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Design and Synthesis of Potent Bivalent Peptide Agonists Targeting the EphA2 Receptor

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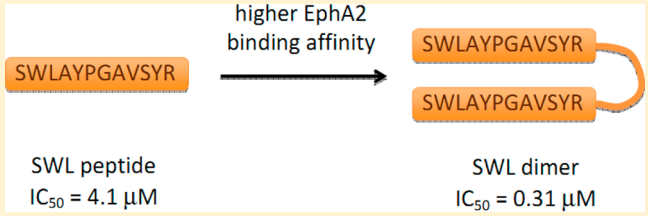
Supporting Information

ABSTRACT: Designing potent and selective peptides and small molecules that target Eph receptor tyrosine kinases remains a challenge, and new strategies are needed for developing novel and potent ligands for these receptors. In this study, we performed a structure−activity relationship study of a previously identified 12 amino acid-long peptide, SWL, by alanine scanning to identify residues important for receptor binding. To further enhance and optimize the receptor binding affinity of the SWL peptide, we applied the concept of bivalent ligand design to synthesize several SWL-derived dimeric peptides as novel ligands capable of binding simultaneously to two EphA2 receptor molecules. The dimeric peptides possess higher receptor binding affinity than the original monomeric SWL peptide, consistent with bivalent binding. The most potent dimeric peptide, a SWL dimer with a six-carbon linker, has about 13-fold increased potency as compared to SWL. Furthermore, similar to SWL, the dimeric peptide is an agonist and can promote EphA2 tyrosine phosphorylation (activation) in cultured cells.



KEYWORDS: *peptide inhibitors, Eph receptors, structure-based drug design, protein−protein interactions*

The Eph receptors comprise the largest family of receptor tyrosine kinases and play critical roles in developmental processes, in the normal physiology of adult tissues, and in the pathogenesis of diseases such as cancer.[1,2] The Eph family is divided in two classes with similar overall structure but different binding affinities for the ephrin ligands. Nine EphA receptors preferentially bind the five A-type ephrins, and five EphB receptors preferentially bind the three B-type ephrins. The ephrin-binding domain of the Eph receptors is located at the amino terminus and is followed by a cysteine-rich region and two fibronectin type III domains, while the cytoplasmic portion contains the tyrosine kinase domain. Whereas other receptor tyrosine kinases bind soluble ligands, the ephrins are tethered to the plasma membrane by either a glycosylphosphatidylinositol anchor (ephrin-A class) or a transmembrane domain (ephrin-B class). Thus, Eph receptors and ephrins regulate cell organization and movement through unique cell contact-dependent bidirectional signal transduction cascades, which are initiated upon dimerization and further clustering of Eph receptor−ephrin complexes.

Among the Eph receptors, EphA2 is perhaps the most widely expressed in different types of cancers and in the tumor vasculature and is therefore regarded as a promising drug target.[3−5] Activation of EphA2 signaling by ligands such as the ephrin-As, the YSA and SWL ephrin-mimetic peptides, or activating antibodies inhibits major oncogenic pathways, such as the Ras-Erk MAP kinase pathway and the Akt-mTORC1 pathway in various cancer cell types.[6−10] Hence, activating EphA2 signaling could be a strategy for inhibiting cancer progression. However, cancer cells expressing high EphA2 levels can become "addicted" to this receptor and its ephrin-dependent or -independent signaling activities, and in these instances, EphA2 promotes cancer cell growth and migration/invasiveness.[8,11−13] Furthermore, EphA2 is highly expressed in tumor endothelials cells, where it promotes tumor angiogenesis by interacting with ephrin-A ligands.[14,15] A better understanding of these diverse functions will help design successful strategies to modulate EphA2 function for anticancer therapy.

Agonistic peptides that target EphA2 with high affinity could be used to stimulate the tumor suppressor activities of the receptor in cancer cells.[6,8−10] Moreover, the peptides could also be used for targeted delivery of chemotherapeutic agents or toxins to tumor cells because ephrins and artificial ligands that cause EphA2 activation promote internalization of the receptor from the cell surface into lysosomes.[16−21] Finally, molecules binding to EphA2 with high affinity can be used for tumor imaging.[22]

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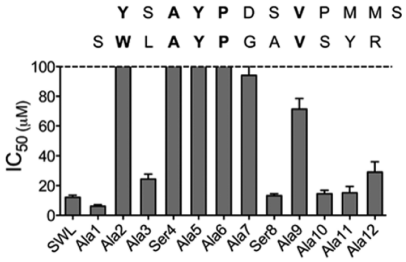
SWL (SWLAYPGAVSYR) is a peptide previously identified by phage display together with another related peptide, YSA (YSAYPDSVPMMS).[7] Both peptides were found to bind in a selective manner to EphA2 and not other Eph receptors. YSA was further characterized, and systematic amino acid replacement to alanine revealed that only six amino acids are critical for high binding affinity to EphA2. Furthermore, analysis of the YSA sequence showed that the C-terminal portion of the peptide is not essential for inhibition of EphA2-ephrin interaction, whereas N-terminal residues are critical for binding activity and EphA2 selectivity.[9] The binding affinity of the YSA and SWL peptides ranges from low micromolar to submicromolar depending on the addition of linkers, biotin, and other groups.[7,20,21] In any case, peptide concentrations of 50−100 μM are typically needed to induce detectable EphA2 activation and internalization into cells.[9,20,21] Thus, it would be desirable to increase the binding affinity of the peptides to make them more potent agonists and targeting molecules. In this study, we have focused on the less characterized SWL peptide. We have identified residues of SWL that are critical for EphA2 receptor binding and explored the effects of dimerization as a strategy to increase peptide potency by making it a bivalent EphA2 ligand.

To identify the SWL residues that are critical for the interaction with EphA2, we performed a systematic alanine scan in which each amino acid residue of SWL was replaced by alanine except for residues A4 and A8, which were replaced by serine. The SWL derivatives were examined in ELISAs for their ability to inhibit the binding of ephrin-A5 fused to alkaline phosphatase (ephrin-A5 AP) to the EphA2 ectodomain fused to the Fc portion of human IgG_1 (EphA2 Fc) and immobilized on protein A-coated wells. This revealed that amino acids W2, A4, Y5, P6, G7, and V9 are critical for EphA2 binding because their replacement with alanine abolishes or strongly impairs the inhibitory activity of SWL (Figure 1). Interestingly, the four residues that are identical in SWL and YSA and the conserved aromatic residues W2 in SWL and Y1 in YSA (in bold in Figure 1) are important for the inhibitory activity of both peptides (Figure 1 and ref 9), suggesting that SWL and YSA interact in a similar manner with EphA2. SWL residue G7 is also important for EphA2 binding, as previously found for the corresponding residue D6 in YSA,[9] despite the different nature of the two residues. In contrast, residues S1, L3, and A8 and the three most C-terminal residues of SWL appear to be less critical for the interaction with EphA2. Therefore, the alanine scan shows that the N terminus of SWL is the portion of the peptide most critical for EphA2 binding, as previously found for YSA.[9] In contrast, replacement of the C-terminal residues with alanine has only a minor or no effect, suggesting that this part of the peptide does not participate in critical interactions with EphA2.

We previously found that a dimeric peptide containing the first nine amino acids of YSA followed by a cysteine forming a disulfide bond upon oxidation is approximately 10-fold more potent as an inhibitor of ephrin binding to EphA2 than the 9-mer peptide without the cysteine.[9] In an effort to also make the related SWL peptide more potent, we therefore replaced S10 with a cysteine, since the alanine scan had shown that this residue is not essential for EphA2 binding. Dimerization through the formation of a disulfide bond between the cysteine residues of two peptide molecule yielded the SWL-C10 dimer peptide (Figure 2). In ELISAs, this dimeric peptide showed only ~3-fold increased ability to inhibit ephrin-A5 AP binding to EphA2 Fc when compared to monomeric SWL (Figure 3A,C), suggesting that this dimerization strategy may not allow bivalent binding.



Peptide	Sequence	IC_{50} ± SE (μM)
SWL	SWLAYPGAVSYR	12 ± 1.4
Ala1	**A**WLAYPGAVSYR	6.3 ± 0.96
Ala2	S**A**LAYPGAVSYR	>>100
Ala3	SW**A**AYPGAVSYR	24 ± 0.96
Ser4	SWL**S**YPGAVSYR	>100
Ala5	SWLA**A**PGAVSYR	>>100
Ala6	SWLAY**A**GAVSYR	>>100
Ala7	SWLAYP**A**AVSYR	94 ± 12
Ser8	SWLAYPG**S**VSYR	13 ± 1.3
Ala9	SWLAYPGA**A**SYR	71 ± 7.1
Ala10	SWLAYPGAV**A**YR	14 ± 2.3
Ala11	SWLAYPGAVS**A**R	15 ± 4.3
Ala12	SWLAYPGAVSY**A**	29 ± 6.9

Figure 1. Alanine scan for the SWL peptide. The histogram shows IC_{50} values calculated from curves of inhibition of ephrin-A5 AP binding to immobilized EphA2 Fc. The table lists the average IC_{50} values and corresponding standard errors (SE) from three measurements. The sequence of the SWL peptide is also shown above the histogram, aligned with the sequence of the related YSA peptide. Residues that are identical in the two peptides and the similarly positioned aromatics W2 in SWL and Y1 in YSA are indicated in bold.

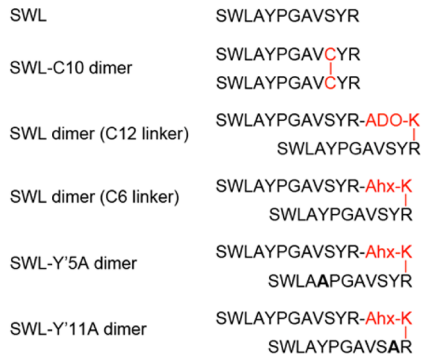


Figure 2. Peptide sequences. SWL is the original monomeric SWL peptide. The SWL-C10 dimer peptide contains two SWL monomers dimerized through a disulfide bond between the cysteines at position 10 (C10), which replace S10 in the original SWL. The SWL dimer (C12 linker) peptide contains two SWL monomers linked through a 12 amino dodecanoic acid−lysine (ADO-K) linker. The SWL dimer (C6 linker) peptide contains two SWL monomers linked through an amino−hexanoic acid−lysine (Ahx-K) linker. SWL-Y′5A and SWL-Y′11A are mutated versions of the SWL dimer (C6 linker) peptide with the alanine replacing a tyrosine indicated in bold.

We therefore explored different linkers that would provide different spacing of the receptor-binding N-terminal portions of dimeric SWL to find one allowing proper positioning in the ephrin-binding pockets of two EphA2 receptor molecules. As a guide, we used manual docking to model a possible binding pose for two SWL sequences dimerized through a linker attached to their less important C termini. The dimeric SWL was docked (without carrying out a peptide structure

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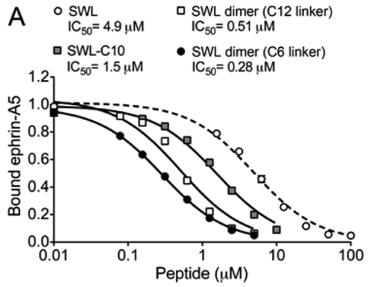

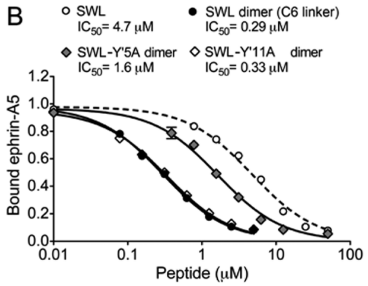

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Peptide	IC_{50} ± SE (μM)
SWL	4.1 ± 0.41
SWL-C10	1.2 ± 0.19
SWL dimer (C12 linker)	0.51 ± 0.030
SWL dimer (C6 linker)	0.31 ± 0.015
SWL-Y'5A (C6 linker)	1.8 ± 0.35
SWL-Y'11A (C6 linker)	0.47 ± 0.17

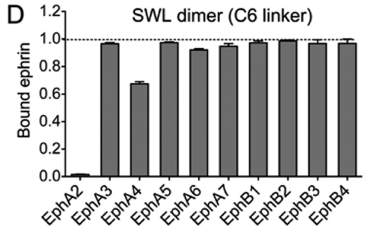

Figure 3. Dimerized SWL peptides are more potent than monomeric SWL. (A and B) Curves measuring inhibition of ephrin-A5 AP binding to immobilized EphA2 Fc. The ephrin-A5 AP signal in the presence of different peptide concentrations was normalized to the signal without peptide. IC_{50} values for the curves shown are indicated. (C) Average IC_{50} values calculated from four independent experiments for each peptide. (D) Measurements of inhibition of ephrin-A5 AP binding to immobilized EphA Fc receptors and ephrin-B2 AP binding to immobilized EphB Fc receptors show that the SWL dimer (C6 linker) peptide selectively inhibits ephrin binding to EphA2. Only some inhibition of EphA4−ephrin-A5 interaction was observed at the 10 μM concentration of SWL used, which is, however, much higher than the IC_{50} value for EphA2. Bound ephrin represents the signal in the presence of 10 μM SWL dimer (C6 linker) peptide normalized to the signal in the absence of the peptide.

conformational search) within the ephrin-binding pockets of two EphA2 ligand-binding domains positioned as observed in crystals of EphA2 ectodomains (including the ligand-binding domain, cysteine-rich region, and first fibronectin type III domain) in complex with the ephrin-A5 ligand (Figure 4A).[23,24] The signaling assemblies observed in the crystals revealed not only EphA2−ephrin-A5 binding interfaces but also interfaces involving the ligand-binding domains and cysteine-rich regions of different EphA2 molecules.[23,24] Importantly, the same signaling assemblies were observed for EphA2 in complex with ephrin-A1 or unbound. Furthermore, site-directed mutagenesis and cell-based signaling studies demonstrated the

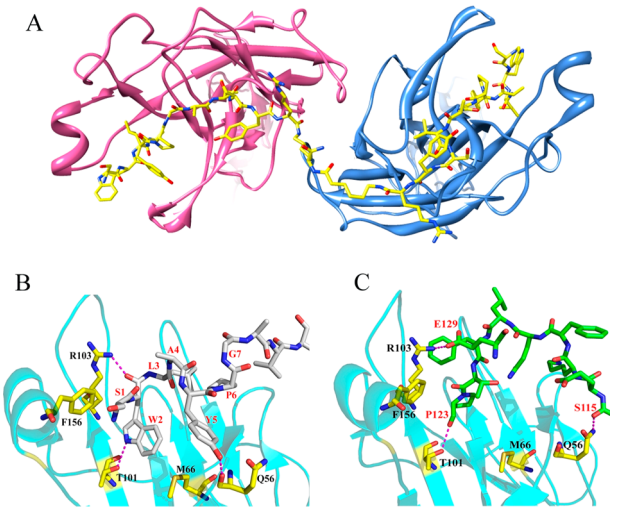

Figure 4. Molecular docking illustrates a potential binding orientation of a bivalent SWL dimer peptide bound to two EphA2 ligand-binding domains. (A) Overall orientation of a SWL dimeric peptide bound to two EphA2 molecules. This model was manually constructed based on the crystal structure of two EphA2 molecules in complex with ephrin-A5 (PDB: 3MX0). The EphA2 ligand-binding domains are shown as pink and blue ribbons, and the SWL dimeric peptide is shown as a stick model with yellow representing carbon atoms, red representing oxygen atoms, and blue representing nitrogen atoms. (B) Zoom-in of SWL residues in the EphA2 ephrin-binding pocket. EphA2 is shown as a cyan ribbon with key residues shown as yellow sticks, and the SWL peptide is shown as gray sticks. Pink dashed lines represent hydrogen bonds. (C) Structure of ephrin-A5 G-H loop residues in the EphA2 ephrin-binding pocket. Ephrin-A5 residues are shown as green sticks.

physiological relevance of the binding interfaces observed in the crystal structures.[23,24]

In the model of the dimeric peptide bound to two EphA2 molecules, SWL residue W2 forms a hydrogen bond with EphA2 T101, the backbone of SWL L3 forms a hydrogen bond with EphA2 R103, and the side chain of SWL Y5 forms a hydrogen bond with EphA2 Q56 (Figure 4B). Furthermore, the phenyl ring of SWL W2 forms an additional hydrophobic interaction with the side chain of EphA2 M66. This model is consistent with the binding arrangement of the ephrin-A5 G−H loop in the EphA2 ephrin-binding pocket in the crystal structure of the complex (PDB: 3MX0), where ephrin-A5 is engaged in hydrogen bond interactions with EphA2 Q56, T101, and R103 (Figure 4C).[23] However, EphA2 mutagenesis studies will be required to unequivocally confirm this model.

From the computer model, we estimated that a C-terminal linker of ∼40 Å would allow the N-terminal portions of the dimeric peptide to reach the ephrin-binding pockets of two EphA2 molecules. Therefore, we selected a hydrophobic six-carbon amino-hexanoic acid (Ahx) linker (Figures 2 and 4A). Additionally, we also used a longer 12-carbon amino dodecanoic acid (ADO) linker for comparison (Figure 2). The dimeric peptides were synthesized starting from a SWL peptide modified with a C-terminal Fmoc-amino hexanoic acid, or Fmoc-amino dodecanoic acid, followed by a lysine. The second SWL moiety was synthesized from C-terminal to N-terminal by coupling it through the γ-amino group of the lysine side chain.

ELISAs measuring inhibition of EphA2−ephrin-A1 binding showed that the SWL dimer (C6 linker) and SWL dimer (C12 linker) peptides are, respectively, ∼13- and ∼8-fold more active

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than monomeric SWL (Figure 3A–C). This suggests that the C6 linker is more suitable for increasing the EphA2 binding affinity of a SWL dimer. Because the SWL dimer (C6 linker) peptide had the highest potency, we further characterized it. This revealed that the dimerization and increased affinity do not affect the high selectivity of the peptide for EphA2 relative to other Eph receptors even at concentrations much higher than the IC_{50} value (Figure 4D).

To demonstrate that the increased affinity of the SWL dimer (C6 linker) peptide is due to its binding to two EphA2 molecules rather than additional interactions with a single receptor molecule, we took advantage of the information from the alanine scan. The scan shows that replacing Y5 with alanine strongly decreases the SWL inhibitory potency, whereas Y11 replacement essentially does not affect potency. We synthesized a SWL-Y′5A dimer in which Y′5 (tyrosine 5 in the peptide moiety attached to the lysine side chain) was replaced by alanine and a SWL-Y′11A dimer in which Y′11 (tyrosine 11 in the peptide moiety attached to the lysine side chain) was replaced by alanine (Figure 2). ELISAs showed that the inhibitory potency of the SWL-Y′5A dimer is significantly reduced ($p < 0.01$ by one-way ANOVA), whereas the potency of the SWL-Y′11A dimer is very similar to the unmodified SWL dimer (Figure 3B). This result is consistent with the SWL-Y′5A peptide binding mostly through the intact peptide moiety, with only a minor contribution from the mutated moiety, and the SWL-Y′11A dimer retaining nearly intact bivalent binding ability. This supports an increased avidity of the SWL dimer, conferred by a decreased dissociation rate due to binding to two EphA2 molecules immobilized on the ELISA wells.[25,26]

SWL was previously shown to promote EphA2 phosphorylation (which is indicative of activation) and downstream inhibition of Erk1/2 MAP kinases and Akt in PC3 prostate cancer cells at a concentration of 50 μM.[9] Consistent with its higher potency in ELISAs, the SWL dimer (C6 linker) peptide can detectably increase EphA2 tyrosine phosphorylation in PC3 cells at concentrations as low as 0.5 μM, even though higher EphA2 tyrosine phosphorylation was observed with 10 and 50 μM (Figure 5). Furthermore, the SWL dimer (C6 linker) peptide at 10 μM activates EphA2 more than the SWL monomer at 50 μM. However, we also found that the SWL dimer (C6 linker) peptide has a ∼8-fold shorter half-life in mouse serum as compared to the SWL monomer (Figure S1 in the Supporting Information). Thus, modifications may be required to decrease the sensitivity of the SWL dimer (C6 linker) peptide to proteases that could generate monomeric or less active peptide. This should result in increased activity of the dimer in biological fluids.

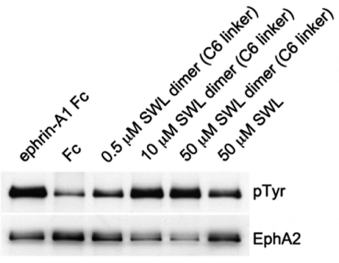

Figure 5. SWL dimer (C6 linker) peptide promotes EphA2 tyrosine phosphorylation at low micromolar concentrations. PC3 prostate cancer cells were stimulated with the indicated concentrations of SWL monomer, SWL dimer (C6 linker) peptide, ephrin-A5 Fc as a positive control, or Fc as a negative control. Immunoprecipitated EphA2 was probed with antiphosphotyrosine antibodies (PTyr) and reprobed with anti-EphA2 antibodies.

EphA2-targeting peptides derived from the SWL peptide identified by phage display but having higher binding affinity, such as the SWL dimer (C6 linker) peptide described here, represent improved molecules that could be used to selectively activate EphA2 tumor suppressor pathways in cancer cells or to deliver chemotherapeutic drugs, toxins, and imaging agents to EphA2-expressing tumor cells and tumor vasculature.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and HPLC data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

S.D. designed and synthesized the dimeric peptides; S.M. performed the SWL alanine scan; I.L. characterized the dimeric peptides; X.H. and X.Y. performed computer modeling; and J.A., E.B.P., and Z.H. oversaw the work. S.M. and I.L. contributed equally to this work.

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Notes

The authors declare no competing financial interest.

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