Chemically Programmed Bispecific Antibody Targeting Legumain Protease and αvβ3 Integrin Mediates Strong Antitumor Effects.

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Supporting Information

1. General Methods – Chemistry.

All solvents and reagents were used as obtained from commercial sources unless otherwise indicated. All starting materials were also obtained from commercial sources. All reactions were performed under argon unless otherwise noted. In the course of aqueous work-ups, organic layers were washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated at 40 °C under reduced pressure ("standard work-up"). ^1H and ^{13}C NMR spectra were recorded on Bruker and Varian instruments 300, 400, and 500 using deuterated chloroform (99.8%D) or DMSO-d6 (99.8%D) as solvents. ^1H Chemical shifts values (δ) are reported in ppm downfield from tetramethylsilane as standard. Mass spectra were measured in positive mode electrospray ionization (ESI) on Agilent LC/MSD TOF instrument. TLC was performed on silica gel 60 F_{254} glass plates; column chromatography was performed using silica gel (35-75 mesh). All final compounds sent for biological assay were further purified by HPLC. Analytical HPLC was performed using a Shimadzu LC-10AD system, equipped with a Waters 484 tunable absorbance detector set at 254, 280, 310 or 360 nm. Synthesis of compound **4** is described in Ref. 1.

2. Linkers. Linkers LNK-**1**, LNK-**2**, and LNK-**4** were prepared as described previously (Ref. 2), or by modifying the methods described therein. LNK-**4** underwent Cu-catalyzed alkyne-azide coupling with linker **VI** affording LNK-**3** (Figure S-1).

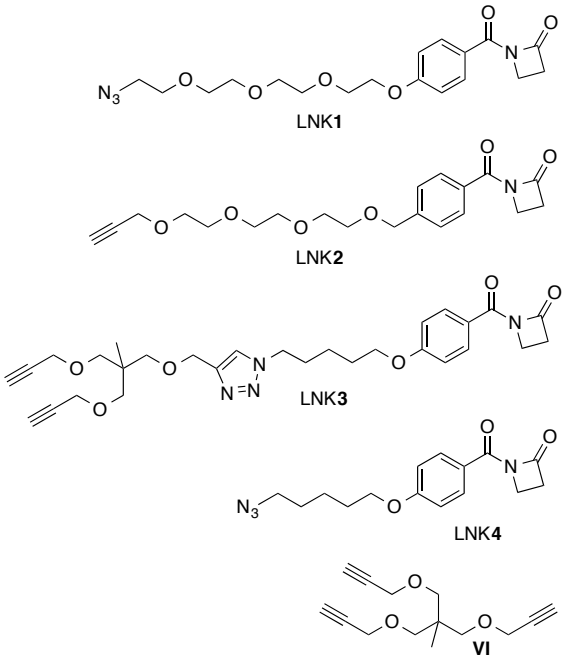


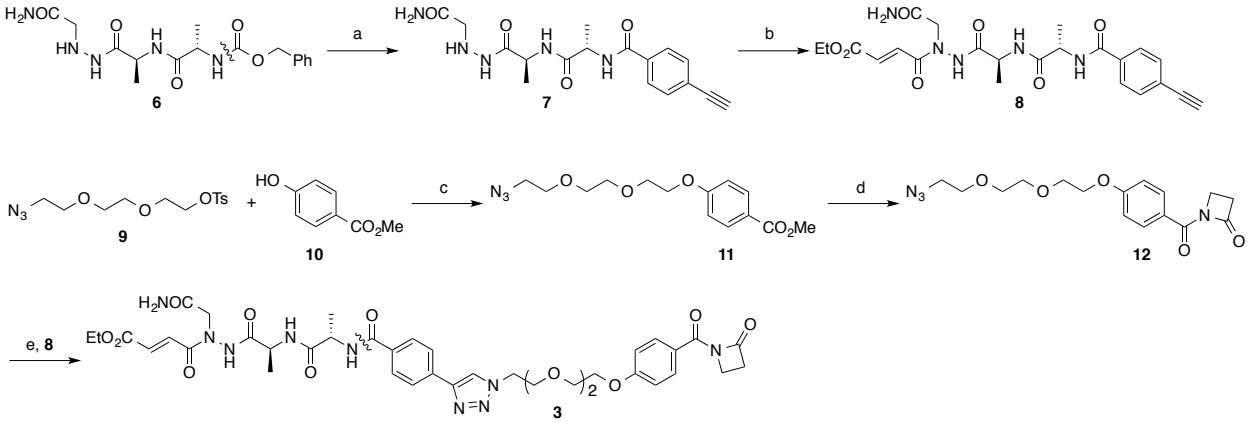
Figure S-1. Structure of mono- and bi-dentate linkers and their precursors.

3. Compound 3. Prepared as shown in Scheme S-1.

3.1. Compound 7. The Cbz protected compound **6** (347 mg, 0.64 mmol) was hydrogenated in presence of Pd/C (34 mg) in MeOH (2 mL) and H_2 atmosphere for overnight at RT in presence of 2 drops of 1N HCl. Pd/C was filtered out using small bed of celite. The organic layer was concentrated under vaccum and taken to next reaction without further purification.

HOBt (105 mg, 0.78 mmol) and EDCI (149 mg, 0.78 mmol) were added sequentially to a solution of the above-described amine (0.64 mmol), 4-acetylene benzoic acid (95 mg, 0.65

mmol), and DIPEA (0.43 mL, 2.59 mmol) in DMF (3 mL), and the reaction mixture was stirred at RT overnight. A saturated solution of NH_4Cl (1 mL) was added and the reaction mixture was worked up using $CHCl_3$ (2 X 30 mL) and water. The combined organic layers were washed with water and brine, and dried over Na_2SO_4. Solvents were removed under vaccum, and the crude residue was purified by silica gel column chromatography to afford compound **7** (186 mg, 80% yield). ^{1}HNMR ($CDCl_3$ + MeOD, 300 MHz): δ 7.85 (d, J = 7.8 Hz, 2H), 7.55 (d, J = 7.8 Hz, 2H), 4.27 (m, 1H), 3.63 (m, 1H), 3.40 (s, 1H), 3.15 (s, 1H), 1.37 (2 x d, J = 7.2 Hz, 6H). MS-ESI: 360.2 $(M+H)^+$.



Scheme S-1. Synthesis of the legumain protease targeting compound **3**. Key: (a) (i) H_2, Pd/C (cat.), MeOH, dil. HCl, RT, (ii) 4-Ethynylbenzoic acid, HOBt, EDCI, DIPEA, DMF, RT; (b) (*E*)-ethyl 4-chloro-4-oxobut-2-enoate, $(COCl)_2$, DMF (cat.), CH_2Cl_2, RT, then compound **7**, $NaHCO_3$, DMAP, THF, RT; (c) K_2CO_3, DMF, 50 ˚C; (d) (i) LiOH, THF-MeOH-H_2O (3:1:1), RT, (ii) $(COCl)_2$, DMF (cat.), CH_2Cl_2, RT, (iii) 2-azetidinone, ^{n}BuLi, THF, -78 ˚C-0 ˚C; (e) Cu wire, Aq. $CuSO_4$ (1 M), CH_3CN, RT.

3.2. Compound 8. Fumaric acid monoethyl ester (63 mg, 0.44 mmol) was treated with $(COCl)_2$ (0.11 mL, 1.30 mmol) in CH_2Cl_2 (1 mL) in the presence of a catalytic amount of DMF (2 drops). After the reaction mixture was stirred at RT for 3 h, it was concentrated in vaccuo and taken to next step without further purification.

A solution of the above-described acid chloride (prepared with 0.44 mmol Fumaric acid monoethyl ester) in THF (1 mL) was cannulated to a stirring mixture of compound **7** (130 mg, 0.36 mmol), solid $NaHCO_3$ (67 mg, 0.79 mmol), and DMAP (5 mg, 0.04 mmol) in THF. The reaction mixture was stirred at RT overnight, and worked-up using $CHCl_3$ (2 X 30 mL) and water. The combined organic layers were washed with water and brine, and dried over Na_2SO_4. Solvents were removed under vaccum, and the crude residue was purified by silica gel column chromatography to afford compound **8** (70 mg, 40% yield). ^{1}HNMR ($CDCl_3$ + MeOD, 300 MHz): δ 7.82 (m, 3H), 7.55 (d, J = 7.8 Hz, 2H), 7.00 (m, 1H) 4.35 (m, 1H), 4.35 (m, 1H), 4.28 (m, 1H), 4.12 (m, 4H), 3.15 (s, 1H), 1.24-1.10 (m, 9H). MS-ESI: 486.2 (M+H).

3.3. Compound 9. Commercially available triethylene glycol (12 mL, 89.90 mmol) was treated with TsCl (8.56 g, 44.95 mmol) in presence of Et_3N (28 mL, 197.78 mmol) in CH_3CN (200 mL) at 0 ˚C under N_2 atmosphere and the resulting mixture was stirred at RT for 5 h. The reaction mixture was quenched with saturated aqueous solution of NH_4Cl (60 mL), concentrated under vaccuo, and worked up using EtOAc (2 X 200 mL) and water. The combined organic layers were washed with water and brine, and dried over Na_2SO_4. Solvents were removed under vaccum, and

the crude residue was purified by silica gel column chromatography to give the previously known monotosylated triethylene glycol (16.5 g) in 60% yield.

NaN_3 (2.12 g, 32.75 mmol) was added to the above-described monotosyl triethylene glycol (5.0 g, 16.37 mmol) was taken in DMF (40 mL), and the reaction mixture was heated under N_2 atmosphere at 60 ˚C for 4 h. DMF was removed, and the mixture was worked-up using EtOAc (2 X 100 mL) and water (50 mL). The combined organic layers were washed with water and brine, and dried over Na_2SO_4. Solvents were removed under vaccum, and the crude residue was purified by silica gel column chromatography to give mono azide triethylene glycol (2.58 g, 90% yield). ^{1}HNMR ($CDCl_3$, 300 MHz): δ 3.72-3.61 (m, 8H), 3.57 (t, *J* = 4.8 Hz, 2H), 3.35 (t, *J* = 4.8 Hz, 2H).

The above-described monoazide triethyleneglycol (2.5 g, 14.28 mmol) was subjected to tosylation using TsCl (2.99 g, 15.71 mmol) and Et_3N (2.98 mL, 21.42 mmol) in CH_3CN (20 mL) for 6h under N_2 atmosphere using the same protocol described above to afford compound **9** (4.14 g, 88% yield). ^{1}HNMR ($CDCl_3$, 300 MHz): δ 7.80 (d, *J* = 7.8 Hz, 2H), 7.34 (d, *J* = 7.8 Hz, 2H), 4.16 (t, *J* = 4.8 Hz, 2H), 3.70 (t, *J* = 4.8 Hz, 2H), 3.64 (t, *J* = 4.8 Hz, 2H), 3.60 (m, 4H), 3.36 (t, *J* = 4.8 Hz, 2H), 2.45 (s, 3H).

3.4. Compound 11. A mixture of compound **9** (1.99 g, 6.06 mmol), methy 4-hydroxybenzoate, **10**, (920 mg, 6.06 mmol) and K_2CO_3 (1.08 g, 7.88 mmol) in DMF (12 mL) was added under N_2 atmosphere at 50°C for 12 h. The reaction mixture was worked up using Et_2O (50 mL) and water, and the combined organic layers were washed with water and brine, and dried over Na_2SO_4. Solvents were removed under vaccuo, and the residue was purified using silica gel column chromatography to give compound **11** (1.49 g) in 80% yield. MS-ESI: 310.2 (M+H).

3.5. Compound 12. Compound **11** (1.4 g, 4.53 mmol) was saponified using LiOH (326 mg, 13.59 mmol) in THF-MeOH-H_2O (3:1:1, 15 ml) at RT for 12h. The reaction mixture was concentrated under vaccuo, acidified using 1N HCl, and worked up using $CHCl_3$ and water. The combined organic layers were washed with water and brine, and dried over Na_2SO_4. Solvents were removed under vacuo, and the crude acid (1.2 g, 90% yield) was taken to next reaction without further purification. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.01 (d, *J* = 8.7 Hz, 2H), 6.93 (d, *J* = 8.7 Hz, 2H), 4.17 (t, *J* = 4.8 Hz, 2H), 3.87 (t, *J* = 4.8 Hz, 2H), 3.74-3.62 (m, 6H), 3.36 (t, *J* = 4.8 Hz, 2H).

The above-described crude acid (1.2 g, 4.06 mmol) was dissolved in CH_2Cl_2 (10 ml), and reacted with $(COCl)_2$ (5 mL) in presence of catalytic amount of DMF (1-2 drops) under N_2 atmosphere at RT for 12 h. Solvents were removed under vaccuo, and the crude residue was taken to next step without further purification.

^{n}BuLi (1.6 M, 2.16 mL, 3.47 mmol) was added dropwise to a solution of the commercially available 2-azetidinone (259 mg, 3.65 mmol) in THF (3 mL) under N_2 atmosphere at -78 ˚C, and the reaction mixture was brought to 0 ˚C within 5 minutes. A solution of the above-described crude acid chloride (4.06 mmol) in THF (4 mL) was cannulated, and the reaction mixture was stirred at 0 ˚C for 30 minutes before it was quenched with an aqueous citic acid solution (3 mL) and worked up using $CHCl_3$ (2 X 20 mL) and water. The combined organic layers were washed with water and brine, and dried over Na_2SO_4. Solvents were removed under vaccuo, and the residue was purified using silica gel column chromatography to give compound **12** (567 mg, 40% yield with respect to **11**). ^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.03 (d, *J* = 8.8 Hz, 2H), 6.95 (d, *J*

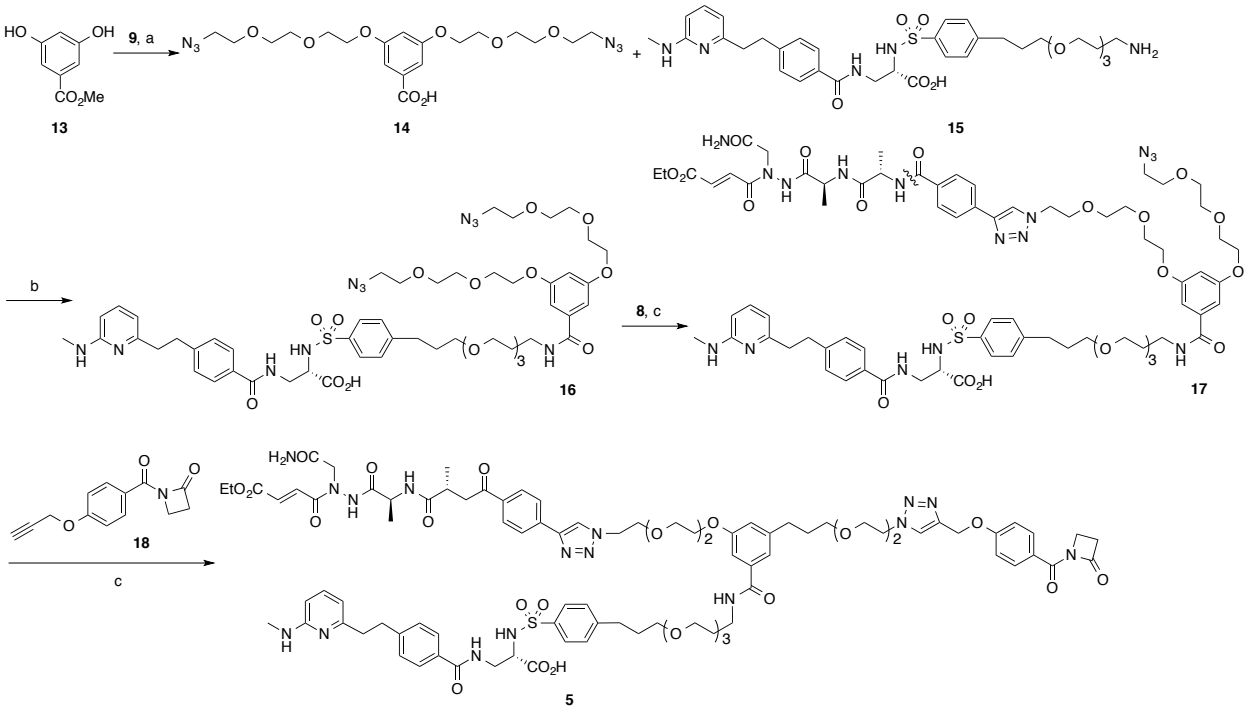
= 8.8 Hz, 2H), 4.18 (t, *J* = 4.8 Hz, 2H), 3.88 (t, *J* = 4.8 Hz, 2H), 3.79-3.65 (m, 8H), 3.37 (t, *J* = 4.8 Hz, 2H), 3.08 (t, *J* = 5.4 Hz, 2H).

3.6. Compound 3. Cu wire and 1M aq Cu_2SO_4 solution (5 μL) were added to a solution of the azide β-lactum **12** (9.2 mg, 0.03 mmol) and alkyne **8** (11.5 mg, 0.03 mmol) in CH_3CN (300 μL), and the mixture was stirred at RT overnight. The resulting 1,3-cycloaddition reaction product **3** (10 mg, 50% yield with respect to **8**) was obtained by concentrating the reaction mixture and purifying over silica gel column. ^{1}HNMR ($CDCl_3$ + MeOD, 300 MHz): δ 8.09 (s, 1H), 7.94-7.90 (m, 2H), 7.83-7.81 (m, 5H), 7.60 (m, 1H), 6.89-6.85 (m, 2H), 4.78 (m, 1H), 4.55 (m, 3H), 4.20 (m, 2H), 4.10 (m, 2H), 3.89 (m, 2H), 3.81-3.72 (m, 4H) 3.67-3.63 (m, 8H), 3.20 (m, 2H), 1.40 (m, 3H), 1.30-1.20 (m, 6H). MS-ESI: 879.2 $(M+H)^+$.

4. Compound 5. Prepared as shown in Scheme S-2.

4.1. Compound 14. A mixture of compounds **9** (1.16 g, 3.53 mmol) and **13** (235 mg, 1.41 mmol), and K_2CO_3 (778 mg, 5.64 mmol) in DMF (6 mL) was heated under N_2 atmosphere at 50 ˚C for 12 h. Reaction mixture was concentrated under vacuuo and worked up using Et_2O and water, washed with brine, and dried over Na_2SO_4. Solvents were removed under vacuuo, and the residue was purified by silica gel column chromatography to give the azido-teriethyleneglycol-ether of compound **9** (637 mg, 94% yield with respect to ester **9**).

The above-described compound (637 mg, 1.32 mmol) was saponified using NaOH (158 mg, 3.97 mmol) in THF:MeOH:H_2O (3:1:1) (5 ml) at RT for 12h. The organic solvent was removed under vaccuo, and the mixture was acidified with 1 N HCl and worked up using $CHCl_3$ and water. The combined organic layers were washed with water and brine, dried over Na_2SO_4, concentrated under vacuuo giving acid **14** (615 mg) that was taken to next reaction without further purification. MS-ESI: 469.2 (M+H), 491.3 (M+Na).



Scheme S-2. Synthesis of the bifunctional compound **5**. Key: (a) (i) K_2CO_3, DMF, 50 ˚C, (ii)

NaOH, THF-MeOH-H_2O (3:1:1), RT; (b) HOBt, EDCI, DIPEA, DMF, RT; (c) Cu wire, Aq. $CuSO_4$ (1 M), CH_3CN, RT.

4.2. Compound 16. EDC (24.5 mg, 0.13 mmol) were added to a solution of compound **14** (36 mg, 0.08 mmol) and NHS (9 mg, 0.08 mmol) in DMF (1 mL), and the mixture was strirred overnight at RT to give the corresponding active ester. Separately, compound **15** was prepared from the corresponding N-Boc derivative (54 mg, 0.08 mmol) and 20% TFA/CH_2Cl_2 at RT, overnight as described previously [1], and dissolved in DMF (1 mL). To this solution, Et_3N (0.06 mL) was added followed by the above-described NHS ester of compound **14**, and the mixture was stirred overnight at RT. The reaction mixture was diluted with water, and extracted using with 10% EtOH/$CHCl_3$. The combined organic layers were washed with water and brine, dried over Na_2SO_4, concentrated under vacuuo, and purified using silica gel column chromatography to give compound **16** (60 mg, 82% yield). MS-ESI: 1073.5 $(M+H)^+$, 537.5.

4.3. Compound 17. The diazide compound **16** (60 mg, 0.05 mmol) and alkyne **8** (7.2 mg, 0.015 mmol) were subjected to 1,3-cycloaddition reaction in presence of Cu wire and 1 M aq Cu_2SO_4 solution (5 µL) in CH_3CN (200 µL) at 50 ˚C (overnight). Solvents were removed under vacuuo, and the residue was purified using silica gel column chromatography to give compound **17** (13 mg, 54% yield with respect to **8**). MS-ESI: 811.5 $(M/2+H)^+$.

4.4. Compound 5. Compound **17** (8 mg, 0.005 mmol) was subjected to 1,3-cycloaddition reaction with the alkyne β-lactum compound **18** (1.1 mg, 0.005 mmol) in the presence of Cu wire and 1M aq Cu_2SO_4 solution (2 µL) in CH_3CN (100 µL) at 50 ˚C (overnight). Solvents were removed under vacuuo, and the residue was purified using preparative TLC to give compound **5** (4.5 mg, in 51% yield). MS-ESI: 925.8 (M/2).

5. Production of cpAb intermediates 38C2-LNK1, 38C2-LNK2, and 38C2-LNK3, cpAbs 38C2-3 and 38C2-4, and cp-bsAb 38C2-5. LNK**1**, LNK**2** and LNK**3** (10 mM in DMSO, 6.5 µl, 10 eq.) were added separately to solutions of Ab 38C2 (2 mg/ml in PBS, pH 6.5, 500 µl) taken in three eppendorfs, and the mixtures were incubated at 37 ˚C for 3 h. Similarly, compounds **3**, **4** and **5** (10 mM in DMSO, 8 µl) were added to solutions of Ab 38C2 (20 mg/ml, 150 µl, 133.4 µM) in PBS (pH 6.5, 850 µl) in three eppendorfs, and the mixtures were incubated at 37 ˚C for 3 h (for 38C2-**3** and 38C2-**5**) and 16 h for 38C2-**4**. After the formation of the cpAbs was confirmed using the Methodol assay, the reaction mixtures were dialyzed using a 10,000 MW cut dialysis bag and PBS (pH 7.4) three times, and filtered using nanofilter giving cpAbs 38C2-**3**, 38C2-**4** or 38C2-**5**. Concentration of the cpAbs was determined using nanoUV, and cpAbs were subsequenly diluted as needed.

5.1. Determination of the cpAb intermediates (38C2-LNK1, 38C2-LNK2, and 38C2-LNK3), cpAbs (38C2-3 and 38C2-4) and cp-bsAb (38C2-5) formation

A solution (1 µM in PBS, pH 7.4) of the above-prepared (Expt. 1) cpAb intermediates 38C2-LNK**1**, 38C2-LNK**2**, and 38C2-LNK**3**, Ab 38C2 alone (1 µM in PBS, pH 7.4) and PBS (pH, 7.4), 100 µl each, or (Expt. 2) cpAbs 38C2-**3**, 38C2-**4**, and and cp-bsAb 38C2-**5**, Ab 38C2 alone (1 µM in PBS, pH 7.4) and PBS (pH, 7.4), 100 µl each, were taken in wells of a 96-well plate. Methodol (10 mM in EtOH, 2 µl) was added in each well, and progress of the retro aldol reaction of methodol giving a fluorescent 6-methoxy-2-naphthaldehyde was measured using a Fluoroscence meter. Fluoroscence was recorded at the emisson wave length λ_{em} 452 against the absorbtion wave length λ_{abs} 330. A complete loss of the catalytic activity in wells containing Ab conjugates confirmed the chemical programming of Ab 38C2.

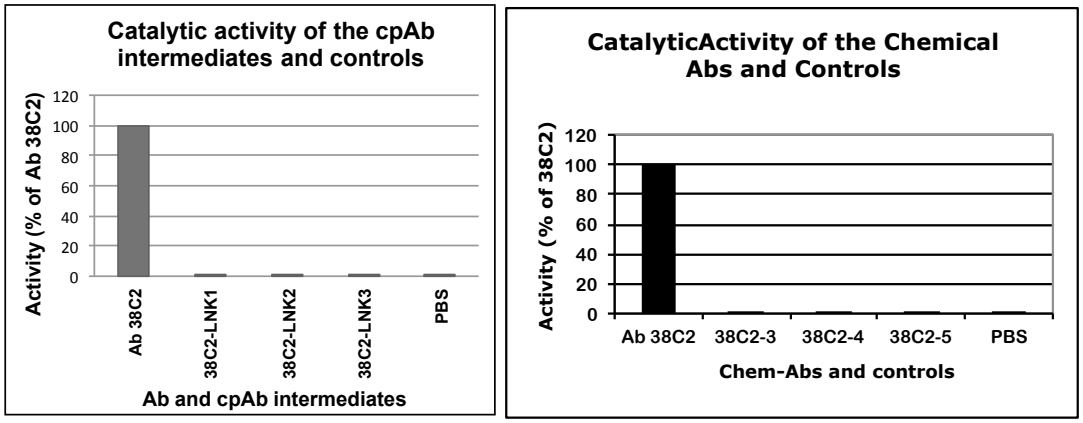


Figure S-2. Determination of the chemical programming of aldolase Ab 38C2 using (Left) linkers and (Right) Ab-PAs.

5.2. Effects of the cpAbs and cp-bsAb on catalytic activity of the Legumain protease. Methods described below.

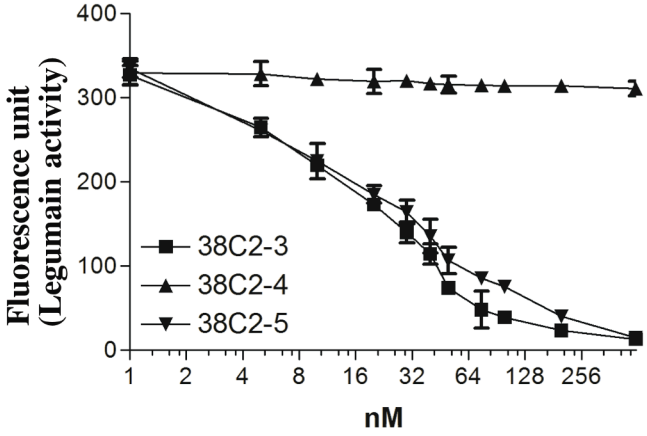


Figure S-3. The inhibition activity assay by 38C2-3, 38C2-4 and 38C2-5. Activated-legumain (20 nM) were incubated with different concentratin of 38C2-3, 38C2-4 and 38C2-5 for 30 min at 4 °C in 0.5 ml assay buffer (50 mM citric acid, 121 mM Na_2HPO_4, pH 6.0, containing 1 mM DTT, 1 mM EDTA, and 0.1% CHAPS). Then the substrate (Z-Ala-Ala-Asn-NHMec 100 μM, in 1.5 ml assay buffer) was added to mixture. The rate of formation of product was followed using a Perkin-Elmer fluorometer. The excitation and emission wavelengths were 360 and 460 nm, respectively.

6. Evaluation of the cpAbs and cpAb-bt.

Material and Methods – Biology.

6.1. Reagents and cell lines. Anti-integrin αvβ3 Ab and FITC conjugated goat anti-mouse Ab were purchased from Chemicon, San Deigo. Human glioblastoma cell line U87, human breast cancer cell line MDA-MB-231, and human umbilical vein endothelial cells (HUVEC) were obtained from American Type Culture Collection (ATCC) and were characterized according to ATCC instructions. The cells were maintained in RPMI-1640 or DMEM medium supplemented with 10% fetal bovine serum in a humidified incubator containing 5% CO_2 at 37 ˚C. All cell lines were used within 20 passages.

6.2. Confocal immunoanalysis. Immunofluorescent staining was performed on human MDA-MB-231 cells, which were coating on cover slip with 10nM EGF under hypoxia cultured for 3 days. The 3% PFA fixed MDA-MB-231 cells and sections from frozen MDA-MB-231 tumor tissue were stained for integrin αvβ3 mouse antibodies and goat legumain antibodies. FITC-conjugated anti-goat and Texas-red conjugated anti-mouse IgG (Vector Laboratories) was used as the secondary reporting reagent. Equal concentration of mouse IgG, goat IgG and secondary antibodies were used as negative control. Nuclei were stained with DAPI. Slides were analyzed by using confocal microscope **(**Zeiss LSM 710 laser scanning confocal microscope).

6.3. Binding of the cpAbs to MDA-MB-231, U87 and Ln-CAP cancer cells using flow cytometry. Cells were detached by trypsinization with 0.25 % (wt/v) trypsin/1 mM EDTA and washed twice with PBS. The cells were resuspended at a concentration of 1.5×10^5 cells/ml and distributed into each tube. The cells were incubated with different cpAbs (15 μg/ml) in binding buffer (1% FCS and 100 nm $MnCl_2$ in PBS) for 2hr at 4 ˚C. Then the cells were washed three times, and incubated with FITC labeled anti mouse antibody (2μg/ml) for 1 hour at 4 ˚C. The cells were washed two times with PBS containing 1% FCS and analyzed using Digital LSR II scanner.

6.4. Binding of the cpAbs to purified integrin αvβ3 and legumain. Immulon 2HB96 well plates (DYNE Technologies) were coated using the purified integrin αvβ3 (0.1 mg/ml, 100 μl) and leugmain protein (0.1 mg/ml, 100 μl) overnight at 4 ˚C. The plates were washed 2 times with PBS and were blocked with the binding buffer (10% BSA in PBS) for 1hr. cpAbs 38C2-**3**, 38C2-**4** and 38C2-**5** were diluted with buffer, and the plates were incubated for 2 hrs at the room temperature. The plates were washed 3 times with washing buffer (2% BSA in PBS). Biotinylated secondary Ab (Vector Lab, 1:500 dilution in binding buffer, 100 nM, 100 μl) were added to plate for 1hr. The plates were washed 4 times with the washing buffer (2% BSA in PBS l). The avidin-horseradish peroxidase reagent (100 μl) was added in each well, and the plate was incubated at 37 ˚C for 45 minutes. After the plate was washed 3 times with washing buffer, the AEC substrate solutions were developed for 10~60 minutes in dark, and the relative absorptions at 405 nM were measured using the UV spectrophotometer.

6.5. Cell proliferation assay. Human U87 cells were plated in 24-well plates in 2% serum containing medium and incubated until the cells adhered. Subsequently, the cells were treated with 1 μM 38C2-**3**, 38C2-**4**, 38C2-**5** and Ab 38C2 alone at 37 ˚C. After 0, 2 and 4 days incubation periods, cell proliferation was determined using the MTT assay as described in the manufacturer's instruction (Promega, San Deigo). Image from each well was scanned using a microscope on day 6.

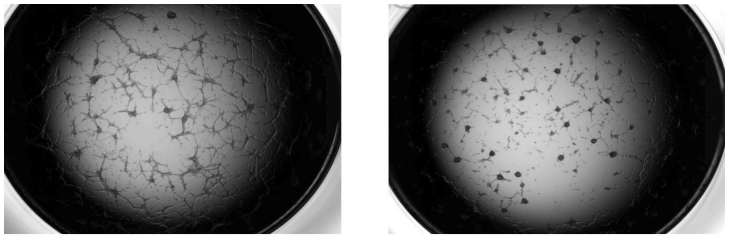


Figure S-4. Cell proliferation assay of Human U87 cells between legumain inhibitor **3** and the cpAb 38C2-**3**.

Similarly, effect of chemical programming was determined using compound **3** alone and cpAb 38C2-**3** in a separate experiment. Indeed, cpAb 38C2-**3** shows superior activity than compound **3** (Figure S-4).

Statistical analysis. All results are expressed as means. Student paired t-test was used to analyze the difference between two groups. Values were regarded significant at $P < 0.05$.

6.6. Inhibition of angiogenesis by programmed Abs in vitro, using the tube formation Assay. The wells of a 96-well microtiter plate were coated with 60 μl of the ice-cold matrigel (Collaborative Biomedical Products), and incubated overnight at 37°C. The next day, 12,000 single-donor human HUVEC cells were layered on top of the gel in EGM-2 complete growth medium with 2% serum supplements and 1 μM 38C2-**3**, 38C2-**4**, or 38C2-**5**. Buffer and Ab 38C2 alone were used as the negative controls. After the plates were incubated for an additional 72 hrs, the image from each well was scanned using a microscope. Images were further analyzed by counting fragments of tubes. Each experiment was carried out in triplicate.

6.7. In vivo tumor growth assay using mouse tumor models. Tumor induction was performed by s.c. injection of 5×10^5 MDA-MB-231 cells in the right flank of eight-week-old Hsd:Athymic nude mice. Four different groups of mice were treated with PBS (200 μl), 38C2-**3** (125 μg in 200 μl), 38C2-**4** (125 μg in 200 μl), and 38C2-**5** (125 μg in 200 μl), respectively starting on day 15 after the tumor challenge. Each mouse was given i.p. injection every 5 days, total 8 injections per mouse. Tumor volumes were measured using micro caliper (Volume = $1/2(\text{Width})^2 \times \text{Length}$). When tumor volume reached 1500 mm^3 in the control groups, tumor was removed. All mice were euthanized on day 80, and lungs were removed and fixed into Bouin's solution. Lung metastases were counted using the anatomy microscope. Tumor growth and signs of physical discomfort were monitored daily including for any gross evidence of tumor necrosis, local tumor ulceration, as well as evidence of toxicity including the mobility of animals, response to stimulus, piloerection, eating, and weight. These procedures have been reviewed and approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute. The Scripps Research Institute maintains an assurance with the Public Health Service and is registered with the Department of Agriculture and is in compliance with all regulations relating to animal care and welfare.

Statistical analysis

Results are expressed as means±s.e.m. Student's paired two-tailed t-test was used to analyze the difference between two groups. Values were regarded significant at $P < 0.01$.

7. Reference:

[1] Guo, F.; Das, S.; Mueller, B. M.; Barbas III, C. F.; Lerner, R. A.; Sinha, S. C. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 11009-14.

[2] Goswami, R. K.; Liu, Y.; Liu, C.; Lerner, R. A.; Sinha, S. C. Synthesis and evaluation of the aldolase antibody-derived chemical-antibodies targeting α5β1 integrin. *Mol. Pharm.* **2013**, *10*, 538-43.