Up-regulation of key molecules for targeted imaging and therapy

Cell culture

The human pancreatic neuroendocrine tumor cells BON-1 (Institute of Pathology, University of Bern, Switzerland) and the human prostate cancer cells PC3 (ATCC, CRL-1435), which both express $sstr_2$ receptors, were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM)/F-12 1:1 containing GlutaMAX™, 10% (vol/vol) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. QGP1 cells (Health Science Research Resources Bank (HSRRB), Japan, #JCRB0183), which express $sstr_2$ receptors at low levels, and AR42J cells (CLS Eppelheim, Germany, #500478), which express $sstr_2$ receptors at high levels, were cultured at 37°C and 5% CO_2 in RPMI containing GlutaMAX™, 10% (vol/vol) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. All cell culture reagents were purchased from Gibco BRL, Life Technologies (USA). All cell lines were amplified, and aliquoted mycoplasma-free, and new aliquots were thawed and cultured every two months.

Radiolabeling of gallium-68-DOTATOC

Gallium-68-DOTATOC was prepared using a germanium-68/gallium-68 generator (IGG100-30M, Eckert-Ziegler, DE). Gallium-68 was eluted with 10 mL 0.1 M hydrochloric acid (HCl) to an in-line cation exchange resin (Strata-X-C, #8B-S029-TAK, Phenomenex, USA), which retains gallium-68. Purified gallium-68 was then eluted with 0.8 mL of 98% acetone/0.02 M HCl. Acetone was evaporated and the residue was re-suspended in 1 mL of 0.2M acetic acid (pH 3). DOTATOC (Chinese Peptide Company # 20-0039, CN) at 0.1 mg/mL in DPBS was mixed 1:1 (vol/vol) with the gallium-68 solution and radiolabeling was carried out at 95°C for 10 min. Gallium-68-DOTATOC was cooled to room temperature before use and diluted with pH 7 DPBS buffer (Life

Technologies # 14190, USA). The radiolabeling yield was then measured by radio-TLC and only batches with radiochemical purities of >97% were used for the experiments.

Reagents and antibodies

Somatostatin-14, forskolin, growth hormone releasing hormone (GHRH), 17β-estradiol, testosterone, dexamethasone, transforming growth factor beta (TGFB), tamoxifen, and trichostatin A were purchased from VWR (Switzerland). Decitabine, azacitidine, scriptaid, phenethyl isothiocyanate (PEITC), valproic acid, vorinostat, phenylbutyrate and tacedinaline were purchased from Sigma Aldrich (Switzerland). Panobinostat, dacinostat and romidepsin were obtained from Selleckchem (USA). The jagged 1 peptide (amino acid sequence: CDDYYYGFGCNKFCRPR) was purchased from ANAWA (Switzerland). The drugs were dissolved and stored as recommended by the manufacturer. The $sstr_2$-specific rabbit monoclonal antibody UMB-1 (SS-8000RM) was bought from Biotrend GmbH (Germany), the $sstr_2$-specific rabbit polyclonal antibody from Genscript (#A01591). The secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (HL) was purchased from Molecular Probes, Inc. (USA). The horseradish peroxidase-conjugated goat anti-rabbit IgG and the horseradish peroxidase substrate kit were purchased from Bio-Rad Laboratories, Inc. (USA).

Real-time qRT-PCR

qRTPCR was performed as previously described (*1*). In brief, RNA from cell lines was extracted with RNeasy Mini Kit (QIAGEN) and treated with deoxyribonuclease (QIAGEN) to eliminate genomic DNA. 4 mcg of purified cell line RNA and 2.2 mcg of purified tumor tissue RNA were treated with gDNA elimination buffer and then reverse-transcribed into first-strand cDNA using oligo (deoxythymidine) primers, with QuantiTect reverse transcriptase (Qiagen). Primers used for quantitative RT-PCR are human $sstr_2$ (Hs00265624_s1), human β-actin (4326315E), and

human β-tubulin 1, Class VI or TUBB1 (Hs00258236_m1) from Applied Biosystems. A 40-cycle PCR was carried out at 60°C annealing temperature in a MicroAmp Optical 96-well plate in BioRad iQ5 real-time PCR detection system. Amplicons were detected using the relevant probes tagged with MGB quencher and FAM (carboxyfluorescein) dye. TaqMan human β-actin (4326315E) control expression assay with probe tagged with MGB and VIC and human β-tubulin 1, Class VI or TUBB1 (Hs00258236_m1) with probe tagged with MGB and FAM (Applied Biosystems) were used as active reference. A total of 125 ng of RNA was loaded into each well and samples were analyzed in duplicates.

Western-blotting

Cells were cultured for 3 days at 37°C and 5% CO_2 in culture medium containing 75 ng/mL decitabine, 500 ng/mL tacedinaline or no test drugs (negative controls). Cells were then washed with PBS and lysed in ice-cold lysis buffer (20 mM HEPES, 150 mM EDTA, 3 mM EGTA, and 4 mg/mL dodecyl β-D-maltoside, pH 7.6) containing protease inhibitors (1 mM PMSF and the protease inhibitor cocktail mini tablets from Pierce (#88668)). 5 x loading buffer (10% SDS, 10mM β-mercapto-ethanol, 20% glycerol, 0.2 M Tris pH 6.8, 0.05% bromophenolblue) was added to the lysate and the samples were then heated at 95°C for 10 min and resolved on a NuPAGE 4-12% gradient SDS-polyacrylamide gel (Life Technologies #NP0335). Resolved proteins were then transferred to nitrocellulose membrane. The gel was subsequently stained with coomassie (0.1% coomassie Brilliant Blue R250, 40% methanol, 10% acetic acid) to visualize the remaining protein. The visible bands on the gel were then used as control for the amount of loaded protein. The membrane was treated for 1 h with 5% nonfat dry milk in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.4), and subsequently incubated overnight at 4 °C with the $sstr_2$-specific primary antibody from Genescript (#A01591) at a dilution of 1:2000 in

TBS-T with 2.5% nonfat dry milk. After incubation, the membrane was washed with TBS-T and the immuno-reactive proteins were detected using the secondary antibodies. Goat anti-rabbit IRDye 680 and goat anti-mouse IRDye 800 (coupled to infra-red dyes, VWR #ROCK605-430-013 and #ROCK605-431-013 respectively) at a dilution of 1:3000 were used. Western blots and coomassie-stained gels were analyzed using a LI-COR Odyssey scanner system (USA).

Enzyme-linked immuno-sorbent assay (ELISA)

A total of 20,000 cells (for 3 days drug treatment), 25,000 (for 2 days drug treatment) and 50,000 (for 1 day drug treatment) per well were seeded in 96-well plates in culture medium containing 75 ng/mL decitabine, 500 ng/mL tacedinaline, 75 ng/mL decitabine plus 500 ng/mL tacedinaline or in medium without drugs (negative controls). In parallel a 96-well plate with the same number of cells and the same drug treatment was prepared for total protein determination by Bicinchoninic Acid Assay (BCA) in order to normalize the $sstr_2$ expression in relation to the total amount of cell protein. Cells were cultivated for 1, 2 and 3 days at 37°C and 5% CO_2. Subsequently, cells were washed 3 times with PS (100 mM phosphate buffer containing 0.15 M sucrose) and then fixed for 15 min at 37°C with 3% formaldehyde in PS. The cells were then washed twice with 0.1 M glycine in PS and twice with PS and permabilized in permeabilization buffer (1% BSA, 0.3% Triton X-100 in PS) at room temperature for 60 min. After permeabilization, cells were incubated with the $sstr_2$-specific primary antibody UMB-1 at a dilution of 1:50 in permeabilization buffer for 2 h at room temperature. Cells were then washed twice with 2% BSA in PS and twice with 2% BSA in PBS and subsequently incubated for 60 min at room temperature with the secondary antibody goat anti-rabbit IgG horseradish peroxidase conjugate (1:2000) in 2% BSA in PBS. After incubation, cells were washed 3 times with PBS and 0.1 mL of horseradish peroxidase mix was added to detect the antibody binding. After

approximately 60 min incubation at room temperature the absorbance at 415 nm was measured using a SpectraMax M4 plate reader (Molecular Devices, LLC, USA). Nonspecific absorbance was determined in cells incubated without primary antibodies and subtracted from the absorbance values of the untreated and drug treated cells. Specific absorbance was then normalized to total amount of protein determined by BCA. The normalized specific absorbance value of the untreated cells was set as 100%.

Immuno-fluorescence microscopy

Immuno-fluorescence was performed as previously described (*2*). Briefly, cells were cultured for 3 d at 37°C and 5% CO_2 on poly-D-lysine (20 μg/mL) (Sigma Aldrich, CH) coated 35-mm four-well plates (Cellstar, Greiner Bio-One GmbH, DE), in growth medium containing 75 ng/mL decitabine, 500 ng/mL tacedinaline, 75 ng/mL decitabine plus 500 ng/mL tacedinaline or no test drugs (negative controls). After this period the cells were rinsed twice with PS (100 mM phosphate buffer containing 0.15 M sucrose), fixed and permeabilized for 7 min with methanol at -20°C, rinsed twice with PS, and then blocked for 60 min at room temperature with PS containing 0.1% BSA. Subsequently, cells were incubated for 60 min at room temperature with the $sstr_2$-specific primary antibody UMB-1 at a dilution of 1:50 in PS. Afterwards, cells were washed 3 times for 5 min with PS containing 0.1% BSA and incubated with the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (H-L) diluted in PS (1:600) for 60 min at room temperature in the dark. After washing 3 times with PS containing 0.1% BSA the cells were embedded with 1:1 PS: glycerol and covered with a glass cover slip. Cells were imaged using a Nikon Eclipse TS 100 immunofluorescence microscope and a Nikon DS-Fi1 camera.

Immunohistochemistry

BON-1 xenografts of decitabine treated or not treated mice were collected and fixed in formalin for at least 24 h. The fixed tissue was processed as conventional 2 µm ($sstr_2$) thick paraffin wax sections using the $sstr_2$-specific primary antibody UMB-1 (BioTrend, SS-8000-RM). The immunohistochemical staining for $sstr_2$ was performed on an automated staining system (Leica Bond III; Leica Biosystems, Nunningen, Switzerland) at the Institute of Pathology of the University of Bern, Switzerland. Antigen retrieval for UMB-1 was performed by heating EDTA based Bond Epitope Retrieval Solution 2 (Leica Bond™, AR9640) at 95°C for 40 minutes. The primary antibody UMB-1 was incubated for 60 minutes at a dilution of 1:25. Visualization was performed using the compact polymer dextran-peroxidase complex method, which yielded a brown staining signal. Receptor visualization was performed using the EnVision™ detection system from Dako (EnVision™ FLEX + Rabbit (LINKER) and the EnVision™ FLEX /HRP) yielding a brown staining signal. The counterstaining was performed with hemalum. Samples were imaged using a Nikon Eclipse TS 100 immunofluorescence microscope and a Nikon DS-Fi1 camera.

Uptake assay and Drug Screening

Uptake experiments were used to identify drugs that up-regulate $sstr_2$, and to subsequently establish a dose-response relationship for all identified drugs. Thereby, BON-1 cells were used for the screening and PC3 cells were used for validation. The uptake experiments were based on previously described assays (*3*). In brief, BON-1 were seeded in 12-well plates (200×10^3 and 30×10^3 cells respectively) and then treated with the test drugs at their respective pharmacological serum concentration for 3 days (**Supplemental Table 1**). Afterwards, 0.35 MBq of gallium-68-DOTATOC was added to the medium and cells were incubated for 2 h at 37°C. Plates were then chilled on ice, media was removed and wells were washed 3 times with PBS. Media and PBS

buffer were collected (non-internalized fraction). Then, cold glycine buffer (pH 2.8) was added to each well for 5 min and wells were washed again 3 times with PBS. Glycine wash and PBS wash were collected (unspecific cell-bound fraction). Finally, cold RIPA buffer (25mM Tris/HCl, 150mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) was added to each well for 20 min. The resulting cell lysates (internalized fraction), media and washes were transferred to 5 mL conical tubes and radioactivity was measured using a Wizard^2 2470 automatic gamma counter (Perkin Elmer, USA). The relative amount of protein present in the lysate was assessed using a BCA kit (#23227, Pierce, USA) and a SpectraMax M4 plate reader (Molecular Devices, USA). Cell numbers per well were assessed from the relative amount of protein and were used for normalization. Untreated cells were used to measure the baseline uptake of gallium-68-DOTATOC. Only drug doses resulting in >50% viable cells were included into the analysis. Subsequently, dose-response relationships in BON-1 cells were established by treating the cells with the most efficacious drugs at concentrations ranging from 0.75 – 75 ng/ml, and assessing gallium-68-DOTATOC uptake as described above. Then, dose-response relationships in PC3 cells were established with the same protocol.

In-vitro treatment

Cells were cultured in 75 cm^2 flasks for 3 days in medium containing 75 ng/mL decitabine plus 500 ng/mL tacedinaline or no test drugs. Then, cells were incubated with 5 MBq/mL lutetium-177-DOTATOC for 2 h, and subsequently washed with PBS. Cells were then treated with trypsin, pelleted in a 1.5 mL Eppendorf tube and incubated for 3 h in culture medium. Every 30 min, the pellet was gently re-suspended to avoid cell attachment. Cells were then counted and seeded at 50,000 cells/mL/well in 12-well plates. After 12 h, cells were washed with PBS, 250 µL lysis buffer was added to each well, and protein amount was quantified by BCA.

Animal studies

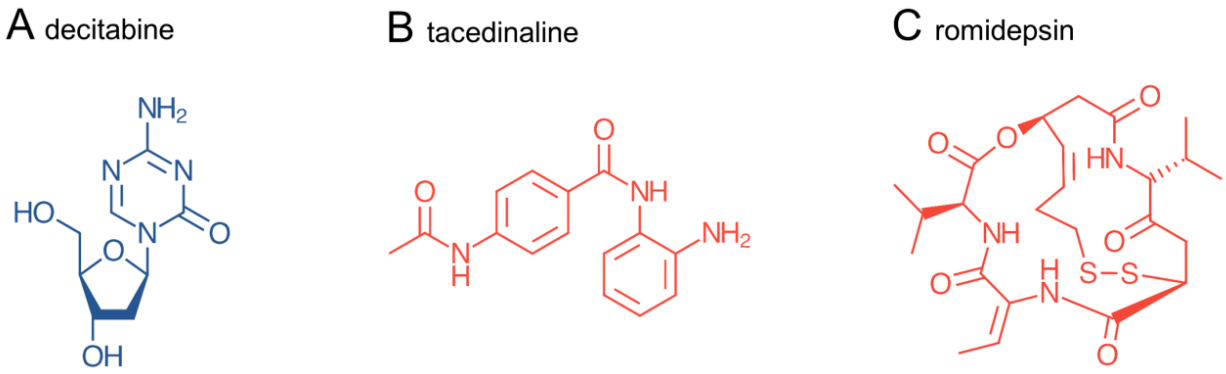
Six weeks old athymic Nude-Foxn1^{nu} mice were subcutaneously injected in the femoral region with $5x10^6$ BON-1 or PC3 cells in 50 µL PBS. *In-vivo* studies were performed when the xenografts had reached approximately 5 mm diameter. After that, subcutaneous injections with 50 µL of decitabine at three different concentrations (0.002, 0.02 and 0.2 mg/kg body weight) in the three treatment groups or with 50 µL of PBS in the control group were performed on day 1, day 3 and day 6 of the experiment. On day 9, 10 MBq of gallium-68-DOTATOC in 150 µL PBS were injected via the tail vein for the biodistribution study. For the blocking experiments, an additional 100-fold excess of non-radiolabeled DOTATOC was added. After 2 h, mice were sacrificed and organs and tumors were removed and weighed. Tracer uptake in each organ was measured using a Wizard^2 2470 automatic gamma counter (Perkin Elmer, USA). Activity concentrations were computed as percent of decay-corrected injected activity per gram of tissue (%ID/g).

In-vivo imaging

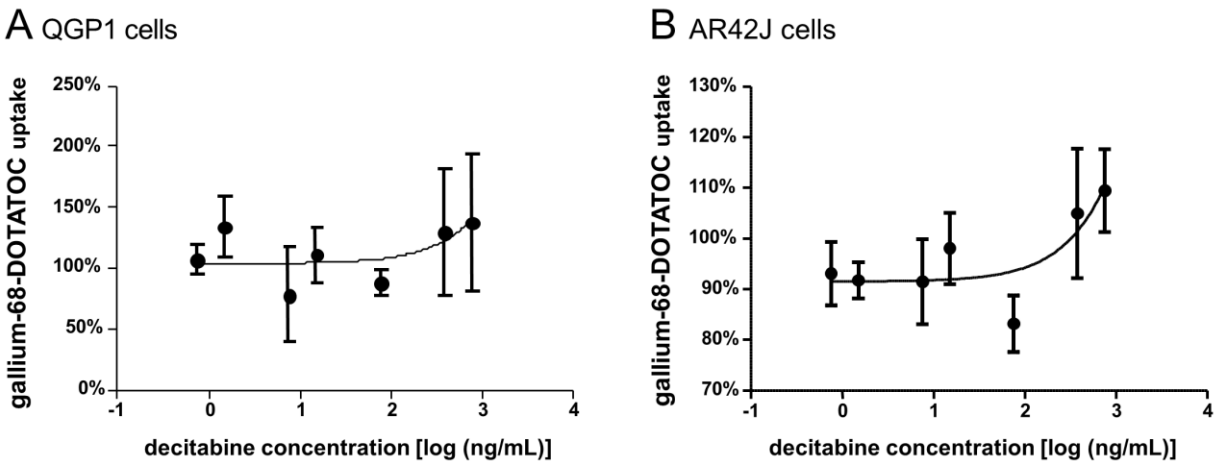
Six weeks old athymic Nude-Foxn1^{nu} mice were subcutaneously injected in the femoral region with 5x10^{6} BON-1 cells in 50 µL PBS. After the xenografts had reached approximately 5 mm diameter, subcutaneous injections with 50 µL of 0.2 mg/kg body weight decitabine or PBS were performed 3 times over a period of 9 days. Subsequently, imaging studies were performed 2 h after injection of 10 MBq of gallium-68-DOTATOC in 150 µl PBS on a positron emission tomography (PET)/computed tomography (CT) scanner (mCT 128, Siemens, DE). CT scanning was performed with 5 mm slice thickness, 2.5 mm increment, 120kV, 50mAs/slice and 0.75s rotation time. PET imaging was performed using gallium-68, was acquired with 3 iterations in 21 subsets, 200 matrix, 5.0 Gauss filter and was reconstructed with True X TOF algorithm. Siemens software was used to display PET and CT images. All animal experiments were conducted with respect to the Swiss animal experiments law, and were approved by the local authorities.

Supplemental Table 1. Screened Test Drugs and their Concentrations

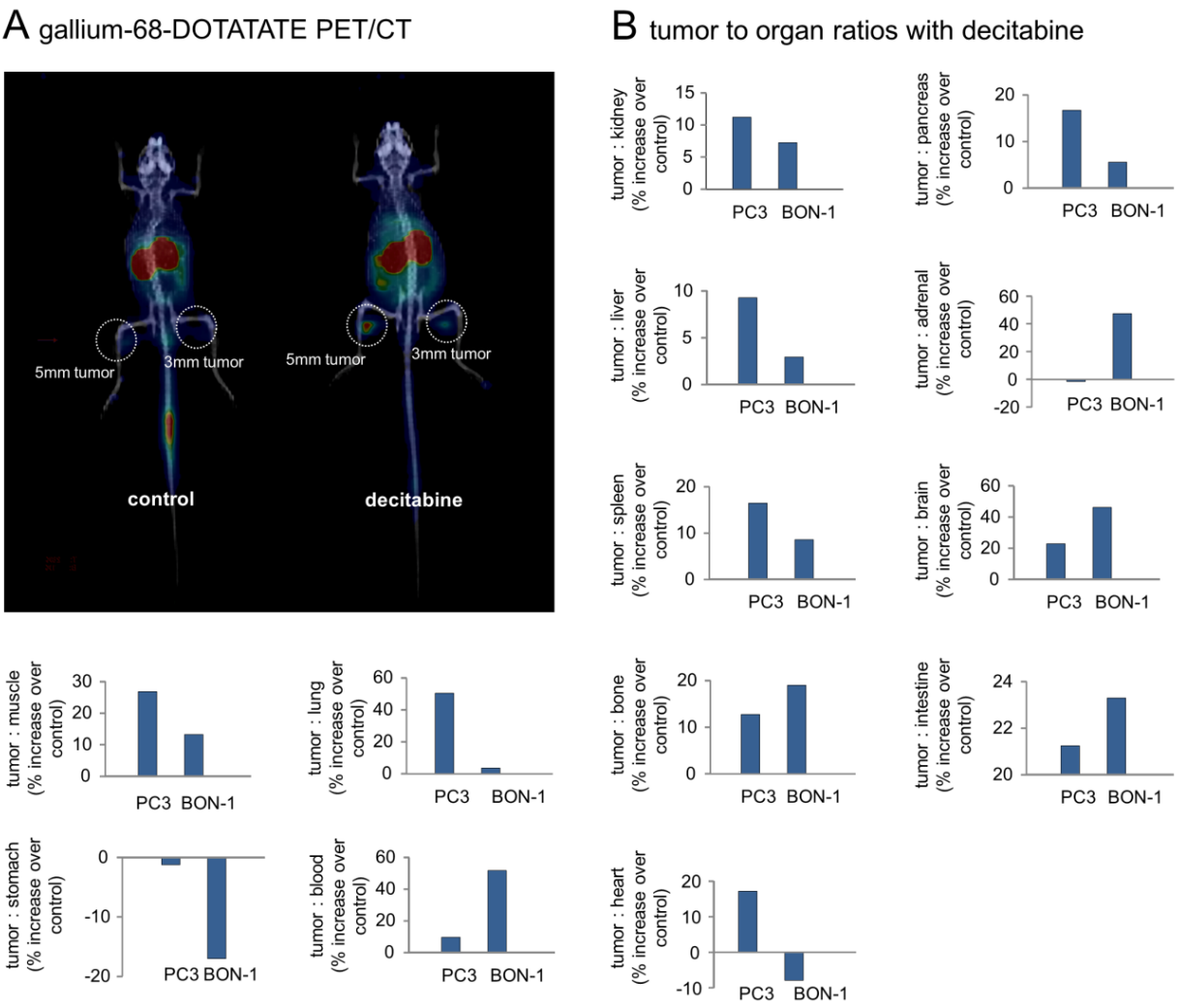
Test Drugs	Tested Concentration	Reference
Miscellaneous Test Drugs		
Testosterone	10 ng/mL	(*4*)
Transforming growth factor beta	5 ng/mL	(*5*)
Growth-hormone-releasing Hormone (GHRH)	50 pg/mL	(*6, 7*)
Dexamethasone	4 ng/mL	(*4*)
Jagged 1 peptide	0.1 mg/mL	(*4, 8, 9*)
17β-Estradiol	50 pg/mL	(*10-14*)
Forskolin	40 ng/mL	(*6, 15*)
Phenethyl Isothiocyanate (PEITC)	163 ng/mL	(*16-18*)
Somatostatin 14	25 pg/mL	(*19, 20*)
Tamoxifen	500 ng/mL	(*16, 17, 21*)
DNMT-Inhibitors		
Azacitidine/Vidaza	75 ng/mL	(*8*)
Decitabine/Dacogen	75 ng/mL	(*8*)
HDAC-Inhibitors		
Scriptaid	500 ng/mL	(*6, 8*)
Dacinostat	1 ng/mL	(*8*)
Trichostatin A	50 ng/mL	(*8*)
Panobinostat	400 pg/mL	(*8*)
Vorinostat	250 ng/mL	(*8*)
Phenylbutyrate	100 µg/mL	(*8*)
Valproic Acid	100 µg/mL	(*8, 9*)
Romidepsin	160 pg/mL	(*8*)
Tacedinaline	500 ng/mL	(*8*)



Supplemental Figure 1. Test drugs. The chemical structures of the DNMT inhibitor decitabine (A) and the HDAC inhibitors tacedinaline (B) and romidepsin (C) used for up-regulating $sstr_2$.



Supplemental Figure 2. Uptake assay in QGP1 and AR42J cells. Dose-dependent effects of decitabine on uptake of the $sstr_2$ ligand gallium-68-DOTATOC in QGP and AR42J cells. No significant effect of decitabine has been observed in these two cell lines.



Supplemental Figure 3. Positron emission tomography & Biodistribution experiments. (A) Positron emission tomography with the $sstr_2$ ligand gallium-68-DOTATOC with and without decitabine-based $sstr_2$ up-regulation. (B) Change of tumor-to-organ ratios in gallium-68-DOTATOC biodistribution after decitabine treatment in PC3 and BON-1 tumors, representing the key factor for imaging contrast in $sstr_2$ targeted imaging or targeted radiopeptide therapy. Gallium-68-DOTATOC biodistribution in not treated PC3 and BON-1 cells is set as baseline. (BON-1, n = 21; PC3, n = 30).

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