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In silico Designing and Optimization of EPO Mimetic Using Combinatorial Library

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Abstract: ***Erythrpoitin receptor (EPOR) is a cytokine receptor protein, which on activation by binding to erythropoietin (EPO), triggers the intracellular cascade regulating differentiation and proliferation of erythropoietic progenitor cells into mature erythrocytes. EPO is a 34 KD glycoprotein hormone which specifically binds to EPOR and facilitates downstream signalling maintaining an adequate systemic availability of RBCs. For use of blood transfusion in various blood disorders such as anaemia, recombinant EPO and other mimetics have been reported, however, they have been found to be associated with certain risks and less potent to cure diseases. Targeting the binding site of EPOR to design small molecules and peptides which can retain the full agonist activity of the protein EPO may be a promising approach. Using computational approaches, large pool of diverse libraries can be analyzed to bind EPOR efficiently. In this study, the development of small EPO mimetic with therapeutic potential has been explored by using computational approaches. Initially, combinatorial library was designed in two classes, the first class of chemical compounds 'Lib-C' designed by dimer formation of SMND309 by using different linkers and a second class of library 'Lib-P' designed by interaction sites of EPO mimetic human monoclonal antibody ABT007 to EPOR and previously reported mimetic EPO mimetic peptide 1 (EMP1) and ERB1-7. SMND-309 is a novel derivative of salvianolic acid B which activates the EPO receptor, and then stimulates JAK2/STAT3 pathway and regulates erythropoiesis. The screening of combinatorial library resulted in efficient mimetic with the docking Glide score of -7.970 and $Energy_{total}$ score of -479.7 for chemical compound and peptide respectively, which are comparatively better results to known chemical compound SMND309 and known mimetics EMP1 and ERB1-7. Resulting chemical compound was found to be having a high binding affinity of -41.849 and peptide mimetic was found to stable and hydrophilic in nature.***

Keywords: Erythropoietin, Erythropoietin receptor, Mimetic, Library designing, Docking

1. Introduction

Anaemia is an abnormal reduction in the number of red blood cells (RBCs) in blood circulation. It occurs from bleeding, or degeneration or insufficient production of RBCs. Erythropoiesis is the process of production of RBCs, which is regulated by a number of growth factors EPO, Stem cell factor, Fms-like tyrosine kinase 3 (Flt3), vascular endothelial growth factor (VEGF), Interleukin 3 (IL3), thrombopoietin (TPO) **[1].** EPO are common to generate variable yield of erythropoietic progenitor cells. If the body needs more oxygen, for instance, the kidney triggers the release of the hormone erythropoietin (EPO), a glycoprotein hormone, which acts in the bone marrow for the growth of erythroid progenitor cell to increase the formation of red blood cells. EPO exerts its erythropietic effects through interaction with EPOR which is expressed by erythropoietic progenitor cells predominately in the kidney **[2], [3], [4]**. EPOR is a member of the cytokine receptor super family and possess signalling efficiency, dependending on the receptor orientation **[5],** and mechanism of signal transduction in which activation is believed to be achieved is through ligand induced homodimerization.

Initially, severe anemia that accompanies chronic renal failure was managed by regular blood transfusions in every 2-3 weeks. Blood transfusions were used to treat severe anaemic patients with sickle cell disease, thalassemia, myelodysplastic syndromes, or other type of anaemia **[6].** However, regular RBCs transfusions to a patient have risks of infection. Frequent blood transfusions can result in iron overload, leading to heart and liver damage **[7], [8]**. In 1967, for treating anaemia Androgen therapy had been shown to potentiate erythropoiesis **[9],** but the effect was meager and posed risks of side effects. In 1977, major breakthrough that transformed the therapeutic field came with the successful purification of small amounts of human EPO from aplastic anaemia **[10].** In 1983, the gene for human EPO was isolated and cloned **[11]**. In the consequent years, to avoid frequent blood transfusions, injections of recombinant erythropoietin were used to increase the number of erythrocytes. Use of recombinant EPO, for cure of anaemia significantly improved the recovering capacity of patients, but despite the enormous success, therapy was inconvenient and expensive **[12].** Hence in consequent years, researchers tried to design EPO mimetics since EPO mimicking peptides of small size can be produced in large numbers with an ease. Mimetics were designed on the basis of minimal binding region or functional epitope only involved in interaction with its specific receptor and efforts were made to identify mimetic of small size which could mimic the role of the EPO. EPO mimetic peptide 1 (Emp1) was designed which was a 20 amino acid sequence**.** Consequent studies on peptide EMP1 revealed that the minimal peptide sequence of 13 amino acids was sufficient to trigger the activation of EPOR and a cascade signalling **[13].** Another mimetic ERB1-7 was designed, which was a cyclic peptide of 18 amino acids belonging to the EPO mimetic family **[14].** It was reported to be less efficient in binding with EPOR and to be associated with other problems as mentioned in Table 1 to activate the target receptor **[15].** Hence, study is required to find out the efficient and target specific EPO mimetics which can efficiently activate and regulate erythropoiesis.

Table 1: Different therapeutics used for the anaemic condition with their associated problems. Initially, for treating anaemic disease direct blood transfusion and androgen therapy were administered. Breakthrough arrived when recombinant protein therapy came to be administered, however, it was expensive and inconvenient. Hence, in upcoming years, small peptide and chemical compounds mimicking the erythropoietin role came into highlight.

Anemia therapeutics	*Disadvantages*	*References*
Blood transfusion	Need regular transfusions and hence, donor and poses risks of infection and side effects.	**[8]**
Androgen therapy	Poses risks of side effects and inconvenient processing.	**[9]**
Administration of recombinant EPO or darbepoetin alfa	Thrombosis due to regular dosing, associated with safety concern, inconvenience and cost associated with chronic administration of EPO.	**[12], [16]**
EPO mimetic peptide 1 (EMP1)	EMP1 is 20 amino acid sequence; less potent than the natural hormone EPO.	**[13]**
Minimal peptide EMP1	Core region of EMP1consisted of 13 amino acids mimic the EPO role; less potent than natural EPO hormone.	**[13]**
ERB1-7	ERB1-7 consisted of 18 amino acid sequence peptide but it is reported to less potent.	**[14]**
Erythropoietin receptor derived peptide (ERP)	ERP although binding to the receptor domain, but other than the EPO hormone binding site, mechanism of binding to receptor EPOR unclear.	**[17]**

Previously, mimetics were derived by varying the sequences predicted from the interaction sites of ligand and receptor

protein, but they resulted in less potency to activate the receptor protein EPOR. New methods for ligand discovery are based on combinatorial procedures by assembling numbers of compounds to produce diverse molecules for binding molecular targets with higher specificity and efficiency **[18].** In 1997, dimerization of 2 EPO mimetic peptides was reported to strongly increase the activity **[19], [20].** In the consequent years, it was found that dimerization of EPO proteins results in enhanced erythopoietic activity both in vitro and in vivo **[21].** Simultaneously, with chemical compounds it was reported that designing dimers of small molecules using different linkers enhances the binding efficiency and specificity **[22].** On the basis of these methods, we have designed the combinatorial library in two classes, first class is library of chemical compounds named as 'Lib-C' formed by series of monomer analogues and dimers using various linkers of SMND309 compound. SMND-309 is a novel derivative of salvianolic acid B. SMND-309 works by controlling the effects of ischemia and reperfusion injury on brain by lowering the infarct volume, increasing the survival of neurons, improving neurological function and promotes angiogenesis by increasing the levels of erythropoietin (EPO), erythropoietin receptor (EPOR), phosphorylated JAK2, phosphorylated STAT3, Vascular endothelial growth factor (VEGF) and VEGF receptor 2 (Flk-1) in the brain **[23].** SMND-309 first activates EPO receptor then stimulates JAK2/STAT3 signalling pathway, which up-regulates the expression of VEGF through EPOR receptor JAK/STAT3 signalling pathways **[24]**. SMND309 has not been reported to activate this signalling effectively, for which the possible reason could be less efficient binding to EPOR. A compound which can bind to the target efficiently would be advantageous to activate the receptor.

Second class of the library consisted of oligopeptides, named as 'Lib-P'. It was formed by oligopeptides derived from interaction sites of monoclonal antibody ABT007, and reported mimetics EMP1, minimal EMP1 peptide and ERB1-7 with EPOR. Human agonist antibody ABT007 F_{ab} fragment binds to EPOR extracellular domain and activates it to regulate erythropoiesis **[25]**. These mimetics were reported to activate receptor less efficiently and were also associated with certain disadvantages mentioned in the Table 1. Unique binding site of monoclonal antibody ABT007 to EPOR and randomly varying the known mimetics and their various combinations is a possible approach to design EPO mimetic, which in future can help design a mimetic with better efficient binding to the receptor.

Here, in this study combinatorial library were designed by dimerization of chemical compound SMND309 and the peptides derived from ABT007-EPOR complex interaction sites and by known mimetics EMP1, ERB1-7. Combinatorial library was screened for efficient EPO mimetic by docking analysis using Glide docking (Maestro server) for chemical compounds and HEX 6.9 for peptides. Shortlisted compounds were further studied for physicochemical properties. Binding affinities of chemical compounds were determined using PRIME-MM-GBSA. Oligopeptides were evaluated for molecular weight, pI and hydrophobicity using Peptide protein calculator and Cello predictor.

2. Methodology

Table 2: Various tools, servers, databases and software used for the study.

Tools	**Description**	**Use**
Chemdraw	Tool for drawing 2D structures of chemical compounds and calculation of various properties.	Chemdraw was used for drawing the 2D structure of SMND309 molecule and dimer compounds of the library
Glide	Grid base ligand docking tool.	Glide was used to determine the binding score for ligand SMND309 and chemical compounds with receptor protein EPOR.
LigPrep	LigPrep produces a single, low-energy, 3D structure with correct chiralities of processing input compound structure.	For preparation of the chemical ligand molecule for docking analysis.
Prepwizard	Protein preparation tool for docking study. It removes water molecules and unwanted heteromolecules.	Prepwizard was used for preparation of EPOR protein.

XP visualizer	Application for prediction of physicochemical properties	Physicochemical properties of chemical compounds were predicted.
Prime MM-GBSA	Determines the binding affinity of ligand molecule	This tool was used for determining the binding affinity/activity between the chemical compounds and receptor protein EPOR.
Pepfold	Tool for prediction of *de novo* 3D structure of oligopeptides.	Pepfold was used for predicting 3D structure of the peptide sequences.
HEX 6.9	Protein – protein docking server.	Peptide sequences were docked with receptor.
Protein peptide calculator	Identifies various properties of a peptide sequence.	Shortlisted peptide sequences were run through the Protein peptide calculator for determining physicochemical properties.
Cello predictor	Determines the sub-cellular localization of target protein.	Shortlisted peptides were run through this tool for predicting the sub-cellular localization.
Pymol	For 3D view of proteins and analysis of PDB structure.	EPO-EPOR & EMP1-EPOR complexes and other 3D structure viewed and analyzed.
Aliphatic index	Determines the relative volume occupied by aliphatic amino acids.	Aliphatic index was used to determine the hydrophilic region and stability of shortlisted peptide mimetic.
Protein Data Bank (PDB)	PDB consists of 3D structures of biomolecules and their complexes.	1EER, 1ERN, 1CN4 and many other 3D structures were retrieved from PDB and used for structure and interaction analysis.

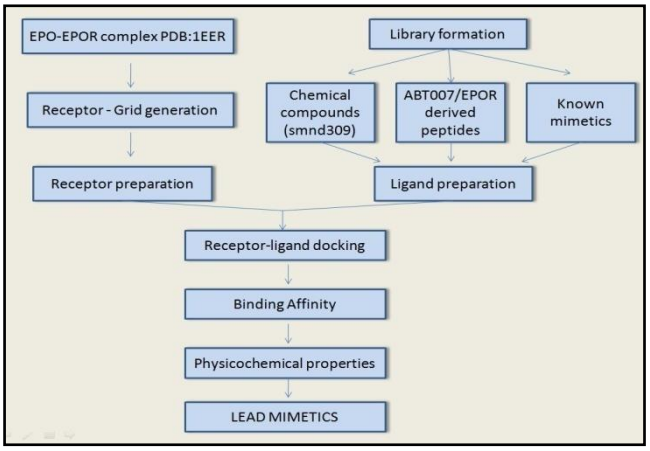


Figure 1: Flow chart of methodology - EPO-EPOR complex was analysed to determine the interaction site and receptor grid was generated. Combinatorial library was generated by dimerization of chemical compound using different linkers and previously known mimetics. Prepared receptor protein and library compounds were docked. Compounds were shortlisted on the basis of their docking results and further analyzed for the physicochemical properties.

2.1 EPO-EPOR Complex Analysis

Three dimensional (3D) structure of EPO-EPOR complex was retrieved from Protein Data Bank (PDB ID: 1EER)**.** PDB file was analyzed to find out the interacting residues and to determine interactions responsible for EPO-EPOR complex formation.

2.2 Library Formation

Two types of libraries were formed, namely Lib-C and Lib-P, which represent library for chemical compounds and peptides respectively. Lib-C was formed from the monomer analogues either by randomly adding carbons and functional groups to the SMND309 compound, or by dimerization of the SMND309 compound using various carbon chain linkers. Lib-P was designed by predicting peptide sequences on the basis of interaction sites of antibody ABT007-EPOR and the various combinations of known mimetics. Residues of ABT007 chains- light chain and heavy chain residues that were interacting with the active site of receptor with high affinity were selected for designing a new mimetic oligopeptides. Light chain of ABT007 was found to be interacting with EPOR by the residues Arg25, Arg26, Asn29, Glu31, Ala32, Glu33. Heavy chain interacts via Tyr27, Asn29, Ser34, Tyr48, Tyr90, Leu91 **[25].** Another subclass of Lib-P was designed by analogues of known mimetics EMP1, ERP, ERB1-7, all of which activate EPOR and are involved in the consequent regulation. Random changes in amino acid sequence in different location were done to enhance the binding affinity.

2.3 Receptor Protein Preparation

The 3D structure of the EPOR extracellular domain region was retrieved from RSCB Protein Data Bank (PDB ID: 1ERN). Native EPOR protein was prepared using the PrepWiz tool of Schrödinger Molecular Modelling Server. Repeated chains, water molecules and other unnecessary heteromolecules were removed from the protein structure and hydrogen molecules were added for the purpose of docking analysis.

2.4 Receptor Grid Generation

Prior to the docking analysis, receptor grid generation is an essential step. The grid was generated by defining the residues which were involved in the interaction between EPO and EPOR as the centroid of the grid. These residues were predicted by analyzing the EPO-EPOR complex. The parameters that were set for a receptor grid generation were Vander Waals scaling factor 1.00, charge partial cut off 0.25 and OPLS_2005 force field.

2.5 Ligand preparations

Designed chemical compounds and peptide sequences were prepared for interaction analysis with the receptor protein EPOR.

2.5a. Ligand preparation of chemical compounds

For ligand preparation, chemical compounds were drawn in ChemDraw tool and chemical properties of the compounds

were calculated. Ligands were prepared for docking**.** LigPrep was used for energy-minimization of ligands and generation of possible structures at pH 7.0 with OPLS_2005 force field, thereby achieving the correct protonated state. The chemical compound SMND309 was retrieved from National Center for Biotechnology Information (NCBI) and prepared.

2.5b. Ligand preparation of peptide sequences

3D structures of Lib-P peptide sequences were predicted by Pepfold peptide structure prediction server. It is de novo peptide structure prediction tool **[26]**. PepFold returns a PDB file of target peptides. Output PDB files of ligands were prepared using PrepWiz.

2.6 Interaction analysis of EPOR and Lib-C

For analysis of chemical compounds (Lib-C) with EPOR receptor, GLIDE docking module was used. GLIDE is commercially available molecular docking software which is used to analyze the interaction between the receptor protein molecules and chemical ligand molecules **[27].** For docking study, prepared receptor protein EPOR and ligand molecule were used. The generated grid was selected for specifying the domain of the receptor protein. Scoring parameter GlideXP was selected in order to get a good correlation between good poses. Glide returns Glide score, lipophilic fraction, H-bond and expos penalty. Glide score is based on the binding energy value of ligand to receptor. Lipophilic fraction indicates the hydrophobicity of the chemical compounds and Expos penalty is an obstacle which arises during interaction of ligand with receptor.

2.7 Interaction analysis of EPOR and Lib-P

The docking of Lib-P with EPOR was done by using the HEX 6.9 (protein docking server). Hex 6.9 is an interactive protein docking and molecular superposition program **[28].** Receptor protein 1ERN and oligopeptide were imported, grid was set and HEX was run. The result of HEX is E_{total}. The value of E_{total} is usually associated with values of four energy terms (E_{ele}, E_{vdw}, E_{pol}, E_{apol}), where E_{ele}, E_{vdw}, E_{pol}, and E_{apol} denote the electrostatic interaction energy, van der Waal interaction energy, polar component of the ligand desolvation energy, and nonpolar part of the cost of ligand desolvation energy, respectively.

2.8 Binding affinity of chemical compounds

Compounds were shortlisted on the basis of GLIDE score and were further evaluated for thier binding affinities using Prime MM-GBSA. Minimization of the protein-ligand complex was executed by the optimal function of the PRIME by applying OPLS force field and the binding energy value of the receptor-ligand protein complex of docked poses were calculated.

2.9 Physicochemical properties of peptides

Peptides were shortlisted on the basis of total energy (E_{total}) score and were further evaluated for physicochemical properties using a Protein peptide calculator which determine the molecular weight, isoelectric pH, hydrophilic nature and hydrphobicity, many other properties required for the stability and reliability of protein sequences.

2.9a Sub-cellular localization

TargetP predicts the subcellular location of eukaryotic proteins. The location assignment is based on the predicted presence of any of the N-terminal sequences. Subcellular localization of shortlisted peptides was also determined by Cello predictor. It determines the localization of peptides after secretary pathway.

3. Results

3.1 Binding Site Identification of EPO-EPOR Complex

EPOR interacts with EPO at two different sites- site 1 and site 2. First site of EPOR is hydrophobic in nature, mainly due to Phe93 which is responsible for nonpolar interactions; its side chain also consisted of hydrophilic amino acids which were involved in the interaction with the ligand (Figure 4). Identified interacting residues from the 3D structure as shown in the Figure 2 were - Leu59, Glu60, Asp61, Thr90, Ser91, Ser92, Phe93, Val94, Pro95, Leu96, Ileu113, His114, Ileu115, Asn116, Ser152, His153, Glu202, Pro203, Ser204, Phe205.

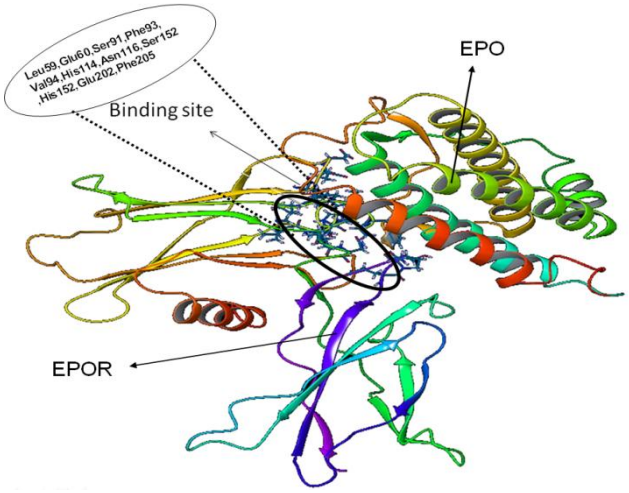


Figure 2: 3D structure of the EPO-EPOR complex. EPOR Chain-A is bound to EPO protein, which is showing the interacting amino acid residues responsible for the formation of complex. Hydrophobic amino acids are responsible for nonpolar interaction in the complex. The side chains are stabilized by other hydrophilic amino acid residues.

3.2 Receptor protein preparation

EPOR had two chains A and chain B. Both chains had similar sequences and for docking analysis duplicated chain was deleted and chain B was deleted and EPOR- chain A was prepared. ECD region (~34-246) of EPOR is responsible for binding with EPO. EPOR chain A prepared for docking analysis by removing the duplicate chain B, water molecules and any other unwanted ligands from the PDB structure of EPOR.

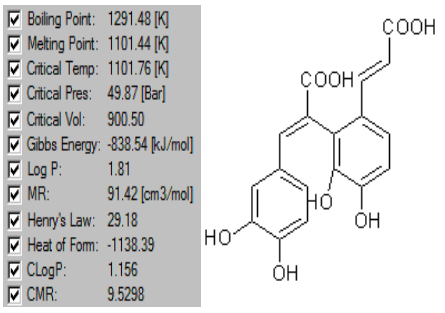
3.3 Interaction analysis of EPOR and Lib-C

Table 3 - Lib-C and EPOR docking analysis results, various analogues of SMND309 monomers and dimers linked via

different linker chains were studied. Among them, Dimer 4 compound showed minimal binding energy value with the receptor protein EPOR. In addition, other properties and parameters responsible for the interaction were also identified.

Chemical compounds	Glide Score	Expos penalty	Activity	H-bond	Electrostatic rewards	Lypophillic term & fraction
SMND309	-4.080	0.2	-4.1	-1.9	-0.6	-1.4
Monomer-1	-3.686	0.2	-3.7	-1.8	-0.5	-1.5
Monomer-2	-4.867	0.4	-4.9	-2.4	-0.8	-2.3
Dimer-1	-5.081	0.5	-5.1	-1.6	-1.9	-2.1
Dimer-2	-5.607	0.4	-5.6	-2.4	-1.4	-2.2
Dimer-3	-7.066	1.4	-7.1	-4.5	-1.3	-2.9
Dimer-4	**-7.970**	**0.5**	**-8.0**	**-5.2**	**-1.6**	**-2.9**
Dimer-5	-6.727	0.6	-6.7	-2.3	-1.6	-3.4
Dimer-6	-5.957	0.8	-6.0	-3.1	-1.3	-2.3
Dimer-7	-7.973	2.0	-8.0	-5.4	-3.5	-1.2
Dimer-8	-6.508	0.2	-6.5	-1.9	-1.7	-3.0
Dimer-9	-7.166	1.3	-7.2	-5.7	-0.9	-2.0
Dimer-10	-7.621	1.7	-8.6	-4.8	-1.9	-3.6
Dimer-11	-6.709	3.2	-6.8	-5.8	-2.0	-2.3
Dimer-12	-6.707	1.4	-6.7	-4.3	-1.1	-2.7
Dimer-13	-4.784	1.9	-4.8	-3.2	-1.2	-2.3

From docking studies of chemical compounds, Dimer 4 showed better binding efficiency than the reference chemical compound SMND-309 and other analogues as mentioned in the Table 3. Lipophipic efficiency parameter was used to evaluate the drug likeliness. ClogP was found to be 3.46 and logP value (Partition coefficient) was 3.20, both determine the hydrophobocity and hydrophilicity of the compound. Dimer 4 was designed by linking with short carbon chain and SMND-309 compound which is a metabolite of salvianolic acid, for which, the structures have been shown in the Figure 3.



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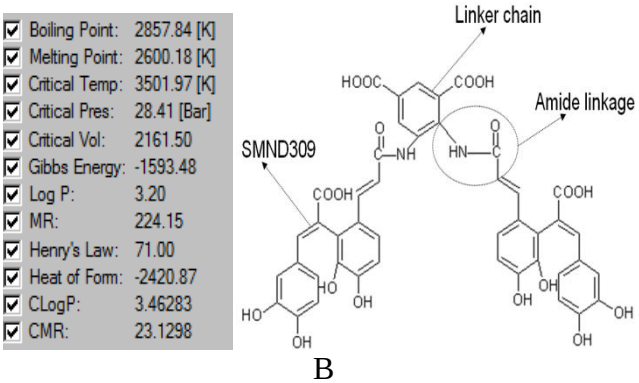


B

Figure 3 A) Chemical structure of SMND 309 (2E) -2-{6-[(E) -2-carboxylvinyl] -2,3-dihydroxyphenyl} -3-(3,4-dihydroxyphenyl) propenoic acid) with molecular formula $C_{18}H_{14}O_8$. **B)** Dimer4 was designed by two monomers of SMND309, linked via CO=NH bonding (amide linkage) and their chemical properties were determined by using the Chemdraw.

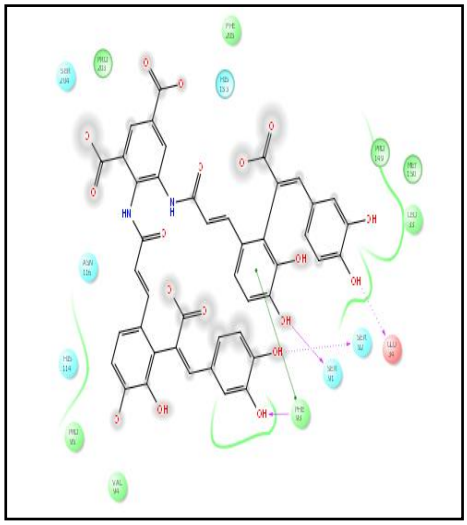


Figure 4: Two dimensional view of hydrophobic and hydrophilic interactions between the lead compound Dimer04 and receptor protein EPOR. Green color residue representing the hydrophobic molecules. Blue color residues show polar amino acids involved in the interaction of ligand chemical compound and the receptor protein EPOR.

Hydrophobic interaction is mainly stabilized by Phenylalanine, Serine, Valine and the hydrophilic interactions are stabilized by Histidine and Aspargine as shown in Fig. 4. Phe93 is involved in hydrogen bonding with backbone and Π-Π stacking with the ligand; Ser91 forms hydrogen bonds with backbone at OH group; Ser92, Glu34 form the hydrogen bond with OH-group of side chain; Arg111 and Gly207 form the hydrogen bond with the backbone of the receptor protein. Chemical compounds were further evaluated to determine the binding energy of the compound with the receptor and results have been shown in Table 4. Lead chemical compound Dimer4 showed good binding score (-41.849).

Table 4: Binding affinity analysis of library of chemical compounds (Lib-C) with EPOR. In Lib-C, compound Dimer4 showed better binding affinity than reference chemical compound SMND309 and its monomer analogues.

CHEMICAL	*MMGBSA dG*	*CHEMICAL*	*MMGBSA dG*
SMND309	-23.245	Dimer 07	-37.344
Monomer 01	-22.369	Dimer 08	-31.302
Monomer 02	-328.124	Dimer 09	-23.132
Monomer 03	-24.063	Dimer 10	-31.165
Dimer 04	**-41.849**	Dimer 11	-34.278
Dimer 05	-33.663	Dimer 12	-24.409
Dimer 06	-30.024	Dimer 13	-28.374

The graph between Glide Score and lipophilic fraction shows that with increasing glide score, a lipophilic fraction values increase, which has been shown in Figure 5. Lipophilic fraction represents the molecules of compounds which have importance for stability of the chemical compound and also required to move through the membrane. Triangle in green color represents the reference compound SMND309, dark blue color boxes represent the SMND309 analogues (monomer) compound and dimer compound joined by linkers have been shown here in magenta color. Dimer-4 compound shows good Glide score and lipophilic fraction values.

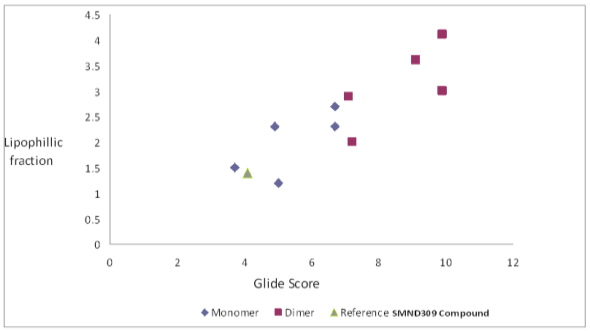


Figure 5: Graph between Glide scores and lipophillic values of shortlisted compound Dimer-4 and reference compound SMND-309 and SMND-309 analogues.

3.4 Interaction analysis of EPOR and Lib-P

The results of the interaction of Lib-P peptides with EPOR have shown in Table 5; Peptide-23 showed better E_{total} score than the reference compound SMND309 and monomer compounds. Interaction of Peptide-23 with EPOR has been shown in Figure 06. Receptor residues- Lys10, Arg32, Arg35 Glu147, His 153, Ileu154, Tyr156, Ser210 were found to be interacting with the peptide sequence. Arg and Glu forms H-bond with the peptide. Physicochemical properties of Peptide-23 were determined. As shown in the Figure 07, Peptide-23 has molecular weight 2174.30, isoelectric pH 6.77, hydrophilic nature 32% and hydrophobicity 16%.

Peptide-23 was predicted to have mitochondrial targeting peptide (mTP) value 0.079, secretory pathway signal peptide (SP) value 0.035 and reliability class (RC) value 01 using the non-plant networks shown in Table 06. According to TargetP algorithm Peptide-23 possesses the good peptide prediction.

Table 5: Different known and predicted peptides with their scores. Peptide-23 has better Energy total score than other known or predicted peptides.

Type	*Sr. No.*	*Oligopeptides*	*Etotal*	*pI*
Known mimetic	1	EMP1-GGTYSCHFGPLTWVCKPQGG	-459.4	8.23
	2	ERB7-DREGCRRGWVGQCKAWFN	-426.6	8.83
	3	YSCHFGPLTW	-402.5	7.36
	4	YSCHFGPLTWVCK	-437.6	8.23
Predicted mimetic	5	CRRNEAE	-336.4	6.37
	6	CCRRNEAEC	-390.5	6.18
	7	RRNEYYAYS	-397.6	9.07
	8	RREASAHY	-368.7	9.35
	9	RNEASHYC	-394.9	7.36
	10	CRRNEASHY	-397.5	8.53
	11	CRNEAESHY	-427.8	5.48
	12	CYNSYHYLC	-355.1	7.25
	13	CCYNSAHYLC	-372.4	7.17
	14	YYNASYHLL	-359.3	7.53
	15	CYYNNASHL	-371.7	7.35
	16	CYSCHLCY	-351.3	7.17
	17	CCYSCAHL	-351.2	7.17
	18	CCYSCNNASHL	-386	7.17
	19	CRRNAECLTW	-383	8.23
	20	CRRNEAERRNC	-412	8.83
	21	RREAERRSHL	-425.5	12.0
	22	CRREAESSYC	-365.5	6.29
	23	**CRNEAESHHYCYYNNASHL**	**-479.7**	6.77
	24	CYYNNASHLRREAERRSHL	-447.6	9.33
	25	CRNEAESHYCCYNSAHYLC	-460.9	6.47
	26	ILVGTLIVLIPVLIVLVFLYWQ	-455.6	6.02

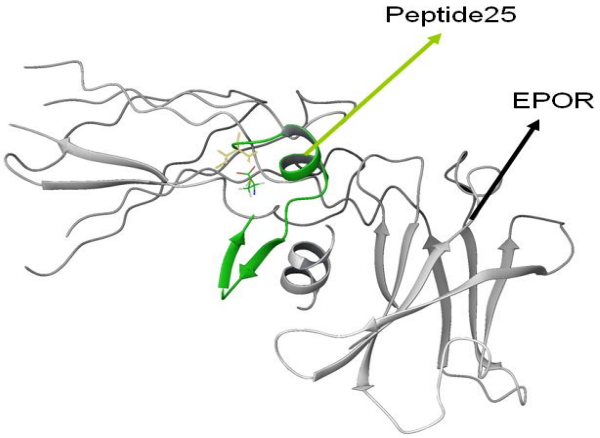
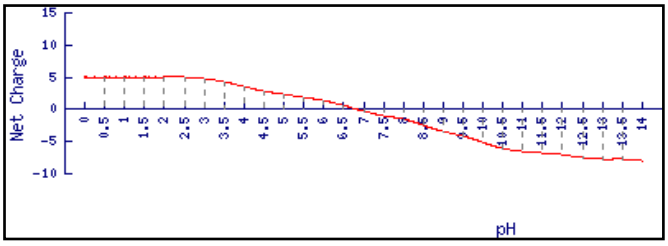
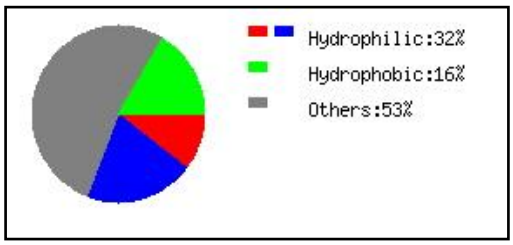


Figure 6: 3D structure of interaction between Peptide-23 and EPOR. The Peptide-23 ligand is shown in green colour and EPOR in gray.



A



B

Figure 7: A) Analysis of isoelectric point of oligopeptide-23. B) Determination of interactive properties of oligopeptide-23.

Table 6: Cello predictor results of Peptide-23 for determining the localization in the cell.

Nuclear	1.057 *	Peroxisomal	0.030
Mitochondrial	2.044 *	ER	0.026
Extracellular	0.379	Cytoskeletal	0.020
Cytoplasmic	0.389	Lysosomal	0.030
Plasma membrane	0.452	Golgi	0.014

4. Discussion

Using the computational analysis, large library can be generated and evaluated. Computationally shortlisted mimetic can be further used for *in vitro* and *in vivo* studies to overcome laborious work, time and expense. In this study,

efficient mimetics of EPO were designed using diverse library. Combinatorial library was designed in two classes- Lib-C for chemical compounds and Lib-P for oligopeptide sequences. Lib-C was designed by monomer analogues and by linking two monomers of SMND309 compound using different linker chains. Lib-P was designed by peptides designed from binding sites of antibody ABT007-EPOR and dimer of known mimetics EMP1, ERB1-7 and minimal peptide sequence involved in EPOR activation. Library tested for interaction with extra cellular domain region of EPOR. For an interaction analysis, receptor EPOR extra cellular domain region (PDB 1ERN) was prepared by removing water and any other unnecessary heteromolecules. EPO-EPOR active site was analyzed using PDB structure (1EER) of the complex in Maestro server. EPOR interacts with two regions to EPO forming the receptor dimer. Receptor binding sites possess hydrophobic amino acids, mainly Phe93, which is responsible for hydrophobic interaction with the ligand molecules. EPOR hydrophobic region surrounded by hydrophilic amino acids play important role in the interactions. Knowing the interaction sites of the EPOR, grid was generated and docking for binding energy analysis was done using Glide module. From Lib-C analysis, Dimer4 showed better binding energy as compared to previously known mimicking compound. This study reports better binding efficiency (-7.970) than the binding energy of a SMND309 compound (-4.081) which was reported to activate the EPOR. A second class of library- Lib-P was studied by the rigid docking approach carried out successfully by HEX6.9 on a correlation type of shape in 3-D FFT mode. HEX gives output as E_{total} value -479.7 of the Peptide-23, which was better than E_{total} values of known mimetics EMP1, ERB1-7, minimal peptide sequence which were -459.4, -426.6 and -437.6 respectively. Further, Dimer4 and Peptide-23 were analyzed for their physicochemical properties and stability. The binding affinity of Dimer4 was found to be -41.849 using Prime-GBSA. Peptide mimetic was found to be 32% hydrophilic in nature, which shows its

stability in the water and hydrophobicity was 16%, certainly required for crossing the cell membranes and globular structure of protein stability. Moreover, both natures- hydrophilic and hydrophobic are important in binding of mimetics to EPOR, as found in the binding of EPO-EPOR complex. Localization of small peptide was predicted using Cello predictor predicts by secretary pathway to nuclear and mitochondrial where it can bind to its receptor molecules. Biological activity and physicochemical properties of reported mimetics show appropriate results for binding to EPOR. In future, reported mimetics possessing important role can be used for ex vivo erythrocyte generation and many other applications such as treatment of anaemia and in the field of regenerative medicine.

5. Conclusion

In silico study using computational tools gives rise to a convenient way to modify the target chemical compounds and peptides, which have helped to improve chemical/peptide stability and further their therapeutic potential. Here, an attempt was made to design novel EPO mimetics (chemical compound and small peptide sequence)

which would activate the EPOR cytokine protein and consequent signalling using computational approach. EPO is an essential factor for the viability and proliferation of erythroid progenitors and primary cause of the anaemia in chronic kidney disease (CKD). We identified that Dimer4 chemical compound and Peptide-23 possess high binding affinity towards the receptor protein EPOR. The predicted mimics have shown acceptable physicochemical properties and biological properties with high solubility and stability. In future, above designed mimetics (small peptides and chemical compound) would be promising candidates for the EPO mimicking in the field of treatment of anaemia. This would reduce the time and expenses of *in vivo* and *in vitro* experiments.

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7. Conflict of Interest

Authors have no conflict of interest regarding the publication of the paper.

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