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Computational Design and Experimental Characterization of a Novel β -Common Receptor Inhibitory Peptide

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Abstract

In short-term animal models of ischemia, erythropoietin (EPO) signaling through the heterodimeric EPO receptor (EPOR)/ β -common receptor (β CR) is believed to elicit tissue protective effects. However, large, randomized, controlled trials demonstrate that targeting a higher hemoglobin level by administering higher doses of EPO, which are more likely to activate the heterodimeric EPOR/ β CR, is associated with an increase in adverse cardiovascular events. Thus, inhibition of long-term activation of the β CR may have therapeutic implications. This study aimed to design and evaluate the efficacy of novel computationally designed β CR inhibitory peptides (β IP). These novel β IPs were designed based on a truncated portion of Helix-A from EPO, specifically residues 11 to 26 (VLERYLLEAKEAEKIT). Seven novel peptides (P1 to P7) were designed. Peptide 7 (P7), VLERYLHEAKHAEKIT, demonstrated the most robust inhibitory activity. We also report here the ability of P7 to inhibit β CR-induced nitric oxide (NO) production and angiogenesis in human umbilical vein endothelial cells (HUVECs). Specifically, we found that P7 β IP completely abolished EPO-induced NO production. The inhibitory effect could be overcome with super physiological doses of EPO, suggesting a competitive inhibition. β CR-induced angiogenesis in HUVEC's was also abolished with treatment of P7 β IP, but P7 β IP did not inhibit vascular endothelial growth factor (VEGF)-induced angiogenesis. In addition, we demonstrate that the novel P7 β IP does not inhibit EPO-induced erythropoiesis with use of peripheral blood mononuclear cells (PBMCs). These results, for the first time, describe a novel, potent β CR peptide inhibitor that inhibit the actions of the β CR without affecting erythropoiesis.

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Keywords

β -common receptor; CD131; CSF2RB; Erythropoietin; EPO; Competitive Inhibitor

1. Introduction

Erythropoietin (EPO) and the cytokines human interleukin (IL)-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) receptors are members of the hematopoietin receptor superfamily. These receptors signal through heterodimers of a cytokine-specific alpha chain and the common β chain or the β -common receptor (β CR) [1, 2]. The human β CR is a glycoprotein with an extracellular region composed of 427 amino acids and a 437 amino acid intracellular domain [3–5]. The ectodomain of the β CR exists as an intertwined homodimer resulting in an arch like structure the overall structure consists of four such domains [4]. The β C-subunits that intertwine are interlocked by swapping of the β -strand of domain 1 of one β C-subunit and domain 3 of the other subunit [1]; domain 4 is positioned adjacent to the membrane. Although initially the β CR was believed to be only essential for high binding efficiency of the respective cytokines [6], it is now clear that the β CR is the principal signal transducing subunit and has an important functional role.

Binding of the ligand to its cognate alpha-chain results in recruitment of the β CR to form a heterodimer, which initiates the downstream signaling cascade. The β CR is thought to activate three principle signaling pathways: janus kinase/signal transducer and activator of transcription (JAK/STAT), mitogen-activated protein kinase (MAPK) pathway, and phosphatidylinositol 3-kinase (PI3K) pathway [7–10] to regulate a variety of cellular processes including proliferation and differentiation. We have shown that activation of the β CR, occurring with high doses of erythropoietin, can result in induction of nitric oxide (NO) and angiogenesis via vascular endothelial growth factor receptor-2 (VEGFR-2) [11]. Excessive VEGFR-2 activation has been shown to accentuate inflammation [12] and atherosclerosis [12–14] in animal models. However, EPO has been shown to exert tissue protection in states of ischemia [15] and sepsis [16, 17], which was suggested to be dependent on the presence of the β CR [17]. These data suggest that although in the short-term activation of the β CR might have tissue protective effects, over the long run activation of the β CR may have adverse cardiovascular effects and may promote cancer metastasis due to the β CR's ability to increase angiogenesis via upregulation of VEGF and its interaction with VEGFR-2 [11]. Upregulation of IL-3 receptor and constitutive phosphorylation of the β CR serine 585 has been demonstrated in patients with acute myeloid leukemia (AML). Further, IL-3 is thought to amplify inflammation in sepsis [18]. These data suggest that inhibition of the β CR could have therapeutic utility to mitigate the side effects witnessed with long term β CR activation on the cardiovascular system, cancer metastasis, and in sepsis.

In the present study, we describe the computational design of a novel β CR inhibitory peptide (β IP), which is a potent and selective inhibitor of the β CR in vitro, and the experimental characterization of the peptide effects on erythropoiesis with use of peripheral blood mononuclear cells (PBMCs). Taken together, this study provides direct evidence that the P7

β IP is a competitive peptide inhibitor of the β CR by inhibiting nitric oxide (NO) and angiogenic potential of the β CR without affecting erythropoiesis.

2. Materials and Methods

2.1. Tissue Culture and Reagents

All tissue culture reagents and human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Basel, Switzerland). Vascular endothelial growth factor (VEGF) was obtained from R&D (Minneapolis, MN). Interleukin (IL)-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were obtained from PreProtech (Rocky Hill, NJ) and Human recombinant erythropoietin (EPO) from Zydus (Cadila, India). EPO stimulating peptide (ESP), which was derived from a 11-amino acid peptide sequence of the Helix-B of EPO which interacts with the β CR [19] also known as either pHBSF or ARA290, and the β CR inhibitory peptide (β IP) were synthesized by the core facility.

2.2. Computational Details

Structural alignments were performed on the jCE/jFATCAT Structure Alignment Server V. 2.11.

Free energy calculations were performed using GROMACS [20] with the same protocol that was previously developed by Dahan et al. to design phospholipid (PL) based prodrugs for inflammatory bowel disease drug targets [21]. The proteins and peptides were modeled with the AMBER ff-03 force field and the TIP3P model described water molecules. All calculations were done in a periodical box of water with dimension 60×60×60 Å. Free energy calculations were done using the Thermodynamic Integration [22, 23] and Umbrella Sampling/WHAM methods [24].

2.3. Design of Novel β CR Peptides

Due to the lack of X-ray structures for the β CR/EPO complex, we have used the β CR/GM-CSF interface region from the X-ray structure of the cytokine receptor complex (PDB code: 4NKQ). The jCE/jFATCAT Structure Alignment Server V. 2.11 was used for the sequence alignment (Figure 1A, top) and structural overlay (Figure 1A, bottom left) between a mutant EPO (PDB code: 1EER, chain A), which the glycosylation site asparagine residues have been replaced with lysine, [25] and GM-CSF (PDB code: 4NKQ, Chain C) [26] in complex with β CR. We found that Helix-A in EPO aligns well with helix-A in GM-CSF that binds strongly to β CR. From the x-ray structure of GM-CSF bound to β CR (both 2GMF.pdb and 3CXE.pdb), it is clear to see that helix-A of GM-CSF interacts with β CR. In particular, the β CR residues Y39, Y365 and Y421 (in the D1/D5/D6 elbow region) interact strongly with helix-A of GM-CSF, which is the basis for the alignment we used here. Additionally, E21 has been identified as a critical residue on GM-CSF. By mutating E21, GM-CSF turns into an antagonist of the β CR [3]. Based on this structural analysis, we extracted a portion of Helix-A from EPO consisting of 16-amino acids (VLERYLLEAKEAEKIT) starting from residues 11 to 26 as a template to design seven novel β IPs. We introduced single and double point mutants to the 16-amino acids peptides from EPO and calculated free energy changes associated with the mutations. Computational models of the protein and peptides were

prepared using the GROMACS software package [20] and the procedure is explained in detail elsewhere [21].

The Free Energy Perturbation (FEP) [27] calculations using the Thermodynamic Integration (TI) method [22, 28] were used to compute the difference in free energy (ΔG) between the wild-type and mutant states, where negative (ΔG) values indicate the increase in binding affinity of the peptide to the protein (β CR). This value is a combination of the peptide states, both in the solvent (water box) as well as bound to the protein (β CR) environments.

To save computational cost, we used a truncated model of β CR that consists of 105 residues (from H331 to T436) and a truncated part of Helix-A from EPO, which include a portion of the low affinity binding site (site 2) for the EPOR, with 16-amino acids (VLERYLLEAKEAEKIT) from residues 11 to 26 to perform the FEP-TI calculations. We introduced several charged, polar, hydrophobic and hydrophilic amino acids to replace Leucine (L) at site-7 and found that the singly protonated Histidine (at the delta nitrogen atom) (L7H) establishes a strong π - π stacking interaction with the singly protonated H367 (at the N δ atom) in β CR, which, in turn, enhanced the binding affinity of the peptide ($\Delta G = -1.08 (\pm 0.19)$ kcal.mol⁻¹) to the protein. Therefore, we chose the optimized VLERYLHEAKEAEKIT peptide model as the starting structure to introduce double mutants and we built seven β IP models with different amino acid combinations at sites 7 and 11 involving residues E, H, Y and N (Figure 1B).

We found that substitution of Glutamic acid (E) with Histidine (H, singly protonated at the N δ atom) at site 11 (E11H) formed an edge-to- π interaction with Y365 and H367 residues in β CR (Figure 1, bottom right), which, in turn, doubled the binding affinity of the peptide ($\Delta G = -2.57 (\pm 0.74)$ kcal.mol⁻¹) to the protein. To further stabilize the peptide-protein interactions, Lysine, at site 14 (K14) of the β IP was found to form strong hydrogen bonds with E366. Therefore, the 16-aa peptide model comprising of VLERYLHEAKHAEKIT (molecular weight: 1937.21 Da), was selected as a suitable β IP for experimental characterization.

2.4. Assay of Nitric Oxide Production

Bioavailable NO was determined as previously described [11]. Briefly, after treatment with EPO alone or in combination with the novel β IP (1 mM or 10 μ M) for 3 hours, human umbilical vein endothelial cells (HUVECs) were incubated with 5 μ M 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate (Invitrogen, Waltham, MA) for 30 minutes at 37°C in the dark. Excess extracellular probe was removed by washing in Hanks balanced salt solution followed by incubation for 10 minutes at room temperature to allow for probe de-esterification. DAF-FM fluorescence increases by approximately 160-fold when it reacts with NO. Green fluorescence was measured using an inverted microscope Axiovert 200 (Carl Zeiss, Jena, Germany) equipped with CCD camera and image acquisition/analysis software AxioVision (Version 4.5). Images were acquired every 1-minute for at least 20 minutes, and fluorescence intensity was measured in 20 to 30 cells per field in at least 6 fields per experiment.

2.5. Angiogenesis Assay

To demonstrate the effectiveness of β IP to inhibit angiogenesis of endothelial cells, we analyzed tube formation with respective treatments alone or in combination with the novel β IP (1 mM). Briefly, HUVECs were plated on a 48-well tissue culture plate pre-coated with 250 μ L of Cultrex Basement Membrane Extract (Trevigen, Gaithersburg, MD) in 300 μ L endothelial basal medium-2 supplemented with 1% fetal bovine serum with or without treatment(s) of VEGF (100 ng/mL or 52.6 nM), EPO (50 mIU/mL or 11.45 fM; Conversion was calculated by 1IU = 8 ng = 0.229 pM) ESP (25 ng/mL or 20 nM), IL-3 (25 ng/mL or 1.7 nM), IL-5 (25 ng/mL or 1 nM), or GM-CSF (25 ng/mL or 1.8nM). After 20 hours of incubation in a 5% CO₂ humidified atmosphere at 37°C, the 3-dimensional structures formed by the cells in this matrix were examined using an inverted phase-contrast microscope. Tube-like structures were quantified by measuring the sum of the lengths of all tubules per field using the image analysis software AxioVision, Version 4.5. Three randomly selected low-power fields were examined for each sample.

2.6. PBMC cell culture and erythroid expansion

To demonstrate whether β IP had an effect on erythropoiesis, we analyzed erythroid expansion with and without the novel β IP (1 mM). Peripheral blood mononuclear cells (PBMCs) from a healthy adult donor were purchased from StemCell Technologies (Vancouver, Canada). PBMC were seeded at a density of 0.5×10^6 cells/ml in StemSpan SFEM II medium (StemCell Technologies) supplemented with or without StemSpan Erythroid Expansion Supplement (StemCell Technologies) and 1% (vol/vol) P/S. Cells were cultured for 8 days at 37°C in 5% CO₂ incubator with the media being changed every two days [29]. PBMCs are treated with peptide inhibitor (25 μ M) from Day 2.

2.7. RNA extraction and gene expression analysis

Hemoglobin β (HBB), transferrin receptor (CD71), and glycophorin A (GYPA) mRNA level were assessed using qPCR. Total RNA was extracted from 1×10^5 erythroid blast cells using the RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Total RNA (0.5 μ g) was reverse transcribed into cDNA using Iscript cDNA synthesis kit (Bio-Rad, Hercules, California) following the manufacturer's instructions. The qPCR was performed with 1 μ L cDNA and 1.5 μ M of each forward and reverse primer in a 10 μ L reaction volume using SYBR green in CFX manager 3.0 (Bio-Rad). Data were normalized to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) mRNA.

Primer sequences:

GAPDH-F: GAAGGTGAAGGTCGGAGTCA

GAPDH-R: GAGGTCAATGAAGGGGTCAT

HBB-F: GCACGTGGATCCTGAGAACT

HBB-R: GCCACCACTTTCTGATAGGC

CD71-F: TCTGCTATGGGACTATTGCTG

CD71-R: CTGTTGCAGCCTTACTATACG

GYPA-F: GTACCACTGAGGTGGCAATG

GYPA-R: AAGGCACGTCTGTGTCAGGT

2.8. Statistical and data analysis

One-way ANOVA with tukey's multiple comparison test was used to detect differences in experiments using GraphPad Software version 6.0 (La Jolla, CA). All data are presented as means $\pm$ SEM. Significance was defined as $P < 0.05$.

3. Results

3.1. P7 β CR Inhibitory Peptide Inhibits EPO Stimulation of Nitric Oxide in HUVEC

We have previously demonstrated that in human bone marrow angiogenic cells EPO leads to the stimulation of NO production via the heterodimeric β CR/EPOR [11], this was confirmed by Su et al. in human endothelial cells [30]. To demonstrate the efficacy of the computationally designed novel β IPs to block the β CR, we tested their ability to block EPO stimulated NO in HUVECs, by quantifying bioavailable NO using DAF-FM. We tested 7 putative β IPs. Although multiple β IPs significantly decreased bioavailable NO production, P7 β IP was the most efficacious peptide in decreasing bioavailable NO, thus all subsequent work utilized P7 β IP (Figure 2).

3.2 The inhibition of the β CR by P7 β CR Inhibitory Peptide is Competitive in Nature

The P7 β IP was designed to be specific to the binding site of EPO to the β CR. This would result in competitive inhibition of the β CR. To confirm this, we analyzed whether the inhibition of the β CR by P7 β IP could be overcome with increasing doses of EPO. We found that P7 β IP at 10 μ M could be overcome with 5 IU/mL of EPO treatment for 3 hours (Figure 3).

3.3. β CR-Induced Angiogenesis is Blocked with Novel P7 β CR Inhibitory Peptide

We have previously shown that activation of the β CR leads to phosphorylation of VEGFR2, AKT and eNOS, all important mediators of angiogenesis. To examine if P7 β IP could inhibit the angiogenic effects of β CR activation, we examined the ability of P7 β IP to inhibit tube formation induced by EPO and in HUVECs. We found that P7 β IP was able to block tube formation by all hematopoietin signaling cytokines to background levels (Figure 4). We have previously shown that VEGFR-2 activity depends on the presence of the β CR. Importantly, the P7 β IP did not affect VEGF-induced tube formation.

3.4. Novel P7 β CR Inhibitory Peptide does not Inhibit Erythropoiesis

To confirm that the novel P7 β IP did not interfere with the erythropoietic effects of EPO, we examined the effects of P7 β IP on expression of HBB, CD71, and GYPA after treatment with a PBMC – expansion supplement. We found that P7 β IP did not inhibit the ability of EPO to increase expression of HBB, CD71, or GYPA (Figure 5) in erythroid-enriched PBMCs. In addition, when we treated the PBMC for 7 days with P7 β IP, there was no increased expression of HBB, CD71, or GYPA (Figure 5).

4. Discussion

To our knowledge, there are currently no peptide inhibitors of the β CR. P7 β IP was developed in-silico based on the crystal structure of the Helix-A regions of both GM-CSF and EPO to act as a competitive inhibitor of the β CR (Figure 1). We demonstrated that the P7 β IP could inhibit EPO mediated NO production (Figure 2 and 3) and endothelial tube formation (Figure 4). The P7 β IP also inhibited IL-3, IL-5, and GM-CSF induced tube formation with similar potency. Additionally, we observed that low dose P7 β IP could be overcome with super-physiological doses of EPO (Figure 3), suggesting a competitive inhibition. Moreover, we also describe that P7 β IP does not lead to an increase in erythropoiesis nor does it block the erythropoietic effects of EPO (Figure 5), suggesting that the novel P7 β IP solely binds to the β CR. Furthermore, P7 β IP did not appear to display signs of cytotoxicity to cells during prolonged treatments, however, further evaluation to the cytotoxicity of the peptide are warranted. Taken together, our results describe a novel, selective inhibitory peptide of the β CR.

Previously, we have demonstrated that the β CR is responsible for EPO-induced production of bioavailable NO [11]. Additionally, we and others have shown that EPO [15, 31] and other β CR signaling cytokines [32, 33] increase angiogenesis through activation of the β CR, although this has been suggested to be cell type dependent [34], and requires the presence of vascular endothelial growth factor receptor (VEGFR)-2 [11]. Although the ability of VEGF to increase NO in bone marrow cells required the presence of the β CR, the P7 β IP did not block VEGF-induced angiogenesis. Thus, while VEGFR-2 signaling, in some way, is dependent on the presence or structural integrity of the β CR [11], the inhibitor does not interfere with this signaling. This is advantageous since VEGFR-2 signaling is critical to endothelial health [35–37].

While the P7 β IP was designed to target the binding of EPO to the β CR, it blocked the effects of all ligands known to interact with the β CR. Additionally, there was no effect on erythropoiesis, suggesting that P7 β IP is selective for the β CR and does not interact with the homodimeric EPO receptor. It has been reported that patients receiving elevated doses of EPO to achieve a higher hemoglobin target have an increased incidence of morbidity and mortality from heart disease and stroke [38–40]. We hypothesize that blockade of the β CR with P7 β IP, in patients receiving elevated doses of EPO may negate the increased morbidity and mortality without altering erythropoiesis by EPO.

In conclusion, to the best of our knowledge, this is the first computational and experimental study to demonstrate the design and characterization of a novel peptide inhibitor of the β CR. This P7 peptide has been shown to be effective in the inhibition of the β CR thus inhibiting downstream actions, such as angiogenesis and bioavailable NO production, without effecting erythropoiesis. These mentioned findings highlight the potential of this peptide as a novel therapeutic to decrease the harmful side effects of EPO therapy in patients undergoing chemotherapy or patients with CKD. In this regard, further tests aimed at evaluating the efficacy of the peptide in vivo are warranted.

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Abbreviations:

EPO	erythropoietin
βCR	beta common receptor
IL	interleukin
GM-CSF	granulocyte macrophage colony stimulating factor
JAK	janus kinase
STAT	signal transducer and activator of transcription
MAPK	mitogen-activated protein kinase
PI3K	phosphatidylinositol 3-kinase
βIP	βCR inhibitory peptide
VEGF	vascular endothelial growth factor, HUVEC, human umbilical vein endothelial cells, PBMC, peripheral blood mononuclear cells
VEGFR-2	vascular endothelial growth factor receptor 2
NO	nitric oxide
AML	acute myeloid leukemia
ESP	erythropoietin stimulating peptide
FEP	free energy perturbation
TI	thermodynamic integration
DAF-FM	5 μM 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate
HBB	Hemoglobin β CD71, transferrin receptor
GYPA	glycophorin A
GAPDH	glyceraldehydes 3-phosphate dehydrogenase
eNOS	endothelial nitric oxide synthase

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Research highlights
 β IP Inhibits EPO Stimulation of Nitric Oxide in HUVEC

- The inhibition of the β CR by P7 β IP is Competitive in Nature
- β CR-Induced Angiogenesis is Blocked with Novel β IP
- Novel β CR Inhibitory Peptide does not Inhibit Erythropoiesis

Figure 1.

β IP Sequence. A) Sequence alignment (top) and structural alignment (bottom, left) between GM-CSF chain C and EPO chain A. Computational design (bottom, right) of the binding of EPO-derived peptide model (orange) with β CR (green). Substitution of Glutamic acid (E) with Histidine (H, singly protonated at the N δ atom) at site 11 (E11H) formed an edge-to- π interaction with Y365 and H367 residues in β CR (Figure 1A, bottom right), which, in turn, doubled the binding affinity of the peptide ($\Delta G = -2.57 (\pm 0.74)$ kcal.mol $^{-1}$) to the protein. To further stabilize the peptide-protein interactions, Lysine, at site 14 (K14) of the β IP was found to form strong hydrogen bonds with E366. Therefore, the 16-aa peptide model comprising of VLERYL**H**EAK**H**AEKIT (molecular weight: 1937.21 Da), was selected as a suitable β IP for experimental characterization. B) Peptide sequences of tested inhibitors.

Figure 2.

Ability of novel β CR inhibitory peptides to block EPO-stimulated production of bioavailable NO in cultured endothelial cells. HUVECs were treated with the indicated β CR inhibitory peptide (1 mM) for 30-minutes prior to being incubated with 50 U/mL EPO for 3 hours before measuring bioavailable NO with DAF-FM diacetate. All values are means $\pm$ SEM; one-way ANOVA followed by tukey's multiple comparison test. * $P < 0.05$ when compared to control; # $P < 0.05$ when compared to EPO. Results shown are representative of three independent experiments.

Figure 3.

Inhibition by P7 βIP can be overcome with super-physiological doses of EPO. HUVECs were treated with the P7 βIP (10μM) for 30-minutes prior to being incubated with increasing doses of EPO for 3 hours before measuring bioavailable NO with DAF-FM diacetate. All values are means ± SEM; one-way ANOVA followed by tukey's multiple comparison test. *P<0.05 when compared to control; #P<0.001 when compared to control. §P<0.01 when compared to EPO (1IU/mL). Results shown are representative of three independent experiments.

Figure 4.

The P7 β IP inhibits β CR mediated tube formation. HUVECs were plated on a pre-coated tissue culture plate and adding the respective treatments with or without P7 β IP at 1M. After 20 hours of an inverted phase-contrast microscope was used to visualize 3 fields randomly selected low powered fields. All values are mean $\pm$ SEM; one-way ANOVA followed by tukey's multiple comparison test. Shown is representative of three different experiments.

* $P < 0.05$ when compared to no treatment. # $P < 0.05$ when compared to respective treatment.

Results shown are representative of three independent experiments.

Figure 5.

The P7 βIP does not interfere with EPO-induced erythropoiesis in PBMCs. PBMCs were plated for 8 days with or without PBMC expansion supplement with P7 βIP treatment starting at day 2. A) mRNA levels of hemoglobin β (HBB). B) mRNA levels of the transferrin receptor (CD71). C) mRNA levels of glycophorin A (GYPA). All values normalized to respective GAPDH and corresponding PBMC – empty levels. Data are all mean ± SEM; one-way ANOVA followed by tukey's multiple comparison test. *P<0.05 when compared to PBMC – empty. **P<0.01 when compared to PBMC – empty. ****P<0.0001 when compared to PBMC – empty. Results shown are representative of two independent experiments with three biological replicates per group per experiment.