Increasing Tumor Accessibility with Conjugatable Disulfide-Bridged Tumor-Penetrating Peptides for Cancer Diagnosis and Treatment

Supplementary Issue: Targeted Therapies in Breast Cancer Treatment

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ABSTRACT: Tumor-homing peptides with tissue-penetrating properties increase the efficacy of targeted cancer therapy by delivering an anticancer treatment to the tumor interior. LyP-1 (CGNKRTRGC) and iRGD (CRGDKGPD) are founding members of this class of peptides. The presence of the cysteines forming the cyclizing disulfide bond complicates conjugation of these peptides with other molecules, such as drugs. Here, we report the synthesis of conjugatable disulfide-bridged peptides and their conjugation to biologically important molecules. We have synthesized the LyP-1, iRGD, and CRGDC (GACRGDCLGA) peptides with a cysteine or maleimidohexanoic acid added externally at N-terminus of the sequences. Subsequent conjugation to payloads yielded stable compounds in which the tumor-homing properties of the peptide and the biological activity of the payload were retained.

KEYWORDS: tumor targeting, homing peptides, tumor-penetrating peptides, conjugatable disulfide peptides, cyclic peptide conjugation nanoworm conjugates, oligonucleotide conjugates

Introduction

Synaptic (docking-based, ligand-mediated, active) targeting is a promising approach that uses targeting elements, such as homing peptides, for selective delivery of drug payloads to diseased tissues. 1,2 These peptides bind to various cell surface receptors, eg, integrins, that are specifically expressed in tumors. These targeting systems largely depend on the homing efficiency of the peptide used in the peptide–payload conjugates. We have previously targeted tumors with a blood clot-binding pentapeptide, CREKA (Cys–Arg–Glu–Lys–Ala), which we coupled to magnetically active iron oxide nanoparticles through a thioether linkage using the N-terminal cysteine. 3,4 We have also utilized the CREKA targeting system for atherosclerotic plaques. 5 Members of another class of tumor-homing peptides from our laboratory possess tissue-penetrating properties, and as a consequence, particularly efficiently accumulate in the tumor tissue. 6,7 These peptides initially bind to a primary, tumor-specific receptor and are then proteolytically cleaved, acquiring an affinity for a second receptor, neuropilin-1 (NRP-1), which triggers the tissue-penetration process. The primary receptor for LyP-1 is p32, a mitochondrial protein that is frequently upregulated and expressed at the cell surface in tumors, particularly in breast cancers. iRGD, 8 like other RGD peptides, initially binds to αv β3 integrins, which are specifically expressed on tumor endothelial cells (and most tumor cells), and like LyP-1 is then processed to a truncated, NRP-1-binding form.

We wanted to conjugate tumor-penetrating peptides to payloads using strategy similar to the one we used with CREKA. However, these peptides are typically cyclic, and the cyclizing disulfide bond poses problems with regard to the coupling chemistry.
Furthermore, coupling of peptides with functionally important amino acid side chains requires modifications to impart chemoselectivity in order to preserve the biological activity of the peptide.

In the literature, a number of conjugation protocols have been reported but chemoselective conjugation of disulfide-bridged peptides has not been well studied. The available methods either are not amenable to preparative scale synthesis or have not been described in detail. Most published methods use carbodiimide reagents to make amide bonds, which are often not chemoselective due to reactive groups, such as amines, acids, and hydroxyls. Other ligation techniques, such as the Staudinger reaction and click chemistry, use external reagents that could alter the peptide structure or biological activity of the conjugate. Traceless Staudinger and copper-free click reactions use intermediates that are obtained synthetically in low yields and are commercially expensive. Tailor-made synthesis of disulfide-bridged peptides for conjugation, which have been attempted in the literature, either fall short of preparative value or have not been clearly described.

Kotamraju et al synthesized disulfide-bridged peptides with various modifications. Ye et al have reported the synthesis of iRGD with a thiol functionality for optical imaging.

We have also been extensively reporting the application of disulfide-bonded cyclic peptides to direct micelles and paclitaxel-albumin nanoparticles to target tissues, especially breast cancers, and to coat cell culture plates for growing human embryonic stem cells. Herein, we report the methods we have been using to synthesize these modified peptides in our laboratory and their characterization. The activation of the extra cysteine functionality of this modified peptide and its utility in oligonucleotide conjugation is further described here. We show that significant scrambling of disulfide bonds did not occur during the synthesis, the desired products could be isolated in good yields, and the functional properties of the peptides were preserved through the conjugation process.

**Methods**

**General materials.** Fmoc-amino acid derivatives with different side-chain protections; the reagents N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate, (HBTU), 1-hydroxybenzotriazole (HOBr), 2,4,6-collidine, pipеразине, and trifluoroacetic acid, and trifluoroacetic acid; and solvents N,N,N′-dimethylpyrrolidinone (NMP), N,N,N′-dimethylethlamide (DMF), dichloromethane (DCM), and acetonitrile were purchased from commercial sources. Other starting materials, namely, 5(6)-carboxyfluorescein (FAM), 5(6)-carboxytetramethylrhodamine (Rd), and linkers N,N,N′,N′-tetramethylcysteamine, succinimide ester and maleimide-5KPEG NHS were purchased from various sources. HeLa pLuc 705 cells, kindly provided by R. Kole and B. Leblue, were grown in Dulbecco’s modified Eagle’s medium with GlutaMAX supplemented with 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, and 200 μg/mL hygromycin (Invitrogen) at 37°C, 5% CO₂ in an incubator. All animal experiments were performed according to procedures approved by the Animal Research Committee at the University of California, Santa Barbara, Santa Barbara, and the Sanford Burnham Prebys Medical Discovery Institute, San Diego.

**Peptide synthesis.** Peptides were synthesized on solid phase using the Fmoc/Bu strategy on a microwave-assisted automated peptide synthesizer (Liberty, CEM Corporation). The amino acids were coupled using HBTU-collidine reagent system in DMF. Fmoc deprotection was performed with 5% piperazine in NMP containing with two microwave energy cycles for 30 and 180 seconds at 75°C under nitrogen (N₂). The coupling of amino acids except Arg and Cys was done for five minutes with 25 W microwave energy at 75°C under N₂. Fmoc-Arg(Pbf)-OH was double coupled with 0 W microwave energy for 25 minutes and with 20 W microwave energy for five minutes at 75°C. All the cysteine residues were coupled for a total of six minutes at 50°C with 0 and 20 W microwave energy for two and four minutes, respectively. The modified peptides were synthesized in different methods as described below. The protected peptide resins were then cleaved with TFA:trisopropylsilane (TIS):water (95:2.5:2.5%) to yield the crude peptides, which were analyzed by reverse phase high-performance liquid chromatography (HPLC) using Gilson’s Analytical HPLC (Phenomenex Jupiter Proteo, 250 mm × 4.60 mm column) and purified on Gilson’s Semi-preparative HPLC (Phenomenex, Jupiter Proteo, 250 mm × 15 mm column). The solvents used were A, 0.1% TFA in water, and B, 0.1% TFA in 60% acetonitrile in water. The peptides were characterized by electron spray ionization mass spectroscopy (ESIMS) on a Waters Micromass QTOF2 tandem mass spectrometer. The data on different peptides are compiled in Table 1.

**Synthesis method A.** The peptides were assembled on rink amide MBHA resin with Fmoc-Cys-(S-Bu)-OH on the C-terminus and Fmoc-Cys(Mmt)-OH on the N-terminus. Furthermore, the third cysteine was introduced in the sequence with Fmoc-Cys(Trt)-OH on the N-terminus spaced from the sequence with 6-aminohexanoic acid. All other residues had standard Fmoc-synthesis compatible protecting groups. FAM was coupled with 25 W microwave energy for five minutes at 75°C under N₂, and Rd was coupled manually using DIC–HOBr. After the completion of synthesis, the thiotolyl protection on the C-terminus cysteine was removed using 50 equiv of tri(n-butyl)phosphine (TBP) in 4 mL of NMP:water (9:1). The free thiol, thus liberated, was activated using 2,2′-Dithiobis (5-nitropyridine) (DTNP) in DCM for one hour. The peptide was cyclized by treating the peptide resin with 1% TFA and 5% TIS in DCM two times for 10 minutes each, and the resin was dried. Upon cleavage with 95% TFA, 2.5% TIS, and 2.5% water, the modified peptides were obtained in 20% yields (based on the initial resin loading) after purification. The peptides were purified by HPLC using...
Conjugatable disulfide-bridged tumor-penetrating peptides

Breast Cancer: Basic and Clinical Research 2015:9(s2)

a solvent system of A, 0.1% TFA in water, and B, 0.1% TFA in a mixture of 60% acetonitrile and 40% water at a flow rate of 10 mL/minute on a Alltima C18 column (250 mm × 22 mm, 10 μm particle size, and 100 Å pore size). The peptides were analyzed by HPLC using a solvent system of A, 0.1% TFA in water, and B, 0.1% TFA in a mixture of 60% acetonitrile and 40% water at a flow rate of 1 mL/minute on a Alltima C18 column (250 mm × 4.60 mm, 10 μm particle size, and 100 Å pore size). The purified peptide products were characterized by ESIMS-time of flight (TOF) and MSMS-TOF.

Synthesis method B. The peptide was assembled on rink amide MBHA resin with Fmoc-Cys(S-tBu)-OH on the C- and N-termini. The third cysteine was introduced in the sequence with Fmoc-Cys(Trt)-OH on the N-terminus spaced from the sequence by 6-aminohexanoic acid. All other residues had standard Fmoc/tBu-synthesis compatible protecting groups. After the completion of synthesis, the thiobutyl protection on the cysteines was removed using 50 equiv of TBP in 4 mL of NMP:water (9:1). The free cysteines liberated were converted to cystine on the resin using 1 equiv of CBr₄ and 2 equiv of Et₃N in NMP at room temperature in two hours. Upon cleavage with 95% TFA, as described in method A, yielded the conjugatable peptide in 20–25% yields based on the initial resin loading.

Synthesis method C. The peptide was assembled on rink amide MBHA resin with Fmoc-Cys(Acm)-OH on the C- and N-termini. The third cysteine or maleimide was introduced in the sequence with Fmoc-Cys(Trt)-OH on the N-terminus spaced from the sequence by 6-aminohexanoic acid or with ε-maleimidocaproic acid. All other residues had standard Fmoc/tBu-synthesis compatible protecting groups. After the synthesis, the peptides were cyclized using thallium(III) trifluoroacetate (Tl(tfa)₃) in DMF with 5% anisole scavenger. Upon cleavage with 95% TFA, as described in method A, yielded the conjugatable peptide in 15% yields based on the initial resin loading.

Synthesis of peptides with nitro-pyridine sulfenyl (Npys)-activated cysteine. The peptides with free N-terminal cysteine and 5 equiv of DTNP were dissolved in TFA:water (4:1) and stirred under N₂ atmosphere for 15 minutes. The reaction mixture was concentrated and dissolved in water and filtered to remove the insoluble solids. The filtrate was lyophilized and purified by HPLC using a gradient of 40% B–80% B over 45 minutes to yield the final product. The peptide characteristics are listed in Table 1.

Preparation of nanoworms (NWs). NWs were coated with peptides 3 and 5 were prepared as described earlier.

In vivo NW injections. To analyze NW biodistribution, mice bearing orthotopic 22Rv1 tumors were injected with the peptides 3 and 5 or PBS into the tail vein (5 mg of iron/kg body weight). The mice were sacrificed after five hours of NW injection by cardiac perfusion with PBS under anesthesia, and tissues were collected for further histological analysis as described earlier.
Preparation of FAM-X-C(iRGD)REKA conjugate. 6-maleimidocaproyl-iRGD peptide 6 (2.5 mg, 0.0021 mmol) was reacted with FAM-X-CREKA (2.7 mg, 0.0025 mmol) in water for four hours under N₂ atmosphere. The reaction mixture was purified by semipreparative HPLC and analyzed by analytical chromatography with a gradient of 25% B-70% B over 45 minutes. The purified peptide had the following characteristics: HPLC—retention time 35 minutes and purity 98% and ESIMS—m/z calc 2215.16 and found 2216.24 [M+H]+.

In vivo FAM-X-C(iRGD)REKA conjugate injection. Mice bearing MCF10Ca1A human breast cancer xenograft tumors were intravenously injected with FAM-X-CREKA, FAM-X-iRGD, or FAM-X-C(iRGD)REKA conjugates, and tumor sections were processed as described earlier.

Conjugation of peptide 9 to antisense (as)-RNA. FAM-Cys(oligonucleotide)-X-LyP-1 conjugate (10). Peptide 9 was synthesized with Npys modification as described earlier in the synthesis section. 2′-O-methyl as-RNA was purchased from Integrated DNA Technologies with a 5′ SH-C6 modification for attachment to the peptide. The peptide and the nucleotide were incubated at room temperature in 0.5 M NH₄OAc buffer at pH 5 for 15 minutes. The conjugation was monitored on gel electrophoresis, which showed a slower moving band compared to the unconjugated nucleotide. The complete absence of the starting material indicated the completion of the reaction.

Splice correction assay. Cells were plated overnight on a 96-well plate at a concentration of 10000 cells/well. Small quantities of lipofectamine 20202 were complexed with unconjugated nucleotide and peptide-conjugated nucleotide at various concentrations and incubated on the cells in serum-free media. Expression of luciferase was assayed using Luciferase Assay Kit (Promega).

Immunocytochemistry. Splice-corrected cells were fixed using 4% paraformaldehyde and blocked using 1% FBS. The cells were incubated with antiluciferase antibody (1:1000; Molecular Probes) for one hour at room temperature. Secondary antibody 647 donkey antigoat (1:1000; Molecular Probes) was used for detection. The fluorescein peptides were labeled with 5(6)-carboxyfluorescein (FAM) or 5(6)-carboxytetramethylrhodamine (Rd). Initially, for the synthesis of iRGD peptides 1 and 2, methoxytrityl (Mmt) - and thiobutyl (S-Bu)-protected cysteines were used at the N- and C-termini, respectively, with a trityl (Trt) group protection on the extra cysteine to introduce the disulfide bond selectively as shown in Figure 1.

After the synthesis, peptide resin was treated with 50 equiv of TBP in NMP:water (9:1) twice for one hour each as per Beekman et al protocol. Gongora-Benitez et al observed that proximity of S-Trt groups influenced the S-Bu deprotection resulting in incomplete or no deprotection with DMF as the solvent. We have observed a complete removal of S-Bu groups in NMP—water mixture though the cysteine was on the C-terminus. The resin-bound partially deprotected peptide resin was reacted with 10 equiv of DTNP in DCM to activate the free thiol and cyclized using 1% TFA in DCM. Cleavage using a standard 95% TFA cocktail released the peptide containing both the intended disulfide bridge and a free cysteine as intended in 15–20% yields after purification.

Looking to improve the synthesis, we adapted bisprotection strategy, wherein the two cysteines in the original sequence were protected with S-Bu and the external cysteine was protected with Trt group. After the synthesis, the S-Bu groups were removed using 50 equiv of TBP in NMP:water (9:1) twice for one hour each as done earlier for one S-Bu. Test cleavage and monitoring the deprotection with ESIMS indicated the removal of both the S-Bu groups. Subsequently, the partially deprotected peptide was cyclized using a modification of a literature method. The resin-bound partially deprotected peptide was treated with 1 equiv of CBr₄ and 2 equiv of Et₃N. The standard cleavage yielded the desired free cysteine peptide 3 in 15–20% yields based on the initial resin loading as shown in Figure 2. HPLC chromatograms of the fluorescein peptides displayed two distinct peaks due to the isomeric mixture, 5(6)-carboxylfluorescein, that was used. Both the peaks showed identical fragmentation pattern in the TOF MSMS. To establish this further, we synthesized this modification and acetylated iRGD peptide 2 without fluorescein, which exhibited a single peak confirming that the peaks observed were from the fluorescein isomers. We further succeeded in extending this method to the synthesis of peptides 5(6)-FAM-CRGDC (4) and 5(6)-Rd-CRGDC (5).

The structures of the peptides synthesized using the above methods were confirmed by mass spectrometry as shown in Table 1. ESIMSMS spectra of cysteine-modified peptides 1 and 3 displayed major peaks at m/z 462 and 575 corresponding to b₂ and b₁ fragments, FAM-Cys and FAM-Cys-(6-aminohexanoyl), respectively, while m/z 644 (FAM-Cys-(6-aminohexanoyl)-Cys, b₄ fragment dethiolated)
Conjugatable disulfide-bridged tumor-penetrating peptides

Figure 1. Directed disulfide bond formation in IRGD peptide with a third cysteine. (a) t-Butylphosphine in NMP:water (9:1), (b) 2,2-dithiobis(5-nitropyridine) (DTNP) in DCM, (c) 1% TFA in DCM, and (d) 95:2.5:2.5.

Figure 2. Disulfide bond formation in LyP-1 peptide using CBr₄. (a) t-Butylphosphine in NMP:water (9:1), (b) CBr₄ (1 equiv), and (c) TEA:TiS:water (95:2.5:2.5).
indicated the presence of the desired disulfide bridge. Furthermore, CRGDC peptide 4 ESIMSMS displayed peaks at m/z 703, 632, 575, and 462 arising from $b_2$, $b_3$, $b_4$, and $b_5$ fragmentation, respectively. Thus, the external cysteine in this peptide modification remained free for conjugation.

Seeking to improve the method further, we chose $S$-acetamidomethyl ($S$-Acm) protection for the cyclizing cysteines, and a Trt group was used for the third cysteine. The $S$-Acm-protecting groups were removed, and the cysteines were cyclized in situ using $\text{Ti(OTf)}_3$. For this protection, $\text{Ti(OTf)}_3$ was the reagent of choice for deprotection and in situ cyclization. The cyclization was completed at 0°C in seven to nine hours depending on the sequence. We have not found the scrambled disulfide products that may be expected from the cleavage of Cys(Trt) in detectable levels. This selectivity in reactivity of Trt group toward $\text{Ti(OTf)}_3$ was found to be in agreement with Albericio et al.\textsuperscript{34}

The three methods all aimed at obtaining a peptide that has a disulfide bridge and an extra cysteine gave similar yields and purities. So any one of the methods can be chosen for synthesizing these modifications. Furthermore, we have replaced the cysteine and fluorescein at the N-terminus with EMCA to obtain the maleimide-functionalized iRGD (6) as shown in Figure 3. Cleavage and purification gave the pure peptides in about 15% yields with >90% HPLC purity by this method.

In order to increase the rate and selectivity of the conjugation, we chose to activate the extra cysteine in the peptide with a Npys group to facilitate an asymmetric disulfide bond formation with a thiol group-bearing cargo. This strategy aids a heterodimeric disulfide formation at a faster rate and at low pH, which minimizes cysteine scrambling. We modified the method reported by Rabanal et al.\textsuperscript{35} for heterodimerization. We treated peptides 1–3 with DTNP in TFA instead of acetic acid to obtain peptides 7–9 with an Npys-activated thiol group in 30 minutes as shown in Figure 4.

The peptide products were >95% pure by HPLC and showed the expected characteristics in ESIMS. The peptides labeled with 5(6)-FAM showed four well-resolved peaks corresponding to four geometrical isomers (Supplementary Files 1–4). This is due to the positioning of the Npys substitutions relative to FAM on the cysteine α-amino group across the plane of symmetry along the disulfide bond between the cysteine and the Npys groups. This was confirmed by synthesizing the Npys-activated peptide iRGD without the FAM label, which appeared as a single peak in the HPLC.

**Peptide conjugation through Michael addition.** To examine peptide stability, peptide 1 was reacted with 0.6 equiv of 1,4,7,10-tetraazacyclododecane-1,4,7-tris-acetic acid-10-maleimidoethyacetamide (DOTA) in water. After four hours,
Figure 5. Combining CRGDC NWs with LyP-1 NWs enhances homing to different areas of tumor. A mixture of iron oxide NWs coated with rhodamine-labeled CRGDC or FAM-labeled LyP-1 was intravenously injected (2.5 mg iron/kg of each NW preparation) into nude mice bearing orthotropic 22Rv1 tumors. The mice had been preinjected with Ni liposomes to reduce uptake by the reticuloendothelial system. The mice were perfused through the heart five hours later, tumors were harvested and sectioned, and the sections were examined by confocal microscopy. Nuclei were stained with DAPI (blue). Scale bars, 200 μm. (A) CRGDC (peptide 5) NWs (red), (B) LyP-1 (peptide 3) NWs (green), (C) panels (A) and (B) superimposed, and (D) DAPI (blue) co-staining with C.

mass spectra of the crude reaction mixture showed the desired product. ESIMS of the excess peptide displayed the same fragmentation pattern as peptide 1, indicating the stability of the modified peptide under the conjugation conditions. Furthermore, to determine the functional integrity of these modifications, iron oxide NWs6,36 coated with peptides 3 and 5 were tested in vivo. As expected, histology showed bright green fluorescence from the peptide 3 NWs in the interior parts of the tumor, while tumor vasculature showed red color, indicating the presence of peptide 5 NWs (Fig. 5). No tumor fluorescence was seen when an inert control peptide FAM-X-KAKEC was injected (not shown). These results show that the conjugated peptides retain their tumor-homing properties. Moreover, the two peptides showed strikingly different preferences in their homing properties, retaining their affinity for tumor lymphatics and tumor macrophages (peptide 3)11 and tumor blood vessels (peptide 5).2

To test the effect of the maleimide functionalization, maleimide-iRGD (peptide 6) was reacted with FAM-X-CREKA in water for four hours to obtain an iRGD-CREKA conjugate. Histological studies showed that the conjugate extravasated into the tumor interior, whereas the CREKA peptide alone homed only to the tumor vasculature (Fig. 6). The penetration into tumor interior is a function of iRGD,14 whereas CREKA (peptide 5) remains bound to the vascular wall of tumor vessels.6

Conjugation through a disulfide link. A disulfide linkage is well suited for intracellular delivery, as it is cleaved by cytoplasmic glutathione, releasing the active payload. The Npys-activated product 9 was reacted with thiol-modified as-RNA in 0.5 M NH₄OAc at pH 5 for 10 minutes. The reaction mixture showed a slower-migrating species in polyacrylamide gel electrophoresis than the original oligonucleotide (Fig. 7A), indicating successful conjugation resulting in formation of a FAM-Cys(oligonucleotide)-X-LyP-1 conjugate (10). To study the activity of the conjugate, we used an as-RNA splice correction system that leads to the expression of luciferase when the intact nucleotide is delivered into the cytoplasm.37

Figure 6. Homing of peptide-coated nanoworms to breast cancer xenografts. Mice bearing MCF10Ca1A human breast cancer xenograft tumors were intravenously injected (A) FAM-X-CREKA, (B) FAM-X-iRGD, or (C) FAM-X-C(iRGD)REKA conjugates, and tumor sections were processed as in Figure 5. NWs are green; blood vessels visualized with anti-CD31 are red. Arrows point to vascular structures that show colocalization of nanoparticle and CD31 signals. FAM-X-CREKA is associated with blood vessels, but because it binds to clotted plasma proteins within tumor vessels,6 there is little colocalization (yellow color) with the endothelial cells marked with CG31. iRGD causes extravasation into tumor parenchyma of the other two peptides, resulting in even less blood vessel localization than seen with FAM-X-CREKA. Nuclei were stained with DAPI (blue). Representative results from three experiments are shown.
We observed a dose-dependent increase of luciferase expression with increasing concentrations of the conjugate, whereas the free as-RNA was inactive (Fig. 7). Hence, the conjugation of the as-RNA to the peptide resulted in cytoplasmic delivery and maintained the function of the nucleotide.

Conclusion
In summary, we have successfully synthesized and demonstrated the structural and functional integrity of disulfide-bridged peptides with a free thiol or maleimide functionalities for conjugation through thioalkyl and disulfide linkages. We have demonstrated their conjugation to different cargoes, and that the nanoparticle, and oligonucleotide, conjugates have the desired activities and efficacies. These results establish chemoselective conjugation of peptides having sensitive disulfide bridges to a variety of payloads. Thus, this is a powerful tool for disulfide-bridged peptide-mediated imaging, targeted drug delivery, and other applications.

Author Contributions
Conceived and designed the experiments: VRK and ER. Contributed data and data analysis: VRK, SS, PK, LA, JP, and ER. Wrote the first draft of the article: VRK. Contributed to the writing of the article: VRK, SS, PK, LA, and ER. Agree with the article results and conclusions: VRK, SS, PK, LA, JP, and ER. Jointly developed the structure and arguments for the article: VRK, SS, PK, LA, JP, and ER. Made critical revisions and approved the final version: VRK and SS, PK, LA, JP, and ER. Made critical revisions and approved the final article: VRK, SS, PK, LA, JP, and ER. All authors reviewed and approved the final article.

Supplementary Materials
Supplementary file 1. HPLC chromatogram of peptide 1.
Supplementary file 2. HPLC chromatogram of peptide 2.
Supplementary file 3. HPLC chromatogram of peptide 3.
Supplementary file 4. HPLC chromatogram of peptide 8.

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Conjugatable disulfide-bridged tumor-penetrating peptides

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