Enzyme Specific Activation of Benzoquinone Ansamycin Prodrugs Using HuCC49ΔCH2−β-Galactosidase Conjugates

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To activate prodrugs for cancer treatment, an anti-TAG-72 antibody (HuCC49ΔCH2) was used for delivery of an activation enzyme (β-galactosidase) to specifically activate a geldanamycin prodrug (17-AG−C2−Gal) against colon cancer. The geldanamycin prodrug 17-AG−C2−Gal was synthesized by coupling a galactose-amine derivative with geldanamycin at the C-17 position. Molecular docking with two different programs (Affinity and Autodock) showed that the prodrug (17-AG−C2−Gal) was unable to bind to Hsp90; however, the product (17-AG−C2), enzymatically cleaved by β-galactosidase conjugate, bound to Hsp90 in a similar way as geldanamycin and 17-AG. The computational docking results were further confirmed in experimental testing by the tetrazolium [3-(4,5-dimethythiazol-2-yl)]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium assay and mass spectrometry. HuCC49ΔCH2 was chemically conjugated to β-galactosidase. The antibody−enzyme conjugate was able to target tumor antigen TAG-72 with the well-preserved enzymatic activity to activate 17-AG−C2−Gal prodrug. The released active drug 17-AG−C2 was demonstrated to induce up to 70% AKT degradation and enhance anticancer activity by more than 25-fold compared to the prodrug.

Introduction

The challenge facing all cancer chemotherapy is its relatively low ability to target tumor cells versus normal cells. The site-specific activation of prodrugs in tumors is a new strategy to achieve high efficacy and specificity and decrease toxicity in normal tissues. First, an activating enzyme will be specifically delivered to the tumor sites by an antibody−enzyme conjugate; subsequently, an inactive prodrug will be administered. The inactive prodrug will be selectively activated by the antibody−enzyme conjugate in the vicinity of the tumor site to achieve better anticancer efficacy, while the inactive prodrug in normal tissues will decrease the toxicity of the therapeutic drug. One of the challenges of the antibody−enzyme conjugates used for prodrug activation is the immunogenicity of the antibody−enzyme conjugate, which limits multiple application.[1,2] In addition, high specificity and affinity of antibody for tumor antigen is required to deliver enough enzyme to the tumor site for prodrug activation.

Monoclonal antibodies against tumor-associated glycoprotein (TAG-72)[a] have been used in tumor targeting in radioimmunoguided surgery (RIGS). TAG-72 is a high molecular weight (300−1000 kDa) glycoprotein with mucin properties. It is expressed in several epithelial-derived carcinomas.[3,4] Both the first (B72.3) and second (murine CC49) generations of anti-TAG-72 monoclonal antibodies had demonstrated excellent tumor targeting in colorectal and breast cancers.[5] However, the clinical application of ^{131}I-mCC49 in RIGS was compromised by the development of human anti-mouse antibody (HAMA) response.[6] Thus, a humanized HuCC49ΔCH2 was produced with the deletion of the constant region CH2 as the third-generation antibody.[7,8] A pilot clinical trial in 21 patients with recurrent or metastatic colorectal cancer was performed in our previous study.[9] HuCC49ΔCH2 generated no HAMA response in all 21 patients postinjection at 4−12 weeks. The HuCC49ΔCH2 levels in tumors at various metastatic sites (including liver, abdominal wall, lymph node, pelvis, kidney, pancreas, stomach, small intestine, and colon) were 5−10-fold higher than the blood and normal tissue levels during days 5−21 after antibody injection, while the HuCC49ΔCH2 levels in normal organs and blood decayed to undetectable levels over 5−20 days. Therefore, HuCC49ΔCH2 is an ideal candidate for tumor targeting in humans with high specificity and affinity for tumors without immunogenecity.

However, despite the successful tumor detection with anti-TAG-72 antibodies in RIGS, surgical resectability rates for patients with recurrent colorectal cancer is only 12.5−60%. For instance, in our three clinical trials of 260 patients with recurrent colorectal cancer, 48−62% patients had resectable cancer, while 38−52% patients are unresectable.[10−13] Even in the patients with resectable cancer, it is also very challenging for a surgeon to detect and remove all occult metastatic lesions.

Furthermore, for patients with recurrent colorectal cancer, systemic chemotherapy (5-fluorouracil and leucovorin) yields response rates of only 15−35% with no significant survival benefit.[10,13−16] A new treatment modality is required to improve the overall survival rate for patients with advanced recurrent and unresectable colorectal cancers.

Geldanamycin (GA) provides a new mechanism for cancer therapy by inhibiting molecular chaperone Hsp90 and down-

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[a] Abbreviations: RIGS, radioimmunoguided surgery; TAG-72, tumor-associated antigen-72; GA, geldanamycin; 17-AAG, 17-(allylamino)-17-demethoxygeldanamycin; 17-AG, 17-amino-17-demethoxygeldanamycin; 17-DMAG, 17-(dimethylaminoethylamino)-17-demethoxygeldanmycin; 17-AG−C2−Gal, 17-amino-17-demethodygeldanamycin−C2-galactose.

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regulating many oncogenic targets simultaneously via the proteasomal degradation.[17−19] Hsp90 is overexpressed 2−10-fold higher in various human cancers compared to normal tissues.[20] Hsp90 modulates the folding and assembly of many oncogenic proteins in cancer cells. These oncogenes include AKT, v-Src, Raf-1, Bcr-Abl, ErbB2, mutant P53, and HIF-1α.[21,22] GA binds to the conserved ATP binding pocket at the N-terminus of Hsp90,[23,24] inhibits ATP-dependent chaperone activity,[25−27] and thus locks Hsp90 in the intermediate complex. This inhibits Hsp90-mediated protein conformational refolding and maturation. Therefore, the premature client proteins are subsequently ubiquitous and targeted for proteasomal degradation.[28−33] This process down-regulates expression of many oncogenic proteins in cancer cells.[24]

The antitumor activity of geldanamycin (GA) has long been recognized. However, preclinical evaluation of GA has demonstrated severe dose-limiting toxicity, and thus clinical evaluation for GA was halted.[34] To identify a better drug candidate, GA has been modified to generate many analogues. 17-Allylaminogeldanamycin (17-AAG) and 17-(dimethylaminoethylamino)-17-demethoxygeldanmycin (17-DMAG) are GA derivatives currently in clinical trials at the National Cancer Institute.[35−39] Although 17-AAG and 17-DMAG have shown good efficacy, they have also shown dose-limiting toxicity (hepatotoxicity, gastrointestinal toxicity, and nephrotoxicity). Clinical phase I studies showed that 17-AAG caused grade 3 hepatoxicity at 56 mg/m^2. At a dose of 80 mg/m^2, grade 3 nausea and vomiting, perirectal bleeding, diarrhea, anemia, anorexia, thrombocytopenia, and transaminitis occurred.[40,41] 17-DMAG also produced similar dose-limiting toxicities at 12 mg/m^2 in rats and dogs.[42,43]

In our previous study, we prepared a series of inactive geldenamycin carbohydrate prodrugs by a four-step reaction using trichloroacetimidate derivatives of sugar as starting material. These prodrugs were synthesized by conjugating galactose derivatives to geldanamycin at the C17 position through a linker chain with various lengths (17-AG−Gal) and these were confirmed to be enzymatically activated by exogenous β-galactosidase to exhibit their anticancer activity in vitro.[44] In this study, we intended to utilize anti-TAG-72 antibody (HuCC49ΔCH2) for targeted delivery of β-galactosidase for 17-AG−Gal prodrug activation in tumors. Only results from one specific prodrug with a two-carbon linker (17-AG−C2−Gal) are presented here since the results were quite similar for those prodrugs. The HuCC49ΔCH2 was chemically conjugated with β-galactosidase. The conjugate was evaluated by antigen binding (and tumor targeting) ability and enzymatic activity on colon cancer cells, while the activation of 17-AG−C2−Gal prodrug by the conjugate was tested via cytotoxicity assay and Hsp90 client protein (AKT) degradation. The HuCC49ΔCH2−β-galactosidase conjugate proved itself a promising delivery tool for glycosylated geldanamycin prodrug activation in tumors.

Chemistry

Synthesis of Geldanamycin Prodrugs. We hypothesized that modification of the geldanamycin with bulky galactose on C-17 inactivated its activity, while exogenous β-galactosidase reactivated the prodrugs. As reported in a previous publication,[44] we have synthesized a series of geldanamycin−carbohydrate prodrugs. One prodrug (17-AG−C2−Gal) was used in this study (Figure 1).

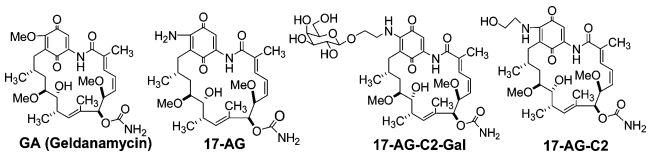

Figure 1. Structure of geldanamycin (GA), 17-amino-17-demethoxygeldanamycin (17-AG), prodrug 17-AG−C2−Gal, and active drug 17-AG−C2.

Molecular Docking of Geldanamycin (GA), 17-Aminogeldanamycin (17-AG), 17-AG−C2−Gal, and 17-AG−C2 into the Nucleotide-Binding Domain of Human Hsp90. Geldanamycin (GA) binds to the amino-terminal domain ATP binding site of Hsp90 inhibiting the chaperone activity of the protein. The derivative 17-aminoallylgeldanamaycin (17-AAG) showed similar anticancer activity as geldanamycin, while 17-AG is an active metabolite of 17-AAG.[45] The cocrystallized structure of the human Hsp90−geldanamycin complex displays an extensive hydrogen-bonding network between the Hsp90 protein, geldananmycin, and solvent within the binding pocket. The direct Hsp90 protein−geldanamycin interactions include hydrogen-bonding between Asp93 and the C-7 carbamate group, the backbone nitrogen of Phe138 with the amide of the ansa ring, the amine of Lys58 interacts with the C-11 hydroxyl group, and the amine of Lys112 hydrogen bonds with the C-21 ketone.[24]

It was proposed in our previous publication that 17-AG was similar to geldanamycin in binding to Hsp90, while the selective leaving groups at the C-17 position in the prodrug 17-AG−C2−Gal blocked the ability of the benzoquinone ansamycin to bind to Hsp90. However, when the prodrug was reactivated by exogenous β-galactosidase to release 17-AG−C2, it regained the ability to bind Hsp90 and exhibited anticancer activity.[44] To confirm our hypothesis, we utilized two distinct computational-based molecular docking programs: Affinity, which allowed both the ligand and binding pocket to be flexible during the simulation, and Autodock, where the flexible ligands were docked into the binding site of a static protein, to simulate the binding of the benzoquinone ansamycins, geldanamycin, 17-AG−C2−Gal, 17-AG−C2, and 17-AG in the nucleotide-binding domain of Hsp90. These docking simulations demonstrated that geldanamycin bound to Hsp90 in a similar overall conformation to that found in the isolated cocrystallized structure obtained from the RSCB Protein Data Bank (PDB code 1YET). The energies associated with the binding of geldanamycin to Hsp90, as determined by the Affinity and Autodock programs, were found to be −34.9 and −11.9 kcal/mol, respectively, using the two different docking methods (Figure 2A). Similarly, 17-AG showed identical binding configuration to Hsp90 compared to geldanamycin, which formed a H-bond with amino acids Lys112, Lys58, Asp93, Thr184, and Asp54 in Hsp90 (Figure 2B). The binding energies of 17-AG to Hsp90 were −34.1 kcal/mol (Affinity) and −12.1 kcal/mol (Autodock). However, the prodrug compound, 17-AG−C2−Gal, was unable to bind to Hsp90 in any conformation, as the *cis*- or *trans*-amide isomers, using the Affinity docking program with either the human cocrystallized Hsp90−geldanamycin (PDB code 1YET) or the human open Hsp90 (PDB code 1YES) structures. The Autodock simulation gave a binding conformation for the *cis*-amide isomer of GA−C2−Gal but in a completely different orientation than that observed for gelanamycin (Figure 2C) and the associated poor binding energy of +83.1 kcal/mol. This clearly indicated that GA−C2−Gal binding to Hsp90 was highly unfavorable. Interestingly, the product of GA−C2−Gal cleavage by the antibody−β-galactosidase conjugate, GA−C2, bound to Hsp90 in a conformation similar to that of the Hsp90−geldanamycin complex (Figure 2D,E) with an additional hydrogen-bond

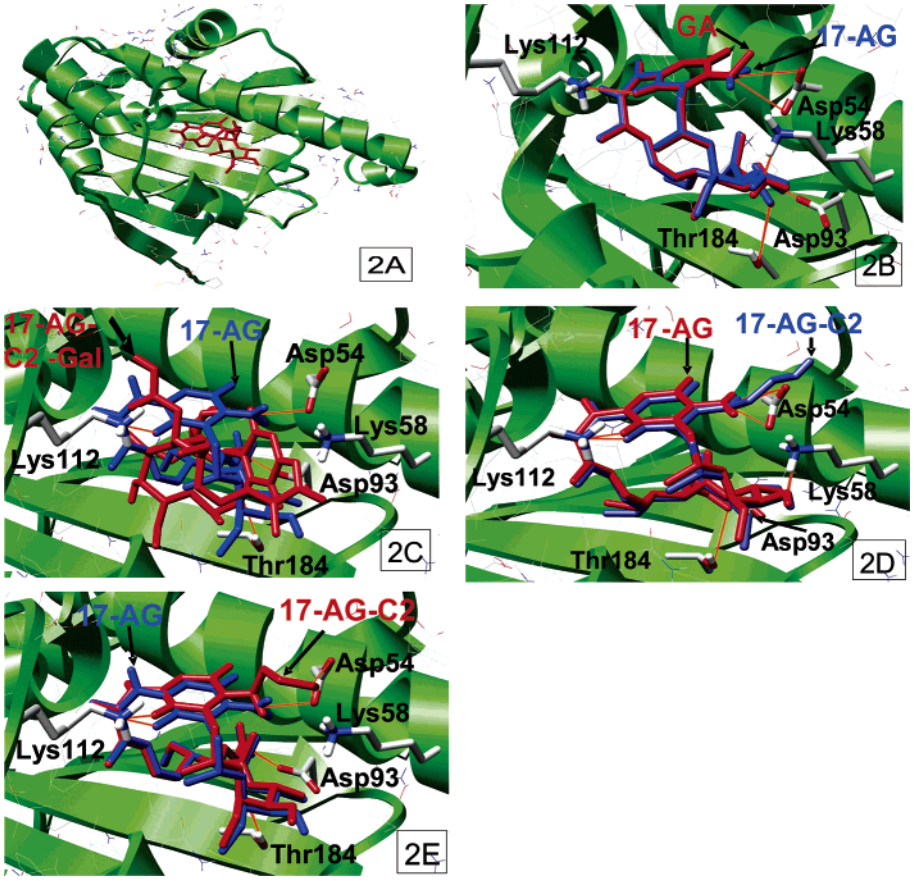

Figure 2. Molecular docking of Hsp90 binding of geldanamycin (GA), 17-amino-17-demethoxygeldanamycin (17-AG), prodrug 17-AG−C2−Gal, and active drug 17-AG−C2: (A) geldanamycin binds to Hsp90 (Autodock); (B) GA and 17-AG bind to Hsp90 (Affinity); (C) 17-AG and 17-AG−C2−Gal bind to Hsp90 (Autodock); (D) 17-AG and 17-AG−C2 bind to Hsp90 (Affinity); (E) 17-AG and 17-AG−C2 bind to Hsp90 (Autodock).

interaction between Asp54 and the 17-amino group. The energy associated with this binding, as determined by Affinity (Figure 2D) and Autodock (Figure 2E), was −27.4 and −12.1 kcal/mol, respectively.

Conjugation of HuCC49ΔCH2 to *β*-Galactosidase. To deliver a drug-activating enzyme (*β*-galactosidase) into tumors, we utilized the humanized anti-TAG-72 antibody (HuCC49ΔCH2) for tumor targeting. Therefore, we conjugated HuCC49ΔCH2 and *β*-galactosidase for future prodrug activation in cancers. For *β*-galactosidase modification, a free thiol was generated on the *β*-galactosidase using SATA (*N*-succinimidyl *S*-acetylthioacetate) followed by the release of the SH with hydroxylamine; this process did not result in significant loss of enzymatic activities. For antibody modification, a maleimide functional group was generated on antibody HuCC49ΔCH2 using MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester) without affecting antigen binding affinity. Then the HuCC49ΔCH2−*β*-galactosidase conjugate was generated upon combination of the activated proteins under nitrogen. The conjugate was purified through a Sephadex G-150 column, and one major peak was obtained. The antibody−enzyme conjugate was assayed for its enzymatic activity and ability to bind to TAG-72 antigen in the following experiments.

Biology

TAG-72 Expression in Different Cancer Cell Lines and in Different Organs in LS174T Xenograft Model in Vivo. To select a cancer cell line for the testing of antibody−enzyme conjugate, we screened the TAG-72 expression by Western blotting in various colon cancer cell lines: colon cancer cells

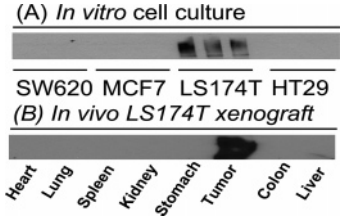

Figure 3. TAG-72 expression levels in different cancer cell lines (A) and different organs in LS174T xenograft model (B).

(SW620, HT-29, and LS174T) and breast cancer cell (MCF-7). Only LS174T cells had stable high TAG-72 expression among these four cell lines in vitro (Figure 3A). Therefore, LS174T cells were used as positive control, while SW-620 and HT-29 cells were negative control in the following experiments. To confirm TAG-72 expression in tumors and normal tissues in the xenograft model in vivo, different organs were collected from LS 174T xenograft mice and homogenized for Western blot detection. As expected, only tumor tissues demonstrated strong signals for TAG-72 expression, while TAG-72 expression was not detected in normal organs, including heart, lung, spleen, kidney, stomach, colon, and liver (Figure 3B).

Characterization of Antibody−Enzyme Conjugates for Specific TAG-72 Binding. To test whether the conjugation process affects the HuCC49ΔCH2 binding specificity and affinity to TAG-72, immunostaining was performed on LS 174T cells and human colon cancer tumors. LS174T cells were fixed and stained with the HuCC49ΔCH2−*β*-galactosidase conjugate followed by blocking. Anti-*β*-galactosidase primary antibody and FITC-labeled secondary antibody was used to detect the

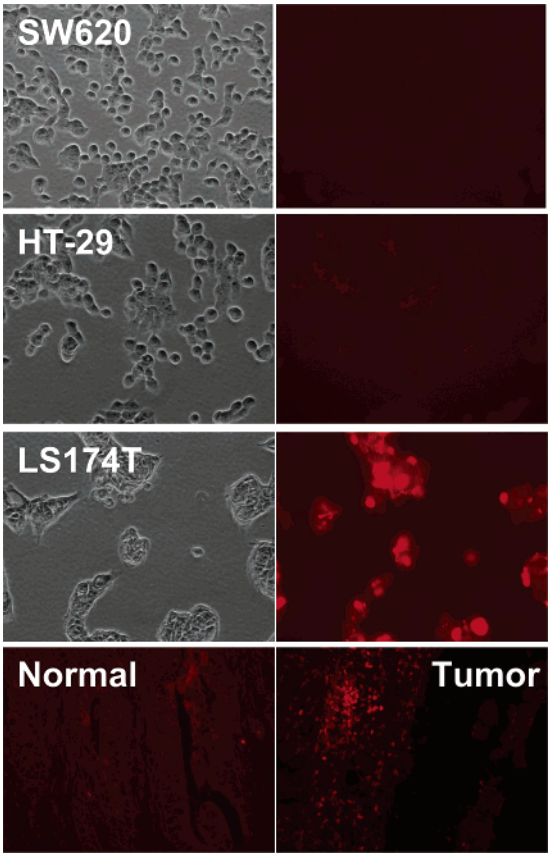

Figure 4. Antigen binding capacity of HuCC49ΔCH2−β-galacosidase in SW620, HT-29, LS174T cells, and human colon cancer tissues and normal tissues. The cells or tissues were treated with antibody−enzyme conjugate. The bound antibody−enzyme conjugate was detected with primary antibody against β-galactosidase and FITC-labeled secondary antibody.

bound antibody−enzyme conjugate. The result showed that antibody−enzyme conjugate only bound to TAG-72-positive cancer cells (LS174T) but not to TAG-72-negative cancer cells HT-29 and SW-620 (Figure 4). Similarly, antibody−enzyme conjugate bound to human colon cancer tissues, while no binding was seen in normal tissues. These results suggested that HuCC49ΔCH2−β-galactosidase conjugate maintained a binding specificity and affinity similar to HuCC49ΔCH2 for tumors expressing TAG-72.

Characterization of Antibody−Enzyme Conjugates for β-Galactosidase Activity. To confirm that HuCC49ΔCH2−β-galactosidase had a similar enzymatic activity as β-galactosidase when bound to TAG-72 in cancer cells, an Elisa assay was performed in LS174T (TAG-72-positive) and SW620 and HT-29 cancer cells (TAG-72-negative). Briefly, the PBS, free enzyme control, or conjugate was incubated with different cells for 1 h followed by extensive wash. The enzymatic activity was determined by the color development of β-galactosidase substrate (*o*-nitrophenyl-β-D-galacopyranoside, ONPG). The result showed that antibody−enzyme conjugate only bound to LS174T cells to exhibit strong enzymatic activity, but not on SW620 and HT-29 cells (Figure 5). This result was in agreement with the previous observation that TAG-72 was only highly expressed on LS174T cells but not on SW620 and HT-29 cells. These results suggested that HuCC49ΔCH2−β-galactosidase conjugate preserved enzymatic activity as well as TAG-72 binding affinity and specificity.

Enzymatic Cleavage of 17-AG−C2−Gal Prodrug. To confirm that the prodrug 17-AG−C2−Gal can be cleaved by

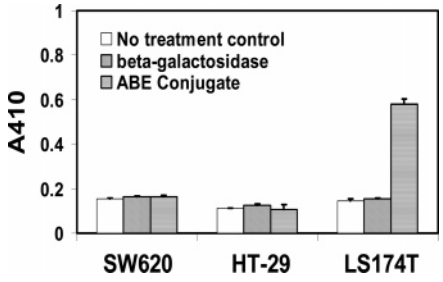

Figure 5. Enzymatic activity of HuCC49ΔCH2−β-galacosidaseconjugate. SW620, HT-29, and LS174T cells were seeded in a 96-well plate overnight. PBS as no treatment control, staining buffer containing β-galacosidase, and HuCC49ΔCH2−β-galacosidaseconjugate incubated with the cells followed by extensive wash. The enzymatic activity was determined by the color development of the β-galactosidase substrate (ONPG) at 410 nm.

the antibody−enzyme conjugate to release 17-AG−C2, the prodrug was incubated with antibody−enzyme conjugate (2 units) in PBS. The compounds were then extracted for analysis by mass spectrometry. The spectra of GA−C2−Gal from PBS reaction buffer (Figure 6A) clearly showed a parent compound peak (*m*/*z* 774). After treatment with antibody−enzyme conjugate, the parent compound peak disappeared and a new peak at *m*/*z* 612 was observed and confirmed to be 17-AG−C2 (Figure 6B). These data indicated that 17-AG−C2−Gal was cleaved by antibody−enzyme conjugate to release 17-AG−C2.

17-GA−C2−Gal Prodrug Activation for Anticancer Activity in Vitro. To test if antibody−enzyme conjugate (HuCC49ΔCH2−β-galactosidase) would bind to cancer cells (LS174T) to activate 17-AG−C2−Gal prodrug for anticancer activity, we utilized the MTS (tetrazolium [3-(4,5-dimethythiazol-2-yl)]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) assay to measure the cytotoxicity of prodrug and active drug in TAG-72-positive cancer cells LS 174T. The result showed that the IC_{50} of geldanamycin (GA) in LS174T cells was 0.45 μM, while IC_{50} of 17-AG−C2−Gal was greater than 25 μM (concentrations above 25 μM were not tested). After LS174T cells were incubated with antibody−enzyme conjugate (2 units) for 1 h followed by extensive wash process, the 17-AG−C2−Gal prodrug was added to the cells, its activation and subsequent anticancer activity were measured with the MTS assay. The results showed that the combination of 17-AG−C2−Gal prodrug (0.01−25 μM) with antibody−enzyme conjugate showed dose-dependent cell-killing effects. The IC_{50} of 17-AG−C2−Gal after conjugate treatment was around 1.12 μM, which was more than 25-fold lower than that of prodrug alone (Figure 7). However, the prodrug (0.01−25 μM) did not show significant anticancer activity with β-galactosidase, since the enzyme alone (with no binding ability to the cancer cells) was washed off the cells during the experiment. The results indicate that HuCC49ΔCH2−β-galactosidase binds to the LS174T cells and activates 17-AG−C2−Gal prodrug to yield its anticancer activity.

Enzyme-Specific Activation of 17-AG−C2−Gal Prodrug To Induce Hsp90 Client Protein Degradation. To confirm if the reactivated 17-AG−C2−Gal prodrug (by HuCC49ΔCH2−β-galactosidase conjugate) will bind to Hsp90 and induce Hsp90 client protein degradation (such as AKT), we used Western blotting to determine the total AKT levels after 2 and 10 μM 17-AG−C2−Gal prodrug treatment for 24 h (following incubation with antibody−enzyme conjugate or β-galactosidase for 1 h and thorough wash) in LS174T cells. 17-AG−C2−Gal treatment following 1 h of incubation with antibody−enzyme conjugate significantly induced AKT degradation (up to 70%).

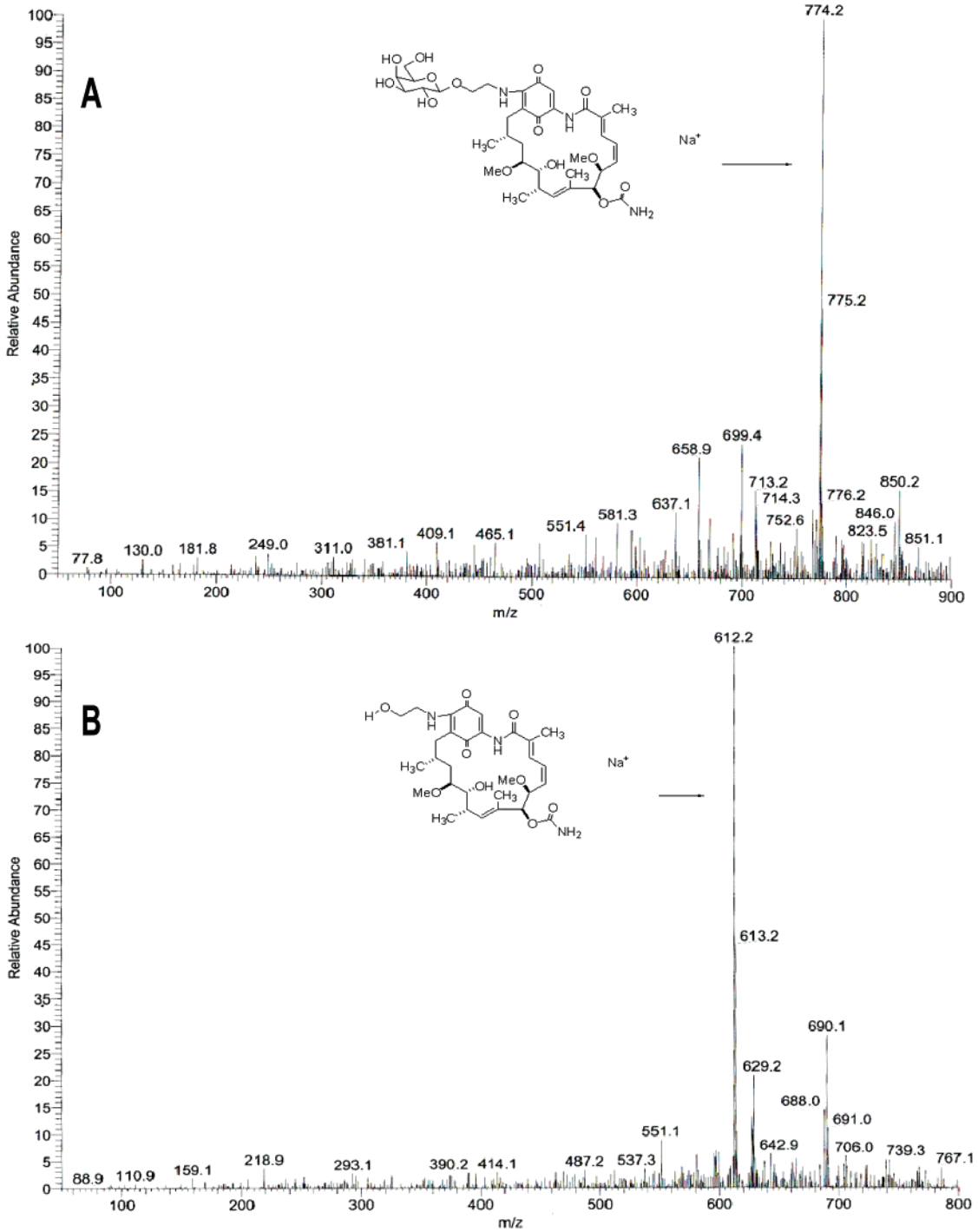

Figure 6. Confirmation of 17-AG−C2−Gal prodrug (A) and active drug 17-AG−C2 (B) by HuCC49ΔCH2−β-galactosidase using mass spectrometry.

This effect was comparable to the effect of 17-AG, which caused 70−80% AKT degradartion at 2 and 10 μM concentrations with or without antibody−enzyme conjugate pretreatment. However, in the absence of antibody−enzyme conjugate, the prodrug at both 2 and 10 μM concentrations did not induce significant AKT level change, as evidenced by similar a Akt:β-actin ratio (>90%) compared to the control (100%). These results suggest that the antibody−enzyme conjugate binds to cancer cells and activates 17-AG−C2−Gal prodrug. The released active drug 17-AG−C2 enters the cancer cells, binds to Hsp90, and induces AKT degradation (Figure 8).

Conclusion

In summary, the prodrug (17-AG−C2−Gal) was shown unable to bind to Hsp90 by molecular docking, and the C-17 glycosylation of 17-AG thus converted it to an inactive prodrug. The product (17-AG−C2) upon enzymatic cleavage by β-galactosidase bound to Hsp90 similarly to geldanamycin and 17-AG to exhibit anticancer activity, as shown by docking and experimental testing. These data suggest that it is feasible to use enzymatic cleavage of the inactive prodrug 17-AG−C2−Gal to release an active drug and thus achieve controlled drug activation for cancer treatment. This result was consistent with the previous publications from other labs.[35,46]

HuCC49ΔCH2−β-galactosidase conjugate was successfully constructed and applied in the target drug delivery of geldanamycin prodrug in the current study. The immunostaining showed that the antibody-enzyme conjugate specifically bound to tumor antigen TAG-72 on LS174T cancer cells. The bound antibody-

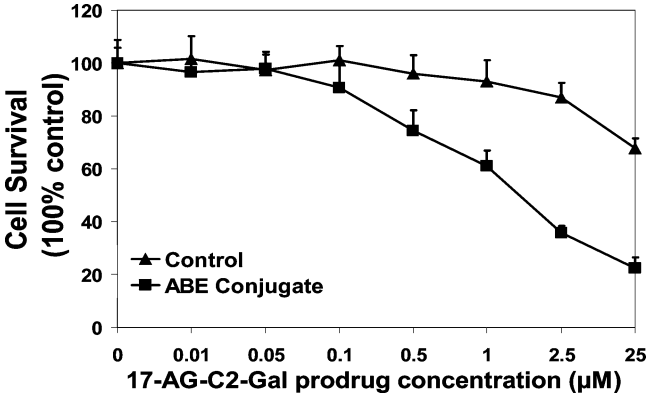

Figure 7. HuCC49ΔCH2−β-galactosidase conjugate binds to LS 174T cells and activates 17-AG−C2−Gal prodrug for cytotoxicity in MTS assay.

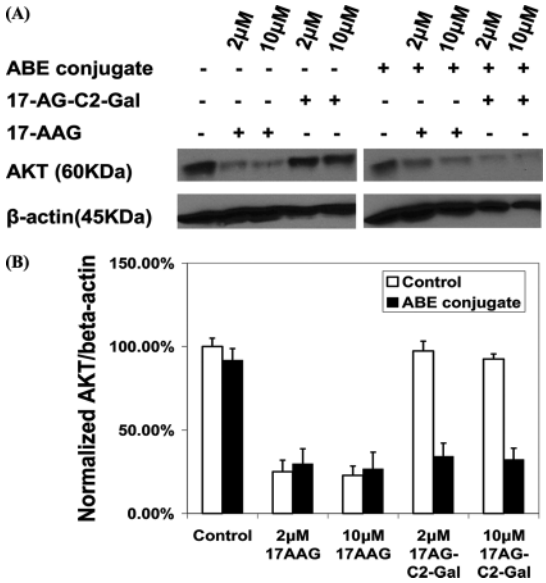

Figure 8. HuCC49ΔCH2−β-galactosidase conjugate binds to cancer cells and activates 17-AG−C2−Gal to release active drug 17-AG−C2 that enters the cancer cell and binds to Hsp90 to induce AKT degradation.

enzyme conjugate retained the enzymatic activity to activate 17-AG−C2−Gal prodrugs. The released active drug 17-AG−C2 entered cancer cells, bound to Hsp90 to induce significant AKT degradation (up to 70%), and therefore enhanced anticancer activity by more than 25-fold compared to the prodrug. These data suggest that anti-TAG-72 antibody HuCC49ΔCH2 could be utilized to deliver drug activation enzyme (β-galactosidase) to cancer for site-specific activation of geldanamycin prodrugs. Finally, it is proposed here that other glycosylated prodrugs in addition to geldanamycin prodrugs could also be used with this antibody-enzyme conjugate and further studies will be carried out in our lab.

Experimental Section

Molecular Docking Methods. The crystallographic coordinates of the amino-terminal domain of the 1.9 Å structure of human Hsp90 cocrystallized with geldanamycin (PDB ID 1YET) and the 2.2 Å structure of open human Hsp90 (PDB ID 1YES)[24] were obtained from the RCSB Protein Data Bank. The *cis*-amide isomers of the geldanamycin, 17-AG, 17-AG−C2, and 17-AG−C2−Gal structures were constructed and assigned the correct atom type and bond order, from the cocrystallized geldanamycin structure. The *trans*-GA−C2−Gal structure was generated from the 17-azetidinyl-17- demethoxygeldanamycin structure obtained from the Cambridge Structural Database.[47]

Molecular docking simulations using the Affinity docking program[48,49] were performed on a SGI Octane 2 workstation using the Insight II software package (Version 2005, Accelrys Inc., San Diego, CA). Hydrogen atoms were added to the protein structures and the ionizable residues were corrected for physiologic pH. The binding site was defined as whole residues within an interface 6 Å radius subset encompassing the ligand-binding domain, using the cocrystallized geldanamycin structure as a reference ligand, and the potentials and charges of the Hsp90-ligand complex were initially corrected using CVFF.[50] The Grid docking Affinity job was run using the default parameters for the grid setup, and the Hsp90−ligand complex was minimized using the conjugate gradient method (1000 iterations) with 50 structural outputs specified. The resulting Hsp90−ligand complexes were evaluated on the basis of the following criterion: the total energy as output by Affinity, the interaction energy as calculated by the Docking Module (both van der Waals and electrostatic energies were calculated with a specified cutoff of 8 Å), the number of hydrogen-bond interactions between protein and ligand, and the positioning of the ligand in the binding site. The top ranked Hsp90−ligand complexes from the Affinity docking simulations were subject to a force-field-based minimization to a convergence of 0.001 kcal/mol, and the potentials and charges of the Hsp90−ligand complex were corrected using CHARMM force field parameters as implemented in the CHARMM program.[51]

A further molecular docking study was also performed using the Autodock program (version 3.0.7) [52] on an Octane 2 workstation using Sybyl 7.1 (Tripos Inc.). The functional Hsp90 binding pocket was selected, the remaining amino acid residues were removed, the polar hydrogens were added, and Kollman charges were assigned to the selected binding pocket. 3-D affinity grids covering the entire binding pocket were calculated for each of the following atom types: C, A (aromatic C), N, O, S, H, and e (electrostatic) using Autogrid3. For the ligands, geldanamycin, GA−2C, and GA−2C−Gal, all hydrogens were added and Gasteiger charges [53] were assigned, and the rotatable bonds were determined via AutoTors. For each ligand, the docking parameters were as follows: trials of 50 dockings, random starting position and conformation, rotation step ranges of 50°, and 1 million energy evaluations.

Conjugation of HuCC49ΔCH2 to β-Galactosidase. Enzyme Modification. The enzyme was modified with the amine-reactive reagent SATA (Pierce, Rockland, IL) to generate protected sulfhydryl groups. The protected sulfhydryl groups were then released by hydroxylamine (Pierce, Rockland, IL). Four milligrams of β-galactosidase was combined with 0.05 mg of SATA in 500 μL of PBS buffer. The resulting solution was stirred for 1 h at room temperature. The modified enzyme was then resolved from reagent through a 1 × 13-cm G-25 medium column equilibrated with PBS buffer. Mixing 1.0 mL of modified enzyme solution with 100 μL hydroxylamine deacetylation solution followed by incubation at room temperature for 2 h gave the final sulfhydryl modified enzyme, which was desalted through a G-25 column.

Antibody Modification. Five milligrams of HuCC49ΔCH2 was combined with 0.3 mg of MBS (Pierce, Rockland, IL) in 500 μL of PBS buffer. This was allowed to react with stirring for 1 h. The modified antibody was purified through a 1 × 13 cm G-25 medium column.

Coupling of Antibody with Enzyme. The modified enzyme was mixed with the modified antibody. The resulting mixture was then carefully adjusted to pH 7.4. The reaction was kept under anaerobic conditions (under N_2) with stirring at room temperature for 2 h. The concentrated conjugate was purified from aggregate, unreacted enzyme or antibody, and small molecules by chromatography on a Sephadex G-150 column.

Cell Culture and Human Colon Cancer Tissues. MCF7, SW620, HT-29, and LS174T were cultured in RPMI 1640 or DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acid, and penicillin (100 units/mL)/streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO_2

and 95% air at 37 °C. The culture mediums were changed every 2−3 days. Human colon cancer tissues and the normal control tissues from the same patient were obtained as fresh tissue or paraffin-embedded tissue from the Ohio State University James Cancer Hospital.

Immumonohistochemistry. Paraffin embedded tissue was sectioned at 4 μm and placed on positively charged slides. Slides with specimens were then placed in a 60 °C oven for 1 h, cooled, deparaffinized, and rehydrated with xylenes and graded ethanol solutions to water. Tissues were antigen-retrieved using citrate buffer in a vegetable steamer. Also, tumor cells such as LS174T and HT-29 were seeded in the chamber slides for at least 12 h before the immunohistochemistry detection. The HuCC49ΔCH2−β-galactosidase conjugate was incubated with the slides at a dilution of 1:500−1:1000. Slides were then subsequently incubated with primary mouse-anti-β-galactosidase antibody and FITC-labeled rabbit-anti-mouse IgG for 2 h. The staining was examined under a microscope after extensive washing.

ELISA for β-Galactosidase Enzyme Activity Detection. SW620, HT-29, and LS174T cells (0.1 million) were seeded in 96-well plates. Then the cells were allowed to grow for 24 h. The bulk of the culture medium was decanted and the residual medium was removed by blotting the plate on a paper cloth. Diluted antibody−enzyme conjugate (100 μL) was added to the cells followed by incubation at 37 °C for 1 h. The plate was washed thoroughly three to five times. Fresh ONPG (Pierce, Rockland, IL) (150 μL) solution was added as substrate, followed by incubation at 30 °C for 30 min or until appropriate color develops. Then 5 μL of stop solution was applied and the absorbance at 410 or 405 nm was recorded.

Enzymatic Cleavage Study of 17-AG−C2−Gal Using Mass Spectrometry. GA−C2−Gal with or without 2 units of HuCC49ΔCH2−β-galactosidase were incubated for 3 h at room temperature followed by extraction with acetonitrile. The extract was dried by nitrogen gas and dissolved in 90% acetonitrile containing 0.1% formic acid. ESI mass spectra were recorded using a LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA). Samples were introduced into the ion source by direct infusion at 3.0 mL/min using a syringe pump on the LCQ instrument.

17-AG−C2−Gal Prodrug Activation by HuCC49ΔCH2−β-Galactosidase To Exhibit Anticancer Activity. A total of 2000−5000 LS174T cells were cultured in a 96-well plate for 24 h. PBS (100 μL) and PBS staining buffer containing enzyme only or antibody−enzyme conjugate were added to the cell culture and the mixture incubated for 1 h. Then the cells were thoroughly washed three to five times. Prodrug 17-AG−C2−Gal (0, 0.01, 0.05, 0.1, 0.5, 1, 2.5, and 25 μM) was administered to the cells, and the cells were incubated for 72 h. After 3 days, MTS (tetrazolium [3-(4,5-dimethythiazol-2-yl)]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) (2 mg/mL) and phenazine methosulfate (PMS, 25 μM) were added directly to the cell culture and incubated for 2 h at 37 °C. The absorbance of formazan (the metabolite of MTS by viable cells) was measured at 490 nm to quantify the number of surviving cells.

Western Blot Analysis. The 50−70% confluent LS174T cells were quickly washed and incubated with free β-galactosidase enzyme or HuCC49ΔCH2−β-galactosidase conjugate in PBS containing 2% FBS for 1 h. The cells were thoroughly washed and incubated with 10 μM 17-AG−C2−Gal for 24 h. All cells were collected in media, centrifuged, washed twice with phosphate-buffered saline (PBS), lysed in cell lysis buffer (50 mM, pH 7.6 Tris-HCl, 250 mM NaCl, 5 mM EDTA, 2 mM Na_3VO_4, 50 mM NaF) with 1% protease inhibitor cocktail (P8340, Sigma) for 30 min on ice, and sonicated three times for 20 s. Protein concentration was determined using the BCA protein assay method (Pierce). The cell lysates were incubated with 2× SDS loading buffer and boiled for 5 min. Then 30 μg of protein was subjected to electrophoresis in 10% SDS−polyacrylamide gels (Bio-Rad). The protein was transferred to nitrocellulose membrane and incubated with different primary antibodies, anti-AKT, anti-Hsp90, and anti-β-actin monoclonal antibody [1:1000 diluted in 5% milk Tris-buffered saline with 0.1% Tween-20 (TBS-T)] at room temperature for 1 h. The membrane was washed four times with TBS-T for 15 min and then incubated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA) for 1 h at room temperature. An enhanced chemiluminescence system ECL (Amersham) was used to detect AKT and β-actin expression level. Also, tumor cells such as HT-29, SW-620, and LS174T and human colon cancer tissue samples obtained by homogenization were detected for TAG-72 expression.

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