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Synthesis of the next-generation therapeutic antibodies that combine cell targeting and antibody-catalyzed prodrug activation

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An obstacle in the utilization of catalytic Abs for selective prodrug activation in cancer therapy has been systemic tumor targeting. Here we report the generation of catalytic Abs that effectively target tumor cells with undiminished prodrug activation capability. Ab conjugates were prepared by covalent conjugation of an integrin αβ3-targeting antibody to catalytic Ab 38C2 through either sulfide groups of cysteine residues generated by reduction of the disulfide bridges in the hinge region or surface lysine residues not involved in the catalytic activity. Using flow cytometry, the Ab conjugates were shown to bind efficiently to integrin αβ3-expressing human breast cancer cells. The Ab conjugates also retained the retro-aldol activity of their parental catalytic Ab 38C2, as measured by methodol and doxorubicin (dox) prodrug activation. Complementing these Ab conjugates, an evolved set of dox prodrugs was designed and synthesized. Dox prodrugs that showed higher stability and lower toxicity were evaluated both in the presence and absence of the integrin αβ3-targeting 38C2 conjugates for cell-killing efficacy by using human breast cancer cells. Our study reveals that cell targeting and prodrug activation capabilities can be efficiently combined for selective chemotherapy with novel dox prodrugs.

Monoclonal Abs with aldolase activity have emerged as highly efficient catalysts for a number of chemical transformations, particularly aldol and retro-aldol reactions (1). The excellent retro-aldol activity (2, 3) of Abs 38C2 (4) and 93F3 (5) has allowed us to design, synthesize, and evaluate prodrugs of various chemotherapeutic agents that can be activated by retro-aldol reactions (6–10). In a syngeneic mouse model of neuroblastoma, systemic administration of an etoposide prodrug and intratumor injection of Ab 38C2 inhibited tumor growth (11). However, to evaluate the utility of these Abs for selective chemotherapy in a systemic setting, it will be necessary to go one step further and equip the Ab with a tumor-recognition device to target the catalytic Ab to the malignant cells. In previous studies, known as Ab-directed enzyme prodrug therapy or the Ab-directed abzyme prodrug therapy approach (12, 13), enzymes or catalytic Abs were directed to tumor cells by chemical conjugation or recombinant fusion to targeting Abs. As a potentially more efficient and chemically defined alternative, we have used a strategy in which the catalytic Ab is conjugated to a targeting device located outside the combining site, thereby leaving the active site available for the prodrug activation. Here we report chemical constructions in which Ab 38C2 is conjugated to a synthetic small-molecule-targeting device that mediates binding of the Ab to integrin αβ3, a tumor and tumor vasculature cell-surface receptor (14). The conjugate of Ab 38C2 to an integrin αβ3-binding synthetic small molecule is expected to selectively localize the Ab to the tumor and/or the tumor vasculature and trigger prodrug activation at that site. Complementing these efforts, we also describe the design, synthesis, and evaluation of a set of doxorubicin (dox) prodrugs that, when tested with an integrin αβ3-expressing human breast cancer cell line, are less toxic than previously reported prodooxorubincins (prodoxs) and are activated more efficiently by Ab 38C2. Collectively, our studies open the way for the construction of a new class of therapeutic Abs that contain both targeting and drug activating moieties.

Results and Discussion

αβ3 Integrin-Targeting Catalytic Ab Conjugates. Earlier, we prepared numerous Ab 38C2 arginine-glycine-aspartic acid peptidomimetics, including compound 1, en route to the corresponding diketone derivatives that were used for the construction of noncatalytic 38C2 constructs (15–18). The central idea in these constructs was to conjugate a linker to a targeting device such that the linker was covalently attached to the Ab-combining site, and the targeting functionality extended beyond the surface of the Ab. In these conjugates, the diketone or the vinylketone compounds reacted in the Ab-binding sites through the reactive lysine residues to form an enamino or Michael-type adduct, respectively, thereby displaying the conjugated peptidomimetics. These Abs bound to cells that expressed αβ3 integrin, including human breast cancer cells MDA-MB-231, human Kaposi’s sarcoma, and human melanoma. Thus, this is a convenient way to covalently attach organic ligands to Abs, but in the process, the catalytic activity of the Ab is destroyed. Although such constructs may be very useful in that they endow organic compounds with the relatively long half-lives and/or effector functions of Abs, they do not take advantage of the Ab’s catalytic potential. We anticipated that a conjugate of 1 outside the active site of 38C2 would also bind integrin αβ3-expressing cells and possibly retain the catalytic activity of the Ab. Therefore, starting from amine 1, compounds 2 and 3 were prepared and conjugated to 38C2, affording 38C2-2 and 38C2-3 conjugates (Fig. 1).

In 38C2-2, compound 2 was conjugated through the reduced sulfide bonds in the Ab hinge region, whereas the activated ester 3 reacted to surface lysine residues of the Ab in 38C2-3.§ The formation of 38C2-2 and 38C2-3 conjugates was confirmed by
mass spectral analysis as well as determining their binding to MDA-MB-231 cells. As shown in Fig. 2, the mass spectra (MALDI-MS) of 38C2-2 and 38C2-3 recorded an increase of 1,761 and 1,049 mass units, respectively, suggesting that on average 2.0 and 1.5 molecules of 2 and 3 were attached to 38C2 in 38C2-2 and 38C2-3 conjugates. Next, the binding of conjugates 38C2-2 and 38C2-3 to MDA-MB-231 cells was determined as described (17). For comparison, we used the previously reported 38C2 conjugate (17) (prepared from 38C2 and the diketone derivative of 1) and 38C2 alone as the positive and negative controls, respectively. As shown in Fig. 2B, the 38C2-2 and 38C2-3 conjugates and the positive control showed efficient binding to MDA-MB-231 cells. In contrast, 38C2 alone did not show any binding to these cells.

Once binding of the 38C2 conjugates to the cells was verified, it was necessary to determine that the conjugation chemistry did not destroy the catalytic activity of the Abs. To examine the catalytic activity of conjugates 38C2-2 and 38C2-3, we used methodol 4 as a substrate, because it was known to undergo 38C2-catalyzed retro-aldol reaction to produce the fluorescent aldehyde 5. Untreated 38C2 and PBS were used in the control experiments (20), and the progress of reaction was monitored by using a fluorescence reader (Fig. 3). As shown in Fig. 3, both 38C2-2 and 38C2-3 constructs retained ~50% catalytic activity with respect to the untreated 38C2.

**Synthesis of Prodox Partners for the Ab Catalysts.** The next task was to develop prodrugs of dox that could be selectively activated by Ab 38C2. Earlier, several prodrugs of dox, 6, including prodoxs 7-8 (Fig. 4d), were prepared and evaluated (6, 7, 21). On treatment with a catalytic amount of 38C2, all prodoxs were activated. Prodox 8 was activated faster than 7, probably because of the longer linker. However, the background activation also increased with 8 because of the uncatalyzed hydrolysis of the aromatic carbamates. Obviously, a longer but stable linker was needed. Therefore, use of 4-aminobenzyl alcohol derived linkers as in prodoxs 9-12, instead of the 4-hydroxyethyl-2-methoxyphenoxy-carbonyl-N’N’-dimethylthelyenediamine of 8, was examined (Fig. 4B). Conceivably, this functionality should reduce the background reaction of the aromatic carbamate but not compromise the rate of prodrug activation. Thus, the 38C2-catalyzed retro-aldol reaction of 9-12 and β elimination of the resultant intermediates, followed by decarboxylation, would afford intermediate I. Release of dox and 4-aminobenzylalcohol from intermediate I was known to be facile (22).

Alternatively, a new kind of aromatic-aldol linker could be conceived that would be obtained from 4-hydroxycacetophenone and connected to dox 6 through a carbamate functionality as in prodoxs 13-14 (Fig. 4C). Such an aldol linker would undergo the Ab 38C2-catalyzed activation at a much faster rate in comparison to an analogous aliphatic aldol linker, affording the electronically deficient ketone intermediate II. In fact, aldol compounds prepared from an aromatic ketone, such as 6-methoxy-2-methylcarbonyl naphthaleine or 4-methoxycacetophenone, were among those substrates that were activated by 38C2 at >1 per min (2). The enhanced reactivity of the carbamate function in II would accelerate its hydrolysis to produce free dox. In the latter approach, however, one main concern was the noncatalyzed
hydrolysis of the aromatic carbamate. With all these considerations in mind, first we concentrated on the second set of prodrugs, 13-14, which differ from each other only in the substituent on the phenyl ring. Here, the methoxynaphthol ring in 14 was introduced to modulate the background reactivity of the aromatic carbamate functions in the prodrugs.

Syntheses of prodrugs 13-14 were achieved by reacting dox hydrochloride with the aldon linkers 20a and 20b (Scheme 1A), which were prepared from phenols 18a-18b, via 19a-19b [see supporting information (SI)]. Next, we examined the activation of prodrugs 13-14 by using a catalytic amount of 38C2. As expected, activation of 13-14 to produce intermediate II was
quite fast; however, no dox was produced from these prodrugs. Instead, a compound 17 was produced from both 13-14 (Fig. 4C). Obviously, the intramolecular attack by the vicinal hydroxy function of prodox prevailed over an intermolecular attack by water. Considering that the hydroxy and amine or carbamate functions in dox or prodox are in syn position, thereby accelerating the formation of 17, we prepared an analogous prodrug 22 by using epirubicin, 21. Notably, the hydroxy and amine or carbamate functions in 21 and 22 lie in anti position and should not form the cyclic carbamate. In this case, the 38C2-catalyzed conversion of the prodrug to the ketone intermediate III was slow, and no epirubicin was reproduced.

Because compounds 13-14 and 22 were not suitable for the prodrug therapy by using aldolase Abs, we focused on prodoxs 9-12, in that prodox 11 and 12 were the cyclopentanone analogs of 9 and 10, respectively. Because cyclopentanone was found to be a very efficient donor in the 38C2-catalyzed aldol reaction (23), it was anticipated that prodrugs 11-12 could undergo the retro-alald reaction faster than 9-10. The prodoxs 9-12 were synthesized by using dox hydrochloride and the aldol linkers, 25a-25b and 29a-29b, respectively (Scheme 1B). Syntheses of linkers 25a-25b were achieved by alcohols 24a-24b starting from 15a-15b and the previously described nitrophényl carbonate 23 (6). Linkers 29a-29b were prepared from an aldol compound 26 that was prepared by Mukaiyama aldol reaction (24) of 4-acetoxy-2-butanone with 1-trimethylsililxypentene, by intermediates 27 and 28a-28b (see SI).

We analyzed dox release from prodox 7 and 9-12 (100 μM solution) in the presence of a catalytic amount (1 μM solution) of Ab 38C2. Under identical conditions at 37°C, prodrugs 9-12 produced dox with an average rate of up to 50 times faster than the previously reported prodrug 7. Thus, ≈10–30 times excess of dox were produced in 5 h from prodox 9-12, respectively, as compared with that from prodox 7 over the identical time periods. Interestingly, however, complete consumption of either 7 or 9-12 was not seen, even when the reaction mixture was left at 37°C for an extended period. We anticipated that the critical lysine residues in the Ab 38C2-binding sites underwent conjugate addition to methyl vinyl ketone (MVK), which was produced during the prodrug activation, thereby inhibiting the catalytic activity of the Ab (25). To test this, Ab 38C2 (1 μM solution) was treated with a prodrug linker 30 (100 equivalent) that possessed the aldol–Michael motif (Eq. 1) or with MVK (100 equivalent), and the activity of the mixtures was analyzed. Buffer alone was used as the negative control.

The catalytic activities of the Ab mixtures and Ab and buffer alone were determined by using the conversion of aldehyde 5 (200 μM solution) to aldehyde 5, as described above. Ab 38C2 was completely deactivated in <1 h when incubated with 30 or MVK (see SI). These observations were pertinent to our studies, because prodox 9-12 also produced MVK on activation with 38C2. However, whereas the production of MVK is a problem for studies in vitro, it should not be a problem in vivo, where it is expected to be rapidly cleared by cellular uptake and/or dilution into the large volume of total body fluids. This reasoning is supported by the fact that a solution containing 1 μM concentration of 38C2 and 10 equivalent of MVK required >24 h to achieve 90% inactivation of 38C2. An intriguing but as-yet-unproven possibility is that, as a Michael acceptor, any MVK taken up by the neoplastic cells could augment the cytotoxic effect of the activated prodrug, thereby affording combination therapy. Indeed, one can think of these constructs as triple therapy involving the prodrug, MVK, and the effector function of the Ab itself.

Tumor Cell Killing by Prodoxs 9–12 in the Presence and Absence of Abs. We evaluated toxicity of prodox 9-12 as compared with the previously described prodox 7 (6) and dox by using human breast cancer cells, MDA-MB-231. An analysis of the results showed that, under the described conditions, dox was toxic to cells at IC50 = 5 μM (Fig. 5A). Prodox 10 and 12 were also quite toxic with an IC50 value of 20 μM for both of them. In contrast, prodox 9 and 11 did not show any appreciable toxicity up to 100 μM concentration (Fig. 5B and C). Therefore, we used prodox 9 and 11 for further experiments.

Prodox 9 and 11 were compared with 7 in the presence and absence of Ab 38C2 in a cell proliferation assay by using MDA-MB-231 cells as described earlier (9). Both prodox 9 and 11 were shown to inhibit cell growth in a fashion similar to 7 in the presence of 1 μM Ab 38C2 concentration. The cytotoxicity profiles of 7, 9, and 11 in the presence of 38C2 were virtually identical to 6 (Fig. 5A and C). The identical cytotoxicity profile of prodox 7 to 9 or 11 in the presence of 38C2, however, was in contrast to our earlier observation that 7 was activated slower than 9 and 11, suggesting that the large amount of Ab used masked the differences in prodrug activation among the various analogues. This led us to speculate that Ab concentration could be further reduced in these experiments, without changing the cytotoxicity profile of prodox 9 and 11. Indeed, when Ab concentration was reduced to 0.1 or 0.033 μM, differences among the various prodrugs became quite clear (Fig. 5D). These data suggested that prodox 9 or 11 would be better than 7 when studies in vivo are carried out.

To determine the efficacy for cell killing of the Ab conjugates that contained the targeting moiety, we compared 38C2 to the 38C2 conjugates 38C2-2 and 38C2-3 (Fig. 6). As evident from Fig. 6A and B, prodox 11 showed the highest efficacy both in the presence of 38C2 and its constructs, 38C2-2 or 38C2-3. The previously described prodox 7 was less efficient than both 9 and 11. Therefore, of all of the prodoxs so far tested in vitro, 11 seems the best companion for the targeting Ab catalysts. Although we have not optimized the 38C2 concentration that will be required for the prodrug activation in vivo, it is evident from Fig. 5D that 38C2 could be used at even less than a concentration of 0.033 μM, because prodox 11 showed identical efficacy when 38C2 was used at 0.033- and 0.1-μM concentrations.

Conclusion
Ab conjugates were prepared by using Ab 38C2 and a small-molecule antagonist of integrin αvβ3. The conjugates bound efficiently to cells expressing integrin αvβ3 and catalyzed prodrug activation. In addition, a set of dox prodrugs with improved stability and lower toxicity was synthesized. In vitro evaluations using these Ab conjugates together with the dox prodrugs revealed that cell targeting and prodrug activation capabilities could be efficiently combined. We anticipate that prodox 11 and Ab conjugates, 38C2-2 or 38C2-3, may be an appropriate com-
bination for in vivo use as antitumor and/or antiangiogenic therapeutic Abs.

Materials and Methods
Ab, Cell Lines, Reagents, and Prodrugs. The generation and purification of mouse Ab 38C2 have been described elsewhere (4). Human breast cancer cell line MDA-MB-231 was obtained from American Type Culture Collection, (Manassas, VA). The cells were cultured in Leibovitz L15 medium supplemented with 2 mM l-glutamine, and 10% FCS at 37°C in a CO2-free environment. FITC-conjugated goat anti-mouse Ab was purchased from Chemicon, Temecula, CA. The Cell Titer 96 AQeas One Solution Cell Proliferation Assay kit was purchased from Promega, Madison, WI. Syntheses of prodrugs are described in the SI.

Preparation of the Integrin αβ2-Targeting Ab 38C2 Conjugates. The preparation of 38C2-2 conjugate is as follows: Ab 38C2 (1 mg/ml, 3 ml) in PBS buffer (pH 7.4) was reduced by using DTT solution (0.14 μmol) at 37°C for 3 h under argon. The solution was dialyzed by using PBS buffer, pH 6.0, under argon. To this solution, compound 2 (0.18 mg/0.2 μmol in 10 μl of dimethylformamide) was added, and the mixture was left at 4°C for 16 h. The reaction mixture was dialyzed by using PBS buffer (pH 7.4) to afford the 38C2-2 conjugate.

The preparation of the 38C2-3 conjugate was as follows: a solution of pentane-2,4-dione (2 μl of 100 mM solution in CH3CN) was added to 38C2 (1 mg/ml, 3 ml) in PBS buffer (pH 7.4) at room temperature to temporarily block the reactive lysine residues in the Ab 38C2-binding sites. After mixing the solution for 2 h, a solution of 3 (0.19 mg in 50 μl of CH3CN) was added, and the mixture was left at room temperature with continuous mixing for 16 h. The resultant 38C2-3 conjugate was reactivated by dialyzing the mixture using PBS (pH 7.4) containing hydrazine (1%), and then using PBS (pH, 7.4) alone.

Evaluation of the Binding of 38C2 Conjugates to Integrin αβ2-Expressing Cells. Binding of 38C2-2 and 38C2-3 was evaluated by using integrin αβ2-expressing cells, as described (17). Briefly, aliquots of 100 μl containing 1 × 10⁶ cells were distributed into wells of a V-bottom 96-well plate for indirect immunofluores-

Fig. 5. Effect of dox and prodox 10 and 12 (A), dox and prodox 9 (B), prodox 7 and 11 (C), and dox and prodox 7, 9, and 11 (D) on human breast cancer cells, MDA-MB-231, in vitro, in the absence or presence of a catalytic amount of Ab 38C2. Ab 38C2 was used at a 1 μM concentration in B and C. Cells (20,000) were used and developed by using the Cell Titer 96 AQeas One Solution Cell Proliferation Assay kit after 72 h of incubation with drug, prodrug or prodrug/38C2 combination. In D, dox was used at 10 μM concentration. The y axis shows cell density in a linear scale, and the x axis shows the dox or prodox concentration in a logarithmic scale in A–C and Ab concentration in a linear scale in D.
cence staining. After centrifugation for 2 min, they were resuspended by using 100 μl of the Ab conjugates (38C2-2 and 38C2-3), Ab 38C2 alone, and the previously described chemically programmed 38C2 construct (5 μg/ml cp38C2) in flow cytometry buffer. After incubating for 1 h, the complex samples were centrifuged, washed twice, and resuspended by using 100 μl of a 10 μg/ml solution of FITC-conjugated goat anti-mouse polyclonal Abs in flow cytometry buffer. After these samples were further incubated for 45 min at room temperature, flow cytometry was performed by using a FACScan instrument.

**Evaluation of the Catalytic Activities of 38C2 Conjugates.** A solution of 38C2 and its conjugates, 38C2-2 and 38C2-3 (98 μl of 0.6 μM solution in PBS) and buffer alone (98 μl) were transferred in four different wells of a 96-well fluorescence measuring plate. Methyldol (4; 2 μl of 10 mM solution in CH3CN) was added to the Ab, Ab conjugates, and buffer-containing wells, and the rate of formation of 6-methoxynaphthaldehyde, 5, was determined by using a fluorescence reader.


**Evaluation of the Prodrug-Mediated Cellular Toxicity in the Absence and Presence of 38C2 Conjugates.** Stock solutions (10 mM) of dox 6 and prodoxes 7 and 9–12 were prepared in DMSO and stored at 4°C. The cell growth assay was carried out by using MDA-MB-231 human breast cancer cells (obtained from American Type Culture Collection). Briefly, cells were plated at a density of 20,000 and 3,000 (used for experiments shown in Figs. 5 and 6, respectively) per well in 96-well tissue culture plates and maintained in culture. Prodrugs were added to the cells 24 h after plating, making 0.01 to 100 μM final concentrations for the prodrugs. For the Ab experiments, prodox and 38C2 or 32C2 conjugates were mixed just before adding to the cells. After prodox addition, the cells were maintained at 37°C in 5% CO2 for 72 or 120 h and developed by using the Cell Titer 96 AQueous One Solution Cell Proliferation Assay kit. Results are depicted in Fig. 5. In experiment (Figs. 5D and 6B), dox and prodoxes were used at a 10 μM concentration, and in Fig. 6A, they were used at 5 μM concentration.

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