



β -Lactam-based approach for the chemical programming of aldolase antibody 38C2

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ABSTRACT

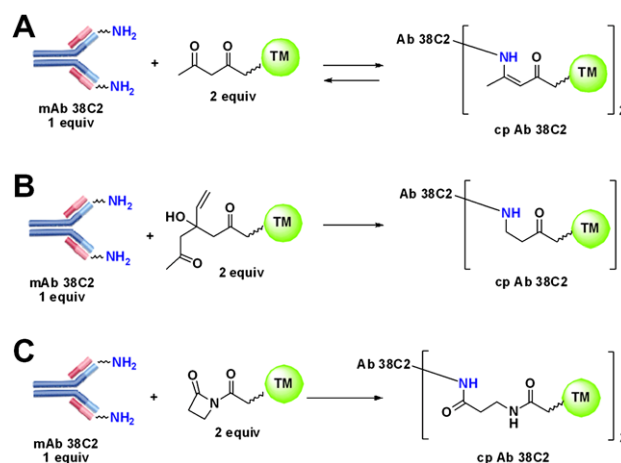
Irreversible chemical programming of monoclonal aldolase antibody (mAb) 38C2 has been accomplished with β -lactam-equipped targeting modules. A model study was first performed with β -lactam conjugated to biotin. This conjugate efficiently and selectively modified the catalytic site lysine (LysH93) of mAb 38C2. We then conjugated a β -lactam to a cyclic-RGD peptide to chemically program mAb 38C2 to target integrin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$. The chemically programmed antibody bound specifically to the isolated integrin receptor proteins as well as the integrins expressed on human melanoma cells. This approach provides an efficient and versatile solution to irreversible chemical programming of aldolase antibodies.

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Monoclonal antibody (mAb) 38C2 was generated by reactive immunization with a 1,3-diketone hapten.¹ This antibody possesses a lysine residue at position 93 of the heavy chain, LysH93, that has an unusually low pK_a of ~ 6.0 and is essential for the catalytic mechanism of the antibody.² mAb 38C2 can be chemically programmed to selectively bind to cells or other targets by selective reaction of LysH93 with β -diketone (Scheme 1A) or an acetone aldol adduct of a vinylketone (Scheme 1B) linker equipped with a targeting ligand or module.^{3–8} Many of our studies have used antagonists of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins as targeting modules for chemical programming of aldolase antibody specificity.^{3–7} In the first case, a reversible covalent attachment of a diketone compound is achieved through the formation of an imine, which isomerizes to the more stable enaminone derivative. In the latter case, an acetone aldol adduct of the vinylketone is used as a prolinker, because the vinylketone linker is highly reactive. mAb 38C2 catalyzes the retro-aldol reaction of the prolinker to give free vinylketone linker, which then undergoes Michael addition, in situ, with the catalytic Lys residue in the antibody-binding site. This approach provides irreversible attachment of the targeting modules; however, preparation of the prolinker equipped targeting modules requires complicated multistep synthesis and is not compatible with a wide range of substrates.

In earlier studies we had explored a β -lactam-based modification strategy for covalent attachment of thiazolium cofactor into the active site of aldolase antibodies in order to prepare the first

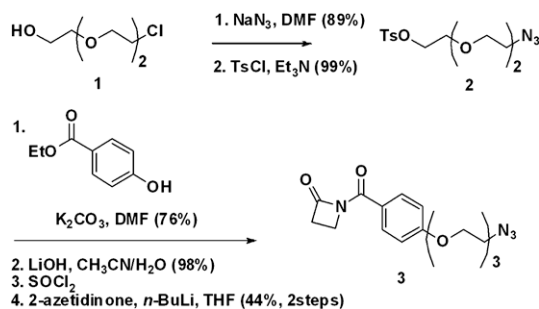
thiazolium-dependent catalytic antibodies.⁹ Given the success of this study and the chemical stability of the resulting covalent modification, we were encouraged to examine this approach as a route to chemically programmed antibodies. Here we report the selective reaction of the catalytic site LysH93 with β -lactam-equipped targeting modules (Scheme 1C); the synthesis of the targeting module was straightforward and this strategy provides for irreversible covalent attachment of targeting modules. We designed



Scheme 1. Different approaches for the chemical programming of mAb 38C2 using a reactive moiety linked to a targeting module (TM).

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Scheme 2. Synthesis of the heterobifunctional linker module **3**.

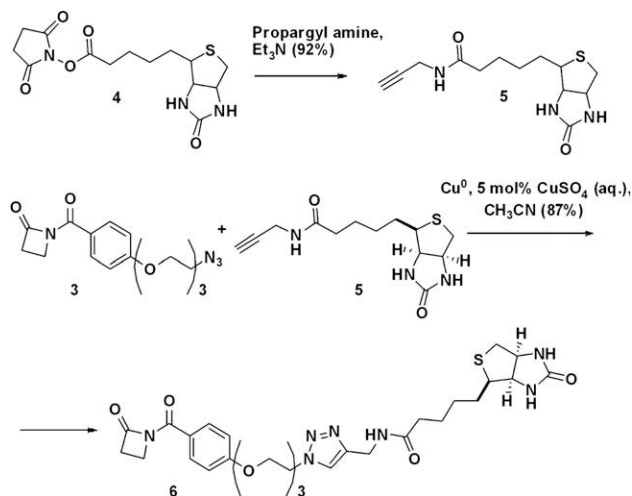
our linker molecules with three features in mind: First, 1-acyl β -lactams react efficiently with LysH93.⁹ Second, the narrow and highly lipophilic catalytic site of mAb 38C2 should bind tightly to a phenyl ring based on the crystal structure of the antibody.² Third, given the disposition of LysH93 at the bottom of the 11-Å deep active site, a linker of appropriate length should be incorporated to allow functional display of the targeting module outside the active site of the antibody.

The heterobifunctional β -lactam targeting module **3** was synthesized in six steps starting from inexpensive starting materials. In brief, 2-(2-(2-chloroethoxy)ethoxy)ethanol **1** was treated with sodium azide in DMF to afford the corresponding azido alcohol in 89% yield (Scheme 2). Subsequent tosylation provided tosyl azide **2** in quantitative yield. Reaction of **2** with ethyl 4-hydroxybenzoate in the presence of potassium carbonate in DMF provided azido ester in 76% isolated yield. The ester was hydrolyzed using lithium hydroxide followed by a reaction with neat thionyl chloride to give the corresponding acid chloride. The crude acid chloride was used immediately for the reaction with 2-azetidinone in the presence of *n*-butyl lithium to afford the desired heterobifunctional module **3** in 44% isolated yield.

We selected biotin as a model targeting module to evaluate the β -lactam-based approach for chemical programming of mAb 38C2. Biotin containing **6** was prepared as follows: biotin *N*-hydroxy-succinimide ester **4** was coupled with propargyl amine to give alkynyl biotin **5** in 96% yield (Scheme 3).

The subsequent copper (I)-catalyzed azide alkyne cycloaddition reaction^{10–15} of β -lactam module **3** and alkyne **5** furnished the desired targeting module **6** in 87% isolated yield.

Chemical programming of the mAb 38C2 with **6** was performed essentially as described.³ Only 2 equiv of the targeting module **6** were used (1 equiv per active site) and complete loss of catalytic



Scheme 3. Synthesis of the β -lactam-biotin conjugate **6**.

activity of mAb 38C2 was observed after 2 h at room temperature. Catalytic activity was measured by analysis of the retro-aldol reaction of methodol (Fig. 1A).³ Aliquots were removed at different timepoints and the reaction was quenched by immediate removal of any unbound small molecules by desalting using Zeba spin desalting columns (Pierce Inc.); the resulting purified antibody was tested in the methodol-based catalytic activity assay. MALDI-TOF analysis of the biotin programmed mAb 38C2 compared

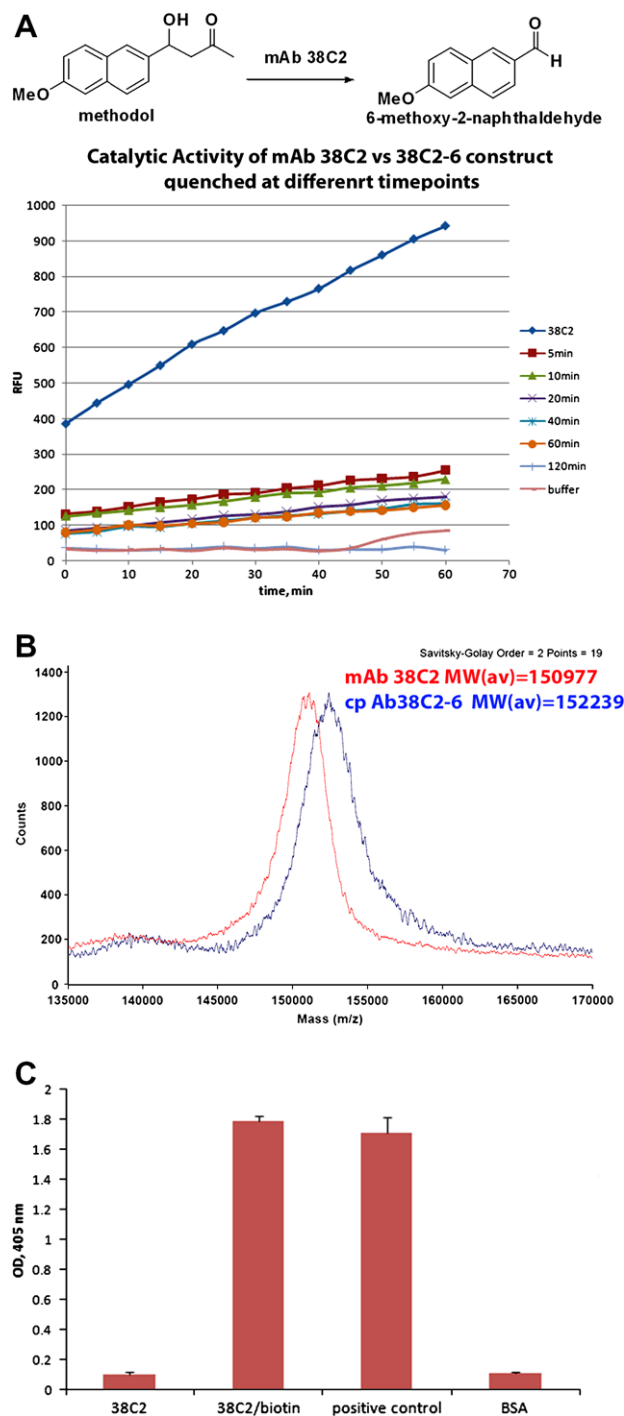
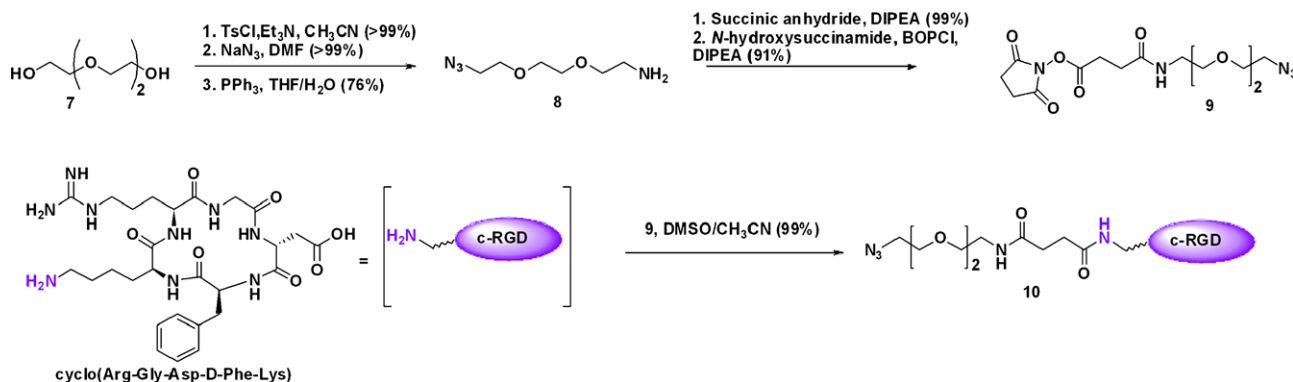
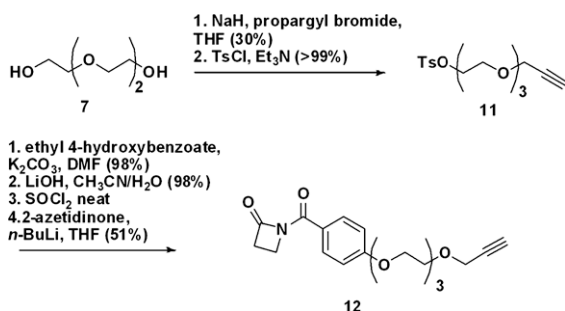


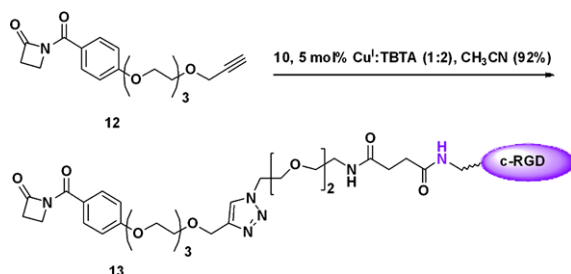
Figure 1. Analysis of the formation of mAb 38C2 chemically programmed with **6** (cp38C2-6). (A) The methodol-based catalytic activity assay of aliquots removed and quenched by desalting at indicated timepoints. (B) Overlay of MALDI-TOF analysis of mAb 38C2 and cp38C2-6. (C) Results of the ELISA assay showing streptavidin-HRP binding of mAb 38C2 alone, cp38C2-6, commercially available biotinylated mouse Ab (positive control), and BSA.



Scheme 4. Synthesis of the azido-functionalized cyclic-RGD module 10.



Scheme 5. Synthesis of the heterobifunctional linker module 12.

Scheme 6. Synthesis of the β -lactam-equipped cyclic-RGD conjugate 13.

to that of mAb 38C2 gave an additional proof of the selective functionalization of catalytic site LysH93 in the context of the IgG (Fig. 1B).⁹ Analysis of tryptic digests confirmed LysH93 labeling. To confirm that the chemically programmed antibody displayed

biotin in a manner to allow for its binding to streptavidin, we also used a streptavidin ELISA (Fig. 1C). We then chemically programmed the antibody to recognize integrin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in order to explore the compatibility of the β -lactam linker strategy with a substantially more functionalized targeting module. Heterobifunctional linker 9 containing azide and NHS functionalities was prepared starting from triethyleneglycol 7 (Scheme 4).

Triethylene glycol was bis-tosylated and converted to the corresponding bis-azide in excellent yield. The bis-azide was selectively reduced using an established procedure to give amino azide 8.¹⁶ Reaction of 8 with succinic anhydride and subsequent coupling with *N*-hydroxysuccinimide provided the desired heterobifunctional linker 9. Linker 9 was selectively conjugated with the ϵ -amino group of the lysine moiety in the cyclic-RGD peptide (cyclo(Arg-Gly-Asp-D-Phe-Lys)) to give module 10. We also prepared linker module 12 (Scheme 5) in order to introduce the β -lactam functionality into our integrin targeting module.

Triethylene glycol 7 was treated with propargyl bromide in the presence of sodium hydride to afford a mixture of mono- and bis-propargylated products that were separated by column chromatography. Mono-propargylated alcohol was then tosylated to afford tosyl alkyne 11. Compound 11 was reacted with ethyl 4-hydroxybenzoate in the presence of potassium carbonate in DMF to afford the alkynyl ester in 98% yield without need for purification. The ester was hydrolyzed using lithium hydroxide and converted to the acid chloride by treatment with neat thionyl chloride. The crude acid chloride was used immediately for the reaction with 2-azetidinone in the presence of *n*-butyl lithium to afford the desired heterobifunctional linker 12 in 51% isolated yield.

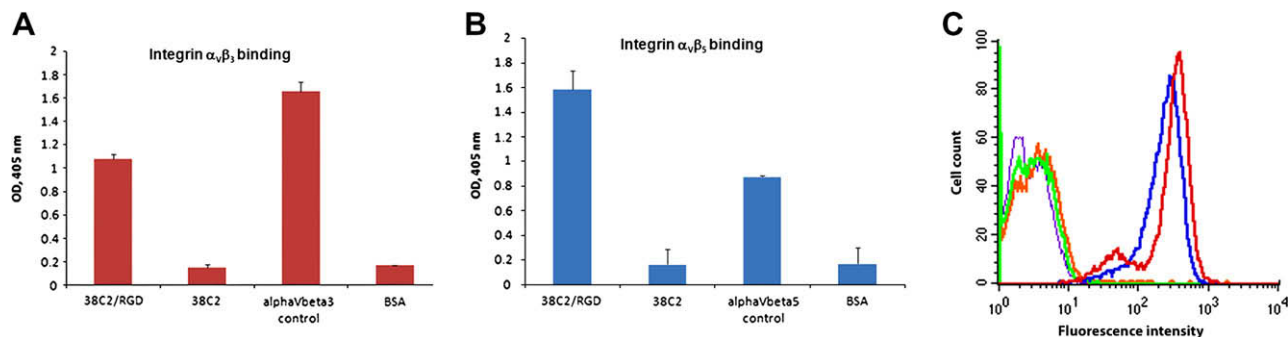


Figure 2. cp38C2 binds to integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Chemicon). (A) ELISA demonstrating preferential binding of cp38C2 (38C2/RGD) to integrin $\alpha_v\beta_3$. Controls were unprogrammed mAb 38C2, antibody specific for the integrin $\alpha_v\beta_3$ (LM609) and BSA as a negative control antigen. (B) ELISA demonstrating preferential binding of cp38C2 to $\alpha_v\beta_5$. Here the integrin $\alpha_v\beta_5$ -specific antibody P1F6 was used as a positive control (C) Analysis of cp38C2 binding to human melanoma M21 cells. Autofluorescence of M21 cells is represented as purple line; cells were stained with cp38C2 (red line), unprogrammed mAb 38C2 (green line), LM609 antibody (blue line) that acts as a positive control, and secondary antibody alone (orange line). Bound antibodies were detected with Cy5-conjugated donkey anti-mouse IgG.

The integrin targeting module **13** was synthesized in a copper (I)-catalyzed azide alkyne cycloaddition reaction of the heterobifunctional module **12** with the azido-targeting module **10** (Scheme 6). The resulting β -lactam-equipped targeting module was incubated with mAb 38C2 to provide chemically programmed (cp) antibody. Binding of the cp38C2 to human integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ was confirmed by ELISA (Fig. 2A and B).^{3,7} Importantly, the chemically programmed antibody recognized the integrins on living cells: cp38C2 bound to human melanoma M21 cells that express both integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on their surface (Fig. 2C).⁷ As expected, unprogrammed catalytic antibody 38C2 did not bind to M21 cells.

In conclusion, we have prepared β -lactam-equipped targeting modules and demonstrated that these modules can be used to prepare chemically programmed antibodies. The modular strategy and use of bioorthogonal click chemistry for the preparation of β -lactam-equipped targeting modules allowed straightforward access to these valuable immunotherapeutics. Further studies of the therapeutic effect of targeting integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors with chemically programmed mAb 38C2 are underway and will be reported in the due course.

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