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

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Abstract

To activate prodrugs in cancer, an anti-TAG-72 antibody (HuCC49 Δ CH2) was used for delivery of an activation enzyme (β -galactosidase) to specifically activate geldanamycin prodrug (17-AG-C2-Gal) in colon cancer. The geldanamycin prodrug 17-AG-C2-Gal was synthesized by coupling galactose-amine derivative with geldanamycin at 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 enzymatically cleaved product (17-AG-C2) by β -galactosidase conjugate bound to Hsp90 in a similar way to geldanamycin and 17-AG. The computational docking results were further confirmed in experimental testing by MTS assay and Mass Spectrometry. HuCC49 Δ CH2 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 the relatively low target ability to 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, the specificity and high affinity of antibody to tumor antigen is required to deliver enough enzymes to the tumor site for prodrug activation.

Monoclonal antibodies against tumor associated glycoprotein (TAG-72) have been used in tumor targeting in radioimmunoguided surgery (RIGS). TAG-72 is a high molecular weight (300

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–1000kDa) 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 cancers⁵. However, the clinical application of ¹³¹I-mCC49 in RIGS was compromised by the development of human anti-mouse antibody (HAMA) response⁶. 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 study⁹. HuCC49ΔCH2 generated no HAMA response in all 21 patients post-injection 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 to 10-fold higher than the blood and normal tissue levels during day 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 immunogenicity.

However, despite the successful tumor detection with anti-TAG-72 antibodies in RIGS, surgical resectability rate for patients with recurrent colorectal cancer is only 12.5 to 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 unresectable¹⁰⁻¹³. 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% to 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-regulating many oncogenic targets simultaneously via the proteasomal degradation¹⁷⁻¹⁹. Hsp90 is over-expressed 2 to 10-fold higher in various human cancers compared to normal tissues²⁰. 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-1 α ^{21,22}. GA binds to the conserved ATP binding pocket at the N-terminus of Hsp90^{23,24}, inhibits ATP-dependent chaperone activity²⁵⁻²⁷, and thus locks Hsp90 in the intermediate complex. This inhibits Hsp90-mediated protein conformational refolding and maturation. Therefore, the premature client proteins are subsequently ubiquitinated and targeted for proteasomal degradation²⁸⁻³³. This process down-regulates expression of many oncogenic proteins in cancer cells²⁴.

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 halted³⁴. To identify a better drug candidate, GA has been modified to generate many analogs. 17-allylaminogeldanamycin (17-AAG) and 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) are GA derivatives currently in clinical trials at the National Cancer Institute³⁵⁻³⁹. 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 hepatotoxicity at 56 mg/m². At a dose of 80 mg/m², grade 3 nausea and vomiting, peri-rectal bleeding, diarrhea, anemia, anorexia, thrombocytopenia, and transaminitis occurred^{40,41}. 17-DMAG also produced similar dose-limiting toxicities at 12 mg/m² in rats and dogs^{42,43}.

In our previous study, we have prepared series of inactive geldanamycin 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 C17 position through a linker chain with various lengths (17-AG-Gal) and were confirmed to be enzymatically activated by exogenous β -galactosidase to exhibit their anticancer activity in vitro⁴⁴. In this study, we intended to utilize anti-TAG-72 antibody (HuCC49 Δ CH2) for targeted delivery of β -galactosidase for 17-AG-Gal prodrug activation in tumors. Only results from one specific prodrug with 2 carbon linker (17-AG-C2-Gal) were presented here since the results were quite similar for those prodrugs. The HuCC49 Δ CH2 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 HuCC49 Δ CH2- 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 with bulky galactose to C-17 of the geldanamycin inactivated its activity, while exogenous beta-galactosidase reactivated the prodrugs. As reported in previous publication⁴⁴, we have synthesized a series of geldanamycin-carbohydrate prodrugs. One prodrug (17-AG-C2-Gal) was used in this study (Fig 1).

Molecular docking of geldanamycin (GA), 17-amino-geldanamycin (17-AG), 17-AG-C2-Gal, and 17-AG-C2 into the nucleotide-binding domain of human Hsp90

Geldanamycin (GA) bound to the amino-terminal domain ATP binding site of Hsp90 inhibiting the chaperone activity of the protein. The derivative 17-amino-allyl-geldanamycin (17-AAG) showed similar anticancer activity with geldanamycin, while 17-AG is an active metabolite of 17-AAG⁴⁵. The co-crystallized structure of the human Hsp90-geldanamycin complex displays extensive hydrogen-bonding network between the Hsp90 protein, geldanamycin 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 ketone²⁴.

It was proposed in our previous publication that 17-AG was similar to geldanamycin in binding to Hsp90, while the selective leaving groups at 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 beta-galactosidase to release 17-AG-C2, it regained the binding ability to Hsp90 and exhibited anticancer activity⁴⁴. 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, GA-2C-Gal, GA-2C 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 co-crystallized 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 kcal/mol and -11.9 kcal/mol, respectively, using the two different docking methods (Fig 2A). Similarly, 17-AG showed identical binding configuration to Hsp90 compared to geldanamycin which formed H-bond with amino acids Lys112, Lys58, Asp93, Thr184, and Asp54 in Hsp90 (Fig 2B). The binding energies of 17-

AG to Hsp90 were -34.1 kcal/mol (Affinity program) and -12.1 kcal/mol (Autodock program). However, the prodrug compound, GA-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 co-crystallized 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 (Fig 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 the Hsp90-geldanamycin complex (Fig 2D, 2E) with an additional hydrogen-bond interaction between Asp54 and the 17-amino group. The energy associated with this binding, as determined by the Affinity (Fig 2D) and Autodock (Fig 2E) programs, was -27.4 kcal/mol and -12.1 kcal/mol, respectively.

Conjugation of HuCC49 Δ CH2 to β -galactosidase

In order to deliver a drug activating enzyme (β -galactosidase) into tumors, we utilized the humanized anti-TAG-72 antibody (HuCC49 Δ CH2) for tumor targeting. Therefore, we conjugated HuCC49 Δ CH2 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 HuCC49 Δ CH2 using MBS (m-maleimidobenzoyl-N-hydroxysuccinimide ester) without affecting antigen binding affinity. Then the HuCC49 Δ CH2- β -galactosidase conjugate was generated upon combination of the activated proteins under nitrogen. The conjugate was purified through 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 (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* (Fig 3A). Therefore, LS174T cells were used as positive control, while SW-620 and HT-29 cells as negative control in the following experiments. To confirm that TAG-72 expression in tumors and normal tissues in 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 (Fig 3B).

Characterization of antibody-enzyme conjugates for specific TAG-72 binding

To test whether the conjugation process affects the HuCC49 Δ CH2 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 HuCC49 Δ CH2- β -galactosidase conjugate followed by blocking. Anti- β -galactosidase primary antibody and FITC-labeled secondary antibody was used to detect the 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 (Fig 4). Similarly, antibody-enzyme conjugate bound to human colon cancer tissues, while no binding was seen

in normal tissues. These results suggested that HuCC49 Δ CH2- β -galactosidase conjugate maintained the similar binding specificity and affinity as HuCC49 Δ CH2 for tumors expressing TAG-72.

Characterization of antibody-enzyme conjugates for β -galactosidase activity

To confirm that HuCC49 Δ CH2- β -galactosidase had the similar enzymatic activity as β -galactosidase when it bound to TAG-72 in cancer cells, Elisa assay was performed in LS174T (TAG-72 positive) and on negative SW620 and HT-29 cancer cells (TAG-72 negative). Briefly, the PBS, free enzyme control or conjugate was incubated with different cells for one hour 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 (Fig 5). This result was in agreement with the previous observation that TAG-72 only highly expressed on LS174T cells, but not on SW620 and HT-29 cells. These results suggested that HuCC49 Δ CH2- 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 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 using Mass Spectrometry. The spectra of GA-C2-Gal from PBS reaction buffer (Fig 6A) clearly showed 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 (Fig 6B). These data indicated that 17-AG-C2-Gal was cleaved by antibody-enzyme conjugate to release 17-AG-C2.

17-AG-C2-Gal prodrug activation for anticancer activity *in vitro*

To test if antibody-enzyme conjugate (HuCC49 Δ CH2- β -galactosidase) would bind to cancer cells (LS174T) to activate 17-AG-C2-Gal prodrug for anticancer activity, we utilized MTS assay to measure the cytotoxicity of prodrug and active drug in TAG-72 positive cancer cells LS 174T. The result showed that IC_{50} of geldanamycin (GA) in LS174T cells were 0.45 μ M; while IC_{50} of 17-AG-C2-Gal was greater than 25 μ M (concentrations above 25 μ M were not tested). After LS174T cells were incubated with antibody-enzyme conjugate (2 units) for one hour followed by extensive wash process, the 17-AG-C2-Gal prodrug was added to the cells, its activation and subsequent anticancer activity was measured with MTS assay. The results showed that combination of 17-AG-C2-Gal prodrug (0.01–25 μ M) 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 μ M, which was more than 25-fold lower than that of prodrug alone (Fig 7). However, the prodrug (0.01 – 25 μ M) 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 HuCC49 Δ CH2- β -galactosidase binds to the LS174T cells and activates 17-AG-C2-Gal prodrug for 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 HuCC49 Δ CH2- β -galactosidase conjugate) will bind to Hsp90 and induce Hsp90 client protein degradation (such as AKT), we used western blot to determine the total AKT levels after 2 and 10 μ M 17-AG-C2-Gal prodrug treatment for 24 hrs (following incubation with antibody-enzyme conjugate or β -galactosidase

for one hour and thorough wash) in LS174T cells. 17-AG-C2-Gal treatment following 1 hour of incubation with antibody-enzyme conjugate significantly induced AKT degradation (up to 70%). This effect was comparable to the effect of 17-AG which caused 70–80% AKT degradation 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 evidenced by similar Akt: β -actin ratio (>90%) compared to 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 (Fig 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 enzymatically cleaved product (17-AG-C2) 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 inactive prodrug 17-AG-C2-Gal to release an active drug and thus achieve the controlled drug activation in cancer. This result was consistent with the previous publications from other labs^{35,46}.

HuCC49 Δ CH2- 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-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 HuCC49 Δ CH2 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 \AA structure of human Hsp90 co-crystallized with geldanamycin (PDB: 1YET) and the 2.2 \AA structure of open human Hsp90 (PDB: 1YES)²⁴ were obtained from the RCSB Protein Data Bank. The cis-amide isomers of the geldanamycin, 17-AG, GA-C2, GA-C2-Gal structures were constructed and assigned the correct atom type and bond order, from the co-crystallized geldanamycin structure. The trans-GA-C2-Gal structure was generated from the 17-azetidiny-17-demethoxygeldanamycin structure obtained from the Cambridge Structural Database⁴⁷.

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, USA). 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 \AA radius subset encompassing the ligand-binding domain, using the co-crystallized geldanamycin structure as a reference ligand and the potentials and charges of the Hsp90-ligand complex were initially corrected using CVFF⁵⁰. The Grid docking Affinity job was run using the default parameters for the grid setup, 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 based on 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 8Å, 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 forcefield based minimization to a convergence of 0.001kcal/mol, the potentials and charges of the Hsp90-ligand complex were corrected using CHARMM force field parameters as implemented in the CHARMM program⁵¹.

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, and 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 charges⁵³ 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 (N-succinimidyl S-acetylthioacetate, Pierce, Rockland, IL, USA) to generate protected sulfhydryl groups. The protected sulfhydryl groups were then released by hydroxylamine (Pierce, Rockland, IL, USA). Four mg β-galactosidase was combined with 0.05mg of SATA in 500μL PBS buffer. The resulting solution was stirred for 1 hour at room temperature. The modified enzyme then resolved from reagent through a 1 × 13-cm G-25 medium column equilibrated with PBS buffer. Mix 1.0mL modified enzyme solution with 100μL hydroxylamine deacetylation solution followed by incubation at room temperature for two hours. The final sulfhydryl modified enzyme was desalted through G-25 column.

Antibody modification

Five mg HuCC49ΔCH2 were combined with 0.3mg MBS (m-maleimidobenzoyl-N-hydroxysuccinimide ester, Pierce, Rockland, IL, USA) in 500μL PBS buffer. This was allowed to react with stirring for 1hour. The modified antibody was purified through 1×13cm 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 pH7.4. The reaction was kept under anaerobic condition (under N₂) and stirring at room temperature for 2 hours. The concentrated conjugate was purified from aggregate, unreacted enzyme or antibody, and small molecules by chromatography on 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% non-essential amino acid and Penicillin (100 units/mL)/Streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO₂ 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.

Immunohistochemistry

Paraffin embedded tissue was sectioned at 4 microns and placed on positively charged slides. Slides with specimens were then placed in a 60 °C oven for 1 hour, cooled, and deparaffinized and rehydrated through 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 hours before the immunohistochemistry detection. The HuCC49 Δ CH2- 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 hrs. The staining was examined under microscope after extensive washing.

ELISA for β -galactosidase enzyme activity detection

0.1 million SW620, HT-29 and LS174T cells were seeded in the 96 well plates. Then the cells were allowed to grow for 24 hrs. The bulk of the culture medium was decanted and the residual medium was removed by blotting the plate on paper cloth. 100 μ L of the diluted antibody-enzyme conjugate was added to the cells followed by incubation at 37 degree for 1 hour. The plate was washed thoroughly for three to five times. 150 μ L fresh ONPG (o-Nitrophenyl- β -D-galacopyranoside, Pierce, Rockland, IL, USA) solution was added as substrate, followed by incubation at 30°C for 30mins or until appropriate color develops. Then 5 μ L 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 HuCC49 Δ CH2- β -galactosidase were incubated for 3 hours 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, USA). 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 HuCC49 Δ CH2- β -galactosidase to exhibit anticancer activity

A total of 2,000–5,000 LS174T cells were cultured in 96-well plate for 24 hours. 100 μ L PBS, PBS staining buffer containing enzyme only or antibody-enzyme conjugate were added to the cell culture and incubated for 1hour. Then the cells were thoroughly washed for three to five times. Prodrug 17-AG-C2-Gal (0, 0.01, 0.05, 0.1, 0.5, 1, 2.5 and 25 μ M) was administered to the cells and incubated for 72 hours. After 3 days, MTS (tetrazolium [3-(4,5-dimethylthiazol-2-yl)]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (2 mg/ml) and phenazine methosulfate (PMS, 25 μ M) were added directly to the cell culture and incubated for 2 hours 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

50–70% confluent LS174T cells were quickly washed and incubated with free β -galactosidase enzyme or HuCC49 Δ CH2- β -galactosidase conjugate in PBS containing 2% FBS for 1 hour. The cells were thoroughly washed and incubated with 10 μ M 17-AG-C2-Gal for 24 hours. All cells were collected in media, centrifuged and washed twice with phosphate-buffered saline (PBS), and lysed in cell lysis buffer (50mM PH 7.6 Tris-HCl, 250mM NaCl, 5mM EDTA, 2mM Na₃VO₄, 50mM NaF) with 1% protease inhibitor cocktail (P8340, Sigma) for 30 min

on ice, and sonicated three times for 20 seconds. Protein concentration was determined using BCA protein assay method (PIERCE). The cell lysates were incubated with 2x SDS loading buffer and boiled for 5 minutes. 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 hr. The membrane was washed 4 times with TBS-T for 15 minutes, and then incubated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA) for 1 hr 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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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-demethoxygeldanamycin; 17-AG-C2-Gal, 17-(amino)-17-demethoxygeldanamycin-C2-galactose..

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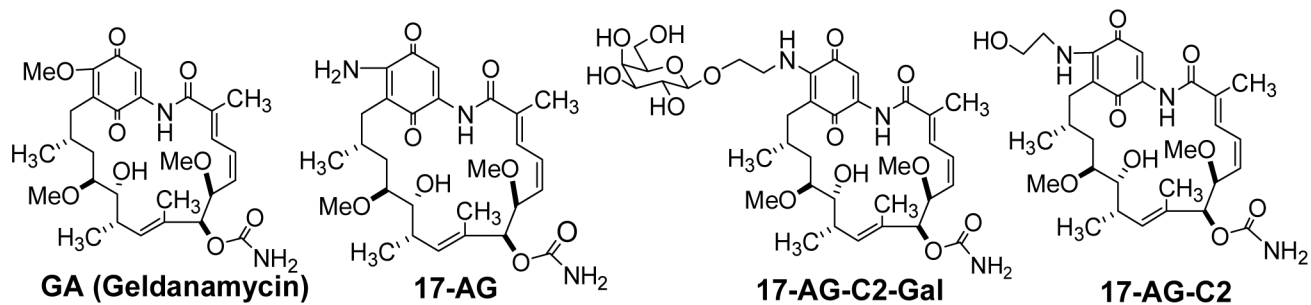


Fig 1. Structure of geldanamycin (GA), 17-(amino)-demethoxygeldanamycin (17-AG), prodrug 17-AG-C2-Gal, and active drug 17-AG-C2.

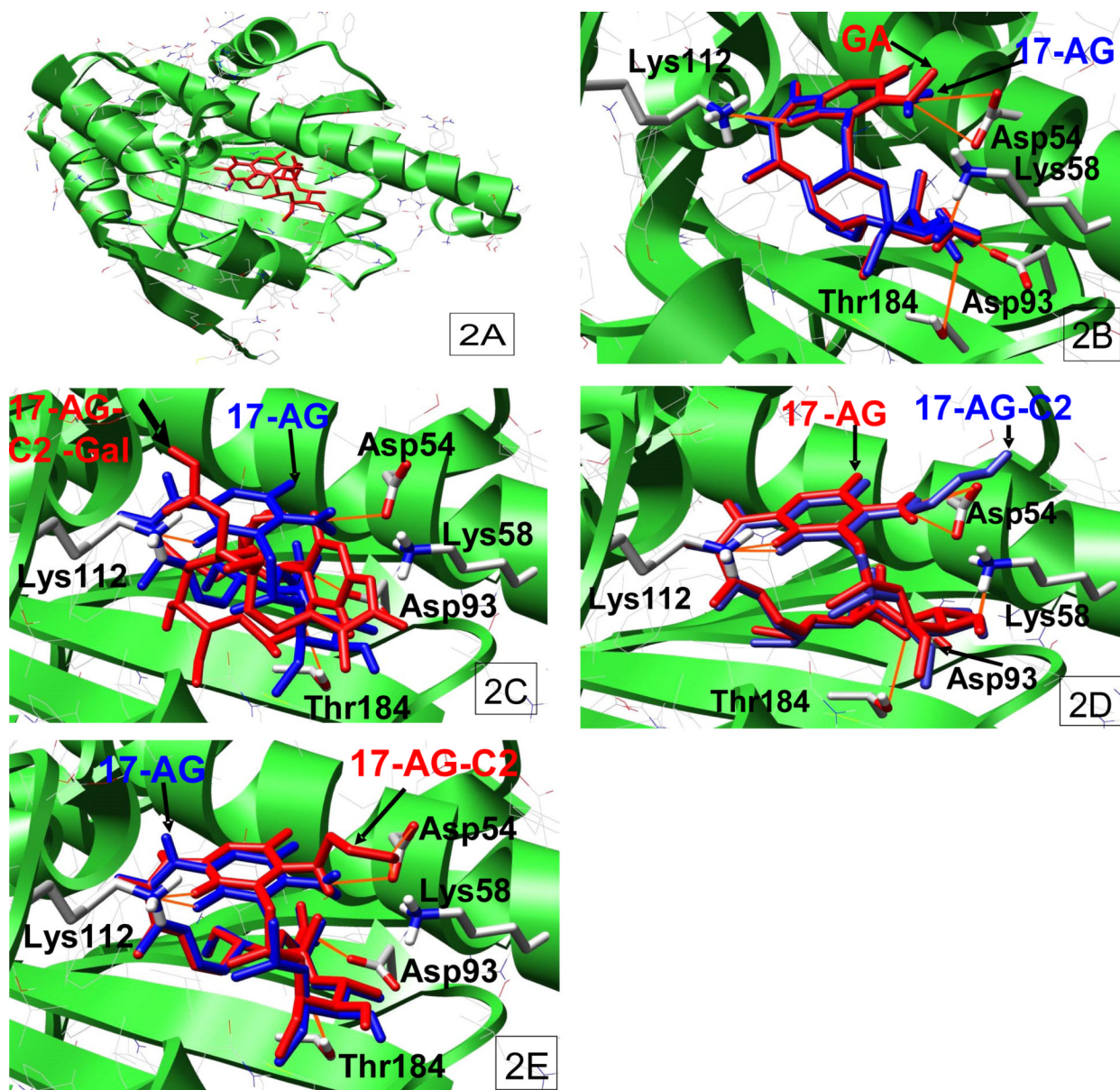
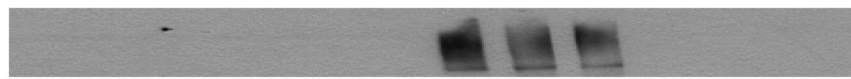


Fig 2. Molecular Docking of Hsp90 binding of geldanamycin (GA), 17-(amino)-demethoxygeldanamycin (17-AG), prodrug 17-AG-C2-Gal, and active drug 17-AG-C2. **2A**, Geldanamycin binds to Hsp90 (Autodock); **2B**, GA and 17-AG binds to Hsp90 (Affinity); **2C**, 17-AG and 17-AG-C2-Gal bind to Hsp90 (Autodock);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

(A) *In vitro* cell culture



SW620 MCF7 LS174T HT29

(B) *In vivo* LS174T xenograft



Heart Lung Spleen Kidney Stomach Tumor Colon Liver

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

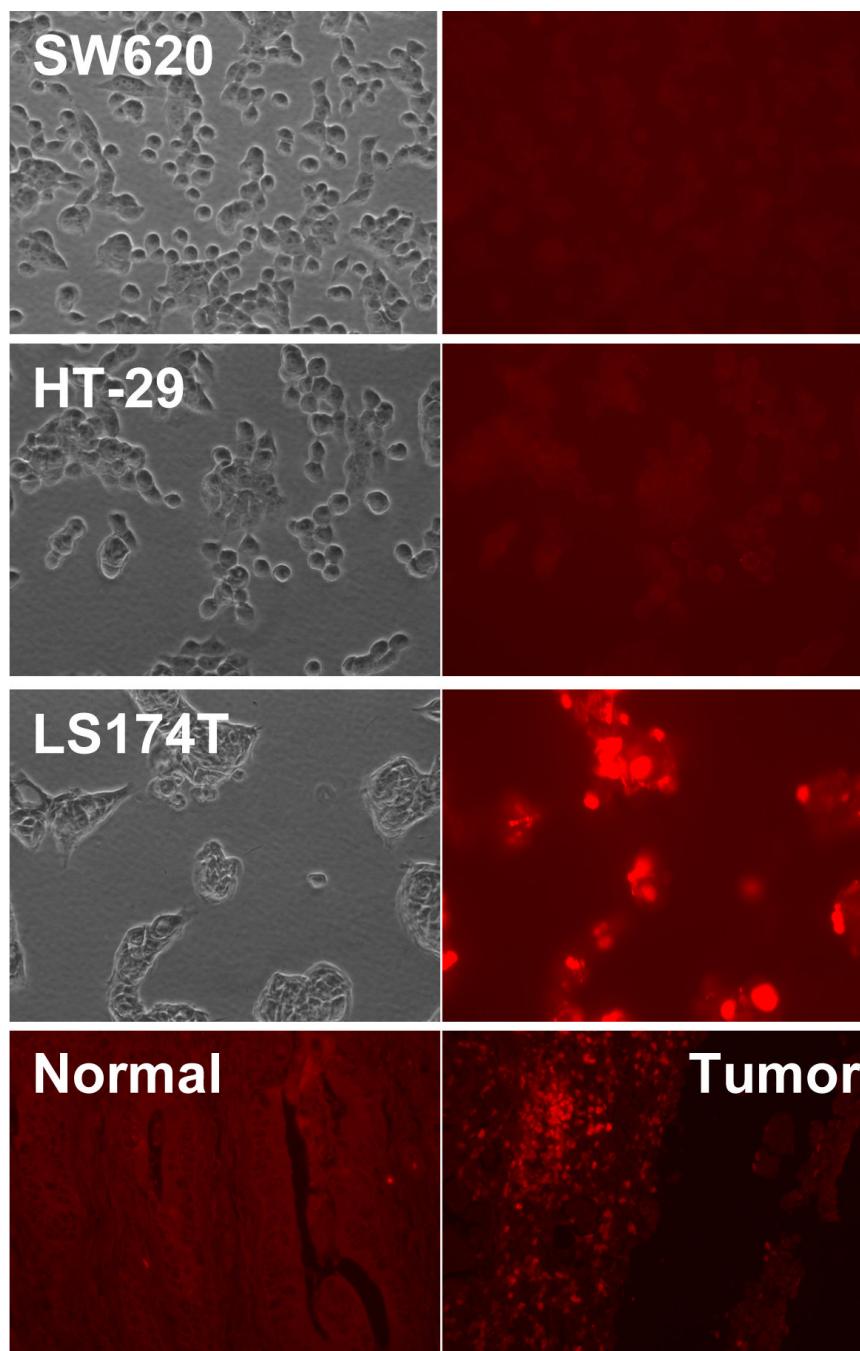


Fig 4. Antigen binding capacity of HuCC49 Δ CH2- β -galactosidase in SW620, HT-29, LS174T cells, and human colon cancer tissues and normal tissues. The cells or tissues were treated with antibody-enzyme conjugate. The bound antibody-enzyme conjugate was detected with primary antibody against β -galactosidase and FITC-labeled secondary antibody.

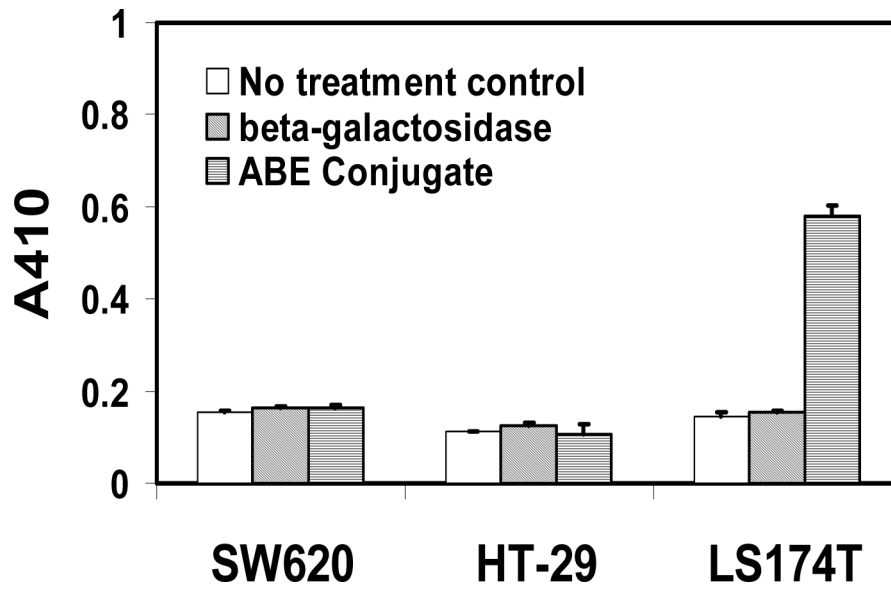


Fig 5. Enzymatic activity of HuCC49 Δ CH2- β -galactosidaseconjugate. SW620, HT-29, and LS174T cells were seeded in 96-well overnight. PBS as no treatment control, staining buffer containing β -galactosidase, and HuCC49 Δ CH2- β -galactosidaseconjugate incubated the cells followed by extensive wash. The enzymatic activity was determined by the color development of the β -galactosidase substrate (o-Nitrophenyl- β -D-galacopyranoside, ONPG) at 410nm.

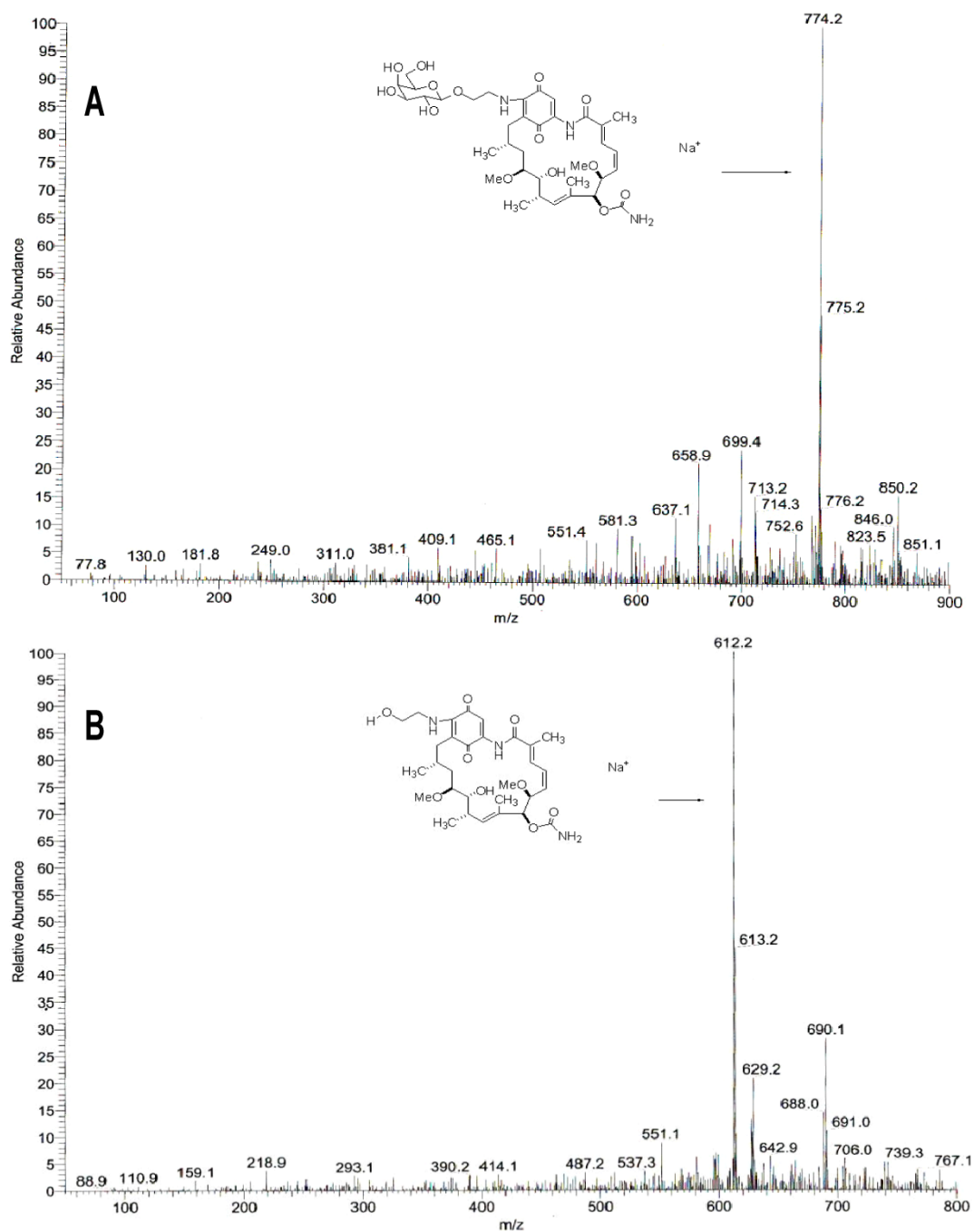


Fig 6. Confirmation of 17-AG-C2-Gal prodrug (A) and active drug 17-AG-C2 (B) by HuCC49 Δ CH2- β -galactosidase using Mass Spectrometry.

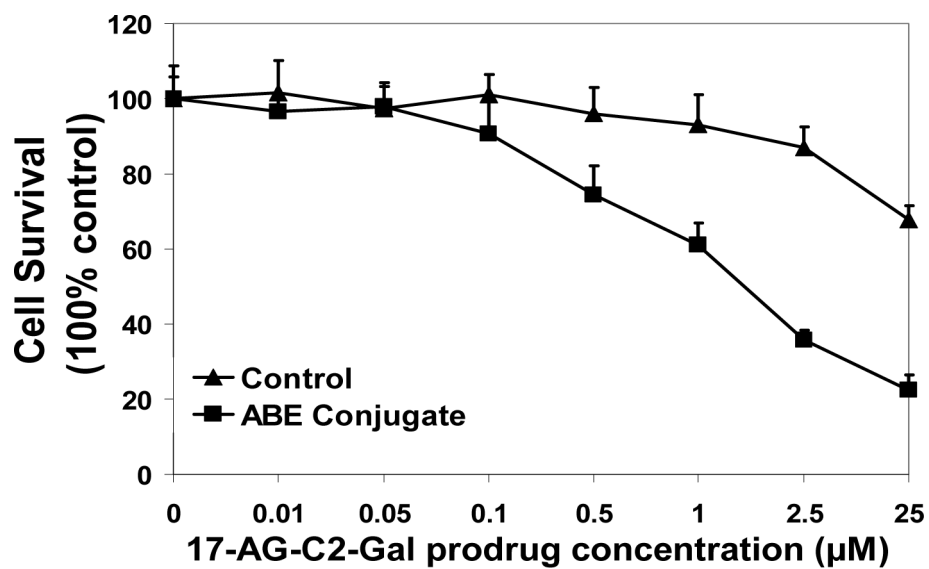
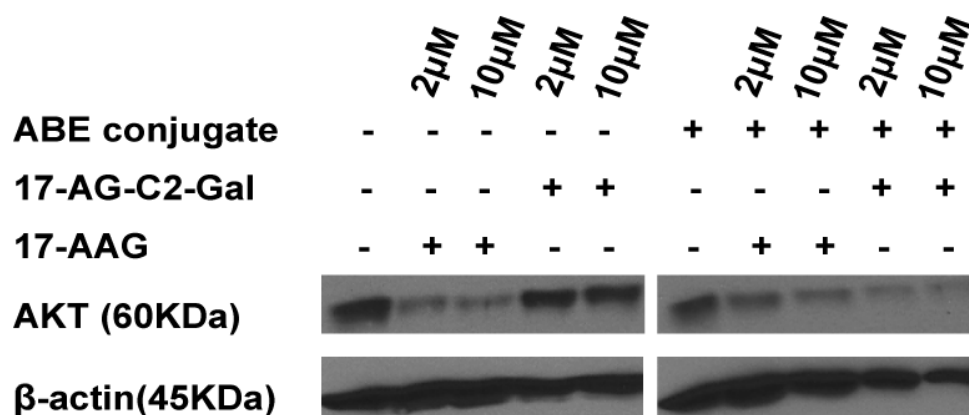
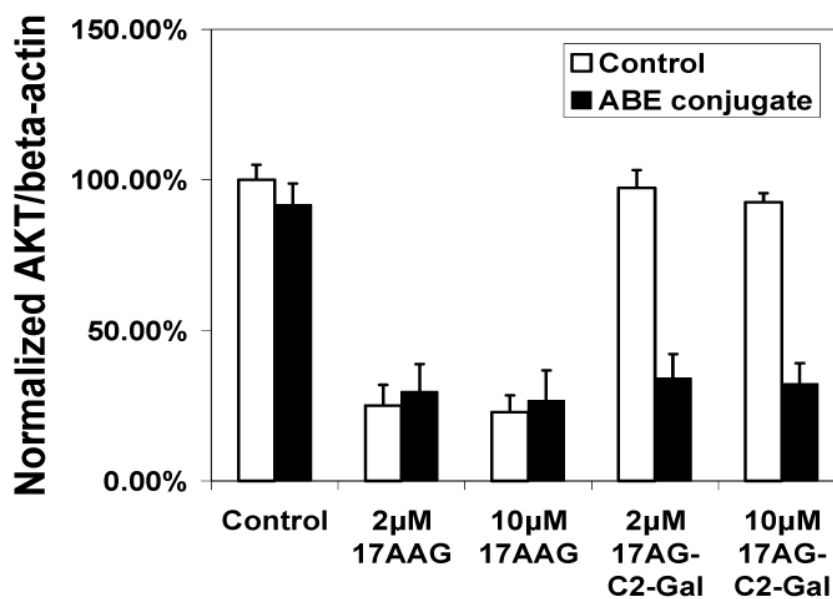


Fig 7. HuCC49 Δ CH2- β -galactosidase conjugate binds to LS 174T cells and activates 17-AG-C2-Gal prodrug for cytotoxicity in MTS assay.

(A)



(B)

**Fig 8.**

HuCC49 Δ CH2- 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.