**SUPPLMENTARY INFORMATION**

# Integrin-Specific Binding Macrocyclic RGD Peptides Functionalized with Dinitrophenol as Multivalent Antibody-Recruiting Molecules to Enhance Cytotoxicity in Cancer Immunotherapy

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## Experimental Section

### Materials

Sortase A was obtained from genetically engineered expression according to the reference. [1] Amino acids and resin with Fmoc protection were purchased from ChangZhou KangLong Biochem Co.,Ltd (China). Peptide synthesis and clevage reagents were from Adamas (China). Azido-PEG3-OH, 2,4-Dinitrofluorobenzene, 6-Heptynoic acid, Fetal Bovine Serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) were all obtained from Adamas (China). MCF7 cell lines was kindly supplied by Professor Wu Zhimeng (Jiangnan University, China). M21 cell lines was purchased from ShangHai JingYuan Biochem Co.,Ltd (China). Rabbit anti-DNP IgG antibody (Cat.No. A-6430) and its Alexa Fluor 488 conjugated version (Cat.No. A-11097) were acquired from Thermo Fisher (USA). Rabbit complement (Cat.No. 234400-M) was obtained from Sigma Aldrich. LDH assay kit was from Beyotime (China). CCK8 assay kit was from Adamas Life (China). Unless specified, chemicals were purchased from commercial suppliers and used without additional purification. Analytical RP-HPLC was conducted on Agilent 1200 with a C18 column (Diamonsil, 5 μm 250×4.6 mm). Preparative RP-HPLC was performed on a Waters 1525 with a preparative C18 column (Diamonsil Diol, 5 μm 250×10.0 mm). ESI-MS were recorded on Shimadzu LCMS 9030. MALDI-TOF-MS were conducted with a Bruker ultrafleXtreme.The $^{1}$H NMR spectra were acquired using an Agilent DD2 600 MHz NMR Spectrometer, and confocal laser scanning microscopy (CLSM) images were captured with an OLYMPUS FV3000. Flow cytometry analyses were performed on BD FACSMelody.

### Synthesis of cyclic RGD-peptide Cyclo[$G_5$RGDKcLPK($N_3$)T]

0.3 mmol Fmoc-Ser(tBu)-Wang resin (loading: 0.34 mmol/g) were swollen with DMF. Following washes with DMF and DCM, the resin was treated with 25% piperidine in DMF for 30 min to remove the Fmoc. Fmoc-protected amino acids (3 eq), TBTU (3 eq) and DIPEA (6 eq) in DMF were added to synthesis tube and reacted for 2 h. Once all amino acids were incorporated, the peptide on the resin was cleaved using 95% trifluoroacetic acid (TFA) with 2.5% triethylsilane ($Et_3SiH$) and 2.5% water. The raw peptides underwent purification via preparative HPLC, employing a linear gradient of 20%-60% aqueous acetonitrile with 0.1% TFA for 30 min at a flow rate of 1 mL min$^{-1}$. The pure product was characterized by analytical RP-HPLC with a linear gradient of 20%-60% aqueous acetonitrile containing 0.1% TFA for 30 min at a flow rate of 1 mL

$min^{-1}$. The mass of final product was measured using ESI-MS.

For peptide cyclization, 0.5 mM peptide and 5 μM Sortase A were mixed in Srt A reaction buffer containing 0.3 M Tris-HCl at pH 7.5, 150 mM NaCl, and 5 mM $CaCl_2$. The mixture was incubated at 37 °C for 4 h.The final cyclic peptides were purified using preparative HPLC. The pure cyclic peptide was characterized by analytical RP-HPLC with a linear gradient of 20%-60% aqueous acetonitrile containing 0.1% TFA for 30 min at a flow rate of 1 mL $min^{-1}$. The mass of cyclic peptide was measured using ESI-MS.

**Synthesis of Azide-PEG3-DNP**

Azido-PEG3-OH (1.9 g, 11 mmol), 2,4-Dinitrofluorobenzene (1.86 g, 10 mmol) and potassium carbonate (1.65 g, 0.65 mmol) were dissolved in DMF. The solution was stirred and refluxed at 70 °C for 15 h, then washed three times with saturated saline and extracted three to four times with ethyl acetate. The organic layer was concentrated under reduced pressure. The residue was then purified by column chromatography (EtoAc/MeOH, 8:1) to give 2.7 g final product with 80% yield. $^{1}$H-NMR (600 MHz, Chloroform-d) δ 8.71 (d, J = 2.8 Hz, 1H), 8.40 (dd, J = 9.2, 2.8 Hz, 1H), 7.27 (d, J = 9.3 Hz, 1H), 4.41- 4.38 (m, 2H), 3.96- 3.93 (m, 2H), 3.75-3.72 (m, 2H), 3.68-3.63 (m, 4H), 3.36 (t, J = 5.0 Hz, 2H). $^{13}$C NMR (151 MHz, Chloroform-*d*) δ 157.12 (s), 140.51(s), 139.36(s), 129.30(s), 122.12(s), 115.22(s), 71.44(s), 71.00(s), 70.86(s), 70.37(s), 69.38(s), 50.98(s). MS: Calculated MW, 341.0972; observed, 342.1048 $[M+H]^+$, 359.1316$[M+NH_4]^+$, 364.0870$[M+Na]^+$.

**Synthesis of multivalent antibody-recruiting molecules (D1, D2 and D3)**

0.6 mmol Rink-Amide-MBHA-Resin (loading 0.326 mmol/g) were swollen with DMF in a 50 mL synthesis tube overnight. The resin was exposed to 40 mL of 25% piperidine in DMF for 30 min. After washing with DCM and DMF 3-5 times, Fmoc-Lys (Mtt)-OH (3 eq), TBTU (3 eq), and DIPEA (6 eq) were introduced into the tube and shaken for 2 hours. After removing Fmoc with 25% piperidine, Fmoc-Lys (Fmoc)-OH (3 eq), TBTU (3 eq), and DIPEA (6 eq) were added to react for 2 h. The coupling of Fmoc-Lys (Fmoc)-OH was required once for the synthesis of D1, twice for D2, and three times for D3. 6-heptynoic acid (3 eq) was then coupled by addition of TBTU (3 eq), and DIPEA (6 eq) for 2 hours, followed by Fmoc removal. Thereafter, copper sulfate (0.3 eq), sodium ascorbate (0.3 eq), and Azide-PEG3-DNP (1.25 eq) were dissolved in a tetrahydrofuran water solution (v:v = 1:1) and added to the resin. The reaction was shaken for 72 h at 40 °C.

Subsequently, the Mtt protective group was removed from the resin using a solution of acetic acid, 2,2,2-trifluoroethanol, and dichloromethane (10%:20%:70%) for 2 hours and repeated three times. Then, 6-heptynoic acid (3 eq), TBTU (3 eq), and DIEA (6 eq) were added and the mixture was shaken for 2 h. Finally, copper (II) sulfate (1 eq), sodium ascorbate (1 eq), and the cyclic peptide Cyclo[$G_5$RGDKcLPK($N_3$)T] (1 eq) were dissolved in a tetrahydrofuran water solution (1:1, v: v) and added to the tube. The mixture was shaken for 72 hours at 35 ˚C.

The above dried peptide resin was treated with a 95% trifluoroacetic acid cleavage solution containing 2.5% (v/v) triethylsilane ($Et_3SiH$) and 2.5% (v/v) water. The mixture was stirred for 2 hours to liberate the peptide from the resin. Following resin filtration, the filtrate was dried under reduced pressure. The crude peptide was purified through preparative RP-HPLC using a C18 column. Elution was performed with a gradient of 30%-90% acetonitrile over 60 min at a flow rate of 0.5 mL $min^{-1}$ and detected at a wavelength of 220 nm. The target peaks were collected and subsequently lyophilized. The final product was characterized using analytical RP-HPLC and mass spectrometry.

**Surface Plasmon Resonance Analysis (SPR)**

A fresh solution of 0.1 M EDCI [1-Ethyl-3- (3-dimethylaminopropyl) carbodiimide, EDCI] was mixed with an equal volume of 0.4 M NHS (N-Hydroxysuccinimide, NHS). The sensor chip (Series S Sensor Chip CM5, GE Healthcare, USA) was reacted with the mixture for 15 min at flow rate of 30 μL/min. 20 μg/mL anti-DNP IgG antibody were prepared in 10 mM sodium acetate buffer. The solution was then injected to the activated surface and left to react for 15 min in 30 μL/min flow. The excess of NHS-esters is deactivated by injecting 1.0 M ethanolamine hydrochloride in pH 8.5 for 15 min. Clean the surface from non-specifically bound molecules with an injection of 2 M NaCl with 0.01 M NaOH solution. The **D1-D3** solution (0.395 μM, 0.78 μM, 1.5625 μM, 3.125 μM, 6.25 μM, 12.5 μM, 25 μM and 50 μM) were prepared in 1×HBS EP buffer (HEPES buffered saline). The solution was injected to sensor chip to binding at flow rate of 30 μL/min. Each concentration was performed three times. Combination and dissociation curves of each concentration were fitted with a Langmuir model using Biacore T200 Evaluation Software to determine the equilibrium dissociation constant $K_D$.

**Confocal Laser Scanning Microscopy Analysis**

Cells were cultured in 12-well plates at 37 ˚C until reaching 40-70% confluence. Subsequently, the

medium was removed, and the cells were treated with 200 μL of 100 nM multivalent DNP antibody recruitment molecules (D1, D2, D3) or cyclic RGD peptide [Cyclo($G_5$RGDKcLPK($N_3$)T)], along with 20 μg/mL Alexa 488-conjugated rabbit anti-DNP antibody, for 30 minutes at 37 ˚C. Following this incubation, the cells were washed three times with PBS. A 200 μL aliquot of 4% paraformaldehyde solution was then added to fix the cells at room temperature for 10 minutes. After additional washes with PBS buffer, the cells were stained with 200 μL of PBS containing 5 μg/mL DAPI for 15 minutes at 37 ˚C in the dark. The cells were washed with PBS 3 times again. Cell fluorescence images were acquired using confocal fluorescence microscopy.

**Flow cytometry**

Cells were grown and collected using trypsin-EDTA, then resuspended in a flow cytometry buffer (1% FBS in PBS) and diluted to $5\times10^5$ cells/mL. A volume of 100 μL of the cell suspension were mixed with 100 μL of different concentrations of multivalent ARMs (D1, D2, D3) or cyclic RGD peptide (final concentration of 50 nM) or isotype antibody. The blank group was added 100 μL of flow cytometry buffer. The mixtures were kept in the dark at 4 ˚C for 30 minutes, then washed 2-3 times with flow cytometry buffer. The cells were incubated on ice for 30 minutes with 100 μL of Alexa488-conjugated rabbit anti-DNP antibody in flow cytometry buffer at a final concentration of 20 μg/mL, then washed three times with flow cytometry buffer. Finally, cells were resuspended in 200 μL of flow cytometry buffer. Fluorescence was measured by flow cytometry, and the data were processed using FlowJo X software.

For determination of αvβ3 and αvβ5 integrins expression levels, 80% confluence cells were detached using trypsin-EDTA and re-suspended in PBS containing 1% FBS. Cell aliquots ($5\times10^5$ cells) were treated with monoclonal antibody PE conjugate (PE-anti-αvβ3 antibody and PE-anti-αvβ5 antibody, Sigma-Aldrich) or isotype control (Thermo Fisher) at the same concentration (14 μg/mL) for 1 h at 4 ˚C. PBS group without monoclonal antibody was used as negative control. The cells were then washed with flow cytometry buffer and measured by flow cytometry. The data were processed using FlowJo X software.

**Cell adhesion assays**

96-well plates were coated with 100 μL integrin solution in coating buffer (5 μg/mL fibrinogen for M21, 1 μg/mL vitronectin for MCF7) at 4 ºC overnight. Plates were then blocked with 150

μL/well 1% BSA in PBST (Phosphate Buffered Saline with Tween 20, PBST) buffer at 37 °C for one hour and followed by 3× washing with 500 μL PBST buffer. Harvested cells were plated to the plates at a given concentration (50000/well for M21 and 30000/well for MCF7). A serial dilution of **D1-D3** were added and incubated with cells for two hours at 37 °C. Non-adhered cells were removed with PBS (Phosphate Buffered Saline, PBS). Cell viability assay was determined by CCK8 assay kit. Experiments were carried out in triplicate. The adhesion inhibition $IC_{50}$ was calculated using OriginPro 8 software based on the sigmoidal dose-response equation.

**ADCC assay**

Cells (8000 cells/well) were seeded into 96-well plates and grown for 12 hours. After discarding medium and washing with ADCC buffer (phenol-free RPMI 1640 medium containing 5% FBS), cells were incubated with multivalent DNP antibody recruitment molecules or cyclic RGD peptides (0.8, 4, 20, 100 nM) and 20 μg/mL rabbit anti-DNP antibody in ADCC buffer at 37 ˚C for 30 min. After washing with ADCC buffer, 200 μL of human peripheral blood mononuclear cells (PBMC) were subsequently introduced to each well at an effector-to-target (E/T) ratio of 15: 1 (determined by dose-dependent experiments). [2]After incubating for 4 hours at 37 ˚C, 60 μL of the supernatant was moved to another 96-well plate. Lactate dehydrogenase (LDH) release was detected at 490 nm using the LDH detection kit. Cytotoxicity was determined by the following formula.

$$\mathrm{ADCC\%}=\left(\frac{\mathrm{A_{experimental}}-\mathrm{A_{spontaneous}}}{\mathrm{A_{maximum}}}\right)\times 100\%$$

Where $A_{experimental}$ represents the absorption of LDH released by cells respectively treated with multivalent DNP antibody recruitment molecules or cyclic RGD peptides in the presence of anti-DNP IgG antibody and PBMC; $A_{spontaneous}$ represents the absorption of LDH spontaneously released by PBMC (PBMC may undergo minor lysis due to handling procedures); $A_{maximum}$ represents the absorption of LDH released by cells treated with 1% Triton X-100.

**CDC assay**

Cells (5000 cells / well) were seeded into 96-well plates and cultured at 37 ˚C for 12 h. After discarding medium, the cells were incubated with 100 μL of multivalent DNP antibody recruitment molecules or RGD cyclic peptide (0.8, 4, 20, 100 nM) in presence of 20 μg/mL rabbit anti-DNP antibody for 1 h at 37 ˚C. Following washing twice with DPBS, cells were incubated

with 100 μL of 1% rabbit total complement at 37 ˚C for 4 h. The absorbance was detected at 450 nm using a CCK-8 kit, and cytotoxicity was calculated according to the following formula:

$$\mathrm{CDC\%}=\left(1-\frac{A_{experimental}-A_{maximum}}{A_{negative}-A_{maximum}}\right)\times 100\%$$

Where $A_{experimental}$ denotes the OD450 value of cells treated with multivalent DNP antibody recruitment molecules or RGD cyclic peptide and anti-DNP IgG antibody; $A_{negative}$ denotes the OD450 value of cells treated with DPBS and anti-DNP IgG antibody; $A_{maximum}$ denotes the OD450 value of cells completely lysed with 1% Triton X-100.

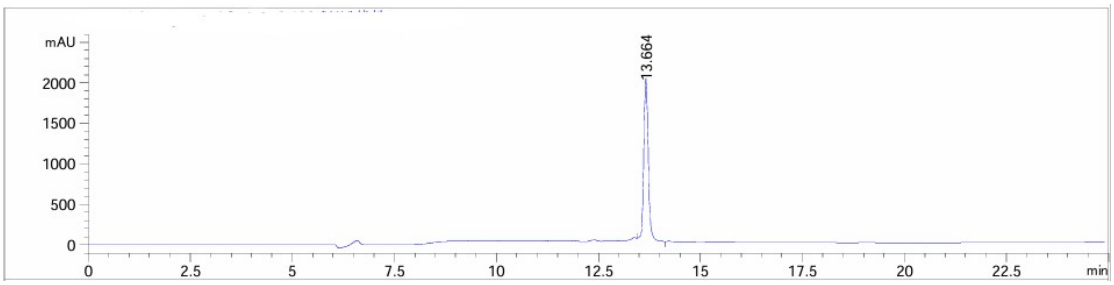


Figure S1. HPLC spectrum of the pure peptide $G_5$RGDKcLPK($N_3$)TGGS.

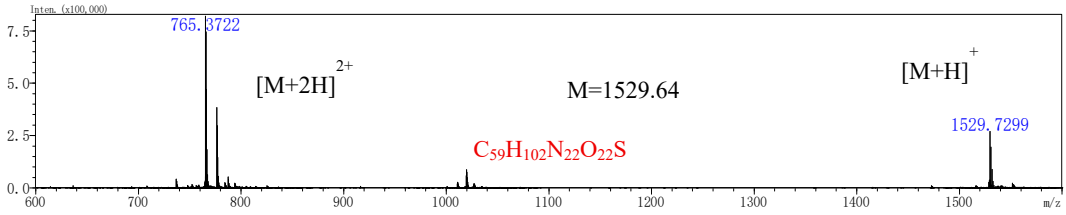


Figure S2. ESI-MS spectrum of the linear peptide $G_5$RGDKcLPK($N_3$)TGGS.

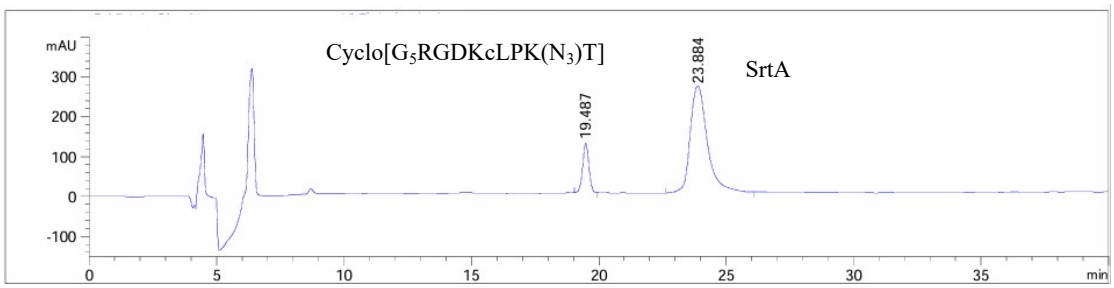


Figure S3. HPLC traces for the reaction of Srt A-mediated cylization.

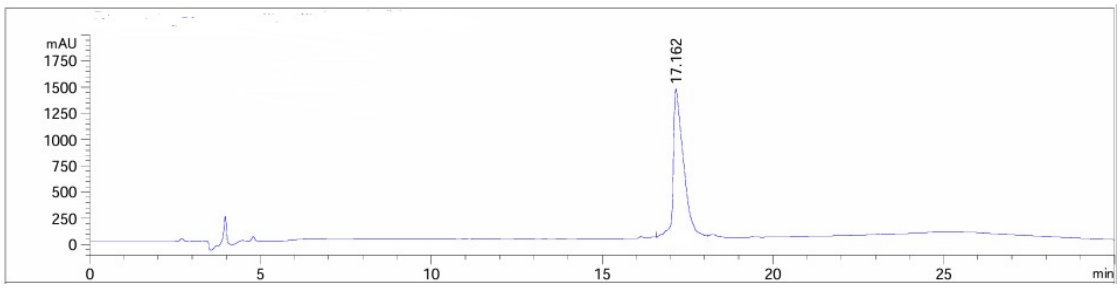


Figure S4. HPLC spectrum of pure cyclic RGD peptide Cyclo[$G_5$RGDKcLPK($N_3$)T].

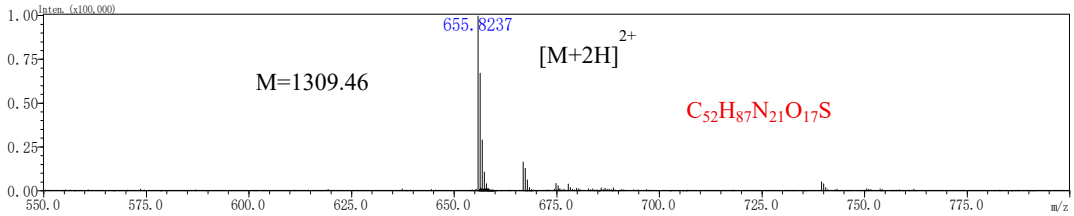


Figure S5. ESI-MS spectrum of the cyclic RGD peptide Cyclo[$G_5$RGDKcLPK($N_3$)T].

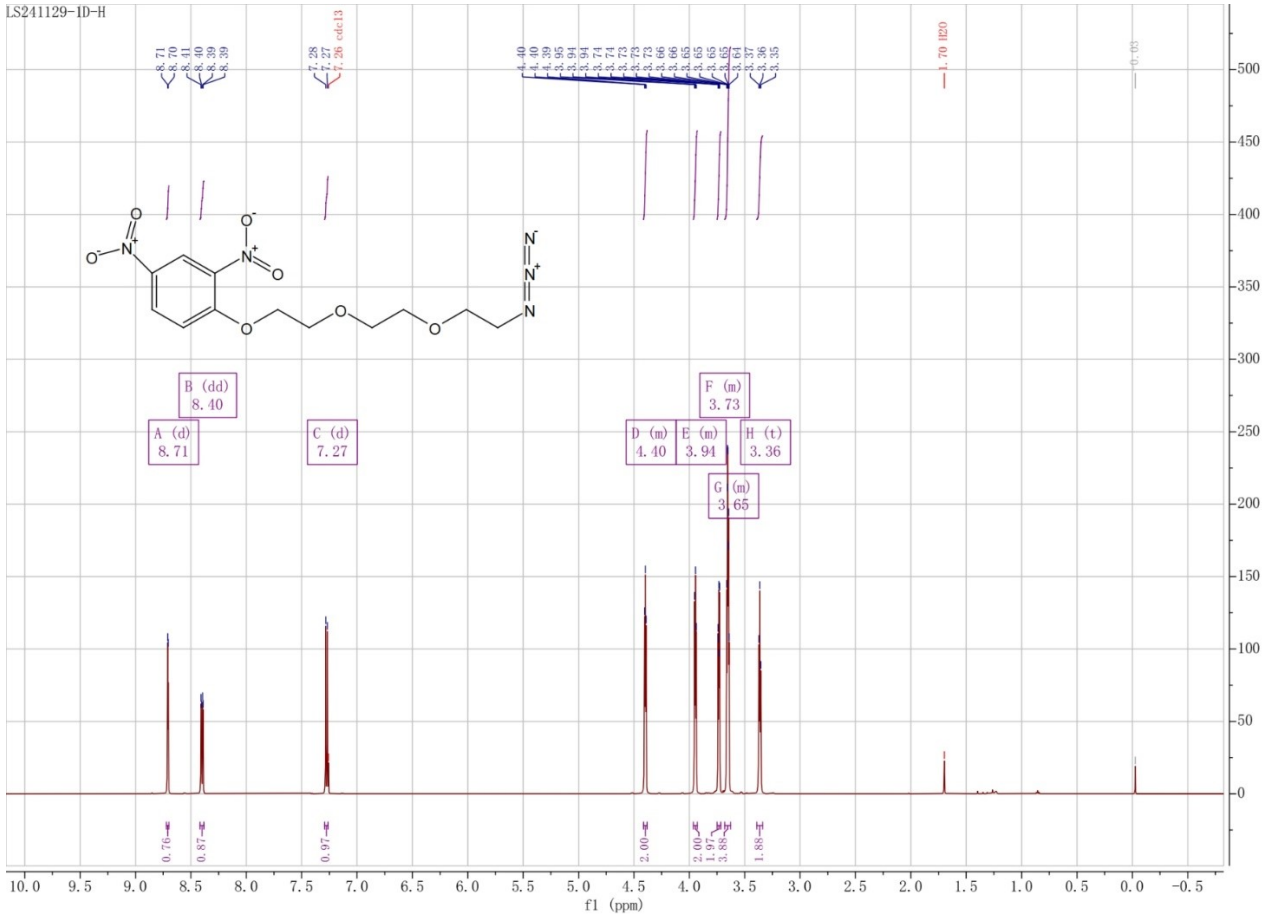


Figure S6. $^{1}$H-NMR spectrum of compound Azide-PEG3-DNP.

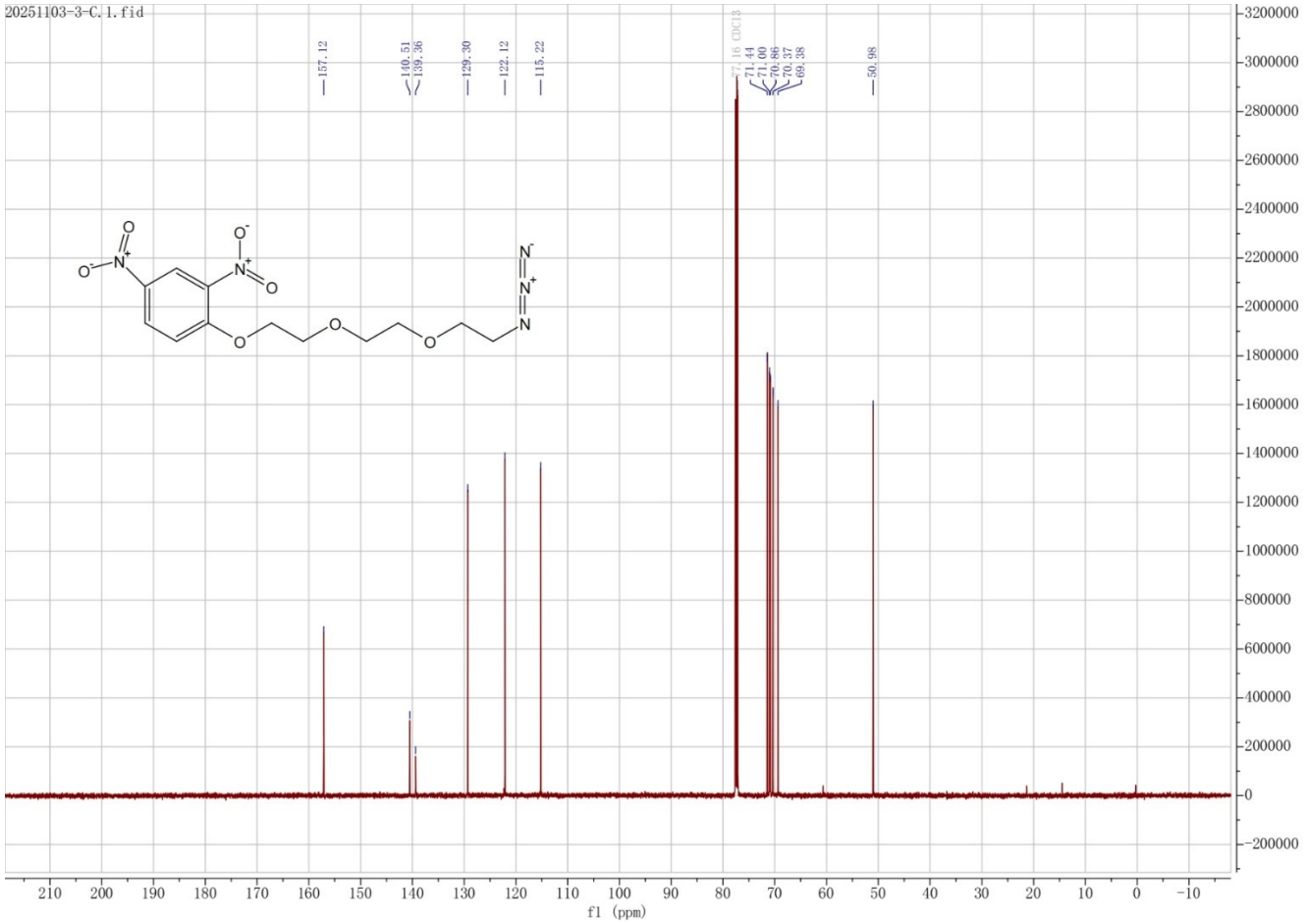


Figure S7. $^{13}$C-NMR spectrum of compound Azide-PEG3-DNP.

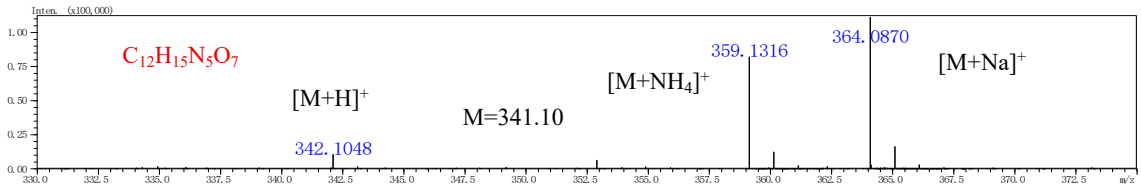


Figure S8. ESI-MS spectrum of compound Azide-PEG3-DNP.

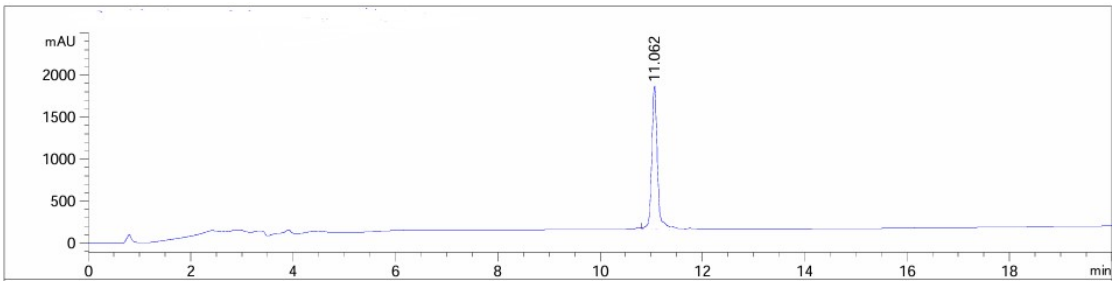


Figure S9. HPLC spectrum of pure compound D1.

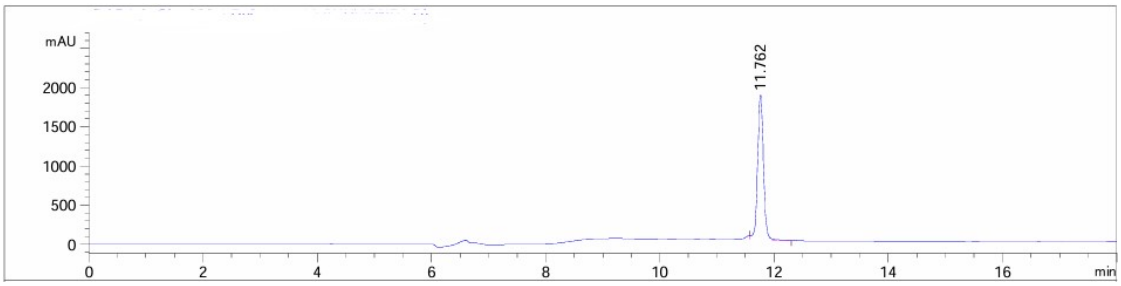


Figure S10. HPLC spectrum of pure compound D2.

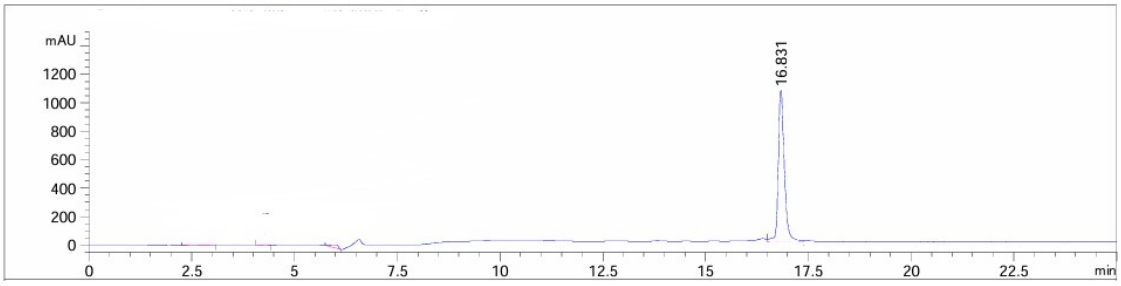


Figure S11. HPLC spectrum of pure compound D3.

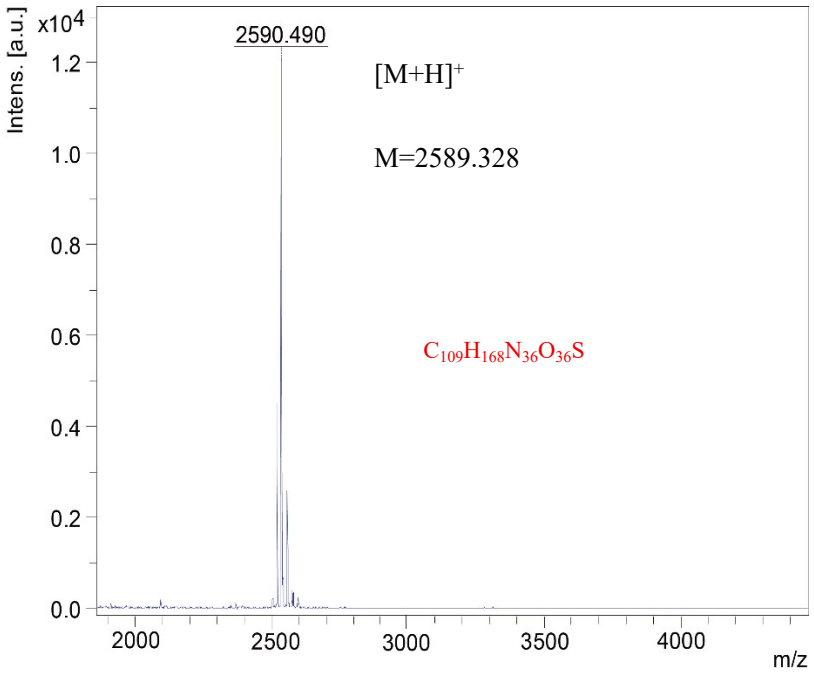


Figure S12. MALDI-TOF analysis of D1.

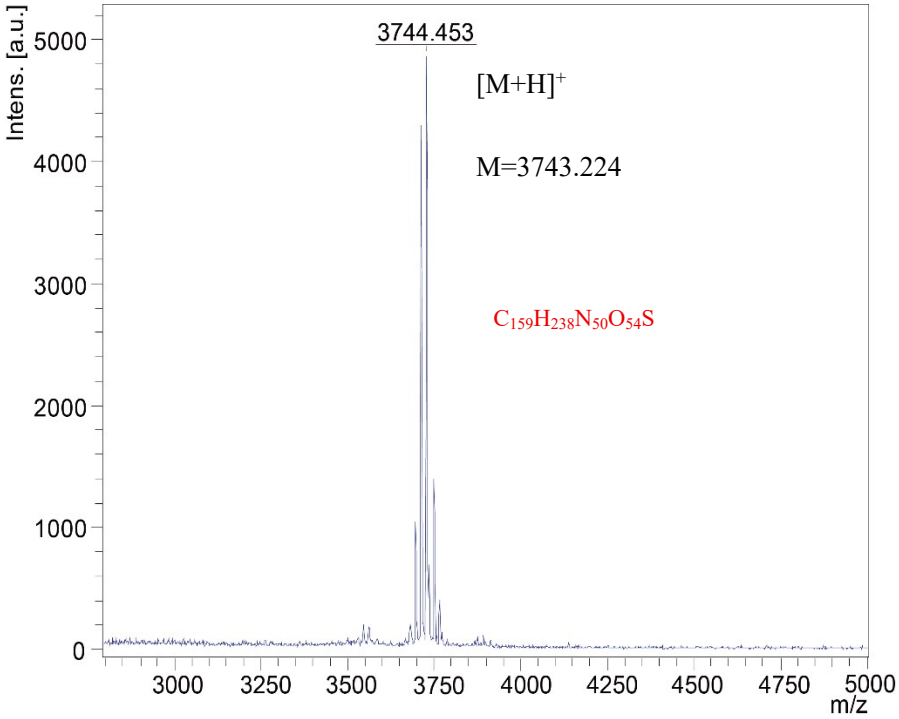


Figure S13. MALDI-TOF analysis of D2.

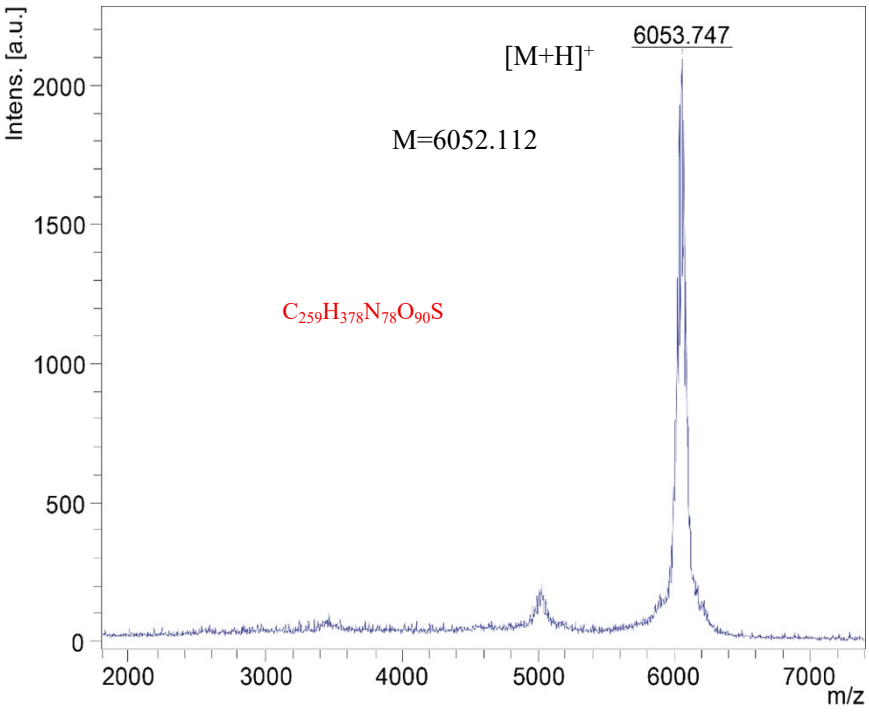


Figure S14. MALDI-TOF analysis of D3.

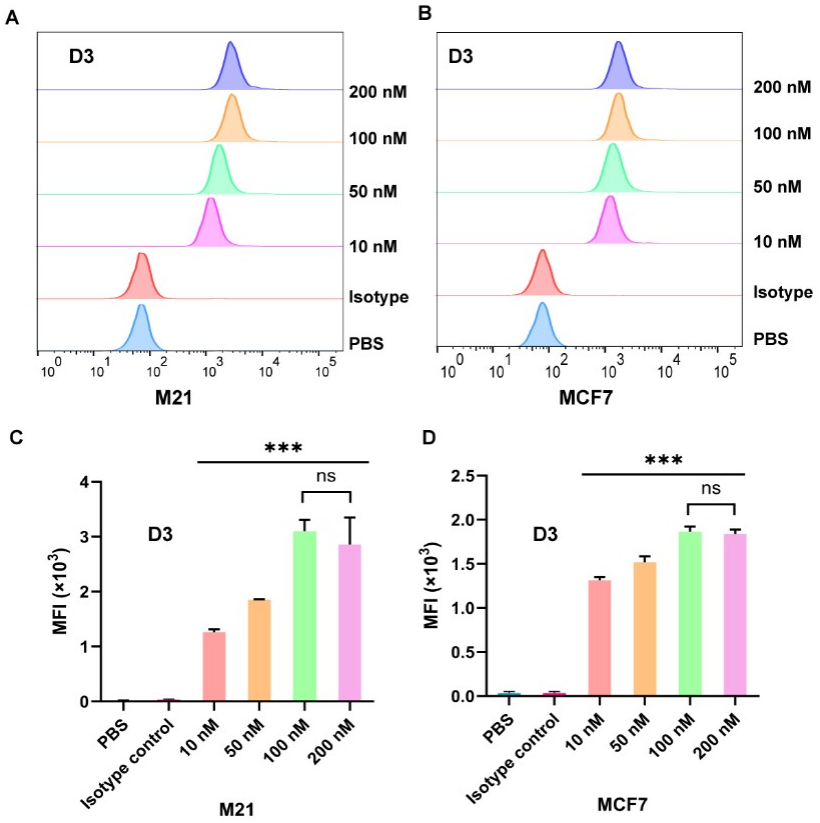


Figure S15. Dose-response analysis of D3-mediated tumor-specific binding and antibody recruitment.

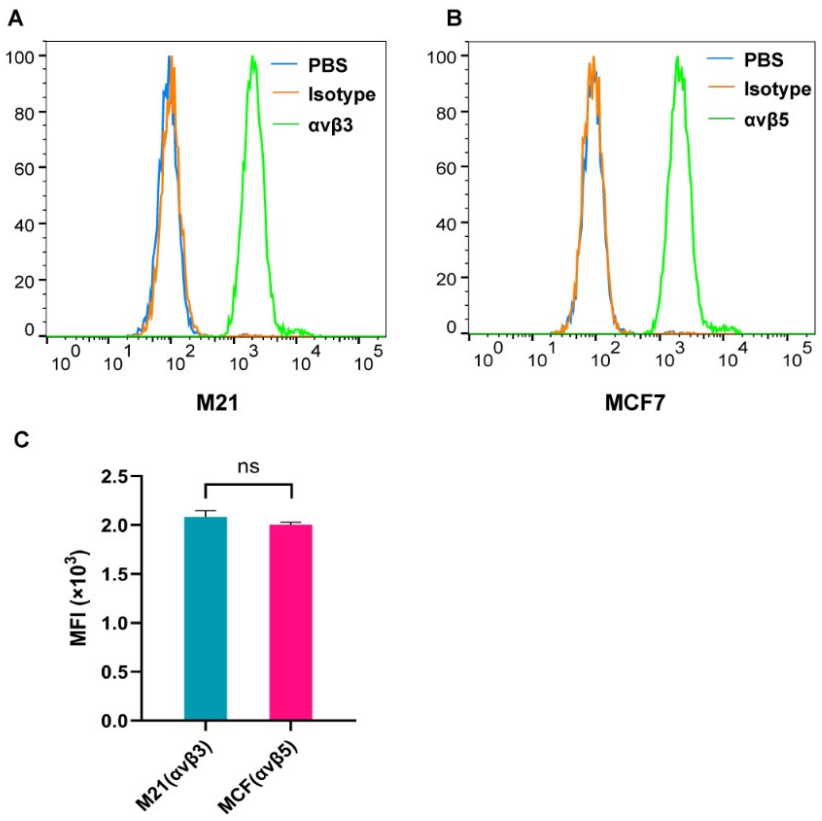


Figure S16. Flow cytometry analysis of the relative integrin expression levels in M21 and MCF7 cell lines.

Table S1. Characterization data of the peptide compounds.

| Compound | Theoretical MW | Detected MW | Retention time (time) | Purity(%) |
|---|---|---|---|---|
| $G_5$RGDKcLPK($N_3$)TGGS | 1529.64 | 765.3722 $[M+2H]^{2+}$ | 13.664 | 99 |
| Cyclo[$G_5$RGDKcLPK($N_3$)T] | 1309.46 | 655.8237 $[M+2H]^{2+}$ | 17.162 | 99 |
| D1 | 2589.328 | 2590.490 $[M+H]^{+}$ | 11.062 | 99 |
| D2 | 3743.224 | 37445.4536 $[M+H]^{+}$ | 11.762 | 99 |
| D3 | 6052.112 | 6053.747 $[M+H]^{+}$ | 16.831 | 99 |

Table S2. $K_D$ of the multivalent ARMs D1-D3.

| **Compounds** | $K_D$(μM) |
|---|---|
| D1 | 0.09±0.02 |
| D2 | 0.11±0.02 |
| D3 | 0.15±0.04 |

Table S3. Cell adhesion inhibition activity of multivalent ARMs D1-D3 in different cell lines.

| **Compounds** | **$IC_{50}$ (,μM)** | | **Selectivity** |
|---|---|---|---|
| | M21($\alpha_v\beta_3$) | MCF7($\alpha_v\beta_5$) | ($\alpha_v\beta_5/\alpha_v\beta_3$) |
| Cyclo[G5RGDKcLPK(N3)T] | 0. 82±0.20 | 10.12±3.21 | 12.34:1 |
| D1 | 0.74±0.24 | 13.11±5.42 | 17.71:1 |
| D2 | 1.16±0.36 | 18.01±5.39 | 15.52:1 |
| D3 | 0.91±0.25 | 11.89±6.19 | 13.06:1 |

## references


1. H. Hong, Z. Zhou, K. Zhou, S. Liu, Z. Guo and Z. Wu, *Chem. Sci*, 2019, **10**, 9331-9338.
2. H. Lin, K. Zhou, D. Li, H. Hong, Y. Xie, L. Gong, Y. Shen, Z. Zhou, J. Shi and Z. Wu, *ChemMedChem*, 2021, **16**, 2960-2968.