Preparation of integrin $\alpha(v)\beta(3)$ -targeting Ab 38C2 constructs

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This protocol describes the preparation of Ab constructs using agents that target cells expressing integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, and the monoclonal aldolase Ab 38C2. The targeting agents are equipped with a diketone or vinylketone linker, and selectively react through the reactive Lys residues in the Ab binding sites to form 38C2 conjugates or chemically programmed 38C2 (i.e., cp38C2). The targeting agent possessing a diketone linker reacts with the Lys residues forming an enaminone derivative. By contrast, the vinylketone linker is used as the corresponding acetone adduct (i.e., a pro-vinylketone linker), and this pro-adaptor undergoes a 38C2-catalyzed retro-aldol reaction to produce the vinylketone linker, which forms a Michael-type adduct with the Lys residues. The Ab construct formation is achieved in <1 h for the diketone compounds at ambient temperature, and in 2–16 h using the pro-vinylketone linker at 37 °C. The 38C2 constructs are retargeted to cells over-expressing integrins, and are potential candidates for immunotherapy.

INTRODUCTION

Abs have emerged as an important class of therapeutics for the treatment of various diseases, including cancer^{1,2}. Numerous Abs are already in use and many more are currently under development. Abs can easily be obtained from different sources; for example, Abs can be derived from patients themselves, from synthetic combinatorial libraries or from humanized chimeric Abs, all of which can be used in humans. Notably, one common feature of Abs is that they are highly specific to a single target and, because of their clonal nature, a different Ab is required for each target. We have devised a new approach in which we modify the binding sites of a single Ab, 38C2, allowing it to be retargeted to more than one receptor^{3–7}. Such Ab constructs are useful for adapter immunotherapy.

Ab 38C2 possesses a pair of reactive Lys residues⁸ in its binding sites that allow it to covalently react with a compound equipped with a diketone or vinylketone function. A diketone compound reacts with the reactive Lys residues in the Ab 38C2 binding sites forming a Schiff base, which isomerizes to the more stable enaminone derivative (Fig. 1a). Similarly a vinylketone compound reacts with the Lys residues in the binding sites affording the corresponding Michael-type adducts (Fig. 1b). Therefore, the non-targeting catalytic Ab 38C2 could be selectively modified in its binding sites using a small-molecule inhibitor of the cell-surface receptors, and the resultant 38C2 constructs or the chemically programmed 38C2 (cp38C2) would function like a cell-targeting Ab.

Indeed, numerous antagonists of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, which are equipped with a diketone (e.g., 1) or an acetone aldol adduct of a vinylketone linker (e.g., 2), reacted selectively with 38C2 in its binding sites to produce the cell-targeting cp38C2 when treated with ~0.5 equiv. 38C2 (Fig. 2)^{4,7}. In the latter case, an aldol linker was used as a prolinker (i.e., an acetone aldol adduct of the vinylketone), because the vinylketone linker was highly reactive. Ab 38C2 catalyzed the retro-aldol reaction of the prolinker to give free vinylketone linker, which then underwent Michael addition, *in situ*, with the reactive Lys residues in the Ab binding sites. An analogous construct formation or chemical programming was also realized

using the humanized Ab 38C2, and the $\alpha_v\beta_3$ - and $\alpha_v\beta_5$ -targeting antagonist diketone⁹.

Retargeting of an Ab has also been achieved by conjugating the targeting agents to the Fc region in a conventional manner. Their effect can be similar; however, unlike the diketone or vinylketone strategy, a conventional conjugate is not homogenous¹⁰. In another example, an Ab can be retargeted using a targeting agent–hapten conjugate, against which the Ab has been elicited without forming a formal conjugate¹¹. A comparative study of these conjugates is needed to determine the advantages and limitations of one approach compared with the others.

The above-mentioned cp38C2 constructs bound strongly to integrins $\alpha_v\beta_3$ - and $\alpha_v\beta_5$ -expressing cancer cells, such as human Kaposi's Sarcoma (SLK), human melanoma (M21) and human breast carcinoma (MDA-MB-231 and MDA-MB-435)^{3–5,7}. The constructs inhibited the growth of the primary tumor and/or metastases of these human cancers as well as several others, such as human colon cancer (SW1222), which lack expression of $\alpha_v\beta_3$.

Figure 1 | Regioselective modification of Ab 38C2. Conjugation of the reactive Lys residues in Ab 38C2 binding sites with (a) the diketone compounds via the Schiff base and enaminone bond formation, and (b) vinylketone by the Michael reaction.

Figure 2 | Synthesis of 38C2 constructs (cp38C2). Conjugate formation of Ab 38C2 with integrin-targeting Arg-Gly-Asp (RGD) mimetics equipped with (a) a diketone (1) or (b) a pro-vinylketone (2) linker.

Mechanistically, the $\alpha_v\beta_3$ - and $\alpha_v\beta_5$ -integrin-targeting 38C2 constructs caused toxicity to cells not only by antagonizing the integrins or causing antiangiogenic effects, but also by instigating Ab-dependent cellular and complement-mediated cytotoxicities⁵.

Experimental design

For the formation of Ab 38C2 constructs, we prepared numerous $\alpha_v\beta_3$ - and $\alpha_v\beta_5$ -integrin-targeting molecules possessing a linker arm, and converted them to their diketone or provinylketone derivatives. However, a detailed protocol for the syntheses of all these compounds is beyond the scope of this article (one should refer to the original publications)^{4,7}. Here, we provide methods for the transformation of a representative $\alpha_v\beta_3$ - and $\alpha_v\beta_5$ -targeting

molecule possessing a linker arm to its diketone or provinylketone derivatives, for conjugate formation of the latter with Ab 38C2 and for analysis of the resultant constructs. Specifically, the following are described: syntheses of compounds 1 and 2 starting from the antagonist derivative 3a, and acids 7 and 9 (Fig. 3); a method for the formation of cp38C2 using 38C2 and 1 or 2; and characterization of the resultant constructs using a fluorescence assay.

The $\alpha_v\beta_3$ - and $\alpha_v\beta_5$ -integrin-targeting compound possessing a linker arm, 3a, was designed on the basis of a previously reported $\alpha_v\beta_3$ and $\alpha_v\beta_5$ inhibitor, 3 (ref. 12), and was prepared using acid 4 (ref. 7) and amine 5 (ref. 7). Compound 3a was conjugated to the diketone or provinylketone linker (7 or 9) as shown in Figure 3. Thus, the Boc-protecting group in compound 3a was deprotected

Figure 3 | Syntheses of the $\alpha_v\beta_3$ - and $\alpha_v\beta_5$ -integrin-targeting RGD mimetics, 1 and 2, equipped with a diketone or provinylketone function. a, TFA, anisole and CH_2Cl_2 ; b, NHS, EDC, DMAP and CH_2Cl_2 ; c, CH_3CN and triethylamine.

using trifluoroacetic acid (TFA) to give compound **6** in the form of its TFA salt. Separately, acids **7** and **9** were activated using *N*-hydroxysuccinimide (NHS) and 1-[3-dimethylaminopropyl]-3-ethylcarbodiimide hydrochloride (EDC) to give the corresponding NHS esters, **8** and **10**, which were reacted with compound **6** under basic conditions to afford compounds **1** and **2**, respectively. Products were purified by silica column chromatography using a mixture of methanol–dichloromethane (MeOH–CH₂Cl₂) with increasing concentrations of MeOH (5–20%). The purified small molecules equipped with these linkers were ready for conjugation with 38C2. For the construct formation, Ab 38C2 (1 equiv.) was treated with compound **1** (2 equiv.) or **2** (2.5 equiv.) for 0.5–24 h at room temperature (23 °C) or 37 °C to give the corresponding 38C2 constructs, which could be used without additional treatments.

Analysis of cp38C2 constructs

Formation of the Ab constructs is mainly analyzed by determining their catalytic activities using methodol as a substrate¹³. As both Lys residues of 38C2 are blocked by the diketone or vinylketone compounds in 38C2 constructs, the latter are non-catalytic and do not catalyze the retro-aldol reaction of methodol, **11**. By contrast, it is efficiently converted to the corresponding aldehyde, **12**, using a catalytic amount of 38C2. The formation of **12** can be quantified using a fluorometer. The catalytic activity of 38C2 constructs, 38C2-1 and 38C2-2, is determined using a 96-well plate that can be used with a microplate spectrofluorometer, and compared with Ab 38C2 as a positive control or buffer as a negative control. An abbreviated method is included in the procedure, and the results obtained are shown in **Figure 4**. Thus, the production of aldehyde **12** from **11** (200 μM) using catalytic Ab 38C2 (1 μM) is evident from the increase in fluorescence with time. By contrast, the reaction of **11** with 38C2-1 or 38C2-2 conjugates did not show an increase in fluorescence, thereby confirming the conjugate formation. These experiments have been repeated numerous

Figure 4 | Catalytic activity of 38C2 constructs, 38C2-1 (prepared from compound **1**) and 38C2-2 (prepared from compound **2**) compared with unmodified 38C2 and buffer alone. 38C2 and its constructs were used at a concentration of 1 μM and methodol was used at 200 μM. Progress of the retro-aldol reaction of methodol to produce 6-methoxynaphthaldehyde was monitored using a spectrofluorometer. The x-axis shows the time after methodol addition and the y-axis represents the raw fluorescence data.

times without contradictory results. Therefore, a single experiment is enough to confirm the noncatalytic activity of 38C2 conjugates. It is, however, advisable to carry out this experiment twice before making a decision about the conjugate formation when these studies are performed for the first time.

Confirmation of the conjugate formation between 38C2 and compounds **1** or **2** can also be established using matrix-assisted laser desorption/ionization (MALDI) spectrometry, similar to that previously reported using 38C2 Fab, and analogs of compounds **1** and **2** (ref. 4). In these spectra, an increase of approximately twice the molecular weight of the diketone or vinylketone compounds to that of Ab 38C2 was noted. As an example, **Figure 5** shows mass spectra of Ab 38C2 (weight-averaged molecular mass (M_w) = 150,951) and cp38C2-2 (M_w = 153,513) obtained from the reaction of 38C2 with compound **2**. The conjugate formation was also established by ELISA experiments, and by flow cytometry to record the binding to cells expressing integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. These methods are described elsewhere^{3–5,7,9}.

Note regarding reagents

All common solvents for the syntheses of compounds **1**, **2** and their precursors were obtained from Fishers' Scientific or Sigma-Aldrich. Methylene chloride was dried over CaH₂, and benzene was dried over Na and distilled.

Figure 5 | Mass spectral analysis of cp38C2. Comparison of untreated 38C2 (M_w = 150,951) and 38C2-2 (M_w = 153,513) conjugates by MALDI-MS analysis.

MATERIALS

REAGENTS

- Anisole (Sigma-Aldrich, cat. no. 29629-5)
- Triethylamine (Sigma-Aldrich, cat. no. 47128-3)
- TFA (Sigma-Aldrich, cat. no. 30203-1)
- DMSO (Sigma-Aldrich, cat. no. 27685-5)
- NHS (Sigma-Aldrich, cat. no. 13067-2)
- EDC (Sigma-Aldrich, cat. no. 16146-2)
- Dimethylaminopyridine (DMAP; Sigma-Aldrich, cat. no. 52281-3)
- Silica gel 60, particle size 40–63 μm (EMD; Fisher Scientific, cat. no. EM-1345)
- Thin-layer silica gel plates on glass, 0.2 mm thickness (EMD; Fisher Scientific, cat. no. M5554-7)
- Anhydrous sodium sulfate (Na_2SO_4 ; Sigma-Aldrich, cat. no. 23931-3)
- Ab aldolase 38C2 (Sigma-Aldrich, cat. no. 47995-0)

EQUIPMENT

- Magnetic hotplate stirrer (IKA, RCT Basic; ChemGlass)
- Teflon-coated magnetic stir bars (ChemGlass)
- Plastic syringes (Fisher Scientific)
- Reusable and disposable hypodermic syringe needles (Fisher Scientific)

- Rotary evaporator (Buchi)
- Prep-thin-layer chromatography (TLC) plate coated with silica gel (Fisher Scientific)
- Glass chromatographic columns (ChemGlass)
- Filter paper (Fisher Scientific)
- Filter funnel (Fisher Scientific)
- Separatory funnels (ChemGlass)
- High-vacuum pump (<1 mm Hg; Fisher Scientific)
- Microplate spectrophotometer (Spectra Max Gemini; Molecular Devices)

REAGENT SETUP

Compounds 3a, 7 and 9 The synthesis of compound **3a** using acid **4** and amine **5** is described in ref. 7 and the synthesis of compound **9** is described in ref. 4; for convenience, they are also shown in **Figure 6**. For the detailed processes, investigators should consult the original papers. If requested, the processes and small amounts of compounds **1**, **2**, **3a**, **7** and **9** will also be made available.

EQUIPMENT SETUP

Analysis of cp38C2 constructs This requires a microplate spectrophotometer equipped with a computer running the SOFTmax Pro 2.6.1 application, and a 96-well plate compatible with the fluorescence reader.

PROCEDURE

Conversion of compound 3a to 6

- 1] Dry a 10-ml single-neck round-bottomed flask containing a Teflon-coated magnetic stir bar in an oven at 150°C .
- 2] Take the flask out of the oven, cap it with a rubber septum and insert a nitrogen inlet with a fairly strong nitrogen flow and a vent using disposable syringe needles. Allow the flask to cool to 23°C .
- 3] Add compound **3a** (200 mg, 0.24 mmol) to the flask, cap with a rubber septum and insert a nitrogen inlet with a disposable syringe needle.
- 4] Add 2 ml dry CH_2Cl_2 to the flask using a plastic syringe fitted with a 20-gauge hypodermic needle, and turn the magnetic stirrer on.
- 5] Add 0.5 ml anisole and 0.5 ml TFA (slowly over 1 min) sequentially to the flask using a plastic syringe fitted with a 20-gauge hypodermic needle.

Figure 6 | Synthesis of the precursors of compounds **1** and **2**. **(a)** Compound **3a**: a, Et_3N and $\text{THF-H}_2\text{O}$ (1:2), RT; b, (i) Br_2 , 4M NaOH , 0°C , then 85°C , (ii) isobutene, conc. H_2SO_4 , (iii) benzylchloro-formate, aq. NaHCO_3 -ether (1:1); c, (i) $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$, CuI , Et_3N , EtCN , (ii) 10% (wt/wt) Pd-C , H_2 , EtOH ; d, $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$, CuI , Et_3N , EtCN ; e, (i) H_2 , 10% (wt/wt) Pd-C , THF , (ii) Aq. NaOH , MeOH ; f, EDC, HOBT, NMM, DMF. **(b)** Acid **7**: g, LDA, -78°C ; h, H_2 , 10% (wt/wt) Pd-C , glutaric anhydride, THF , RT. **(c)** Acid **9**: i, (i) ethylene glycol, PTSA $\cdot \text{H}_2\text{O}$, benzene, reflux, (ii) LiAlH_4 , ether, RT, (iii) $(\text{COCl})_2$, DMSO, CH_2Cl_2 , -78°C , then Et_3N , (iv) vinylmagnesium bromide, THF , 0°C ; j, $\text{Pd}(\text{OAc})_2$, $n\text{-Bu}_4\text{NCl}$, NaHCO_3 , DMF, 80°C ; k, (i) H_2 , 10% (wt/wt) Pd-C , THF ; (ii) vinylmagnesium bromide, THF ; l, (i) glutaric anhydride, THF , (ii) 1N HCl , THF .

! CAUTION TFA is corrosive (see material safety data sheet at <http://www.sigmaaldrich.com/catalog/search/ProductDetail/SIAL/302031>).

- 6| Continue stirring for 16 h at 23 °C and check the completion of the reaction using thin-layer silica gel plates and CH₂Cl₂/MeOH (9:1; retention factor (*R_f*) = 0.5 for **3a** and 0.2 for **6**).
- 7| After completion of the reaction, turn the stirrer off, and remove the nitrogen inlet and the rubber septum.
- 8| Take the magnetic stir bar out, wash it with CH₂Cl₂ (1 ml) into the flask, and evaporate solvents under reduced pressure on a rotary evaporator keeping the bath temperature <30 °C.
- 9| Add 5 ml ethyl acetate (EtOAc) and evaporate solutions using the rotary evaporator as described in Step 8. Repeat the process twice to remove TFA.
- 10| Dry the yellow residue using a vacuum pump (<1 mm Hg) overnight to give key intermediate **6** as a TFA salt. This product can be used to prepare **1** and **2** without purification.
- **PAUSE POINT** Compound **6**·TFA is freshly prepared from **3a** when needed, and can be kept overnight at –20 °C.

Syntheses of NHS esters **8** and **10**

- 11| Dry a 25-ml single-neck round-bottomed flask containing a Teflon-coated magnetic stir bar in an oven at 150 °C.
- 12| Take the flask out of the oven and cap it with a rubber septum. Insert a nitrogen inlet with a fairly strong nitrogen flow and a vent using disposable syringe needles. Allow the flask to cool to 23 °C.
- 13| Weigh 415 mg compound **7** (1.3 mmol, 1.0 equiv.) or 450 mg compound **9** (1.3 mmol, 1.0 equiv.) into the flask, and recap with the septum attached to the nitrogen inlet.
- 14| Transfer 5 ml dry CH₂Cl₂ into the flask using a plastic syringe fitted with a 20-gauge hypodermic needle, and turn the magnetic stirrer on.
- 15| Add 298 mg EDC (1.56 mmol, 1.2 equiv.) and 180 mg NHS (1.56 mmol, 1.2 equiv.) into the flask, and add 8.0 mg DMAP (0.065 mmol, 0.05 equiv.) sequentially to the resulting solution.
- 16| Recap the flask with the septum attached to the nitrogen inlet, and continue stirring for 4 h at 23 °C.
- 17| Monitor the reaction by TLC using thin-layer silica gel plates (solvent system: EtOAc/hexanes, 4:1; *R_f* = 0.70 for compound **8** and *R_f* = 0.55 for **10**).
- 18| Remove the septum and nitrogen inlet after the reaction has completed (4 h).
- 19| Remove the solvent under reduced pressure on a rotary evaporator to obtain the crude residue keeping the bath temperature <40 °C.
- 20| Pack a chromatographic column (2.5 cm i.d. × 15.5 cm length) with ~45 g dry silica gel and cover the top of the column further with a layer of sand (~1 cm thick). Equilibrate the column with hexanes.
- 21| Dissolve the residue from Step 19 in CH₂Cl₂, and load the solution on the top of the silica column using a pipette.
- 22| Elute the column with EtOAc/hexanes (20, 50, 80 and 100%; 200 ml each) under minimal air pressure and collect the fractions (10 ml each) using test tubes.
- 23| Identify the fractions containing the desired products by TLC using silica gel plates and 80% EtOAc/hexanes (*R_f* = 0.70 for **8** and 0.55 for **10**). Collect all fractions showing the desired compounds into a round-bottomed flask.
- ▲ **CRITICAL STEP** NHS esters are susceptible to hydrolysis. It is advisable to perform the chromatography in one continuous step — do not let the compounds sit on the silica gel for a prolonged period.
- 24| Remove the solvents under reduced pressure on a rotary evaporator, and dry the residue using a high-vacuum pump (<1 mm Hg) to give pure product **8** (typical yield: 430 mg, 80%) or **10** (typical yield: 450 mg, 78%).
- **PAUSE POINT** Compounds **8** and **10** can be stored at –20 °C under argon and dry conditions for several weeks; however, it is advisable to prepare them fresh and use as soon as possible for the best results.

Syntheses of compounds **1** and **2**

- 25| Fit a Teflon-coated magnetic stir bar to the flask containing 150 mg crude compound **6** (0.24 mmol) obtained in Step 10.

- 26| Cap the flask with a rubber septum. Insert a nitrogen inlet with a disposable syringe needle.
- 27| Add 2 ml CH₃CN to the flask and turn the magnetic stirrer on.
- 28| Add 0.25 ml TEA to the reaction mixture.
- 29| Weigh out 150 mg NHS ester **8** (0.36 mmol, 1.5 equiv.) for compound **1** or 160 mg NHS ester **10** (0.36 mmol, 1.5 equiv.) for compound **2** in a separate vial, dissolve it in CH₃CN (1 ml) and add it into the reaction mixture using a plastic syringe fitted with a 20-gauge hypodermic needle.
- 30| Continue stirring for 4 h at 23 °C, turn the stirrer off and remove the nitrogen inlet and rubber septum.
- 31| Dilute the reaction mixture with 30 ml EtOAc, and transfer the mixture to a separatory funnel.
- 32| Wash the organic layer with a 1:1 (vol/vol) mixture of 10% citric acid and brine (5 ml each), and dry the organic layer with anhydrous Na₂SO₄ (5–10 g).
- 33| Filter the mixture under gravity through a fluted filter paper on a glass filter funnel and collect the filtrate in a round-bottomed flask.
- 34| Remove the solvent under reduced pressure on a rotary evaporator to get the crude residue.
- 35| Pack a chromatography column with silica gel and cover the top of the column further with a layer of sand (~1 cm thick). Equilibrate the column with CH₂Cl₂.
- 36| Dissolve the residue from Step 34 in CH₂Cl₂, and load the solution on top of the silica column using a pipette.
- 37| Elute the column with MeOH/CH₂Cl₂ (0–25%) under minimal air pressure, and collect the fractions using test tubes.
- 38| Identify the fractions containing desired product **1** or **2** by TLC using silica gel plates and 20% MeOH/CH₂Cl₂ (*R_f* = 0.45 for **1** and 0.37 for **2**). Collect all the fractions showing **1** and **2** separately into round-bottomed flasks.
- 39| Remove the solvents under reduced pressure on a rotary evaporator, and dry the residue using a high-vacuum pump (<1 mm Hg) to give pure product **1** (typical yield: 167 mg, 75%) or **2** (typical yield: 181 mg, 75%).
- **PAUSE POINT** Compounds **1** and **2** were stored for several weeks under an argon atmosphere in a refrigerator at –20 °C with no sign of any appreciable decomposition. Stock solution of 38C2 (10 mg ml^{–1}, 66.7 μM solution) in PBS buffer (pH 7.4), and compounds **1** (9.3 mg ml^{–1}, 10 mM solution) and **2** (9.6 mg ml^{–1}, 10 mM solution) in CH₃CN, can be kept at –20 °C for an extended period of time.

Formation of Ab 38C2 constructs (cp38C2s)

- 40| Using a micropipette, transfer 37.5 μl Ab 38C2 (10 mg ml^{–1} solution in PBS) solution to two separate 1.5 ml Eppendorf tubes and appropriately label. Keep the Ab solution cold using an ice bath.
- 41| Transfer 2 μl of the 10 mM stock solutions of compound **1** and **2** into two separate 1.5 ml Eppendorf tubes containing 18 μl of PBS buffer (pH 7.4), making the end concentration of the solutions 1 mM. Appropriately label them, and vortex. Keep the solutions cold using an ice bath.
- 42| Transfer 10 μl of the solution of compound **1** from Step 41 to the first Ab 38C2 solution from Step 40, add 2.5 μl of PBS (pH 7.4) to it, and vortex. Similarly, transfer 12.5 μl of the solution of compound **2** from Step 41 to the second Ab 38C2 solution from Step 40, and vortex.

- 43| Incubate the solution containing **1** for 0.5 h at room temperature (23 °C), and that containing **2** for 16 h at room temperature followed by 2 h at 37 °C.

■ **PAUSE POINT** The incubation time can be prolonged to 16 h at room temperature (23 °C) for both solutions. However, the solution containing **2** has to be further incubated at 37 °C for 2 h to ensure the complete formation of construct 38C2-**2**.

Analysis of cp38C2 constructs

- 44| Dilute Ab 38C2 and the 38C2 constructs in PBS (pH 7.4; 1 μM solution in Ab, 100 μl each). Use an appropriate volume of the stock solution of Ab 38C2 (incubated at 37 °C for 2 h) for comparison with the solutions obtained in Step 43.
- 45| Start the spectrofluorometer, and set the program to read fluorescence using wavelength of excitation (λ_{ext}) = 330 nM and wavelength of emission (λ_{em}) = 452 nM.
- 46| Transfer 98 μl solutions of Ab and constructs prepared in Step 44 into three different wells of a 96-well plate compatible with the fluorescence reader, and transfer 98 μl PBS buffer (pH 7.4) into another well.

47| Add methodol (2 μ l from 10 mM stock solution) 200 μ M to the four wells containing Ab, constructs and buffer, and measure the formation of 6-methoxynaphthaldehyde for 60 min starting at 0 min. A representative example is shown in **Figure 4**.

48| Dilute the mixture (3.3 μ l) from Step 43 using 46.7 μ l buffer, vortex to give 38C2 constructs with 3.3 μ M in Ab and use as needed after further dilution.

? TROUBLESHOOTING

● TIMING

Compound 6 Step 1, 30 min; Step 2, 10 min; Steps 3–6, 16.5 $\pm$ 1 h (overnight reaction); Steps 7–9, 30 min; Step 10, 20 $\pm$ 1 h (overnight)

Compounds 8 and 10 Step 11, 30 min; Step 12, 10 min; Steps 13–16, 4.5 $\pm$ 0.5 h; Steps 17–19, 30 min; Steps 20–24, 3 h

Compounds 1 and 2 Steps 25–30, 4.5 $\pm$ 0.5 h; Steps 31–34, 1 h; Steps 35–39, 3 h

38C2 constructs (cp38C2s) Preparing the stock solutions, 30 min; Steps 40–42, 30 min; Step 43, 1 h for 38C2-1 and 2.5 h for 38C2-2, or 16.5 $\pm$ 1 h (overnight reactions)

? TROUBLESHOOTING

In general, the processes described here have been repeated numerous times and no difficulties have been noted. Nevertheless, some potential problems might arise if proper care is not taken, as illustrated by the following examples: (i) the NHS esters **8** and **10** are water sensitive, and yields of **1** and **2** might vary depending upon the quality of compounds **8** and **10**; (ii) if compounds **1** and **2** are eluted with traces of silica gel, which might occur during their purification on silica gel using MeOH/CH₂Cl₂ (0–25%) as an eluting solvent, the silica gel can be removed by dissolving the compound in 5% MeOH/CH₂Cl₂ and filtering through a syringe filter (PTFE, 0.2 μ m; Thomson Instrument Company, cat. no. TIC-6550225); (iii) if compounds **1** and **2** are not pure, construct formation between 38C2 and compounds **1** or **2** will not be efficient; (iv) amine- and thiol-containing buffer might impair the 38C2 construct formation, and should therefore be avoided.

ANTICIPATED RESULTS

Compounds **1** and **2**, and analogous compounds, should selectively conjugate in Ab 38C2 binding sites to afford highly homogenous chemically programmed 38C2-constructs, which target to cells expressing integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$.

Analytical data

Compound 1. ¹H NMR (600 MHz, CD₃OD/CDCl₃): δ 7.73 (d, J = 8.3 Hz, 2H), 7.68 (d, J = 8.3 Hz, 2H), 7.54 (t, J = 8.3 Hz, 1H), 7.43 (m, 2H), 7.22 (dd, J = 7.9, 5.3 Hz, 2H), 7.10 (m, 2H), 6.53 (d, J = 8.8 Hz, 1H), 6.47 (d, J = 7.4 Hz, 1H), 3.83 (dd, J = 7.4, 4.8 Hz, 1H), 3.68 (dd, J = 13.6, 5.3 Hz, 1H), 3.63 (dd, J = 13.6, 7.5 Hz, 1H), 3.58 (m, 2H), 3.53 (m, 4H), 3.40 (t, J = 6.6 Hz, 2H), 3.36 (t, J = 5.3 Hz, 2H), 3.31 (m, 2H), 2.99 (m, 2H), 2.93 (m, 2H), 2.89 (s, 3H), 2.85 (t, J = 7.9 Hz, 2H), 2.80 (s, 1H), 2.64 (t, J = 7.4 Hz, 2H), 2.55 (t, J = 7.9 Hz, 2H), 2.36 (t, J = 7.4 Hz, 2H), 2.26 (t, J = 7.4 Hz, 2H), 2.14 (s, 1H), 1.99 (s, 3H), 1.95 (q, J = 7.0 Hz, 2H), 1.79 (m, 2H).
MS (ESI) (m/z) [$M+H^+$] 929, [$M-H$][−] 927.

Compound 2. ¹H NMR (500 MHz, CDCl₃): 7.70 (d, J = 8.1 Hz, 2H), 7.57 (d, J = 7.8 Hz, 2H), 7.48 (dd, J = 7.4, 1.2 Hz, 1H), 7.37 (d, J = 8.5 Hz, 2H), 7.16 (d, J = 8.1 Hz, 2H), 7.02–6.99 (m, 4H), 6.35 (d, J = 8.8 Hz, 2H), 6.28 (d, J = 7.4 Hz, 2H), 5.78 (dd, J = 17.2, 10.6 Hz, 1H), 5.20 (dd, J = 17.2, 1.1 Hz, 1H), 5.11 (dd, J = 10.6, 1.1 Hz, 1H), 3.82 (m, 1H), 3.73–3.31 (m, 12H), 3.06–3.00 (m, 2H), 2.91–2.46 (m, 11H), 2.27 (t, J = 7.4 Hz, 2H), 2.18 (t, J = 6.6 Hz, 2H), 2.09 (s, 3H), 1.92–1.88 (m, 2H), 1.81–1.63 (m, 4H).
MS (ESI) (m/z) [$M+H^+$] 957, [$M+Na^+$] 979.

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