PET Imaging of β-Glucuronidase Activity by an Activity-Based $^{124}$I-Trapping Probe for the Personalized Glucuronide Prodrug Targeted Therapy

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ABSTRACT

Beta-glucuronidase (βG) is a potential biomarker for cancer diagnosis and prodrug therapy. The ability to image βG activity in patients would assist in personalized glucuronide prodrug cancer therapy. However, whole-body imaging of beta-glucuronidase activity for medical usage is not yet available. Here, we developed a radioactive βG activity-based trapping probe for positron emission tomography (PET). We generated a $^{124}$I-tyramine conjugated difluoromethylphenol beta-glucuronide probe (TrapG) to form $^{124}$I-TrapG that could be selectively activated by βG for subsequent attachment of $^{124}$I-tyramine to nucleophilic moieties near βG-expressing sites. We estimated the specificity of a fluorescent FITC-TrapG, the cytotoxicity of tyramine-TrapG and the serum half-life of $^{124}$I-TrapG. βG-targeting of $^{124}$I-TrapG in vivo was examined by micro-PET. The biodistribution of $^{131}$I-TrapG was investigated in different organs. Finally, we image the endogenous βG activity and assess its correlation with therapeutic efficacy of 9-aminocamptothecin glucuronide (9ACG) prodrug in native tumors. FITC-TrapG showed specific trapping at βG-expressing CT26 (CT26/mβG) cells but not CT26 cells. The native TrapG probe possessed low cytotoxicity. $^{124}$I-TrapG preferentially accumulated in CT26/mβG but not CT26 cells. Meanwhile, micro-PET and whole body autoradiography results demonstrated that $^{124}$I-TrapG signals in CT26/mβG tumors were 141.4 folds greater than in CT26 tumors. Importantly, Colo205 xenografts in nude mice that express elevated endogenous βG can be monitored by using infrared glucuronide trapping probes (NIR-TrapG) and suppressed by 9ACG prodrug treatment. $^{124}$I-TrapG exhibited low cytotoxicity allowing long-term monitoring of βG activity in vivo to aid in the optimization of prodrug targeted therapy.
INTRODUCTION

β-Glucuronidase (βG) can catalyzes the hydrolysis of β-D-glucuronic acid residues for breakdown of mucopolysaccharides (e.g. heparan sulfate) in lysosomes (1). It has been widely used as an attractive enzyme for reporter imaging (2-4) and cancer prodrug therapies (5-8). Several glucuronide prodrugs have been used in antibody-directed enzyme prodrug therapy (ADEPT) (9-11) and gene-directed enzyme prodrug therapy (GDEPT) (6, 12, 13) as well as directly in prodrug monotherapy, which relies on the elevated levels of βG found in the tumor microenvironment (14-16) to selectively convert prodrug into active drug. For instance, Albin and colleagues reported that the level of βG human breast tumors was up to 6 times greater than that in normal tissues (17). Sperker and colleagues also reported that pancreatic adenocarcinoma exhibited higher βG levels than did normal pancreatic tissue (14). Furthermore, human tumor xenografts also showed elevated levels of βG including human breast (MCF-7, BT20 and HS578T), colon (HT29 and SW480), and small cell lung cancer (OH3 and SW2) cell lines (18). Elevated levels of βG present in the tumor environment are believed to be due to tumor overexpression (14) and release from necrotic tumor tissues (19) or tumor-infiltrating immune cells (15). Since βG has been considered as a tumor marker (14, 15, 20), it would be very useful to image βG activity in vivo to personalize glucuronide prodrug treatment and greatly improve exogenous or endogenous βG-based targeted therapy.

We have previously demonstrated that a fluorescein di-β-D-glucuronide probe (FDGlcU) can be applied for assessment of βG activity in vivo. However, the fluorescent product FDGlcU rapidly leaked from βG-expressing sites and exhibited poor penetrative properties, limiting imaging to subcutaneous tumors but not deeper tumors (2). To overcome these problems, we also developed fluorescent glucuronide
trapping probes by conjugating a fluorescein isothiocyanate (FITC, λex = 495 nm, λem = 519 nm) and the near infrared dye IR-820 isothiocyanate (NIR, λex = 710 nm, λem = 820 nm) with the difluoromethylphenol beta-glucuronide trapping moiety to form FITC-TrapG and NIR-TrapG probes, respectively. Only the highly signal penetrating NIR-TrapG with near infrared spectrum properties can be employed in long-term tracking (for about 72 hours) of βG activity in deep liver tissues; the FITC-TrapG cannot be. NIR-TrapG is a good probe to monitor βG activity in small animals (21) but the near infrared fluorescent probes are still not feasible for human use since the large thickness of the human body causes a decrease in fluorescence emission and optical imaging systems for medical usage are not available. Development of a positron emission tomography (PET) glucuronide probe to image βG activity will provide a novel method to allow optimization of the protocols for βG-based personalized cancer therapy. Indeed, we previously described a 124I-phenolphthalein-glucuronide probe (124I-PTH-G) to image βG activity in vivo by micro-PET (3). The conversion of 124I-PTH-G by βG caused the in situ precipitation of 124I-PTH due to its hydrophobic property. However, this probe only allows short-term in vivo imaging (about 3 hours) and may induce carcinogenesis based on a feed study of phenolphthalein in B6C3F1 mice and F344/N rats (22).

In the present study, we developed a 124I-tyramine-difluoromethylphenol-glucuronide probe (124I-TrapG) for imaging βG activity by micro-PET. 124I-TrapG can be converted by βG to form the quinine methide derivative of 124I-Trap to quickly react with any nearby nucleophile moiety such as membrane proteins (23) (Fig. 1B). The specificity of the probe was tested in vitro by incubating a FITC-TrapG probe with either CT26/mβG (CT26 cancer cells engineered to express membrane-anchored βG) or parental CT26 cells. The
specificity and cytotoxicity of TrapG were also examined by incubating the probe with CT26/mβG or CT26 cells. For in vivo imaging studies, the $^{124}$I-TrapG was injected into mice bearing both CT26/mβG and parental CT26 tumors to assess intratumoral βG activity by micro-PET. We also examined the biodistribution of $^{124}$I-TrapG in various tissues. To estimate whether TrapG probes can be employed to detect endogenous βG activity in vivo, immunodeficient mice bearing Colo205 (human βG$^{\text{high}}$) and SW620 (human βG$^{\text{low}}$) tumors were injected with NIR-TrapG for optical imaging. Finally, to evaluate whether the endogenous βG can specifically activate glucuronide prodrugs, mice bearing Colo205 or SW620 tumors were injected with 9-aminocamptothecin glucuronide (9ACG) for βG-mediated prodrug therapy. 9ACG is a water-soluble substrate of βG that displays antineoplastic activity after being hydrolyzed by βG to release a topoisomerase I inhibitor, 9AC. Our results indicate that $^{124}$I-TrapG can systemically image the location and the expression of βG in vitro and in vivo, promoting βG usage as a reporter gene for future clinical therapy protocols.
Materials and Methods

Reagents, Cells and Mice

The 9ACG was synthesized as described (24). D-saccharic acid 1,4-lactone monohydrate (SAL) was purchased from Sigma-Aldrich. We previously generated CT26/mβG cells (2) in 2007. CL1-5 cells (25) were obtained from Dr. Cheng-Wen Wu’s laboratory (Institute of Biomedical Sciences) in 2003. CT26, SW480, Hela, HCC36, SW620 and Colo205 cells were obtained from ATCC (American Type Culture Collection) in 2002. All these cell lines were maintained in Dulbecco’s minimal essential medium (DMEM) (Sigma-Aldrich) supplemented with 10% heat-inactivated bovine calf serum, 100 unit/mL penicillin and 100 μg/mL streptomycin at 37°C in an atmosphere of 5% CO2. The cell lines were not authenticated by our laboratory. All cell lines were propagated for less than 6 months after resuscitation. Six to eight-week-old female BALB/cByJNarl and BALB/cAnN.Cg-Foxn1nu/CrlNarl (BALB/c nude) mice were purchased from the National Laboratory Animal Center, Taipei, Taiwan. Mice were pretreated with 0.2% Lugol solution (Sigma-Aldrich) in their drinking water for 2 days prior to injection of radioiodinated probes, as previously described (3) to reduce thyroid uptake. All animal experiments were conducted in specific pathogen-free conditions and in accordance with guidelines approved by the Animal Care and Use Committee of the National Yang-Ming University.

Radioiodination of $^{131}$I or $^{124}$I-TrapG

Compound 5 shown in supplementary methods (Tyramine-TrapG) was labeled with iodine 124 (124I) NaI (3.7 – 37 MBq; IBA Molecular) or $^{131}$I NaI solution (Nuclear Science and Technology Development Center, National Tsing Hua
University, Taiwan) using chloramine-T methods as previously described (3). The specific activity of $^{131}$I-TrapG and $^{124}$I-TrapG were about 500 MBq/mol with > 95% purity as determined at radio-thin-layer chromatography. The radiochemical yields of $^{131}$I-TrapG and $^{124}$I-TrapG were 65% and 70%, respectively.

**Analysis of Cell Surface Trapping of FITC-TrapG Probe**

CT26 and CT26/mβG cells were stained with 0.5 μM FITC-TrapG probes in serum-free PBS (pH=6.5) in the presence or absence of SAL (0.5 mg/mL) for 30 min at 37°C. Unreactive probes were removed by washing with cold PBS twice. The surface fluorescence of $10^5$ viable cells was measured on a BD™ LSR II flow cytometer (Becton Dickinson) and analyzed with Flowjo (Tree Star). Similarly, cells ($3 \times 10^5$ cells) were incubated with 0.69 μM FITC-TrapG in pH 6.5 PBS for 45 min at room temperature. The cells were boiled in reducing SDS buffer, electrophoresed on a SDS-PAGE and transferred to PVDF membranes. Membranes were sequentially stained with mouse anti-FITC antibody followed by HRP-conjugated goat anti mouse antibody (Jackson ImmunoResearch Laboratories). Bands were visualized by ECL detection (Thermo Scientific).

**Cytotoxicity of the TrapG Probe**

Graded concentrations of Tyramine-TrapG or the glucuronide prodrug (p-hydroxyaniline mustard glucuronide, BHAMG) (7) were added to the CT26 cells ($1 \times 10^4$ cells/well) with or without recombinant *E. coli* β-glucuronidase (eβG) (2 μg/well) at 37°C for 24 hours. 37 kBq $^3$H-thymidine was added to the cells during the last 12-hour period of culture. Results are expressed as percent of $^3$H-thymidine incorporation as compared to untreated cells by the following formula:
% of control = 100 × (cpm sample – cpm background) / (cpm control – cpm background)

**The Specificity of \textsuperscript{124}I-TrapG**

Graded concentrations of \textsuperscript{124}I-TrapG in PBS were added to the CT26 and CT26/mβG cells (8 × 10\textsuperscript{4} cells/well) at room temperature for 1 hour. Likewise, the cells were incubated with 0.74 MBq of \textsuperscript{124}I-TrapG for various times. Following washing with PBS (supplemented with 0.495 mM MgCl\textsubscript{2} and 0.9 mM CaCl\textsubscript{2}, pH=3.0) to remove non-bound probe. The radioactivity of the cells was measured in a 1470 Wizard gamma counter (Wallac).

**Liver Function Evaluation**

Groups of BALB/c mice (n = 6) were i.v. injected with PBS or 40 mg/kg TrapG probes. Blood was collected in serum separation 7 days post-injection. Liver injury was assessed by measuring serum AST, ALT, and TBIL levels using a Fuji Dri-Chem 3500 biochemistry analyzer (Fujifilm).

**Serum Half-life of \textsuperscript{124}I-TrapG Probe**

BALB/c mice (n=3) were i.v. injected with 1.85-MBq \textsuperscript{124}I-TrapG, and blood samples were collected at different time points. The blood samples were weighed and assayed for radioactivity in a gamma counter. The radioactivity in the samples is expressed as the percentage of injected dose per gram of tissue. The elimination half-life (t\textsubscript{1/2}) of \textsuperscript{124}I-TrapG was calculated by a two phase exponential decay equation with use of software (Prism 4; Graphpad Software).
**Imaging of β-Glucuronidase Activity In Vivo**

BALB/c mice (n=3) bearing 200 mm³ CT26/mβG (right hind leg) and control CT26 (left hind leg) tumors were i.v. injected with 3.7 MBq of ¹²⁴I-TrapG. The whole-body micro-PET imaging of pentobarbital-anesthetized mice was performed at 1, 8 and 20 hours post-injection using a micro-PET R4 scanner (Concorde Microsystems). To test the specificity of ¹²⁴I-TrapG in vivo, the mice were i.p. injected with SAL inhibitors (1 g per kilogram of body weight) 1 hour before ¹²⁴I-TrapG injection. Fully 3-dimensional list-mode data were collected using an energy window of 350 ~ 750 keV and a time window of 6 ns. Image pixel size was 0.85 mm transaxially with a 1.21 mm slice thickness. BALB/c nude mice (n=3) bearing 100 mm³ SW480, SW620 and Colo205 tumors or CL1-5, Hela and HCC36 tumors were also i.v. injected with 100 μg of NIR-TrapG (21). Whole-body optical images were acquired on an IVIS Spectrum imaging system (Caliper Life Sciences) at 24 h after probe injection at an acquisition time of 10 s.

**In Vivo Antitumor Activity**

Groups of 5 BALB/c nude mice bearing 100 mm³ subcutaneous SW620 and Colo205 tumors in their right flank were i.p. injected with doses of 50 mg/kg 9ACG or PBS on days 1, 3 and 6. Tumor volumes were measured every 2-3 days. Tumor sizes were calculated according the formula: length × width × height × 0.5. The relative tumor volume was determined as % =100% × (tumor size / initial tumor size).

**Whole-Body Autoradiography in Mice**

Mice (n=3) were i.v. injected with 1.85 MBq of ¹³¹I-TrapG 3 hours before the
mice were sacrificed by means of chloroform inhalation. Animals were dipped into isopentane at liquid nitrogen temperatures and embedded on a cryostat holder (7 × 5 cm) in 4% carboxymethylcellulose. The frozen carcass was sliced into 30 μm whole-body sections then attached on microscopic slides. The whole-body sections were applied to an imaging plate (BAS cassette 2040; Fujifilm). After 36 hours exposure, the phosphor images were acquired with a FLA5000 reader (Fujifilm).

**Biodistribution of the Radioiodinated TrapG Probe**

BALB/c mice (n = 4) bearing established CT26 and CT26/mβG tumors (200 mm³) on the right and left shoulders, respectively, were i.v. injected with 1.85 MBq of ¹³¹I-TrapG. The mice were sacrificed at 1, 8, 20 and 40 hours post-injection, and the radioactivity in selected tissues was measured in a multi-channel gamma–counter. The biodistribution of the probe was expressed as % injected dose per gram tissue. The tumors were harvest at 20 hours after ¹²⁴I-TrapG injection then embedded in tissue embedding medium (Tissue-Tek O.C.T.) at -80°C, and sectioned into 5 μm slices. Consecutive sections were either directly placed onto a phosphor imaging plate for autoradiography and detected by Typhoon 9410 phosphorimager (GE Healthcare Life Sciences) or stained with β-glucuronidase Reporter Gene Staining Kit (Sigma-Aldrich) as previously described (21).

**Reverse transcription polymerase chain reaction (RT-PCR)**

SW620 and Colo205 tumor tissue samples from tumor-bearing nude mice were snap-frozen in liquid nitrogen before grinding the samples into fine powders. Total RNA was extracted by using the NucleoSpin® RNA isolation kit (MACHEREY-NAGEL) according to the manufacturer's protocol. Five micrograms
of total RNA were reverse transcribed by using the SuperScript III RT-PCR system with oligo(dT) primers (Invitrogen). Two microliters of cDNA sample were amplified by PCR and visualized on an agarose gel. The primers

\[ 5'\text{-TCACCCAAGAAGCAGCCCTTC-3'} \quad \text{and} \]
\[ 5'\text{-CTAGCTGGAAATGTTCGCTGC-3'} \quad \text{for mouse } \beta\text{G;} \]
\[ 5'\text{-CTGGCGCTGCCGCAGTTCTTCAA-3'} \quad \text{and} \]
\[ 5'\text{-GGTGAAACCCTGCAATCGTT-3'} \quad \text{for human } \beta\text{G;} \]
\[ 5'\text{-GACCACAGTCCATGCCATCACT-3'} \quad \text{and} \]
\[ 5'\text{-TCCACCACCTGTGCTGTAG-3'} \quad \text{for human GAPDH genes were used in PCR.} \]
RESULTS

Synthesis of $^{131}\text{I}$-TrapG and $^{124}\text{I}$-TrapG

The synthesis of $^{131}\text{I}$-TrapG and $^{124}\text{I}$-TrapG are shown in Figure 1A. Methyl 1-O-(2-difloromethyl-4-amino)-2,3,4-tri-O-acetyl-$\beta$-D-glucopyranuronate (1) was prepared as described previously (21). To prepare Tyramine-TrapG, tyramine was coupled with compound 1 via an amide linkage to obtain compound 3. The acetyl groups in 3 were removed by treatment with sodium methoxide and the methyl ester was changed to carboxylic acid via methyl trimethylsilanoate in 1N hydroxychloric acid to give compound 5, Tyramine-TrapG. To prepare $^{131}\text{I}$-TrapG and $^{124}\text{I}$-TrapG, Tyramine-TrapG was labeled by iodine 124 ($^{124}\text{I}$) NaI or $^{131}\text{I}$ NaI solution and purified on a Sep-Pak PLus C18 cartridge. Details of synthesis are described in the supplementary methods.

The Specificity of the TrapG Probe in $\beta$-Glucuronidase-expressing Cells

To examine whether the activated TrapG probes could be specifically trapped on $\beta$G-expressing cells, parental CT26 and CT26/m$\beta$G cells (CT26 cells engineered to express membrane-tethered $\beta$G on their surface, 93 kDa) were stained with 0.5 μM FITC-TrapG probes in the presence or absence of SAL inhibitors. After washing with PBS, FITC that remained attached to cells was detected on a flow cytometer. As shown in Figure 2A, CT26/m$\beta$G cells were specifically bound to FITC-TrapG probes in the absence of SAL inhibitors as compared to the CT26 cells. Meanwhile, the addition of SAL inhibitors completely suppressed the fluorescence retained by CT26/m$\beta$G, indicating that the activation of FITC-TrapG depended on $\beta$G activity (Fig. 2A). To verify that TrapG could be covalently crosslinked to the cell surface after reaction with $\beta$G, FITC-TrapG was added to CT26 or CT26/$\beta$G cells and
analyzed by anti-FITC western blot. Compared to the control cells, only CT26/mβG samples showed bands which were detected by anti-FITC antibodies, consistent with covalently crosslinking of FITC to the cell surface proteins since non-covalently bound FITC would dissociate from proteins boiled in reducing SDS-PAGE electrophoresis buffer (Fig. 2B). We conclude that activated FITC-Trap can covalently label bystander nucleophiles on βG-expressing cells.

**Toxicity, Specificity and Half-Life of the TrapG Probe**

To estimate the cytotoxicity of TrapG to cells, graded concentrations of tyramine-TrapG and BHAMG (an anti-cancer glucuronide prodrug as positive control) were added to CT26 cells with or without the addition of 2 μg eβG. The incorporation of 3H-thymidine into cellular DNA was measured as an index of cell viability. Figure 3A shows that 200 μM tyramine-TrapG and BHAMG were not cytotoxic to CT26 cells in absence of βG. In the presence of βG, tyramine-TrapG displayed some toxicity at concentrations greater than about 100 μM whereas BHAMG was much more cytotoxic with an IC_{50} value of about 5 μM. These results indicate that native tyramine-TrapG exhibited low cytotoxicity to cells with or without βG activation.

To examine the specificity of 124I-TrapG, CT26/mβG or CT26 cells were incubated with graded concentrations of 124I-TrapG in the presence or absence of the βG inhibitor SAL. In addition, the cells were also incubated with 124I-TrapG for different time periods. After washing cells with acidic PBS buffer, the radioactivity retained on the cells was measured on a γ-counter. Figures 3A and 3B show that addition of SAL completely blocked the radioactivity retained by cells expressing membrane-tethered βG, showing that the activation of 124I-TrapG depended on βG activity. In addition, the radioactivity (cpm) retained by CT26/mβG cells after
washing with acid PBS was 1.6-10.3-fold higher than CT26 cells and was both dose and time dependent (Fig. 3B and 3C). These results indicate that $^{124}$I-TrapG could be selectively activated by $\beta$G, resulting in covalent retention of $^{124}$I-TrapG at $\beta$G-expressing cells.

To investigate the toxicity of TrapG probes in vivo, mice were i.v. injected with tyramine-TrapG (40 mg/kg) or PBS. The blood samples were collected and analyzed to evaluate the haematological parameters 7 days after tyramine-TrapG injection. The liver injury was determined by measuring the serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (TBIL). As shown in Figure 3D, when compared to the PBS treated mice, the serum levels of AST ($p = 0.37$), ALT ($p = 0.19$) and TBIL ($p = 0.93$) in tyramine-TrapG treated mice were not significantly increased. These results suggest that tyramine-TrapG did not provoke hepatic toxicity in vivo.

To evaluate the pharmacokinetics of $^{124}$I-TrapG, BALB/c mice were i.v. injected with $^{124}$I-TrapG and the radioactivity in serum samples collected at defined times was measured on a $\gamma$-counter. Figure 4A shows that $^{124}$I-TrapG elimination from the blood followed two-phase exponential decay kinetics, with an initial half-life of 7.3 minutes and a terminal half-life of 57.3 minutes.

**Micro-PET Imaging of $\beta$-Glucuronidase Activity in Forced $\beta$G-overexpressing Tumors with $^{124}$I-TrapG**

To evaluate whether $^{124}$I-TrapG could serve as a micro-PET probe for imaging $\beta$G-expressing tumors in vivo, BALB/c mice (n=3) bearing established subcutaneous CT26/m$\beta$G and control CT26 tumors were i.v. injected with $^{124}$I-TrapG for micro-PET imaging at 1, 8 and 20 hours after probe injection. Enhanced signals of
$^{124}$I-TrapG were observed in CT26/mβG tumors but not CT26 tumors (Fig. 4B). The region of interest ratio of CT26/mβG to CT26 tumors were 3.3, 21 and 141.4 at 1, 8 and 20 hours, respectively, suggesting that $^{124}$I-TrapG was preferentially hydrolyzed into $^{124}$I-Trap by βG-mediated activation. The radioactivity accumulated in CT26/mβG tumors could last up to 20 hours, indicating that long-term imaging of βG activity can be achieved by using the βG-activated trapping $^{124}$I-TrapG probe. To confirm whether conversion of $^{124}$I-TrapG is βG dependent, mice bearing CT26/mβG and CT26 tumors were i.p. injected with the βG inhibitor SAL before probe injection for PET imaging. Figure 4B shows that SAL completely obstructed the generation and accumulation of radioactivity in CT26/mβG tumors, suggesting that the conversion of $^{124}$I-TrapG to $^{124}$I-Trap was blocked by suppression of βG activity. A previous study showed that intraperitoneal administration of SAL in mice can prevent CPT-11 induced mucosa damage by inhibiting bacterial βG (26). Although the pre-treatment of SAL hampered the accumulation of cell-associated radioactivity in CT26/mβG tumors (Fig 4B), strong abdominal signals were still observed. This result indicates that conversion of the fecal excreted $^{124}$I-TrapG by intestinal βG is not the major source of the abdominal signals. To estimate the co-localization of βG activity and specific $^{124}$I-TrapG retention, CT26 and CT26/mβG tumors were frozen sectioned 20 hours after the injection of $^{124}$I-TrapG in tumor bearing mice. The adjacent tumor sections were either directly exposed to phosphor imaging for autoradiography or stained with X-GlcA substrate to visualize βG activity. Figure 4C shows that autoradiography and βG activity matched in adjacent CT26/mβG tumor sections.

**Biodistribution of the $^{131}$I-TrapG in Mice**
To investigate the biodistribution of $^{131}$I-TrapG \textit{in vivo}, BALB/c mice (n = 4) bearing established CT26/mβG and CT26 tumors were i.v. injected with $^{131}$I-TrapG and then examined by measuring radioactivity of the probe in organs at 1, 8, 20, 40 hours after probe injection. Consistent with the micro-PET analysis, mean radioactivity of CT26/mβG tumors was significantly higher than the CT26 tumors (Fig. 5A). The accumulation of radioactivity in CT26/mβG tumors were 2.5, 4.9, 7.1 and 9.6 fold higher than CT26 tumor at 1, 8, 20 and 40 hours, respectively. Notably, 15% and 15.7% of the total $^{131}$I-TrapG were found in the gallbladder and urine, respectively at 1 hour after injection (Fig. 5A). At 40 hours, 9.3% and 1.3% of the total $^{131}$I-TrapG were found in the gallbladder and urine, respectively. This result suggests that $^{131}$I-TrapG undergoes both renal and biliary excretion. $^{131}$I-TrapG was eliminated rapidly by renal excretion but metabolized slowly by biliary excretion. To confirm this result, whole mouse sections were detected by autoradiography at 40 hours after probe injection. Figure 5B shows that higher radioactivity accumulated in CT26/mβG tumors as compared to control CT26 tumors. However, we also found non-targeted $^{124}$I-TrapG in the urine, gallbladder, liver and intestines which may be caused by the biliary excretion of $^{124}$I-TrapG into the intestinal tract.

**Imaging of β-Glucuronidase Activity in Endogenous βG-overexpressing Tumors for Targeted 9ACG Prodrug therapy**

To further investigate whether TrapG probes can be employed to image different levels of endogenous βG activity in tumors, mice bearing SW480, HCC36, CL1-5, SW620, Hela or Colo205 tumors were i.v. injected with NIR-TrapG and imaged on an IVIS optical imaging system at 24 hours after injection. As shown in Figure 6A and Supplementary Figure S1, Colo205, CL1-5 and Hela (human βG \textsuperscript{high}) tumors
displayed more fluorescent intensity than SW620, SW480 and HCC36 (human βG low) tumors. These results suggest that TrapG probes can specifically image the different levels of βG activity in tumors. To investigate whether NIR-TrapG tumor imaging and 9ACG cancer therapy were contributed by increased βG in the tumor microenvironment, the mouse βG expression levels in human SW620 and Colo205 tumors were determined by RT-PCR. As shown in Figure 6B, Colo205 tumors displayed elevated mouse βG as compared to the SW620 tumors, indicating that the conversion of TrapG probes and 9ACG prodrugs relies on extracellular βG in the tumor microenvironment. To estimate the antitumor potency of endogenous βG combined with 9ACG prodrug treatment, BALB/c nude mice bearing SW620 (human βG low) or Colo205 (human βG high) tumors were i.p. injected with PBS or 9ACG (50 mg/kg) on days 1, 3 and 6. 9ACG treatment significantly (p < 0.05) suppressed the growth of Colo205 tumors as compared to PBS treatment (Fig. 6C, left panel). In contrast, treatment of 9ACG in SW620 tumors did not significantly delayed tumor growth (Fig. 6C, right panel). These results showed that the therapeutic efficacy of 9ACG prodrug is correlated with the expression levels of endogenous βG thereby suggesting that the TrapG-based PET imaging system might be employed to improve βG-mediated personalized antitumor treatments.
DISCUSSION

We have successfully developed a 124I-tyramine-difluoromethylphenol-glucuronide probe (124I-TrapG) for in vivo monitoring of βG enzyme activity by micro-PET imaging. The in vitro and in vivo results demonstrated that the 124I-TrapG probe can be activated by βG to radioactively image βG-expressing cells or tumors. In the cytotoxicity assay, native tyramine-TrapG displayed modest cytotoxicity in the presence of βG. We also found that the therapeutic efficacy of 9ACG prodrug is correlated to the βG levels in tumors which are determined by TrapG imaging. These results indicate that 124I-TrapG may be a useful probe for the detection of βG activity by PET technology to optimize βG-based targeted therapies in the clinic.

Although 124I-TrapG can be specifically converted by βG and thereby trapped in βG-expressing tumors, we also observed strong abdominal signals in mice. Two major causes may result in this background signals. First, liver cells can absorb xenobiotic glucuronides from the circulation by organic anion-transporting polypeptides such as estradiol-17-β-D-glucuronide and telmisartan acylglucuronide (27-29). Second, glucuronide conjugates can be transported to the intestines via biliary excretion. To diminish this effect, several methods have been employed, including depletion of intestinal microbes by antibiotics (30, 31) and inhibition of bacterial βG by chemical inhibitors (32, 33). Therefore, we treated mice with antibiotics or a βG inhibitor before 124I-TrapG injection. However, the abdominal signals were not reduced by either elimination of intestinal bacteria (data not show) or inhibition of bacterial βG activity (Fig. 4B). However, biliary excretion may be reduced by repeated injections of acetaminophen to induce Mrp3 expression and provoke the clearance of glucuronide conjugates through the urinary pathway (34).
βG is considered as a tumor marker (14, 15, 20) and a prodrug-activating enzyme for cancer prodrug therapies (35, 36). Connors and Whisson demonstrated that high βG levels present in plasmacytomas were correlated with higher drug sensitivity of the tumors (37). Indeed, we also found that Colo205 xenografts exhibited high levels of endogenous βG activity based on the imaging results using TrapG probes. Meanwhile, several studies have found that βG accumulates in the necrotic areas of human cancers (14, 18, 38, 39). In addition, glucuronide-conjugates usually exhibit enhanced solubility and decreased cell-permeability (3) that greatly increases the utility and specificity of pro-drugs or pro-probes relying on selective hydrolysis by βG enzyme. Many βG-activity based prodrugs have been developed for cancer-targeted therapies, including 9ACG (24), BHAMG (40), daunorubicin-GA3 (DNR-GA3) (35), and glucuronide conjugated doxorubicin (DOX-GA3, HMR1826) (35). Thus, the βG activity based ¹²⁴I-TrapG imaging system may provide a powerful tool for tracking tumor associated βG activity by PET imaging to improve βG-based personalized anticancer therapies.

Development of a functional PET probe to image βG activity in patients would improve βG-based targeted therapy. PET imaging is a sensitive, noninvasive, and clinical used technology for detecting organ function and disease diagnosis. The high penetration of radioactive signals facilitates deep tissue/organ imaging (41, 42). Antunes and colleagues generated 1-O-(4-(2-¹⁸fluoroethyl-carbamoyloxymethyl)-2-nitrophenyl)-O-β-D-glucopyronuronate ([¹⁸F]-FEAnGA) for in vivo tracking βG activity by PET imaging (43). However, the optimal radioactive accumulation of [¹⁸F]-FEAnGA was 2-fold higher in βG-tumor than in control tumors at 1 hour (4). We have also previously developed a


124I labeled βG-based hydrophobic conversion probe (124I-PTH-G) which can specifically accumulate at βG-expressing tumors. However, the optimal imaging efficiency of 124I-PTH-G was only 3.6-fold greater in βG-expressing tumors than in control tumors at 1 hour (3). These studies indicate that both [18F]-FEAnGA and 124I-PTH-G rapidly diffuse away from βG-expressing sites and limit their utility for clinical use. In the present study, we describe a new 124I-TrapG probe for monitoring of βG enzyme activity by micro-PET imaging. The optimal radioactive accumulation of 124I-TrapG was 141.4-fold higher in βG-expressing tumors than in control tumors at 20 hours after probe injection. These results indicate that PET imaging of 124I-TrapG is potentially useful for long-term monitoring of βG activity in vivo.

An imaging probe should display low toxicity, high specificity and strong sensitivity. We previously demonstrated that a near infrared glucuronide trapping probe (NIR-TrapG) is an activity-based probe that allows specific and direct detection of βG activity in preclinical models (21). Additionally, the glucuronide trapping probe did not inhibit βG enzyme activity which can improve substrate activation, imaging intensity and might not affect the conversion of βG-based prodrugs (43, 44). Here, we also verified that TrapG probe exhibits relatively low toxicity (IC50 > 200 μM). By contrast, FIAU, the substrate for HSV-tk, is much more toxic (IC50 = 0.073 μM) (45). Moreover, the glucuronide trapping moiety (difluoromethylphenol) can be conjugated with various probes for multi-imaging systems, such as fluorescent dyes (FITC and IR-820) for optical imaging (21) and radioactive isotopes (124I) for micro-PET imaging. In the future, the glucuronide trapping probe could also be linked to clinical used diethylenetriamine pentaacetic acid (DTPA) (46) for SPECT imaging or 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA) (47), ultra-small paramagnetic iron oxide (USPIO) particles (48) and
1,4,7-tricarboxymethylene-1,4,7,10-tetraazacyclododecane (DO3A) (49) for MR imaging. The $^{124}\text{I}$-TrapG PET probe displays low toxicity, high sensitivity, non-inhibition of enzyme activity and long-term accumulation, which may facilitate monitoring $\beta$G activity in the clinical setting. Future developments to reduce the signals in the gastrointestinal tract should further refine and promote the use of the $\beta$G reporter system in laboratory and clinical medicine.

The $\beta$G activity-based PET probe, $^{124}\text{I}$-TrapG, is useful for noninvasive imaging of $\beta$G-expression in the living body. The $^{124}\text{I}$-TrapG possesses several advantages including: (1) the low toxicity of TrapG should allow clinical imaging, (2) high specificity and signal amplification due to the catalytic hydrolysis of probes by $\beta$G, (3) possibility of generating a wide range of imaging probes by attachment of TrapG groups and (4) retention of probes upon $\beta$G conversion leading to long-term imaging in vivo. Based on these advantages, $^{124}\text{I}$-tyramine-TrapG may be paired with $\beta$G-based prodrugs to generate a personalized therapy system.
Authors’ Contributions

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References

FIGURE LEGENDS

Figure 1. Chemical synthesis and β-glucuronidase mediated activation of tyramine-TrapG probes. A, chemical structure and synthesis of $^{131}$I-TrapG and $^{124}$I-TrapG probes. B, βG-mediated activation of the $^{124}$I-TrapG probe releases the $^{124}$I-difluoromethylphenol moiety that may crosslink nucleophiles nearby the site of βG expression in vitro and in vivo.

Figure 2. Specificity of the FITC-TrapG probe. A, Functional binding of FITC-TrapG probes on CT26 and CT26/mβG cells was detected by flow cytometry after staining the cells with FITC-TrapG probes with or without SAL inhibitors. B, cell lysates prepared from CT26 or CT26/mβG cells that were treated with FITC-TrapG were electrophoresed on a SDS-PAGE and immunoblotted with anti-FITC antibody.

Figure 3. Cytotoxicity and specificity of TrapG. A, CT26 cells were incubated with graded concentrations of BHAMG (○), BHAMG with eβG (●), tyramine-TrapG (□), or tyramine-TrapG with eβG (■) for 72 hours. Cell viability was measured by determining incorporation of $^3$H-thymidine into cellular DNA. B, graded concentrations of $^{124}$I-TrapG were incubated with CT26 cells (●), CT26/mβG cells (○), CT26 cells with SAL (■), or CT26/mβG cells with SAL (■) for 60 min before the cells were washed and bound radioactivity was measured. C, 0.74 MBq/mL of $^{124}$I-TrapG (50 μL/well) was incubated with CT26 cells (●), CT26/mβG cells (○), CT26 cells with SAL (■), or CT26/mβG cells with SAL (■) for the indicated times (5, 15, 30, 60, 120 and 180 mins). The radioactivity of $^{124}$I-TrapG treated cells was
measured on a γ-counter. Results represent the mean of results from 3 separate wells. Mean ± SD. D, Balb/c mice were i.v. injected with 40 mg/kg PBS or TrapG. The serum concentrations of aspartate aminotransferase (AST) (■), alanine aminotransferase (ALT) (●), and total bilirubin (TBIL) (▲) were measured 7 days after TrapG administration. Bars, SD (n = 6).

Figure 4. *In vivo* micro-PET imaging of β-glucuronidase by 124I-TrapG. A, BALB/c mice (n=3) were i.v. injected with 0.37 MBq of 124I-TrapG. Serum samples were collected at the indicated times and directly measured on a gamma counter. The radioactivity in serum samples was expressed as % injected dose/g tissue. Mean ± SD.

B, BALB/c mice bearing CT26/mβG tumors (solid arrow) and control CT26 tumors (dashed arrow) were i.v. injected with 3.7 MBq of 124I-TrapG in the presence or absence of D-saccharic acid 1,4-lactone monohydrate (SAL). Micro-PET images were acquired at the coronal and transverse planes of the mice at 1, 8 and 20 hours post-injection of the probe. Scale bar: 1 cm. C, CT26/mβG and CT26 tumors were resected at 20 h after 124I-TrapG injection for autoradiography (phosphor imaging) and βG activity (X-GlcA staining). Scale bar: 1 mm.

Figure 5. Biodistribution of 131I-TrapG. BALB/c mice bearing CT26/mβG tumors and parental CT26 tumors were i.v. injected with 1.85 MBq of 131I-TrapG. A, selected organs and tumors were harvested after 1 (white bars), 8 (black bars), 20 (gray bars) and 40 (hatched bars) hours. Radioactivity in the organs was measured by a γ-counter and normalized for sample weight. B, the autoradiography of whole-body sections were scanned by a radioisotope image analyzer at 40 hours after 131I-TrapG injection. The biodistribution of 131I-TrapG is expressed as % injected dose/g tissue. Data
represent mean ± SD from 4 mice at each time point.

**Figure 6. In vivo imaging of endogenous β-glucuronidase by TrapG probes for β-glucuronide prodrug therapy.** A, BALB/c nude mice bearing SW620 and Colo205 tumors were i.v. injected with 100 μg of NIR-TrapG. Optical images were acquired at 24 hours post-injection of the probe. B, total RNA samples prepared from Colo205 and SW620 tumor tissue samples were used as templates for RT-PCR by using mouse βG (upper panel), human βG (middle panel) or human GAPDH (lower panel) gene-specific primers. The human GAPDH served as a loading control and the DNA plasmids containing mouse βG or human βG genes (p-mβG and p-hβG) served as positive controls. C, BALB/c nude mice bearing 100 mm³ s.c. Colo205 (left panel) or SW620 (right panel) tumors were i.p. injected with PBS (●) or 50 mg/kg 9ACG (○) on days 1, 3 and 6. Bars, SEM (n = 4). Arrows, treatment schedule. Significant differences in mean tumor size in mice treated with 9ACG are indicated: *, p < 0.05.
A

Chemical Structure and Synthesis of Tyramine-TrapG, $^{125}$I-TrapG and $^{131}$I-TrapG

Reagents:
i. Succinic anhydride, TEA, CH$_2$Cl$_2$, rt, 1.5 h
ii. Tyramine, TEA, HOBt, PyBOP, rt, 2 h.
iii. NaOCH$_3$, CH$_3$OH, rt, 1 h.
iv. Potassium trimethylsilyllorionate, THF, 1N HCl.
v. Iodine-124, NaI, Ammonium acetate, Chloramine-T solution, rt, 2 min.
vi. Iodine-131, NaI, Ammonium acetate, Chloramine-T solution, rt, 2 min.

B

Figure 1
Figure 2
Figure 3
Figure 6
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PET Imaging of β-Glucuronidase Activity by an Activity-Based 124I-Trapping Probe for the Personalized Glucuronide Prodrug Targeted Therapy

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