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Selective Cancer Therapy by Extracellular Activation of a Highly Potent Glycosidic Duocarmycin Analogue

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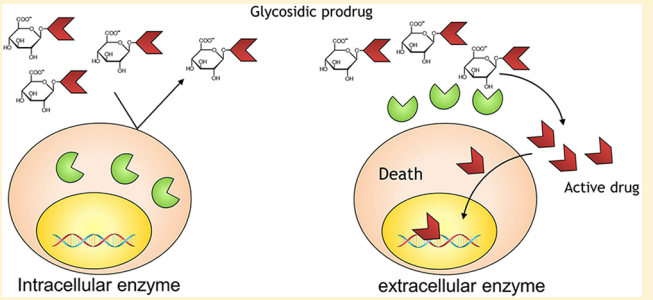
ABSTRACT: Conventional cancer chemotherapy is limited by systemic toxicity and poor selectivity. Tumor-selective activation of glucuronide prodrugs by beta-glucuronidase in the tumor microenvironment in a monotherapeutic approach is one promising way to increase cancer selectivity. Here we examined the cellular requirement for enzymatic activation as well as the in vivo toxicity and antitumor activity of a glucuronide prodrug of a potent duocarmycin analogue that is active at low picomolar concentrations. Prodrug activation by intracellular and extracellular beta-glucuronidase was investigated by measuring prodrug **2** cytotoxicity against human cancer cell lines that displayed different endogenous levels of beta-glucuronidase, as well as against beta-glucuronidase-deficient fibroblasts and newly established beta-glucuronidase knockdown cancer lines. In all cases, glucuronide prodrug **2** was 1000−5000 times less cytotoxic than the parent duocarmycin analogue regardless of intracellular levels of beta-glucuronidase. By contrast, cancer cells that displayed tethered beta-glucuronidase on their plasma membrane were 80-fold more sensitive to glucuronide prodrug **2**, demonstrating that prodrug activation depended primarily on extracellular rather than intracellular beta-glucuronidase activity. Glucuronide prodrug **2** (2.5 mg/kg) displayed greater antitumor activity and less systemic toxicity in vivo than the clinically used drug carboplatin (50 mg/kg) to mice bearing human lung cancer xenografts. Intratumoral injection of an adenoviral vector expressing membrane-tethered beta-glucuronidase dramatically enhanced the in vivo antitumor activity of prodrug **2**. Our data provide evidence that increasing extracellular beta-glucuronidase activity in the tumor microenvironment can boost the therapeutic index of a highly potent glucuronide prodrug.



KEYWORDS: *prodrugs, glycosides, beta-glucuronidase, cancer therapy, tumor microenvironment*

INTRODUCTION

Although traditional chemotherapeutic agents have been used for cancer therapy for decades, poor selectivity against cancer cells limits their therapeutic effectiveness. Enzyme prodrug therapy is a promising strategy to improve the therapeutic index of chemotherapy.[1−3] This approach seeks to develop prodrugs that can be selectively activated by enzymes present in the tumor microenvironment. Glucuronide prodrugs possess advantages for enzyme prodrug therapy based on: (a) increased water solubility due to the charged glucuronide moiety; (b) increased polarity that lowers cellular permeability, which in turn, reduces uptake and activation in normal tissues; and (c) good in vivo stability due to low levels of beta-glucuronidase in serum.[4−8] Novel protein−drug conjugates and heterodimeric prodrugs that are activated by beta-glucuronidase promise to further extend the potential of glucuronide prodrugs for cancer treatment.[9,10]

Increased concentrations of beta-glucuronidase have been reported in solid tumors.[6,11] Elevated beta-glucuronidase levels in the tumor microenvironment are believed to result from the release of lysosomal beta-glucuronidase from necrotic or apoptotic cancer cells as well as from tumor-infiltrating monocytes and neutrophils.[12−14] Beta-glucuronidase is also associated with tumoral metastatic potential.[15] Beta-glucuronidase activity in tumors can also be artificially elevated by immunoenzyme therapy[16−20] or by expressing beta-glucuronidase in cancer cells.[21,22] The design of drugs that can be selectively activated by beta-glucuronidase in the tumor microenvironment is therefore a rational and promising approach to increase cancer chemotherapy efficacy.

Duocarmycin SA (Figure 1)[23] is a member of a group of antineoplastic agents which includes yatakemycin[24] and CC1065[25] with low picomolar potency. Their potent antineoplastic activity is believed to derive from their ability to bind and alkylate DNA in AT-rich regions of the minor groove,[26] although recent investigations have suggested another target exists for these compounds.[27−29] Duocarmycin SA, however, displays side effects including hepatotoxicity and myelosuppression in vivo.[30,31] A series of glycoside prodrugs of seco-analogues of duocarmycin SA were previously synthesized to decrease systemic toxicity and enhance tumor selectivity.[32−36]

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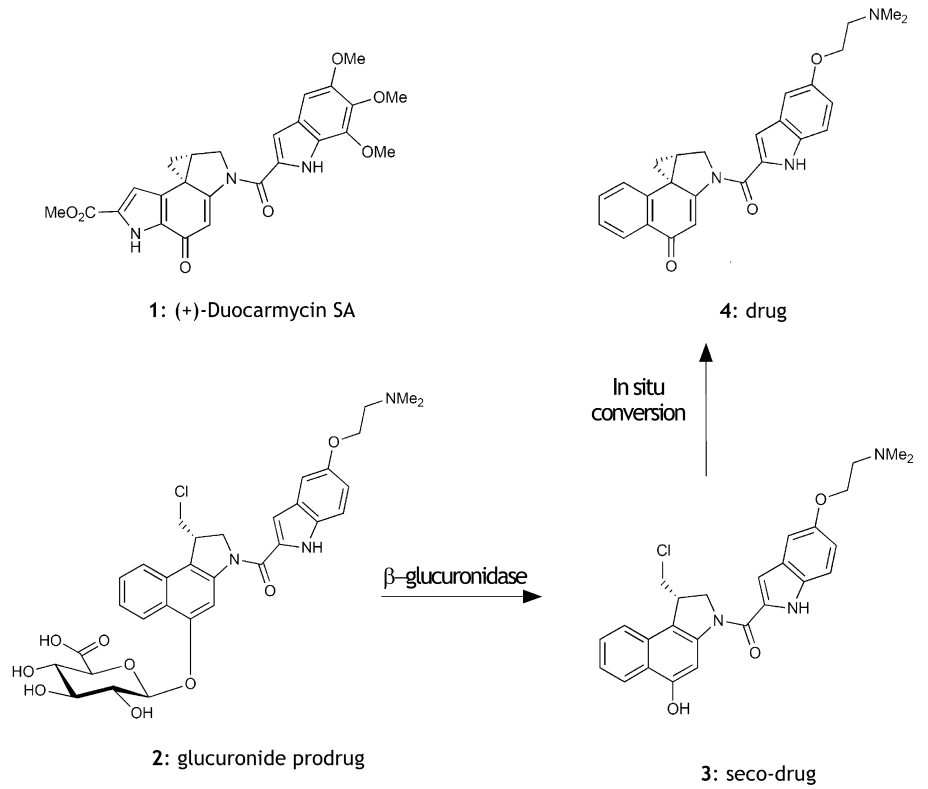

Figure 1. Structures of (+)-Duocarmycin SA (**1**), glucuronide prodrug (**2**), seco-drug (**3**), and cytotoxic drug (**4**).

Compound **2**, a glucuronide derivate of the seco-drug **3**, exhibits high QIC_{50} values (IC_{50} of prodrug/IC_{50} of prodrug in the presence of beta-glucuronidase) and good stability in human serum.[37,38] The seco-drug **3**, derived from **2** by removal of the sugar moiety, is transformed in situ with a half-life of 12.2 ± 0.7 min in human serum at pH 7.4 into the cyclopropyl derivative **4**,[39] which is the final drug responsible for the high cytotoxicity of these compounds.[40]

Beta-glucuronidase can be present inside cancer cells and tumor-infiltrating immune cells as well as extracellularly in the tumor microenvironment. Given the high potencies of prodrug **2** and the cyclopropyl derivative **4**, entry of even a small amount of **2** into cells could result in significant anticancer activity. It is therefore unclear if cellular sensitivity to prodrug **2** is primarily determined by intracellular beta-glucuronidase activity or if beta-glucuronidase must be present in the extracellular environment to effectively activate prodrug **2**. In addition, the in vivo antitumor activity of compound **2** has never been examined. Here, we investigated the cellular requirements for prodrug **2** cytotoxicity as well as the in vivo toxicity and antitumor activity of prodrug **2** against human tumor xenografts for cancer monotherapy and enzyme prodrug therapy.

MATERIALS AND METHODS

Cell Lines. LS174T human colon adenocarcinoma (ATCC CCL-188), Caski human cervical carcinoma (ATCC CRL-1550), and HT-29 colorectal adenocarcinoma (ATCC HTB-38) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). CL1-5 human lung adenocarcinoma cells were kindly provided by Pan-Chyr Yang (Academia Sinica, Taipei, Taiwan).[41] EJ human bladder carcinoma cells were a gift from Konan Peck (Academia Sinica, Taipei, Taiwan).[42] Murine MPS VII skin 3521 fibroblasts (beta-glucuronidase deficient) and 3522 fibroblasts (normal beta-glucuronidase levels) isolated from heterozygous mice were kindly provided by Mark Sands (Washington University, School of Medicine, St. Louis, MO, USA). LS174T, CL1-5, HT-29, EJ, and Caski cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated bovine serum, 2.98 mg/mL HEPES, 1 mg/mL sodium bicarbonate, 100 units/ml penicillin, and 100 μg/mL streptomycin in a 5% CO_2 humidified atmosphere in air at 37 °C. 3521 and 3522 cells were cultured in high glucose Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing the same supplements. Cancer cell lines were infected with retroviral particles expressing membrane-anchored murine beta-glucuronidase and selected with G418 to create stable surface beta-glucuronidase-expressing cell lines as previously described.[43]

shRNA Mediated Downregulation of Endogenous Beta-Glucuronidase. The vectors pCMV-ΔR8.91, pMD.G, and a shRNA expression plasmid (pLKO.1) which targets the sequence 5′-CCGAATCACTATCGCCATCAA in human beta-glucuronidase were obtained from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). Recombinant lentiviral particles were produced by cotransfection of 2.25 μg pCMV-ΔR8.91, 0.25 μg pMD.G, and 2.5 μg PLKO.1 using TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI, USA) in 6×10^5 HEK 293T cells (ATCC CRL-11268). The virus suspension was collected at 40 and 64 h after transfection. For lentivirus transduction, lentiviral particles were suspended in culture medium containing 5 μg/mL polybrene (Sigma-Aldrich). Human cancer cell lines were seeded in 6-well plates (1×10^5 cells/well) one day before viral infection. Lentivirus containing medium was added to the cells and then centrifuged

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for 1.5 h (500 × g, 32 °C). Stable cell lines were selected in puromycin (5 μg/mL, Sigma-Aldrich).

DNA Plasmid Construction. A furin-2A (F2A) based bicistronic expression strategy was used to link an enhanced green fluorescence protein (eGFP) to *E. coli* beta-glucuronidase.[44] The eGFP gene was amplified by PCR from pTY-EFeGFP (generously provided by Dr. Lung-Ji Chang, University of Florida, USA) with the primers 5′-ATCGAAGCTTGCCACCATGGTGAGCAAGGGCGAGGAGCTG-3′ and 5′- ATCGCTCGAGCTTGTACAGCTCGTCCATGCCGAG-3′, respectively. The PCR product was digested with *Hin*dIII and *Xho*I restriction enzymes and cloned in pLNCX-anti-PEG-eB7.[45] A cDNA fragment encompassing an HA epitope tag and full *E. coli* beta-glucuronidase gene was amplified from pLNCX-e*β*G-e-B7[43] with the primers 5′-GACCAGCCACAACCATGGTTTATCCATATGATGTTCCAGATTATGC-3′ and 5′-GAGTTGTCGACTCATTGTTTTCCTCCCTGCTG-3′ to introduce a *Bst*XI site at the 5′-end and a *Sal*I site at the 3′-end, respectively. The PCR product was digested with *Bst*XI and *Sal*I and ligated behind the eGF-F2A sequence to generate the retroviral vector pLNCX-eGFP-F2A-HA-e*β*G for cytosolic expression of *E. coli* beta-glucuronidase. CL1-5 cells were infected with retroviral particles expressing membrane-anchored *E. coli* beta-glucuronidase (pLNCX-e*β*G-e-B7[43]) or cytosolic *E. coli* beta-glucuronidase (pLNCX-eGFP-F2A-HA-e*β*G) and selected with G418 to create stable cell lines as described.[43]

Preparation of Recombinant Adenoviral Vectors. Ad-*α*DNS and Ad-*β*G were prepared as previously described.[22]

Flow Cytometry. Cells were stained with a rat monoclonal antibody against murine beta-glucuronidase (7G7)[43] or a rat monoclonal antibody against HA (Roche, Mannheim, Germany) followed by a goat antibody against rat IgG (H+L) conjugated with FITC (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). For cells expressing *E. coli* beta-glucuronidase, cells were stained with 1E8 antibeta-glucuronidase monoclonal antibody[17] and FITC-labeled goat antimouse Fc antibody (Jackson ImmunoResearch Laboratories). The surface immunofluorescence of 10^4 viable cells was measured with a FACSCaliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and fluorescence intensities were analyzed with Flowjo V7.2.5 (Tree Star Inc., Ashland, OR, USA).

In Vitro Cytotoxicity. About 5000 to 10 000 cells per well were plated in 96 well microtiter plates overnight. Graded concentrations of compound **3** or **2** with or without 1 μg of *E. coli* beta-glucuronidase in 200 μL of medium were added for 24 h in triplicate. The cells were then washed twice with sterile phosphate-buffered saline (PBS), and fresh medium was added for another 24 h. Fresh medium containing 1 μCi per well ^{3}H-thymidine was added 16 h before the cells were harvested on glass-fiber filters, and the radioactivity was measured on a Topcount scintillation counter. Results are expressed as percent of ^{3}H-thymidine incorporation compared with untreated cells.

Beta-Glucuronidase Activity Assays. To measure endogenous beta-glucuronidase activity, cells were harvested with trypsin, washed twice with cold PBS, and then suspended in PBS/0.1% Tween 20. Cells were frozen at −80 °C overnight and broken by sonication (XL-2020, Misonix Inc., Farmingdale, NY, USA). The cell lysate was clarified by centrifugation at 15 000 × *g* for 30 min at 4 °C. The enzymatic activity of beta-glucuronidase was measured in triplicate by incubating 20 μL of cell lysate, 10 μL of 2.5 mM 4-methylumbelliferyl *β*-D-glucuronide (Sigma-Aldrich), and 70 μL of reaction buffer (50 mM Bis-Tris, 50 mM triethanol amine, 100 mM acetic acid, 100 ng/mL bovine serum albumin, pH 7.0) in a microtiter plate for 60 min at 37 °C. The reaction was terminated by adding an equal volume of stop buffer (1 M glycine, 0.5 M sodium bicarbonate, pH 11). The fluorescence of 4-methylumbellferone (4MU) was measured at excitation/emission wavelengths of 355/460 nm in a Gemini EM microplate spectrofluorometer (Molecular Device, Sunnyvale, CA, USA). The acquired readings were converted to product concentrations by a pre-established standard curve. One unit of specific enzymatic activity corresponds to generation of one nanomole of 4MU per milligram total protein per hour (nmol·mg^{-1}·h^{-1}).

To assay surface beta-glucuronidase activity, CL1-5 or CL1-5/m*β*G cells were washed twice with PBS and suspended in cellular reaction buffer (50 mM bis-Tris, 25 mM glucose, 85.6 mM NaCl, 5.4 mM KCl, 0.6 mM $MgSO_4$, 1.3 mM $CaCl_2$, pH 7) containing 0.25 mM 4-methylumbelliferyl *β*-D-glucuronide and incubated at 37 °C for 30 min. The supernatant was collected after centrifuging at 500 × *g* for 5 min at 4 °C. The fluorescence of 4MU was measured after adding equal volume of stop buffer.

In Vivo Antitumor Activity. Groups of BALB/c nu/nu female mice (n = 8) bearing 50−100 mm^3 subcutaneous CL1-5 tumor in their right flank were intravenously injected with PBS, 2.5 mg/kg prodrug **2** or 50 mg/kg carboplatin on days 7, 10, 13, 16, and 19. Body weights and tumor sizes were measured every 2−3 days. For adenovirus-mediated gene delivery experiments, mice were intratumorally injected with recombinant adenovirus (10^9 pfu/mouse) expressing beta-glucuronidase or *α*DNS single-chain antibody (negative control) in 50 μL of PBS on days 8, 13, and 19, followed by i.v. administration of PBS, a single 5 mg/kg dose of prodrug **2** or 2.5 mg/kg dose of prodrug **2** administrated on two consecutive days. Body weights and tumor sizes were followed every 2−3 days. The tumor volume was calculated according to the formula: length × width × height × 0.5.

RESULTS

In Vitro Cytotoxicity of 2 and 3. Table 1 shows the in vitro cytotoxicity of the prodrug **2** and the corresponding seco-drug **3** to several human cancer cell lines. Prodrug **2** produced IC_{50} values ranging from 10 to 82 nM. Compound **3** was over a thousand-fold more cytotoxic with IC_{50} values ranging from 12 to 46 pM. Addition of beta-glucuronidase to cleave the

Table 1. In Vitro Cytotoxicity of 2 and 3[a]

	compound **3**	compound **2**	compound **2**+*β*G[c]	
cell line	IC_{50}[b] (pM)	IC_{50} (pM)	IC_{50} (pM)	QIC_{50}[d]
HT-29	22.0 ± 1.3	81,900 ± 8900	3.68 ± 1.9	1900
CL1-5	13.8 ± 1.7	18,900 ± 6200	15.1 ± 1.5	1300
Caski	3.87 ± 0.53	10,100 ± 2400	3.61 ± 0.81	2500
EJ	15.4 ± 5.3	82,100 ± 1100	54.3 ± 33	1500
LS174T	7.31 ± 0.72	28,900 ± 7200	11.6 ± 2.8	2400

[a]Drugs were incubated with human cancer cells for 24 h. Results represent mean IC_{50} values ± s.d. of triplicate determinations. [b]IC_{50} is the concentration of test compound that inhibits incorporation of ^{3}H-thymidine into cellular DNA by 50%. [c]A sample of 1 μg of *E. coli* beta-glucuronidase was added with compound **2** to each well. [d]QIC_{50} is equal to IC_{50} of prodrug/IC_{50} of prodrug in the presence of beta-glucuronidase.

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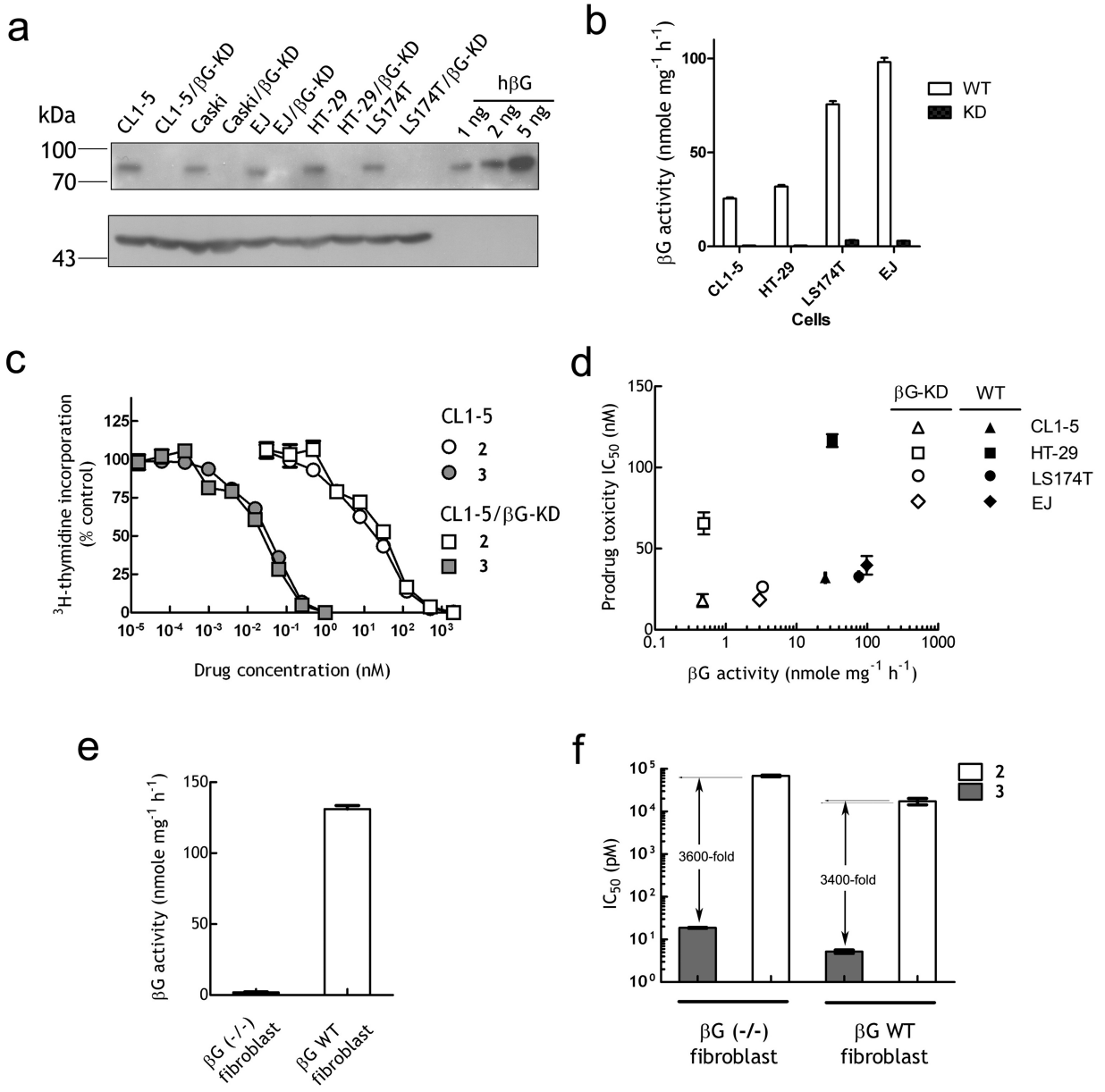

Figure 2. Prodrug **2** cytotoxicity does not strongly depend on intracellular beta-glucuronidase activity. (a) Beta-glucuronidase levels in several human cancer lines were knocked down by lentiviral expression of specific shRNA. Beta-glucuronidase levels in parental and knock-down cell lines were determined by immunoblotting cell lysates with antihuman beta-glucuronidase rabbit serum (upper panel) or antibeta-actin antibody (lower panel) as a loading control. Defined amounts (1, 2, and 5 ng) recombinant human beta-glucuronidase was also immunoblotted. (b) The specific beta-glucuronidase activity in lysates prepared from parental (WT) or knockdown (KD) cells are shown as mean values of triplicate determinations. Bars, SEM. (c) CL1-5 and CL1-5/βG-KD (beta-glucuronidase knockdown) cell drug sensitivities were measured by ^{3}H-thymidine incorporation. Results shown mean values of triplicate determinations. Bars, SEM. (d) Drug sensitivity to prodrug **2** (IC_{50} values) are plotted against beta-glucuronidase activity of wild-type and beta-glucuronidase knockdown cancer lines. (e) Mean values of beta-glucuronidase activity in lysates prepared from beta-glucuronidase deficient fibroblasts or matched littermate beta-glucuronidase positive fibroblasts are shown. Bars, SEM. (f) Drug sensitivity of MPS VII beta-glucuronidase-deficient fibroblasts and beta-glucuronidase-positive fibroblasts were measured by ^{3}H-thymidine incorporation.

glucuronide moiety from **2** produced similar cytotoxicity as **3**, indicating that prodrug **2** could be enzymatically converted to **3**. We conclude that **2** behaves as a glucuronide prodrug, with QIC_{50} values ranging from 1300 to 2500.

Prodrug 2 Cytotoxicity Is Insensitive to Intracellular Beta-Glucuronidase Levels. The in vitro cytotoxicity data indicated that beta-glucuronidase enzymatic activity is required to activate prodrug **2** and release the seco-drug **3** which then forms the cyclopropyl derivative compound **4** in situ,[40] which is the final highly cytotoxic drug. Because beta-glucuronidase can be present inside cancer cells or extracellularly in the tumor microenvironment, we investigated whether the intracellular beta-glucuronidase activity of cancer cells affects their sensitivity to compound **2**. Toward this goal, we first used shRNA to knock down beta-glucuronidase levels in several cancer cell lines. Immunoblotting of the cells showed that beta-glucuronidase protein levels were clearly reduced in the knockdown cells as compared to the parental wild-type cells (Figure 2a). Beta-glucuronidase activities in cell lysates of the knockdown cell lines were also reduced by over 95% as

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compared to the parental cells (Figure 2b). shRNA-mediated reduction of beta-glucuronidase by 98% in CL1-5 human lung cancer cells did not significantly alter cellular sensitivity to **2** or **3** (Figure 2c). Similarly, knockdown of intracellular beta-glucuronidase levels in other cancer cell lines only marginally affected cellular sensitivity to prodrug 2 (Figure 2d) and linear regression analysis of parental and beta-glucuronidase knockdown cells found that sensitivity to prodrug **2** did not correlate (R^2 = 0.006) with intracellular beta-glucuronidase activity. Along the same lines, beta-glucuronidase-deficient fibroblasts, which possess only background levels of beta-glucuronidase activity (Figure 2e), were about as resistant to prodrug **2** (3600 times less sensitive than to compound **3**) as were matched beta-glucuronidase-positive fibroblasts (3400-times less sensitive than to compound **3**) (Figure 2f). We conclude that intracellular beta-glucuronidase activity is not a major determinant of cellular sensitivity to prodrug **2**.

Extracellular Beta-Glucuronidase Can Effectively Enhance Cancer Cell Sensitivity to Prodrug 2. Cellular insensitivity of prodrug **2** to intracellular beta-glucuronidase levels indicates that the charged glucuronide group of the prodrug hinders its passive diffusion through the plasma membrane of cells. To examine if extracellular beta-glucuronidase could enhance the cytotoxicity of **2** to cancer cells, murine beta-glucuronidase was fused to a truncated cell receptor previously shown to effectively target recombinant proteins to the surface of mammalian cells (Figure 3a).[43] Beta-glucuronidase was stably expressed on CL1-5 cells as determined by immunofluorescence staining of viable cells with a monoclonal antibody specific for beta-glucuronidase (Figure 3b). Surface beta-glucuronidase was active on CL1-5 cells as determined by effective hydrolysis of the substrate 4-methylumbelliferyl β-D-glucuronide (Figure 3c). Surface beta-glucuronidase-expressing and parental cells displayed similar sensitivities to **3**, but cells expressing beta-glucuronidase on their surface were substantially (~80-fold) more sensitive to prodrug **2** as compared to parental cells (Figure 3d), consistent with effective prodrug activation by extracellular but not intracellular beta-glucuronidase.

Mammalian beta-glucuronidases display maximal activity at pH 4–4.5. Thus, intracellular beta-glucuronidase (in lysosomes) likely displays much greater activity than membrane anchored beta-glucuronidase, which is exposed to neutral pH. Although this supports the notion that extracellular beta-glucuronidase is more important for prodrug 2 activation, to more carefully investigate the effect of beta-glucuronidase location on sensitivity to prodrug **2**, we constructed vectors to generate stable CL1-5 cells that express *E. coli* beta-glucuronidase in the cytosol or on the plasma membrane (Figure 4a). *E. coli* beta-glucuronidase displays maximal catalytic activity at pH 7, making this enzyme an appropriate choice for direct comparison of intracellular versus extracellular prodrug activation. *E. coli* beta-glucuronidase was expressed at modest but detectable levels on CL1-5/surface eβG cells but not on parental CL1-5 cells (Figure 4b, right panel). Likewise, green fluorescence protein, indicative of cytosolic *E. coli* beta-glucuronidase, was detected in CL1-5/cytosolic eβG cells but not parental CL1-5 cells (Figure 4b, left panel). Lysates prepared from CL1-5/cytosolic eβG cells displayed about 3-fold greater beta-glucuronidase activity as compared to lysates prepared from CL1-5/surface eβG cells (Figure 4c). By contrast, CL1-5/surface eβG cells were significantly more sensitive to prodrug **2** as compared to parental CL1-5 cells,

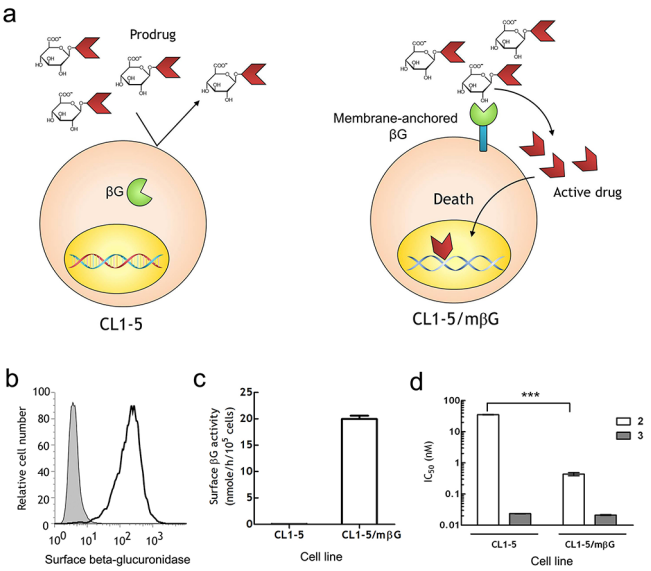

Figure 3. Surface displayed beta-glucuronidase effectively sensitizes cells to prodrug **2**. (a) The charged glucuronide group on prodrug **2** may hinder passage through the plasma membrane to contact intracellular beta-glucuronidase in CL1-5 cells. Membrane-anchored beta-glucuronidase, on the other hand, can effectively hydrolyze prodrug **2** to **3**, which can enter and kill CL1-5/mβG cells. (b) Live parental CL1-5 cells (gray area) and beta-glucuronidase-expressing CL1-5/mβG cells (solid lines) were immunofluorescence stained for beta-glucuronidase and then analyzed on a flow cytometer. (c) Extracellular beta-glucuronidase activities on live CL1-5 or CL1-5/mβG cells were determined by incubated live cells with 4-methylumbelliferyl β-D-glucuronide at 37 °C for 30 min. Results represented the mean of triplicate determinations ± SEM. (d) CL1-5 or CL1-5/mβG cells were incubated with graded concentrations of prodrug **2** or compound **3** in triplicate for 48 h before ^{3}H-thymidine incorporation was measured. Bars, SEM. Significant differences in mean IC_{50} values of CL1-5 or CL1-5/mβG cells treated with compound **2** are indicated: ***, $p \leq 0.0005$.

whereas CL1-5/cytosolic eβG cells and parental CL1-5 cells exhibited similar sensitivity to prodrug **2** (Figure 4d). We conclude that cellular sensitivity to prodrug **2** is primarily determined by extracellular beta-glucuronidase activity rather than by intracellular beta-glucuronidase levels.

In Vivo Toxicity and Therapeutic Efficacy of Prodrug 2 against Human Cancer Xenografts. Table 2 shows the in vivo toxicity of **2** to BALB/c nu/nu mice as monitored by loss of body weight. A single i.v. injection of 5 mg/kg prodrug **2** produced about 8% weight loss, whereas 2.5 mg/kg prodrug **2** did not cause weight loss. Doses of 10 mg/kg prodrug **2** and above produced significant loss of body mass and were deemed toxic. To investigate the antitumor efficacy of prodrug **2** for cancer prodrug monotherapy, nude mice bearing CL1-5 tumors (50–150 mm^3) were i.v. injected with vehicle (PBS), prodrug **2** (2.5 mg/kg), or carboplatin (50 mg/kg, which is close to previously determined maximum tolerable dose[46,47]) every three days for five times. Mice treated with prodrug **2** displayed a significant reduction of tumor size as compared to the PBS treated tumors, with better antitumor activity than the carboplatin-treated mice (Figure 5a). Moreover, in contrast to carboplatin-treated mice, there was no weight loss in mice treated with prodrug **2** (Figure 5b). This data indicates that prodrug **2** displays modest but significant antitumor activity with minimal toxicity.

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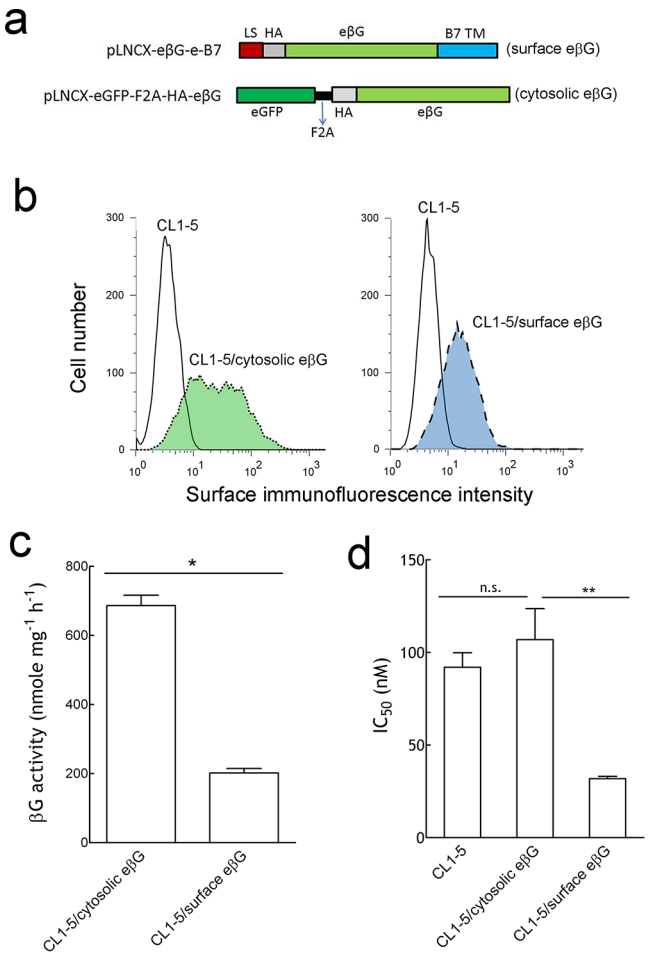

Figure 4. Comparison of sensitivity to prodrug **2** of cells expressing cytosolic versus membrane *E. coli* beta-glucuronidase. (a) Schematic of transgenes for the expression of *E. coli* beta-glucuronidase on the surface (pLNCX-eβG-B7) or in the cytosol (pLNCX-eGFP-F2A-HA-eβG). LS, signal peptide; HA, HA epitope tag; eβG, *E. coli* beta-glucuronidase; B7 TM, juxtamembrane extracellular, transmembrane, and cytosolic domains of the murine B7-1 antigen; eGFP, enhanced green fluorescence protein; F2A, self-cleaving 2A peptide. (b) Live parental CL1-5 cells (open area) and CL1-5/cytosolic eβG cells were analyzed for fluorescence from eGFP (left panel). Live CL1-5 cells (open area) and CL1-5/surface eβG cells were immunofluorescence stained for *E. coli* beta-glucuronidase (right panel). (c) Beta-glucuronidase activities of CL1-5/cytosolic eβG or CL1-5/surface eβG cells were determined by incubated cell lysates with 4-methylumbelliferyl β-D-glucuronide at 37 °C for 60 min at pH 7. Results represented mean of triplicate determinations ± s.d. (d) CL1-5, CL1-5/cytosolic eβG or CL1-5/surface eβG cells were incubated with graded concentrations of prodrug **2** for 2 h before ^{3}H-thymidine incorporation was measured. Bars, s.d. Significant differences between mean values are indicated: *, $p \leq 0.05$; **, $p \leq 0.005$.

GDEPT Therapy of Human Cancer Xenografts. We examined if an adenoviral vector-mediated GDEPT approach could enhance the antitumor activity of prodrug **2**. CL1-5 human lung cancer cells were transduced with defined numbers of adenoviruses expressing membrane-anchored murine beta-glucuronidase (Ad-βG) or a membrane-anchored α-dansyl single-chain antibody (Ad-αDNS) as a negative control. Immunofluorescence staining of the cells demonstrated dose-dependent expression of beta-glucuronidase on the surface of viable CL1-5 cells as shown by positive staining with an anti-HA epitope antibody or a monoclonal antibody against beta-glucuronidase (Figure 6a). Similar levels of α-dansyl single-chain antibody were expressed on CL1-5 cells infected with control Ad-αDNS virus particles (Figure 6b). Cancer cells transduced with Ad-βG (MOI = 100) displayed about 1300-fold augmented sensitivity to **2** as compared to uninfected cells (Figure 6c). By contrast, cancer cells transduced with Ad-αDNS (MOI = 100) displayed similar sensitivity as uninfected cells to prodrug **2**. We conclude that adenoviral-mediated expression of beta-glucuronidase on the surface of CL1-5 cells can effectively potentiate the cytotoxicity of prodrug **2**.

To evaluate the therapeutic effect of Ad-βG treatment combined with prodrug **2**, mice bearing 50−100 mm^3 CL1-5 tumors received three rounds of treatment consisting of intratumoral injection with 10^9 pfu Ad-βG or 10^9 pfu Ad-αDNS followed 24 h later by i.v. injection with PBS or a total of

Table 2. In Vivo Toxicity of Compound 2 to Nude Mice

	body weight (%) ± s.d.[a]		
dose (mg/kg)	Day 5	Day 7	Day 9
vehicle	103 ± 1.1	107 ± 1.5	106 ± 0.4
2.5	104 ± 1.1	105 ± 0.8	104 ± 0.2
5	92 ± 0.0	97 ± 0.8	101 ± 1.0
10	75 ± 0.4	82 ± 0.1	87 ± 0.4
20	75 ± 1.0	71 ± 0.9	72 ± 3.3

[a]Body weight changes ± standard deviation for each group (n = 2) are shown as mean weights on the indicated days relative to mean body weights on day 0.

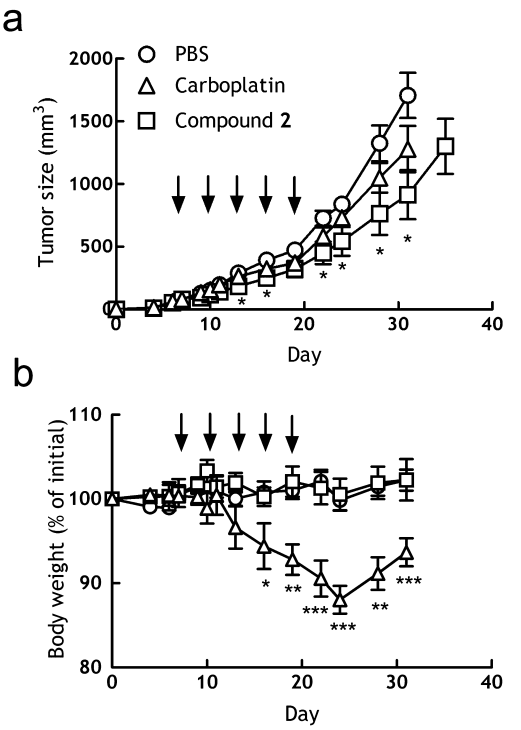

Figure 5. Antitumor activity of compound **2** against human lung cancer tumors in nude mice. (a) Mice bearing 50−100 mm^3 subcutaneous CL1-5 tumors were i.v. injected with PBS, compound **2** (2.5 mg/kg) or carboplatin (50 mg/kg) on days 7, 10, 13, 16, and 19. Significant differences in mean tumor size in mice treated with PBS or **2** are indicated: *, $p \leq 0.05$. (b) Mean body weights of each group of mice are shown. Bars, SEM (n = 8). Significant differences in mean body weight in mice treated with PBS or carboplatin are indicated: *, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.0005$.

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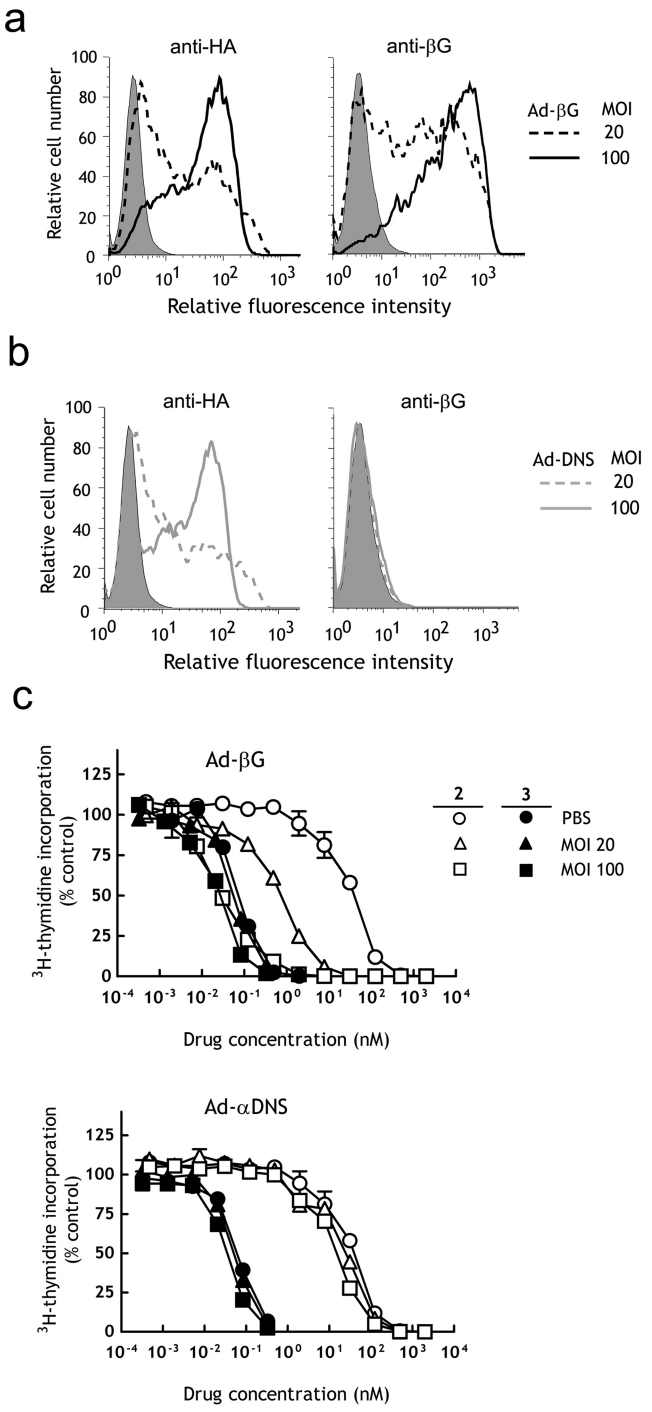

Figure 6. Adenovirus-mediated expression of beta-glucuronidase on the surface of cancer cells enhances sensitivity to prodrug **2**. CL1-5 cells were untreated (shaded) or transduced with (a) Ad-*β*G at MOI 20 (black dashed line) or 100 (black solid line) or (b) Ad-*α*DNS at MOI 20 (gray dashed line) or 100 (gray solid line). Expression levels were determined in a flow cytometer after immunofluorescence staining with an antibody against the HA epitope tag present at the N-terminus of the recombinant fusion proteins (left panels) or a monoclonal antibody against beta-glucuronidase (right panels). (c) Cells infected with Ad-*β*G (upper panel) or Ad-*α*DNS (lower panel) at MOI 20 or 100 were incubated with graded concentrations of **2** or **3** in triplicate for 48 h. ^{3}H-thymidine incorporation was determined as above.

5 mg/kg prodrug **2** over the next two days (Figure 7a). Strong suppression of tumor growth was observed for combined

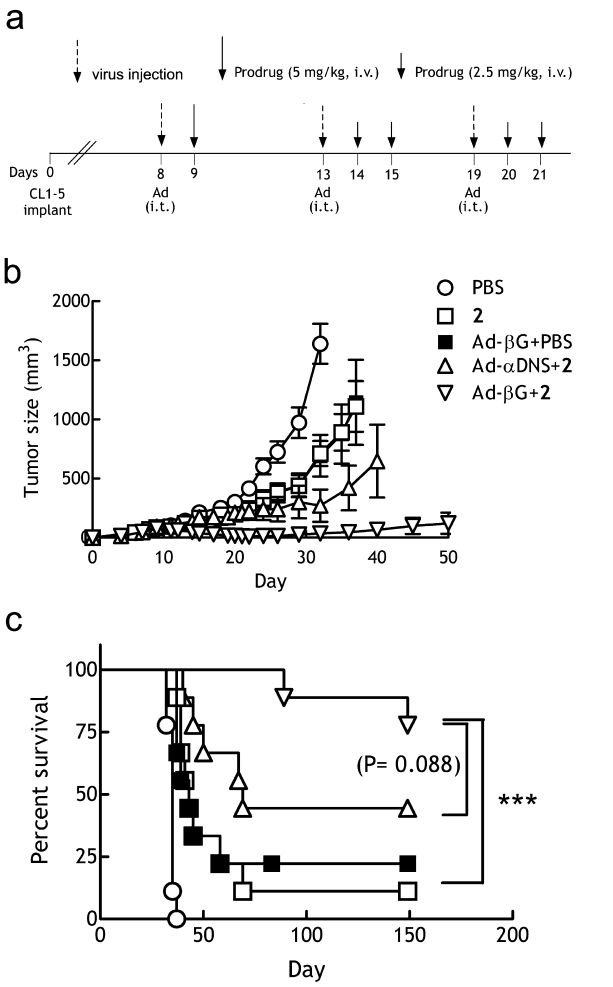

Figure 7. Enhanced antitumor activity of prodrug **2** in combination with adenovirus-mediated beta-glucuronidase delivery. (a) Schematic representation of the treatment schedule. (b) Mice bearing 50−100 mm^3 s.c. CL1-5 tumors were i.v. injected with vehicle (PBS), prodrug **2** alone (**2**), 10^9 pfu Ad-*β*G and PBS (Ad-*β*G + PBS), 10^9 pfu Ad-*α*DNS and prodrug **2** (Ad-*α*DNS + **2**) or 10^9 pfu Ad-*β*G and prodrug **2** (Ad-*β*G + **2**) at the times and doses illustrated in Figure 7a. The results show mean tumor sizes for each group. Bars, SEM. (c) Overall survival curves for each group described in Figure 7b. Statistical differences between survival times were determined using the Mantel-Cox log rank test: ***, $p \leq 0.001$.

treatment of mice with Ad-*β*G and **2** as compared with PBS alone, **2** alone, or with Ad-*β*G alone (Figure 7b). Mice treated with vehicle (PBS) had a median survival time of 35 days (n = 9). Mice treated with Ad-*β*G alone or prodrug **2** alone had slightly longer median survival times of 43 days (n = 9). Mice treated with control virus Ad-*α*DNS and **2** had median survival times of 69 d (Figure 7c), whereas mice treated with Ad-*β*G and prodrug **2** produced median survival exceeding 150 days with seven of nine mice apparently cured of CL1-5 tumors. These results indicate that extracellular conversion of **2** to **3** in the tumor microenvironment can effectively enhance prodrug **2** antitumor activity.

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DISCUSSION

In this study, we found that cellular sensitivity to the glucuronide prodrug **2** depended on the level of extracellular rather than intracellular beta-glucuronidase. Prodrug **2** produced a better therapeutic index than a conventional antineoplastic agent in cancer monotherapy. Furthermore, adenoviral-mediated expression of beta-glucuronidase on the surface of cancer cells increased cellular sensitivity to **2** by 3 orders of magnitude. Treatment of human lung cancer xenografts with adenoviral vectors expressing membrane-anchored beta-glucuronidase in combination with systemically administered prodrug **2** produced dramatic antitumor activity and long-term cures in seven of nine mice. Collectively, our results indicate that prodrug **2** exhibits appropriate properties for cancer monotherapy and therapeutic approaches that increase the level of extracellular beta-glucuronidase in the tumor microenvironment.

Duocarmycins and CC-1065 are a class of potent natural antitumor products isolated from *Streptomyces* species that are active at the picomolar level.[23,25,48,49] CC-1065 and related synthetic analogues such as adozelesin, carzelesin, and bizelesin, however, have limited therapeutic efficacy due to excessive systemic toxicity in preclinical studies.[50−52] Prodrugs of anticancer agents can be designed to display conditional activity against cancer cells and reduced toxicity to normal tissues, thereby increasing the therapeutic index. Thus, a series of anticancer prodrugs was developed by conjugating carbohydrate moieties to duocarmycin analogues.[32−36,53] In addition to reducing systemic toxicity, the glycosidic prodrugs displayed enhanced water solubility and increased in vivo stability by preventing the spontaneously conversion of seco-drug into the cyclopropyl form,[54] which can alkylate DNA in normal cells before the drug reaches the tumor microenvironment.

Evaluation of a panel of human cancer cell lines for cytotoxic sensitivity showed that all cancer cells were at least a thousand fold less sensitive to prodrug **2** than to the product of enzymatic hydrolysis **3**. In addition, by examining a series of beta-glucuronidase knockdown cells, beta-glucuronidase deficient fibroblasts, and membrane-anchored beta-glucuronidase cell lines, we found that cellular sensitivity to prodrug **2** primarily correlated with the presence of extracellular rather than intracellular beta-glucuronidase. These results indicate that the charged glucuronide moiety present on prodrug **2** prevents passive diffusion across the plasma membrane to contact intracellular beta-glucuronidase. The low in vivo toxicity of prodrug **2** can thus be explained by high prodrug stability, low beta-glucuronidase activity in the blood, and inability of the prodrug to enter normal cells.

Prodrug **2** displayed modest but significant antitumor activity against human lung xenografts. Importantly, the therapeutic index of prodrug **2** was greater than a commonly used antineoplastic agent as demonstrated by better tumor suppression with less systemic toxicity. We hypothesized that increasing the extracellular level of beta-glucuronidase would further enhance the antitumor activity of prodrug **2** based on the results of our in vitro studies. Indeed, a replication-deficient adenovirus encoding beta-glucuronidase fused to an optimized transmembrane domain derived from the B7-1 antigen for expression on the cell surface[22] enhanced cellular sensitivity to prodrug **2** by over a thousand fold. The antitumor activity of prodrug **2** was also greatly improved by intratumoral injection of the adenoviral vector expressing membrane-anchored beta-glucuronidase with complete tumor remission observed in seven of nine mice. Interestingly, the antitumor effect of prodrug **2** was also enhanced, although to a lesser degree, by intratumoral injection of an adenoviral vector expressing a control protein on cells. Although not investigated here, it is possible that macrophages infiltrating tumors in response to adenoviral administration[55,56] might release beta-glucuronidase in the tumor microenvironment and activate prodrug **2**.[14] In addition, release of intracellular beta-glucuronidase from cancer cells undergoing apoptosis or necrosis due to adenoviral infection might also increase the sensitivity of bystander cancer cells to prodrug **2**.[57]

In summary, our report provides evidence that a highly potent glucuronide prodrug of a seco-analogue of duocarmycin SA produced significant antitumor activity against human lung xenografts with less toxicity than a conventional antineoplastic agent used for lung cancer treatment. Furthermore, primary tumors were eliminated by prodrug **2** in combination with an adenoviral vector expressing membrane anchored beta-glucuronidase. Other strategies to increase the level of beta-glucuronidase in the tumor microenvironment, such as systemic administration of antibody-enzyme conjugates,[20,58] bacteria engineered to overexpress beta-glucuronidase,[59] beta-glucuronidase loaded nanoparticles,[60] and systemically administered conditionally replicating adenoviruses or oncolytic viruses[61] are also expected to potentiate the antitumor activity of prodrug **2** and may be useful for the treatment of metastatic tumors.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Kratz, F.; Muller, I. A.; Ryppa, C.; Warnecke, A. Prodrug strategies in anticancer chemotherapy. *ChemMedChem* **2008**, *3* (1), 20−53.

(2) Tietze, L. F.; Krewer, B. Antibody-directed enzyme prodrug therapy: a promising approach for a selective treatment of cancer based on prodrugs and monoclonal antibodies. *Chem. Biol. Drug Des.* **2009**, *74* (3), 205−211.

(3) Tietze, L. F.; Schmuck, K. Prodrugs for targeted tumor therapies: recent developments in ADEPT, GDEPT and PMT. *Curr. Pharm. Des.* **2011**, *17* (32), 3527−3547.

(4) Leu, Y. L.; Roffler, S. R.; Chern, J. W. 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* (18), 3623−3628.
(5) Cheng, T. L.; Chou, W. C.; Chen, B. M.; Chern, J. W.; Roffler, S. R. Characterization of an antineoplastic glucuronide prodrug. *Biochem. Pharmacol.* **1999**, *58* (2), 325−328.
(6) de Graaf, M.; Boven, E.; Scheeren, H. W.; Haisma, H. J.; Pinedo, H. M. Beta-glucuronidase-mediated drug release. *Curr. Pharm. Des.* **2002**, *8* (15), 1391−1403.
(7) Prijovich, Z. M.; Chen, B. M.; Leu, Y. L.; Chern, J. W.; Roffler, S. R. Anti-tumour activity and toxicity of the new prodrug 9-aminocamptothecin glucuronide (9ACG) in mice. *Br. J. Cancer* **2002**, *86* (10), 1634−1638.
(8) Cheng, T. C.; Roffler, S. R.; Tzou, S. C.; Chuang, K. H.; Su, Y. C.; Chuang, C. H.; Kao, C. H.; Chen, C. S.; Harn, I. H.; Liu, K. Y.; Cheng, T. L.; Leu, Y. L. An activity-based near-infrared glucuronide trapping probe for imaging beta-glucuronidase expression in deep tissues. *J. Am. Chem. Soc.* **2012**, *134* (6), 3103−3110.
(9) Grinda, M.; Clarhaut, J.; Tranoy-Opalinski, I.; Renoux, B.; Monvoisin, A.; Cronier, L.; Papot, S. A Heterodimeric Glucuronide Prodrug for Cancer Tritherapy: the Double Role of the Chemical Amplifier. *ChemMedChem* **2011**, *6* (12), 2137−2141.
(10) Legigan, T.; Clarhaut, J.; Renoux, B.; Tranoy-Opalinski, I.; Monvoisin, A.; Berjeaud, J.-M.; Guilhot, F.; Papot, S. Synthesis and Antitumor Efficacy of a β-Glucuronidase-Responsive Albumin-Binding Prodrug of Doxorubicin. *J. Med. Chem.* **2012**, *55* (9), 4516−4520.
(11) Sinhababu, A. K.; Thakker, D. R. Prodrugs of anticancer agents. *Adv. Drug Delivery Rev.* **1996**, *19* (2), 241−273.
(12) Murdter, T. E.; Sperker, B.; Kivisto, K. T.; McClellan, M.; Fritz, P.; Friedel, G.; Linder, A.; Bosslet, K.; Toomes, H.; Dierkesmann, R.; Kroemer, H. K. Enhanced uptake of doxorubicin into bronchial carcinoma: beta-glucuronidase mediates release of doxorubicin from a glucuronide prodrug (HMR 1826) at the tumor site. *Cancer Res.* **1997**, *57* (12), 2440−2445.
(13) Boyer, M. J.; Tannock, I. F. Lysosomes, lysosomal enzymes, and cancer. *Adv. Cancer Res.* **1993**, *60*, 269−291.
(14) Juan, T. Y.; Roffler, S. R.; Hou, H. S.; Huang, S. M.; Chen, K. C.; Leu, Y. L.; Prijovich, Z. M.; Yu, C. P.; Wu, C. C.; Sun, G. H.; Cha, T. L. Antiangiogenesis targeting tumor microenvironment synergizes glucuronide prodrug antitumor activity. *Clin. Cancer Res.* **2009**, *15* (14), 4600−4611.
(15) van Zanten, A. P.; Twijnstra, A.; van Benthem, V.; Hart, A. A.; Ongerboer de Visser, B. W. Cerebrospinal fluid beta-glucuronidase activities in patients with central nervous system metastases. *Clin. Chim. Acta* **1985**, *147* (2), 127−134.
(16) Chen, B. M.; Chan, L. Y.; Wang, S. M.; Wu, M. F.; Chern, J. W.; Roffler, S. R. 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* (3), 392−402.
(17) Cheng, T. L.; Chen, B. M.; Chan, L. Y.; Wu, P. Y.; Chern, J. W.; Roffler, S. R. Poly(ethylene glycol) modification of beta-glucuronidase-antibody conjugates for solid-tumor therapy by targeted activation of glucuronide prodrugs. *Cancer Immunol. Immunother.* **1997**, *44* (6), 305−315.
(18) Chen, B. M.; Cheng, T. L.; Tzou, S. C.; Roffler, S. R. Potentiation of antitumor immunity by antibody-directed enzyme prodrug therapy. *Int. J. Cancer* **2001**, *94* (6), 850−858.
(19) Houba, P. H.; Boven, E.; van der Meulen-Muileman, I. H.; Leenders, R. G.; Scheeren, J. W.; Pinedo, H. M.; Haisma, H. J. Pronounced antitumor efficacy of doxorubicin when given as the prodrug DOX-GA3 in combination with a monoclonal antibody beta-glucuronidase conjugate. *Int. J. Cancer* **2001**, *91* (4), 550−554.
(20) Chen, K. C.; Wu, S. Y.; Leu, Y. L.; Prijovich, Z. M.; Chen, B. M.; Wang, H. E.; Cheng, T. L.; Roffler, S. R. A humanized immunoenzyme with enhanced activity for glucuronide prodrug activation in the tumor microenvironment. *Bioconjugate Chem.* **2011**, *22* (5), 938−948.
(21) Chen, K. C.; Cheng, T. L.; Leu, Y. L.; Prijovich, Z. M.; Chuang, C. H.; Chen, B. M.; Roffler, S. R. Membrane-localized activation of glucuronide prodrugs by beta-glucuronidase enzymes. *Cancer Gene Ther.* **2007**, *14* (2), 187−200.
(22) Huang, P. T.; Chen, K. C.; Prijovich, Z. M.; Cheng, T. L.; Leu, Y. L.; Roffler, S. R. Enhancement of CPT-11 antitumor activity by adenovirus-mediated expression of beta-glucuronidase in tumors. *Cancer Gene Ther.* **2011**, *18* (6), 381−389.
(23) Ichimura, M.; Ogawa, T.; Takahashi, K.; Kobayashi, E.; Kawamoto, I.; Yasuzawa, T.; Takahashi, I.; Nakano, H.; Duocarmycin, S. A. a new antitumor antibiotic from Streptomyces sp. *J. Antibiot.* **1990**, *43* (8), 1037−1038.
(24) Tichenor, M. S.; Kastrinsky, D. B.; Boger, D. L. Total synthesis, structure revision, and absolute configuration of (+)-yatakemycin. *J. Am. Chem. Soc.* **2004**, *126* (27), 8396−8398.
(25) Martin, D. G.; Biles, C.; Gerpheide, S. A.; Hanka, L. J.; Krueger, W. C.; McGovren, J. P.; Mizsak, S. A.; Neil, G. L.; Stewart, J. C.; Visser, J. CC-1065 (NSC 298223), a potent new antitumor agent improved production and isolation, characterization and antitumor activity. *J. Antibiot.* **1981**, *34* (9), 1119−1125.
(26) Boger, D. L.; Johnson, D. S.; Weiya, Y. (+)- and ent-(-)-Duocarmycin SA and (+)- and ent-(-)-N-BOC-DSA DNA Alkylation Properties. Alkylation Site Models That Accommodate the Offset AT-Rich Adenine N3 Alkylation Selectivity of the Enantiomeric Agents. *J. Am. Chem. Soc.* **1994**, *116*, 1635−1656.
(27) Tietze, L. F.; Krewer, B.; Frauendorf, H. Investigation of the transformations of a novel anti-cancer agent combining HPLC, HPLC-MS and direct ESI-HRMS analyses. *Anal. Bioanal. Chem.* **2009**, *395* (2), 437−448.
(28) Tietze, L. F.; Krewer, B.; Major, F.; Schuberth, I. CD-spectroscopy as a powerful tool for investigating the mode of action of unmodified drugs in live cells. *J. Am. Chem. Soc.* **2009**, *131* (36), 13031−13036.
(29) Wirth, T.; Schmuck, K.; Tietze, L. F.; Sieber, S. A. Duocarmycin analogues target aldehyde dehydrogenase 1 in lung cancer cells. *Angew. Chem., Int. Ed. Engl.* **2012**, *51* (12), 2874−2877.
(30) Baraldi, P. G.; Bovero, A.; Fruttarolo, F.; Preti, D.; Tabrizi, M. A.; Pavani, M. G.; Romagnoli, R. DNA minor groove binders as potential antitumor and antimicrobial agents. *Med. Res. Rev.* **2004**, *24* (4), 475−528.
(31) Nagamura, S.; Asai, A.; Kobayashi, E.; Gomi, K.; Saito, H. Studies on duocarmycin SA and its derivatives. *Bioorg. Med. Chem.* **1997**, *5* (3), 623−630.
(32) Tietze, L. F.; Major, F.; Schuberth, I. Antitumor agents: development of highly potent glycosidic duocarmycin analogues for selective cancer therapy. *Angew. Chem., Int. Ed. Engl.* **2006**, *45* (39), 6574−6577.
(33) Tietze, L. F.; Major, F.; Schuberth, I.; Spiegl, D. A.; Krewer, B.; Maksimenka, K.; Bringmann, G.; Magull, J. Selective treatment of cancer: synthesis, biological evaluation and structural elucidation of novel analogues of the antibiotic CC-1065 and the duocarmycins. *Chem.—Eur. J.* **2007**, *13* (16), 4396−4409.
(34) Tietze, L. F.; Schuster, H. J.; Krewer, B.; Schuberth, I. Synthesis and biological studies of different duocarmycin based glycosidic prodrugs for their use in the antibody-directed enzyme prodrug therapy. *J. Med. Chem.* **2009**, *52* (2), 537−543.
(35) Tietze, L. F.; Krewer, B. Novel analogues of CC-1065 and the duocarmycins for the use in targeted tumour therapies. *Anticancer Agents Med. Chem.* **2009**, *9* (3), 304−325.
(36) Tietze, L. F.; von Hof, J. M.; Muller, M.; Krewer, B.; Schuberth, I. Glycosidic prodrugs of highly potent bifunctional duocarmycin derivatives for selective treatment of cancer. *Angew. Chem., Int. Ed. Engl.* **2010**, *49* (40), 7336−7339.
(37) Tietze, L. F.; Schuster, H. J.; Schmuck, K.; Schuberth, I.; Alves, F. Duocarmycin-based prodrugs for cancer prodrug monotherapy. *Bioorg. Med. Chem.* **2008**, *16* (12), 6312−6318.
(38) Tietze, L. F.; Schmuck, K. Prodrugs for targeted tumor therapies: recent developments in ADEPT, GDEPT and PMT. *Curr. Pharm. Des.* **2011**, *17* (32), 3527−47.
(39) Schmuck, K. Synthese, fluormarkeirung und biologische evaluation von duocarmycin-analaga für eine selektive krebstherapie. Ph.D. Thesis, Sierke Verlag Göttingen, Göttingen, 2011.

(40) Tietze, L. F.; Haunert, F.; Feuerstein, T.; Herzig, T. A Concise and Efficient Synthesis of seco-Duocarmycin SA. *Eur. J. Org. Chem.* **2003**, *2003* (3), 562−566.
(41) Chu, Y. W.; Yang, P. C.; Yang, S. C.; Shyu, Y. C.; Hendrix, M. J.; Wu, R.; Wu, C. W. Selection of invasive and metastatic subpopulations from a human lung adenocarcinoma cell line. *Am. J. Respir. Cell Mol. Biol.* **1997**, *17* (3), 353−360.
(42) Marshall, C. J.; Franks, L. M.; Carbonell, A. W. Markers of neoplastic transformation in epithelial cell lines derived from human carcinomas. *J. Natl. Cancer Inst.* **1977**, *58* (6), 1743−1751.
(43) Chen, K. C.; Cheng, T. L.; Leu, Y. L.; Prijovich, Z. M.; Chuang, C. H.; Chen, B. M.; Roffler, S. R. Membrane-localized activation of glucuronide prodrugs by beta-glucuronidase enzymes. *Cancer Gene Ther.* **2007**, *14* (2), 187−200.
(44) Fang, J.; Qian, J. J.; Yi, S.; Harding, T. C.; Tu, G. H.; VanRoey, M.; Jooss, K. Stable antibody expression at therapeutic levels using the 2A peptide. *Nat. Biotechnol.* **2005**, *23* (5), 584−590.
(45) Chuang, K. H.; Wang, H. E.; Cheng, T. C.; Tzou, S. C.; Tseng, W. L.; Hung, W. C.; Tai, M. H.; Chang, T. K.; Roffler, S. R.; Cheng, T. L. Development of a Universal Anti−Polyethylene Glycol Reporter Gene for Noninvasive Imaging of PEGylated Probes. *J. Nucl. Med.* **2010**, *51* (6), 933−941.
(46) Shalinsky, D. R.; Brekken, J.; Zou, H.; Bloom, L. A.; McDermott, C. D.; Zook, S.; Varki, N. M.; Appelt, K. Marked antiangiogenic and antitumor efficacy of AG3340 in chemoresistant human non-small cell lung cancer tumors: single agent and combination chemotherapy studies. *Clin. Cancer Res.* **1999**, *5* (7), 1905−1917.
(47) Boven, E.; Verschraagen, M.; Hulscher, T. M.; Erkelens, C. A.; Hausheer, F. H.; Pinedo, H. M.; van der Vijgh, W. J. BNP7787, a novel protector against platinum-related toxicities, does not affect the efficacy of cisplatin or carboplatin in human tumour xenografts. *Eur. J. Cancer* **2002**, *38* (8), 1148−1156.
(48) Takahashi, I.; Takahashi, K.; Ichimura, M.; Morimoto, M.; Asano, K.; Kawamoto, I.; Tomita, F.; Nakano, H.; Duocarmycin, A. a new antitumor antibiotic from Streptomyces. *J. Antibiot.* **1988**, *41* (12), 1915−1917.
(49) Igarashi, Y.; Futamata, K.; Fujita, T.; Sekine, A.; Senda, H.; Naoki, H.; Furumai, T. Yatakemycin, a novel antifungal antibiotic produced by Streptomyces sp. TP-A0356. *J. Antibiot.* **2003**, *56* (2), 107−113.
(50) Li, L. H.; DeKoning, T. F.; Kelly, R. C.; Krueger, W. C.; McGovren, J. P.; Padbury, G. E.; Petzold, G. L.; Wallace, T. L.; Ouding, R. J.; Prairie, M. D.; et al. Cytotoxicity and antitumor activity of carzelesin, a prodrug cyclopropylpyrroloindole analogue. *Cancer Res.* **1992**, *52* (18), 4904−4913.
(51) Carter, C. A.; Waud, W. R.; Li, L. H.; DeKoning, T. F.; McGovren, J. P.; Plowman, J. Preclinical antitumor activity of bizelesin in mice. *Clin. Cancer Res.* **1996**, *2* (7), 1143−1149.
(52) Cacciari, B.; Romagnoli, R.; Baraldi, P. G.; Da Ros, T.; Spalluto, G. CC-1065 and the duocarmycins: recent developments. *Exp. Opin. Ther. Pat.* **2000**, *10* (12), 1853−1871.
(53) Tietze, L. F.; von Hof, J. M.; Krewer, B.; Muller, M.; Major, F.; Schuster, H. J.; Schuberth, I.; Alves, F. Asymmetric synthesis and biological evaluation of glycosidic prodrugs for a selective cancer therapy. *ChemMedChem* **2008**, *3* (12), 1946−1955.
(54) Baird, R.; Winstein, S. Neighboring Carbon and Hydrogen. LI. Dienones from Ar1θ-3 Participation. Isolation and Behavior of Spiro(2,5)octa-1,4-diene-3-one. *J. Am. Chem. Soc.* **1963**, *85*, 567−578.
(55) Lu, W.; Fidler, I. J.; Dong, Z. Eradication of primary murine fibrosarcomas and induction of systemic immunity by adenovirus-mediated interferon beta gene therapy. *Cancer Res.* **1999**, *59* (20), 5202−5208.
(56) Cao, G.; Su, J.; Lu, W.; Zhang, F.; Zhao, G.; Marteralli, D.; Dong, Z. Adenovirus-mediated interferon-beta gene therapy suppresses growth and metastasis of human prostate cancer in nude mice. *Cancer Gene Ther.* **2001**, *8* (7), 497−505.
(57) Subramanian, T.; Tarodi, B.; Chinnadurai, G. p53-independent apoptotic and necrotic cell deaths induced by adenovirus infection: suppression by E1B 19K and Bcl-2 proteins. *Cell Growth Diff.* **1995**, *6* (2), 131.
(58) Bosslet, K.; Czech, J.; Hoffmann, D. Tumor-selective prodrug activation by fusion protein-mediated catalysis. *Cancer Res.* **1994**, *54* (8), 2151−2159.
(59) Cheng, C. M.; Lu, Y. L.; Chuang, K. H.; Hung, W. C.; Shiea, J.; Su, Y. C.; Kao, C. H.; Chen, B. M.; Roffler, S.; Cheng, T. L. Tumor-targeting prodrug-activating bacteria for cancer therapy. *Cancer Gene Ther.* **2008**, *15* (6), 393−401.
(60) Hudson, L. D.; Fiddler, M. B.; Desnick, R. J. Immunologic aspects of enzyme replacement therapy. An evaluation of the immune response to unentrapped, erythrocyte- and liposome-entrapped enzyme in C3H/HeJ Gush mice. *Birth Defects* **1980**, *16* (1), 163−178.
(61) Hess, M.; Stritzker, J.; Hartl, B.; Sturm, J.; Gentschev, I.; Szalay, A. Bacterial glucuronidase as general marker for oncolytic virotherapy or other biological therapies. *J. Transl. Med.* **2011**, *9* (1), 172.