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Multiple catalytic aldolase antibodies suitable for chemical programming

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ABSTRACT

Chemical programming of nine murine antibodies with catalytic aldolase activity was examined using compounds, equipped with diketone or pro-vinyl ketone linkers that inhibit integrin adhesion receptor functions. The results showed that most Abs were programmed using the diketone compounds in a manner similar to previously reported catalytic antibody 38C2. On the other hand, only those antibodies, which catalyzed the retro aldol reaction of the pro-vinyl ketone linkers efficiently, were programmed. Conjugated to integrin targeting compounds, at least three new antibodies, including 84G3, 85H6, and 90G8, exhibited high specific binding to human tumor cells expressing integrin $\alpha_v\beta_3$.

Chemical programming of the catalytic monoclonal aldolase antibody (Ab) 38C2[1] has emerged as a powerful strategy that equips the Ab with novel functions and redirects to desired targets by modifying the Ab binding sites using a targeting agent (TA).[2] In turn, the Ab provides the TA with an enhanced serum half-life and affinity for their targets. Key to the chemical programming of Ab 38C2 is the presence of the reactive lysine residues in the binding sites,[3] which react covalently and selectively with a compound having a diketone or vinyl ketone function (Fig. 1A, B). In the resulting Ab constructs, the compounds are conjugated to Ab through an enaminone or the β-amino alcohol function.[4] Because vinyl ketone is less stable and highly reactive, one can use its acetone aldol instead. The reactive vinyl ketone group is regenerated on treatment with 38C2, before reacting with the Ab. In this manner, we prepared and evaluated a series of chemically programmed Ab 38C2 (i.e., cp38C2 or 38C2-**x**) constructs targeting $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins.[2,4,5] The representative examples are 38C2-**1**, 38C2-**2** and 38C2-**3**, prepared from compounds **1**–**3** (Fig. 1C). These constructs inhibited the growth of primary and metastatic tumors, in vivo, including human Kaposi's Sarcoma (SLK), melanoma (M21), and breast cancer (MDA-MB-231), more effectively than the compound alone.[2,4,6] In a similar manner, numerous cp38C2s were constructed using a single Ab 38C2 and multiple TAs, including the endothelin A antagonists.[7] Indeed, at least three Ab conjugates, CVX-045, CVX-060, and CVX-096, based on a single humanized 38C2,[8] have entered clinical trials (http://www.covx.com). These studies inspired us to develop new Ab platform and identify a series of 'generic aldolase Abs' for the chemical programming. The desired Abs will have properties that commensurate with the need. First, we decided to examine an inventory of nine additional aldolase Abs that were available at Scripps.[1,9] Here, we report the preliminary results of our studies, including chemical programming of all nine Abs using TAs **1**, **2**, or **3**, analysis of the resulting cpAbs: Ab-**1**, Ab-**2** and Ab-**3**, and binding of the cpAbs to cells expressing (or deficient in) integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$.

All nine aldolase Abs, 33F12, 84G3, 84G11, 85A2, 85C7, 85H6, 90G8, 92F9 and 93F3, were obtained from the immunizations against different β diketone haptens like Ab 38C2. 'These Abs also possessed the reactive lysine residues in their binding sites' was previously determined by reacting them with 2,4-pentanedione and determining the enaminone formation (λ_{max} = 318 nm) in the UV spectra. Several Abs were also investigated and were shown to catalyze the aldol and retro aldol reactions of a wide variety of substrates.[1,9] Therefore, we examined chemical programming of all nine aldolase Abs using TAs **1**, **2**, and/or **3**. Typically, a solution of an Ab and the TA (100 μl, 20 μM in Ab and 50–60 μM in TA) was incubated for 1–2 h at room temperature for compounds **1** or **3**, and 2–16 h at 37 °C for **2**. Chemical programming of the Abs was ascertained using both the UV analyses of the Ab and its conjugates and determination of catalytic activities of the resulting cpAbs vs the untreated Abs after some dilution. In UV spectra, all Ab conjugates prepared from compound **1** showed a distinct new peak at λ_{max} 318 nm, confirming the formation of the enaminone

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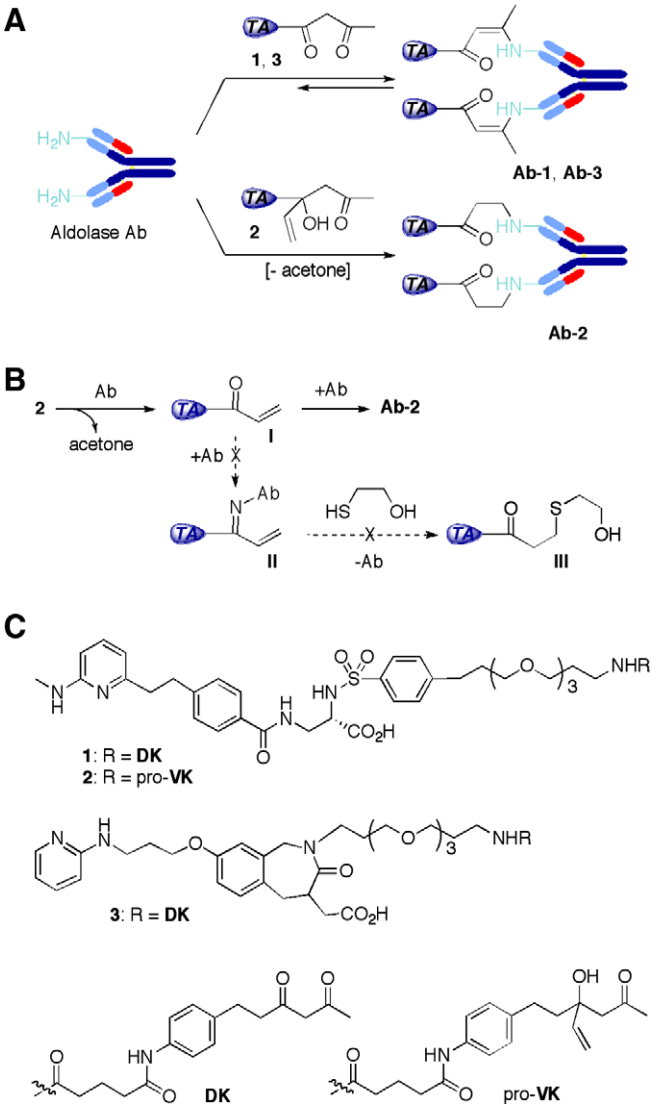

Figure 1. Construction of the anti- $\alpha_v\beta_3$ integrin cp38C2s (or cpAbs) by the conjugation of compounds **1**, **2** or **3** to Ab 38C2 (or other aldolase Abs), and (B) formation of the vinyl ketone derivative **I** by the aldolase Ab catalyzed retro aldol reaction of the pro-vinyl ketone **2** and an alternative mode of the conjugate formation, and (C) Structure of the integrin $\alpha_v\beta_3$ inhibitors, **1**–**3**, with a diketone or the pro-vinyl ketone linker.

function.[1] Whereas, some Ab solutions prepared with compound **2** showed a fairly weak and flat hump at 300-340 nm, and Ab alone did not discern any peak in this region (see: Fig. 2 for UV spectra of the representative samples).

Alternatively, Ab-catalyzed retro-aldol reaction of methodol was used as an indirect method for determining the construct formation between the Abs and compounds **1**, **2**, or **3**.[10] First, relative rates of the retro aldol reaction of methodol (200 μM) using the catalytic amounts (1 μM) of all aldolase Abs to produce 6-methoxy-2-naphthaldehyde were determined using the fluorescence meter. As shown in Figure 3, all Abs, except 85C7 and to a lesser extent 33F12, were catalytic. In contrast the conjugates formed from these Abs and compound **1** or **3** were inactive and did not catalyze the activation of methodol. Several reaction mixtures using Abs and compound **2** were also noncatalytic. Based on the combined UV and activity data, we concluded that most Abs were programmed using compounds **1** and **3**, and several Abs were also programmed using compound **2**. Abs that did not catalyze or weakly catalyzed the activation of **2** to produce the vinyl ketone

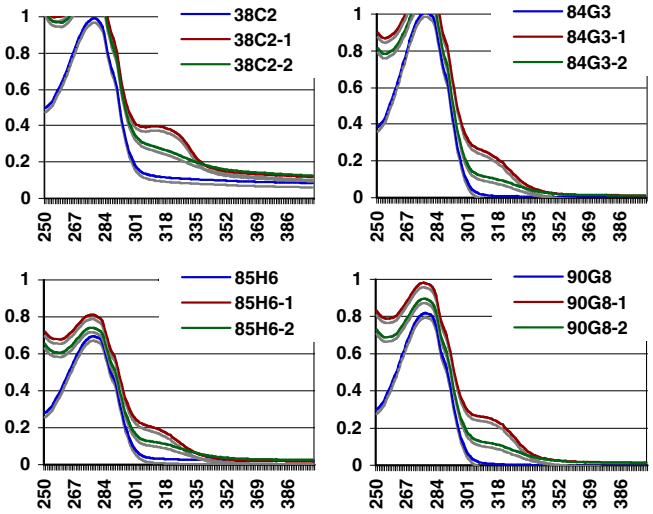

Figure 2. UV spectra of Abs 38C2, 84G3, 85H6, 90G8 and the cpAbs (conjugates of the Abs with compounds **1** and **2**). Abs and cpAbs were diluted to 3–5 μM before running the UV spectra.

I were unlikely to be programmed efficiently, and were therefore excluded from further investigation.

In order to confirm that 38C2-**2** was indeed formed by a Michael-type addition of the lysine residue to the vinyl ketone (**I**) and not through the dienamine (**II**) formation, we examined the conjugate formation in the presence and absence of mercaptoethanol. We anticipated that vinyl ketone **I** would react with mercaptoethanol to give thio-Michael adduct **III**, unless the former reacted with the Ab immediately to give 38C2-**2**, most likely before the substrate dissociated from the binding pocket (Fig. 1C). Indeed, we found that the conjugate formation between Ab 38C2 and compound **2** was not affected by the addition of mercaptoethanol, whether it was added at the start of the conjugate formation experiment or after the conjugate was formed, nor did mass spectral analysis show the formation of any Michael adduct **III** in the reaction mixture. These facts were in favor of the formation of 38C2-**2** and not the alternative conjugate **II**, as the latter should react with mercaptoethanol to give **III** and free Ab. Evidently, conjugates formed from compound **2** and other aldolase Abs, which catalyzed the activation of **2** to **I**, were also the Michael-type adducts, that is, Ab-**2**.

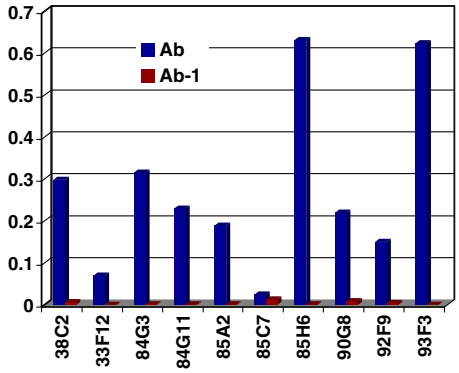

Figure 3. Relative rate of retro aldol reaction of methodol in the presence of aldolase antibodies and the corresponding cpAbs. Methodol (10 mM in EtOH, 2 μl) was added to a solution of Abs or cpAbs (1 μM, 98 μl) in PBS buffer, and the progress of aldol reaction was determined by reading the fluorescence (λ_{max} of excitation, nm; λ_{max} of emission, nm) of the produced 6-methoxy-2-naphthaldehyde using a fluorescence reader. *Y*-axis represents the relative rate of the production of the aldehyde, and *X*-axis shows the Ab and the corresponding *cpAb*.

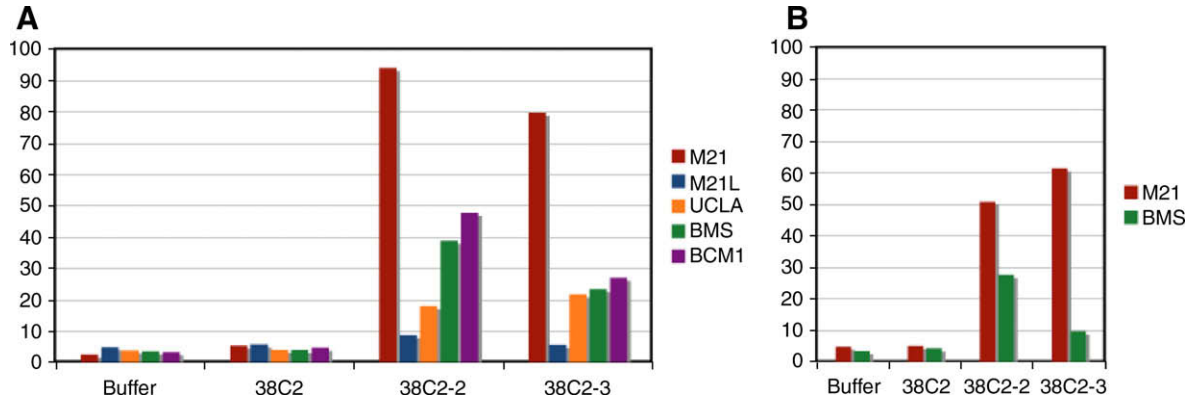

Figure 4. Flow cytometry analysis data of binding of the *cp38C2s*, viz. 38C2-**2** and 38C2-**3** to cells of human M21 melanoma, BMS and BCM1 breast cancer, or UCLA-P3 lung carcinoma cells in the presence of (A) $MnCl_2$ or (B) $CaCl_2$. M21 expresses high levels and BMS and BCM1 intermediate levels of integrin $\alpha_v\beta_3$. M21-L lacks $\alpha_v\beta_3$ expression, and UCLA-P3 expresses high levels of integrin $\alpha_v\beta_5$ but no $\alpha_v\beta_3$. Ab 38C2, and buffer alone were used as the negative controls. Cells were separately incubated with 38C2, 38C2-**2** or 38C2-**3** (20 μg/ml) in TBS buffer (with/without 0.1 mM $MnCl_2$ or 2 mM $CaCl_2$) for 45 min on ice, washed and then incubated with the FITC-labeled anti mouse goat Ab before analyzing by flow cytometry. *Y*-axis shows the mean fluorescence intensities.

To analyze the cell surface binding of the cpAbs, we first used 38C2-**2** and 38C2-**3**, and confirmed their binding to integrin $\alpha_v\beta_3$ using a series of human tumor cells, including M21, M21-L (melanoma), UCLA-P3 (lung carcinoma), BMS and BCM1 (breast cancer). M21 and M21L melanoma cells share a genetic background but differ in their α_v integrin expression, which is high in M21 and missing in M21-L.[11] M21 expresses high levels of $\alpha_v\beta_3$ and low levels of $\alpha_v\beta_5$. BMS and BCM1 are also variant cells related to each other, and both express integrin $\alpha_v\beta_3$ and some $\alpha_v\beta_5$.[12] UCLA-P3 cells express $\alpha_v\beta_5$ but no $\alpha_v\beta_3$.[13] Thus, these cell models served to determine the binding specificity of the antibody conjugates and if the integrin target was recognized in a cation dependent and integrin activation dependent manner. We analyzed binding of 38C2-**2** and 38C2-**3** to the cells in our diagnostic panel in the presence of Ca^{2+} or Mn^{2+}. Requirement of Ca^{2+} indicates cation dependence and modulation of binding in Mn^{2+} indicated impact of integrin activation.[14] The results are shown in Figure 4.

Evidently, in the presence of $MnCl_2$, the Ab conjugates 38C2-**2** and 38C2-**3** bound strongly to M21 cells that express high levels of $\alpha_v\beta_3$ integrin reinforcing our previous observations,[2,4,6] but not to M21L cells deficient in this and other α_v integrins (Fig. 4A). The conjugate also bound to cells BMS and BCM1, which express integrin $\alpha_v\beta_3$ at a lower level than M21, and to a significantly lesser extent to UCLA cells, which carry high levels of $\alpha_v\beta_5$ but no $\alpha_v\beta_3$.

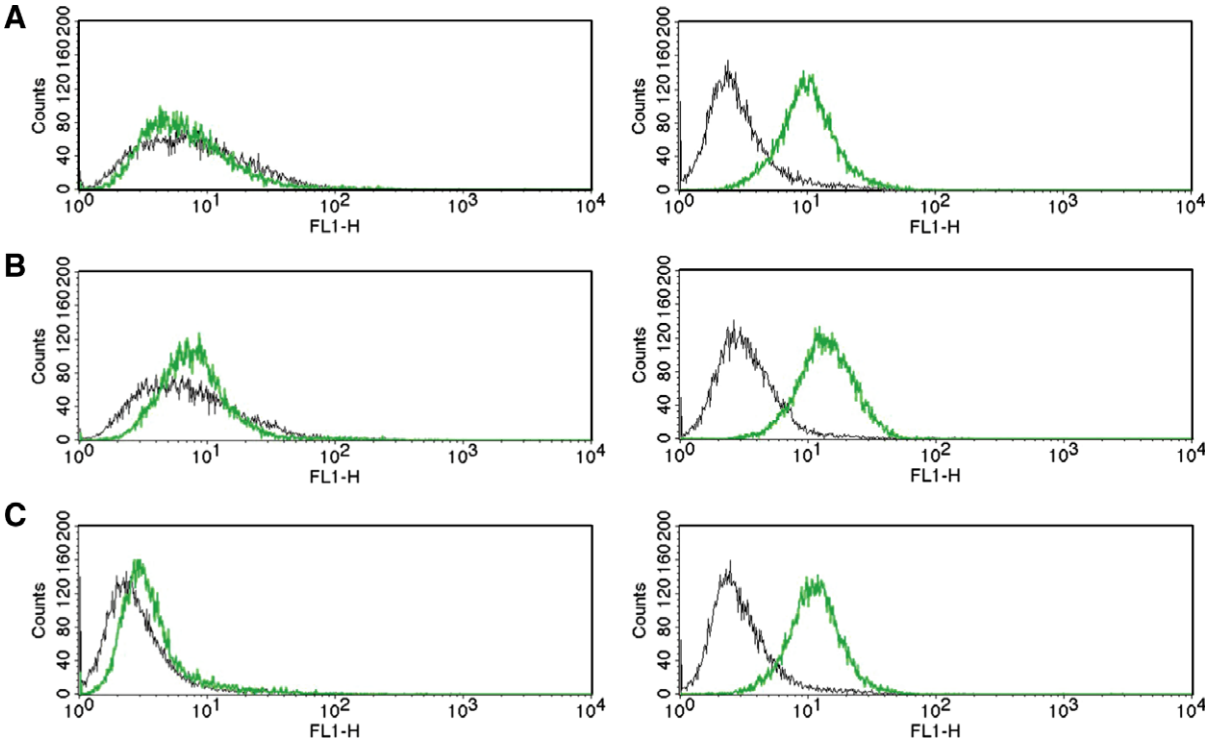

Figure 5. Flow cytometry analysis of binding of the cpAbs (A) 84G3-**1**, (B) 85H6-**1**, and (C) 90G8-**2** to $\alpha_v\beta_3$-positive M21 cells (green line) or α_v integrin-negative M21-L cells (black line). The cells were incubated with the individual Ab and cpAb, and then incubated with the FITC-labeled anti mouse goat Ab before analyzing by flow cytometry. Shown in Left Panel are the histograms using Ab alone, and in the right panel using the cpAbs. The *Y*-axis gives the number of events in linear scale, the *X*-axis the fluorescence intensity in logarithmic scale.

Thus, the Ab conjugates 38C2-**2** and 38C2-**3** clearly target integrin $\alpha_v\beta_3$, and shows some reactivity with the related integrin $\alpha_v\beta_5$. Even though the integrins expressed by the tumor cells were fully activated by exogenous addition of Mn^{2+}, the unconjugated Ab 38C2 showed no binding activity, clearly demonstrating the binding specificity of the Ab conjugate. To assess the ability of the Ab conjugates to recognize the integrin at its endogenous activity level, binding of 38C2-**2** and 38C2-**3** to $\alpha_v\beta_3$ positive M21 and BMS cells was analyzed in the presence of Ca^{2+}, which does not activate integrins but supports cation dependent integrin ligand binding. Figure 4B shows that the Ab conjugate clearly and specifically recognized $\alpha_v\beta_3$ on the tumor cells without a requirement of exogenous activation, thus indicating a high binding affinity to the integrin which is expressed by M21 and BMS tumor cells in an intrinsically activated functional state. 'The cp38C2s bound only to the activated integrins and in the cation dependent manner' was further confirmed by repeating the binding experiments in the absence of metal ions, which did not show binding to any cells, including M21, BMS, BCM1, and UCLA-P3 (data not shown).

Subsequently, the binding properties of the new cpAbs were determined on M21 and M21-L cells using the same flow cytometry approach. The experiments were conducted using one set of cpAbs, including 33F12-**1**, 84G3-**1**, 84G11-**2**, 85A2-**2**, 85C7-**1**, 85H6-**1**, 90G8-**2**, 92F9-**1** and 93F3-**1**, which were prepared from the respective antibodies and either compound **1** or **2**. Only those cpAbs from compound **2** were used, which were fully conjugated as observed by determining their catalytic activity. All cpAbs showed positive binding to M21 cells and were negative on M21-L cells, except 92F9-**1** which did not bind to either cell line. Shifts in the flow cytometry histogram with the positive binders were noted in the following order: 84G3-**1** ~ 85H6-**1** ~ 90G8-**2** > 85A2-**2** > 33F12-**1** ~ 84G11-**2** > 85C7-**1** ~ 93F3-**1**. At least three cpAbs, including 84G3-**1**, 85H6-**1**, and 90G8-**2** showed the largest shifts in the flow cytometry histogram indicating the best binders of cells expressing integrin $\alpha_v\beta_3$ (Fig. 5).

There are several important points that could not be addressed in this Letter, and would be worth finding out in the future. For example, all aldolase Abs could react with other reactive functional groups, including β-lactam and γ-lactone, like Ab 38C2.[15] Second, Ab 93F3 possessed two lysine residues in each binding sites, and we did not confirm whether both lysine residues could take part in conjugation. Also, the conjugate prepared from 93F3 and the TAs with diketone function did not show binding comparable to other Abs. We wonder whether cp93F3 undergoes a rapid disassembly to release TA during the experiments. Third, we have constructed numerous cp38C2s with high affinities for cells expressing the $\alpha_v\beta_3$ integrin, and determined their inhibitory effects on tumor growth and metastasis in a number of cancer models.[6,16] One can anticipate different levels of Ab-mediated immune response from one Ab to another, and similarly of one cpAb to other. Therefore, a comparative study of one of the efficient cpAb, such as cp84G3, to cp38C2 should provide useful information.

In conclusion, we confirmed that, like Ab 38C2, most aldolase Abs were programmed using a TA equipped with a diketone linker, such as **1**. Highly active Abs, 84G3, 85H6, and 90G8, were also programmed using the targeting agent equipped with the pro-vinyl ketone function, such as **2**. Many conjugates showed high binding to cells expressing integrin $\alpha_v\beta_3$, thus preserving the binding specificity of the conjugated compound, and underlining their importance as the therapeutic Abs. Further studies with the selected Abs are in progress, and will be reported in due course.

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