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A Putative Single-Photon Emission CT Imaging Tracer for Erythropoietin-Producing Hepatocellular A2 Receptor

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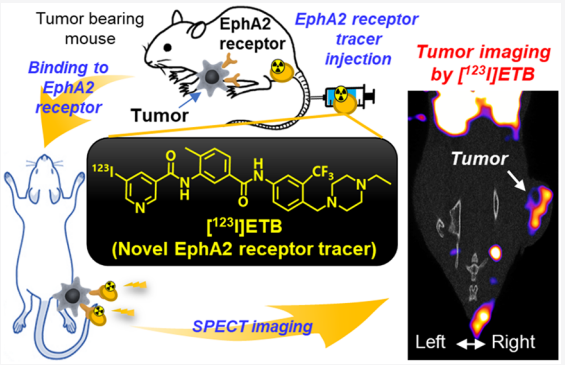
ABSTRACT: Erythropoietin-producing hepatocellular (Eph) receptors are receptor tyrosine kinases involved in cell−cell contact. The EphA2 receptor is associated with cancer proliferation and migration. Therefore, EphA2 receptor imaging has the potential for cancer diagnosis. Here, we synthesized *N*-(5-((4-((4-ethylpiperazin-1-yl)methyl)-3-(trifluoromethyl)-phenyl)carbamoyl)-2-methylphenyl)-5-[^{123}I]iodonicotinamide ([^{123}I]ETB) and evaluated it as an imaging tracer for single-photon emission computed tomography (SPECT) imaging of the EphA2 receptor. [^{123}I]ETB was designed on the basis of ALW-II-41-27, an inhibitor of EphA2 receptor kinase. Nonradioactive ETB was also synthesized and has been shown to efficiently inhibit EphA2 receptor kinase activity *in vitro* (IC_{50}: ETB, 90.2 ± 18.9 nM). A cell-binding assay demonstrated that [^{125}I]ETB binds specifically to the EphA2 receptor. The *ex vivo* biodistribution study of [^{125}I]ETB in U87MG tumor-bearing mice also revealed tumor uptake (2.2% ID/g at 240 min). In addition, [^{123}I]ETB uptake in tumors was visualized via SPECT/CT imaging. On the basis of the above, [^{123}I]ETB can be considered a potential SPECT imaging tracer for the EphA2 receptor.



KEYWORDS: *EphA2 receptor, SPECT imaging tracer, ALW-II-41-27, I-123, docking simulation*

Erythropoietin-producing hepatocellular (Eph) receptor, a family of receptor tyrosine kinases,[1] is divided into nine EphA and five EphB subgroups defined in the human genome.[2,3] Eph receptors and their ephrin ligands are bound to cell membranes, and their binding causes dimerization and subsequent activation.[4−7] On the basis of these mechanisms, it was hypothesized that the Eph receptor is related to migration and permeation of cancer cells. This study focused on the EphA2 receptor as it was determined to be particularly overexpressed in cancer cells than in normal cells. Furthermore, the EphA2 receptor has been reportedly expressed in various types of cancer, including breast and prostate cancer and malignant gliomas.[8−10] Additionally, cell migration was increased by introducing a vector of EphA2 receptor into a cancer cell line that does not express the EphA2 receptor.[11] Although our understanding of the EphA2 receptor has advanced, its detailed mechanisms are yet to be fully understood.[7,11,12] Therefore, a method for performing noninvasive measurement of the distribution and density of the EphA2 receptor in tumors could provide useful information for diagnosing cancer, which in turn can contribute to the development of therapeutic drugs. From the viewpoint of sensitivity and quantitativity, positron emission tomography (PET) and single-photon emission computed tomography (SPECT) are widely used as noninvasive imaging techniques. The first EphA2-targeted PET imaging tracer based on peptide has recently been reported by Mudd et al.,[13] and its potential usefulness as an imaging tracer has been discussed. Antibodies against EphA2 receptor that are labeled with Cu-64 and Zr-89 have been also proposed for PET.[14] We decided to develop a SPECT imaging tracer because of its high clinical versatility. Given the high number of SPECT instruments installed in medical facilities, development of SPECT imaging tracers is of great significance for clinical versatility. In addition, since a small molecule imaging tracer for EphA2 receptor has not yet been reported, we decided to develop an imaging tracer based on a small molecule. Herein, we report on an analogue of a well-established EphA2 receptor inhibitor that can be successfully labeled with I-123 and serve as a potential EphA2 receptor-specific SPECT imaging tracer. Several compounds have been reported as EphA2 receptor inhibitors (Figure 1).[15−17] Among these, we decided to use ALW-II-41-27 as a scaffold, as it was reported to bind to EphA2 receptor

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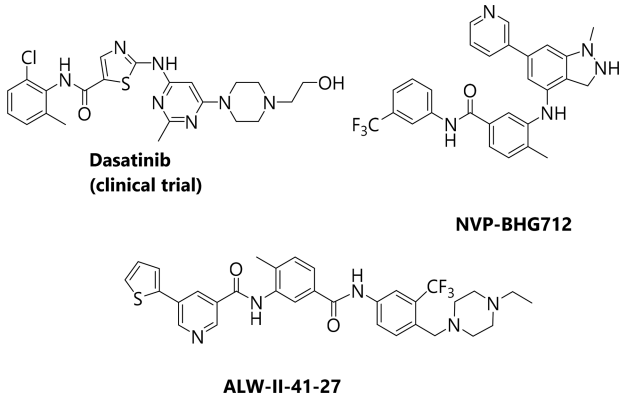

Figure 1. Chemical structures of previously reported EphA2 receptor inhibitors.

kinase with high affinity (IC_{50} = 11 nM) and also efficiently inhibit cancer progression.[15,18,19] We also decided on radioiodination as the radiolabeling method of choice, in order to achieve minor conformational changes to our highly potent scaffold. In this context, gamma-emitting I-123 was selected for the development of our SPECT imaging tracer candidate because of its superior qualities for SPECT imaging.

We used a computational scientific approach by means of Molecular Operating Environment (Chemical Computing Group, Montreal, Canada)[20] to choose the best-suited iodine position in candidate compounds based on ALW-II-41-27. Because its large size, iodine needs adequate space available in order to ensure binding of the final imaging tracer to EphA2 receptor. The computer simulation predicted that ALW-II-41-27 (Figure 2A) can bind to the ATP pocket of the EphA2 receptor with −8.74 kcal/mol stability (Figure 2C) and form hydrogen bonds with multiple amino acids at the vicinity (Figure 2E). We decided to substitute the thiophene group with iodine because of their comparable sizes (Figure S2) and dispensability for EphA2 receptor inhibition,[15] which led to the compound *N*-(5-((4-((4-ethylpiperazin-1-yl)methyl)-3-

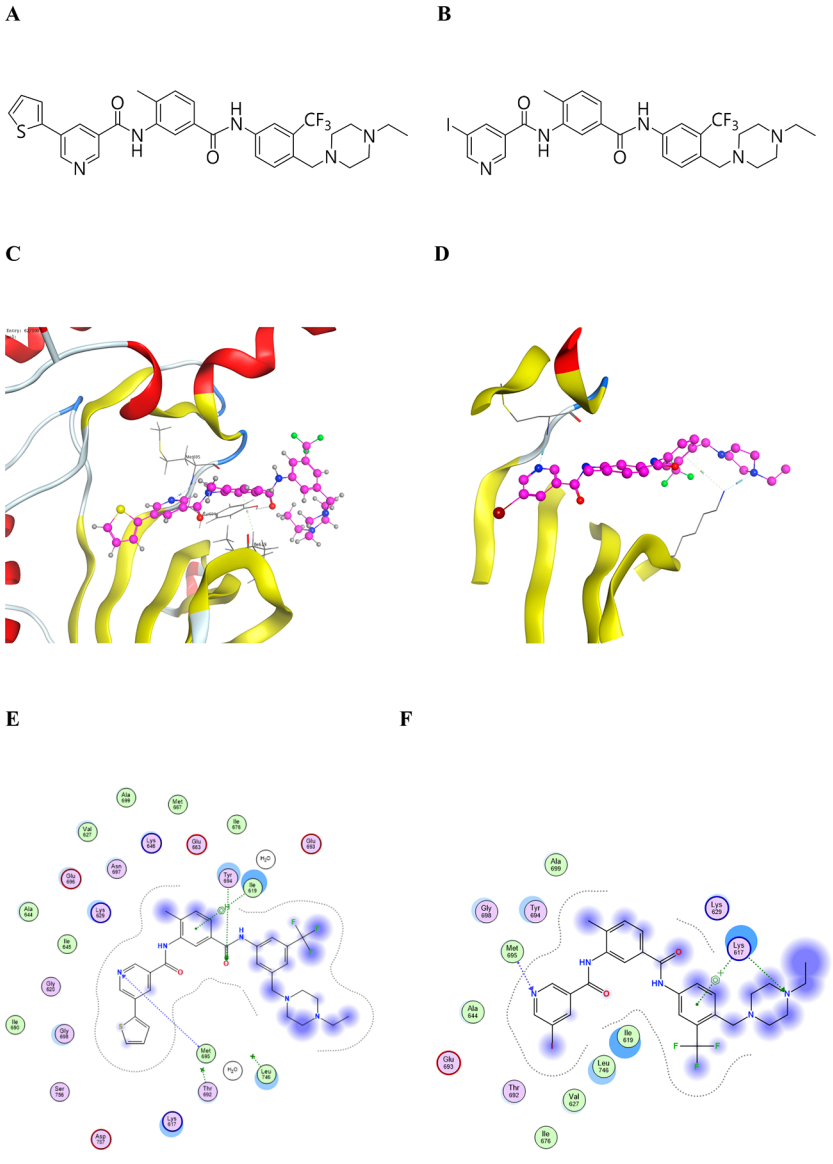

Figure 2. Chemical structures of inhibitors and 3D and 2D binding poses. (A, B) Chemical structures of ALW-II-41-27 and ETB. (C, D) Proposed binding mode of ALW-II-41-27 and ETB in complex with EphA2 receptor and (E, F) interaction of ALW-II-41-27 and ETB with amino acids.

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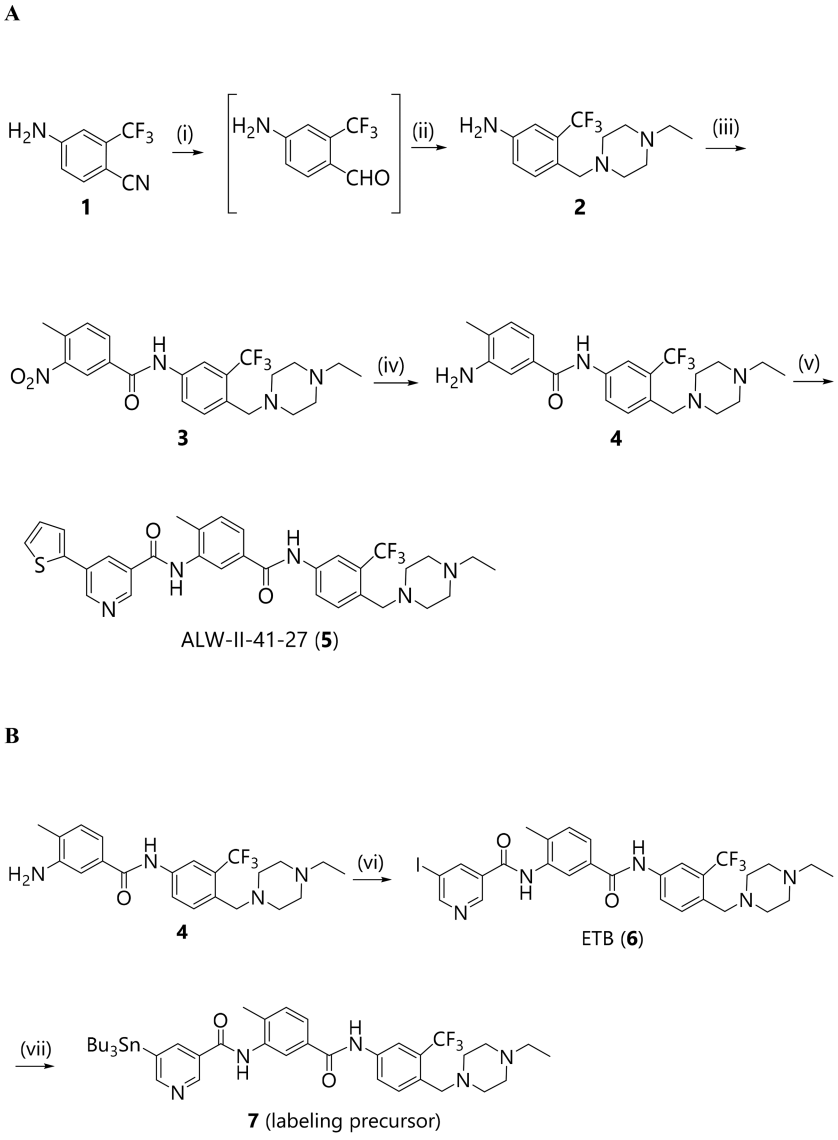

(i) DIBAL-H, THF; (ii) 1-ethylpiperazine, PPTS, toluene/AcOEt reflux, then $NaBH(OAc)_3$; (iii) 4-methyl-3-nitro benzoic acid, HATU, DMAP, DIPEA, CH_2Cl_2; (iv) H_2, Pd/C CH_2Cl_2; (v) 5-thiophen-2-yl nicotinic acid, HATU, DMAP, DIPEA, CH_2Cl_2; (vi) 5-iodo nicotinic acid, HATU, DMAP, DIPEA, CH_2Cl_2; (vii) $(PPh_3)_2Cl_2Pd$, Bis(tributyltin), 1,4-dioxane.

Figure 3. Synthetic schemes. (A) ALW-II-41-27 and (B) nonradioactive ETB and labeling precursor.

(trifluoromethyl)phenyl)carbamoyl)-2-methylphenyl)-5-iodo-nicotinamide (ETB) (Figure 2B). Computed simulation showed that ETB had a similar stability value (−8.14 kcal/mol) to that of ALW-II-41-27 for the EphA2 receptor (Figures 2D, 2F).

ALW-II-41-27 (reference standard) was synthesized using a previously reported method, as shown in Figure 3A.[21−23] The reduction of 4-amino-2-(trifluoromethyl)benzonitrile (**1**) with diisobutylaluminum hydride, followed by reductive amination with 1-ethylpiperazine, led to compound **2**. Aniline **2** was then reacted with 4-methyl-3-nitrobenzoic acid to obtain amide **3**. The nitro group of **3** was reduced to aniline **4**; subsequently, amidation with 5-thiophen-2-yl nicotinic acid led to ALW-II-41-27.

Nonradioactive ETB and tributyltin derivative **7** (labeling precursor) were synthesized as shown in Figure 3B. Aniline **4** was reacted with 5-iodo nicotinic acid to obtain nonradioactive ETB. Tributyltin derivative **7** was then obtained from ETB using Stille cross-coupling. ALW-II-41-27, nonradioactive ETB, and compound **7** were synthesized from compound **1** in 5.3%, 12.1%, and 9.6% yields, respectively.

The ability of the synthesized compounds to inhibit EphA2 receptor kinase activity was analyzed using an ADP-Glo kinase inhibition assay. As shown in Figure 4, nonradioactive ETB was observed to inhibit EphA2 receptor kinase activity to the same extent as ALW-II-41-27 (IC_{50}: ALW-II-41-27, 67.2 ± 18.9 nM; ETB, 90.2 ± 18.9 nM). These results validated our drug design using computational chemistry.

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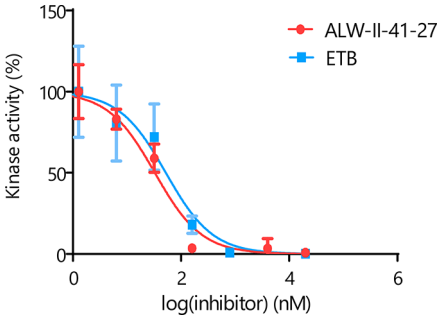

Figure 4. Dose-dependent inhibition of EphA2 receptor kinase using ALW-II-41-27 and ETB.

Radioiodinated [^{125}I]ETB was obtained by iododestannylation reaction with sodium [^{125}I]iodide and an oxidant, as per the methods described by Kimura et al. (Figure 5A).[24] Following purification with preparative HPLC, [^{125}I]ETB was obtained in 70.2 ± 1.8% (2.9−3.1 MBq) radiochemical yield and high radiochemical purity (>99%) (Figure 5B). Analytical HPLC indicated that the retention time of [^{125}I]ETB corresponded with that of nonradioactive ETB, as shown in Figure 5B. [^{123}I]ETB was then synthesized using sodium [^{123}I]iodide utilizing a similar method to [^{125}I]ETB. The radiochemical yield of [^{123}I]ETB was determined to be 30.1 ± 5.6% (25.6−59.1 MBq), while the radiochemical purity of [^{123}I]ETB was >99% (Figure S23). Given the longer half-life of I-125, I-125 was used for the *ex vivo* biodistribution studies instead of I-123.

The physical properties of [^{125}I]ETB were subsequently evaluated in order to verify suitability for use in *in vivo* testing. The octanol/water partition coefficient (log $D_{o/w}$) of [^{125}I]ETB was determined by measuring the distribution of the radiolabeled compound in 1-octanol and phosphate-buffered saline (pH 7.4). The log $D_{o/w}$ of [^{125}I]ETB was found to be 2.9 (Table S1), indicating moderate lipophilicity and solubility in saline. The stability of [^{123}I]ETB in mouse plasma was determined using radio-TLC analysis. [^{123}I]ETB was determined to be stable in mouse plasma at 37 °C for up to 4 h (94.5 ± 1.5%) (Figure S24).

For the *in vitro* and *ex vivo* evaluation of [^{125}I]ETB, the EphA2 receptor-positive U87MG cell line was used. Western blot analysis confirmed expression of the EphA2 receptor in U87MG cells and tumor xenograft tissues (Figure 6).

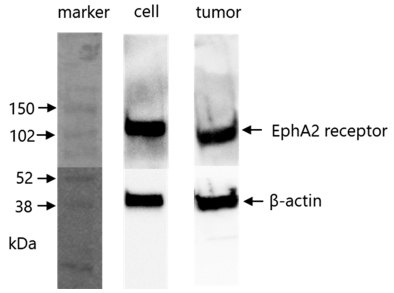

Figure 6. Expression of EphA2 receptor. EphA2 receptor expression in U87MG cells and tumor xenograft tissues. *β*-Actin was used as a loading control.

[^{125}I]ETB uptake in U87MG cells is demonstrated in Figure 7. The amount of cellular uptake of [^{125}I]ETB has been determined to increase with time (154.7 ± 3.9 cellular uptake ratio (CUR)%/mg protein at 60 min, 160.4 ± 19.7 CUR%/mg protein at 120 min). On the contrary, the uptake of [^{125}I]ETB significantly decreased with the addition of 0.1 μM of inhibitor ALW-II-41-27 (51.6% reduction at 60 min; 44.7% reduction at 120 min; $p < 0.05$). This result indicated that cellular uptake of [^{125}I]ETB is specific and EphA2-mediated. Because of its limited water solubility and toxic effect on cells,[18] the effective concentration of ALW-II-41-27 for receptor blockade was considered to be insufficient.

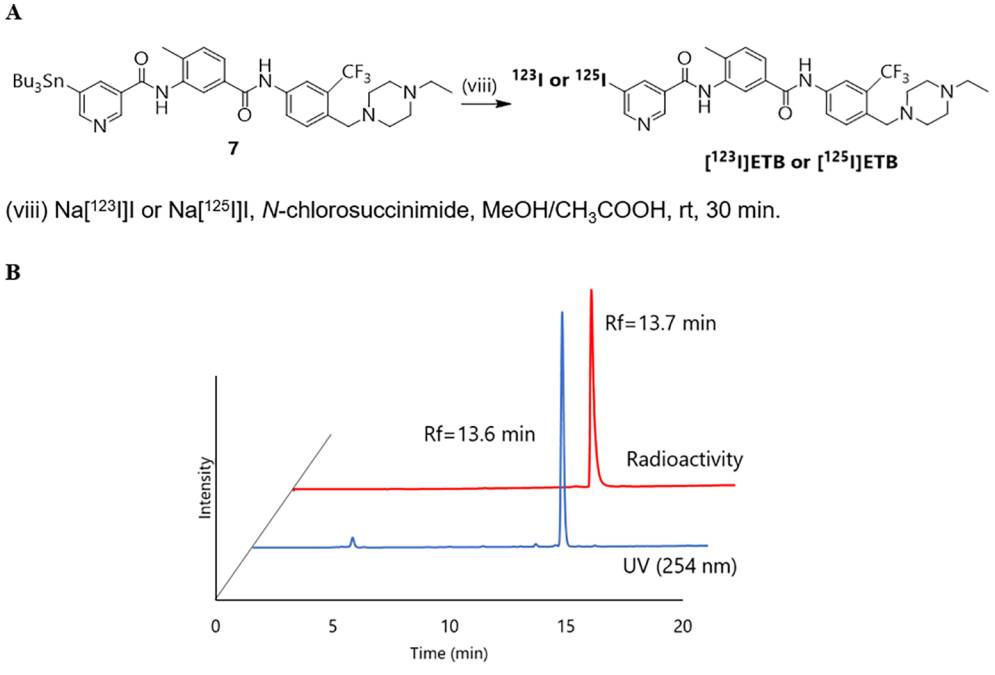

Figure 5. Radiosynthesis scheme and HPLC analysis. (A) Synthesis scheme of [^{123}I]ETB or [^{125}I]ETB and (B) HPLC analysis of nonradioactive ETB (UV) and [^{125}I]ETB (radioactivity).

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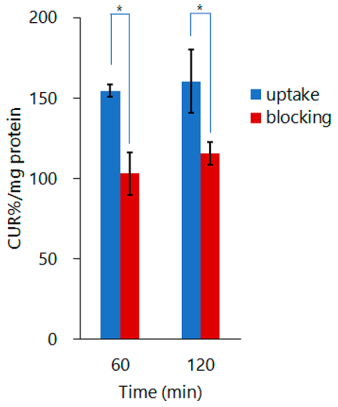

Figure 7. Cell-binding assay. [^{125}I]ETB CUR%/mg protein in U87MG cells. Data are represented as mean ± SD; n = 5. *: p < 0.05 by Student's t test.

We studied the *ex vivo* biodistribution of [^{125}I]ETB in U87MG tumor-bearing mice after intravenous injection of 37 kBq/100 μL [^{125}I]ETB. [^{125}I]ETB was administered to U87MG tumor-bearing mice without anesthesia using a mouse holder. The time-course accumulation of [^{125}I]ETB in the tissues of U87MG tumor-bearing mice is presented in Figure 8 as % injected dose per gram of tissue (% ID/g). The uptake of [^{125}I]ETB by the experimental tumors was evident, reaching 2.2 ± 0.3% ID/g at 240 min postinjection. [^{125}I]ETB was cleared rapidly from the blood, and a low level of radioactivity was observed in the heart and muscle. High renal accumulation was also observed (27.3% ID/g at 60 min postinjection), which decreased with time due to excretion (18.3% ID/g at 240 min postinjection). Furthermore, high accumulation and retention of [^{125}I]ETB in the liver was also confirmed, which can be attributed to the following reasons: (1) the compound is highly lipophilic and therefore easily taken up and retained by the liver; (2) the EphA2 receptor is highly expressed in the liver.[25] In addition to the liver, [^{125}I]ETB showed high accumulation and retention in the pancreas, in accordance with reports confirming EphA2 receptor expression in this tissue along with the lungs.[26,27] Radioactivity in the thyroid gland was found to be minimal at all time points tested (<0.1% ID), confirming radiochemical stability *in vivo* (absence of free radioiodide) and consistent with the stability results in mouse plasma. Table 1 showed the

Table 1. Tumor/Blood and Tumor/Muscle Ratios of [^{125}I]ETB in U87MG Tumor-Bearing Mice after Injection

	time after injection (min)			
	30	60	120	240
tumor/blood	5.6	5.6	8.4	11.7
tumor/muscle	1.4	1.8	2.6	4.2

tumor/blood and tumor/muscle ratios for each time point (tumor/blood = 11.7 at 240 min, tumor/muscle = 4.2 at 240 min; Table 1). Compared with the previously reported [^{89}Zr]Zr-deferoxamine-*p*-NCS-DS-8895a, [^{125}I]ETB was superior to [^{89}Zr]Zr-deferoxamine-*p*-NCS-DS-8895a in tumor/blood ratio at an early time point ([^{125}I]ETB:11.7 at 240 min, [^{89}Zr]Zr-deferoxamine-*p*-NCS-DS-8895a:3.62 at 7 day[14]).

SPECT/CT imaging was conducted in U87MG tumor-bearing mice. [^{123}I]ETB (33.62 MBq/140 μL) was administered intravenously to nonanesthetized U87MG tumor-bearing mice, and the mice were maintained in a nonanesthetized condition for 205 min after administration. A 5 min CT scan on an X-CUBE scanner (MOLECUBES, Ghent, Belgium) was initially performed for attenuation correction followed by a 60 min SPECT scan on γ-CUBE scanner (MOLECUBES, Ghent, Belgium) under anesthesia using isoflurane. As shown in Figure 9, the implanted U87MG tumors in the right limbs were clearly visualized in both dorsal and caudal images. Furthermore, in accordance with the *ex vivo* distribution data, a high accumulation of [^{123}I]ETB in the kidney was also observed.

In conclusion, we designed and synthesized an ALW-II-41-27 derivative that can be labeled with gamma-emitting I-123, [^{123}I]ETB, aiming toward the development of a potential SPECT imaging tracer targeting EphA2 receptors *in vivo*. Nonradioactive ETB is shown to inhibit EphA2 receptor kinase activity as efficiently as its parent ALW-II-41-27. The cell-binding assay performed in EphA2 receptor-positive U87MG cells, along with *in vitro* blocking with the parent ALW-II-41-27, demonstrated the specific binding of [^{125}I]ETB to the EphA2 receptor. *Ex vivo* biodistribution studies in U87MG tumor-bearing mice showed that [^{125}I]ETB is taken up by the tumor, while SPECT/CT imaging using [^{123}I]ETB in the same animal model confirmed *ex vivo* biodistribution data with a clear delineation of U87MG tumors at 205 min after injection. In view of the above, [^{123}I]ETB can be further investigated for

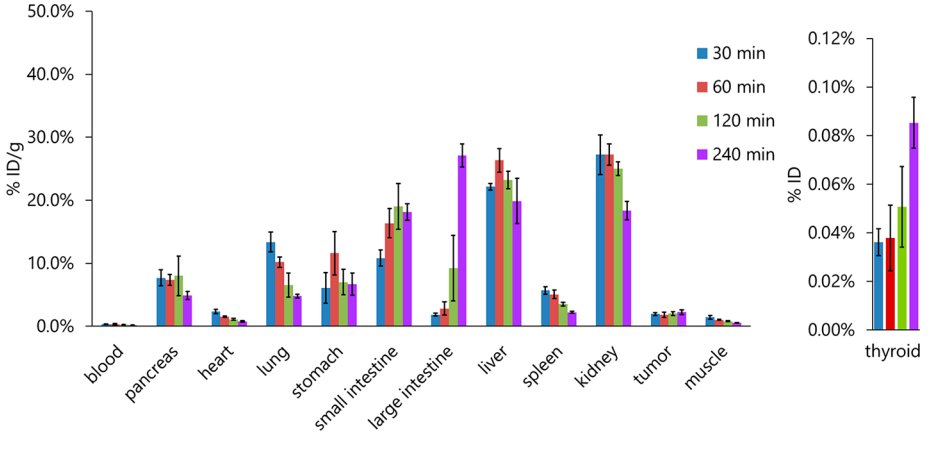

Figure 8. *Ex vivo* biodistribution data in U87MG tumor-bearing mice. *Ex vivo* biodistribution of [^{125}I]ETB in U87MG tumor-bearing mice at 30, 60, 120, and 240 min after injection. [^{125}I]ETB uptake in normal tissues and tumor. Data are represented as mean ± SD; n = 5.

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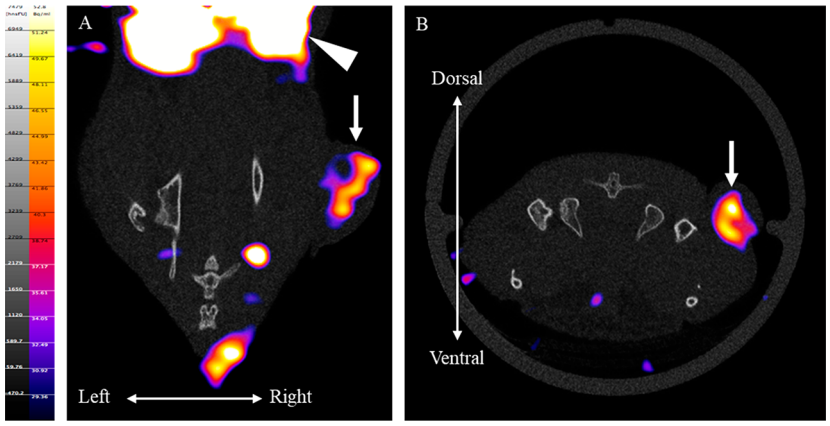

Figure 9. SPECT imaging of U87MG tumor-bearing mouse. Representative [^{123}I]ETB SPECT/CT imaging in tumor-bearing mouse at 205 min after injection: (A) dorsal view and (B) caudal view image. The tumor (arrow) and the kidney (arrowhead) are shown.

its potential as a SPECT imaging tracer for EphA2 receptor-rich malignancies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.1c00030.

Materials and methods, mass spectra, and NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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