Evans Blue Attachment Enhances Somatostatin Receptor Subtype-2 Imaging and Radiotherapy

Rui Tian¹², Orit Jacobson²*, Gang Niu², Dale O. Kiesewetter², Zhantong Wang², Guizhi Zhu², Ying Ma², Gang Liu¹ and Xiaoyuan Chen²*

1. State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics & Center for Molecular Imaging and Translational Medicine, School of Public Health, Xiamen University, Xiamen 361102 China.
2. Laboratory of Molecular Imaging and Nanomedicine, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, Maryland, USA.

* Corresponding authors: Orit Jacobson (orit.jacobsonweiss@nih.gov); Xiaoyuan Chen (shawn.chen@nih.gov)
Supplementary Information

General

Synthesis and characterization of DOTA-Maleimide-EB (EB) derivative was previously reported by us (1). TATE peptide containing Dde protecting group, TATE(Dde), was purchased from CSBio Company, Inc. (Menlo Park, CA). S-Tritylmercaptoacetic acid NHS ester was synthesized following the published procedure (2). DOTA-TATE was synthesized using a minor modification of the published procedure (1, 3). Analytical high-performance liquid chromatography (HPLC) used Phenomenex Luna C8 column (5 μm, 4.60×150 mm) with a gradient system starting from 95% solvent A (50mM NH₄OAc, pH 6.5) and 5% solvent B (CH₃CN) and changing to 65% solvent B at 35 min at flow rate of 1 mL/min. The ultraviolet absorbance was monitored at 254 and 600 nm. Compounds were purified on Higgins column (C-18, 5μm, 250 x 20 mm) using gradient system as described above except changes in the solvents [solvent A: 0.1% trifluoroacetic acid (TFA)/H₂O, solvent B: 0.1% TFA/CH₃CN] and flow rate of 12 mL/min. LC-MS analysis utilized analogous procedures to those reported (1).

Synthesis of thiolated TATE (TATE-SH)

TATE(Dde) (1 eq.) was dissolved in 1 mL dimethylformamide (DMF). Then S-Tritylmercaptoacetic acid NHS ester (1.1 eq.) was added, followed by addition of 5 eq. of diisopropylethylamine (DIPEA). The reaction was mixed for 4-5 h at room temperature until full conversion into protected TATE was observed by HPLC analysis. Then 2% (w/v) of Hydrazine was added and the reaction mixture stirred for additional 0.5 h. Lysine deprotection
was confirmed by LCMS and the crude mixture was purified using HPLC. The peptide was collected and lyophilized overnight. Deprotection of S-trityl from TATE employed TFA:H₂O:1,2-ethanedithiol (90:5:5) at room temperature for 1 h to give TATE-SH. LC-MS analysis confirmed mass of 1119 [M-H]⁻. The crude mixture was purified on HPLC to give TATE-SH in a chemical purity >95% and a chemical yield of 38-40%.

**Synthesis of EB-TATE**

TATE-SH (1 eq) was dissolved in 10% (v/v) Dimethyl sulfoxide (DMSO) and 90% of 0.1% (w/v) Na-ascorbate in PBS and mixed with 1 eq of EB derivative dissolved in 0.1% Na-ascorbate (w/v) in PBS to a total volume of 1-2 mL. The reaction was stirred for 2 h at room temperature and purified by HPLC to give EB-TATE in a chemical purity >95% and a yield of 65-70%. LC-MS analysis confirmed mass of 2328 [M-H]⁻.
Radiochemistry

\(^{86}\text{Y}\) was purchased from University of Wisconsin and NIH Cyclotron Facility. \(^{90}\text{Y}\) was purchased from Perkin-Elmer. 10 \(\mu\text{L}\) of \(^{86}\text{YCl}_3\) (37 MBq) and/or \(^{90}\text{YCl}_3\) (111 MBq) were diluted with 0.5 \(\text{mL}\) 0.4 M ammonium acetate pH 5.6. Then EB-TATE or DOTA-TATE (40 nmol in 10-20 \(\mu\text{L}\)) was added and the reaction mixed for 30 min at 37\(^\circ\text{C}\). Purity of the products was assayed by radio-TLC (AR-2000 Bioscan scanner), using iTLC plates (Fisher) and 0.1 M Citric acid pH 5 as the developing solvent. \(R_f\) of free \(^{86/90}\text{Y} \sim 0.9\); \(R_f\) of \(^{86/90}\text{Y-EB-TATE}\) and TATE \~ 0.1. The compounds were labeled in radiochemical purity \(> 95\%\) and specific activity ranging from 6.6-6.8 mCi/\(\mu\text{mol}\) for \(^{86}\text{Y-EB-TATE/TATE}\) to 140-150 mCi/\(\mu\text{mol}\) for \(^{90}\text{Y-EB-TATE/TATE}\). Synthesis of \(^{64}\text{Cu-DOTA-TATE}\) was done in the same manner. Synthesis of \(^{68}\text{Ga-DOTA-TATE}\) was conducted according to the published procedure (3).

Albumin binding assay

\textit{In vitro} albumin binding affinity of EB-TATE was evaluated by competitive study against \(^{86}\text{Y-EB-TATE}\) using streptavidin agarose beads (Thermo Fisher Scientific) and biotinylated human serum albumin (Protein Mods). 200 \(\mu\text{L}\) of beads slurry were washed five times with PBS by centrifuge and then resuspend in PBS and mixed with 600 \(\mu\text{L}\) biotinylated-HSA (1 mg/mL, 3-molar excess) for 1 h at room temperature. Then the mixture was extensively washed with PBS to remove excess of biotinylated-HSA. Next, the labeled beads were incubated with radiolabeled 7.4 KBq \(^{86}\text{Y-EB-TATE}\) and increasing concentrations (1 nM-100 \(\mu\text{M}\)) of unlabeled EB-TATE for 1.5 h at room temperature in a 96-well membrane plate. After the incubation, the plate was washed several times with PBS and the radioactivity bound to the
beads was measured using a gamma-counter. Each time point was measured in triplicate.

**90Y-EB-TATE Biodistribution**

1.85 MBq of 90Y-EB-TATE was intravenous injected to HCT116/SSTR2 tumor bearing mice. At days 1 (n = 5), 4 (n = 6), 7 (n = 6) and 12 (n = 7) post-injection, mice were sacrificed and tumor, heart, lung, liver, spleen, intestine, kidney, muscle, bone and blood were collected. The tissues were minced, weighed and dissolve in 1-1.5 mL of SOLVABLE (Perkin-Elmer) in 20 mL scintillation vials. The vials were heated to 50-55 °C for 3 h with agitation every 30 min and then left at room temperature for 24 h. After tissue dissolution, 200 µL of 30% H2O2 were added and the mixture, incubated at 50-55 °C for 1 h. After cooling, 16 mL of scintillation cocktail (ULTIMA Gold, Perkin-Elmer) were added to each vial. Radioactivity was measured by a beta counter (WinSpectral, Perkin-Elmer) and the results were calculated as percent injected dose per gram tissue (%ID/g).

**In vivo stability**

Mice bearing HCT116/SSTR2 tumors were each injected with 1.85 MBq of 90Y-EB-TATE, tumors were collected at different time points post-injection, minced, and dissolved in 50 µL of CH3CN:CH3OH (1:1). The slurry solution was centrifuge for 1-2 min at 10,000 rpm. An aliquot was taken and analyzed by iTLC plate, as described in the radiochemistry methodology section above.
Figure Legends

Supplementary Fig. S1. Representative immunofluorescence staining (X20) of anti-SSTR2 (green) and nucleus (DAPI, blue) in tumor tissues from HCT116/SSTR2, HCT116 and AR42J xenografts.

Supplementary Fig. S2. (A) Cell binding affinity assays of TATE vs. EB-TATE in AR42J cells. IC_{50} of TATE (red curve): 3.46 ± 0.88 nM, and IC_{50} of EB-TATE (blue curve): 9.16 ± 0.09 nM. Results are shown as average ± SD of triplicates. (B) Uptake and internalization of ^{86}Y-EB-TATE and ^{86}Y-TATE in AR42J (SSTR2^+) cells in the presence of 1% (w/v) of human serum albumin. Blocking studies were evaluated at 24 h. Results are shown as average ± SD of triplicates. ***P < 0.001. (C) Binding affinity assay of ^{86}Y-EB-TATE and biotinylated-albumin using streptavidin agarose beads. IC_{50} of EB-TATE 1.92 ± 0.34 µM. Results are shown as average ± SD of triplicates.

Supplementary Fig. S3. (A) Time-activity curves of ^{86}Y-EB-TATE and ^{86}Y-TATE in different organs over time, calculated from PET quantification. (B) Biodistribution of ^{86}Y-EB-TATE and ^{86}Y-TATE in HCT116/SSTR2 and HCT116 xenografts at 48 h post injection.

Supplementary Fig. S4. (A) Uptake of ^{86}Y-EB-TATE in AR42J tumor model with and without co-injection of EB-TATE excess (block) over time. Results are presented as %ID/g mean and max values. (B) Biodistribution of ^{86}Y-EB-TATE in AR42J xenografts at 48h post injection with or without unlabeled EB-TATE. *p<0.05, **P < 0.01, ***P < 0.001.

Supplementary Fig. S5. Area under the curves (AUCs) of EB-TATE (blue) and TATE (orange) calculated from the radioactivity accumulation in the tumor over time.
**Supplementary Fig. S6.** Representative pictures of tumor-bearing mice before radionuclide therapy (A) during the treatment when the tumor is still visible (B) and at the end of the study, at day 180 when the tumor almost disappeared (C) or completely eradicated (D). Red arrows indicate tumor location. Enlargement of the tumor region before (blue) and after (green) treatment are shown in the lower panel.

**Supplementary Fig. S7.** $^{68}$Ga-DOTA-TATE PET imaging of HCT116/SSTR2 xenograft after radionuclide tumor therapy of (A) mice injected with saline and (B) mice injected 3.7 MBq $^{90}$Y-EB-TATE, about 80 days post initial treatment when the tumor started to re-grow. Images were acquired 60 min after injection of $^{68}$Ga-DOTA-TATE. White arrows indicate tumor location.

**Supplementary Fig. S8.** Representative immunofluorescence staining (X 20) of anti-SSTR2 (green) and nucleus (DAPI, blue) in mice bearing HCT116/SSTR2 tumor model after radionuclide tumor therapy (A) with saline and (B) with 3.7 MBq of $^{90}$Y-EB-TATE that was sacrificed at day 77 after tumor partial re-growth.

**Supplementary Fig. S9.** Representative immunofluorescent staining (X 20) of anti-SSTR2 (green) and nucleus (DAPI, blue), 1, 7 and 14 days after injection of saline (HCT116/SSTR2 tumor model, upper panel) or 7.4 MBq of $^{90}$Y-EB-TATE in HCT116 (middle panel) and HCT116/SSTR2 (lower panel) xenografts.

**Supplementary Fig. S10.** Body weight change of HCT116/SSTR2, HCT116 tumor bearing mice during the radiotherapy study. Red arrows indicate injection of the radiotherapeutic dose.

**Supplementary Fig S11.** Body weight change of AR42J tumor bearing mice during the radiotherapy study. Red arrow indicates injection of the radiotherapeutic dose.
Supplementary Fig. S12. Representative H&E staining of different organs from mice after radionuclide therapy (day 14).

Supplementary Fig. S13. Representative H&E staining of bone (A) and kidneys (B) from mice 3 to 4 months after radionuclide therapy.

Supplementary Fig. S14. Biodistribution of $^{90}$Y-EB-TATE in HCT116/SSTR2 xenografts at different time points post injection (n=5-7 for each time point).

Supplementary Fig. S15. (A) Representative radioTLC analysis for $^{90}$Y-EB-TATE and uncoordinated free $^{90}$Y using iTLC plate and 0.1 M citric acid at pH 5 as developing solvent. (B) Representative radioTLC analysis of extracts from HCT116/SSTR2 tumors as a function of time following injection of $^{90}$Y-EB-TATE.
Figure S1
Figure S2
Figure S3
Figure S4

Tumor (Mean)

**Tumor (Max)**

AR42J Block
AR42J No Block

Time post injection (h)

% ID /g

Time post injection (h)

% ID /g

AR42J Block
AR42J No Block

% ID /g

Tumor Heart Lung Liver Spleen Stomach Intestine Pancreas Kidneys Muscle Bone Blood

ns ns ns ns ns ns ns ns ns ns ns

*ns ns ns ns ns ns ns ns

Figure S4
Figure S5

HCT116/SSTR2
(SSTR2⁺)

AUC

Hours post injection

86Y-TATE
86Y-EB-TATE
Figure S6

HCT116/SSTR2
(SSTR2⁺)
Figure S7

Before treatment

After treatment

HCT116/SSTR2

68Ga-DOTA-TATE

68Ga-DOTA-TATE
Figure S8

A

DAPI          SSTR2          Merge

B

HCT116/SSTR2

Figure S8
Figure S9

Day 1  Day 7  Day 14

Saline
HCT116/SSTR2

HCT116

HCT116/SSTR2

50µm  50µm  50µm
50µm  50µm  50µm
50µm  50µm  50µm
Figure S10

Table: Body weight (g) over time for different groups and treatments:

- **HCT116/SSTR2 (SSTR2\(^+\)) Saline**
- **HCT116 (SSTR2\(^-\)) Saline**
- **HCT116/SSTR2 (SSTR2\(^+\)) \(^{90}\)Y-EB-TATE 7.4MBq**
- **HCT116/SSTR2 (SSTR2\(^+\)) \(^{90}\)Y-EB-TATE 3.7MBq**
- **HCT116/SSTR2 (SSTR2\(^+\)) \(^{90}\)Y-TATE 7.4MBq**
- **HCT116 (SSTR2\(^-\)) \(^{90}\)Y-EB-TATE 7.4MBq**

Legend:
- **One dose**

Start therapy markers are indicated before the respective time points.
Figure S11

Graph showing body weight (g) over time (Day) for different treatments:
- AR42J Saline
- AR42J \(^{90}\)Y-TATE 7.4 MBq
- AR42J \(^{90}\)Y-EB-TATE 3.7 MBq
- AR42J \(^{90}\)Y-EB-TATE 7.4 MBq

Start therapy marker is indicated on the graph.
Figure S12
Figure S13

A

Control

Bone marrow

7.4 MBq $^{90}$Y-EB-TATE (AR42J)

Bone marrow

B

Control

Blood vessel

Glomerular Capsule

7.4 MBq $^{90}$Y-EB-TATE (AR42J)
Figure S14
Figure S15
References

1. Chen H, Jacobson O, Niu G, et al. Novel "Add-On" Molecule Based on Evans Blue Confers Superior Pharmacokinetics and Transforms Drugs to Theranostic Agents. J Nucl Med 2017;58: 590-7.

2. Chen X, Li L, Liu F, Liu B. Synthesis and biological evaluation of technetium-99m-labeled deoxyglucose derivatives as imaging agents for tumor. Bioorg Med Chem Lett 2006;16: 5503-6.

3. Lang L, Li W, Guo N, et al. Comparison study of $[^{18}\text{F}]\text{FAI-NOTA-PRGD2}$, $[^{18}\text{F}]\text{FPPRGD2}$, and $[^{68}\text{Ga}]\text{Ga-NOTA-PRGD2}$ for PET imaging of U87MG tumors in mice. Bioconjug Chem 2011;22: 2415-22.