythropoiesis-stimulating agents (ESAs) and EPOR. According to this hypothesis, several preclinical studies indicate the existence of EPO-induced proliferation of cancer cells, migration and invasion of cancer cells, stimulation of chemotaxis, increase in drug resistance, interference of apoptosis, and protection from ischemic injury. In addition, the presence of EPOR mRNA or EPOR protein has been observed in several tumor types among different organs (Bennett et al., 2016; Uversky & Redwan, 2016). EPO induces proliferation, migration, and invasion of several cancer cells including glioma cells (Alural, Ayyildiz, Tufekci, Genc, & Genc, 2017; Peres et al., 2011). Besides, EPO has suppressed sensitivity in glioma cells toward chemotherapeutic agents (Alural et al., 2017; Peres et al., 2011). The patients with tumors which have EPORs might not be affected from the harmful proliferative effects of ESAs (Erslev, 1993; Uversky & Redwan, 2016). Although it is not determined how ESAs affect the tumor control, two potential mechanisms were propounded by McKinney and Arcasoy for tumor progression by rhEPO therapy (McKinney & Arcasoy, 2011). rhEPO may have an effect locally in tumor cells or other cell types found in tumor microenvironment, and rhEPO may alter tumor biology by raising the systemic effects or increasing specific systemic toxicities (Debeljak et al., 2014).

Another unwanted effect of ESAs is an increase in arterial blood pressure, possibly caused by hypertension in the treatment of patients with chronic kidney disease (CKD) (Provatopoulou & Ziroyiannis, 2011). Rise in blood pressure can be connected to increase in blood viscosity (Jelkmann, 2013; Provatopoulou & Ziroyiannis, 2011).

Nearly all biopharmaceuticals induce an immune response, but their effects can be remissible or serious. Antibodies to therapeutic proteins can also react with endogenous molecules to increase toxicity, while they can be efficient for the treatment of the target tissue (Uversky & Redwan, 2016). Similarly, pure red cell aplasia was emerged from patients with CDK who receive recombinant erythropoietin in late 1990s (Casadevall, Eckardt, & Rossert, 2005). Immunogenicity studies have shown that pure red cell aplasia is caused by the arising of autoantibodies against rhEPO (Hermanson et al., 2016; Uversky & Redwan, 2016).

The studies provide an evidence that thromboembolic complications and death risk were increased with the ESAs treatment. Endothelial cells

are activated by EPO to express adhesion molecules which induce plug accumulation in vessels with platelets and leucocytes by thrombus formation (Jelkmann, 2013). ESA treatment increased stroke risk in a clinical study involving 4038 patients with type 2 diabetes and CKD (Pfeffer et al., 2009).

4.4 Neuroprotective Effects of Recombinant Human Erythropoietin

EPO and EPOR are expressed by other tissues, including heart, kidney, and central and peripheral nervous system. Several in vitro and in vivo studies demonstrated neuroprotective, cardioprotective, and renoprotective effects of EPO (Nekoui & Blaise, 2017).

EPOR is expressed in neuronal cell lines, primary cultured hippocampal and cortical neurons, and murine hippocampal and cerebral cortical areas (Masuda et al., 1993; Morishita, Masuda, Nagao, Yasuda, & Sasaki, 1997). Western blotting experiment by using protein samples obtained from PC12 cells revealed that the size of EPOR was smaller than in erythroid cells (Masuda et al., 1993). Recent studies with new antibodies against EPOR developed using novel genetic strategies also confirmed EPOR expression in nervous system (Maxwell et al., 2015; Ott et al., 2015). Conditional knockdown of EPOR leads to embryonic and adult neurogenesis deficits, suggesting that EPO and EPOR are essential for embryonic neural development and regeneration of neurons in ischemic injury (Tsai et al., 2006). EPO expression was detected in primary astrocyte cell culture and culture supernatant, supporting that EPO is produced and secreted by astrocyte (Masuda et al., 1994). EPO and EPOR are expressed by Schwann cells in rat sciatic nerve and their expression increased in crush injury (Li, Gonias, & Campana, 2005). In vitro neuroprotective effects were first demonstrated by Sakanaka et al. against glutamate toxicity in 1997 (Morishita et al., 1997). After 1 year, in vivo neuroprotective effects of EPO against ischemic damage in Mongolian gerbils have been reported (Sakanaka et al., 1998). Then, various in vitro and in vivo preclinical studies showed a wide range of neuroprotective effects of EPO in acute and chronic degenerative disorders (Sargin et al., 2010). These promising preclinical results encouraged the EPO clinical studies in neurodegenerative disorders.

4.5 Clinical Failure of Recombinant Human Erythropoietin in Neurological Disorders

EPO and EPOR have been shown to exist and function in several nonerythroid tissues including the brain, heart, and vascular endothelium. In vitro and in vivo studies demonstrated that EPO exerts tissue protective effects in a number of conditions (Solling, 2012). Thus, it was considered that the

tissue protective effects of EPO may translate to humans. But, only a few multicentered and large clinical trials with EPO have been published until today. Clinical trials with EPO have been carried out to reduce tissue damage especially in cardiac ischemia, stroke, and kidney diseases (Lund, Lundby, & Olsen, 2014). A large-scale multicentered study with 522 stroke patients determined that EPO increases mortality without any positive effect on neurological outcome (Ehrenreich et al., 2009). EPO also did not exhibit cardioprotective and nephroprotective effect in most of clinical studies (Solling, 2012). Tissue protective effects of EPO in preclinical studies may not have been transferred to the clinic due to various reasons. Major reason for this discrepancy could be the differences in anatomy and physiology between rodents and humans (Reagan-Shaw, Nihal, & Ahmad, 2008; Shanks, Greek, & Greek, 2009). Differences in the dosage and timing of treatment may also explain clinical translation failure (Lund et al., 2014). rhEPO has been used in animal studies at higher doses than human studies. Lower dose of EPO than used in animal studies does not exhibit therapeutic effect in clinical studies. Lack of replication and reproducibility in experimental animal studies with EPO due to inappropriate methodology is common (Steward, Popovich, Dietrich, & Kleitman, 2012). The absence of ideal animal models that represent all the features of the diseases is another factor limiting the translation of knowledge from preclinical studies to the clinic (Mak, Evaniew, & Ghert, 2014). Another limitation in preclinical evaluation is the inability to consider comorbid diseases and possible interactions with other drugs.

5. CHEMICALLY MODIFIED AND NONERYTHROPOIETIC ERYTHROPOIETIN ANALOGS

The emergence of side effects related to EPO therapy has led to the development of modified forms of EPO with no erythropoietic effect but with retained tissue protective effect. EPO molecule could be altered by several ways such as carbamoylation, amidation, and acylation (Chen, Yang, & Zhang, 2015; de Lange et al., 2017; Torup & Leist, 2006). Carbamoylated EPO (CEPO) exhibits neuroprotective effect in wide range of animal models of neurological disorders including ischemic stroke, spinal cord injury, and diabetic neuropathy binding to CD131/EPO heteroreceptor (Chen et al., 2015). The results of completed clinical safety studies with CEPO are not published yet. Glutaraldehyde EPO (GEPO) was obtained by chemical modification of the lysine residues in rhEPO (Chattong et al., 2013). GEPO

interacts with heterotrimeric receptor of EPO and increases BCL2 expression (Chattong et al., 2013). GEPO protects kidney cells against silver nanoparticle exposure and ischemia reperfusion injury (Chattong et al., 2013; Sooklert et al., 2016). The effects of GEPO in the nervous system have not been evaluated to date. Neuro-EPO is a low sialic acid-containing EPO that exhibits neuroprotective effect in experimental focal cerebral ischemia and Alzheimer's disease (Rodriguez Cruz, Strehaiano, Rodriguez Obaya, Garcia Rodriguez, & Maurice, 2017; Teste et al., 2012). Randomized open-label safety study with intranasal Neuro-EPO in healthy volunteers was reported with no safety issues (Lagarto et al., 2012). Phase I/II clinical trials have just started in the treatment of stroke and spinocerebellar ataxia (World Health Organization, 2017a, 2017b). Less acidic modified EPO glycoform called neuropoietin does not exhibit significant in vivo hematopoietic activity and retains neuroprotective effect in vitro (Mattio et al., 2011). The ability of the neuropoietin to pass blood–brain barrier (BBB) faster than rhEPO gives advantages to neuropoietin for faster penetration into damaged tissue (Etcheverrigaray et al., 2011). In vivo studies evaluating neuroprotective effect of neuropoietin in animal model of neurodegenerative disorders have not performed yet.

Although chemically modified nonerythropoietic analogs of EPO circumvent its adverse effects, high expense, and require parenteral administration, potential development of antidrug antibodies and promotion of tumorigenicity should be considered especially in chronic applications (Collino, Thiemermann, Cerami, & Brines, 2015).

6. GROWTH FACTOR MIMETIC PEPTIDE DRUGS

Growth factor mimetic peptides are promising candidates in the treatment of various diseases with low or no side effects of growth factors. They have advantages as a therapeutic molecule in terms of oral usage, longer half-life, crossing BBB, and less immunogenicity. One of the growth hormone mimetic peptides, P021, is an 11-mer ciliary neurotrophic factor (CNTF) mimetic peptide generated by Kazim and Iqbal (2016). Its half-life is longer than 3 h, and it has higher stability in artificial intestinal fluid and the capacity to cross the BBB. Perorally treatment of $3 \times$ Tg Alzheimer's disease mice with P021 for 12 months decreased tau pathology (Kazim & Iqbal, 2016). Thrombopoietin mimetic peptides are one of first-generation growth factor mimetics entering clinical use. They are used for the treatment of immune thrombocytopenic purpura (ITP), hepatitis C-associated thrombocytopenia, and myelodysplastic syndrome (Gutti et al., 2016).

Shortly after recombinant EPO, peptide and nonpeptide erythropoiesis-inducing agents were developed. EMP-1 is a first developed synthetic peptide with ESA activity (Johnson et al., 1998). EMP-1 did not exhibit potent erythropoietic activity. But, it is very useful to study the activation mechanisms of EPOR (Guarnieri, 2017).

Continuous erythropoietin receptor activator (CERA) is a 60-kDA molecule; its half-life has been extended to 135 h by modification in formulation (Gutti et al., 2016). Erythropoietic activity of CERA is not superior to rhEPO and darbepoetin (Saglimbene et al., 2017).

Peginesatide is another peptide ESA that has potent erythropoietic activities. It is synthetic PEGylated dimeric peptide in which the primary sequence is unrelated to EPO (Gutti et al., 2016). Peginesatide binds and activates EPOR and stimulates erythropoiesis in vitro and in vivo. Phase III studies reported higher cardiovascular events and mortality in patients treated with peginesatide (Hermanson et al., 2016; Locatelli, Choukroun, Truman, Wiggenhauser, & Fliser, 2016). Peginesatide was withdrawn from the market due to unexplained serious adverse drug reactions (anaphylactic reactions) in February 2013 (Hermanson et al., 2016).

7. ERYTHROPOIETIN MIMETIC PEPTIDES

10 EPO mimetic peptides have been recently discovered. They were demonstrated to be beneficial in in vitro and in vivo models of neurological disorders (Tables 4 and 5).

7.1 EMP-1

EMP-1 is a member of cyclic peptide family with low erythropoietic activity. It was isolated and characterized by Wrighton et al. in 1996 (Wrighton et al., 1996). EMP-1 is a 2-kDa agonistic peptide whose sequence does not have any homology with EPO's primary sequence (Middleton et al., 1999; Wrighton et al., 1996). EMP-1 can bind EPOR and lead to tyrosine phosphorylation in several erythroid and neuronal cell lines.

The effects of EMP-1 on neuronal cells were evaluated in vitro studies too (Kawakami et al., 2000, 2001), where EMP-1 was shown to inhibit Ca^{2+}-induced dopamine release in a concentration-dependent manner (Kawakami et al., 2000). The maximum effect was seen at 10 μM concentration of EMP-1. Western blotting showed that EMP-1 increased JAK2 phosphorylation and dephosphorylation of GAP-43 which has an important role in the regulation of neurotransmitter release from presynaptic neurons. EMP-1

Table 4 In Vitro Neuronal Effects of EPO Mimetic Peptides

Peptide	Cell Type	Insult Model	Dose	Time	Effects	Signaling Mechanism	Reference
EMP-1	PC12	Ca^{2+} induction	0–10 μM	10 min	Inhibits DA release, dephosphorylates GAP-43	JAK2	Kawakami, Iwasaki, Sato, and Takahashi (2000)
	Rat CGNs Rat hippocampal neurons	Ca^{2+} induction, chemical ischemia (glucose-free 1 mM KCN)	0–25 μM	10 min	Suppresses Glu release and neuronal death	NA	Kawakami, Sekiguchi, Sato, Kozaki, and Takahashi (2001)
Epopeptide AB	NS20Y SK-N-MC PC 12	Serum starvation	0–5 ng/mL	0–48 h	Stimulates differentiation, inhibits neuronal death	ERK1/2	Campana, Misasi, and O'Brien (1998)
ARA 290	Rat primary motoneurons	Kainic acid	1.8 nM	48 h	Protects against kainic acid-induced excitotoxic death	NA	Brines et al. (2008)
Epobis	Rat CGNs Rat hippocampal neurons	Low K^+ and kainic acid	0.1–10 μM	24 h	Stimulates neurite outgrowth, inhibits neuronal death	JAK2/STAT5	Pankratova et al. (2012)
	Rat motor neurons	None	0.04–0.098 μM	24 h	Stimulates neurite outgrowth	NA	Dmytriyeva, Pankratova, Korshunova, and Walmod (2016)
	Rat microglia	LPS	0.9 μM	24 h	Suppresses TNF-α secretion	NA	

Continued

Table 4 In Vitro Neuronal Effects of EPO Mimetic Peptides—cont'd

Peptide	Cell Type	Insult Model	Dose	Time	Effects	Signaling Mechanism	Reference
Epotris	Rat CGNs Rat hippocampal neurons	Low K^+ and kainic acid	0.1–10 μM	24 h	Stimulates neurite outgrowth, inhibits of neuronal death	JAK2/STAT5	Pankratova et al. (2012)
JM-4	MOG peptide enriched T cells PC12		1 ng/mL	72 h	Suppresses proinflammatory cytokine production	NA	Yuan, Wang, Lu, Maeda, and Dowling (2015)
	PC12	$A\beta_{25–35}$ peptide	1 ng/mL	2 h pretreatment (26 h)	Inhibits neuronal death	NA	Yuan et al. (2015)
MK-X	PC12	H_2O_2	0–5 ng/mL	0–7 h	Attenuates neuronal death	ERK1/2, AKT, P38	Yoo, Cho, Moon, Yu, and Moon (2016)
	Rat cortical neuron	Glutamate-induced excitotoxicity	1 pM	15 min to 24 h	Ameliorates mitochondrial damage, inhibits caspase-dependent and -independent cell death pathways	JAK2/STAT5, ERK1/2, AKT	Yoo et al. (2017)

Table 5 In Vivo Neuronal Effects of EPO Mimetic Peptides

Peptide	Model Organism	Disease Model	Dose	Time	Administration Route	Outcome	Reference
Epopeptide AB	Mouse	None	20 μg/day	NA	Right superior gluteus muscle (7 days)	Induces motor endplate sprouting	Campana et al. (1998)
	Mongolian gerbil	3-min forebrain ischemia	4/20 ng/day	Just after ischemia/ reperfusion	Lateral ventricle with osmotic minipump (7 days)	Protects against neuron death	Nagao et al. (2005)
ARA 290	Rat	Middle cerebral artery occlusion	1.5 nmol/kg	Immediately after a 1-h arterial occlusion	IV	Reduces infarct volume	Brines et al. (2008)
	Rat	Sciatic nerve compression injury	0.3 nmol/kg	Immediately after compression of sciatic nerve	IV (single injection)	Reduces sciatic nerve compression-induced damage	
	Rat	Diabetic retinal edema	1.5 nmol/kg	After confirming diabetes	IP (5 days per week, for 3 weeks)	Prevents development of retinal edema	

Continued

Table 5 In Vivo Neuronal Effects of EPO Mimetic Peptides—cont'd

Peptide	Model Organism	Disease Model	Dose	Time	Administration Route	Outcome	Reference
	Rat	Status epilepticus	1.5 nmol/kg, IV; 10 mg/kg, SC	2–5 min following status epilepticus	IV, SC	Promotes neuronal differentiation and survival, attenuated cognitive deficits in epileptic animals	Seeger et al. (2011)
	Akita mouse	Diabetic autonomic neuropathy	36.1 μg/kg	After 15–16 weeks	SC (5 days per week, for 7 weeks)	Decreases neuritic dystrophy in celiac sympathetic ganglia	Schmidt et al. (2011)
	Rat	Sciatic nerve dissection-induced neuritis	30 or 120 μg/kg	1, 2, 3, and 4 days following surgery	IP	Prevents development of mechanical allodynia	Pulman, Smith, Mengozzi, Ghezzi, and Dilley (2013)
ARA 290	Rat	Spared nerve injury-induced chronic neuropathic pain	3, 10, 30, and 60 μg/kg	1, 3, 6, 8, and 10 days postsurgery	IP	Reduces allodynia, suppresses microglial reactivity	Swartjes et al. (2014)
	Rat	Experimental autoimmune neuritis	30 mg/kg	14 days	IP	Reduces paraparesis, inflammation, enhances nerve repair, promotes phagocytic activity	Liu et al. (2014)

	Rat	EAE	35 or 70 μg/kg	Daily from Day 7 to Day 18	IP	Reduces disease severity, decreased number of inflammatory cells in spinal cord, and helper T cells in lymph nodes	Chen et al. (2014)
	Mouse	Capsaicin-induced pain in DRG and TG dissection	1 and 10 nM	15 min before capsaicin	SC	Inhibits TRPV1 channel activity, and relieves the mechanical hypersensitivity induced by capsaicin	Zhang, Yu, and Zhang (2016)
	Rat	Traumatic brain injury	60 μg/kg	3- and 12-h following injury	IP	Decreases TBI-induced pulmonary injury	Liu et al. (2017)
Epobis	Rat	EAE (MBP and CFA)	10 mg/kg	10 days after EAE induction	SC (5 days)	Delays the clinical signs of EAE, improves social memory	Dmytriyeva et al. (2016)
Epotris	Rat	Kainic acid-induced seizures	10 mg/kg	2–48 h pre	SC (single dose)	Attenuates seizures, decreases neurodegeneration and mortality	Pankratova et al. (2010)
	Rat	Status epilepticus	10 mg/kg	2–48 h pre	SC (single dose)	Attenuates microglial activation in the thalamus and modulates hippocampal neurogenesis	Zellinger et al. (2011)

Continued

Table 5 In Vivo Neuronal Effects of EPO Mimetic Peptides—cont'd

Peptide	Model Organism	Disease Model	Dose	Time	Administration Route	Outcome	Reference
JM-4	Mouse (SJL/J, C57BL/6)	EAE (PLP peptide/MOG peptide 35–55)	250 μg/kg/day	24–48 h after the onset of neurologic signs	IV	Reduces clinical sign of EAE, demyelination, axonal damage, and immune cells in peripheral lymphatics	Yuan et al. (2015)
	Mouse	TBI (cortical impact injury)	10 μg/animal	15 min, 3, 9, or 24 h after TBI	IP (repeated)	Suppresses neuron death, improves neurological deficits	Wang et al. (2016)
MK-X	Mouse	Cerebral ischemia	3.6 μg/kg	2 h after	IP (single)	Decreases the infarction area, crosses the BBB	Yoo et al. (2017)

also inhibits Ca^{2+}- and chemical ischemia-induced Glu release from cerebellar granule neurons (CGNs) via EPOR–JAK2 activation (Kawakami et al., 2000). EMP-1 suppressed neuronal death induced by the chemical ischemia for 20 min in hippocampal slices culture.

7.2 Epopeptide AB

Epopeptide AB was identified by Campana et al. in 1998 (Campana et al., 1998). Epopeptide AB has 17 aa residues in the predicted A–B loop of EPO. It dose-dependently induces neurite outgrowth and choline acetyltransferase activity of neuroblastoma cells with the maximal dose of 250 pg/mL. Functional study with EPOR antibody confirmed that differentiation induction with Epopeptide AB is EPOR mediated. This study also exhibited neuroprotective effect against serum starvation-induced neuronal cell death. Epopeptide AB had no effect on cell proliferation in EPO responsive mouse spleen cells and human erythroleukemic TF-1 cells, suggesting lack of erythropoietic activity of this peptide. 20 μg/mL Epopeptide AB injection into gluteus muscle induced motor endplate sprouting in vivo (Campana et al., 1998).

The continuous infusion of Epopeptide AB with minipump for 7 days into the lateral ventricle of mongolian gerbils just after ischemia/reperfusion protected neurons from ischemic damage (Nagao et al., 2005). Epopeptide AB increased the response latency in the step-down passive avoidance task and hippocampal CA1 neuron density in ischemic injury.

7.3 ARA 290

ARA 290 [also called as helix B surface peptide (HBSP)] is an 11-mer peptide, computationally modeled based on the three-dimensional structure of EPO's helix B, responsible for EPO's interaction with the EPOR/βCR heteromer (Brines et al., 2008). It was shown that ARA 290 lacks hematopoietic properties of EPO (Brines et al., 2008). To gain favorable storage conditions, HBSP was modified by the spontaneous cyclization of N-terminal glutamine. The pharmaceutical product pyroglutamate HBSP (p-HBSP) is stable at least 2 years at 4°C or up to 12 months at 25°C (Collino et al., 2015).

ARA 290 has been extensively investigated by in vitro, in vivo, and clinical studies. The first in vitro study which shows neuroprotective effects was examined by Brines et al. in motor neurons. ARA 290 at 1.8 nM concentration inhibited kainic acid (KA)-induced excitotoxic cell death (Brines et al., 2008). In the same study, Brines et al. also reported the protective roles of ARA 290 in middle cerebral artery occlusion injury model. In this study,

administration of peptide 1 hour after occlusion adequately reduced infarct volume. Regarding traumatic brain injury (TBI), a recent study reported that ARA 290 also prevented from acute lung injury following TBI (Liu et al., 2017). In this study, ARA 290 was administered 3 and 12 h after weight drop-induced TBI model in rats and the author found decreased total bronchoalveolar lavage fluid protein, attenuated pulmonary injury, reduced CD68+ macrophages, and inflammatory markers.

Brines et al. studied the neuroprotective roles of ARA 290 in sciatic nerve compression injury in sciatic nerve compression model. Single IV injection of ARA 290 immediately after compression successfully reduced compression-induced damage. In addition, it has been reported that ARA 290 was able to prevent mechanical allodynia in both the spared nerve injury model, based on sciatic nerve surgical transection, and neuritis model in rats (Pulman et al., 2013). In this study, ARA 290 was administered intraperitoneally for 4 days post-op. In peripheral nervous system injuries, local delivery methods such as slow releasing with implantable pumps may be an alternative for a better efficiency.

In a model of status epilepticus in rats, ARA 290 notably promoted neuronal differentiation and survival, and attenuated cognitive deficits in epileptic animals (Seeger et al., 2011). In this model ARA 290 was given 2–5 min following status epilepticus by both IV and SC ways. Subcutaneous administration was repeated 24 and 72 h following status epilepticus.

ARA 290 has also been investigated in animal models of neuropathy. The first study in neuropathy models showed the reduction in the microglial activation and therefore the suppression of the neuroinflammatory response and chronic neuropathic pain (Swartjes et al., 2014). In this study, ARA 290 was tested in spared nerve injury-induced chronic neuropathic pain and administered intraperitoneally on 1, 3, 6, 8, and 10 days post-op. In another neuropathic pain model induced by capsaicin, ARA 290 was administered 15 min before capsaicin and inhibited transient receptor potential vanilloid 1 (TRPV1) channel activity, which serves as the receptor for capsaicin (Zhang et al., 2016). Moreover, ARA 290 caused the relief of capsaicin-induced mechanical hypersensitivity. The effect of ARA 290 was also investigated in neuritic dystrophy and neuropathy in Akita diabetic mouse (Schmidt et al., 2011). Akita mice, which has spontaneous point mutation of insulin 2 gene, is a genetic model of type 1 diabetes, which became diabetic at 3–4 weeks of age. ARA 290 treatment of these mice started at age 18–19 weeks via SC route. After 7 weeks, the effect of ARA 290 on metabolic parameters and sympathetic ganglia was evaluated with measurement of body weight, blood glucose, hemoglobin A1c (HbA1c), and histological analysis of sympathetic ganglia.

ARA 290 reduced neuritic dystrophy without affecting metabolic parameters and neurodegeneration (Liu et al., 2014).

There are also solid data on the neuroprotective effects of ARA 290 in experimental autoimmune encephalomyelitis (EAE) model, mimicking the human multiple sclerosis. Therapeutic administration of ARA 290 to rats reduced severity and prevented demyelination in the EAE, presumably by suppressing the expression of inflammatory cytokines in the spinal cord, repressing lymphocyte proliferation, and modifying T helper cell differentiation outcomes (Chen et al., 2014). ARA 290 also suppressed clinical findings of experimental allergic neuritis (EAN), enhanced nerve regeneration and remyelination, and inhibited nerve inflammation (Liu et al., 2014).

There is some evidence showing the beneficial effects of ARA 290 in animal models of diabetes mellitus and its complications, such as diabetic retinal edema models in rats. Regarding diabetic retinal edema, after confirming diabetes in rats, intraperitoneal ARA 290 treatment has been applied for 3 weeks and 5 days per week (Brines et al., 2008). In this model, ARA 290 treatment effectively prevented development of retinal edema in diabetes. Reengineered ARA 290 into elastin biopolymers accelerated wound healing via inhibiting apoptosis of keratinocytes and increasing angiogenesis in the wound bed in diabetic mice (Devalliere et al., 2017).

The promising data obtained from preclinical studies encouraged researchers to launch some clinical studies for neurological disorders (Table 6). The initial clinical studies were commenced in patients with sarcoidosis or type 1 or 2 diabetes mellitus. The first clinical study on ARA 290 was published by Heij et al. in 2012 (Heij et al., 2012). This double-blind, randomized, placebo-controlled trial was performed with 22 sarcoidosis patients. 2 mg intravenous ARA 290 was administered on Monday, Wednesday, and Friday for four consecutive weeks. After 1 week of treatment, a significant reduction in pain scores was observed. At the end of treatment period, small fiber neuropathy screening list scores were improved, while brief pain inventory score and fatigue assessment scale scores were reduced. No safety issues or side effects were reported by clinical or laboratory assessments. In another study on sarcoidosis patients, 26 subjects were recruited and asked to perform injection of 4 mg ARA 290 for 28 days (Dahan et al., 2013). As opposed to first trial, subcutaneous formulation of ARA 290 was utilized to allow daily self-injection of drug. This study resulted in increased density of both dermal and retinal nerve fibers in the ARA 290-treated group. Furthermore, a significant improvement in neuropathic pain and autonomic symptoms, with no effect on hemoglobin concentrations, was reported.

Table 6 Clinical Studies With ARA 290

Patient Group	Study Type	*N* (D/P)	Dose	Route	Time to Treatment	Follow-Up	Methods	Outcome	Side Effects	Reference
Small fiber neuropathy in sarcoidosis	Single-site, double-blind, placebo-controlled	22 (12/10)	2 mg	IV	3 days per week for 4 weeks		Self-report, QST	Improves symptoms and quality of life, recovery in QST	None reported	Heij et al. (2012)
Small nerve fiber loss and damage in sarcoidosis	Single-site, double-blind, placebo-controlled	38 (21/17)	4 mg	SC	Daily for 28 days	12 weeks	Self-report, QST, skin biopsy, 6-min walk test, CCM	Increases median nerve fiber area in retina and skin, and small fiber neuropathy screening list score, elevation in thermal pain threshold, and exercise capacity	Weight loss (1 patient)	Dahan et al. (2013)
Type 2 diabetes with neuropathic symptoms	Single-site, double-blind, placebo-controlled	48 (24/24)	4 mg	SC	Daily for 28 days	56 days	Self-report, QST, CCM	Increases mean corneal fiber density, improves HbA1c and lipid profiles, decreases neuropathic symptoms and QST	No difference between ARA 290 and placebo	Brines et al. (2015)

Healthy volunteers	Double-blind, randomized, parallel group	36	2 mg	IV	Single	After 6–7 days	Affective symptoms and mood states evaluation and fMRI	Suppresses neural responses to happy faces in fusiform gyrus and recognition of happy and disgust facial expressions, enhances categorization of positive and negative words and attention to positive emotional pictures	None reported	Cerit et al. (2015)
Sarcoidosis-associated small nerve fiber loss and neuropathic pain	Double-blind, randomized, placebo-controlled, two centers	64 (48/16)	1, 4, and 8 mg	SC	Daily for 28 days	Week 2 and 4	Self-report, QST, CCM, retinal tomography, skin biopsy	Increases small nerve fiber abundance in the cornea and skin, fiber length in 4 mg group, improves functional capacity	Suicidal ideation (1 patient), injection site pain, diarrhea, fatigue, nausea, headache	Culver et al. (2017)

CCM, corneal confocal microscopy; *QST*, quantitative sensory testing.

Furthermore, a double-blind, randomized, and controlled trial of subcutaneous ARA 290 was performed in type 2 diabetes with neuropathic symptoms (Brines et al., 2015). In this study, 42 patients were recruited and 4 mg subcutaneous ARA 290 was injected daily for 28 days. Neuropathic symptoms, HbA1c levels, and lipid profiles were monitored throughout the 56-day observation period. ARA 290 administration resulted in decrease in PainDetect questionnaire scores and improvement of HbA1c and lipid profiles. Their observations suggest that ARA 290 may improve metabolic control and diminish neuropathic symptoms in type 2 diabetes patients. Similar to these findings, parent protein EPO protects pancreatic β cells, increases angiogenesis in the islet, and reduces inflammation (Choi et al., 2010). EPO is also involved in the regulation of glucose and energy metabolism through the hypothalamic–pituitary axis (Dey & Noguchi, 2017).

The another published clinical study on ARA 290 was performed to test antidepressant properties. In this trial, 36 healthy volunteers were admitted and single dose of 2 mg intravenous ARA 290 was injected to ARA 290-receiving group (Cerit et al., 2015). Patients were examined by categorization, free recall, recognition, visual probe tasks, and functional magnetic resonance imaging or functional MRI (fMRI) analysis. The results of fMRI analysis suggest that ARA 290 caused lowered neural responses to happy faces in fusiform gyrus and recognition of happy and disgust facial expressions. Furthermore, ARA 290 enhanced categorization of positive and negative words and increased attention to positive emotional pictures. Also, in this study no potential safety issues and side effects were reported.

The effect of ARA 290 (cibinetide as marketing name) was evaluated in sarcoidosis-associated small nerve fiber loss which quantified with corneal confocal microscopy (Culver et al., 2017; Oudejans, Niesters, Brines, Dahan, & van Velzen, 2017; van Velzen et al., 2014). There, ARA 290 enhanced small nerve fiber in the cornea and skin (Culver et al., 2017).

The results of a phase II clinical trial (EudraCT number 2015-001940-12) in diabetic macular edema with ARA290 have not been published yet (EU Clinical Trials Register, 2017).

7.4 Epobis

Epobis is composed of an 18-mer sequence motif that mimics the C-terminal part of the AB loop of EPO. Epobis was synthesized as a tetramer by Pankratova et al. (2012). Epobis induced neurite outgrowth in CGNs and hippocampal neurons via EPOR with an optimal concentration of 1 μM.

It stimulates neurite outgrowth in primary cultures of rat motor neurons at a lower dose (maximal stimulation at 0.33 μM) (Dmytriyeva et al., 2016). STAT5 signaling pathway is activated in hippocampal neurons by Epobis (Pankratova et al., 2012). It also protected primary neurons against low potassium- and KA-induced apoptotic cell death (Pankratova et al., 2012). Apart from neurons, Epobis alters microglial responses. Treatment of primary rat microglia with 0.9 μM Epobis decreased lipopolysaccharide (LPS)-induced tumor necrosis factor-alpha (TNF-α) secretion (Dmytriyeva et al., 2016). Peptide was detectable in cerebrospinal fluid (CSF) 2 h after a single SC injection of Epobis, suggesting that it can cross BBB. Epobis treatment at 10-mg/kg dose did not decrease clinical signs and microglial activation in EAE induced by complete Freund's adjuvant (CFA), but it delayed onset of EAE clinical findings. Additionally, Epobis improved social memory in healthy adult rats at long-term period.

7.5 Epotris

Epotris is a tetrameric 20-amino acid-containing peptide which corresponds to helix C region of EPO (Pankratova et al., 2010). Epotris did not demonstrate hematopoietic effect both in vitro and in vivo. It induced neurite outgrowth both in hippocampal neurons and CGNs with an optimal concentration of 0.3 mM. Functional studies by using EPOR short-hairpin RNA and JAK2 inhibitor showed that neurotrophic effect of Epotris is mediated by EPOR and the downstream JAK2–STAT5 signaling pathway. Epotris also protected primary neurons against low potassium- and KA-induced neuronal cell death.

Systemically administered biotinylated Epotris was also detected in CSF 2 h after SC injection, suggesting it can cross the BBB (Pankratova et al., 2010). Epotris exhibited a significant delay in seizure onset, decreased frequency of high-grade seizures, and mortality in a mouse model of KA-induced seizure (Pankratova et al., 2010). Peptide protected neurons against KA-induced degeneration and attenuated status epilepticus-associated expansion of the hippocampal neuronal progenitor cells (Pankratova et al., 2010; Zellinger et al., 2011). Epotris also diminished the microglial activation in the thalamus (Zellinger et al., 2011).

7.6 JM-4

JM-4 is a 19-mer peptide derived from the AB loop of EPO (Yuan et al., 2015). JM-4 blocked amyloid beta 25–35 ($A\beta_{25–35}$)-induced apoptotic cell death in PC12 cell line in vitro (Yuan et al., 2015). Peptide passed BBB after

IV injection of radiolabeled 3H-JM4 in normal mouse (Wang et al., 2016). JM-4 peptide demonstrated neuroprotective effect in EAE and TBI model (Wang et al., 2016; Yuan et al., 2015). JM-4 therapy for 7 days delayed the onset of disease and reduced clinical signs of both proteolipid protein (PLP)- and myelin oligodendrocyte glycoprotein (MOG)-induced EAE (Yuan et al., 2015). Histopathological analysis showed that JM-4 prevented demyelination and axonal damage. JM-4 treatment suppressed inflammatory cells in peripheral lymphatics and proinflammatory cytokine production by MOG peptide-specific T cells. JM-4 therapy decreased neuronal cell death and lesion size, and improved functional recovery in TBI (Wang et al., 2016).

7.7 MK-X

MK-X was derived from α-helix C in the low-affinity binding site of EPO by Yoo et al in 2016 (Yoo et al., 2016). MK-X protected PC12 cells from hydrogen peroxide-induced cell death with an optimal concentration of 1.2 ng/mL. MK-X peptide also decreased reactive oxygen species (ROS) production of PC12 cells. It activated ERK1/2 and Akt signaling pathways, inhibited p38 phosphorylation, but did not lead to JAK2–STAT5 activation under the oxidative stress conditions. It also inhibited mitochondrial dysfunction and caspase-dependent and -independent cell death caused by glutamate-induced oxidative stress (Yoo et al., 2017). Both JAK2–STAT5 and ERK1/2 signaling pathways contribute to its neuroprotective effects. Single injection of MK-X (3.6 μg/kg) reduced infarction area in mouse model of ischemic stroke induced by middle cerebral artery occlusion (Yoo et al., 2017).

7.8 NP1 and NP2

NP1 is a 28-mer peptide originated from helix B region of EPO. NP2 is a shorter peptide (18 mer) originated from helix A region (Colella et al., 2011). Gene delivery of this EPO mimetic peptide into subretina exhibits protective effect against light-induced and genetic retinitis pigmentosa degeneration. Subretinal delivery of NP1 and NP2 gene encoding vectors did not alter hematocrit levels in rats and mice, suggesting that these peptides do not have erythropoietic activity in vivo.

7.9 AF41676 and AF43136

AF41676 and AF43136 are dimeric nonerythropoietic peptides, which have structural relationship to the peptide portion of peginesatide (Kiss et al., 2016).

Intravenous administration of AF41676 and AF43136 5 min before the onset of reperfusion decreased infarct size in acute myocardial infarction in rats. The effect of these peptides in the CNS has not been studied yet.

7.10 Cyclic Helix B Peptide

Cyclic helix B peptide (CHBP) is a proteolysis-resistant novel peptide which derived from HBSP (Yang et al., 2014). Its metabolic stability and half-life are higher than HBSP. CHBP protected kidney tissue against ischemia reperfusion and prevented renal allograft rejection (Yang et al., 2014, 2015). It also exhibits protective effects against mitochondrial dysfunction and apoptosis in mesenchymal stem cells via Nrf2/Sirt3/FoxO3a pathway activation (Wang et al., 2017). Neuroprotective effects of CHBP have not been evaluated.

8. FUTURE PERSPECTIVES AND CONCLUSION

Peptide-based drugs continue to attract increasing interest due to their easy synthetic production, controlled metabolism, and specific and high-affinity binding capacities. Moreover, the costs and immunogenic features of peptides are lower than protein drugs. Despite these favorable properties, their poor pharmacokinetic features (low permeability, bioavailability, stability, and oral bioavailability) and lack of appropriate routes of administration are disadvantages of the therapeutic use of peptides. Recent scientific and technological advancements in peptide drug discovery contribute to the recovery of suboptimal properties of peptide-based drugs. New tools available for the computational design of peptide drugs, cell-free high-throughput screening and production methods, peptidomics approaches, novel formulation technologies, and innovative drug delivery strategies such as nanocarriers and cell-penetrating peptides will be helpful in improving the drug-like properties of the peptides, while maintaining their target potency. Another strategy for enhancing the effects of peptide drugs could be the design and development of multifunctional peptides. Multifunctional peptides that target multiple EPO receptors may exhibit strong beneficial effects. Combined therapy with EPO mimetic peptides and nonpeptide selective activator of EPO receptor, STS-E412, could also enhance their therapeutic effects (Miller et al., 2015). Accurate identification of dose, dosing frequency, duration of therapy, therapeutic window, and administration timing in preclinical studies improve the success of peptide drugs in clinical trials. Pharmacogenomic evaluation, personalized drug dosage, and monitorization of pharmacokinetics and pharmacodynamics of peptides are helpful approaches for succeeding with peptide clinical trials. As an important

basic research area, EPO mimetic peptide drugs may be used to clarify the activation mechanisms of EPOR and EPOR-associated receptors.

All in all, peptide-based drugs are promising means in the treatment of neurodegenerative and neuroinflammatory disorders with unknown cure.

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