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Design, synthesis, and activity evaluation of novel erythropoietin mimetic peptides

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ABSTRACT

The approval of the erythropoietin (EPO) mimetic peptide drug peginesatide in 2012 was a breakthrough for the treatment of secondary anemia. However, due to severe allergic reactions, peginesatide was recalled a year later. In this study, 12 novel peptides were designed and synthesized by substituting specific amino acids of the monomeric peptide in peginesatide, with the aim of obtaining new EPO mimetic peptides with higher activities and lower side effects than the parent compound. Their cell proliferation activities were evaluated, and the structure-activity relationships were analyzed. Five compounds had equal cell proliferation activity to the control peptide. Among them, one compound showed a higher *in vivo* activity than the control peptide, with no obvious side effects.

Secondary anemia is a symptom of many diseases such as cancer and chronic renal failure, and its main cause is erythropoietin (EPO) deficiency.[1–5] At present, drugs for the treatment of secondary anemia have mostly focused on recombinant human erythropoietin (rHuEPO).[6–10] Meanwhile, there are many new anti-anemic drugs under clinical development, including hypoxia inducible factor (HIF) stabilizers, activin traps and EPO gene therapy.[11] Several rHuEPO drugs have been approved and widely used in clinic. However, rHuEPO may produce antibodies that interact with endogenous EPO due to their sequence homology, resulting in pure red cell aplasia (PRCA).[12]

In 1996, a series of EPO mimetic peptides (EMPs) were obtained by Wrighton and his coworkers using random phage display peptide libraries and selective affinity purification methods.[13] The signaling pathways activated by these peptides appeared to be identical to those induced by the natural ligand, but the amino acid sequences of these peptides were not found in the primary sequence of EPO. The structure-activity relationships were analyzed by Ala scanning, and the activity was greatly enhanced through conjugation of two monomeric peptides.[14,15] In 2012, the PEGylated EPO mimetic peptide peginesatide was approved by the FDA for the treatment of patients with renal failure-induced anemia. Due to the absence of sequence homology with endogenous EPO, peginesatide does not cause PRCA and can even cure patients with PRCA.[16–18] Regrettably, peginesatide was soon recalled in 2013 due to severe allergic reactions.[19–21]

The structure of peginesatide includes three parts, as shown in Fig. 1: two identical monomeric peptides, a linker for conjugation, and two PEG chains. The activity was greatly enhanced through conjugation of the monomeric peptides, and the pharmacokinetic behavior was improved by two PEG chains, prolonging the half-life.[15,22,23] However, pegylation is possible one of the severe allergic reactions causal factors.[19] Other possible causes for peginesatide anaphylaxis include the use of phenol and the particle formation in multi-use vials.[20,21]

The structure-activity relationship (SAR) of peginesatide has not been reported yet. The conjugation of two monomeric peptides would greatly enhance the activity as a none covalent peptide dimer was seen spanning the cleft between two molecules of EPO receptor extracellular domain.[15] However, we found the tendency of the conjugate potency was consistent with that of the monomeric peptides, which were much easier to synthesize and purify, as well as the SAR study. In this work, the SAR of the monomeric peptides of peginesatide was analyzed by site-specific substitutions of amino acids. We tried to find novel EMPs with higher activities and lower side effects by using another possible way to solve the existing problem. In EMPs, 10-Pro participates in the formation of beta turns, playing an important role in the interaction of the peptide with the receptor; and 13-Trp or other aromatic amino acids form a hydrophobic core with 93-Phe, 150-Met, and 205-Phe of the EPO receptor.[14,24] Therefore, 10-Pro and 17-Pro were first chosen to be replaced by N-Me Gly and N-Me Ala, both of which were N-Me amino

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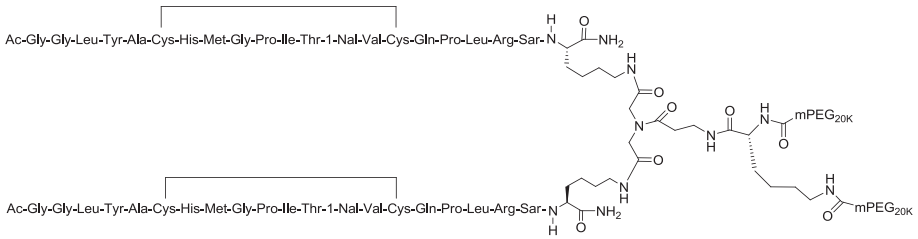

Fig. 1. Structure of peginesatide.

Table 1
Sequences and cell proliferation activities of new EPO mimetic peptides.

Ac-GGLX1X2CHMGX3ITX4VCQX5LX6(MeG)K-NH_2

Entry	Peptide sequence	Rationals for substitutions	EC_{50} (nM)
rHuEPO	–	–	0.015
H1	Ac-GGLYACHMGPIT(1-Nal)VCQPLR(MeG)K-NH_2	–	11
H2	Ac-GGLYACHMG**(MeA)**IT(1-Nal)VCQPLR(MeG)K-NH_2	N-Me amino aicds	26
H3	Ac-GGLYACHMG**(MeG)**IT(1-Nal)VCQPLR(MeG)K-NH_2	N-Me amino aicds	31
H4	Ac-GGLYACHMGPIT(1-Nal)VCQ**(MeA)**LR(MeG)K-NH_2	N-Me amino aicds	12
H5	Ac-GGLYACHMGPIT(1-Nal)VCQ**(MeG)**LR(MeG)K-NH_2	N-Me amino aicds	42
H6	Ac-GGLYACHMGPIT**F1**VCQPLR(MeG)K-NH_2	Aromatic amino acids	540
H7	Ac-GGLYACHMGPIT**F2**VCQPLR(MeG)K-NH_2	Aromatic amino acids	240
H8	Ac-GGLYACHMGPIT**F3**VCQPLR(MeG)K-NH_2	Aromatic amino acids	540
H9	Ac-GGLYACHMGPIT**(2-Nal)**VCQPLR(MeG)K-NH_2	Aromatic amino acids	540
H10	Ac-GGLYACHMGPIT(1-Nal)VCQPL**(Cit)**(MeG)K-NH_2	Isosterism	31
H11	Ac-GGL**Y1**ACHMGPIT(1-Nal)VCQPLR(MeG)K-NH_2	D-amino acids	NA
H12	Ac-GGLY**A1**CHMGPIT(1-Nal)VCQPLR(MeG)K-NH_2	D-amino acids	NA
H13	Ac-GGLYACH**M1**GPIT(1-Nal)VCQPLR(MeG)K-NH_2	D-amino acids	NA

Ac: acetyl; 1-Nal: 1-naphthyl alanyl; 2-Nal: 2-naphthyl alanyl; MeG: N-methyl glycyl; MeA: N-methyl alanyl; F1: 4-Br phenyl; F2: 4-Cl phenyl; F3: 2-F phenyl; Cit: citrullyl; Y1: D-Tyr; A1: D-Ala; M1: D-Met; NA: no activity.

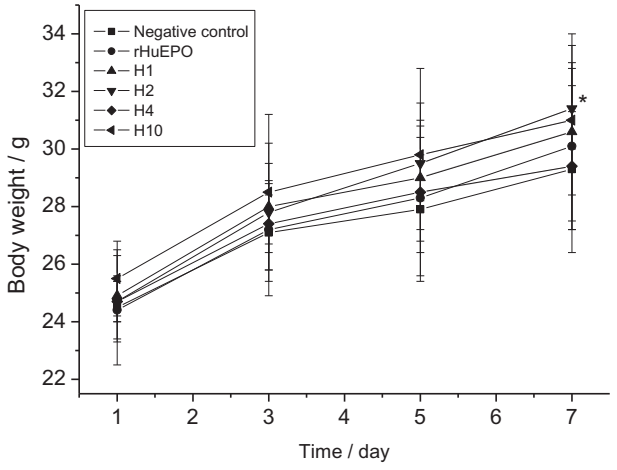

Fig. 2. Body weight (g) changes of mice in six different groups (Mice were administered subcutaneously once every day for 7 days. The dosage of rHuEPO was 125×10^{-6} μmol/kg, while the dosages of 4 EMPs were all 2.1 μmol/kg. $^{*}p < 0.05$ compared with the negative control group).

acids and can promote the formation of beta turns. Next, 13-1-Nal was substituted with other aromatic amino acids like halogen-substituted Phe and 2-Nal. Finally, 4-Tyr, 5-Ala, and 8-Met were replaced with the corresponding D-amino acids; and 19-Arg, which contains a guanidine group, was replaced with citrulline, which contains a urea group. We hoped that the compound stability against enzymes would be improved by introducing unnatural amino acids. Altogether, 12 new compounds were designed and synthesized, and their cell proliferation activities were measured in human TF-1 erythroblasts with a high expression of EPO receptor (Table 1). The *in vivo* activities of the analogues with high cell proliferation activities were further evaluated in healthy mice.

All peptides were synthesized by solid-phase synthesis using Fmoc chemistry on Rink amide resin with HBTU/HOBt/DIEA as condensation reagents. Crude linear peptides without purification were cyclized in 20% DMSO/H_2O solution at a concentration of 0.1 mM. After cyclization, the products were purified by medium-pressure liquid chromatography and lyophilized. High-resolution mass spectra were used to identify the molecular weights, and HPLC was applied to determine the purity.

Next, the cell proliferation activities were measured in human TF-1 erythroblasts with a high expression of EPO receptor.[25,26] As shown in Table 1, the monomeric peptide of peginesatide (H1) exhibited an EC_{50} value of 11 nM, and five new compounds showed similar activity to H1, with EC_{50} values ranging from 12 nM to 42 nM. H6–H9 showed 20–50 × lower activity, and H11–H13 showed no activity.

In EMPs, 10-Pro participates in the formation of beta turns, playing an important role in the interaction of the peptide with the EPO receptor.[14] As shown in Table 1, the replacement of 10-Pro with N-Me Ala (H2) or N-Me Gly (H3) led to a decrease of activity by about three times. This may be because Pro is more helpful in promoting beta turn formation compared to N-Me Ala and N-Me Gly. Similarly, replacement of 17-Pro with N-Me Ala (H4) led to a slightly reduced activity, and substitution with N-Me Gly (H5) led to a decrease of activity by about three times.

The replacement of 13-1-Nal with other aromatic amino acids, including Phe(4-Br), Phe(4-Cl), and Phe(2-F), resulted in analogues H6, H7, and H8, respectively. The aromatic amino acid at position 13 in EMP1 has been reported to form a hydrophobic core with 93-Phe, 150-Met, and 205-Phe of the EPO receptor.[24] However, the cell proliferation activities of H6, H7, and H8 were reduced by 20–50 times compared to that of H1. This finding may be due to the decrease of hydrophobicity with the amino acid substitution, compared to that of 1-Nal. In

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