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Investigation of the interactions between the EphB2 receptor and SNEW peptide variants

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Abstract

EphB2 interacts with cell surface-bound ephrin ligands to relay bidirectional signals. Overexpression of the EphB2 receptor protein has been linked to different types of cancer. The SNEW (SNEWIQPRLPQH) peptide binds with high selectivity and moderate affinity to EphB2, inhibiting Eph-ephrin interactions by competing with ephrin ligands for the EphB2 high-affinity pocket. We used rigorous free energy perturbation calculations to reevaluate the binding interactions of SNEW peptide with EphB2 receptor, followed by experimental testing of the computational results. Our results provide insight into dynamic interactions of EphB2 with SNEW peptide. While the first four residues of the SNEW peptide are already highly optimized, change of the C-terminal end of the peptide has the potential to improve SNEW binding affinity. We found that a PXSPY motif that can be similarly aligned with several other EphB2 binding peptides.

Keywords

EphB2 receptor; ephrin ligands; protein stability; protein dynamics; inhibitor design; molecular dynamics simulations

Introduction

Eph (Erythropoietin-producing hepatoma) tyrosine kinase cell surface receptors [1-7] are multidomain transmembrane proteins that interact with a variety of cellular targets and can

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induce distinct responses in different cell types [7-10]. As transmembrane proteins, Eph receptors contain an intracellular and an extracellular region. Both classes of Eph receptors contain an N-terminal domain to which the ephrin ligand binds, a cysteine-rich region, and two fibronectin type III repeats on their extracellular region. A sterile alpha motif, tyrosine kinase domain, and C-terminal PDZ-binding motif are present on the intracellular region [10]. Each Eph receptor has a ligand-binding domain on its extracellular region, which displays high-affinity for an ephrin ligand. There are sixteen Eph receptors and nine ephrin ligands, which are classified as either EphA or EphB receptors and ephrinA or ephrinB ligands, respectively. Eph receptors and ephrin ligands are classified based on their sequences and how they are bound to the cell membrane. EphrinA ligands connect via a glycosylphosphatidylinositol linkage, while ephrinB ligands are transmembrane bound. Eph receptors tend to bind to ligands of the same subclass, with some exceptions. Eph receptor function can be modulated by heterooligomerization of A and B type Eph receptors [11] and by cis as opposed to trans interactions [12].

The roles of Eph/ephrin have been characterized in embryogenesis [6, 13, 14] and carcinogenesis [15-19]. Eph silencing or overexpression can have devastating effects on the health of an organism. For example, the absence of ephrinB2 during development leads to early embryonic death due to a failure to develop a proper cardiovascular system. The absence of EphB2 receptor and the ephrinB3 ligand can lead to cleft palate, impaired development of the heart, problems in urorectal development, and irregular development of the hippocampus [20]. Deregulated expression of Eph receptors and the corresponding ephrin ligands in cancer patients are linked to reduced survival rates [21-23]. Therefore, therapeutic targeting of the Eph receptors is of great importance [24], as it has the potential to reduce progression of deadly diseases [19, 25-27]. Small peptides have been identified to target Eph receptors [28-30]. Using phage display library screening, several peptides that bind EphA4 were identified [3, 31, 32]; some are promiscuous and some are more selective. Most peptides were antagonistic, i.e. blocked receptor-ligand binding, but two EphA2 binding peptides (SWL and YSA) acted as agonists, inducing EphA2 tyrosine phosphorylation [33]. Two available Eph receptor-peptide crystal structures provide insight into the characteristics of Eph-peptide interactions. The TNYL-RAW peptide is a strong EphB4 binder, with K_d of 71 nM. The EphB2-SNEW (SNEWIQPRLPQH) peptide complex [32] has a different Eph binding pocket conformation, and the SNEW peptide was shown to have an extended random conformation in the EphB2-SNEW peptide complex [1]. The SNEW peptide has been tested experimentally and has been shown to bind to the EphB2 receptor and inhibit Eph-ephrin binding [32, 34] with moderate affinity (IC₅₀ of ~15 μM) and high selectivity. While TNYL-RAW and several other peptides can bind Eph receptors in the nM range [29], SNEW peptide is the peptide with the highest affinity to bind to EphB2 receptor. The improvements of the binding affinity and other physic-chemical properties of the EphB2-SNEW system would help to better target EphB2 receptor related cancer. EphB2 is an important drug target. Overexpression of the EphB2 receptor has been linked to ovarian, prostate, breast, gastric, and colorectal cancers, as well as mesothelioma and melanoma [35-38]. Furthermore, based on the identification of EphA2 agonist peptides, it is possible that EphB2 agonist peptides could be identified. SNEW peptide motif has been

linked with imaging radiotracers to facilitate the early detection of EphB2-overexpressing tumors[39].

Conformational dynamics is an important factor that underlies the recognition and activation of Eph receptors [2, 4, 40-42]. The EphB2 receptor's ligand-binding domain is composed of its D-E, G-H and J-K loops. The ephrin ligand binds to this pocket with its own G-H loop. For example, in the EphA4-EphrinA2 complex, the JK loop is in a coil conformation, while it changes to α -helix in the EphA4-EphrinB2 complex. The conformational changes in the binding loop region illustrated that conformational dynamics of these loop facilitates cross-class ephrin signaling (42), and provides specificity in ligand interactions. In this study, we used molecular dynamics simulations to understand the conformational dynamics of EphB2-SNEW (SNEWIQPRLPQH) peptide complex. Using the conformational ensemble generated from the replica exchange molecular dynamics simulations and free energy perturbations, we performed step-wise systematical optimization of SNEW peptide sequence. Finally, experimental approaches were used to test the selected peptides optimized with the computational approaches.

Experimental Methods

Classic Molecular Dynamics Simulations

The PDB file 2QBX was used as a starting point for the EphB2 receptor in complex with the original SNEW peptide. However, the last three amino acids, PQH, were missing from this file, and had to be added manually. The ionization states of ionizable sidechains were maintained as in neutral pH.

The simulated molecules were solvated in a TIP3P water box with a margin of at least 10 Å from any point on the edge of the water box. The dimensions of the water box were 55 Å * 60 Å * 70 Å. Sodium and chloride ions were added to the system up to a concentration of 0.15 mol/L. MD simulations were performed using the NAMD package [43] and the CHARMM27 force field [44] with a constant pressure of 1 atm and a temperature of 300K. The simulations start with initial heating stages and followed by 30 nanoseconds of simulation. The time step was 1fs with a SHAKE constraint on all bonds with hydrogen atoms [45]. The Langevin piston method [46-48] with a decay period of 100 fs, and a damping time of 50 fs was used to maintain a constant pressure of 1 atm. The temperature 330K was controlled by Langevin thermostat with a damping coefficient of 10 ps⁻¹[46]. The short-range van der Waals (VDW) interactions were calculated using the switching function, with a twin range cutoff of 10.0 and 12.0 Å. Long-range electrostatic interactions were calculated with the Particle Mesh Ewald method [49] with a cut-off of 12.0 Å for all simulations. The CHARMM program [44, 50] was used to analyze trajectories. The RMSD (root mean squared deviation) analysis of the dynamic trajectories was obtained using the starting crystal structures as the reference.

Replica Exchange Molecular Dynamics (REMD) Simulations

REMD simulations were performed using the NAMD program. Each REMD simulation consists of 48 replicas, with 24 bound state replicas and 24 unbound state replicas. The simulation temperature ranges from 300 K to 400 K with an exponential distribution. The

starting temperatures for 48 replicas are arranged with alternative spacing of bound state replica and unbound state replica, to ensure unbiased sampling. The exchange time among the replicas is 1 ps with an averaged acceptance ratio of 19%. Each replica was equilibrated for 5.5 ns (total simulation time 264 ns), and the last 2 ns of the trajectories were used for obtained low energy ensemble for free energy perturbation simulations.

Free Energy Perturbation (FEP)

Alchemical Free Energy Perturbation Calculations were performed using the NAMD package [43] and the CHARMM27 force field [44]. In FEP, the free energy difference between two thermodynamic states, a and b , is calculated by a series of transformations between intermediate states along a pathway that connects a to b . The hybrid Hamiltonian of the system is evaluated as:

$$H(\lambda)=H_0+\lambda H_a+(1-\lambda)H_b \quad (1)$$

H_a is the Hamiltonian for the group of atoms representative of the initial state, a , and H_b characterizes the final state, b . H_0 is the Hamiltonian for those atoms that do not undergo any transformation during the MD simulation.

For the mutation of morph residues from different charge states (such as Arg to Ala), simultaneous creation or elimination of counter ions (sodium or chloride) was used to maintain net charge balance in simulation system.

Each FEP calculation had 14 step windows ($\lambda = 0, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 0.95, 0.99, 1.0$) for mutating residue a to residue b . Each window was equilibrated from 60 ps (short) to 200 ps (long). Softcore potentials for the Lennard-Jones interactions were used with a shift coefficient of 6.0. Coulomb interactions are switched on after $\lambda > 0.5$. Due to large perturbation of small peptide caused by mutation, it is not guaranteed that the backward mutation can lead to wild type binding position. Therefore, only forward mutation is used. In order to better sample different conformation state, in this work, FEP energies were calculated by averaging 36 FEP calculations of protein-peptide bound conformers and 14 protein-peptide unbound conformers. The difference between these energies, ΔG , was calculated by taking the difference of FEP energy changes of bound and unbound states. The FEP energy of bound states was average value of 36 FEP calculations and that of unbound states was average value of 14 unbound states.

$$\Delta\Delta G = \sum_1^{36} \Delta G_i(bound)/36 - \sum_1^{14} \Delta G_j(unbound)/14 \quad (2)$$

MM-GBSA binding energy calculation

We recalculated the interaction energies between the Eph and SNEW peptide using the MM-GBSA in the Amber software[51, 52]. MM-GBSA methods calculate binding free energies for macromolecules by combining molecular mechanics calculations and continuum solvation models. Conformations from the simulated trajectories were energy optimized for

100 steps used SANDER of amber software. Then the interaction energies between two Eph and Evi phrin of each conformation from the simulated trajectories were calculated. Onufriy's GB (igb=5) method was used in the MM-GBSA setup. Solute dielectric constant was 1.0, and the solution dielectric constant was 78.5. Surface tension value was 0.005. Default value of 0.0 was used for salt concentration. No entropy changes were calculated.

Peptide synthesis

The following peptides were synthesized via Fmoc chemistry by AnaSpec (Fremont, CA) to at least 97% purity as judged by reverse phase HPLC: SNEWIQPRLPQH (SNEW)[32], EPQNHSWPIRQL (scramble), SNEWLLPRSPYK (SNEWLL), SNEWIQPRSPQH (SNEW PRS), SNEWILPRGPYK (SNEWIL).

EphrinB2 preparation

The human Fc (hFc) portion of the hEphrinB2 fusion protein (R&D Systems, Minneapolis, MN) was cleaved from EphrinB2 using Factor Xa (EMD-Millipore, Billerica, MA) at room temperature overnight according to manufacturer's recommendations (2U Factor Xa/50µg EphrinB2-Fc). The free hFc was removed by incubating with protein A agarose (ThermoFisher Scientific, Rockford, IL) overnight at 4°C. Factor Xa was removed using XArrest agarose (EMD-Millipore) following manufacturer's recommendations. Removal of the Fc was confirmed by Western blot.

ELISA

ELISA plates (Immulon 4HBX, ThermoFisher Scientific) were coated overnight at 4°C with cleaved EphrinB2 (as prepared above) at 125ng/mL in 50mM Na-carbonate buffer pH 9.6, and blocked with an anti hFc F_{ab} (goat polyclonal, JacksonImmuno Research, West Grove, PA) at 1:500 in 1% BSA (Sigma-Aldrich, St. Louis, MO) for two hours at 37°C. Soluble hEphB2-Fc (250ng/mL R&D Systems), hEphB3-Fc (500ng/mL, R&D Systems) or hEphB6-Fc (500ng/mL, R&D Systems) was incubated with peptide at varying concentration (1µM-1nM) at room temperature for 30 minutes prior to being applied to the ELISA well for two hours at room temperature. The amount of hEphB2 bound to hEphrinB2 was measured using an anti-hFc HRP antibody (mouse monoclonal, Southern Biotech Birmingham, AL) at 1:15,000 in 0.5% BSA in 0.1% Tween 20, PBS. The ELISA was developed with 3,3',5,5'-Tetramethylbenzidine (TMB) (ThermoFisher Scientific) for 15 minutes and stopped with 1M H₂SO₄ (Sigma-Aldrich). Absorbance at 450nm was measured using POLARstar Optima plate reader (BMG Labtech, Cary, NC). All conditions were tested in triplicate.

Surface plasmon resonance

Biospecific interaction analysis was monitored by surface plasmon resonance using the BIAcore T200 system. EphB2-Fc was immobilized onto the CM5 (carboxymethylated dextran matrix) sensor chip using the amine coupling kit (Biacore), and unreactive groups were blocked by ethanolamine by standard procedure. A continuous flow of SNEW and SNEW mutant peptides (concentrations range from 80 nM to 200 µM) across the sensor chip was monitored. The kinetic parameters were calculated by standard methods according to a

1:1 Langmuir binding model by direct fitting of ligand binding sensograms at multiple concentrations.

Western blotting

Colo 205 cells (ATCC Manassas, VA) were cultured in RPMI 1640 (Corning, Corning, NY) plus 10% FBS (Sigma-Aldrich). One million Colo 205 cells were plated 18 hours prior to the experiment. Peptides were incubated at 500 μ M with Colo 205 cells for two hours under restricted culture conditions (RPMI + 0.5% FBS). Next, clustered EphrinB2-Fc or hFc (clustered with goat anti-hFc antibody (JacksonImmuno Research) for 30 minutes [53]), hEphrinB2-Fc (R&D Systems) or hFc (JacksonImmuno Research) as a negative control, was added to the cells at 1 μ g/mL and incubated at 37°C for 30 minutes. The cells were immediately lysed with TNTG lysis buffer [25mM Tris HCl pH 7.5, 150mM NaCl, 1% Triton X100, 10% Glycerol] containing protease (Complete with EDTA, Roche, Basel, Switzerland) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 2, Sigma-Aldrich). Lysates were clarified by centrifugation for 30min at 20,000 $\times$ g. Lysed samples were then run on a 4-12% gel by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% (w/v) non-fat dry milk in TBS, 0.1% Tween20. The detection rabbit polyclonal antibody recognizes phosphorylated (p)-EphB1^{Y594} and p-EphB2^{Y594} (1:1000, AbCam, Cambridge, MA). The membrane was then stripped (50mM glycine, 150mM NaCl pH 2.5) for 30 minutes and probed for total EphB2 (goat polyclonal 1:1000, R&D Systems) and beta actin (mouse monoclonal C4, 1:1000 Santa Cruz Biotechnology, Dallas, TX) as loading controls. Horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Piscataway, NJ) were added for two hours at room temperature and developed with ECL Prime (GE Healthcare) according to manufacturer's recommendations. Membranes were digitally imaged on LAS4000 (GE Healthcare).

Results

Generated bound state ensemble using Replica exchange Molecular Dynamics Simulations

We first investigate the dynamics of EphB2-SNEW complex using classic molecular dynamics. We compared the RMSD for the protein complex, the peptide by itself and the EphB2 receptor (Figure 1A). While the overall complex is stable, the SNEW peptide has much larger flexibility than the EphB2 receptor (Figure 1A).

We also conducted replica exchange simulation of the EphB2-SNEW complex, with temperature ranges from 300 K to 400 K. Each replica was equilibrated for 5.5 ns (total simulation time 264 ns). The REMD simulation can provide better sampling of the conformational states of the EphB2-SNEW complex. In addition, we explore how well our simulation setup can test the stabilities of the EphB2-SNEW complex in the bound state. The initial conformations for the REMD simulations are equally grouped into bound and unbound systems (Supporting Figure 1B and 1C, respectively). In the 24 bound state replicas, the EphB2-SNEW complex crystal structure was used as starting conformation, as in the classical MD simulations. In the 24 unbound state replicas, the SNEW peptide was removed from the Eph binding pocket and artificially put freely nearby the Eph ligand

binding domain (Supporting Figure 1C). In order to compare the relative stabilities of bound and unbound states, we compare the temperatures of the replicas corresponding to the bound and unbound states in the last 0.5 of simulation. During the REMD simulations, we have seen that the bound states occupied most of the low temperature replicas, indicating that bound states are much more stable than the unbound states (Figure 1B).

36 lowest energy conformations were selected from the REMD simulations of the EphB2-SNEW complex. As can be seen from Supporting Figure 1B, these conformers have large variation around binding site loops in the EphB2 receptor. For the SNEW peptide, the N-terminal residues are very stable, while the C-terminal residues have large conformational changes. Since all unbound states are located in the high temperature replicas, we have run additional MD simulation of unbound EphB2-SNEW in room temperature MD simulations and selected 14 unbound EphB2-SNEW conformers as references for the free energy perturbation calculations.

Free Energy Perturbation (FEP) indicated that the N-terminal of the SNEW peptide is already highly optimized

Experimental binding affinities were measured for several SNEW peptide mutants (Table 1). We first test our FEP protocol by comparing the simulation and experimental results. We select E3Q, Q6L, Q6S, R8A for the FEP simulations with long (200 ps each window) and short (60 ps each window) simulation times. As can be seen from Table 1, the long simulation protocol reproduced the experimental trend for all mutants tested, while the short protocol predicted right the trend for the Q6L, E3Q mutants, but obtained false positive results for Q6S and R8A. Overall, the short protocol can be used for fast screening of possible mutations, to exclude mutations that likely will weaken EphB2-SNEW binding.

Using the short protocol, we extensively screened most positions in the SNEW peptide for possible improvements of the binding affinity (Figure 2). In Figure 2, we illustrate the structure and calculated free energy change of the tested mutations. It is interesting to note that all tested mutations within the first four residues SNEW have positive free energy change, indicating decreasing binding energy. For the position of Ile5, a Leu (I5L) mutation might increase binding affinity. In the crystal structure, Gln6 has no direct interaction with the Eph receptor. Data from our simulations supports earlier findings that mutating the SNEW's sixth residue from glutamine to leucine (Q6L) creates a peptide with a higher binding affinity for the EphB2 receptor. Our ΔG value for this mutation was -0.26, compared to the experimental value of -0.4 ± 0.1 . Several mutations at this position also resulted in negative ΔG values, which indicated a high affinity mutation (Q6C, Q6H, Q6I, and Q6M). As mentioned earlier, Q6S is a false positive result.

We selected several SNEW peptide mutants for molecular dynamics simulations to investigate possible effects of point mutations on the conformational dynamics of the EphB2-SNEW complex. As can be seen in Figure 3, E3Q, Q6L, and Q6S generally stabilized the EphB2-SNEW complex to a certain extent (Figures 3A-3C). However, the SNEW peptide is greatly destabilized by the Q6G mutation (Figure 3D).

The C-terminal of the SNEW peptide can be further optimized to improve EphB2 binding affinity

There are two proline residues in the C-terminal of part of the SNEW peptide (Pro7 and Pro10). Since mutations of these two residues involve backbone change and may have a large error in the FEP calculations, we choose not to test mutations at the Pro7 and Pro10 positions. FEP results for the mutation at the Arg8 position are generally unfavorable (Figure 2), because there is a salt bridge between Arg8 and Glu52 in EphB2. This salt bridge has a longer distance in the crystal structure, but is very close during the MD simulations. The calculated negative free energy value of R8A mutation is a false positive. It seems that for a mutation involving charged residue (as in the R8A mutation) a longer simulation time is needed.

Fast screen at Leu9 position generated interesting results (Figure 2). Since Leu9 binds near a hydrophobic residue (Phe73) in EphB2, mutations to charged residues are not favored (L9D and L9R). The mutation to another aromatic residue Tyr (L9Y) provides no indication of improvement. However, about half of the tested mutations suggest possible improvement. The results of L9G and L9S appear encouraging. Additional MD simulations have shown that the L9S mutation leads to the most stable EphB2 complex observed in this study (Figure 3E), mainly due to the stabilized His12(SNEW)-Glu74(EphB2) interactions (Figure 3I). However, the L9G mutation has increased considerably the peptide flexibility (Figure 3D).

Using the long protocol, we rechecked the I5L, L9S, L9T, and L9G mutations. The Q6L mutation was combined with I5L, L9G, L9S, and L9T mutations (Table 1). Negative ΔG values were obtained for mutations of Q6L-I5L, Q6L-L9S, and Q6L-L9T. The results confirmed that I5L, L9S, and L9T could potentially boost EphB2-SNEW binding affinity.

In an effort to generate peptides with improved binding interactions at the C-terminal, we combined the L9G and L9S with mutations of Gln11 and His12. Starting from SNEW-L9S and SNEW-L9G sequences, we explored variations in Gln11 position (Figure 2, Table 2). For the SNEW-L9G sequence, more than half of the mutations tested can improve the binding energy, suggesting that these mutations can make up for some of the interactions lost by the L9G mutation. For the SNEW-L9S sequence, mutations at the Gln11 position indicated that this is a potential site for improving the binding energy. Note that Q11Y mutation in both SNEW-L9S and SNEW-L9G sequences shows a similar negative free energy change. For the SNEW-L9S sequence, we also screened for possible mutations at the His12 position. Interestingly, none of the tested mutations at the His12 position suggest possible improvement (Table 2), indicating that for SNEW-L9S sequences, it is important to keep the stable His12(SNEW)-Glu74(EphB2) interactions observed during SNEW-L9S-EphB2 simulations (Figure 2).

In the final round of the FEP screen, starting from the SNEW-L9S sequence and Q11 position variations optimized in the earlier steps (for example, L9S-Q11Y and L9S-Q11H) we again searched for possible mutations at the His12 position. While a few mutations were identified with negative free energy change (like L9S-Q11Y-H12K, Figure 2), most tested mutants showed no sign of improvement.

Allosteric coupling of N- and C-terminal residues of SNEW peptide in the SNEW-EphB2 interactions

Four N-terminal residues SNEW are buried in a deep pocket in EphB2, and our FEP calculations indicated that any perturbation of these four residues would decrease the binding energy. Here we examine the dynamics of the first two residues. We analyzed distances in the wild-type protein complex, in addition to the E3Q, L9G, L9S, Q6G, Q6L, Q6S and R8A mutations.

Distances between the first residue on the peptide, Ser1, and the 107th residue of the EphB2 protein, Ser107, were monitored. The nitrogen (N) on Ser1 and the oxygen (OG) on Ser107 were used for the first distance analysis. The graph of the distance between Ser1's nitrogen and Ser107's oxygen showed that this distance was significantly smaller for L9G than for the wild-type complex and SNEW-L9S mutant (SNEWPRS) complex (Figure 3J), indicating the allosteric coupling of N- and C-terminal residues of SNEW peptide in the SNEW-EphB2 interactions. The Q6G mutation and E3Q mutation also shortened the distance between these two residues.

The distance between Ser1 and Asp104 was analyzed. First, we looked at the distance between serine's oxygen (OG) and aspartic acid's oxygen (OD1). Overall, the distance showed high fluctuation, indicating that the oxygen on the serine side-chain appears to have more room to rotate. The L9G mutation increased the distances between these two atoms. This indicated that the L9G mutation rotated the peptide in such a way that brought Ser1 and Ser107 closer, but Ser1 and Asp104 farther apart. Other mutations such as L9S and Q6G, however, did not significantly change this distance. We also analyzed the distance between serine's nitrogen (N) and Asp104-OD1. This distance was largely consistent throughout the mutations that we examined.

We next looked at the distances between asparagine's oxygen (OD1) and lysine's nitrogen (Asn2-Lys166). No significant differences were seen for this distance. We then analyzed distances between the same two amino acids, this time focusing on distances between asparagine's nitrogen (ND2) and lysine's oxygen (O). Very little difference could be seen for this distance as well (Figure 3L), except for E3Q. E3Q had slightly larger distances, partially explaining why E3Q weakened SNEW binding. The distance between Asn2 (ND2) and Val102 (O) was also examined, and found to be consistent with the wild-type distance. This was also the case for the distance between Asn2 (N) and Val164 (O). The results showed the stability and rigidity of asparagine in EphB2's binding pocket.

Calculation of the SNEW-EphB2 binding affinity using MM-GBSA

The binding energy changes predicted from the FEP are based on only one point mutation each time. In order to compare the overall binding energy for the peptides with the multiple introduced mutations, we calculate the binding free energy using the Amber MM-GBSA method. Before calculating binding energies, sixty conformations extracted from the MD trajectories were energy optimized for 100 steps using the SANDER module in the Amber software. Then, the overall binding energy is obtained by averaging the binding energies from the sixty conformations. Seven peptides are selected to run the MM-GBSA binding

energy evaluation after 30 ns MD simulations, and the predicted binding energies are listed in Table 3. When we examined the average binding energy and the lowest binding energy conformers, we found that several designed peptides have potential to have better binding energy than SNEW and its known Q6L variant. We selected three of them (SNEWLL, SNEWPRS, and SNEWIL) to compare with the wild type SNEW peptide using experimental approaches.

Experimental examination of SNEW peptide variants-EphB2 binding interactions

To test experimentally the EphB2-EphrinB2 blocking function of SNEW mutant peptides, we employed a solid-phase sandwich ELISA. In this assay, we measured the specific binding of recombinant EphB2-Fc extracellular domain to recombinant EphrinB2 extracellular domain, with or without addition of SNEW and SNEW mutant peptides. The results show that all peptides display similar dose-dependent blocking of EphB2-EphrinB2 binding. The mutant peptide SNEWLL displayed the lowest degree of blocking activity (Figure 4A). We estimated that the IC₅₀ ranged between 10-50 μ M for all peptides. Biacore documented rapid on and off rates for the SNEW and SNEW mutant peptides. Affinity estimates from these studies ranged from 2 μ M to 10 μ M for all the peptides (Supporting information Figure 2).

SNEW and mutant SNEW peptides blocking activity was specific for EphB2-EphrinB2 as all peptides minimally inhibited EphB3-EphrinB2 binding (Figure 4B) and only slightly inhibited EphB6-EphrinB2 binding at the higher concentrations tested (Figure 4C).

We examined whether the peptide blocking function detected by ELISA resulted in functional inhibition of EphB2 signaling induced by EphrinB2. To this end, we induced EphB2 phosphorylation in the colon carcinoma cell line Colo205, which expresses surface EphB2 (but not or minimally EphB1), by incubation with clustered EphrinB2-Fc alone or in the presence of SNEW and SNEW mutant peptides. As shown in Figure 5A, SNEW, SNEWPRS and SNEWIL similarly inhibited EphB2 phosphorylation, whereas SNEWLL displayed minimal inhibition (Figure 5A, and confirmed by measurement of p-EphB2/EphB2 and p-EphB2/beta-actin ratios). Noteworthy, SNEWLL did not increase EphB2 phosphorylation induced by EphrinB2-Fc (Figure 5A).

These results show that SNEW and SNEW mutant peptides SNEWPRS and SNEWIL similarly block EphrinB2 binding and activation of EphB2.

Discussion and Conclusion

SNEW peptide was originally identified through Phage display screens [32]. Previously, the Monte Carlo combinatorial mutagenesis program package was used to optimize residues 1-9 of the SNEW peptide, with consideration of the changes in energy due to van der Waals forces, hydrophobic interactions, electrostatic interactions, hydrogen bonds and changes in entropy. These energy changes helped create a “docking score” to rate how well the newly-mutated peptide bound to the Eph receptor. The top scoring mutants were chosen for laboratory production. The selected mutations were E3Q, Q6S, Q6L, R8K, R8A and R8H, as well as two double mutants and one triple mutant peptide. The researchers were able to

analyze the binding interactions and measure the change in binding energy and binding affinities experimentally for these mutant SNEW peptides. They discovered that the Q6L mutation resulted in doubling of the binding affinity. Additionally, Q6S and R8K mutations produced a peptide with binding affinity similar to the original peptide sequence [1].

In this work, we used more rigorous computational approaches to reevaluate the binding interactions of SNEW peptide with EphB2 receptor. Our method combined all atom explicit water molecular dynamics simulations, free energy perturbation calculations, and MM-GBSA binding energy evaluations, followed by experimental examination of the computational results. Our results showed that several combinations of mutations have the potential to modify the SNEW peptide sequence and maintain binding affinity for the EphB2 receptor. The data provide insight into how each amino acid interacts within the binding pocket of EphB2. First, mutating any of the first four amino acids produced a mutated peptide with significantly lower binding affinity than the original peptide. By contrast, the C-terminal end of the peptide has the potential to improve SNEW binding affinity biophysical properties.

We have found that the L9S mutation, as well as other mutations involving L9S, may be used to further optimize SNEW peptide. This mutation stabilized the C-terminal end of the SNEW peptide, allowing stronger interaction with the EphB2 receptor. Other combinations of mutations, such as the I5L-Q6L-L9S-Q11Y-H12K, showed significant changes in the orientation of the SNEW peptide in complex with the Eph receptor.

Experimental examination of the computationally-selected peptides provided insights into SNEW-EphB2 binding. The tested SNEW peptide variants display similar inhibitory effects, indicating the merit of further improving computational accuracy. SNEWPRS and SNEWIL have different binding mechanisms at the C-terminal end. While the L9S mutations render the SNEWPRS peptide a rigid binder with stabilized His12-Glu74 interaction, the L9G mutation introduced in the SNEWIL sequence (SNEWILPRGPYK) renders the C-terminus more flexible (Figure 3H). The experimental results from the SNEWIL peptide indicate that it is indeed possible to further optimize the C-terminal sequence of the SNEW peptide. This is an important conclusion, especially when considering that the L9S-Q11Y combination identified in this study generates a PXSPY motif that can be similarly aligned with several other EphB2 binding peptides (Figure 5B). There is no structural information for the EphB2 binding peptides other than SNEW peptides, but if the peptides with the PXSPY motif assume a similar binding mode as shown by the SNEW peptide, it is expected to help in evaluating common structural features of EphB2-peptide binding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

EphB2-SNEW complex is a dynamically flexible molecular system. (A) Root-mean-square deviations (RMSD) for the wild-type observed during classical MD simulation (B) Temperature distributions of 48 replicas in the REMD simulations indicated that the EphB2-SNEW conformers in the bound state are much more stable than unbound SNEW together with EphB2 receptor.

Figure 2. Structural illustration of SNEW-EphB2 binding. The binding energy changes predicted by FEP calculations are listed. The structure was based on crystal structure (PDB cord: 2QBX) of SNEW peptide EphB2 complex. The missing residue of H12 was added and energy minimized. The detail SNEW-EphB2 interactions can also be seen from Figure 4 in reference [1].

Figure 3.

SNEW variants have different conformational dynamics in the EphB2 binding site, and SNEW-L9S is the most stable. (A-H). RMSD of SNEW variants EphB2 complex observed during classical MD simulation. (I-L) Residue distances trajectories of N- and C-terminal residues during classical MD simulation.

Figure 4. Effects of SNEW, SNEWLL, SNEWPRS and SNEWWIL peptides on EphrinB2 binding to EphB2 (A), EphB3 (B) and EphB6 (C). The results of solid-phase ELISA show dose-dependent peptide competition of EphB2 binding to immobilized EphrinB2. Data points reflect the mean of triplicate measurements; standard deviations <10%

Figure 5.

(A) Effects of SNEW, SNEWLL, SNEWPRS and SNEWWIL peptides on EphB2 phosphorylation induced by clustered EphrinB2-Fc in the colon carcinoma cell line Colo2015. Immunoblotting results with specific antibodies to phosphorylated-EphB2. The p-EphB2 specific band is identified the arrow. The membranes were re-probed for total EphB2 and β-actin. (B) Sequence comparison of SNEW peptide variants and other EphB2 binding peptides. The red and italicized letters in panel B highlight our proposed PXSPY motif for the EphB2 binding peptides. Two aligned proline residue are italicized, and the positions of S and Y residue are showing in red font. The five peptides in the lower half of the panel are other phage display identified peptides that bind EphB2 with less binding affinity as SNEW peptide.

Table 1

Comparison of different simulation protocols and experimental binding affinities of SNEW peptide variants.

Mutation	G kcal/mol (long)	G kcal/mol (Short)	G kcal/mol Experimental	K_d (μ m)
Q6S	1.1 ± 0.4	-0.3 ± 0.4	0.3 ± 0.1	11
Q6L	-0.1 ± 0.6	-0.3 ± 0.6	-0.4 ± 0.1	3
R8A	-2.5 ± 4.4	-7.3 ± 3.8		> 50
E3Q	54.5 ± 13.6	7.0 ± 7.0		> 50
R8H	6.0 ± 5.1	6.8 ± 5.2		$\gg 50$
Q6G	1.6 ± 0.4	0.7 ± 0.3		
Q6L-I5L	-0.9 ± 0.4			
Q6L-L9G	0.4 ± 0.2			
Q6L-L9S	-0.2 ± 0.3			
Q6L-L9T	-0.5 ± 0.4			

Table 2

Binding affinities changed predicted by free energy perturbation simulations.

Mutations *	G kcal/mol (long)	Mutations *	G kcal/mol (long)	Mutations *	G kcal/mol (long)
L9G-Q11C	0.3 ±0.3	L9G-Q11K	-0.6 ±1.0	L9G-Q11W	-0.1 ±0.5
L9G-Q11D	1.2 ±0.7	L9G-Q11L	-0.8 ±0.3	L9G-Q11Y	-0.5 ±0.5
L9G-Q11E	0.0 ±0.5	L9G-Q11M	0.3 ±0.3	L9G-Q6I	0.5 ±0.3
L9G-Q11F	0.5 ±0.4	L9G-Q11S	-0.1 ±0.2	L9G-Q6M	-0.3 ±0.3
L9G-Q11H	0.2 ±0.3	L9G-Q11T	-0.8 ±0.4		
L9S-Q11L	0.2 ±0.6	L9S-Q11C	0.2 ±0.3	L9S-H12M	0.4 ±0.4
L9S-Q11Y	-0.2 ±0.7	L9S-Q11S	-0.1 ±0.4	L9S-H12C	0.7 ±0.4
L9S-Q11W	-0.1 ±0.6	L9S-H12L	0.6 ±0.4	L9S-H12S	0.6 ±0.4
L9S-Q11H	-0.6 ±0.6	L9S-H12Y	0.9 ±0.6	L9S-H12Q	1.3 ±0.5
L9S-Q11E	-1.0 ±0.6	L9S-H12W	1.1 ±0.6		
L9S-Q11K	2.9 ±1.4	L9S-H12E	3.9 ±0.8		
L9S-Q11M	0.0 ±0.5	L9S-H12K	2.7 ±0.8		

* The first group of mutations at L9 position only refers to sequence used for SNEW peptide, the FEP energies are the binding energy change caused by the mutations at Q11 or H12 positions.

Table 3
Binding energy calculated by MM-GBSA method

Peptides	Sequence	Binding energy, kcal/mol
SNEW	SNEWIQPRLPQH	-43.5 $\pm$ 5.9
SNEWLL	SNEWLLPRSPYK	-50.7 $\pm$ 10.5
I5L-Q6L-L9S-Q11Y-H12S/	SNEWLLPRSPYS	-41.7 $\pm$ 9.8
SNEW PRS	SNEWIQPRSPQH	-50.72 $\pm$ 6.8
Q6L	SNEWILPRLPQH	-40.77 $\pm$ 11.5
SNEWIL	SNEWILPRGPYK	-49.84 $\pm$ 7.7
I5L-Q6I-L9G-Q11L	SNEWLIPRGPLH	-46.30 $\pm$ 6.4