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A unique mid-sequence linker used to multimerize the lipid-phosphatidylserine (PS) binding peptide-peptoid hybrid PPS1

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Abstract

Ligand multimerizations enhance the binding affinity towards cell surface biomarkers through their avidity effects. Typical linkers connect individual monomeric ligand moieties from one end (e.g., C- or N-terminus of a peptide) and exclusively target protein receptors. The lipid phosphatidylserine (PS) is normally present on the cytoplasmic side of the eukaryotic cell membrane, but in tumors and tumor endothelial cells, this negatively charged PS flips to the outer layer. We recently reported a PS binding peptide-peptoid hybrid (PPS1) that has distinct positively charged and hydrophobic residue-containing regions. The PPS1 monomer is inactive, and upon C-terminal dimerization (PPS1D1), it triggers cytotoxicity. In the current study, a unique series of PPS1 multimeric derivatives were synthesized by switching the linker from the C-terminus to an internal position. The unimportant fourth residue (N-lys) from the C-terminus was utilized to build the linker. The synthesis strategy was developed employing variations of (I) the linker size, (II) the number of positively charged residues, and (III) the number of hydrophobic regions. Cytotoxicity of these new derivatives on HCC4017 lung cancer cells showed that a minimum of two hydrophobic regions was important to retain the activity and that the shortest linker length was optimal for activity.

Graphical abstract

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Keywords

Multimers; Peptoids; Sequence modifications

1. Introduction

Monovalent ligands utilize a limited number of binding mechanisms. Natural (e.g., antibodies) and synthetic multivalent molecular peptides[1-3], peptidomimetics (such as peptoids[4-6]), carbohydrates[7-9], and small molecules[10, 11], present multiple copies of a receptor-binding element, which can therefore bind with high avidity and specificity.[9, 12-17] These can interact with receptors via chelating effects, receptor clustering, subsite binding, and statistical rebinding, all of which can lead to an improved affinity.[18] The formation of homo-multimers is the most common approach used to target multiple copies of a receptor, while hetero-dimerizations[16, 19] are also reported to target two different receptors that simultaneously express on the same cell surface. For almost all of the cases, the homo- or hetero-multimer linkers was either on the C- or N-terminus of the individual monomeric peptide or peptidomimetic units.[1, 3-6, 12, 20, 21]. In this study, we report a unique multimerization procedure using a lipid-phosphatidylserine (PS)-targeted peptide-peptoid hybrid (PPS1)[4, 22] containing linkers built into the internal region of the monomeric sequence.

Although significant progress has been made in cancer therapeutic development, the targeted biomarker proteins are highly heterogeneously expressed, which hinders the development of universally effective anti-cancer drugs.[23-25] Therefore, biomarkers that specifically and universally present on cancer cells and can be easily differentiated from normal cells are needed to be identified and targeted. The anionic lipid, PS, which is normally found in the inner layer of normal cell membranes, has been reported to be transferred to the outer layer of the lipid membrane in cancer and tumor endothelial cells.[26-30] The universal presence of negatively charged PS in the tumor microenvironment suggests a promising target with increased specificity over normal cells.

Our PS targeted PPS1 displayed high specificity of binding towards a panel of cancer cells that express PS, but not to normal cells [4, 22]. Interestingly, a simple C-terminal dimeric version of PPS1, PPS1D1 (Figure 1A), displayed potent *in vitro* cancer cell killing activity as well as *in vivo* tumor burden effects, while the monomeric PPS1 remained inactive[4]. This clearly indicated that the multimerization triggered the cancer cell killing activity of PPS1. Subsequently, the minimum pharmacophore studies of PPS1D1 (by replacing each monomer with alanine/sarcosine, one at a time) using ELISA binding and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell viability assays showed that the first and fourth residues were not necessary for the binding and activity of PPS1D1[31]. This suggested that the presence of positively charged residues at the second and third positions, and all four hydrophobic residues at the N-terminus (residues 5–8) were important for binding and cytotoxicity (Figure 1A). There are previous reports describing the use of cationic residue containing ligands for specific cell surface binding and activities as well.[32-37]

We previously found that PPS1D1 recognized phospholipids with overall negative charge, such as phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylglycerol (PG) in addition to PS, but not neutral or positively charged phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM).[22] We hypothesized that the two positively charged residues at the second and third positions of PPS1D1 may interact with the additional negative charges on the phospholipid head groups, while the hydrophobic region at the N-terminus may interact with long carbon chains of the lipids within the membrane. This provided us a unique opportunity to move the linker into the middle of the sequence, as shown in Figure 1B, because the fourth N-lys residue is not critical for the activity. This separated the two important positively charged and hydrophobic regions into two groups (Figure 1B). Furthermore, we predict that moving this connecting linker into the middle of the sequence may not hinder the target recognition, as PPS1 does not interact with a conventional binding pocket on a protein. Rather, PPS1 may recognize membrane regions/clefts that are comprised of multiple PS-lipid molecules.

2. Results and Discussion

2.1 Overall ligand design

Considering scaffold architectural derivatization, we studied the multimerizations of PPS1 by varying: (I) the linker size (in the middle); (II) positive charges; and (III) the hydrophobic regions, and by converting the unimportant fourth residue to an internal linker (Figure 1B). This strategy positioned the two positively charged lysine residues towards one side of the linker and the four residues of the hydrophobic region on the other side of the linker (Figure 1B). According to the PPS1 sequence, two important positively charged residues were amino acids and four important hydrophobic residues were peptoid residues, which made the actual synthesis less complicated. The number of positively charged regions was kept constant at two, but we varied the number of positively charged residues between two to four. The number of hydrophobic regions, where each contained four residues, was varied one to three. We have designed a complete on-bead solid phase synthesis strategy to enable the synthesis of these compounds on resin beads (Scheme 1). Because no solution phase reactions were involved, this approach provided a facile avenue for synthesizing different numbers of derivatives, while changing the above mentioned parameters. Our main strategy for linkages and branching points was to use orthogonally-protected lysine residues [ivDde-Lys(Fmoc)-OH] (Scheme 1). The remaining strategy involved the utilization of standard solid-phase peptide/peptoid synthesis methodologies.[5, 38, 39]

2.2 The effect of linker transition to the middle

We first wanted to verify how the transition of the linker from the C-terminus to the fourth residue in the middle region would affect the binding and activity. We synthesized the compound PPS1-4P2H1 (Figure 2A) using lysine as the internal linker at the fourth position, which was the equivalent size of the C-terminal linker of PPS1D1. All the other residues remained intact as was found in the PPS1D1 homodimer. This produced a compound with two monomeric sequences of PPS1 uniquely combined at the fourth position. Each of these monomeric units contained two lysines at the second and third positions at one side of the

linker, which was at the fourth position, and four hydrophobic peptoids at the other side of the linker towards the N-terminus (Figure 2A).

The synthesis was completely performed on resin beads as follows: First, the C-terminal three amino acids (methionine and two lysines) were coupled onto the resin using lysines protected with standard Boc groups (Scheme 1, A-C). Then, an orthogonally protected ivDde-Lys(Fmoc)-OH was coupled to obtain compound D (Scheme 1, step 4). After removing the Fmoc group from the side chain of the lysine, another ivDde-Lys(Fmoc)-OH was coupled to obtain compound E (Scheme 1, step 5). Upon removal of the Fmoc group from the side chain of the second lysine, the N-terminal two lysine residues of the second monomeric sequence were added (Scheme 1, F-G). The sequence of adding two lysines was actually moving backwards, which introduced the symmetry and also the two important positively charged residues to this region. At the end of the synthesis, the open end of compound G was blocked, because our plan was to synthesize both hydrophobic regions on the other side in a parallel approach. This position was blocked using Boc-protected glycine and resulted in compound H (Scheme 1, step 8). Next, we removed both the ivDde groups simultaneously to obtain compound I (Scheme 1, step 9), and continued synthesis of both hydrophobic peptoid regions at the same time. The synthesis steps included the coupling of four peptoid residues, one at a time: 4-methoxybenzylamine (N-pmba); (R)-(+)- α -methylbenzylamine (Nmmba); piperonylamine (Npip); and (R)-(+)- α -methylbenzylamine (Nmmba), wherein, each time, they were added to both sequences to obtain compound J (Scheme 1, steps 10-13). Then, the final TFA treatment cleaved the compound from resin and simultaneously removed all the side chain protection groups, including the Boc-protection on glycine (Scheme 1, step 14).

We next determined the cytotoxicity of PPS1-4P2H1 by performing standard MTS assay[4, 6, 22] on HCC4017 lung cancer cells. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, which is known as MTS, a yellow tetrazole, is reduced to purple formazan in living cells. Absorbance of this can be read at 490 nm wavelength using a plate reader. If the compounds used to treat living cells produce cytotoxicity, this color change will be lesser and can be quantified. The cytotoxic effect of compound PPS1-4P2H1 was comparable with PPS1D1 (Figure 2B). As expected, the monomeric sequence PPS1 [Supplementary Figure S7 (A)] and the control non-active compound PC462D1 [Supplementary Figure S9 (A)] had no effect.[4] This confirmed that the linker could effectively be transferred to the midpoint of the sequence without affecting the activity of PPS1D1. Unlike protein binding ligands, this may be possible because of the nature of the lipid arrangements on the cell membrane. Our compound may not bind to a single PS molecule, but perhaps binds to multiple PS molecules embedded within the lipid bilayer. The two important regions necessary for binding (positive and hydrophobic) may be interacting independently with their counterparts, allowing structural changes to be made between these two regions of the PPS1D1. This observation provides valuable information for the future developments of lipid-binding ligand derivatives.

2.3 The effects of the mid-sequence linker length

We next determined how the length of this mid-sequence linker affected the activity. Two identical homo-dimers having the same number of positive and hydrophobic moieties as PPS1-4P2H1 were synthesized with a variable linker length at the 4th position of the middle of the sequence. PPS1-4P2H2 (Figure 3A) was first synthesized with β -alanine as an additional moiety at the linker region. Similarly, PPS1-4P2H3, comprised of 8-amino-3,6-dioxaoctanoic acid (AEEAc) as an additional linker apart from β -alanine, was also synthesized (Figure 3B). In both compounds, the new linker moieties were coupled after the removal of Fmoc from the fourth lysine residue (as shown in scheme 1, E). We next determined the effects of these two compounds on cell viability using MTS assays on HCC4017 lung cancer cells. Both compounds had less cytotoxic activity than that of PPS1D1 (Figure 3C) or PPS1-4P2H1 (Figure 2B and 3C). Overall, the cytotoxic effects of compounds PPS1-4P2H1, PPS1-4P2H2, and PPS1-4P2H3 were declined as the linker length was increased (Figures 2B and 3C). This indicated that the shortest linker produced compounds with the best cytotoxic activity. This may be due to the increased entropic penalty effect of binding energy, as increasing the linker length could increase the flexibility of the types of linkers used in our study. The fluidic nature of the cell membrane and complexities in PS and other lipid arrangements on the cell membrane could also contribute to these results. The reasons could be much more complex, and therefore more focused studies are needed to further evaluate this phenomena.

2.4 The effect of reducing number of hydrophobic regions

We previously reported that truncations to the N-terminal hydrophobic region inactivated PPS1D1.[31] To determine the number of hydrophobic regions necessary for minimal activity, compound PPS1-4P1H was synthesized having positive residues similar to its dimer counterpart, but possessing only one hydrophobic arm (Figure 4A). This compound was synthesized in similar fashion with PPS1-4P2H1 with specific modification to get only one hydrophobic arm in its moiety. Initial steps for the synthesis of PPS1-4P1H were similar as shown in scheme 1, B-D, except the addition of methionine residue (Scheme 1, A).[31] Requirement of one hydrophobic arm rendered a leap in its synthesis from D to F excluding E (Scheme 1) using only one ivDde-Lys(Fmoc)-OH. Then the rest of the steps were followed in Scheme 1 to obtain compound PPS1-4P1H.

Cell viability was determined by using the MTS assay in HCC4017 lung cancer cells. The cytotoxic effect of PPS1-4P1H was compared to that of PPS1D1, PPS1 and PC462D1. Interestingly as shown in Figure 4B, PPS1-4P1H was ineffective in cancer cell killing. This suggested that, although four important positively charged residues were present in the compound (two on each monomer), having only one hydrophobic arm was not sufficient to retain its cytotoxic activity. This strongly suggested that two hydrophobic regions were mandatory for the activity. This observation was similar to that observed for PPS1 and PPS1D1, where PPS1 (simple monomer) was completely inactive, while C-terminal dimerization of PPS1D1 triggered a drastic activity change[4]. Future studies are planned to determine how these unique dimerizations, either at the C-terminus or in the middle, alter cancer cell killing activity.

2.5 The effect of increased number of hydrophobic regions and changes to the positively charged region

After determining that a minimum of two hydrophobic arms was required for activity, we synthesized two new compounds with three hydrophobic arms with variable numbers of positively charged moieties present in the derivative. The linker was again positioned at the 4th residue region, such a way that it would separate the positively charged residues from the hydrophobic region (Figure 5A and B). Compound PPS1-2P3H has two positive charges and three hydrophobic arms (Figure 5A), whereas PPS1-4P3H has four positive charges along with three hydrophobic arms (Figure 5B). The synthesis of PPS1-2P3H and PPS1-4P3H were similar to that of the synthesis of PPS1-4P2H1, with specific modifications used to produce variable positively charged residues at the C-terminal and three hydrophobic regions at the N-terminal. The synthesis scheme for PPS1-4P3H is shown in Scheme 2, with the first five steps being same to scheme 1, to obtain compound E. Next, an additional (third) ivDde-Lys(Fmoc)-OH was added (Scheme 2, step 1) to obtain three amino ends (scheme 2, O) needed to building the three desired hydrophobic regions. The next steps were similar to scheme 1, F-J. Briefly, upon removal of the Fmoc group from the side chain of this third lysine, the N-terminal three residues of the second monomeric sequence were coupled (Scheme 2, steps 2-4). At the end of the synthesis, the open end of compound M was blocked using Boc-protected glycine to yield compound N (Scheme 2, step 5). Next, we removed all three ivDde groups simultaneously to obtain compound O (Scheme 2, step 6), and continued synthesis of all three hydrophobic peptoid regions at the same time yielding compound P (Scheme 2, steps 7-10). Then, the final TFA treatment cleaved the compound from resin, and simultaneously removed all the side chain protection groups, including the Boc-protection on glycine (Scheme 2, step 11). Synthesis of compound PPS1-2P3H was done as for PPS1-4P3H, except two steps described on Scheme 1, step 3, and Scheme 2, step 2, producing only one lysine on N-terminus of each of the monomeric units.

The cytotoxicity of PPS1-2P3H and PPS1-4P3H along with PPS1D1, PPS1, and PC462D1 as control compounds was evaluated in HCC4017 lung cancer cells using the same MTS cell viability assay (Figure 5C) as well as the calcein AM activity assay (Figure 5D) as a secondary functional assay. In living cells, the nonfluorescent calcein AM is converted to a green-fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases. This fluorescence can be read at 485nm using a plate reader. As is on MTS assay, if the compounds used to treat living cells produce cytotoxicity, this change will not occur. PPS1-4P3H, containing four positively charged residues (two each, on two monomeric units) and three hydrophobic regions, showed a 5-fold improvement in cell killing activity compared to that of PPS1D1. PPS1-4P3H had greater cytotoxic effects than PPS1-2P3H, which had only two positively charged residues (one each, on two monomeric units), again confirming the importance of having two positive charges on each monomeric sequence.[31] More importantly, these observations suggested that multimerization of hydrophobic regions provided improved compound activity. These results further confirmed our observation that two regions in the compounds (positive and hydrophobic) are important for binding and activity, and may interact independently. This observation further supports the possibility that our compounds are binding with multiple PS molecules embedded within the lipid bilayer.

3. Conclusions

The MTS cell viability assay data for HCC4017 lung cancer cells described above showed that a minimum of two hydrophobic arms are needed in the derivatives for them to exhibit cytotoxicity effects. The potency decreased with increased linker length (PPS1-4P2H3 < PPS1-4P2H2 < PPS1-4P2H1), indicating that the shorter linkers appeared to be the most effective. Increasing the hydrophobic regions to three at the N-terminus, along with four positively charged residues on two monomeric units (two on each) at the C-terminus, produced compound PPS1-4P3H, with the greatest cancer cell killing activity, showing a 5-fold improvement over its parent counterpart PPS1D1. This indicated that the positive region and hydrophobic region of the compounds independently interact with the negatively charged head group and hydrophobic region of the lipid (PS) respectively and multimerizations of these regions increase the cancer cell killing activity.

4. Experimental Section

4.1 General

5-mL disposable reaction columns (Intavis AG, Tübingen, Germany) were used as reaction vessels for solid-phase synthesis. All primary amines, bromoacetic acid, N,N-diisopropylcarbodiimide (DIC), N,N-diisopropylethylamine (DiPEA), piperidine, trifluoroacetic acid (TFA), dichloromethane (DCM) and N,N-dimethylformamide (DMF) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Novasyn TGR resin, all Fmoc-protected and ivDde protected amino acids and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from EMD Millipore. Hydroxybenzotriazole (HOBt) was purchased from AAPptec (Louisville, KY, USA). All chemical reagents and solvents from commercial sources were used without further purification until otherwise mentioned. Syntheses of peptoids under microwave conditions were performed in a 1000W microwave oven with 10% power. All purifications were completed on a Waters HPLC system, Waters Corporation, MA, USA. Mass spectra were recorded on an Applied Biosystems Voyager DE Pro mass spectrometer using alpha-cyano-4-hydroxycinnamic acid as the matrix.

4.2 Chemistry

4.2.1 Synthesis of PPS1-4P2H1—PPS1-4P2H1 was synthesized on NovaSyn TGR resin utilizing standard Fmoc-peptide synthesis protocol and microwave-assisted peptoid synthesis[38, 39], as described in Scheme 1. The peptide portion with methionine and lysines was completed prior to peptoid synthesis. Internal linker(s) were developed using orthogonally protected Lysine (ivDde-Lys(Fmoc)-OH). Peptoid residues were coupled by using microwave assisted two step peptoid coupling procedure. First reacting with bromoacetic acid and N,N-diisopropylcarbodiimide (DIC), followed by respective amine substitution to couple a peptoid residue. Briefly the steps from the beginning, 200 mg of beads were loaded in 5 mL reaction vessel and swelled in DMF for 30 min. Fmoc-Met-OH (92.9 mg, 5.0 eq), HOBt (5.0 eq), HBTU (5.0 eq) and DiPEA (10.0 eq) in 2 mL of anhydrous DMF was added to the beads and the reaction vessel was shaken overnight (Scheme 1, A). The solution was drained and beads were rinsed with DMF (10 × 2 mL).

Fmoc was deprotected by treating beads with 20% piperidine/DMF twice, each for 10 min. After rinsing the beads, subsequent amino acids Fmoc-D-Lys-(Boc)-OH, Fmoc-Lys-(Boc)-OH, ivDde-Lys(Fmoc)-OH, ivDde-Lys-(Fmoc)-OH, Fmoc-Lys-(Boc)-OH, Fmoc-D-Lys-(Fmoc)-OH and Boc-Gly-OH were coupled under similar reaction conditions, however duration of the reaction was 2.0 hours (Scheme 1, B-H). Next, two ivDde protecting groups were removed by treating beads with 5% Hydrazine/DMF solution three times, each for 10 min (Scheme 1, I). At this point, two hydrophobic regions with four peptoid units in each were synthesized using the two step microwave assisted peptoid synthesis protocol as follows. First, 1 mL of Bromoacetic acid (2.0 M in anhydrous DMF) and N,N-diisopropylcarbodiimide (3.2 M in anhydrous DMF) were added to beads, gently shaken for 30 seconds and placed in the microwave oven for 15 seconds with power set at 10%. The beads were again shaken for 30 seconds and placed in the microwave oven for another 15 seconds. Solution was drained and the beads were washed with DMF (10 × 2 mL). Next, the beads were treated with 2.0 M solution of the first primary amine, 4-Methoxybenzylamine (N-pmba) dissolved in anhydrous DMF (2 mL) and subjected to the same microwave procedure described above. The next three peptoid residues of the hydrophobic region was completed by repeating these two peptoid coupling steps using primary amines, (R)-(α)-Methylbenzylamine (Nmmba), Piperonylamine (Npip) and (R)-(α)-Methylbenzylamine (Nmmba). The synthesized compound was finally cleaved off from beads with the concomitant removal of protecting groups by treating the beads with 2mL solution of 95% TFA, 2.5% water, and 2.5% Triisopropylsilane (TIS) for 2.0 hours. The compound was purified using HPLC. Synthesis was confirmed by MALDI-TOF analysis and purity was confirmed by analytical HPLC.

4.2.2 Synthesis of PPS1-4P2H2—PPS1-4P2H2 was synthesized by using same protocol described above 4.2.1 and shown in Scheme 1 as it was for PPS1-4P2H1, except having β-alanine as an additional moiety at the linker region. This new linker moiety was coupled after the removal of Fmoc from the fourth lysine residue (as shown in scheme 1, E).

4.2.3 Synthesis of PPS1-4P2H3—PPS1-4P2H3 was synthesized by using same protocol described above 4.2.1 and shown in Scheme 1 as it was for PPS1-4P2H1, except having β-alanine and 8-amino-3,6-dioxaoctanoic acid (AEEAc) as additional moieties at the linker region. These new linker moieties were coupled after the removal of Fmoc from the fourth lysine residue (as shown in scheme 1, E).

4.2.4 Synthesis of PPS1-4P1H—PPS1-4P1H was synthesized by using same protocol described above 4.2.1 and shown in Scheme 1 as it was for PPS1-4P2H1, except with specific modification to get only one hydrophobic arm in its moiety. Initial steps for the synthesis of PPS1-4P1H were similar as shown in scheme 1, B-D, except the addition of Methionine residue (Scheme 1, A). Synthesis from compound D to F was completed excluding compound E (Scheme 1) using only one ivDde-Lys(Fmoc)-OH. Then the rest of the steps were followed in Scheme 1 to obtain compound PPS1-4P1H.

4.2.5 Synthesis of PPS1-2P3H—PPS1-2P3H was synthesized by using same protocol described above 4.2.1 and shown in Schemes 1 and 2, except with specific modification to

get only two lysines (one on each monomeric unit) at the C-terminus and three hydrophobic regions at the N-terminus. The first five steps were followed same as described in scheme 1, A-E, except step B (only one lysine added). Next, an additional (third) ivDde-Lys(Fmoc)-OH was added (Scheme 2, K). Then the steps described in Scheme 2 were followed, except adding only one lysine at L-M.

4.2.6 Synthesis of PPS1-4P3H—PPS1-4P3H was synthesized by using same protocol described above 4.2.1 and shown in Schemes 1 and 2, except with specific modification to get only four lysines (two on each monomeric unit) at the C-terminus and three hydrophobic regions at the N-terminus. The first five steps were followed same as described in scheme 1, A-E. Next, an additional (third) ivDde-Lys(Fmoc)-OH was added (Scheme 2, K). Then, the steps described in Scheme 2 were followed.

4.3 Biology

4.3.1 MTS cell viability assay—HCC4017 lung cancer cells (10,000 cells/well) were plated in quadruplicates in a 96 well plate and allowed to attach overnight. Cells were treated with increasing concentrations of test compounds from 1 nM to 50 μ M for 24 hours. 20 μ L of MTS reagent (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, WI) was added to each well and allowed to develop for 2 hours. Absorbance was read at 490 nm wavelength using the plate reader (Spectramax i3, Molecular Devices, CA).

4.3.2 Calcein AM activity assay—HCC4017 lung cancer cells were plated in triplicates in a 96 well plate (10,000 cells/well) and treated with peptoid compounds as described above 4.3.1. After the treatment, the culture media was replaced with 1 $\times$ sterile PBS containing 1 μ M Calcein AM (Molecular Probes, OR) and allowed to incubate for 15 minutes at room temperature. Fluorescence was read at 490 nm using the plate reader (Spectramax i3, Molecular Devices, CA).

4.3.3 Statistical analysis—Statistical analysis was performed by normalizing absorbance and fluorescence values to their negative controls. Data were analyzed using Graphpad Prism 6 analysis software (Graphpad, CA). Dose response curves were generated with a best-fit curve. IC₅₀ was calculated using non-linear regression analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Unique mid-sequence multimerizations of lipid-PS binding ligand-PPS1 is performed.
- Two monomers are linked via non-important 4th residue of peptidomimetic PPS1.
- Linker size, number of positively charged and hydrophobic regions are varied.
- Shortest linker with minimum two hydrophobic regions are important for activity.

Figure 1. (A) Chemical structure and pictorial representation of PPS1D1 homodimers connected to each other at the C-terminus. (B) Pictorial representation of current strategy, involving positively charged peptide residues separated from hydrophobic peptoids via the mid-sequence linker instead of the C-terminal linker.

Figure 2.

Transfer of the linker to the middle (A) Chemical structure of PPS1-4P2H1. (B) MTS cell viability assay results in HCC4017 cells treated with PPS1D1, PPS1, PC462D1, and PPS1-4P2H1 respectively.

Figure 3. The effects of the mid-sequence linker length. (A) and (B) Chemical structures of PPS1-4P2H2 and PPS1-4P2H3 having mid-sequence linkers of variable lengths (C) The MTS cell viability assay results in HCC4017 cells treated with PPS1D1, PPS1, PC462D1, PPS1-4P2H2, and PPS1-4P2H3 respectively.

Figure 4.

The effect of reducing number of hydrophobic regions. (A) Chemical structure of compound PPS1-4P1H with one hydrophobic region. (B) MTS cell viability assay results on HCC4017 cells treated with PPS1D1, PPS1, PC462D1, and PPS1-4P1H respectively.

Figure 5.

The effect of increased number of hydrophobic regions and changes to the positively charged region. (A) and (B) the chemical structures of PPS1-2P3H and PPS1-4P3H, respectively. (C) MTS cell viability assay results for HCC4017 cells treated with PPS1D1, PPS1, PC462D1, PPS1-2P3H, and PPS1-4P3H. (D) Cell viability calcein AM activity determination for HCC4017 cells treated with PPS1D1, PPS1, PC462D1, PPS1-2P3H, and PPS1-4P3H respectively.

Scheme 1. Synthesis route for PPS1-4P2H1 containing the mid-sequence linker

Scheme 2.

Synthesis scheme for PPS1-4P3H having two positive regions and three hydrophobic regions in the moiety.