Electronic Supplementary Information

Beta-galactosidase-responsive Synthetic Biomarker for Targeted Tumor Detection

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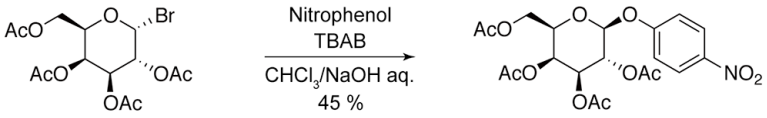
1. Supporting Methods

1-1. Synthesis

General information

Reagents and solvents were purchased from standard suppliers and used without further purification. NMR spectra were measured using a JEOL ECS 400 spectrometer. Chloroform-d_1 (7.26 ppm) or methanol-d_4 (3.31 ppm) was used as the internal standard for ^{1}H NMR. Chloroform-d_1 (77.0 ppm) or methanol-d_4 (49.0 ppm) was used as the internal standard for ^{13}C NMR. Mass spectra (MS) were measured using a Bruker micrOTOF II (ESI).

Synthesis of 1



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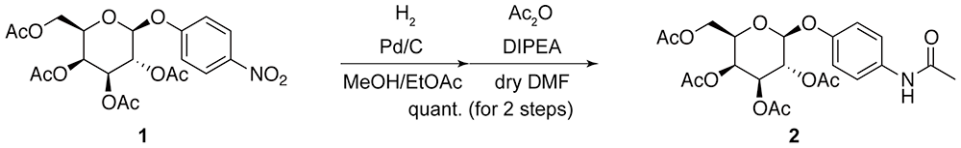
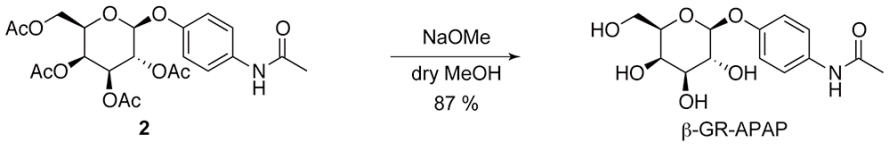
Compound **1** was synthesized following the procedure described in the literature.[1]

Synthesis of 2



Compound **1** (218 mg, 0.465 mmol), in a mixture of MeOH (5 mL) and EtOAc (2 mL), was stirred under hydrogen at room temperature for 5.5 h in presence of 10 wt.% palladium on activated carbon (28.7 mg). The solution was filtered through Celite, and the filtrate was evaporated to give the reductant, quantitatively, as yellowish oil. Subsequently, acetic anhydride (220 μL, 2.33 mmol) was added dropwise to a solution of the residue and N,N-diisopropylethylamine (486 μL, 2.79 mmol) in dry DMF (5 mL) under nitrogen atmosphere. The mixture was stirred at room temperature for 35 h. After evaporation, the mixture was dissolved in EtOAc (20 mL). The organic phase was washed with 0.1 N HCl (3×) and brine (3×), dried over Na_2SO_4 and evaporated to give **2** as a white solid (244 mg, quant.). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.01 (s, 3H), 2.06 (s, 3H), 2.07 (s, 3H), 2.17 (s, 3H), 2.18 (s, 3H), 4.02–4.05 (m, 1H), 4.14–4.25 (m, 2H), 4.98 (d, *J* = 8.4 Hz, 1H), 5.08–5.12 (m, 1H), 5.44–5.49 (m, 2H), 6.97 (d, *J* = 9.2 Hz, 2H), 7.09 (s, 1H), 7.41 (d, *J* = 9.2 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 20.6, 20.7, 24.4, 61.3, 66.9, 68.6, 70.8, 71.0, 100.1, 117.6, 121.5, 133.3, 153.6, 168.2, 169.4, 170.1, 170.2, 170.4.

Synthesis of β-GR-APAP



Under nitrogen atmosphere, 28 wt.% NaOMe in methanol (26 μL) was added dropwise to a solution of **2** (174 mg, 0.363 mmol) in dry methanol (13 mL) on ice. After 10 min, the mixture was stirred at room temperature for 1.5 h. DOWEX cation exchange resin (H form, 2.5 mg) was added to the reaction mixture on ice and stirred for 15 min. After filtration, the filtrate was evaporated to give β-GR-APAP as a white solid (98.6 mg, 87%). ^{1}H NMR (CD_3OD, 400 MHz): δ = 2.10 (s, 3H), 3.55–3.59 (m, 1H), 3.65–3.68 (m, 1H), 3.72–3.81 (m, 3H), 3.90 (d, *J* = 3.6 Hz, 1H), 4.81 (d, *J* = 8.0 Hz, 1H), 7.07 (d, *J* = 8.8 Hz, 2H), 7.43 (d, *J* = 9.2 Hz, 2H). ^{13}C NMR (CD_3OD, 100 MHz): δ = 23.6, 62.4, 70.2, 72.3, 74.9, 77.0, 103.4, 118.1, 122.6, 134.5, 155.8, 171.4. HRMS (ESI): m/z calc. for $C_{14}H_{18}NO_7^-$ $[M-H]^-$ = 312.1083, found = 312.1112.

1-2. LC-MS/MS measurements

General information

Experiments were conducted according to the procedures shown in reference 9 (main text). Briefly, chromatography was performed on an XBridge™ Phenyl column (2.1 × 50 mm, 2.5 μm, Waters) at 45 °C under isocratic condition (99.5:0.5 water:methanol with 0.1% formic acid) with a flow rate of 0.23 mL/min. Under positive electrospray ionization conditions (+2,000 V), all analytes (β-GR-APAP, APAP, APAP-G and APAP-S) were predominantly in the protonated form $[M-H]^+$. The compound-specific operating parameters were as follows: The fragmentor voltage for all analytes was 380 V. The collision energy for β-GR-APAP and APAP was 16 eV and that for APAP-G and APAP-S was 10 eV. The dwell time for all analytes was 180 ms. Mass spectrum was recorded by selected reaction monitoring (β-GR-APAP m/z 314.1 → 152.1; APAP m/z 152.1 → 110.0; APAP-G m/z 328.1 → 152.1; APAP-S m/z 232.0 → 152.1) on a 6490 Triple Quad LC/MS system (Agilent Technologies). The 1200 series LC-MS/MS system was controlled by MassHunter Workstation Software LC/MS Data Acquisition for 6400 Series Triple Quadrupole B.06.00 (Agilent Technologies). Data were processed using MassHunter Workstation Software-Quantitative Analysis Version B.06.00.SP01 (Agilent Technologies). The calibrators for all analytes were prepared in phosphate buffered saline (PBS).

Measurement of culture media (Fig. 2a, S2)

The obtained media were centrifugally filtered through a 5-kDa cutoff filter (Merck Millipore) to remove proteins (9,100 *g*, 4 °C, 3 h). The aliquots were analyzed by LC-MS/MS (injection volume, 1 μL). In Fig. S2, the aliquots (5 μL) were diluted with water (45 μL) for the LC-MS/MS measurements.

Measurements of plasma sample (Fig. 2c, 2d, S1 and S4–S6).

The plasma samples were obtained by centrifugation (1,000 *g*, 4 °C, 15 min). The samples (10 μL) were pre-treated with acetonitrile (50 μL), mixed and centrifuged (9,100 *g*, 4 °C, 10 min). The supernatants (30 μL) were diluted with water (270 μL), and aliquots were analyzed by LC-MS/MS (injection volume, 10 μL).

Instrument detection limit calculation[2]

Instrument detection limit (IDL) was calculated using the following equation.

IDL = t (n-1, 1-α = 0.99) × (S) /100 × (injected amount)

t (n-1, 1-α = 0.99) = the student's t-value appropriate for a 99% confidence level and a standard deviation estimate with n-1 degree of freedom (n = 8)

S = % standard deviation of the replicate analysis

1-3. Cellular experiments

β-GR-APAP to APAP conversion by β-gal labeled LoVo cells (Fig. 2a, S2)

LoVo cells were obtained from the American Type Culture Collection. The cells (0, 0.5, or 1.0×10^6 cells) were grown in a 6-cm dish in a humidified atmosphere of 5% CO_2 at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum and 1% Antibiotic-Antimycotic (penicillin-streptomycin-amphotericin B).

β-gal labeling analysis (Fig. 2a and Fig. S2a)

After the incubation for 18 h (Fig. 2a) or 22 h (Fig S2a), the medium was replaced by fresh DMEM with avidin-β-gal or β-gal (0 or 0.5 μg/mL, 3 mL). After 6 h incubation, the cells were washed thrice with PBS (4 mL) and harvested. The harvested cells were incubated in DMEM containing 20 μM β-GR-APAP (1 mL). The culture media were collected after 24 h.

Endogenous β-gal activity analysis using PETG (Fig. S2b)

After the incubation for 24 h, the medium was replaced by fresh DMEM with 0, 0.3 or 1 mM 2-phenylethyl β-D-thiogalactoside, PETG and 40 μM β-GR-APAP. The culture media were collected after 48 h.

1-4. Animal experiments

Animal experiments (Fig. S1 and S4) were performed according to the Institutional Guidance of National Institute of Technology, Tsuruoka College on Animal Experimentation with permission from the Animal Experiment Committee of National Institute of Technology, Tsuruoka College.

Animal experiments using intraperitoneal tumor model (Fig. 2, S3, S5 and S6) were performed according to the Institutional Guidance of the University of Tokyo on Animal Experimentation with permission from the Animal Experiment Committee of the University of Tokyo.

β-gal detection from plasma sample using β-GR-APAP (Fig. S1)

Female BALB/cAJcl mice were purchased from CLEA Japan, housed in a climate-controlled circadian rhythm-adjusted rack and allowed free access to water and food (CE-2, CLEA Japan). BALB/cAJcl mice (8-week-old, 21–22 g) were administered PBS solution containing purified β-gal (0 or 3 U, 600 μL) intraperitoneally (i.p.). Within 1 min, PBS solution containing β-GR-APAP (3.6 mM, 400 μL) was administered i.p. (n = 3 per group). At 20, 60, 120 and 270 min after injection, blood from the tail vein was collected using a heparin-coated capillary.

Analysis of β-GR-APAP stability in the blood (Fig. S4)

Female BALB/cAJcl mice (13-week-old, 22–23 g) were administered PBS solution containing avidin-β-gal (0 or 30 μg, 300 μL) i.p. (n = 4). After 20–21 h, blood was collected using a capillary and mixed with the β-GR-APAP solution. The mixture was incubated on ice for 3.5 h or 12.5 h. After incubation, plasma was obtained by centrifugation (1,000 *g*, 4 °C, 15 min).

Preparation of intraperitoneal tumor model (Fig. 2b-2d, S3, S5 and S6)

Female BALB/cAJcl nu/nu mice were purchased from CLEA Japan, housed in a climate-controlled circadian rhythm-adjusted room and allowed free access to water and food (CRF-1, Oriental Yeast). Six-week-old mice (day 0, 16–21 g) were used in the experiments. The intraperitoneal tumor model was developed as follows:[3] LoVo cells were harvested after 72 h culture in RPMI 1640 containing 10% heat-inactivated fetal bovine serum and 1% Antibiotic-Antimycotic (penicillin-streptomycin-amphotericin B). Approximately 3.4×10^6 cells in 450 μL PBS solution were injected into the peritoneal cavity of a mouse. Experiments using a mouse model of intraperitoneal tumor were performed after 25 days of dissemination, when numerous small intraperitoneally disseminated tumors had formed, especially around the subphrenic space of hepatic and splenic region and on the mesentery.

X-Gal staining (Fig. S3)

The excised organs and tumor tissues were incubated in X-Gal staining solution (1 mM $MgCl_2$, 3 mM $K_3[Fe(CN)_6]$, 3 mM $K_4[Fe(CN)_6]$, 0.3% Triton-X-100, 0.2% X-Gal in PBS) at room temperature for 1, 2 and 4 h.

Tumor detection using β-GR-APAP *in vivo* (Fig. 2c, 2d and S5)

Normal and tumor-implanted mice were administered PBS solution containing avidin-β-gal (30 μg, 300 μL) i.p. (n = 6 for normal mice and 7 for tumor-implanted mice). After 19.5 h, PBS solution containing β-GR-APAP (3.6 mM, 400 μL) was administered i.p. At 20, 60 and 120 min after injection, blood from the tail vein was collected using a heparin-coated capillary.

Analysis of the effect of tumor implant on the production of APAP-conjugates (Fig. S6)

Normal and tumor-implanted mice were administered PBS solution containing APAP (3.6 mM, 400 μL) i.p. (n = 3 per group). At 20, 60 and 120 min after injection, blood from the tail vein was collected using a heparin coated-capillary.

Statistical analyses

All statistical analyses were performed using the KaleidaGraph version 4.5.1. Mann-Whitney *U* test was used to evaluate data from the experiments, and significance was defined as $*P < 0.05$.

2. Supporting Figures

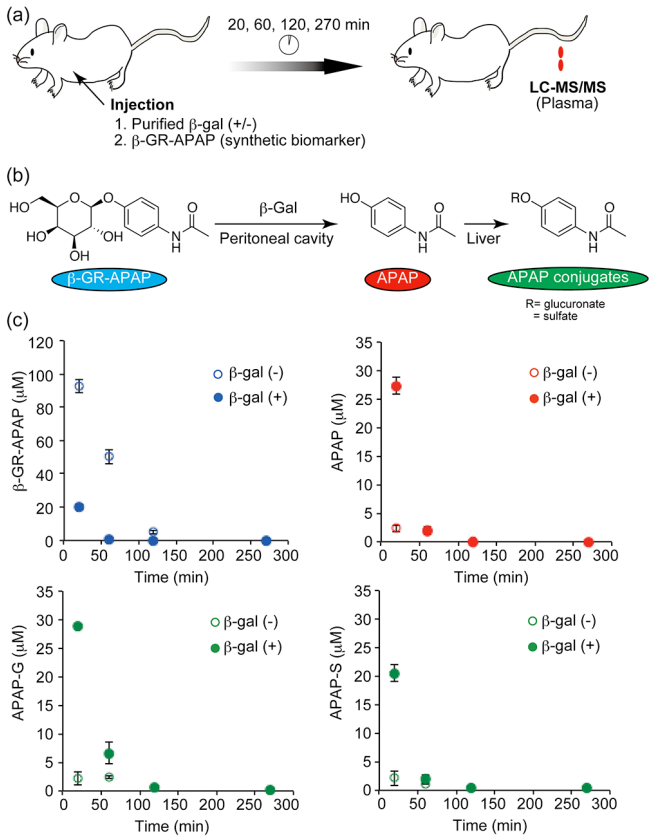


Fig. S1 (a) Schematic of *in vivo* β-gal detection using β-GR-APAP as a synthetic biomarker. (b) Reaction of β-GR-APAP with β-gal, and APAP metabolism in the liver. (c) Plasma concentrations of β-GR-APAP, APAP and APAP conjugates (APAP-G and APAP-S) at 20, 60, 120 and 270 min following β-GR-APAP injection. β-GR-APAP (3.6 mM, 400 μL) was injected into the peritoneal cavity, along with purified β-gal (0 or 3 U). Data are mean ± SEM (n = 3).

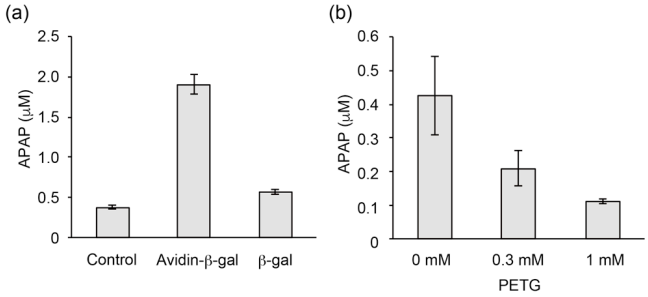


Fig. S2 *In vitro* evaluation of β-GR-APAP to APAP conversion using LoVo cells. (a) The LoVo cells (1.0×10^6) were labeled with avidin-β-gal or β-gal alone (0 or 0.5 μg/mL) and harvested after washing. The harvested LoVo cells were incubated in Dulbecco's modified eagle medium (20 μM β-GR-APAP) for 24 h. Data are mean ± SD (n = 4 per group). (b) The LoVo cells (5.0×10^5) were incubated in the medium containing PETG (0, 0.3, 1 mM) and 40 μM β-GR-APAP for 48 h (n = 3 per group).

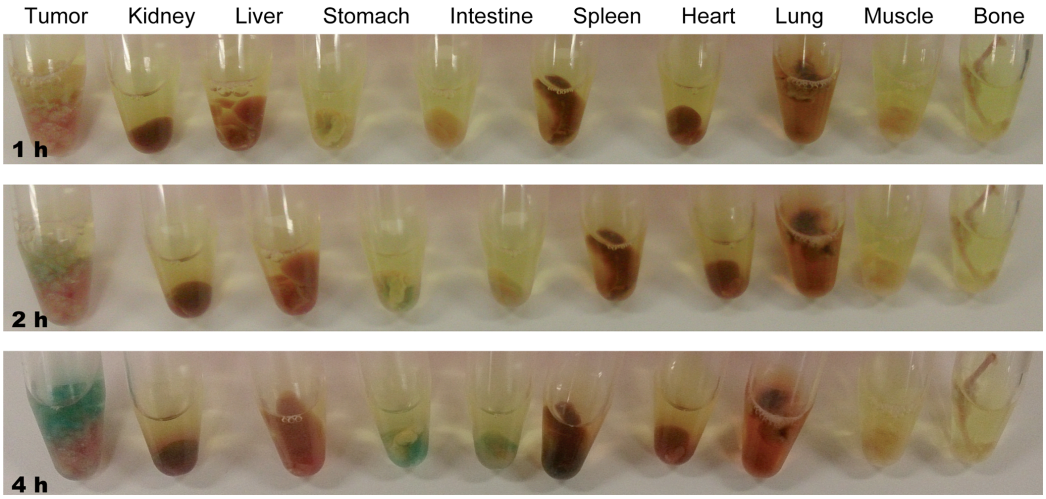


Fig. S3 Representative X-Gal staining of normal and tumor (LoVo) tissues. The blue precipitate was detected in the β-gal-labeled tumor tissue depending on the incubation time. The blue color was also observed in stomach and intestine. This color may be derived from the inefficient tumor β-gal labeling by avidin-β-gal used in this study, or endogenous β-gal activity in these organs.

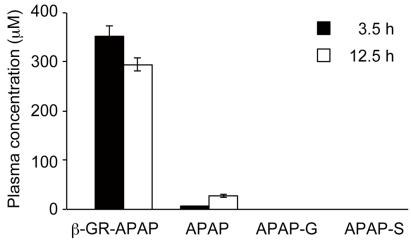


Fig. S4 Stability of β-GR-APAP in the blood. After mixing β-GR-APAP with blood, the latter was incubated for 3.5 or 12.5 h on ice. Plasma concentration of the four species (β-GR-APAP, APAP, APAP-G and APAP-S) was measured by LC-MS/MS. Data are mean ± SD (n = 4).

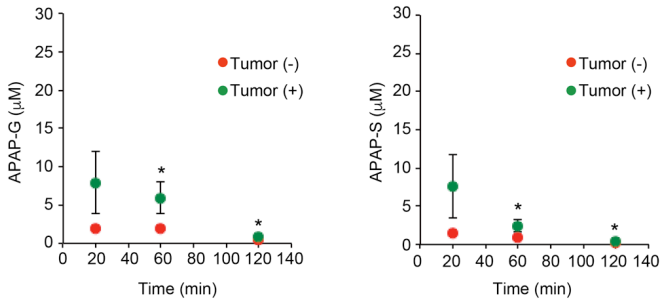


Fig. S5 Plasma concentration of APAP conjugates (APAP-G and APAP-S) at 20, 60 and 120 min following β-GR-APAP injection into normal and tumor-implanted mice. β-GR-APAP (3.6 mM, 400 μL) was injected into the peritoneal cavity at 19.5 h after avidin-β-gal injection (30 μg). Data are mean ± SEM (n = 6, 7). Mann-Whitney *U* test; * $P < 0.05$.

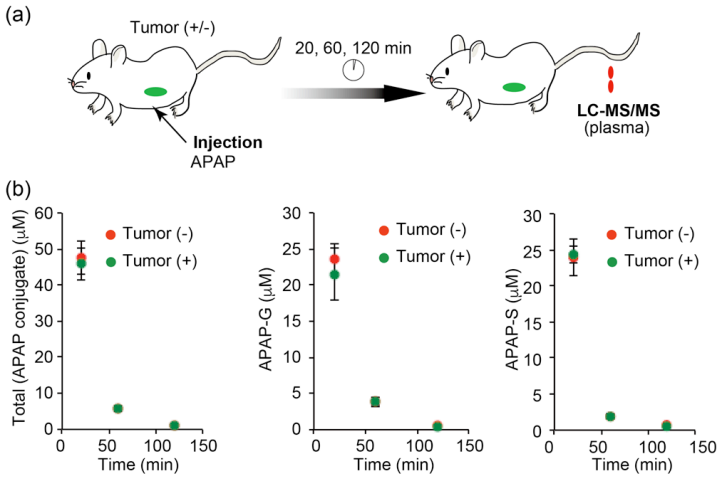


Fig. S6 Plasma concentration of APAP conjugates (APAP-G and APAP-S) at 20, 60 and 120 min following APAP injection. APAP (3.6 mM, 400 μL) was injected into the peritoneal cavity of normal and tumor-implanted mice. Data are mean ± SEM (n = 3).

3. References

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