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A Humanized Immunoenzyme with Enhanced Activity for Glucuronide Prodrug Activation in the Tumor Microenvironment

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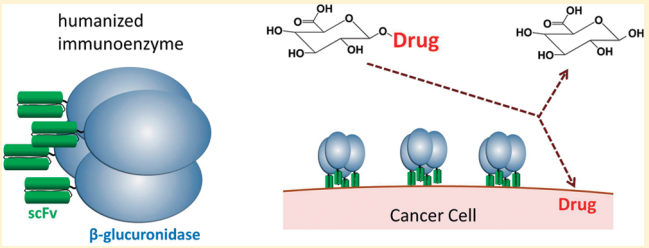
Supporting Information

ABSTRACT: Antibody-directed enzyme prodrug therapy (ADEPT) utilizing β-glucuronidase is a promising method to enhance the therapeutic index of cancer chemotherapy. In this approach, an immunoenzyme (antibody-β-glucuronidase fusion protein) is employed to selectively activate anticancer glucuronide prodrugs in the tumor microenvironment. A major roadblock to the clinical translation of this therapeutic strategy, however, is the low enzymatic activity and strong immunogenicity of the current generation of immunoenzymes. To overcome this problem, we fused a humanized single-chain antibody (scFv) of mAb CC49 to S2, a human β-glucuronidase (hβG) variant that displays enhanced catalytic activity for prodrug hydrolysis. Here, we show that hcc49-S2 displayed 100-fold greater binding avidity than hcc49 scFv, possessed greater enzymatic activity than wild-type hβG, and more effectively killed antigen-positive cancer cells exposed to an anticancer glucuronide prodrug as compared to an analogous hβG immunoenzyme. Treatment of tumor-bearing mice with hcc49-S2 followed by prodrug significantly delayed tumor growth as compared to hcc49-hβG. Our study shows that hcc49-S2 is a promising targeted enzyme for cancer treatment and demonstrates that enhancement of human enzyme catalytic activity is a powerful approach to improve immunoenzyme efficacy.



INTRODUCTION

Conventional chemotherapy represents an important treatment option for advanced cancers, but the limited therapeutic index of most antineoplastic drugs can cause normal tissue toxicity and severe side effects. Antibody-directed enzyme prodrug therapy (ADEPT) seeks to increase tumor selectivity and reduce undesirable side effects of chemotherapy by targeting an antibody–enzyme conjugate (immunoenzyme) to cancer cells for preferential activation of nontoxic anticancer prodrugs in the tumor microenvironment.[1−3] We previously reported that immunoenzymes formed by chemical linkage of anticancer antibodies and *E. coli* β-glucuronidase could selectively activate glucuronide prodrugs at antigen-positive cancer cells, enhance antitumor immune responses, and produce long-term tumor regression in syngenic and xenograft cancer models.[4−9] However, a major impediment to clinical translation of immunoenzymes containing microbial enzymes is their high immunogenicity in patients, which dramatically hinders tumor targeting and prevents effective therapy.[10−12]

Immunoenzymes incorporating human enzymes should be nonimmunogenic but are limited by the relatively low activity of many human enzymes as compared to their bacterial counterparts and the possibility of systemic prodrug activation by endogenous enzymes present in patients.[13] Human β-glucuronidase (hβG) is a good target for creating human immunoenzymes[14−16] because hβG is largely sequestered in lysosomes, which limits hydrolysis of hydrophilic glucuronide prodrugs that do not readily diffuse across the plasma membrane of cells,[17,18] resulting in minimal in vivo prodrug toxicity.[19] hβG, however, displays relatively low enzymatic activity as compared to bacterial βG.[20,21] To preserve the advantages of human enzymes in ADEPT while overcoming the limitation of limited catalytic activity, we previously employed directed molecular evolution to create human enzyme variants with enhanced catalytic activity against anticancer prodrugs.[21] Here, we fused either wild-type hβG or a hβG variant (S2) with enhanced catalytic activity to a humanized single-chain antibody fragment (scFv) of CC49,[22] which binds the TAG-72 antigen expressed on many human tumor cells.[23,24] The humanized immunoenzymes retained enzymatic activity and bound antigen-positive cancer cells

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with high avidity. Importantly, hcc49-S2 in combination with an anticancer prodrug displayed significantly greater antitumor activity against human colorectal xenografts than an equivalent immunoenzyme containing wild-type hβG, demonstrating that enhancement of human enzyme activity can improve the therapeutic efficacy of ADEPT. Our results suggest that creation of highly active humanized immunoenzymes is feasible and may help overcome limitations of using microbial enzymes for ADEPT.

EXPERIMENTAL PROCEDURES

Cell Lines. BALB/3T3 fibroblasts (ATCC CCL-163) were grown in Dulbecco's Modified Eagle's Medium (high glucose) supplemented with 10% bovine serum, 2.98 g/L HEPES, 2 g/L $NaHCO_3$, 100 U/mL penicillin, and 100 μg/mL streptomycin. EJ human bladder carcinoma cells,[25] LS174T human colon adenocarcinoma (ATCC CCL-188), MCF-7 human breast adenocarcinoma (ATCC HTB 22), and HT-29 colorectal adenocarcinoma (ATCC HTB-38) cells were cultured in RPMI-1640 medium containing the same supplements. The cells were free of mycoplasma as determined by PCR.

Reagents and Antibodies. Fluorescein di-β-D-glucuronide (FDGlcU), ELF-97 alcohol and ELF-97 β-D-glucuronide (ELF = enzyme-labeled fluorescence) were from Molecular Probes (Eugene, OR, USA).[26] CPT-11, ovalbumin (A-2512), bovine serum albumin (A-7906), and catalase from bovine liver (C-3155) were from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-6xHis was from LTK BioLaboratories (Bade, Taiwan). Biotinylated anti-HA antibody was from Vector Laboratories (Burlingame, CA, USA). Rhodamine-conjugated streptavidin was from Jackson ImmunoResearch Laboratories (Westgrove, PA, USA). Monoclonal antibody (7G8) against hβG has been described.[27] 7G8 was directly labeled with FITC as described.[28] HAMG was synthesized as described.[29]

Immunoenzymes. To construct a tumor targeting immunoenzyme, the variable regions of the heavy chain (V_H) and light chain (V_L) of a humanized single-chain antibody fragment of CC49 (hcc49 scFv)[22] were assembled with synthetic oligonucleotides P1 to P10 and P18 to P26 for V_H and P10 to P17 and P26 to P34 for V_L (Supporting Information Table 1) by a primerless PCR gene-assembly method.[30] PCR was carried out as follows: 95 °C for 5 min; 22 cycles at 95 °C for 30 s, 63 to 53 °C touchdown for 1 min (decrease 1 °C every 2 cycles), 72 °C for 1 min; 16 cycles at 95 °C for 30 s, 53 °C for 1 min, 72 °C for 1 min; 72 °C for 10 min. The V_H fragment was amplified with primers P1 and P36, and the V_L fragment was amplified with primers P34 and P35 by standard PCR. The V_H and V_L fragments were joined and amplified as hcc49 scFv using primers P1 and P34, which contain Sfi I and Xba I restriction sites, respectively. The PCR products were digested with Sfi I and Xba I and then subcloned into pLHCX-L6-hβG[31] to generate pLHCX-hcc49-hβG-myc-His. The hβG and S2 genes in pLNCX-hβGs-eB7 and pLNCX-S2-eB7[21] were ligated into the *Not*I and *Sal*I sites in pLHCX-hcc49-hβG-myc-His to remove the myc tag and generate pLHCX-hcc49-S2-His and pLHCX-hcc49-S2-His. pLHCX-αDNS-S2-His was generated by transferring the antidansyl scFv gene from pLNCX-DNS-eB7[19] in place of the hcc49 scFv gene present in pLHCX-hcc49-S2-His.

Production of Recombinant Proteins. To generate His-tagged immunoenzyme producer cells, plasmid DNA was co-transfected with pVSVG into GP293 cells (Clontech, Mountainview, CA) to produce recombinant retroviral particles. At 2 days after transfection, the culture medium was added to 3T3 cells in the presence of 8 μg/mL Polybrene. Stable cell lines were selected in medium containing 0.5 mg/mL G418 (Calbiochem, San Diego, CA). Recombinant his-tagged proteins were purified from the stable 3T3 fibroblast lines as described.[31]

Western Blot Analysis. His-tagged recombinant hcc49 scFv and immunoenzymes were boiled in reducing SDS buffer, electrophoresed on a 12.5% SDS-PAGE, and transferred to PVDF membranes. Membranes were sequentially stained with rabbit anti-His or rabbit anti-hβG antibody followed by HRP-conjugated secondary antibody. Bands were visualized by ECL detection (Pierce, Rockford, IL, USA).

HPLC Size Exclusion Analysis. Samples (10 μL, 1 mg/mL) were injected into an Aligent Bio SEC-5 column (300 × 7.8 mm, 300 Å) and separated at 1 mL/min in 150 mM sodium phosphate buffer, pH 7. Protein peaks were detected at 214 nm. Standard proteins included ovalbumin (44 kDa), bovine serum albumin (66 kDa), bovine liver catalase (250 kDa), and recombinant human β-glucuronidase (320 kDa).

Enzyme Assays. The enzyme activities of recombinant wild-type hβG, the hβG variant S2, hcc49-hβG, hcc49-S2, and αDNS-S2 were measured in triplicate with 0.1 mM ELF-97 β-D-glucuronide at 37 °C for 30 min at pH 7 in βG reaction buffer (50 mM bis-tris, 50 mM triethanol amine, 100 mM acetic acid, 100 ng/mL bovine serum albumin). The reaction was terminated by adding an equal volume of stop buffer (2 M tris-HCl, 0.8 M sodium bicarbonate, pH 8). The fluorescence of ELF-97 alcohol was measured at excitation/emission wavelengths of 355/555 nm in a Gemini EM microplate spectrofluorometer (Molecular Device, Sunnyvale, CA). To measure enzyme kinetic values, ELF-97 β-D-glucuronide (2 mM) in pH 4.5 or pH 7 βG reaction buffer was diluted 1:1 with defined concentrations of recombinant proteins. Fluorescence was immediately measured under thermal control at 37 °C for 8−10 min. The measurement was repeated using the same amount of enzyme for different concentrations of substrate over an optimal range. The acquired readings were converted to product concentration by pre-established standard curves. Double reciprocal plots were used to determine K_m and k_{cat}. Kinetic assays were performed at least 3 times and mean values were calculated.

Surface Plasmon Resonance. The binding kinetics of immunoenzymes and CC49 scFv were measured on a BIAcore (GE Lifesciences Uppsala, Sweden). Bovine submaxillary gland mucin was immobilized on the CM5 chips by amine coupling. BSA was immobilized as a negative control. Binding analysis was performed as described.[32]

Flow Cytometer Analysis. Cells were stained with immunoenzymes followed by FITC-conjugated mouse anti-hβG (7G8-FITC).[21] For competition assays, hcc49-S2 was mixed with various amounts of CC49 antibody[33] before staining MCF-7 cells. The surface immunofluorescence of 10^4 viable cells was measured with a FACScaliber flow cytometer (Becton Dickinson, Mountain View, CA, USA) and fluorescence intensities were analyzed with Flowjo (Tree Star Inc., San Carlos, CA, USA).

Immunofluorescence Staining. MCF-7 cells (5×10^5) were cultured overnight in 12-well plates. hcc49-S2 or αDNS-S2 (2 μg/well) was added to the cells at 37 °C for 1 h. After washing 3 times with PBS, the cells were incubated in complete medium at 37 °C. At defined times, the cells were washed with PBS and fixed with 2% paraformaldehyde in PBS for 15 min at room temperature. After washing, the cells were stained with 4 μg/mL 7G8-FITC for 45 min at 4 °C. The cells were viewed under a phase contrast microscope and a digital fluorescence microscope.

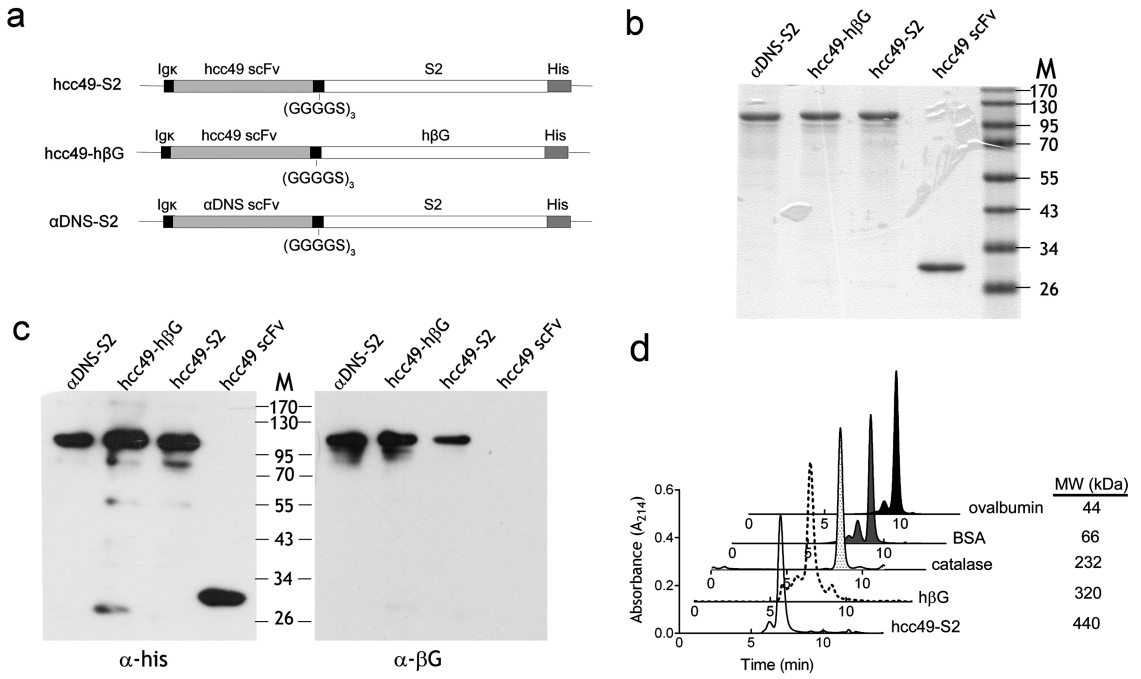

Figure 1. Analysis of purified scFv and immunoenzymes. (a) Schematic of immunoenzyme constructs. (b) Reducing SDS PAGE showing the Coomassie Blue staining of hcc49 scFv and immunoenzymes. (c) Western blots stained with anti-His (left) or anti-hβG (right) antibodies. (d) HPLC size exclusion chromatographs of ovalbumin, bovine serum albumin, catalase, hβG, and hcc49-S2.

^{3}H-Thymidine Incorporation Assay. hcc49-hβG, hcc49-S2, or αDNS-S2 (1 μg/well) were added to LS174T, MCF-7, or HT-29 cells at 37 °C for 1 h. After washing twice with PBS, graded concentrations of *p*-hydroxyaniline mustard (pHAM) or *p*-hydroxyaniline mustard glucuronide (HAMG) were added in triplicate at pH 6.8 for 48 h at 37 °C. The cells were then pulsed for 16 h with ^{3}H-thymidine (1 μCi/well). Results are expressed as percent inhibition of ^{3}H-thymidine incorporation into cellular DNA in comparison to untreated cells.

Radioiodination. Immunoenzymes were labeled with ^{131}I using a variation of the original iodogen method.[34] In brief, 120 μCi sodium [^{131}I]-iodide in 100 μL reaction buffer (0.1 M Na_2HPO_4, pH 6.5) was added to an iodogen precoated tube (Pierce Chemical Co.) followed by 50 μL hcc49-S2 or αDNS-S2 (2 mg/mL in PBS) and incubated for 10 min at room temperature. The reaction was terminated by transferring the reaction mixture to an Eppendorf tube and adding 50 μL reaction buffer. The radiochemical purity of ^{131}I-hcc49-S2 or ^{131}I-αDNS-S2 was 90−92% as measured by TLC using silica gel (Silica gel 60; Merck) and the specific activity was 57−66 Ci/mmol.

In Vivo Imaging and Biodistribution of Radiolabeled Immunoenzymes. BALB/c nu/nu mice bearing LS174T tumors (150−200 mm^3) in their right flank were i.v. injected with 100−115 μCi ^{131}I-hcc49-S2 or ^{131}I-αDNS-S2. The mice were killed after 24, 48, 72, and 96 h, and the radioactivity in the tissues was measured in a multichannel gamma counter. Whole-body scintigraphy of anesthetized mice was performed at 5 and 48 h with a gamma camera (Siemens ECAM+DHC) using a pinhole collimator.

In Vivo Pharmacokinetics. To determine the pharmacokinetics of immunoenzymes, Beige/SCID mice bearing LS174T tumors (150−200 mm^3) in their right flank were i.v. injected with 100 μg hcc49-hβG or hcc49-S2. Blood samples were collected from the tail vein of the mice or by retro-orbital punctuation. Plasma was prepared by centrifugation (5 min, 12 700 × *g*) and further diluted in pH 4.5 βG reaction buffer. The immunoenzyme levels in plasma were determined by measuring βG activity after capture of immunoenzymes on mucin-coated plates or by directly measuring βG activity. The initial and terminal half-lives of the immunoenzymes were estimated by fitting the data to a two-phase exponential decay model with *Prism 5* software (Graphpad Software, San Diego, CA, USA).

Imaging of Immunoenzyme Localization in Mice. BALB/c nu/nu mice (n = 3) bearing subcutaneous LS174T tumors (100−150 mm^3) in their right flank were i.v. injected with 200 μg immunoenzyme. After 2 or 48 h, mice were i.v. injected with 500 μg FDGlcU. After 30 min, mice were sacrificed and organs were excised. Images of tumors, liver, and muscles of mice were obtained on a Xenogen IVIS Spectrum imaging system.

In Vivo Antitumor Activity. Groups of BALB/c nu/nu mice (n = 8) bearing 50−100 mm^3 subcutaneous LS174T tumors in their right flank were intravenously injected with PBS or 500 μg immunoenzymes on days 0 and 8 and intravenously injected with three hourly doses of 70 mg/kg HAMG or PBS on days 1 and 10. Tumor sizes were measured every 2−3 days. Tumor volume was calculated according the formula: length × width × height × 0.5.

Haematological and Biochemical Evaluation. Groups of BALB/c nu/nu mice (n = 4−6) bearing ∼100 mm^3 subcutaneous LS174T tumors in their right flank were intravenously injected with PBS or 500 μg hcc49-S2 on day 0 and intravenously injected with three hourly doses of 70 mg/kg HAMG or PBS on day 1. Blood was collected in serum separation or blood collection tubes on day 8. Serum was obtained by leaving blood at room temperature for 30 min to clot and then centrifuged at 1000 × *g* at 4 °C for 10 min. Hepatic injury was assessed by measuring serum AST, ALT, and TBIL levels using a Fuji Dri-Chem 3500 biochemistry analyzer (Fujifilm, Japan). Hematological toxicity was determined by counting lymphocytes and red blood

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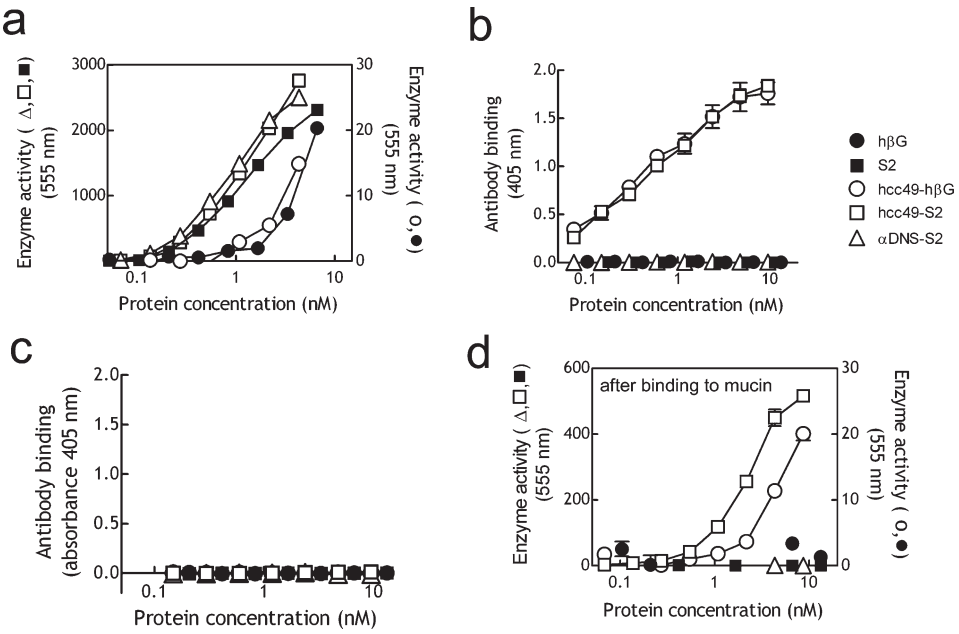

Figure 2. Enzyme and antibody-binding activity of immunoenzymes. (a) Enzyme activity of immunoenzymes and their parent enzymes was determined by measuring the fluorescence generated upon hydrolysis of ELF-97 β-D-glucuronide at pH 7. The left axis shows the activity of S2, hcc49-S2, and αDNS-S2, whereas the right axis displays the activity of hβG and hcc49-hβG. (b,c) Graded concentrations of the indicated recombinant proteins were incubated in mucin (b) or bovine-serum albumin-coated (c) microtiter plates. After washing, binding was determined by direct ELISA. (d) Graded concentrations of immunoenzymes and their parent enzymes were first incubated on mucin-coated plates. The plates were washed and bound βG enzyme activity was determined by measuring the fluorescence generated upon hydrolysis of ELF-97 β-D-glucuronide at pH 7. The left axis shows the activity of S2, hcc49-S2, and αDNS-S2, whereas the right axis displays the activity of hβG and hcc49-hβG. Error bars: SEM, $n = 3$.

cells with a Cell-Dyn hematology analyzer 3700 (Abbott Diagnostic Division, USA).

RESULTS

Expression and Purification of Humanized Immunoenzymes. A humanized cc49 scFv gene was fused to genes coding wild-type hβG or the enhanced activity hβG variant S2 to create hcc49-hβG and hcc49-S2, respectively (Figure 1a). The control immunoenzyme (αDNS-S2), with antigen-binding specificity for the chemical hapten dansyl, was also generated by fusing the antidansyl scFv and S2 genes. The immunoenzyme genes were inserted into a retroviral expression vector (pLHCX) to generate stable 3T3 fibroblast producer cell lines. Immunoenzyme proteins that were purified from the culture medium displayed the expected molecular sizes on a 12.5% SDS-PAGE (Figure 1b). Western blot analysis of purified hcc49 scFv and immunoenzymes with anti-His antibody (Figure 1c, left panel) or anti-hβG antibody (Figure 1c, right panel) showed that the immunoenzymes possessed both scFv and hβG moieties and displayed the expected molecular weights of approximately 110 kDa. Recombinant hcc49 scFv migrated with an apparent molecular weight of 30 kDa. HPLC size exclusion analysis of hcc49-S2 showed that it eluted before hβG and displayed an apparent molecular weight of ~440 kDa (Figure 1d), consistent with a tetrameric structure as found for wild-type hβG.[35]

Immunoenzymes Selectively Bind Antigen-Positive Cancer Cells with High Avidity. The immunoenzymes were characterized for antigen-binding activity and enzymatic activity. βG enzymatic activity was determined by incubating purified proteins with the βG substrate ELF-97 β-D-glucuronide. Hcc49-S2 displayed high enzymatic activity with kinetic properties that were similar to those of the S2 protein (Figure 2a, Table 1). Hcc49-hβG also possessed similar βG activity and kinetic properties to wild-type hβG. Both hcc49-hβG and hcc49-S2 immunoenzymes bound to bovine submaxillary gland mucin-coated 96-well microtiter plates,[36] whereas negligible mucin binding was observed for anti-DNS-S2 (Figure 2b). Binding appeared to be specific, because neither hcc49-hβG nor hcc49-S2 bound to BSA coated plates (Figure 2c). Surface plasmon resonance was used to quantify the binding kinetics of hcc49 scFv and hcc49 immunoenzymes. Hcc49 scFv displayed a K_D of $(4.4 \pm 1.2) \times 10^{-8}$ M (Table 2), which is similar to murine CC49 scFv.[32] The hcc49 immunoenzymes, on the other hand, displayed stronger binding avidity with K_D values of $\sim 4 \times 10^{-10}$ M, likely due to the attachment of four scFv molecules to each βG tetramer. βG activity was also examined after immunoenzymes were allowed to bind to mucin. βG activity of hcc49-S2 was about 20-fold higher than that of hcc49-hβG after binding to mucin-coated plate (Figure 2d). The enhanced enzymatic activity of hcc49-S2 reflects the greater βG activity of S2 (Figure 2a). Taken together, the data show that

Table 1. Kinetic Parameters of Proteins at pH 7.0[a]

enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
S2	0.050 ± 0.006	60 ± 3	1220 ± 160
αDNS-S2	0.055 ± 0.029	53 ± 9	1120 ± 410
Hcc49-S2	0.061 ± 0.032	57 ± 10	1050 ± 360
hβG	2.6 ± 0.57	49 ± 13	20 ± 10
Hcc49- hβG	4.3 ± 0.30	64 ± 13	14 ± 3

[a] Results represent mean values of triplicate determinations ± SD. The substrate was ELF-97 β-D-glucuronide.

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Table 2. Kinetic Constants for Binding of Humanized Proteins to Immobilized Bovine Submaxillary Mucin[a]

	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (M)
Hcc49 scFv	$(6.01 \pm 1.62) \times 10^4$	$(2.54 \pm 0.48) \times 10^{-3}$	$(4.41 \pm 1.21) \times 10^{-8}$
Hcc49-S2	$(4.05 \pm 0.05) \times 10^5$	$(1.80 \pm 0.08) \times 10^{-4}$	$(4.45 \pm 0.20) \times 10^{-10}$
Hcc49-hβG	$(3.36 \pm 0.08) \times 10^5$	$(1.06 \pm 0.30) \times 10^{-4}$	$(3.19 \pm 0.95) \times 10^{-10}$

[a] Results represent mean values of triplicate determinations ± SD.

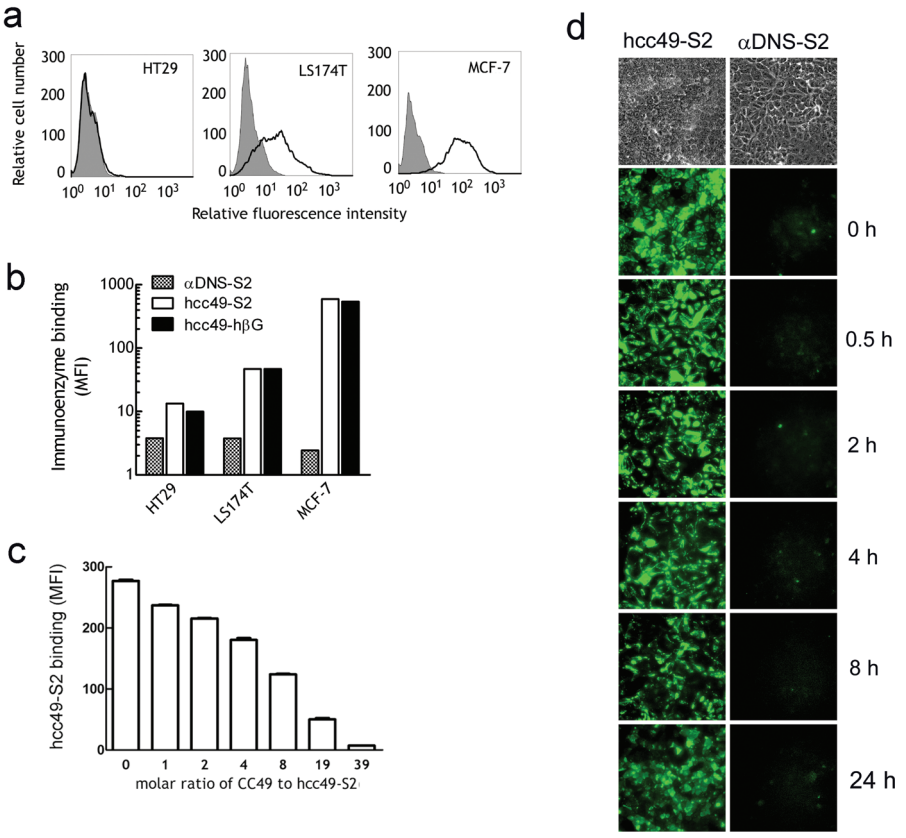

Figure 3. Binding of hcc49-S2 and hcc49-hβG to cancer cells. (a) mAb CC49 (solid line) or isotype control mAb 7G8 (gray filled) were incubated with HT29, LS174T, or MCF-7 cells followed by FITC-labeled goat antimouse second antibody. The results show the fluorescence of 10 000 live cells measured by flow cytometry. (b) HT29, LS174T, and MCF-7 cells were incubated with αDNS-S2 (shaded bars), hcc49-S2 (white bars), or hcc49-hβG (black bars) followed by FITC-labeled anti-hβG antibody before the fluorescence of 10 000 live cells was measured on a flow cytometer. (c) Increasing amounts of CC49 antibody were added with a fixed amount of hcc49-S2 to compete immunoenzyme binding. The binding of hcc49-S2 to MCF-7 cells was determined by flow cytometry. Bars, SEM ($n = 3$). (d) MCF-7 cells were incubated with hcc49-S2 (left panels) or αDNS-S2 (right panels), washed, and then incubated in complete medium at 37 °C. Cells were washed and fixed at the indicated times and then stained with anti-hβG-FITC conjugate to visualize immunoenzyme on the cells.

hcc49-S2 displayed antigen-binding activity with enhanced enzymatic activity as compared to hcc49-hβG.

To examine the binding ability of the immunoenzymes on tumor cell lines, we first utilized mAb CC49[37] to measure TAG-72 expression levels on HT29, LS174T, and MCF-7 cancer cell lines. Figure 3a shows that HT29 cells expressed undetectable levels of TAG-72, LS174T cells expressed moderate levels, and MCF-7 cancer cells expressed high levels of TAG-72, in agreement with previous reports.[38,39] The binding of immunoenzymes to these cancer cells was then examined by FACS analysis. hcc49 immunoenzymes bound HT29 cells at low background levels but selectively bound TAG-72 positive LS174T and MCF-7 cells (Figure 3b). In addition, soluble mAb CC49 competed with hcc49-S2 binding to MCF-7 cells in a dose-dependent fashion (Figure 3c), confirming that murine CC49 antibody and hcc49-S2 bind the identical epitope on TAG-72. To examine the persistence of hcc49-S2 binding to TAG-72 positive cells, MCF-7 cells were incubated with saturating concentrations of hcc49-S2 or αDNS-S2, washed, and then incubated at 37 °C for various times before surface immunoenzyme was detected by anti-hβG-FITC staining. Figure 3d shows that hcc49-S2 was clearly visible on MCF-7 cells even after 24 h, indicating prolonged retention of immunoenzyme on TAG-72 positive cells.

hcc49-S2 Can Effectively Activate a Prodrug at Antigen-Positive Cancer Cells. To examine if the immunoenzymes could preferentially activate glucuronide prodrugs at TAG-72 positive cells in vitro, MCF-7, LS174T, and HT-29 cells were incubated with hcc49 immunoenzymes or αDNS-S2, washed, and then exposed to the glucuronide prodrug HAMG (Figure 4a). None

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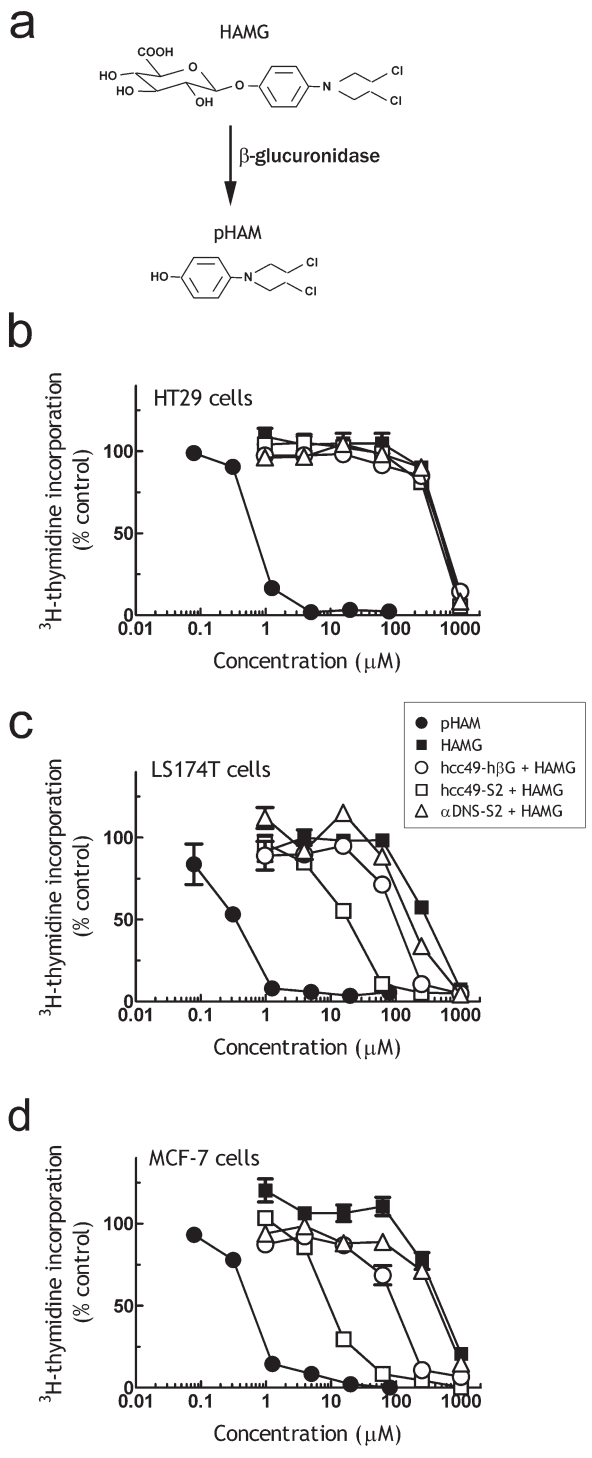

Figure 4. In vitro prodrug activation by immunoenzymes. (a) Hydrolysis of HAMG by βG releases the alkylating agent pHAM. HT-29 (b), LS174T (c), or MCF-7 (d) cells were incubated with PBS or 5 μg/mL immunoenzymes, washed, and then exposed to graded concentrations of pHAM or HAMG. The incorporation of ^{3}H-thymidine into cellular DNA was measured 48 h later. Bars, SEM (n = 3).

of the immunoenzymes increased the cytotoxicity of HAMG to TAG-72 negative HT29 cells (Figure 4b). On the other hand, LS174T (Figure 4c) and MCF-7 (Figure 4d) cells treated with hcc49-hβG and hcc49-S2 displayed augmented sensitivity to HAMG. There was a trend of increased cytotoxicity for cells treated with hcc49-S2 and for MCF-7 cells, indicating that both enhanced enzymatic activity and higher antigen levels promoted prodrug activation. Pretreatment of MCF-7 or LS174T cells with the control αDNS-S2 immunoenzyme produced similar IC_{50} values as prodrug alone, further demonstrating a requirement for antigen binding for effective prodrug activation by immunoenzymes.

Pharmacokinetics of Immunoenzymes in Tumor Bearing Mice. The plasma half-lives of the immunoenzymes were determined after i.v. injection of 100 μg hcc49-S2 or hcc49-hβG in Beige/SCID mice bearing 100−200 mm^3 LS174T tumors. Although MCF-7 cells expressed higher levels of TAG-72 antigen than did LS174T cells, we examined LS174T tumors in all in vivo experiments due to the more reproducible and uniform growth of these tumors. Immunoenzyme concentrations were determined by direct ELISA against mucin-coated microtiter plates (Figure 5a) or by directly measuring βG activity in blood samples (Figure 5b). The initial and terminal half-lives of the immunoenzymes were approximately 0.15 and 2.1 h, respectively, for hcc49-hβG, and 0.11 and 1.9 h for hcc49-S2. Direct measurement of enzymatic activity was more sensitive than ELISA, but both methods gave comparable results, indicating that the immunoenzymes retained both antigen-binding and enzymatic activity in vivo.

Imaging and Biodistribution of Immunoenzymes in Tumor-Bearing Mice. We first examined the tumor localization of the immunoenzymes by whole-body imaging in mice. Nude mice bearing 100−200 mm^3 LS174T xenografts were first imaged 5 and 48 h after i.v. injection of ^{131}I-hcc49-S2 (Figure 5c). After 48 h, ^{131}I-hcc49-S2 showed increased accumulation at the tumor site. ^{131}I-hcc49-S2 also clearly accumulated in the liver (Figure 5c). To further determine the amount of immunoenzymes in tumors, nude mice bearing 100−200 mm^3 LS174T xenografts were i.v. injected with 100 μg radiolabeled hcc49-S2 or αDNS-S2. The tumoral accumulation of ^{131}I-hcc49-S2 was stable after 24 h post injection (Table 3). By contrast, the accumulation of ^{131}I-αDNS-S2 decreased after 24 h post injection, indicating retention of hcc49-S2 but not αDNS-S2 in tumors. Liver uptake for both ^{131}I-hcc49-S2 and ^{131}I-αDNS-S2 was approximately 1% of the injected dose of radiolabeled fusion protein per gram of liver (% ID/g) after 48 and 72 h (Table 3). This might be due to the carbohydrate-mediated hepatic uptake of hβG.[40,41] Although the amount of ^{131}I-labeled hcc49-S2 was higher in the liver than in tumors, i.v. injection of FDGlcU resulted in significantly higher fluorescence signals in tumors as compared to the liver (Figure 5d). This result indicates that hcc49-S2 in the liver is likely sequestered inside cells and is not assessable for hydrolysis of glucuronide substrates. By contrast, the highly fluorescent signal in tumors shows that βG activity of hcc49-S2 is available for glucuronide hydrolysis in the tumor microenvironment.

ADEPT Therapy of Human Cancer Xenografts. To evaluate the therapeutic effect of tumor targeting immunoenzymes combined with prodrug treatment in vivo, BALB/c nu/nu mice bearing LS174T tumors were first i.v. injected with PBS or 500 μg/kg immunoenzyme on days 0 and 8. Mice were i.v. injected with PBS or three hourly doses of 70 mg/kg HAMG on days 1 and 10. Significant suppression of tumor growth with partial regression was observed in the mice injected with hcc49-S2 and HAMG (Figure 6a). By contrast, treatment of mice with hcc49-hβG and HAMG did not significantly suppress tumor growth as compared to treatment with αDNS-S2 and HAMG (p > 0.58). These data indicate that enhancing the enzymatic activity of hβG immunoenzymes resulted in greater therapeutic efficacy. Although previous data showed that liver accumulation of hcc49-S2 did not

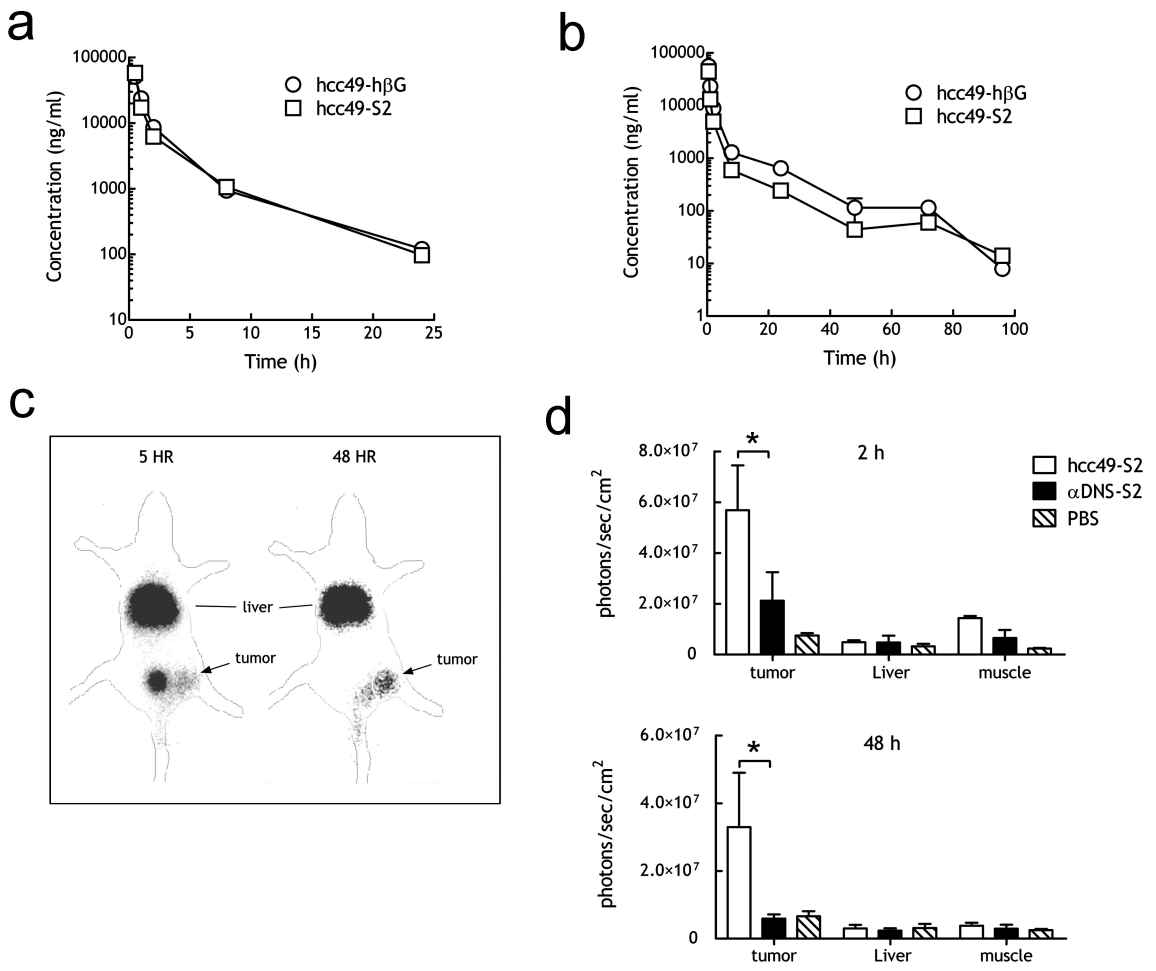

Figure 5. Pharmacokinetics of immunoenzymes in mice. Beige/SCID mice were i.v. injected with 100 μg hcc49-hβG or hcc49-S2. Mean plasma concentrations of the immunoenzymes were determined by measuring βG activity after capture of immunoenzymes on mucin-coated plates (a) or by directly measuring βG activity in serum (b). Bars, SEM (n = 3 mice). (c) Whole-body image of a representative nude mouse bearing a s.c. LS174T xenograft on the right flank at 5 and 48 h after i.v. injection of 100 μg of ^{131}I-hcc49-S2 (89 μCi). The images were obtained from the same mouse. (d) FDGlcU was administrated through the tail vein at 2 h (upper panel) or 48 h (lower panel) after i.v. injection of PBS, hcc49-S2, or αDNS-S2. The fluorescence intensity of tumor or liver was measured 30 min after injection of FDGlcU on an IVIS Imaging System (Xenogen, Alameda, CA, USA). The tumors and liver were analyzed with *Living Image* software. Significant differences between hcc49-S2 and αDNA-S2 fluorescence are indicated: *, $p < 0.05$.

hydrolyze a glucuronide substrate in the liver, we further investigated possible liver toxicity by measuring several hepatic enzyme markers 7 days after administration of immunoenzyme and HAMG. We also measured red blood cells (RBCs) and white blood cells (WBCs) because alkylating agents such as HAMG can cause dose-limiting leucopenia.[5] The mean numbers of RBCs in mice injected with hcc49-S2 and HAMG was slightly but not significantly lower (p = 0.19) than those of vehicle-treated mice (Figure 6b). Importantly, the number of WBCs in mice was not affected by immunoenzyme and prodrug treatment (p = 0.29). Likewise, the serum levels of hepatic enzyme markers were not significantly elevated in mice treated with immunoenzyme and HAMG (p = 0.09, 0.75, and 0.81 for AST, ALT, and TBIL, respectively) as compared to vehicle-treated mice (Figure 6c). Taken together, the hematological and hepatic enzyme measurements indicate that uptake of immunoenzymes in the liver did not cause excessive toxicity.

DISCUSSION

ADEPT is an attractive strategy to selectively treat cancer, but low catalytic activity and/or strong immunogenicity of currently investigated immunoenzymes has hampered clinical adoption. To help solve this problem, we created immunoconjugates linking the scFv fragment of a humanized CC49 antibody to S2, a hβG variant with enhanced enzymatic activity. We found that humanized immunoenzymes retained enzymatic activity and displayed exceptional antigen binding avidity ($K_D \approx 4 \times 10^{-10}$ M). The immunoenzymes could selectively bind and activate anticancer prodrug at TAG-72 positive cancer cells with more effective prodrug activation being achieved with hcc49-S2 as compared to hcc49-hβG. Importantly, the growth of human tumor xenografts in mice was significantly suppressed by treatment with hcc49-S2 and prodrug. Our results offer a promising approach to create highly active humanized immunoenzymes for ADEPT.

The use of hβG in immunoenzymes has been hampered by its relatively low enzymatic activity as compared to microbial sources of βG.[14−16] For example, the activity of *E. coli* βG is about 200-fold greater than hβG at pH 7,[27] and βG from *Staphylococcus* sp. displays about 2000-fold higher activity than hβG.[20] To help overcome this limitation, we recently developed a mammalian surface display system to facilitate the directed

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Table 3. Biodistribution of Humanized CC49 Fusion Proteins (% ID/g) in Nude Mice Bearing LS174T Colon Carcinoma Xenografts

	time (h)			
tissue	24	48	72	96
		hcc49-S2		
liver	4.74 ± 2.23	1.08 ± 0.15	1.16 ± 1.02	0.98 ± 0.25
muscle	0.04 ± 0.03	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01
blood	0.25 ± 0.16	0.05 ± 0.01	0.04 ± 0.01	0.02 ± 0.01
tumor	0.46 ± 0.27	0.37 ± 0.13	0.38 ± 0.20	0.43 ± 0.39
tumor/blood ratio	1.8	7.4	9.5	21.5
		αDNS-S2		
liver	4.30 ± 0.72	2.18 ± 1.02	1.18 ± 0.31	1.22 ± 0.3
muscle	0.10 ± 0.10	0.01 ± 0.00	0.004 ± 0.003	0.004 ± 0.001
blood	0.32 ± 0.08	0.08 ± 0.02	0.03 ± 0.00	0.03 ± 0.001
tumor	0.24 ± 0.10	0.07 ± 0.02	0.04 ± 0.01	0.05 ± 0.02
tumor/blood ratio	0.8	0.9	1.3	1.7

molecular evolution of human enzymes.[21] We successfully generated the hβG variant S2, which displays about 60-fold higher activity against ELF-97G and about 8-fold higher activity against several anticancer prodrugs.[21] Indeed, the immunoenzyme utilizing the S2 hβG variant more effectively activated a glucuronide prodrug at antigen-positive cancer cells (Figure 4) and produced significantly greater antitumor activity in vivo (Figure 6). Although it seems intuitive that more activity enzymes would improve prodrug activation in the tumor microenvironment, mathematical models have predicted that utilization of lower activity enzymes may be advantageous for ADEPT.[42,43] Our results, however, support the development of human enzymes with enhanced catalytic activity as an approach to improve the efficacy of ADEPT. We recently developed a more general screening approach for creating highly active human enzymes and successfully created hβG variants with 30-fold greater activity for anticancer glucuronide prodrugs (manuscript in preparation). Given the high activity of microbial sources of βG, we suspect that hβG variants with much higher catalytic activity can be generated. This approach should also be applicable to enhance the enzymatic activity of other human enzymes for ADEPT including β-galactosidase, β-glucosidase, carboxypeptidase A1, carboxylesterase, α-mannosidase, and purine nucleoside phosphorylase.[44−47]

In addition to enhanced enzymatic activity, hcc49-S2 displayed about 100-fold greater binding avidity for mucin as compared to hcc49 scFv (Table 2). The avidity of hcc49-S2 (K_D = 4.45 × 10^{-10} M) was also greater than that of the intact CC49 IgG molecule (K_D = 8.77 × 10^{-9} M[48]). The high avidity of hcc49-S2 is likely attributable to the tetrameric structure of hβG, which is required for enzymatic activity.[35] Thus, each immunoenzyme contains four scFv molecules (Figure 1d). The high affinity of hcc49-S2 should be beneficial for ADEPT because tumor retention correlates with antibody affinity.[49]

We observed that hcc49-hβG and hcc49-S2 were rapidly cleared from the circulation (Figure 5a,b). Imaging and radiolabeled immunoconjugate data demonstrated rapid liver uptake of the immunoenzyme, likely via mannose receptors (MR) and mannose 6-phosphate receptors (M6PR).[41,50] Fortunately, internalized immunoconjugates did not appear to be available for prodrug activation (Figure 5). On one hand, the rapid clearance of immunoenzyme from the circulation allows the tumor-to-plasma ratio to quickly reach suitable levels for prodrug administration. On the other hand, rapid clearance of immunoenzyme might hamper tumor localization. Chemical modification of hβG to hinder the uptake by MPR and M6PR on liver cells or pegylation of the immunoenzyme are approaches that may prolong immunoenzyme circulation to allow increased accumulation of immunoenzyme in tumor.[8,51,52] Future studies are required to determine the optimal half-life of the immunoconjugates for cancer therapy by ADEPT.

Strong immune response against antibody−enzyme conjugates adversely affects tumor localization and treatment efficacy in clinical trials of ADEPT, even with the administration of immunosuppressive drugs.[10,12] Murine antibodies such as CC49 also induce immune responses in patients that hamper antibody effectiveness.[53,54] Because humanized antibodies are less immunogenic as compared to murine and chimeric antibodies,[55] we utilized a humanized CC49 scFv to construct immunoenzymes.[22] However, reducing the immunogenicity of the enzyme portion of the immunoenzymes is more problematic. Besides using immunosuppressive drugs to blunt antienzyme immune responses,[56] foreign enzymes have been engineered to remove immunogenic epitopes[57,58] and catalytic antibodies have been employed to replace the enzyme portion of the immunoenzyme.[59] However, besides exhibiting limited effectiveness in suppressing strong immune responses against microbial enzymes, immunosuppressive drugs can cause undesirable side effects and may suppress desirable antitumor immune responses in cancer patients.[60] Removal of all immunogenic B cell and T cell epitopes of highly divergent foreign enzymes with retention of activity and stability remains a challenging problem[61,62] and creation of catalytic antibodies with enzymatic activities comparable to natural enzymes is not easily achieved.

Utilization of highly active human enzyme variants is an alternative approach that is expected to reduce immunoenzyme immunogenicity as compared to those incorporating microbial enzymes. For example, the amino acid sequence of *E. coli* βG differs from hβG at 322 positions and differs from mouse βG at 323 positions. Immunoenzymes utilizing *E. coli* βG are therefore highly immunogenic in mice.[7,63] By contrast, the S2 variant differs from hβG by only four amino acids, suggesting that immune responses against hcc49-S2 should be mild as compared to immunoenzymes created with microbial sources of βG. Studies showing that even partial replacement of mouse immunoglobulin sequences (57−72% identity to human immunoglobulin[64]) with the corresponding human immunoglobulin sequences can substantially reduce the immunogenicity of mouse antibodies also suggest that human enzymes with limited numbers of amino acid alterations should be less immunogenic than microbial enzymes.[55,65] However, further studies in appropriate transgenic mouse models or in human subjects will be required to define actual immunogenicity of human enzyme variants.

hcc49-S2 possesses potential advantages for cancer therapy. The high avidity of hcc49-S2 may promote better tumor delivery[66] and facilitate prolonged retention of the immunoenzyme at tumor cells in vivo (Table 3), resulting in high immunoconjugate tumor/blood ratios. The wide distribution of the TAG-72 antigen in colorectal, gastric, pancreatic, ovarian, endometrial, breast, nonsmall cell lung cancer, and prostate carcinomas suggests that hcc49-S2 may be useful for the treatment of many types of cancer.[67] The plethora of anticancer glucuronide prodrugs available also means that effective

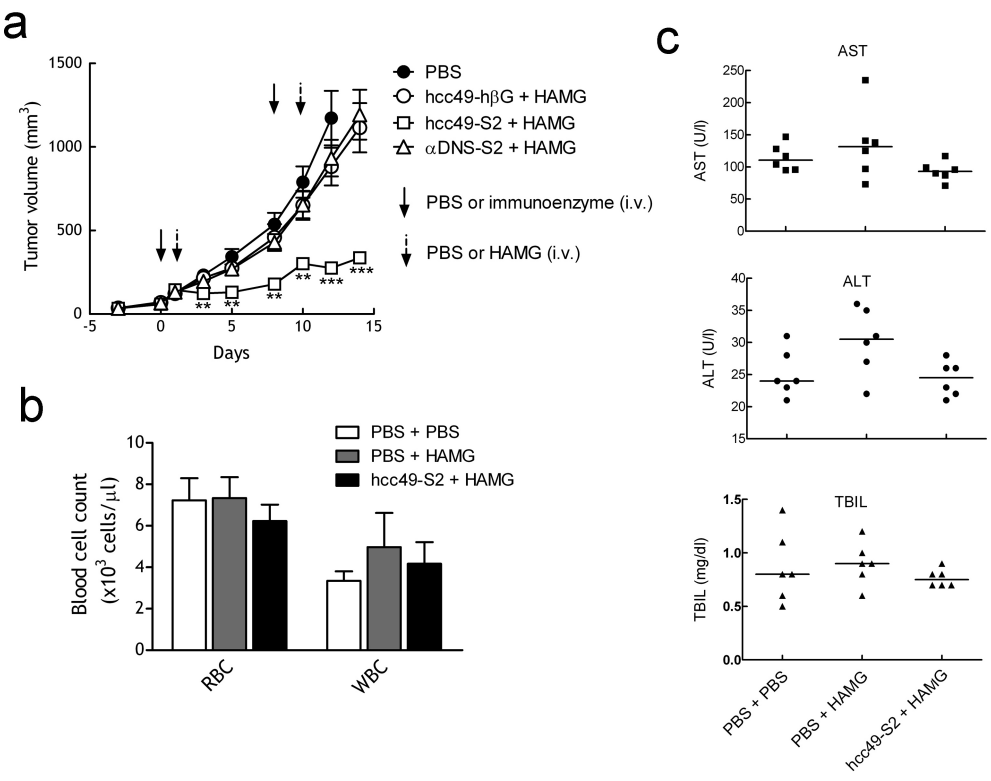

Figure 6. ADEPT with humanized immunoenzymes. (a) Nude mice bearing 50−100 mm^3 s.c. LS174T tumors were i.v. injected with PBS or 500 μg hcc49-hβG, hcc49-S2, or αDNS-S2 on days 0 and 8 and i.v. injected with three fractionated doses of 70 mg/kg HAMG or PBS on days 1 and 10. Bars, SEM (n = 8). Significant differences in mean tumor size in mice treated with hcc49-S2 or hcc49-hβG and HAMG are indicated: **, $p \leq 0.005$; ***, $p \leq 0.0005$. (b, c) Nude mice were i.v. injected with 500 μg hcc49-S2 or PBS, and after 24 h, mice were i.v. injected with three fractionated doses of 70 mg/kg HAMG or PBS. The numbers of the WBCs and RBCs (b) and the serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (TBIL) (c) were measured 7 days after HAMG administration. Bars, SD (n = 4−6).

combinations may be utilized depending on the tumor type under therapy. The enzymatic amplification of drug activity and the relatively fast diffusion of activated drug molecules within solid tumors may also help effectively treat antigen-negative and antigen-escape cancer cell variants.

In conclusion, our study demonstrates that hcc49-S2, a humanized immunoenzyme with enhanced enzymatic activity, displays promising therapeutic effects in combination with glucuronide prodrug in a human tumor xenograft model. Our study suggests that further improvement of treatment efficacy may be achieved by generation of more active enzyme variants and optimization of immunoenzyme pharmacokinetic properties, and possibly by utilization of more potent prodrugs or development of prodrugs with enhanced binding affinity to hβG variants. We hope that further development of highly active humanized immunoenzymes can accelerate the translation of ADEPT from the lab to the clinic.

■ ASSOCIATED CONTENT

Supporting Information. Table 1 shows the oligonucleotide sequences used for scFv assembly. This material is available free of charge via the Internet at http://pubs.acs.org.

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