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ORIGINAL ARTICLE

Membrane-localized activation of glucuronide prodrugs by β-glucuronidase enzymes

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Gene-mediated enzyme prodrug therapy (GDEPT) seeks to increase the therapeutic index of anti-neoplastic agents by promoting selective activation of relatively nontoxic drug derivatives at sites of specific enzyme expression. Glucuronide prodrugs are attractive for GDEPT due to their low toxicity, bystander effect in the interstitial tumor space and the large range of possible glucuronide drug targets. In this study, we expressed human, murine and *Esherichia coli* β-glucuronidase on tumor cells and examined their *in vitro* and *in vivo* efficacy for the activation of glucuronide prodrugs of 9-aminocamptothecin and *p*-hydroxy aniline mustard. We show that (1) fusion of β-glucuronidase to the Ig-like C_2-type and Ig-hinge-like domains of the B7-1 antigen followed by the B7-1 transmembrane domain anchored high levels of active murine and human β-glucuronidase on cells, (2) strong bystander killing of tumor cells was achieved *in vitro* by murine β-glucuronidase activation of prodrug, (3) potent *in vivo* anti-tumor activity was achieved by prodrug treatment of tumors that expressed murine β-glucuronidase and (4) the *p*-hydroxy aniline prodrug was more effective *in vivo* than the 9-aminocamptothecin prodrug. Our results demonstrate that surface expression of murine β-glucuronidase for activation of a glucuronide prodrug of *p*-hydroxy aniline mustard may be useful for more selective therapy of cancer.
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Introduction

Although many anti-neoplastic agents have demonstrated utility in the clinic, drug-induced toxicity to non-cancerous tissues can prevent attainment of therapeutic drug concentrations at cancer cells and lead to premature termination of therapy before achieving complete remission. More selective anti-neoplastic agents could positively influence therapy by reducing the exposure of normal tissues to cytotoxic drugs. GDEPT seeks to increase the therapeutic index of anti-neoplastic agents by selective delivery or expression in cancer cells of a gene that encodes an enzyme that converts a relatively non-toxic prodrug into an anti-neoplastic agent. Preferential activation of prodrugs at transduced cancer cells generates high drug concentrations in tumors while minimizing drug exposure to normal tissues.[1]

Several GDEPT systems have been developed for selective cancer treatment, the most prevalent being herpes simplex thymidine kinase activation of ganciclovir[2,3] and *Esherchia coli*-derived cytosine deaminase activation of 5-fluorocytosine.[4] Phosphorylation of ganciclovir by herpes simplex thymidine kinase is followed by a series of intracellular reactions that produces a triphosphate that is trapped within the target cells. The triphosphate product can compete with deoxyguanosine triphosphate during DNA elongation, thereby inhibiting DNA polymerase and causing single-strand breaks.[5] Ganciclovir, however, is S-phase specific and kills only actively dividing cells,[6] a disadvantage in tumors containing regions of non-proliferating cells. Bystander killing by ganciclovir also relies on gap junctions for transport of the charged triphosphate to neighboring cells, which can limit the bystander effect in tumors since the expression of connexins is often decreased in neoplastic tissues and by hypoxia.[7] Furthermore, low concentrations of ganciclovir can also cause chromosome breaks and sister chromatid exchange.[8] Cytosine deaminase derived from some bacterial and fungal sources is capable of converting the less toxic 5-fluorocytosine to 5-FU. 5-fluouracil (5-FU) is enzymatically converted to 5-FUTP, which is incorporated into DNA and prevents nuclear processing of ribosomal and mRNA, and to 5-fluorouridine-5′-monophosphate, which

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irreversibly inhibits thymidylate synthase.[9] In contrast to ganciclovir, bystander killing by 5-FU is not dependent on gap junctions because this drug can diffuse into and out of cells.[10] 5-FC, however, is relatively hydrophilic and therefore diffuses across the plasma membrane of cells slowly, but is rapidly excreted from some cells.[11] Furthermore, because the inherent sensitivity of tumor cells is an important factor in determining the efficacy of suicide gene therapy with 5-FC,[12] this prodrug is unsuitable for many tumor types. Although suicide gene therapy with either ganciclovir or 5-FC appears to safe, only marginal clinical efficacy has been observed,[13–16] indicating the need for more efficacious GDEPT systems.

β-glucuronidase (βG) is attractive for GDEPT for several reasons. First, a wide variety of glucuronide prodrugs are available, including prodrugs of effective anti-neoplastic agents such as doxorubicin,[17,18] etoposide,[19] camptothecin analogs,[20,21] paclitaxel,[22] docetaxel [23] and alkylating agents.[24,25] In fact, glucuronide derivatives of almost any anti-neoplastic agent can be synthesized by employing linkers between the drug and glucuronide moieties.[22,23] This is a major advantage since the most effective prodrug can be selected for a particular tumor type. Second, glucuronide prodrugs do not readily enter cells due to their charged carboxy group, minimizing interactions with endogenous βG located inside lysosomes.[17] Thus, prodrug activation selectively occurs at sites of exogenous βG expression only if the enzyme is located extracellularly.[26] Surface expression of prodrug-activating enzymes may promote more extensive killing of non-transduced cancer cells since activated drug is not trapped in the cytosol of transduced cells. For example, hydrolysis of glucuronide prodrugs by βG released from cells or targeted to the cell membrane produces potent cytotoxicity as well as bystander killing of neighboring enzyme-negative cells,[27–29] an important consideration given the low transduction efficiencies achievable *in vivo*. Finally, glucuronide prodrugs produce potent anti-tumor activity in antibody-directed enzyme prodrug therapy (ADEPT), in which specific antibodies are employed to deliver βG to cancer cells[30–32] as well as directly in prodrug monotherapy, which relies on the presence of elevated levels of βG in the tumor interstitial space.[33,34]

Although many variables may influence the effectiveness of GDEPT, we hypothesized that maximizing the activity of βG on tumor cells is of primary importance. In the present study, we examined the effectiveness of anchoring βG on cells with different juxtamembrane spacer domains and transmembrane domains (Figure 1a). As different sources of βG display unique kinetic properties, we compared the surface expression, surface activity, *in vitro* prodrug activation and *in vivo* anti-tumor activity of *E. coli* (eβG), murine (mβG), and human (hβG) βG. hβG has previously been expressed as a secreted or membrane anchored form for the selective activation of a glucuronide prodrug of doxorubicin,[28,29] but other βG enzymes have not been examined. Finally, we directly compared the *in vitro* and *in vivo* efficacy of two glucuronide prodrugs, HAMG and 9ACG. HAMG is a glucuronide prodrug that can be enzymatically converted by βG to *p*-hydroxy aniline mustard (pHAM), a potent alkylating agent.[24,35] We previously demonstrated that combined treatment with immunoenzymes and HAMG could cure late-stage malignant ascites[31] as well as cause the regression of human tumors in mice.[32,36] 9ACG is a water soluble prodrug that can be hydrolyzed by βG to release 9-aminocamptothecin (9AC), an anti-tumor alkaloid that inhibits topoisomerase I to produce selective anti-tumor activity.[37,38] 9ACG displays anti-tumor activity against human xenografts.[34] The active prodrug of 9ACG is about 1000 times more potent than the activated form of HAMG, suggesting that these prodrugs may display differences in their effectiveness.

Materials and methods

Reagents and antibodies

The synthesis of pHAM, HAMG, 9AC and 9ACG have been described. [20,35] Rat anti-HA antibody was from Roche (Basel, Switzerland). Mouse anti-β-actin and rabbit anti-BiP were from Sigma Aldrich (St Louis, MO, USA). Horseradish peroxidase (HRP), FITC and rhodamine-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (Westgrove, PA, USA) or Organon Teknika Corp (Durham, NC, USA). Monoclonal antibody 1E8 (IgG_1 against eβG) has been described.[39] To generate monoclonal antibodies (mAb) against hβG (1B3, IgG_1 and 7G8, IgG_{2b}), female Balb/c mice were s.c. injected with 100 μg recombinant hβG in complete Freund's adjuvant, and then boosted at 3–4 week intervals with decreasing doses of hβG in incomplete adjuvant. At 3 days before fusion with FO myeloma cells, the mice were i.p. injected with 30 μg hβG in PBS. Hybridomas were screened by ELISA for clones secreting antibodies that bound hβG but not mβG or eβG. Stable hybridomas were obtained by repeated limiting dilution cloning. A rat mAb against mβG (7G7, IgG) was generated in an analogous fashion by immunizing Lou/c rats with recombinant mβG before splenocytes were fused with FO myeloma cells.

DNA constructs

The hβG cDNA fragment in pLNCX-hβG,[40] the mβG cDNA fragment in pLHCX-mβG[40] and the eβG fragment in pGUS N358S (Clontech, Mountain View, CA, USA) were inserted in place of the 2C11 scFv gene (*Sfi*I/*Sal*I fragment) in p2C11-PDGFR, p2C11-BGP-B7, p2C11-e-B7, p2C11-γ_1-B7 and p2C11-CD44-B7[41,42] to create the corresponding transgenes for surface expression of hβG, mβG and eβG. Removal of the immunoglobulin hinge region in the γ_1 domain of p-hβG-γ1–B7 produced p-hβG-mγ_1-B7. p-hβG-L-e-B7 includes cDNA for a 10 amino-acid linker (GGGGSGGGGS) at the 5′-end of the e-B7 domain. p-hβG-ICAM was generated by inserting the hβG cDNA fragment between the ICAM-1 leader sequence and ICAM-1 transmembrane domain in pLTM-1 (generously provided by Dr Alister Craig, University of Oxford, UK). Insertion of the hβG-e-B7, mβG-e-B7 and eβG-e-B7 transgenes into the retroviral vector pLNCX

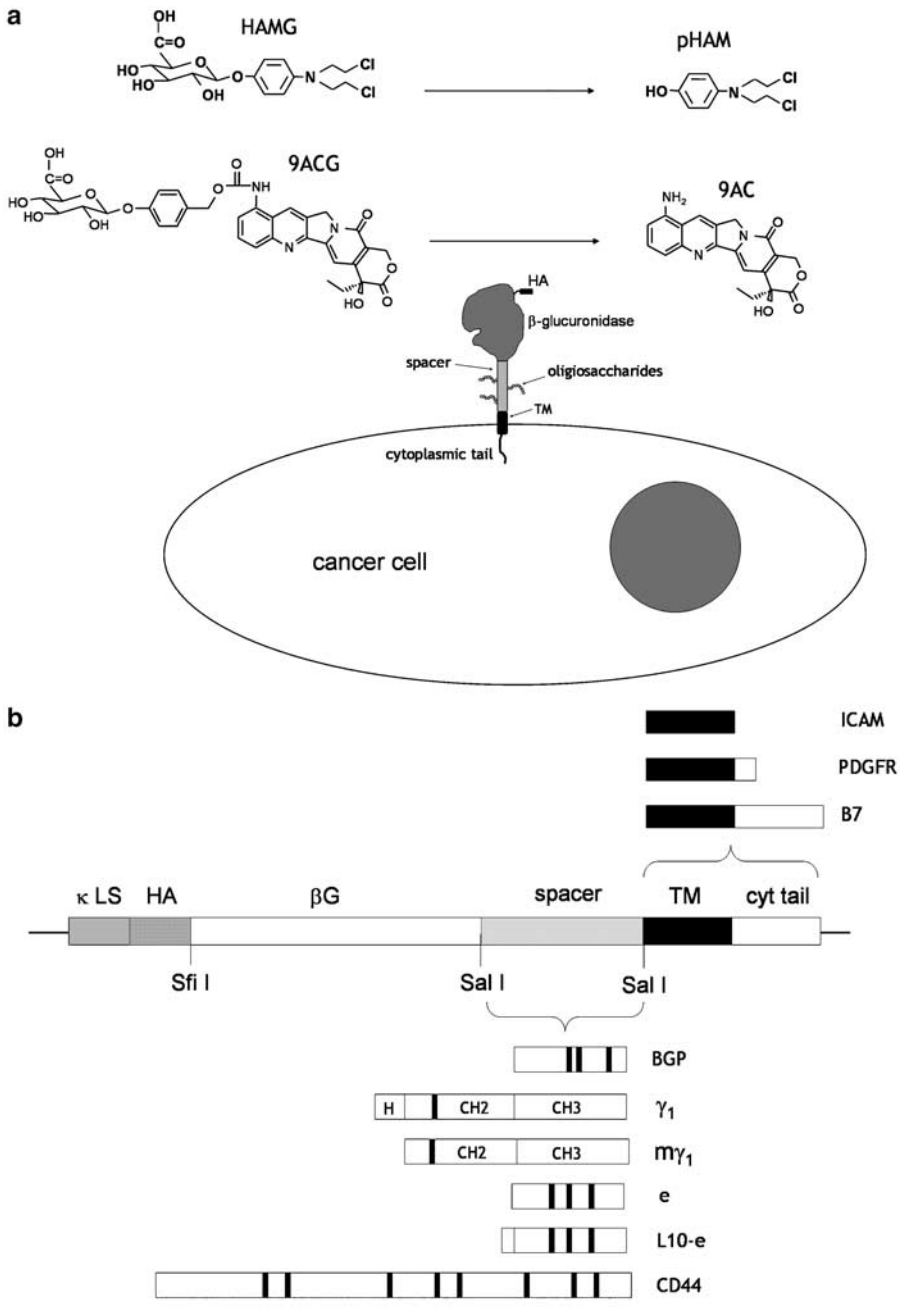

Figure 1 βG activation of glucuronide prodrugs. (**a**) βG is anchored on cancer cells via a spacer domain fused to a TM. The glucuronide prodrugs HAMG or 9ACG can be enzymatically converted by βG to the anti-neoplastic agents pHAM or 9AC, respectively. (**b**) General layout of the transgenes for surface expression of βG. The transgenes code for the immunoglobulin kappa chain leader sequence (κLS) followed by an HA epitope (HA) and the mature βG gene. Sequences coding 'spacer' domains (BGP, γ1, mγ1,e, L10-e and CD44) were derived from cell receptors as described in the text. Black bars indicate the presence of N-linked glycosylation sites. TM were derived from human ICAM-1, the human platelet-derived growth factor receptor or the mouse B7-1 antigen.

(BD Biosciences, San Diego, CA, USA) generated the retroviral vectors pLNCX-hβG-e-B7, pLNCX-mβG-e-B7 and pLNCX-eβG-e-B7. pNeo/GUS and pLS/GUS, which direct the expression of eβG to the cytosol or allow secretion of eβG, respectively, have been described.[26] Expression of all transgenes was under control of the CMV promoter.

Cell lines

Balb/3T3 fibroblasts (CCL-163) and CT26 murine colon carcinoma cells (CRL-2638) were grown in DMEM (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[43] were cultured in RPMI containing the same

supplements. The cells were free of mycoplasma as determined by PCR.

Transfection of βG transgenes

3T3 fibroblasts were transfected with plasmid DNA using Lipofectamine 2000 (Gibco Laboratories, Grand Island, NY, USA). To generate stable cell lines, pLNCX-eβG-e-B7, pLNCX-hβG-e-B7 and pLNCX-mβG-e-B7 were co-transfected with pVSVG in GP293 cells (Clontech) to produce recombinant retroviral particles. At 2 days after transfection, the culture medium was filtered, mixed with 8 μg/ml polybrene and added to EJ or CT26 cells. Stable cell lines were selected in medium containing G418.

Western blot analysis

Transiently transfected 3T3 fibroblasts were boiled in reducing SDS buffer, electrophoresed on a SDS-PAGE and transferred to PVDF membranes. Membranes were sequentially probed with anti-HA antibody or mAb 1B3 against hβG followed by HRP-conjugated secondary antibody. The membranes were stripped and reprobed with anti-β-actin antibody. Bands were visualized by ECL detection (Pierce, Rockford, IL, USA). Relative expression levels of βG were normalized to β-actin band intensities using the shareware program NIH Image (http://rsb.info.nih.gov/nih-image/download.html).

Flow cytometer analysis

Cells were stained with anti-HA antibody followed by FITC-conjugated goat anti-rat $F(ab')_2$ fragment. Alternatively, cells were stained with mAb 1E8 against eβG,[39] mAb 7G8 against hβG or mAb 7G7 against mβG followed by the appropriate FITC-labeled secondary antibody. The surface immunofluorescence of 10 000 viable cells was measured with a FACScaliber flow cytometer (Becton Dickinson, Mountain View, CA, USA) and fluorescence intensities were analyzed with Flowjo V3.2 (Tree Star Inc., San Carlos, CA, USA).

Surface enzyme activity assay

Transiently transfected 3T3 fibroblasts in 96-well microplates were washed once with PBS and immediately assayed for βG activity by adding 200 μl PBS (pH 7.0) containing 0.1% BSA and 0.25 mM 4-methylumbelliferyl β-D-glucuronide for 30 min at 37°C. 150 μl of the reaction mixture were transferred to a 96-well fluorescence microplate and mixed with 75 μl stop buffer (1 M glycine/0.5 M sodium bicarbonate, pH 11). The fluorescence of the wells was measured at an excitation wavelength of 365 nm and an emission wavelength of 455 nm.

Production and characterization of recombinant βG

Recombinant eβG, mβG and hβG were produced as described.[36,40] Enzymatic activities at defined pH values were measured in triplicate as described.[40] One unit βG activity corresponds to the hydrolysis of 1 μmole *p*-nitrophenol β-D-glucuronide per h at 37°C.

^{3}H-thymidine incorporation assay

Graded concentrations of drugs were added in triplicate to cell lines at pH 6.6 or pH 7.4 for 24 h at 37°C. The cells were incubated in fresh medium for 24 h and then pulsed for 16 h with ^{3}H-thymidine (1 μC_i/well). Bystander killing was measured by adding 30 μM pHAM or BHAMG to defined ratios of CT26 and CT26/mβG cells for 24 h and then measuring ^{3}H-thymidine incorporation after incubation in fresh medium for 24 h. Results are expressed as percent inhibition of ^{3}H-thymidine incorporation compared with untreated cells by the following formula:

$$\%\ \text{inhibition} = 100 \times \frac{\text{cpm sample} - \text{cpm background}}{\text{cpm control} - \text{cpm background}}$$

Tumor therapy

Scid-Beige and Balb/c mice were maintained under specific-pathogen free conditions. Groups of 9–11 female Scid-Beige mice were s.c. injected in the right flank with 1×10^7 EJ, EJ/eβG, EJ/mβG or EJ/hβG cells. When tumors reached a mean size of 100–200 mm^3, the mice were intraveneous (i.v.) injected with PBS ($n = 4$–5) or 50 mg/kg 9ACG in PBS ($n = 5$–6). Tumor volumes (length × width × height × 0.5) were measured 11 days after drug administration. Scid-Beige mice bearing established EJ or EJ/mβG tumors ($n = 5$) were i.v. injected on days 15 and 19 with three doses of PBS or 50 mg/kg HAMG at hourly intervals. Groups of six BALB/c mice bearing established CT26 or CT26/mβG tumors (100 mm^3) were i.v. injected on days 7, 9 and 11 with three doses of PBS or 50 mg/kg HAMG at hourly intervals. Tumor volumes were measured biweekly until tumors exceeded 2000 mm^3.

In vivo expression of βG on tumor cells

Resected tumors (200–300 mm^3) were cut into small fragments and digested with 0.5 mg/ml collagenase in Hank's balanced saline solution containing Ca^{2+} and Mg^{2+} for 1 h at room temperature. The cells were cultured in complete medium for 24 h before the cells were immunofluorescence stained for the HA epitope and analyzed by flow cytometry.

Intracellular immunofluorescence staining

Cells on glass coverslips were fixed with 4% paraformaldehyde in PBS for 15 min and then permeabilized with 1% Triton X-l00 in PBS for 10 min. The cells were stained with biotin-labeled goat anti-HA and streptavidin-labeled rhodamine or rabbit anti-BiP and rhodamine-labeled goat anti-rabbit IgG, mounted on slides in 50% glycerol/PBS, and viewed under a digital fluorescence microscope.

Secretion of eβG from fibroblasts

The culture medium from transiently transfected 3T3 fibroblasts was collected after 48 or 60 h. The cells were suspended in 0.1% Triton X-100/PBS, freeze/thawed three times and centrifuged to remove insoluble material. Cell lysate and culture medium were assayed for βG activity at pH 7 for eβG and at pH 4.5 for mβG and hβG.

The measured activities were adjusted to calculate the βG activity originally present in the total cell and medium fractions.

Statistical significance

Statistical significance of differences between mean values was estimated with Excel (Microsoft, Redmond, WA, USA) using the independent *t*-test for unequal variances. *P*-values of <0.05 were considered statistically significant.

Results

Transmembrane and spacer domains can influence βG surface expression

Previous studies have demonstrated that the transmembrane domain (TM) and the juxtamembrane 'spacer' domain employed to anchor single-chain antibodies (scFv) on the plasma membrane of cells can greatly affect cell surface expression levels.[41,42,44] To examine whether the expression of hβG on cells also depended on the TM and spacer domains, a panel of hβG transgenes was constructed with an immunoglobulin κ chain signal sequence and an HA tag at the 3′ end of the mature hβG cDNA (Figure 1b). TM from the human platelet-derived growth factor receptor (PDGFR), the murine B7-1 antigen (B7) or human intracellular adhesion molecule 1 (ICAM-1) were employed to anchor hβG on the cell surface. The PDGFR TM includes six amino acids of the cytoplasmic tail whereas the B7 TM includes the entire 38 amino acid cytoplasmic tail of the B7-1 antigen. Spacer domains introduced between hβG and the B7 TM included the first immunoglobulin-like V-type domain of human biliary glycoprotein I (hβG-BGP-B7), the Ig-like C2-type and Ig-hinge-like domains of the murine B7-1 antigen (hβG-e-B7), the hinge-CH_2-CH_3 domains of human IgG_1 (hβG-γ_1-B7), the CH_2-CH_3 domains of human IgG_1 (hβG-mγ_1-B7) and the extracellular portion of human CD44E (hβG-CD44-B7). The γ_1 domain allows formation of disulfide linked dimers.[41] A sequence coding a 10 amino acid flexible linker (GGGGSGGGGS) was appended to the 3′ end of hβG in hβG-L-e-B7 to examine the influence of additional flexibility between the domains.

We transiently transfected the hβG transgenes into murine 3T3 fibroblasts and detected the protein products by immunoblotting whole cell lysates with an antibody against the HA epitope in the chimeric proteins. The transfected fibroblasts expressed the chimeric proteins at similar levels (Figure 2a). hβG-ICAM does not contain the HA epitope but was detected with anti-hβG antiserum (Figure 2b). Immunofluorescence staining of transiently transfected fibroblasts showed that cells poorly expressed hβG-ICAM, moderately expressed hβG-PDGFR and highly expressed hβG-BGP-B7, hβG-e-B7 and hβG-CD44-B7 (Figure 2c). Repeated transient transfection experiments showed that hβG-e-B7 and hβG-L-e-B7 directed the highest expression of hβG on the cells (Figure 2d, open bars). hβG-e-B7 and hβG-L-e-B7 also displayed the greatest βG enzyme activity on the cells (Figure 2d, solid bars). phOx-e-B7, a scFv that is highly expressed on cells, was included to demonstrate the specificity of the anti-hβG antibody and enzyme activity assays. Comparison of mβG expression on fibroblasts showed that mβG-e-B7 and mβG-CD44-B7 directed about twice the amount of mβG to the cell surface as compared with mβG-BGP-B7 (Figure 2e). Although the expression of eβG was low regardless of the spacer used, βG-CD44-B7 allowed the expression of about twice the levels of eβG on the cell surface as compared to eβG-eB7 (Figure 2f). The enzymatic activity of eβG-CD44-B7, however, was dramatically lower than eβG-e-B7, indicating that the CD44 spacer did not allow proper folding or formation of the eβG tetramer. Taken together, our results demonstrate that the B7-1 spacer and the B7 TM anchored the highest levels of active βG on cells.

Stable expression of mβG, hβG and eβG on EJ carcinoma cells

Retroviral transduction of EJ human bladder cancer cells generated stable EJ transfectants that expressed hβG-e-B7, mβG-e-B7 or eβG-e-B7 (EJ/hβG, EJ/mβG and EJ/eβG cells, respectively). Immunoblotting of whole cell lysates demonstrated that hβG-e-B7 and mβG-e-B7 were expressed at about 2.5-fold higher levels as compared to eβG-e-B7 (Figure 3a). Large amounts of both hβG-e-B7 and mβG-e-B7 were detected on the surface of live EJ transfectants, but eβG-e-B7 levels on the plasma membrane were about 20-fold lower (Figure 3b). Murine fibroblasts (Figure 2c) and murine colon carcinoma CT26 cells displayed similar large differences in the surface levels of hβG and mβG versus eβG (results not shown). Both hβG-e-B7 and mβG-e-B7 were easily visualized on the plasma membrane of cells whereas eβG-e-B7 was only weakly detected (Figure 3c). EJ/mβG cells displayed two- to threefold more βG activity than did EJ/eβG cells and fivefold higher activity than EJ/hβG cells (Figure 3d). As expected, EJ cells did not hydrolyze the glucuronide substrate.

The neutral pH optimum of eβG or a high specific enzyme activity could cause the relatively effective hydrolysis of glucuronide substrates by the low levels of eβG present on EJ/eβG cells. To differentiate these possibilities, we produced and purified recombinant forms of each enzyme to measure accurately their specific activities at defined pH values (Figure 3e). As expected, eβG displayed optimal catalytic activity at neutral pH whereas both hβG and mβG exhibited maximal activities at pH 4–4.5 (Figure 3f). eβG displayed a maximal specific activity of 20 000 U/mg whereas hβG and mβG had maximal specific activities of 1600 and 1100 U/mg (Figure 3g). Thus, eβG possesses intrinsically higher catalytic activity as compared to the lysosomal enzymes.

Membrane-localized prodrug activation

The proliferation of the EJ cell lines after treatment with drugs showed that all the cell lines were similarly sensitive to 9AC with IC_{50} values ranging from 5 to 9 nM (Figure 4a). As expected, EJ cells were relatively

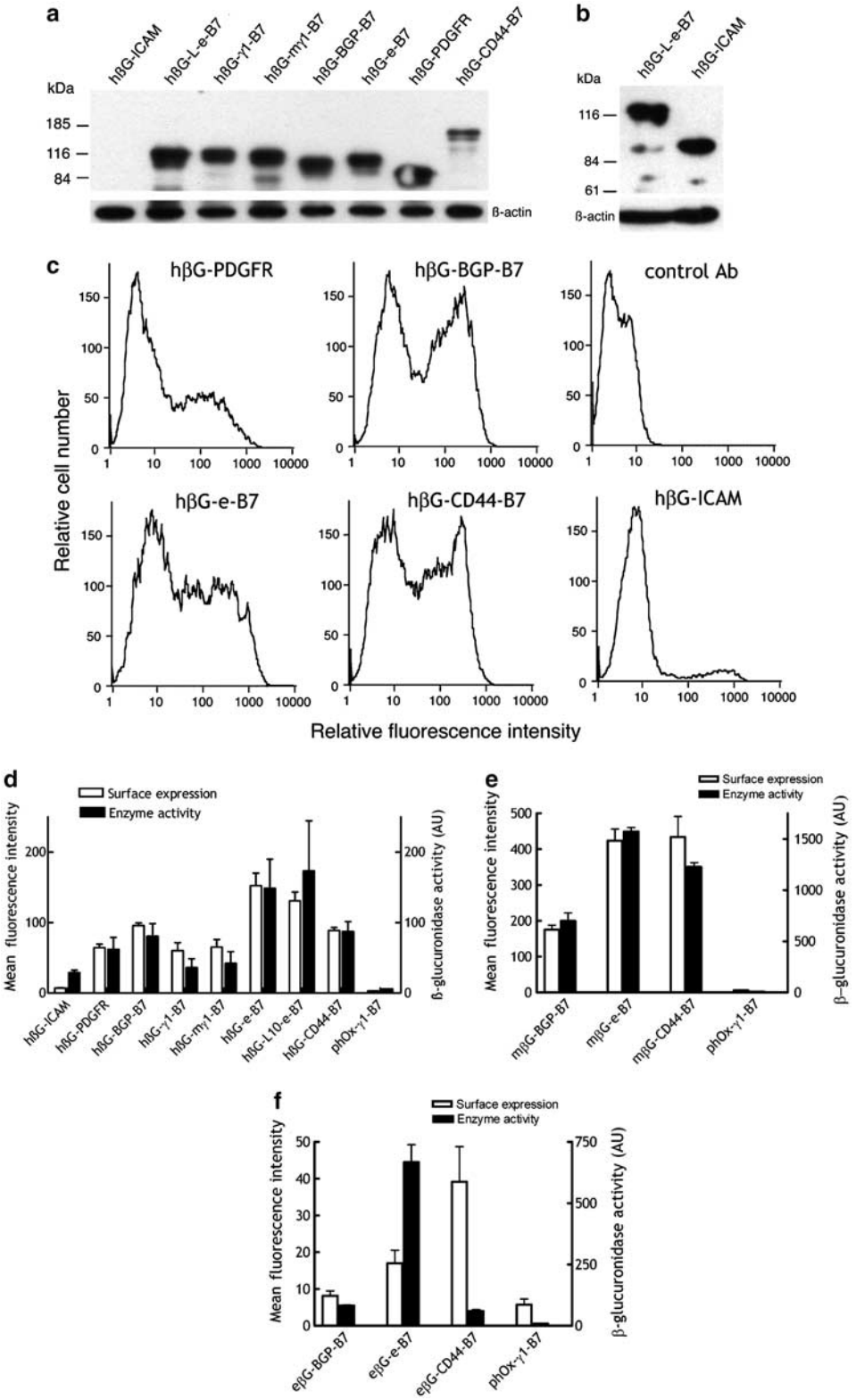

Figure 2 Expression of βG transgenes. (**a**) Cell lysates prepared from 3T3 fibroblasts that were transiently transfected with transgenes coding for the indicated chimeric enzymes were immunoblotted with anti-HA antibody (upper panel) or anti-β-actin antibody (lower panel). (**b**) Transfected cell lysates were immunoblotted with anti-hβG rabbit serum or anti-β-actin antibody. (**c**) Live transiently transfected fibroblasts were immunofluorescence stained for hβG and analyzed on a flow cytometer. (**d–f**) Fibroblasts were transiently transfected with transgenes containing hβG (**d**), mβG (**e**) or eβG (**f**). After 2 days, the cells were immunofluorescence stained for surface βG and analyzed on a flow cytometer (left axis) or directly assayed for βG activity (right axis). Results represent mean values of three determinations. Bars, s.e.

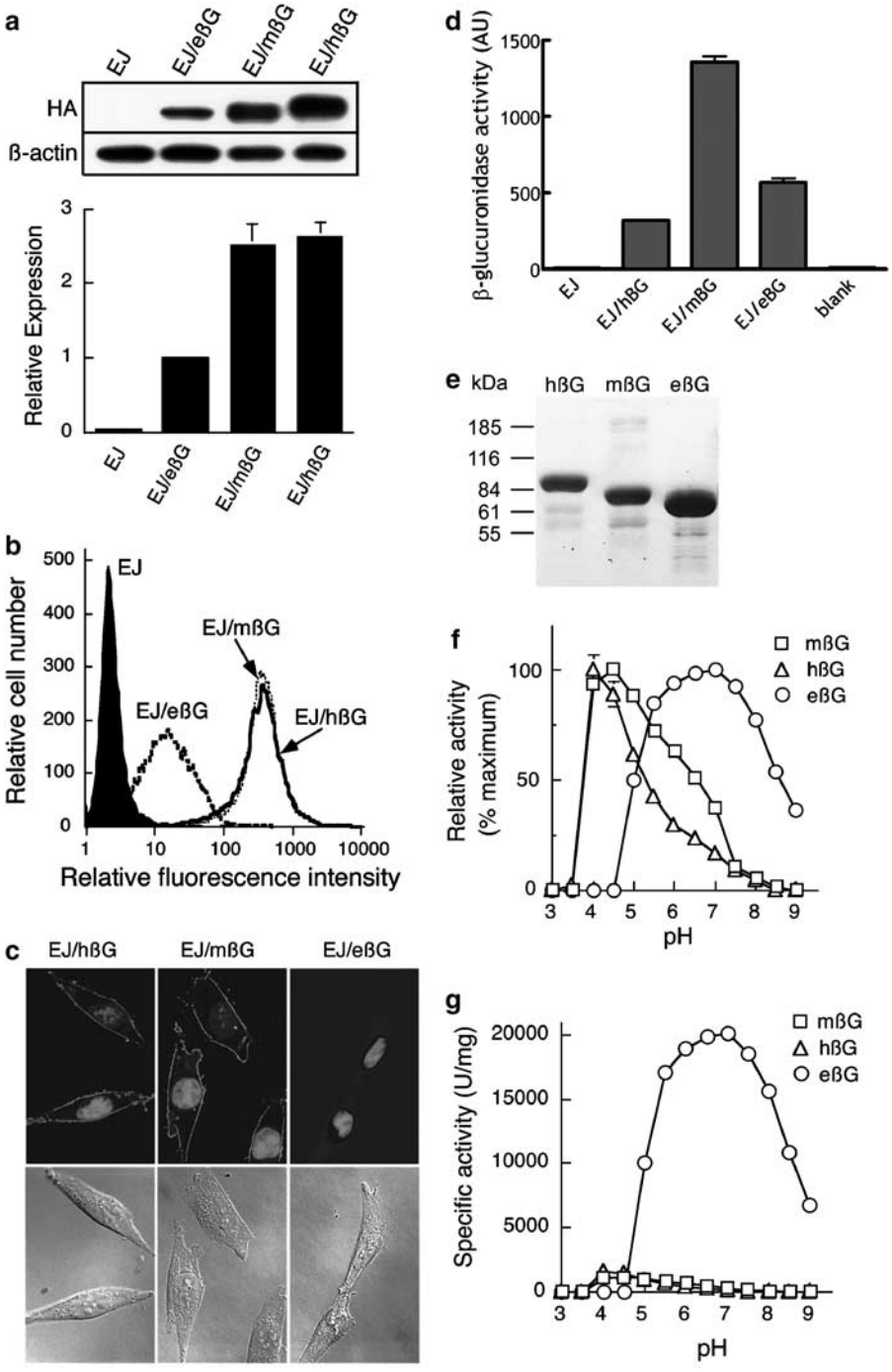

Figure 3 Characterization of βG expression on stable EJ bladder carcinoma cells. (**a**) Stable EJ transfectants were immunoblotted with anti-HA or anti-β-actin antibody. The relative expression of βG was estimated by normalizing the HA band intensity by the intensity of the β-actin band. (**b**) Live EJ cell lines were immunofluorescence stained for the HA epitope and analyzed on a flow cytometer. (**c**) Live EJ cell lines were immunofluorescence stained for the HA epitope (red) and observed under a fluorescence microscope equipped with a CCD detector (upper panels) or under phase-contrast (lower panels). Blue regions show nuclear staining by DAPI. (**d**) EJ cell lines were assayed for βG activity on their surface. Results show the mean values of triplicate determinations. Bars, s.e. (**e**) Purified recombinant hβG, mβG and eβG were electrophoresed on a SDS PAGE and stained with Coomassie blue. (**f**) The relative enzymatic activities (percentage maximum activity) of the recombinant enzymes at the indicated pH values are shown ($n=3$). (**g**) The specific activity of the recombinant enzymes at defined pH values are shown ($n=3$).

insensitive to 9ACG with an IC_{50} value of 820 nM (Figure 4b). EJ/hβG cells were also relatively insensitive to 9ACG, indicating limited hydrolysis of 9ACG by EJ/hβG cells. 9ACG was significantly more cytotoxic to EJ/mβG cells as compared to all other cells (Figure 4b). EJ/mβG cells were also sensitive to HAMG (Figure 4c) with

an IC_{50} value of 19 μM. The IC_{50} value of HAMG to EJ/mβG cells was significantly lower then the IC_{50} values of HAMG to the other cells lines (Figure 4d). These results show that EJ/mβG cells most effectively hydrolyzed both 9ACG and HAMG.

Expression of mβG on EJ cancer cells can enhance glucuronide prodrug anti-tumor activity

Mice bearing established EJ, EJ/eβG, EJ/mβG and EJ/hβG subcutaneous tumors were i.v. injected with PBS or 9ACG and tumor sizes were measured 11 days later. 9ACG significantly suppressed the rate of EJ/mβG tumor growth as compared to treatment with vehicle (PBS) (Figure 5a). By contrast, 9ACG did not significantly suppress the growth of EJ, EJ/hβG or EJ/eβG tumors. 9ACG treatment was well tolerated; there was no difference in the weights of mice treated with 9ACG or PBS (results not shown). Downregulation of eβG expression on EJ/eβG tumors as compared to cultured EJ/eβG cells (Figure 5b) could cause poor response to 9ACG therapy. HAMG also significantly suppressed the growth of EJ/mβG tumors *in vivo* with two of five mice experiencing complete tumor regression (Figure 5c). HAMG had little effect on the growth of EJ tumors, demonstrating that surface mβG was required for prodrug activation.

mβG expression on CT26 colon cancer cells

To investigate further the utility of mβG for activation of glucuronide prodrugs, we generated stable CT26 murine colon carcinoma cells that expressed mβG or hβG on their surface (Figure 6a). hβG expression was lower on CT26/hβG tumor cells as compared with cultured CT26/hβG cells, indicating lost hβG expression *in vivo* (Figure 6b, upper panel). By contrast, mβG expression was higher on the CT26/mβG tumor cells, showing that mβG expression was actually more stable *in vivo* than *in vitro* (Figure 6b, lower panel). HAMG was clearly more toxic to CT26/mβG cells ($IC_{50} = 58\ \mu$M) than to either CT26 or CT26/hβG cells (Figure 6c). As the interstitial space of tumors is often acidic,[45] we examined the cytotoxicity of HAMG to cells at pH 6.6. Figure 6d shows that HAMG was about 80-fold more toxic to CT26/mβG cells ($IC_{50} = 2.8\ \mu$M) than to CT26 cells ($IC_{50} = 220\ \mu$M), demonstrating relatively efficient activation of HAMG by CT26/mβG cells at slightly acidic pH values. As it is difficult to transduce all the cancer cells in a tumor, we also investigated whether activation of HAMG could produce bystander killing of parental CT26 cells. Addition of HAMG to as few as 5% CT26/mβG cells mixed with 95% CT26 cells significantly reduced ^{3}H-thymidine incorporation as compared to prodrug treatment of CT26 cells (Figure 6e), showing that surface activation of HAMG produces potent bystander killing. The *in vivo* anti-tumor activity of HAMG was examined in a mouse subcutaneous tumor model. HAMG also displayed potent anti-tumor activity to established CT26/mβG tumors with two of six mice experiencing complete tumor rejection (Figure 6f). As expected, HAMG did not display anti-tumor activity

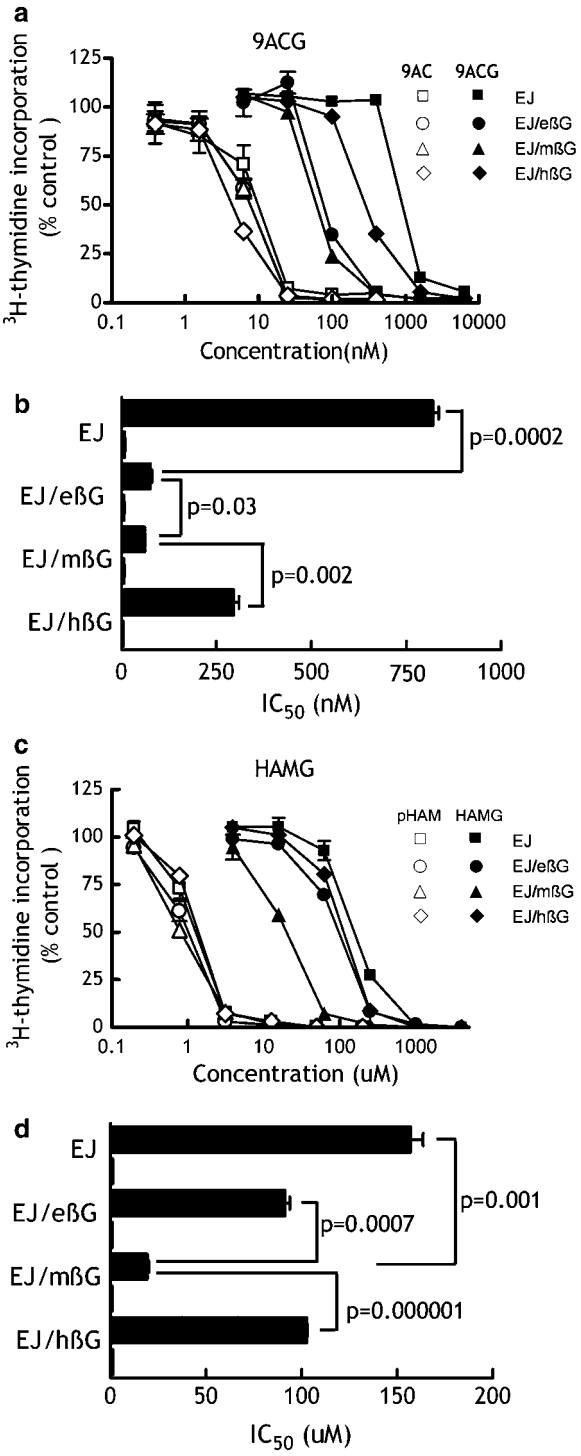

Figure 4 Prodrug activation by EJ cell lines. (**a**) EJ cell lines were incubated with 9AC or 9ACG (**a**) or pHAM and HAMG (**c**) at pH 6.6 before incorporation of ^{3}H-thymidine into cellular DNA was measured ($n = 3$). The IC_{50} values of 9AC and 9ACG (**b**) as well as pHAM and HAMG (**d**) to EJ cell lines are shown. Significant differences (*P*-values) between the IC_{50} values of 9ACG or HAMG to EJ/mβG cells and other cell lines are indicated.

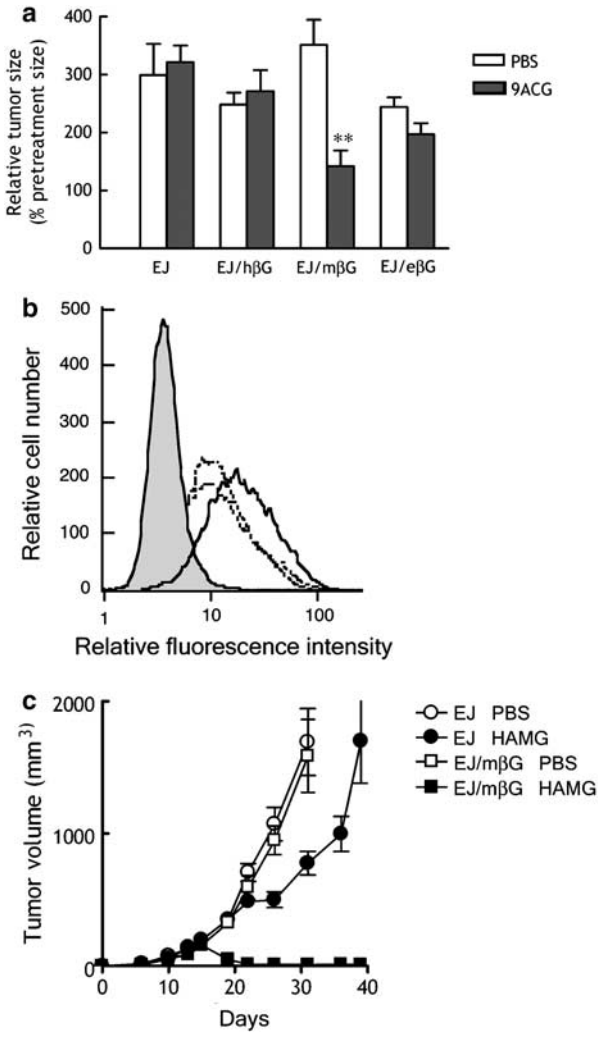

Figure 5 *In vivo* anti-tumor activity of prodrugs to EJ tumors. (**a**) Scid-Beige mice bearing 100–200 mm^3 tumors were i.v. injected with PBS (open bars) or 50 mg/kg 9ACG (solid bars). The results show the mean size of tumors in each group of mice 11 days after 9ACG administration relative to the size of the tumors at the time of prodrug administration. Bars, s.e. 9ACG significantly suppressed the growth of EJ/mβG tumors as compared to treatment with PBS (**$P \leqslant 0.005$). (**b**) Cultured EJ/eβG cells (solid line) or EJ/eβG cells recovered from solid tumors in two Scid-Beige mice (dotted lines) were immunofluorescence stained for the HA epitope or stained with control antibody (solid curve). (**c**) Scid-Beige mice bearing established EJ or EJ/mβG tumors were treated with PBS or three hourly i.v. injections of 50 mg/kg HAMG on days 15 and 19. Results show the mean values of five mice. Bars, s.e.

against CT26 tumors. The therapy was well tolerated with a transient weight loss of 12% in the CT26/mβG group treated with HAMG as compared to a 5% weight loss observed in the CT26/mβG group treated with PBS.

Mammalian cells can secrete eβG

The poor expression of eβG-e-B7 on the surface of various cells suggested that cells retained eβG. Both hβG-e-B7 and mβG-e-B7 displayed similar intracellular distributions with typical golgi localization whereas eβG-e-B7 was more diffusely present in EJ/eβG cells (Figure 7a). Staining for BIP, a resident ER protein, demonstrated colocalization of BIP and eβG-e-B7 (Figure 7b), indicating a predominantly ER localization. By contrast, most mβG-e-B7 did not colocalize with BIP (Figure 7c).

To determine if the ER retention of eβG-e-B7 was an inherent property of eβG, murine fibroblasts were transfected with pLS/GUS or pNeo/GUS (Figure 7d) to allow for secretion or cytosolic expression of eβG, respectively. Cells transfected with pLS/GUS secreted about three-fourths of eβG, showing that eβG can be efficiently secreted from mammalian cells (Figure 7e). By contrast, eβG was largely associated with the cell fraction in cells infected with pNeo/GUS. The total activity of eβG was similar in cells transfected with pLS/GUS or pNeo/GUS, demonstrating that the activity of eβG was retained through the secretory pathway. Thus, retention of eβG-e-B7 in cells is not an inherent property of eβG. Control cells transfected with pLNCX-mβG or pLNCX-hβG displayed equal distribution of βG activity in the medium and cell fraction.

We also examined the possibility that the low expression of eβG-e-B7 was due to rapid shedding of the enzyme from the cell surface. Measurement of βG activities in the culture medium and cell fraction, however, showed that eβG-e-B7 was associated with cells as efficiently as were mβG-e-B7 and hβG-e-B7 (Figure 7f). The total activity of cells transfected with pLNCX-eβG-e-B7 (4.8 U), however, was much lower than the activity of cells transfected with pLS/GUS or pNeo/GUS (600 and 570 U, respectively), indicating loss of eβG catalytic activity in eβG-e-B7.

Discussion

Expression of βG on cancer cells to preferentially activate glucuronide prodrugs is attractive for GDEPT because a wide range of glucuronide prodrugs have been synthesized, glucuronide prodrugs typically display orders of magnitude less toxicity as compared to the activated product,[17,20,24] potent bystander killing of cancer cells may be achieved,[27] and the same gene can potentially be employed for gene expression imaging and therapy.[46] In the present study, we show that maximizing βG activity on cancer cells dramatically enhanced *in vivo* anti-tumor efficacy. We also found that the glucuronide prodrug HAMG was superior to 9ACG for GDEPT.

We anchored βG on the surface of cells by fusion of the enzyme to type I TM. Spacer domains between βG and the TM allow more flexible assembly of βG tetramers and contain glycosylation sites that reduce the shedding of chimeric proteins,[42] presumably by hindering proteolytic cleavage of the receptors. The hβG-e-B7 construct allowed the highest expression of active hβG on fibroblasts. This construct also allowed most effective expression of active mβG and eβG on cells. An analogous chimeric receptor directed the highest levels of a single-chain antibody (scFv) on cells.[42] In contrast to scFv,

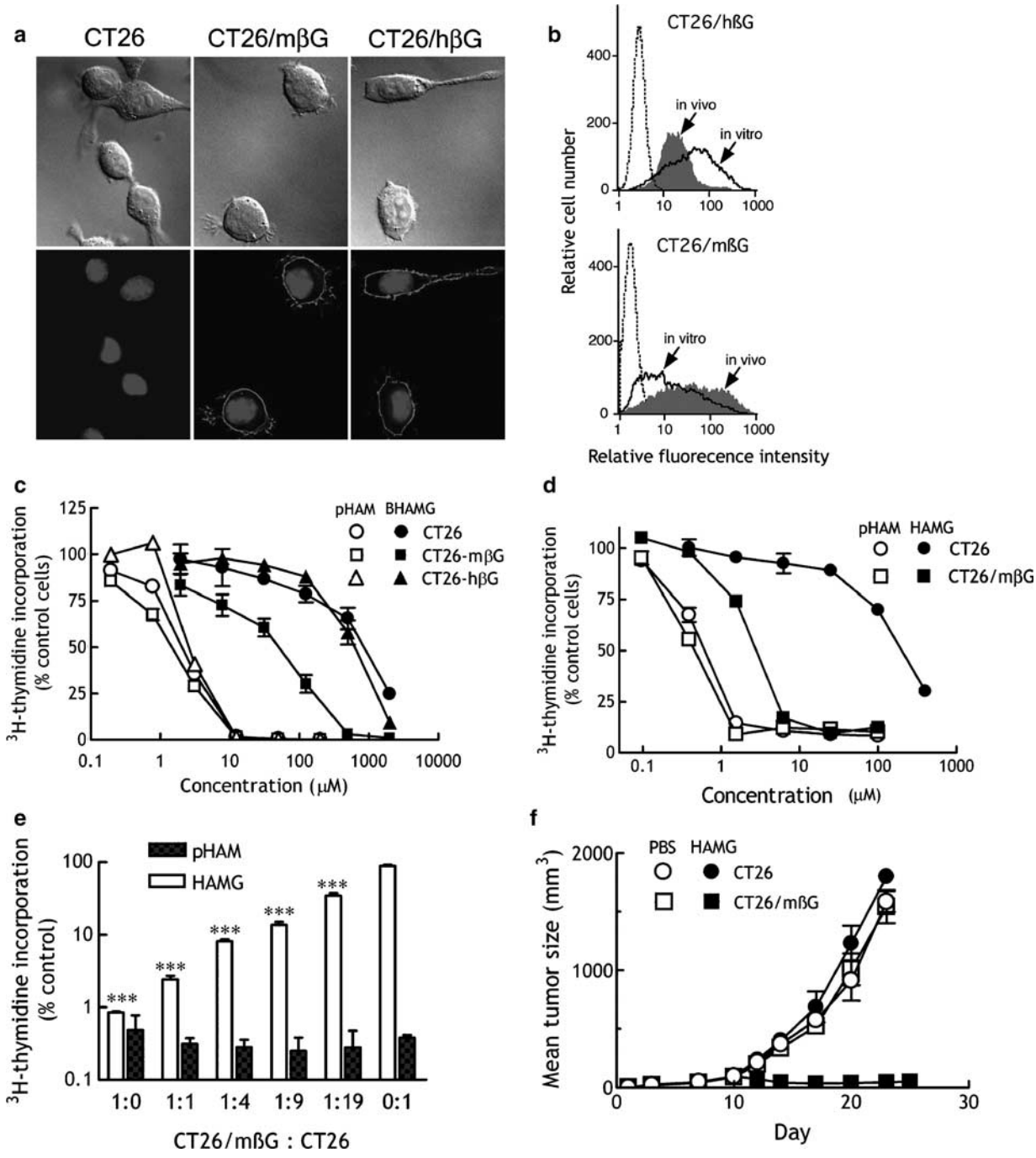

Figure 6 Anti-tumor activity of HAMG to CT26/mβG tumors. (**a**) CT26 cell lines were immunofluorescence stained for the HA epitope (red) and observed under a fluorescence microscope equipped with a CCD detector (lower panels) or under phase-contrast (upper panels). Blue regions show nuclear staining by DAPI. (**b**) CT26/hβG and CT26/mβG cells were cultured two weeks *in vitro* or injected s.c. in BALB/c mice and recovered two weeks later. The cells were then immunofluorescence stained for the HA epitope and cellular fluorescence was quantified on a flow cytometer. (**c**) CT26 cell lines were exposed to pHAM or HAMG at pH 7.4 for 24 h and then ^{3}H-thymidine incorporation was measured 24 h later ($n=3$). (**d**) CT26 cell lines were exposed to pHAM or HAMG at pH 6.6 for 24 h and then ^{3}H-thymidine incorporation was measured 24 h later ($n=3$). (**e**) Bystander killing of CT26 cells was examined by adding pHAM or HAMG to mixtures containing the indicated ratios of CT26/mβG to CT26 cells. ^{3}H-thymidine incorporation was measured 24 h later ($n=3$). Significantly lower incorporation of ^{3}H-thymidine as compared to CT26 cells after treatment with HAMG is indicated (***$P<0.0005$). (**f**) BALB/c mice bearing established CT26 or CT26/mβG tumors were i.v. injected on day 10 with PBS or three fractionated doses of 50 mg/kg HAMG. Results show the mean values of six mice. Bars, s.e.

however, the expression of hβG on cells was relatively insensitive to the nature of the TM and the presence of a spacer domain. For example, attachment of the B7 TM to a scFv resulted in a 15-fold increase in the level of scFv on the cell surface as compared to attachment of the PDGFR TM.[47] Likewise, introduction of the e-B7 spacer between

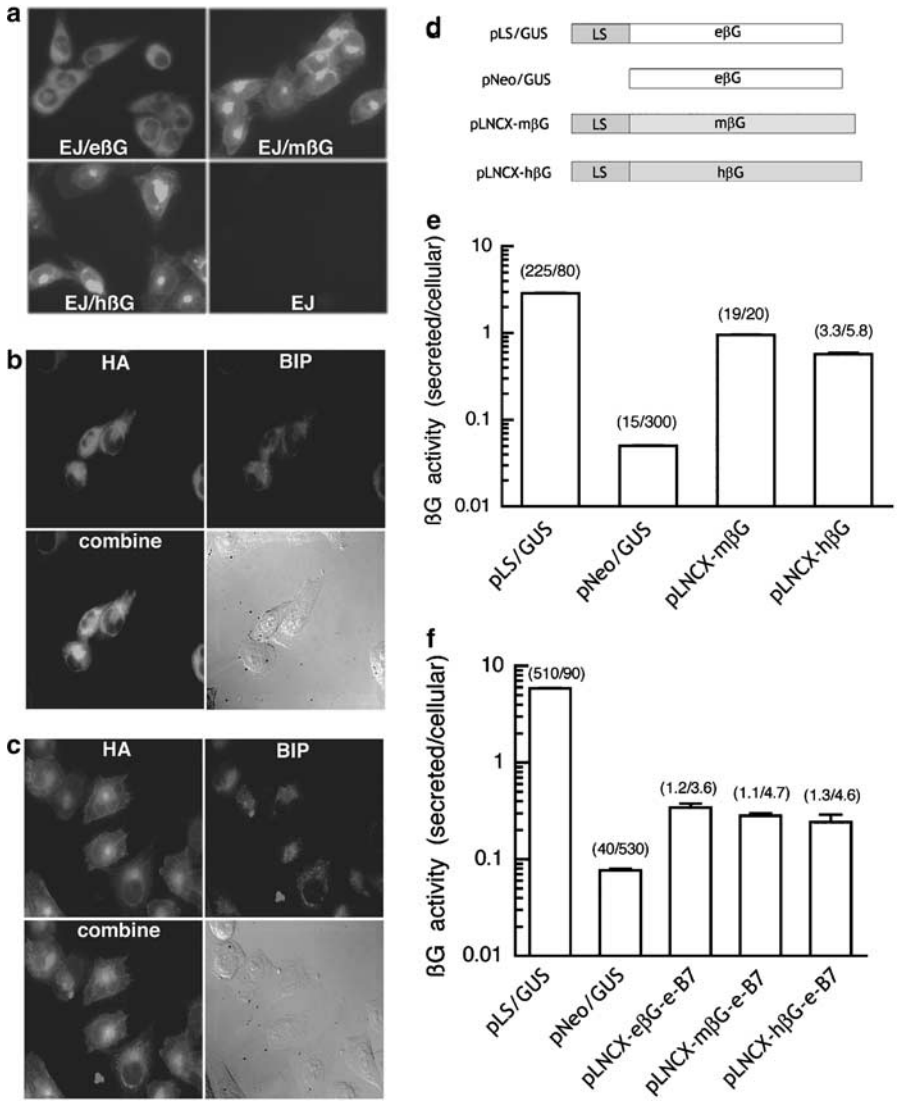

Figure 7 Intracellular localization and secretion of eβG. (**a**) The indicated cells were fixed and permeabilized before the cells were stained for the HA epitope. Fixed and permeabilized EJ/eβG cells (**b**) and EJ/mβG cells (**c**) were stained for the HA epitope (green) and BIP (red). Yellow regions in the combined overlays indicate regions of βG and BIP colocalization. (**d**) Schematic of the trangenes employed for secretion or intracellular expression of βG. (**e**) 3T3 fibroblasts were transiently transfected with the indicated vectors and the ratio of βG activity in the culture medium (secreted) relative to the βG activity in the total cell lysate (cellular) was determined 48 h later ($n=3$) (**f**) 3T3 fibroblasts were transiently transfected with the indicated vectors and the ratio of βG activity in the culture medium (secreted) relative to the βG activity in the total cell lysate (cellular) was determined 60 h later ($n=3$). eβG activity was measured at pH 7 whereas mβG and hβG activities were assayed at pH 4.5. The units of βG activity present in the culture medium and cell lysates (secreted/cellular) are shown above each bar.

the scFv and B7 TM further increased surface expression by about eightfold.[42] In contrast, the expression of hβG-e-B7 on cells was only about 2.5-fold higher than hβG-PDGFR. We attribute these differences to the bulky, tetrameric structure of βG. Accumulation of chimeric receptors on cells depends on their intracellular transport rate and half-life. scFv are highly susceptible to shedding[42] and surface expression can thus be greatly enhanced by the TM (which affects the rate of intracellular transport[44]) and the spacer domain (which hinders shedding of the receptor[42]). By contrast, the bulky structure of βG[48] likely hinders shedding as previously found for another large protein.[44] In addition, shedding of the enzyme requires cleavage of all four 'legs' of the βG tetramer. Stable surface expression allows accumulation of βG on cells even at relatively slow rates of intracellular transport. Thus, the choice of spacer and TM had less impact on the amount of hβG present on the cell surface.

Comparison of three different sources of βG revealed that mβG possessed the best combination of surface expression and activity for GDEPT. Thus, EJ/mβG cells exhibited greater βG activity, were significantly more sensitive to both 9ACG and HAMG and had growth significantly suppressed by two glucuronide prodrugs *in vivo* as compared to cells expressing hβG or eβG. Although cells expressed hβG and mβG at similar levels,

mβG displayed about three times greater enzymatic activity at pH 7. The K_m of mβG is lower than hβG at pH 7 (results not shown), indicating that the effective activity differences between mβG and hβG may be even greater at low prodrug concentrations. Furthermore, the clear superiority of mβG as compared to hβG suggests that even modest increases in expression levels and specific catalytic activity can enhance therapeutic efficacy. Screening for more active βG variants from other species or development of βG mutants with higher enzyme activity appear to be warranted. eβG displayed optimal activity at physiological pH values and possesses higher intrinsic βG activity as compared to either lysosomal βG, but was poorly expressed on the plasma membrane. Furthermore, attachment of spacer or TM domains damaged eβG activity. This was most apparent with eβG-CD44-B7, which produced high surface expression but low βG activity. Immunofluorescence staining of EJ cell lines indicated that eβG was retained in the ER. As mβG can be retained in the ER by complex formation with egasyn,[49] we tested whether eβG retention was an intrinsic property of the bacterial enzyme. Murine fibroblasts, however, secreted eβG more efficiently than hβG or mβG. It thus appears that attachment of spacer or TM domains changed the conformation of eβG and hindered its transit from the ER. We also attempted to anchor eβG to cells by attachment of a type II TM (asialoglycoprotein receptor) with eB7, BGP or CD44 spacers to the N-terminus of eβG but surface expression levels were uniformly poor (results not shown). The mechanism of the low expression of eβG on the cell membrane requires additional investigation.

HAMG displayed better anti-tumor activity than 9ACG against EJ-mβG tumors; 9ACG significantly suppressed the growth of tumors but did not cause complete tumor regression whereas HAMG caused regression of all tumors and cured two mice of established tumors. This result was unexpected because the active form of 9ACG is about 1000 times more potent than the active form of HAMG. We speculate that HAMG may be more effective *in vivo* due to its rapid and uniform modes of action. Alkylating agents such as pHAM, the activated form of HAMG, cross-link DNA and act rapidly. For example, similar IC_{50} values to pHAM are obtained after exposure to cells for 2 or 24 h.[32,36] Furthermore, the cytotoxic effects of alkylating agents are relatively insensitive to the cell cycle status of cells.[50] By contrast, 9AC, the activation product of 9ACG, inhibits topoisomerase I and kills cells in a time-dependent fashion; short exposure to topoisomerase inhibitors is ineffective.[51] Glucuronide prodrugs that produce rapidly acting drug products may therefore be more favorable for GDEPT since glucuronides are rapidly eliminated from the circulation.[52] Mice also tolerated HAMG well. Bolus injections (i.v.) of up to 240 mg/kg HAMG did not produce weight loss in mice. Treatment of mice bearing CT-26/mβG tumors produced modest but transient weight loss. Although not investigated in the present study, we previously found that the normal dose-limiting toxicity of alkylating agents, leukopenia, was greatly reduced in rats treated with a βG immunoconjugate and HAMG as compared to treatment with pHAM.[31] The short half-life of pHAM (on the order of minutes)[26] may help reduce systemic toxicity. A study showing that surface hβG activation of a doxorubicin prodrug produced anti-tumor activity in mice[29] suggests that multiple glucuronide prodrugs may be suitable for GDEPT with βG.

An advantage of GDEPT over ADEPT is the possibility of employing 'immunogenic' enzymes for therapy. In ADEPT, antibody-enzyme fusion proteins containing non-human enzymes induce neutralizing antibodies that curtail repeated treatment cycles.[53] Although tumors in mice lost expression of non-syngeneic βG (eβG and hβG), this may be less important for the transient expression required in clinical settings. For example, non-mammalian enzymes are commonly employed in GDEPT.[3,54] This suggests that mβG-e-B7 could activate glucuronide prodrugs in humans.

In summary, linking βG to the Ig-like C_2-type and Ig-hinge-like domains of the B7-1 antigen (e-B7) followed by the B7-1 TM allowed high-level expression of hβG and mβG on cells. HAMG was more effective than 9ACG for GDEPT and mβG-e-B7 achieved the best compromise between surface expression and catalytic activity. The high specific activity of eβG at physiologically relevant pH values (about 65-fold greater than mβG and 200-fold greater than hβG at pH 7) makes this enzyme highly attractive for GDEPT, but strategies are required to engineer surface forms of this enzyme that promote rapid intracellular transport with retention of enzymatic activity. Finally, we have recently shown that mβG-e-B7 can be employed to image gene expression in mice.[46] The same gene can therefore be employed to first verify successful and proper expression of the transgene in target tissue and then to activate glucuronide prodrugs for therapy.

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References

1 Hamstra DA, Lee KC, Tychewicz JM, Schepkin VD, Moffat BA, Chen M *et al*. The use of 19F spectroscopy and diffusion-weighted MRI to evaluate differences in gene-dependent enzyme prodrug therapies. *Mol Ther* 2004; **10**: 916–928.
2 Moolten FL, Wells JM. Curability of tumors bearing herpes thymidine kinase genes transferred by retroviral vectors. *J Natl Cancer Inst* 1990; **82**: 297–300.
3 Sung MW, Yeh HC, Thung SN, Schwartz ME, Mandeli JP, Chen SH *et al*. Intratumoral adenovirus-mediated suicide gene transfer for hepatic metastases from colorectal

adenocarcinoma: results of a phase I clinical trial. *Mol Ther* 2001; **4**: 182–191.
4 Pandha HS, Martin LA, Rigg A, Hurst HC, Stamp GW, Sikora K *et al*. Genetic prodrug activation therapy for breast cancer: A phase I clinical trial of erbB-2-directed suicide gene expression. *J Clin Oncol* 1999; **17**: 2180–2189.
5 Mesnil M, Yamasaki H. Bystander effect in herpes simplex virus-thymidine kinase/ganciclovir cancer gene therapy: role of gap-junctional intercellular communication. *Cancer Res* 2000; **60**: 3989–3999.
6 Rubsam LZ, Davidson BL, Shewach DS. Superior cytotoxicity with ganciclovir compared with acyclovir and 1-beta-D-arabinofuranosylthymine in herpes simplex virus-thymidine kinase-expressing cells: a novel paradigm for cell killing. *Cancer Res* 1998; **58**: 3873–3882.
7 Nishida M, Futami S, Morita I, Maekawa K, Murota SI. Hypoxia-reoxygenation inhibits gap junctional communication in cultured human umbilical vein endothelial cells. *Endothelium* 2000; **7**: 279–286.
8 Thust R, Tomicic M, Klocking R, Voutilainen N, Wutzler P, Kaina B. Comparison of the genotoxic and apoptosis-inducing properties of ganciclovir and penciclovir in Chinese hamster ovary cells transfected with the thymidine kinase gene of herpes simplex virus-1: implications for gene therapeutic approaches. *Cancer Gene Ther* 2000; **7**: 107–117.
9 Huber BE, Austin EA, Richards CA, Davis ST, Good SS. Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. *Proc Natl Acad Sci USA* 1994; **91**: 8302–8306.
10 Lawrence TS, Rehemtulla A, Ng EY, Wilson M, Trosko JE, Stetson PL. Preferential cytotoxicity of cells transduced with cytosine deaminase compared to bystander cells after treatment with 5-flucytosine. *Cancer Res* 1998; **58**: 2588–2593.
11 Haberkorn U, Oberdorfer F, Gebert J, Morr I, Haack K, Weber K *et al*. Monitoring gene therapy with cytosine deaminase: in vitro studies using tritiated-5-fluorocytosine. *J Nucl Med* 1996; **37**: 87–94.
12 Miller CR, Williams CR, Buchsbaum DJ, Gillespie GY. Intratumoral 5-fluorouracil produced by cytosine deaminase/5-fluorocytosine gene therapy is effective for experimental human glioblastomas. *Cancer Res* 2002; **62**: 773–780.
13 Shand N, Weber F, Mariani L, Bernstein M, Gianella-Borradori A, Long Z *et al*. A phase 1-2 clinical trial of gene therapy for recurrent glioblastoma multiforme by tumor transduction with the herpes simplex thymidine kinase gene followed by ganciclovir. GLI328 European-Canadian Study Group. *Hum Gene Ther* 1999; **10**: 2325–2335.
14 Crystal RG, Hirschowitz E, Lieberman M, Daly J, Kazam E, Henschke C *et al*. Phase I study of direct administration of a replication deficient adenovirus vector containing the *E. coli* cytosine deaminase gene to metastatic colon carcinoma of the liver in association with the oral administration of the pro-drug 5-fluorocytosine. *Hum Gene Ther* 1997; **8**: 985–1001.
15 Freytag SO, Khil M, Stricker H, Peabody J, Menon M, DePeralta-Venturina M *et al*. Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. *Cancer Res* 2002; **62**: 4968–4976.
16 Rainov NG. A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther* 2000; **11**: 2389–2401.
17 Haisma HJ, van Muijen M, Pinedo HM, Boven E. Comparison of two anthracycline-based prodrugs for activation by a monoclonal antibody-beta-glucuronidase conjugate in the specific treatment of cancer. *Cell Biophys* 1994; **24-25**: 185–192.
18 Houba PH, Boven E, van der Meulen-Muileman IH, Leenders RG, Scheeren JW, Pinedo HM *et al*. A novel doxorubicin-glucuronide prodrug DOX-GA3 for tumour-selective chemotherapy: distribution and efficacy in experimental human ovarian cancer. *Br J Cancer* 2001; **84**: 550–557.
19 Schmidt F, Monneret C. Prodrug Mono Therapy: synthesis and biological evaluation of an etoposide glucuronide-prodrug. *Bioorg Med Chem* 2003; **11**: 2277–2283.
20 Leu YL, Roffler SR, Chern JW. Design and synthesis of water-soluble glucuronide derivatives of camptothecin for cancer prodrug monotherapy and antibody-directed enzyme prodrug therapy (ADEPT). *J Med Chem* 1999; **42**: 3623–3628.
21 Angenault S, Thirot S, Schmidt F, Monneret C, Pfeiffer B, Renard P. Cancer chemotherapy: a SN-38 (7-ethyl-10-hydroxycamptothecin) glucuronide prodrug for treatment by a PMT (Prodrug MonoTherapy) strategy. *Bioorg Med Chem Lett* 2003; **13**: 947–950.
22 de Bont DB, Leenders RG, Haisma HJ, van der Meulen-Muileman I, Scheeren HW. Synthesis and biological activity of beta-glucuronyl carbamate-based prodrugs of paclitaxel as potential candidates for ADEPT. *Bioorg Med Chem* 1997; **5**: 405–414.
23 Bouvier E, Thirot S, Schmidt F, Monneret C. First enzymatically activated Taxotere prodrugs designed for ADEPT and PMT. *Bioorg Med Chem* 2004; **12**: 969–977.
24 Wang SM, Chern JW, Yeh MY, Ng JC, Tung E, Roffler SR. Specific activation of glucuronide prodrugs by antibody-targeted enzyme conjugates for cancer therapy. *Cancer Res* 1992; **52**: 4484–4491.
25 Lougerstay-Madec R, Florent JC, Monneret C, Nemati F, Poupon MF. Synthesis of self-immolative glucuronide-based prodrugs of a phenol mustard. *Anticancer Drug Des* 1998; **13**: 995–1007.
26 Cheng TL, Chou WC, Chen BM, Chern JW, Roffler SR. Characterization of an antineoplastic glucuronide prodrug. *Biochem Pharmacol* 1999; **58**: 325–328.
27 Cheng TL, Wei SL, Chen BM, Chern JW, Wu MF, Liu PW *et al*. Bystander killing of tumour cells by antibody-targeted enzymatic activation of a glucuronide prodrug. *Br J Cancer* 1999; **79**: 1378–1385.
28 Weyel D, Sedlacek HH, Muller R, Brusselbach S. Secreted human beta-glucuronidase: a novel tool for gene-directed enzyme prodrug therapy. *Gene Therapy* 2000; **7**: 224–231.
29 Heine D, Muller R, Brusselbach S. Cell surface display of a lysosomal enzyme for extracellular gene-directed enzyme prodrug therapy. *Gene Therapy* 2001; **8**: 1005–1010.
30 Bosslet K, Czech J, Hoffmann D. Tumor-selective prodrug activation by fusion protein-mediated catalysis. *Cancer Res* 1994; **54**: 2151–2159.
31 Chen BM, Chan LY, Wang SM, Wu MF, Chern JW, Roffler SR. Cure of malignant ascites and generation of protective immunity by monoclonal antibody-targeted activation of a glucuronide prodrug in rats. *Int J Cancer* 1997; **73**: 392–402.
32 Cheng TL, Chen BM, Chern JW, Wu MF, Roffler SR. Efficient clearance of poly(ethylene glycol)-modified

immunoenzyme with anti-PEG monoclonal antibody for prodrug cancer therapy. *Bioconjug Chem* 2000; **11**: 258–266.
33 Bosslet K, Straub R, Blumrich M, Czech J, Gerken M, Sperker B *et al*. Elucidation of the mechanism enabling tumor selective prodrug monotherapy. *Cancer Res* 1998; **58**: 1195–1201.
34 Prijovich ZM, Chen BM, Leu YL, Chern JW, Roffler SR. Anti-tumour activity and toxicity of the new prodrug 9-aminocamptothecin glucuronide (9ACG) in mice. *Br J Cancer* 2002; **86**: 1634–1638.
35 Roffler SR, Wang SM, Chern JW, Yeh MY, Tung E. Anti-neoplastic glucuronide prodrug treatment of human tumor cells targeted with a monoclonal antibody-enzyme conjugate. *Biochem Pharmacol* 1991; **42**: 2062–2065.
36 Cheng TL, Chen BM, Chan LY, Wu PY, Chern JW, Roffler SR. Poly(ethylene glycol) modification of beta-glucuronidase-antibody conjugates for solid-tumor therapy by targeted activation of glucuronide prodrugs. *Cancer Immunol Immunother* 1997; **44**: 305–315.
37 Hsiang YH, Hertzberg R, Hecht S, Liu LF. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J Biol Chem* 1985; **260**: 14873–14878.
38 Hsiang YH, Liu LF, Wall ME, Wani MC, Nicholas AW, Manikumar G *et al*. DNA topoisomerase I-mediated DNA cleavage and cytotoxicity of camptothecin analogues. *Cancer Res* 1989; **49**: 4385–4389.
39 Cheng TL, Wu PY, Wu MF, Chern JW, Roffler SR. Accelerated clearance of polyethylene glycol-modified proteins by anti-polyethylene glycol IgM. *Bioconjug Chem* 1999; **10**: 520–528.
40 Wu CH, Balasubramanian WR, Ko YP, Hsu G, Chang SE, Prijovich ZM *et al*. A simple method for the production of recombinant proteins from mammalian cells. *Biotechnol Appl Biochem* 2004; **40**: 167–172.
41 Chou WC, Liao KW, Lo YC, Jiang SY, Yeh MY, Roffler SR. Expression of chimeric monomer and dimer proteins on the plasma membrane of mammalian cells. *Biotechnol Bioeng* 1999; **65**: 160–169.
42 Liao KW, Chen BM, Liu TB, Tzou SC, Lin YM, Lin KF *et al*. Stable expression of chimeric anti-CD3 receptors on mammalian cells for stimulation of antitumor immunity. *Cancer Gene Ther* 2003; **10**: 779–790.
43 Marshall CJ, Franks LM, Carbonell AW. Markers of neoplastic transformation in epithelial cell lines derived from human carcinomas. *J Natl Cancer Inst* 1977; **58**: 1743–1751.
44 Liao KW, Chou WC, Lo YC, Roffler SR. Design of transgenes for efficient expression of active chimeric proteins on mammalian cells. *Biotechnol Bioeng* 2001; **73**: 313–323.
45 Tannock IF, Rotin D. Acid pH in tumors and its potential for therapeutic exploitation. *Cancer Res* 1989; **49**: 4373–4384.
46 Su YC, Chuang KH, Wang YM, Cheng CM, Lin SR, Wang JY *et al*. Gene expression imaging by enzymatic catalysis of a fluorescent probe via membrane-anchored β-glucuronidase. *Gene Therapy* (Submitted).
47 Liao KW, Lo YC, Roffler SR. Activation of lymphocytes by anti-CD3 single-chain antibody dimers expressed on the plasma membrane of tumor cells. *Gene Therapy* 2000; **7**: 339–347.
48 Jain S, Drendel WB, Chen ZW, Mathews FS, Sly WS, Grubb JH. Structure of human beta-glucuronidase reveals candidate lysosomal targeting and active-site motifs. *Nat Struct Biol* 1996; **3**: 375–381.
49 Zhen L, Rusiniak ME, Swank RT. The beta-glucuronidase propeptide contains a serpin-related octamer necessary for complex formation with egasyn esterase and for retention within the endoplasmic reticulum. *J Biol Chem* 1995; **270**: 11912–11920.
50 Murray D, Meyn RE. Cell cycle-dependent cytotoxicity of alkylating agents: determination of nitrogen mustard-induced DNA cross-links and their repair in Chinese hamster ovary cells synchronized by centrifugal elutriation. *Cancer Res* 1986; **46**: 2324–2329.
51 Chen BM, Chen JY, Kao M, Lin JB, Yu MH, Roffler SR. Elevated topoisomerase I activity in cervical cancer as a target for chemoradiation therapy. *Gynecol Oncol* 2000; **79**: 272–280.
52 de Graaf M, Nevalainen TJ, Scheeren HW, Pinedo HM, Haisma HJ, Boven E. A methylester of the glucuronide prodrug DOX-GA3 for improvement of tumor-selective chemotherapy. *Biochem Pharmacol* 2004; **68**: 2273–2281.
53 Sharma SK, Bagshawe KD, Melton RG, Sherwood RF. Human immune response to monoclonal antibody-enzyme conjugates in ADEPT pilot clinical trial. *Cell Biophys* 1992; **21**: 109–120.
54 Freytag SO, Stricker H, Pegg J, Paielli D, Pradhan DG, Peabody J *et al*. Phase I study of replication-competent adenovirus-mediated double-suicide gene therapy in combination with conventional-dose three-dimensional conformal radiation therapy for the treatment of newly diagnosed, intermediate- to high-risk prostate cancer. *Cancer Res* 2003; **63**: 7497–7506.