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An efficient chemical approach to bispecific antibodies and antibodies of high valency

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ABSTRACT

Irreversible chemical programming of monoclonal aldolase antibody (mAb) 38C2 has been accomplished with β-lactam equipped mono- and bifunctional targeting modules, including a cyclic-RGD peptide linked to either the peptide (D-Lys^6)-LHRH or another cyclic RGD unit and a small-molecule integrin inhibitor SCS-873 conjugated to (D-Lys^6)LHRH. We also prepared monofunctional targeting modules containing either cyclic RGD or (D-Lys^6)-LHRH peptides. Binding of the chemically programmed antibodies to integrin receptors α(v)β(3) and α(v)β(5) and to the luteinizing hormone releasing hormone receptor were evaluated. The bifunctional and bivalent c-RGD/LHRH and SCS-783/LHRH, the monofunctional and tetravalent c-RGD/c-RGD, and the monofunctional bivalent c-RGD chemically programmed antibodies bound specifically to the isolated integrin receptor proteins as well as to integrins expressed on human melanoma M-21 cells. c-RGD/LHRH, SCS-783/LHRH, and LHRH chemically programmed antibodies bound specifically to the LHRH receptors expressed on human ovarian cancer cells. This approach provides an efficient, versatile, and economically viable route to high-valency therapeutic antibodies that target defined combinations of specific receptors. Additionally, this approach should be applicable to chemically programmed vaccines.

Chemically programmed antibodies (cpAbs) are a new class of therapeutic molecules that result from the marriage of medicinal chemistry and protein engineering. In this approach an antibody is programmed through chemistry to bind a target of biological interest; the programmed antibody maintains advantages characteristic of both antibodies and the targeting agent. Selective and irreversible chemical programming of the aldolase antibody 38C2 can be accomplished via amide bond formation between the low pK_a catalytic site lysine H93[1,2] and β-lactam equipped targeting modules.[3,4] Early studies focused on the programming of mAb 38C2 with integrin αvβ3 and αvβ5 targeting units.[3,5–9]

It has long been recognized that antibodies with the ability to bind to more than one target protein might have a therapeutic advantage in treating disease. Despite numerous attempts over the past two decades to develop viable approaches to synthesis of bi- and multi-specific antibodies, no such antibodies are approved drugs. Protein engineering approaches for the creation of bispecific antibodies have been challenging due to issues of protein stability and difficulties in obtaining high-level expression. Indeed, only recently have promising breakthroughs been reported.[10] Here we demonstrate a method that provides an efficient route to antibodies with multiple specificities and to antibodies of high valency.

In our model study, we combined integrin αvβ3 and αvβ5 targeting with luteinizing hormone releasing hormone (LHRH) receptor targeting to create a molecule that has the potential for a synergistic double strike against tumor and supporting vasculature. Integrins αvβ3 and αvβ5 are well-established targets for cancer therapy, as these receptors are critical for tumor angiogenesis and metastasis.[11–13] However, cancer therapy would likely be more efficacious if another tumor-associated antigen could also be targeted. In general, co-targeting should allow for more precise targeting of varied cell-types in multiple diseases. The LHRH receptor is a promising target since it is not expressed in most normal tissues whereas binding sites for LHRH are found cells isolated from 52% of human breast cancers, about 80% of human ovarian and endometrial cancers, and 86% of human prostate carcinoma specimens.[14–17] Targeting of cancer cells through two different receptor pathways should make the development of resistance less likely since down regulation of more than one receptor would be required. Further, we expect that the antibody therapeutic should be more potent as the antibody load onto the targeted cells will be increased thereby increasing the effective coupling of bound antibody to the immune system via complement- and cell-dependent mechanisms.

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The linkers used to connect integrin αvβ3 and αvβ5 targeting and LHRH receptor targeting units are unlikely, due to steric reasons, to enable each arm to simultaneously bind more than one receptor. The antibody complexes are expected to bind LHRH receptor and integrin, two LHRH receptors, or two integrins (Fig. 1). In order to dissect the relative contribution of each component of our multifunctional chemically programmed (cp) Ab 38C2, each constituent was independently synthesized as a β-lactam containing targeting unit and directly coupled to antibody 38C2.

Monofunctional integrin targeting unit **4** was synthesized by conjugation of NHS-PEG-azide linker **1** with the ε-amino group of the lysine moiety in the cyclic-RGD peptide[18,19] (cyclo(Arg-Gly-Asp-D-Phe-Lys)), commercially available from Peptides International, Inc., to give corresponding azide **2** (Scheme 1). Copper-catalyzed azide–alkyne cycloaddition[20,21] of **2** with heterobifunctional β-lactam linker **3** provided the desired targeting module **4** in excellent yield. Similarly, the ε-amino group of D-Lys^6 in (D-Lys^6) LHRH[14,17] (commercially available from Bachem) was reacted with **1** followed by click reaction with **3** to provide the monofunctional LHRH-targeting module **6**.

We evaluated two different integrin targeting modules, the cyclic-RGD peptide and the peptidomimetic SCS-873. These molecules were combined with the LHRH targeting unit in the bifunctional β-lactam equipped molecule. Targeting module **9** containing two

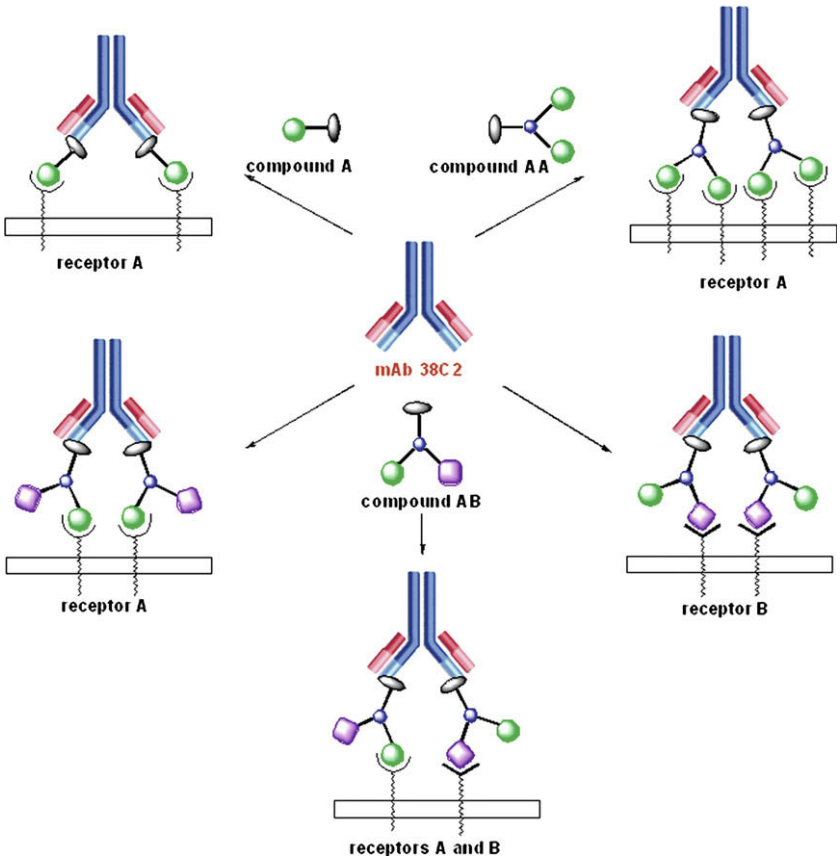
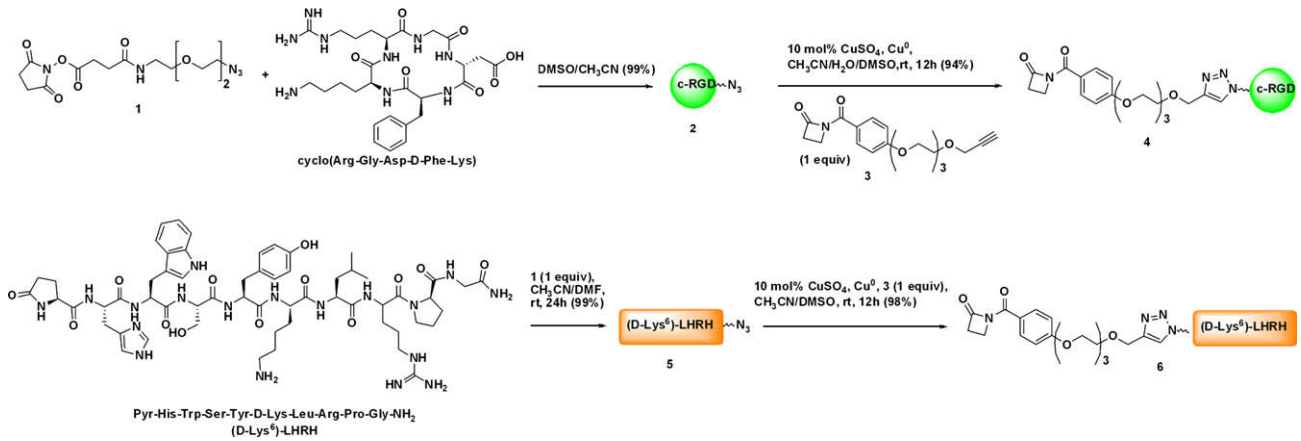
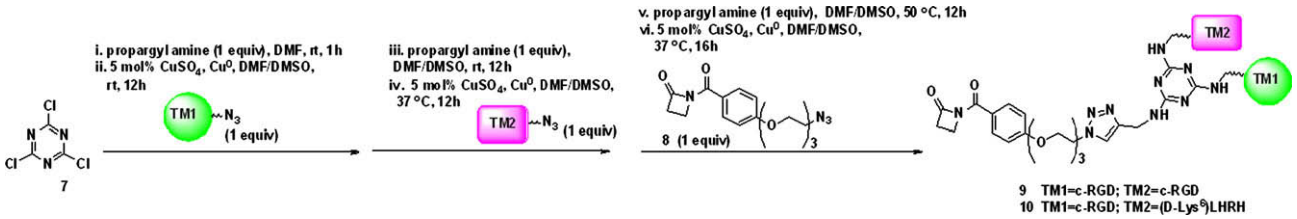


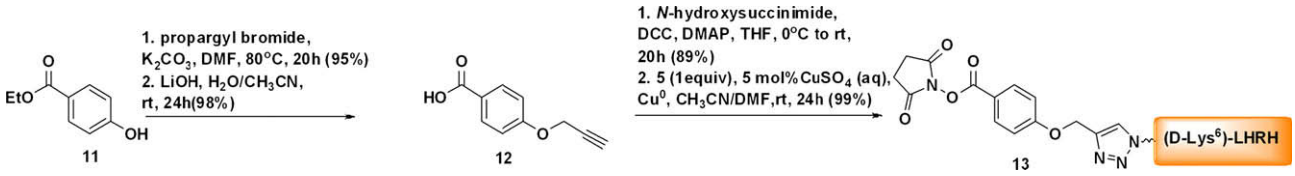
Figure 1. Schematic representation of possible binding modes for a bispecific cpAb and a tetravalent cpAb.



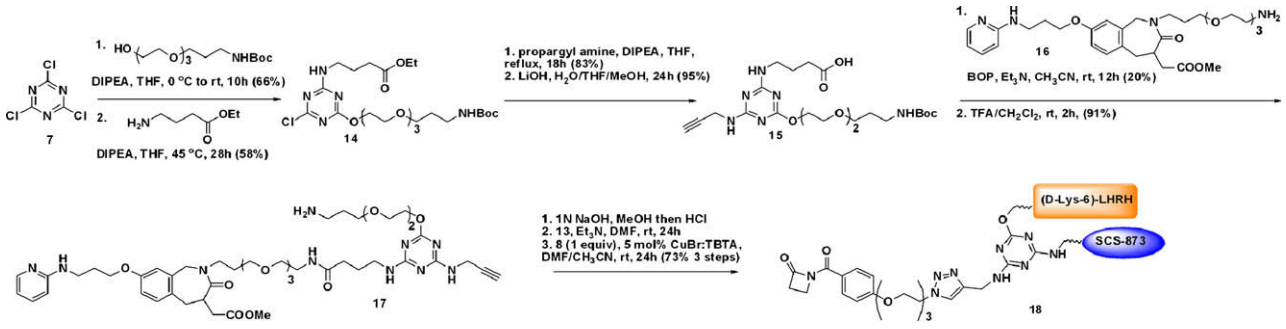
Scheme 1. Synthesis of the targeting modules **4** and **6**.



Scheme 2. Synthesis of the bi/trifunctional targeting modules **9** and **10**.



Scheme 3. Synthesis of building block **13**.



Scheme 4. Synthesis of the targeting module **18**.

cyclic-RGD peptide units was also prepared. Bi- and trifunctional targeting modules **9, 10**, and **18** were prepared on a milligram scale. Cyanuric chloride **7** was used as a platform for sequential functionalization following established procedures.[22–24] Cyanuric chloride was sequentially reacted with propargyl amine in the presence of diisopropyl ethyl amine, followed by click reaction with corresponding azido functionalized peptide as shown in Scheme 2. β-lactam linker **3** was introduced using a click reaction followed by removal of copper with copper scavenging resin and cold ether trituration of the products **9** or **10.**

In order to prepare bi/trifunctional targeting module **18**, the NHS-activated ester **13** of (D-Lys^6)LHRH was prepared (Scheme 3). Ethyl 4-hydroxybenzoate **11** was reacted with propargyl bromide in the presence of potassium carbonate in DMF to afford the alkynyl ester **12** in 95% isolated yield. The ester was hydrolyzed using lithium hydroxide followed by coupling with *N*-hydroxysuccinimide in the presence of DCC and catalytic DMAP. Click reaction of corresponding NHS-alkyne with azido-functionalized LHRH module **5** provided desired activated ester **13**.

Cyanuric chloride **7** was monosubstituted with *tert*-butyl 1-hydroxy-3,6,9,12-tetraoxapentadecan-15-ylcarbamate in the presence of Hunig's base (Scheme 4), followed by subsequent substitution with ethyl 4-aminobutanoate to provide product **13** in 58% isolated yield. Substitution of the last chlorine with propargyl amine in the presence of Hunig's base under reflux in THF was followed by ester hydrolysis with lithium hydroxide to provide intermediate **14**. Free acid was coupled to the SCS-873 methyl ester **16**[8] in the presence of BOP chloride and triethylamine in 20% isolated yield. Subsequent treatment with trifluoroacetic acid in dichloromethane deprotected the amine linker arm to furnish intermediate **17** in 91% isolated yield. Compound **16** was treated with 1 N NaOH to remove the methyl ester from the SCS-873 module followed by coupling of the free amine to activated NHS-ester of LHRH module **13**. Click coupling of the alkynyl subunit contain-

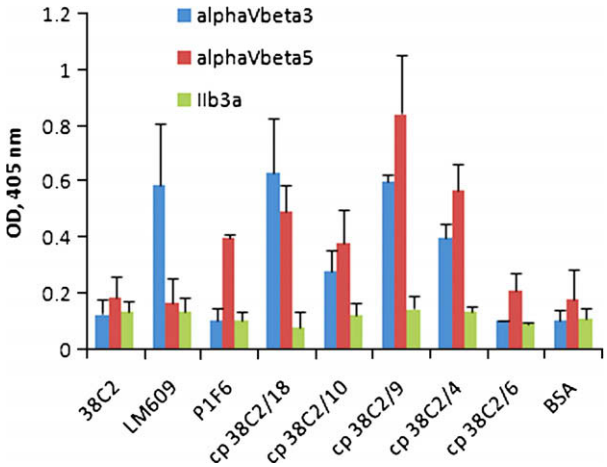


Figure 2. Results of the integrin binding ELISA assay. Unprogrammed mAb 38C2 was used as a negative control, αvβ3 specific antibody LM609 and αvβ5 specific antibody P1F6 (Chemicon) were used as positive controls.

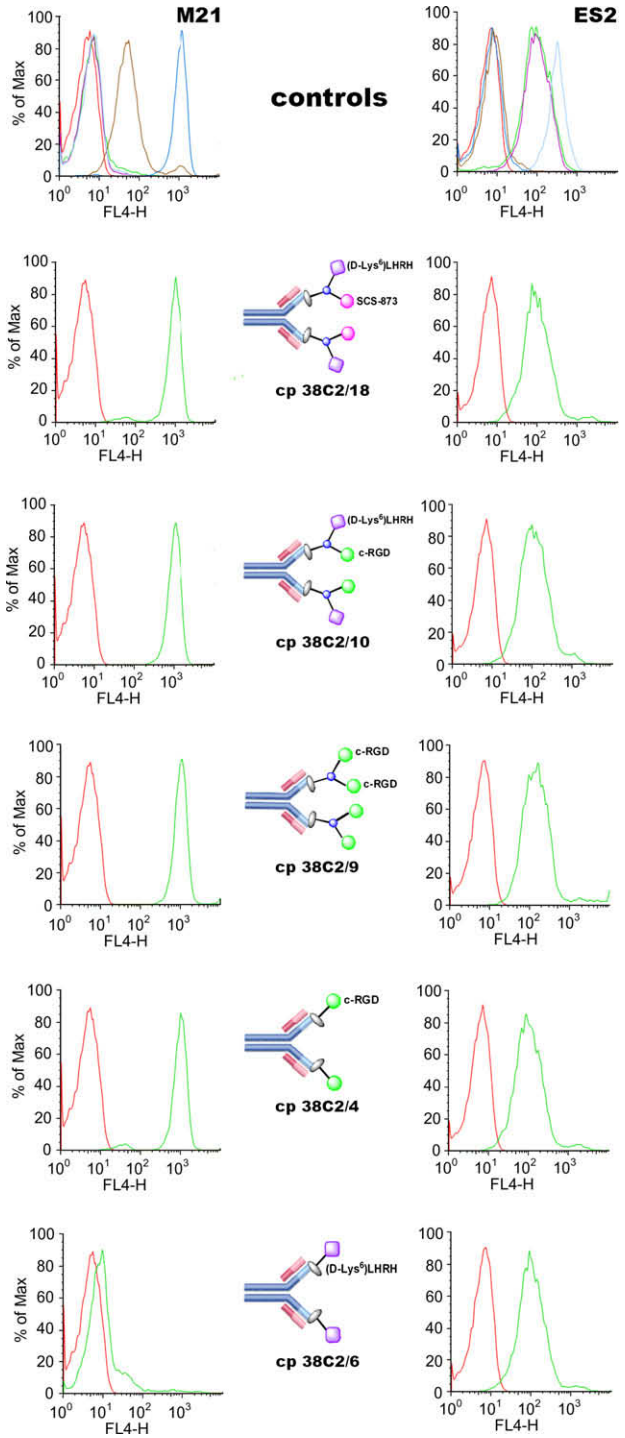


Figure 3. Results of the flow cytometry analysis. Controls for M-21 cell line: cells only (red line); anti-LHRH receptor antibody (Abcam) (green); Cy-5 conjugated secondary anti-mouse antibody (purple); Cy-5 conjugated secondary anti-rabbit antibody (light blue); P1F6 (brown); LM609 (blue). Controls for ES2 cell line: cells only (red line); Cy-5 conjugated secondary anti-mouse antibody (brown); Cy-5 conjugated secondary anti-rabbit antibody (blue); anti-LHRH receptor antibody (9652615, Abcam) (green); P1F6 (purple); LM609 (light blue). Antibody binding experiments: 38C2 binding (red line), cp Ab bindings (green line).

ing SCS-873 and LHRH modules to the β-lactam azide linker **3** completed the synthesis of bi/trifunctional targeting module **18**. Copper has been removed using copper scavenging resin and product **18** has been isolated by cold ether triturating in 71% overall yield for last three steps. All the targeting modules were characterized by HPLC and MALDI-TOF confirming desired molecular weights and good to excellent purities.

Mono- and polyfunctional targeting modules **4**, **6**, **9**, **10**, and **18** were used for the chemical programming of mAb 38C2 as previously described.[2] mAb 38C2 was incubated with 2 equiv of the targeting unit at room temperature for 2–3 h. Complete loss of catalytic activity in the methodol-based catalytic assay[9] indicated Lys H93 labeling. Each of the mono- and bi/trifunctional chemically programmed antibodies was characterized by SEC/MS to confirm incorporation of two targeting units per antibody (see Supplementary data).

The mono- and bi-functional antibodies were tested for integrin binding by ELISA. Chemically programmed antibody constructs 38C2/4, 38C2/9, 38C2/10, and 38C2/18 bound to integrin receptors αvβ3 and αvβ5 but not to IIb3a (Fig. 2), whereas the (D-Lys^6)LHRH-programmed 38C2 did not bind to the integrin receptors. Tetravalent cRGD/cRGD cpAb demonstrated enhanced binding to integrin as compared with bivalent cRGD cpAb (data not shown). Binding to M-21 cells, which express integrin receptor but not LHRH receptor, was evaluated by flow cytometry analysis.[7] Only chemically programmed antibodies containing SCS-873 or cyclic-RGD peptide bound to the M-21 cell surface (Fig. 3). All of the chemically programmed antibodies bound to ES-2 cells, which express both types of receptors (Fig. 3).

In conclusion, we have developed a versatile linker strategy for mono- and bi-functional chemical programming of the aldolase antibody 38C2. This chemistry should also be applicable to cytotoxin delivery. By selection of the targeting unit, this approach allows for the creation of antibodies that simultaneously target several different receptors, such as integrin receptors αvβ3 and αvβ5 and LHRH receptor demonstrated here. This method provides a convenient approach to synthesis of multifunctional antibodies and antibodies of high valency and should be directly applicable to chemically programmed vaccines.[25] We anticipate that the ability to target defined collections of antigens with our approach will allow for more precise targeting of varied cell-types in multiple diseases wherein the net avidity obtained by engaging the various targeting arms drives selectivity to cells expressing the targeted antigen constellation. This level of discrimination is not possible through the simple administration of mixtures of mono-targeting antibodies. Finally, as we develop new types of drugs, economic realities must also be considered. Monoclonal antibody therapy can cost over $100,000.00 per year. Treatment with defined combinations of monoclonal antibodies are expected to show therapeutic advantages, however, the cost of such therapies will preclude their consideration as viable treatments for most individuals. We anticipate that our approach will lead to superior drugs with substantially reduced costs as compared to combinations of monoclonal antibodies, thereby making them an economically viable drug class. Further biological evaluation of the mono- and bi/tri-specific chemically programmed antibodies and vaccines is underway and will be reported in the due course.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.05.047.

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