Preclinical Evaluation of a Novel $^{99m}$Tc-Labeled CB86 for Rheumatoid Arthritis Imaging

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ABSTRACT: Early diagnosis and therapy are crucial to control disease progression optimally and achieve a good prognosis in rheumatoid arthritis (RA). Previous study showed that a technetium-$^{99m}$-labeled TSPO ligand ($^{99m}$Tc-CB256 [2-(5-(2-(bis(pyridin-2-yl)methyl)amino)acetamido)-2-(4-chlorophenyl)H-imidazo[1,2-a]pyridin-3-yl]-N,N-dipropylacetamide] composed of a translocator protein (TSPO) ligand CB86 [(2-(4-chlorophenyl)-8-amino-imidazo[1,2-a]pyridin-3-yl]-N,N-di-N-propylacetamide] and di-(2-picolyl)amine, a bifunctional chelate, was used to image a TSPO-rich cancer cell in vitro; however, few $^{99m}$Tc-CB256 in vivo evaluation has been reported so far probably due to the cytotoxicity of CB256 (ca. 75 times more than analogous CB86). Herein, we describe a novel TSPO targeting radiopharmaceutical consisting of CB86 and diethylenetriaminepentaacetic acid (DTPA), a conventional bifunctional chelating ligand in clinical trials used to prepare $^{99m}$Tc-labeled CB86, and its evaluation as a $^{99m}$Tc-single-photon emission computed tomography (SPECT) probe. The radiosynthesis and characterization of $^{99m}$Tc-DPTA-CB86 including hydrophilicity and stability tests were determined. Additionally, the binding affinity and specificity of $^{99m}$Tc-DTPA-CB86 to TSPO were evaluated using RAW264.7 macrophage cells. Biodistribution and $^{99m}$Tc-SPECT studies were conducted on rheumatoid arthritis (RA) rat models after the injection of $^{99m}$Tc-DTPA-CB86 with or without co-injection of unlabeled DTPA-CB86. The radiosynthesis of $^{99m}$Tc-DTPA-CB86 was completed successfully with the labeling yields and radiochemical purity of 95.86 ± 2.45 and 97.45 ± 0.69%, respectively. The probe displayed good stability in vitro and binding specificity to RAW264.7 macrophage cells. In the biodistribution studies, $^{99m}$Tc-DTPA-CB86 exhibited rapid inflammatory ankle accumulation. At 180 min after administration, $^{99m}$Tc-DTPA-CB86 uptakes of the left inflammatory ankle were 2.35 ± 0.10 percentage of the injected radioactivity per gram of tissue (% ID/g), significantly higher than those of the normal tissues. $^{99m}$Tc-SPECT imaging studies revealed that $^{99m}$Tc-DTPA-CB86 could clearly identify the left inflammatory ankle with good contrast at 30–180 min after injection. Therefore, $^{99m}$Tc-DTPA-CB86 may be a promising probe for arthritis $^{99m}$Tc-SPECT imaging.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by synovial inflammation that results in joint destruction and loss of function and quality of life. Clinical studies have shown that immunological and inflammatory processes resulting in joint destruction have already been set off at the very beginning of RA. Thus, it seems reasonable that therapeutic intervention should start as soon as the diagnostic has been established, with the aim of stopping inflammation before irreversible damage is caused. Currently, clinical diagnosis of RA is based on the 2010 classification criteria proposed by the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR). These criteria include laboratory tests (C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF); and anticitrullinated protein antibodies (ACCP)) and conventional imaging techniques (plain radiography, ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI)). However, they still are not very sensitive and specific to RA. Therefore, the development of noninvasive and highly sensitive and specific tests/imaging techniques is essential for very early detection of RA.

Activated macrophages play key roles in the pathogenesis of RA since they secrete proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and...
interleukin-6 (IL-6), which contribute to synovial inflammation in the early stages of RA and then bone erosion.7 The increase in the number of macrophages in the synovium is an early hallmark of active rheumatic disease.8 So, a specific tracer of such a process would be more specific and possibly also enable earlier detection of RA.

Under normal physiological conditions, translocator protein (TSPO, 18 kDa) levels in macrophages are very low, but a strong increase in TSPO levels occurs in an activated state of macrophages in response to inflammation.9 Hence, TSPO is considered a promising biomarker for inflammatory diseases.10 Previous studies showed that positron emission tomography (PET) imaging based TSPO ligands, such as 11C-(R)-PK11195, 11C-DPA-713, and 18F-DPA-714, can visualize RA.11−13 Although PET has higher resolution and sensitivity, single-photon emission computed tomography (SPECT) holds several advantages over PET including lower cost, more widespread availability, and favorable physical and imaging characteristics (γ ray = 140 keV, half-life = 6.02 h). In addition, the preparation of technetium-99m (99mTc)-labeled tracers is efficient, reproducible, and simple, making its clinical use easy. A 99mTc-labeled TSPO ligand [99mTc-CB256 [2-[(pyridin-2-yl)methyl]amino]acetamido]-2-(4-chlorophenyl)H-imidazo[1,2-a]pyridin-3-yl]-N,N-dipropylacetamide] composed of CB86 [2-(4-chlorophenyl)-8-amino-imidazo[1,2-a]-pyridin-3-yl]-N,N-di-n-propylacetamide and di-(2-picolyl)-amine, a bifunctional chelate agent, was reported to image a TSPO-rich cancer cell in vitro;14,15 however, the cytotoxicity of CB256 is 75 times more than that of analogous CB86 probably due to the cytotoxicity of di-(2-picolyl)amine.14 Therefore, in the present study, diethylenetriaminepentaacetic acid (DTPA), a conventional bifunctional chelating ligand in clinical trials, is used to develop 99mTc-DTPA-CB86 (Figure 1) as a novel 99mTc-SPECT probe for the imaging of rheumatoid arthritis.

**RESULTS AND DISCUSSION**

**Characterizations of CB86 and DTPA-CB86.** PK11195 is a classic ligand of TSPO. Due to the low in vivo specific binding of 11C-PK11195, recent efforts have focused on identifying novel compounds that selectively bind to TSPO with high affinity in an effort to improve the delineation of in vivo specific binding.16 In this regard, many new classes of compounds have been identified that have members that bind...
TSPO specifically with low nanomolar or sub-nanomolar affinity. Among these novel compounds, CB86 has been shown to have a higher affinity (IC$_{50}$ = 1.6 nM) compared to PK11195 (IC$_{50}$ = 2.2 nM). Moreover, DTPA is known as an efficient chelating agent and has been used as an MRI contrast agent as well as a radiopharmaceutical in clinical practices.

The chelator DTPA was attached to the CB86 compound via the chemical reaction between DTPA anhydride and CB86-functionalized amine under mild conditions of pH and temperatures. The retention times of CB86 and DTPA-CB86 on analytical high-performance liquid chromatography (HPLC) were found to be 15.31 and 24.72 min, respectively. The measured molecular weights (MWs) of CB86 and DTPA-CB86 on matrix-assisted laser desorption ionization−time-of-flight mass spectrometry (MALDI-TOF-MS) were 384.53 and 759.03, respectively (calculated MW = 384.17 and 759.30, respectively). The MW for its construct was consistent with the expected MW. The chemical characterization of DTPA-CB86 is shown in the Supporting Information (Figures S1−S3).

Fluorescence Imaging of CB86 in the Activated RAW264.7 Cells. To determine CB86 binding to the TSPO receptor, we prepared coumarin-CB86. As shown in Figure 2, the activated RAW264.7 macrophage cells were stained with MitoRed, a well-established mitochondrial dye, and subjected to confocal fluorescence microscopy. Coumarin-CB86 was found to be localized to mitochondria (Figure 2A), whereas it was barely observed in the presence of CB86 (Figure 2B). These results indicated that CB86 could bind well with the TSPO receptor on the surface of the mitochondrion in the activated RAW264.7 cells.

In Vitro Cytotoxicity of CB86 and DTPA-CB86. In vitro cytotoxicity of CB86 and DTPA-CB86 was determined using the MTT assay in RAW264.7 and 4T1 breast cancer cells (with low TSPO expression). The cells were incubated with different concentrations (0, 1.25, 2.5, 5, 10, 20 μM in Dulbecco’s modified Eagle’s medium (DMEM)) of CB86 and DTPA-CB86 for 24 h, respectively. As shown in Figure 3, the cell survival rates of RAW264.7 and 4T1 cells were not significantly different (P > 0.05) between the groups of CB86 and DTPA-CB86. The cell survival rates were >90% even in the concentration of 20 μM CB86 and DTPA-CB86, indicating that CB86 and DTPA-CB86 were safe to the RAW264.7 and 4T1 cells at the test concentrations. Denora et al. and Choi et al. reported that based on CB86, a new TSPO selective ligand CB256 with di-(2-picolyl)amine could be used to complex the tricarbonyl technetium-99m (99mTc-(CO)$_3$) unit for imaging a TSPO-rich cancer cell in vitro. However, few 99mTc-CB256 in vivo evaluation has been reported so far probably due to the cytotoxicity of CB256.

Radiosynthesis of 99mTc-DTPA-CB86 and log P Determination. The radiolabeling method of DTPA-CB86 was performed as described in our previous methods. Under radio-HPLC, 99mTc-DTPA-CB86 showed a retention time of 25.7 min. The radiolabeling efficiency, radiochemical purity, and specific activity of 99mTc-DTPA-CB86 were 95.86 ± 2.45%, 97.45 ± 0.69%, and 2.6 MBq/nmol, respectively. The lipid−water partition coefficient (log P) of 99mTc-DTPA-CB86 is −1.22 ± 0.04, suggesting that 99mTc-DTPA-CB86 is a water-soluble compound.

In Vitro Stability Analysis. In vitro stability studies showed that more than 90% of 99mTc-DTPA-CB86 remained intact during 1−4 h of incubation in the phosphate-buffered saline (PBS) (Figure 4) or mouse serum (Figure S5), indicating that 99mTc-DTPA-CB86 maintained excellently stability in the PBS or mouse serum.
In *V*tro Cell assays. Cell uptake ratios of $^{99m}$Tc-DTPA-CB86 are shown in Figure 6A. $^{99m}$Tc-DTPA-CB86 accumulated in RAW264.7 cells and reached the highest value of 36.45 ± 2.18% of applied activity at 180 min. When the probe was incubated with large excesses of nonradioactive DTPA-CB86, its uptake levels in RAW264.7 cells were significantly inhibited ($P < 0.05$) at all incubation time points. Moreover, the binding affinity of $^{99m}$Tc-DTPA-CB86 to TSPO was determined through the receptor saturation assay. As shown in Figure 6B, the IC$_{50}$ value of $^{99m}$Tc-DTPA-CB86 was 0.49 nM. Additional, cell efflux studies (Figure 6C) indicated that $^{99m}$Tc-DTPA-CB86 has good cell retention in RAW264.7 cells, with only about 13.99% (decreased from 33.31 ± 2.34 to 19.32 ± 2.01% of the total input radioactivity) of $^{99m}$Tc-DTPA-CB86 efflux observed from 4.5 to 8 h incubation. Overall, these results strongly suggested that labeling did not influence the ability of CB86 to bind specifically to TSPO. These results also warranted the further evaluation of the probe for *in vivo* TSPO-targeted imaging.

**Biodistribution Study.** At 30, 90, and 180 min after administration, the biodistribution profiles of $^{99m}$Tc-DTPA-CB86 are presented in Figure 7. $^{99m}$Tc-DTPA-CB86 exhibited high levels of radioactivity accumulation in the left inflammatory ankle. At 30 min, $^{99m}$Tc-DTPA-CB86 uptakes of the left inflammatory ankle were 1.33 ± 0.16 percentage of the injected radioactivity per gram of tissue (% ID/g), lower than those of the liver (5.56 ± 0.76% ID/g), intestine (3.48 ± 0.59% ID/g), stomach (2.76 ± 0.36% ID/g), lungs (1.51 ± 0.14% ID/g), and blood (1.49 ± 0.17% ID/g). However, the radioactivity uptakes of the left inflammatory ankle increased over time, whereas normal tissues decreased rapidly. $^{99m}$Tc-DTPA-CB86 exhibited rapid inflammatory ankle accumulation and blood clearance, which are the major advantages of using small molecules as imaging agents compared to large long-circulating proteins such as full antibodies or antibody fragments. At 90 min, $^{99m}$Tc-DTPA-CB86 uptakes of the left inflammatory ankle were 2.01 ± 0.18% ID/g and those of the liver were 3.82 ± 0.97% ID/g and of intestine was 2.83 ± 0.63% ID/g. At 180 min, the radioactivity uptakes of the left inflammatory ankle were 2.35 ± 0.10% ID/g, significantly higher than those of the normal tissues, including the liver (2.14 ± 0.23% ID/g). Lower levels of radioactivity were always observed in muscle and bone during 30−180 min postinjection (such as 1.01 ± 0.12 and 0.51 ± 0.16% ID/g at 30 min postinjection, respectively). The kidney and liver showed the highest $^{99m}$Tc-DTPA-CB86 uptake because they are the major organs of metabolism. In agreement with the previous study, radioactivity was found in the lung, heart, intestine, and stomach since these normal organs have moderate TSPO expression. Furthermore, $^{99m}$Tc-DTPA-CB86 provided a high ratio of the left inflammatory ankle to muscle (LIA/M) and left inflammatory ankle to blood (LIA/B) (Figure 7). At 30 min, the ratios of LIA/M and LIA/B were 1.32 ± 0.13 and 1.71 ± 0.16, respectively. Moreover, during 90 to 180 min, the ratios of LIA/M and LIA/B increased gradually over time. It is also interesting to find out that the inflammatory uptake of the
Figure 8. SPECT/CT imaging of 99mTc-DTPA-CB86 in RA rat models co-injected with 0 μg dose (unblock, A) and 300 μg dose (block, B) of DTPA-CB86 at 30, 90, and 180 min after injection (n = 4 for each group).

99mTc-DTPA-CB86 and inflammatory-to-muscle ratio are higher than those of the 18F-DPA-714,11C-DPA-713, and (R)-11C-PK11195.1,12

A high expression of the target in a normal organ might appreciably influence the imaging results, especially when the target level in the lesion is low. After the optimization of spiking doses was administered to saturate the target expression in normal organ, an increase lesion–normal ratio could be achieved.11,22 For in vivo blocking study (Figure 7), 99mTc-DTPA-CB86 was co-injected with a large excess (300 μg) of unlabeled DTPA-CB86 to saturate endogenous and overexpressed TSPO. The co-injection of DTPA-CB86 reduces the uptake of 99mTc-DTPA-CB86 in several tissues including liver, lung, heart, intestine, left inflammatory ankle, etc., indicating that there is a significant difference between blocking and unblocking group in these tissues (P < 0.05), whereas the kidney, muscle, and bone uptakes are not significantly different between the blocking and unblocking group (P > 0.05). 99mTc-DTPA-CB86 provided high ratios of the left inflammatory ankle to muscle (LIA/M) and the left inflammatory ankle to blood (LIA/B) (Figure 7).

SPECT/CT imaging. The biodistribution results indicated that 99mTc-DTPA-CB86 showed good in vivo pharmacokinetics for TSPO-targeted SPECT. Small animal SPECT/CT images acquired at 30, 90, and 180 min after injection of 99mTc-DTPA-CB86 are shown in Figure 8A. 99mTc-DTPA-CB86 accumulated in the left inflammatory ankles at 30 min and then showed a gradual increase of uptake. During 90–180 min after injection, the left inflammatory ankles were clearly visible, with good inflammatory to background contrast. When co-injected with unlabeled DTPA-CB86 (300 μg), the left inflammatory ankles were barely visible on SPECT images at 30–180 min after injection (Figure 8B). Regions of interest (ROI) analysis of SPECT showed a high ratio of the left inflammatory ankle to muscle (LIA/M) for RA rats injected unblocking dose compared to with 300 μg blocking dose at 30–180 min postinjection (Figure 9) (P < 0.05). Evaluation of the probe in these RA rats demonstrated that 99mTc-DTPA-CB86 may be a promising agent for TSPO SPECT imaging.

■ CONCLUSIONS

This study demonstrates that 99mTc-DTPA-CB86 SPECT imaging can identify the activated macrophages in synovitis in RA rat models. 99mTc-DTPA-CB86 SPECT may be useful as a noninvasive imaging technique for clinical management of RA.

■ MATERIALS AND METHODS

Materials. TSPO ligand CB86 was kindly provided by Professor Xuechuan Hong (Wuhan University School of Pharmaceutical Sciences). All chemicals obtained commercially were used without further purification. Mycobacterium tuberculosis H37 Ra (Mtb), incomplete Freund’s adjuvant (IFA), diethylenetriaminepentaacetic acid (DTPA), tin(ii) chloride dehydrate, phenylmethanesulfonyl fluoride (PMSF), and lipopolysaccharide (LPS) were purchased from J&K Chemical Ltd. (Beijing, China). The eluent Na99mTcO4 was obtained from a commercial 99Mo/99mTc generator (China Institute of Atom Energy). WIZARD 2480 γ counter from PerkinElmer Inc. (Waltham, MA). CRC-25R Dose Calibrator from Capintec Inc. (Ramsey, New Jersey). Mouse macrophage RAW264.7 cell lines and mouse breast cancer 4T1 cell lines were obtained from the Cell Culture Center of Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences (Beijing, China). Male Wistar rats, aged 6–8 weeks (200–300 g), were purchased from the Experimental Animal Center of Xiamen University (Xiamen, China). Small animal SPECT/CT imaging studies were performed using a nano-Scan-SPECT/CT scanner (Mediso, Budapest, Hungary).

Synthesis of Coumarin-CB86. CB86 purity and molecular mass were determined by analytic scale reversed-phase high-performance liquid chromatography (HPLC, model: 3000 HPLC System, Dionex Corporation, Sunnyvale, California) and matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF-MS, model: Perspective Voyager-DE RP Biospectrometer, Framingham,
Massachusetts). The preparation of coumarin-CB86 is shown in the Supporting Information (Scheme S1 and Figure S4).

Fluorescence Imaging of CB86 in Living Cells. The RAW264.7 cells stimulated with LPS were incubated in the probe coumarin-CB86 (25 μM) with or without CB86 (10.0 μg) for 2 h at 37 °C and washed with 0.1 M PBS (0.6 mL × 3) before observation. The cells were then stained for 10 min with MitoRed, a well-established mitochondrial dye. The cells were observed using the confocal fluorescence microscope (Olympus FV 1000 Inverted, Olympus, Columbia, South Carolina, USA) with a 63 × oil-immersion objective lens. The excitation wavelength was 496 nm, and emission was collected at 516 ± 10 nm. All experiments were performed in duplicate.

Conjugation of DTPA-CB86. Conjugation of DTPA-CB86 was performed as described in our previous methods.24 CB86 (50 mg) and DTPA (200 mg) were dissolved in DMSO (2 mL) under vigorous stirring at room temperature for 24 h in the dark. The byproduct was removed by filtration to give a reddish-brown filtrate containing DTPA-CB86. The DTPA-CB86 was characterized by HPLC and MALDI-TOF-MS. The chemical characterization of DTPA-CB86 is shown in the Supporting Information (Figures S1–S3).

Cell Viability Assay. Cell viability was analyzed by MTT assay. RAW264.7 cells and 4T1 cells (with low TSPo expression) were seeded in 96-well plates at 1 × 10^4 cells and treated with CB86 or DTPA-CB86 suspensions (100 μL per well), respectively, at different concentrations (0, 1.25, 2.5, 5, 10, 20 μM in DMEM) for 24 h at 37 °C and 5% CO2. Subsequently, 10 μL of 5 mg/mL MTT was added to each well and incubated for an additional 4 h at 37 °C under 5% CO2. The optical density (OD) in each well was measured by a microplate reader (Multiskan Spectrum; Thermo Fisher). The OD at 490 nm was determined. The OD from the wells of the cells cultured with the complete medium was taken as 100% viability. Relative cell viability (%) compared to control cells was calculated using the formula: % viability = OD (treated)/OD (control) × 100%.

Labeling DTPA-CB86 with 99mTc. The radiolabeling method of DTPA-CB86 was performed as described in our previous methods.25 The compound DTPA-CB86 was labeled with 99mTc using SnCl2·2H2O as a reducing agent. Briefly, 100 μg, 100 μL of DTPA-CB86, and 20 μL of SnCl2·2H2O (2 mg/mL in 0.1 M HCl) were mixed in a vial. Next, 185–370 MBq of fresh Na109mTcO4 was added to the mixture. The reaction mixture was then incubated at 100 °C for 30 min to obtain the resulting radiotracer 99mTc-DTPA-CB86. The resulting solution of 99mTc-DTPA-CB86 was purified and analyzed by Sep-Pak C18 cartridge (GE Healthcare, Piscataway, New Jersey) and radio-HPLC (Thermo Scientific, Waltham, MA). The mobile phase is presented below: A: H2O, B: 100% CH3OH; 0–10 min, B: 10%; 10–20 min, B: 90%; 20–30 min, B: 90%; 30–40 min, B: 10%; flow rate: 0.5 mL/min. The synthetic scheme of 99mTc-DTPA-CB86 is shown in Figure 1.

Determination of Lipid–Water Partition Coefficient of 99mTc-DTPA-CB86. To determine the hydrophilicity of 99mTc-DTPA-CB86, the partition coefficient (expressed as log P) was measured as described in our previous methods.25 Two hundred microliters of 99mTc-DTPA-CB86 was added to 1 mL of phosphate-buffered saline (PBS, pH = 7.4) saturated by n-octyl alcohol and 1 mL of n-octyl alcohol saturated by PBS (pH = 7.4). After shaking for 5 min at room temperature, the solution was centrifuged at 3000 rpm for 5 min. Afterward, 100 μL of the organic phase and water phase were counted in a γ counter, respectively. The averaged activities from each phase were used to calculate the log P values. The lipid–water partition coefficient (Po/w) of 99mTc-DTPA-CB86 was calculated as (cpm in organic phase)/(cpm in water phase).

All of the experiments were performed with triplicate samples and reported as mean ± standard deviation.

In Vitro Stability Analysis. In vitro stabilities in phosphate-buffered saline (PBS, pH = 7.4) or mouse serum were determined similarly to our previously described procedures with minor modifications.20 99mTc-DTPA-CB86 (5.55 MBq) in 250 μL of PBS was added to 2.0 mL of PBS or mouse serum and incubated at 37 °C for 1, 2, and 4 h. At each time point, the mixture in the mouse serum 1.85 MBq was precipitated with 300 μL of ethanol and centrifuged at 16 000 for 2 min. The supernatant was transferred to a new Eppendorf tube, and DMF (300 μL) was added to precipitate the residue of serum protein. After centrifugation, the supernatant or the mixture in saline was acidified with 300 μL of buffer A (water + 0.1% trifluoroacetic acid (TFA)) and filtered using a 0.2 μm nylon Spin-X column (Corning Inc. Corning, New York). The filtrates were then analyzed by radio-HPLC under conditions identical to the ones used to analyze the original radiolabeled compound. The percentage of intact 99mTc-DTPA-CB86 was determined by quantifying peaks corresponding to the intact and the degradation products. The assays were repeated twice.

Cell Assays. Cell uptake, blocking, and efflux assays were performed as previously described with minor modifications.20 Briefly, the RAW264.7 cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were maintained in a humidified atmosphere of 5% CO2 at 37 °C, with the medium changed every 2 days. A 70–80% confluent monolayer was detached by 0.1% trypsin and dissociated into a single cell suspension for further cell culture. All experiments were performed in duplicate.

Cell Uptake Assay. The RAW264.7 cells were washed three times with 0.01 M PBS (pH 7.4) and dissociated with 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA). DMEM medium was then added to neutralize trypsin–EDTA. Cells were spun down and resuspended with serum-free DMEM. Cells (0.5 × 10^6) were incubated at 37 °C for 30, 90, 180, and 240 min with 1.85 × 10^−2 MBq 100 μL of 99mTc-DTPA-CB86 in 0.5 mL of the serum-free DMEM medium. The nonspecific binding of the probes with RAW264.7 cells was determined by co-incubation with 10.0 μg of unlabeled DTPA-CB86. At each time point, after supernatants were removed, the cells were washed with PBS and then lysed with 1 mL of NaOH (1 M) for 5 min. The radioactivity of the lysates was measured using a γ counter, and the cell uptake (counts/min) was normalized to the percentage of binding for analysis using Excel (Microsoft Software Inc., Redmond, Washington). All experiments were performed in duplicate.

Binding Affinity Assay. The RAW264.7 cells (2 × 10^6) were plated on 24-well plates 1 day before the experiment. After washing twice with DMEM, the cells were incubated at 25 °C for 3 h with 1.11 × 10^−2 MBq 100 μL 99mTc-DTPA-CB86 in 300 μL of DMEM with concentrations of unlabeled DTPA-CB86 ranging from 10−13 to 10−6 mol/L. After incubation, the cells were washed with cold PBS three times and detached with 1 mL of NaOH (1 M) for 5 min. The radioactivity in the cells was measured using a γ counter and corrected for physical decay. The data were analyzed using GraphPad Prism (GraphPad Software Inc. San Diego,
Californi), and the half-maximal inhibitory concentration (IC₅₀ value) of ⁹⁹mTc-DTPA-CB86 was measured using a least-square fitting routine. All experiments were performed in duplicate.

**Cell Efflux Study.** The RAW264.7 cells in separate 24-well plates were incubated with 1.11 × 10⁻² MBq 100 μL of ⁹⁹mTc-DTPA-CB86 at 37 °C for 240 min. After washing twice with PBS, the cells were then incubated with a culture medium for 30, 90, 180, and 240 min again to monitor the radioactivity efflux. At each time point, the cells were washed, lysed, and counted using a γ counter. The cell retention rate of radioactivity was expressed as a percentage of the total input radioactive dose.

**Induction of RA.** The animal procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Zhongshan Hospital Xiamen University. Experimental RA was induced in male Wistar rats aged 6–8 weeks, weighted 200–300 g according to the method previously described with some modifications. First, complete Freund’s adjuvant (CFA) was prepared as follows: incomplete Freund’s adjuvant (IFA) (20 mL) was added dropwise, with continuous mixing, to finely crushed Mycobacterium tuberculosis H37 Ra (Mtbd) (100 mg). The resulting oily preparation (Mtbd: 5 mg/mL) was temporarily stored at −20°C. Second, the induction of RA was performed for each rat by intradermal injection at the paw of the left hind limb with 100 μL of CFA. Finally, the development of the joint inflammation (the left ankle) was supervised at least 3 times a week. The joint thickness (the left ankle) was evaluated using the method of the left RA ankle joint with a digital vernier caliper (Exploit Technology CO., LTD., Taiwan, China). The maximum inflammation was achieved after 13–15 days after the injection of CFA, and then the mice were subjected to *in vivo* biodistribution and ⁹⁹mTc-SPECT imaging studies.

**Biodistribution Study.** The animal procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Zhongshan Hospital Xiamen University. RA rats (*n* = 4 for each group) were injected with ⁹⁹mTc-DTPA-CB86 (0.37 MBq, 100 μL) through the tail vein. At 30, 90, and 180 min after injection, the mice were sacrificed, and the left inflammatory ankles and normal tissues of interest were removed and weighed; their radioactivity was measured in a γ counter. The radioactivity uptake in the left inflammatory ankles and normal tissues was expressed as a percentage of the injected radioactivity per gram of tissue (% ID/g). To study the *in vivo* TSPO targeting specificity of ⁹⁹mTc-DTPA-CB86, based on the previous studies, unlabeled DTPA-CB86 (300 μg) was co-injected with ⁹⁹mTc-DTPA-CB86 in RA rats (*n* = 3 for each group) via a tail vein, and biodistribution studies were conducted at 180 min after injection. The radioactivity ratios of the left inflammatory ankle to blood (LIA/B) and the left inflammatory ankle to muscle (LIA/M) were calculated.

**SPECT/CT Imaging.** Small animal SPECT/CT imaging of RA rats was performed using a nanoscan-SPECT/CT preclinical imager (Mediso, Hungary). The RA rats (*n* = 4 for each group) were injected with ⁹⁹mTc-DTPA-CB86 (0.37 MBq, 100 μL) with or without co-injection of unlabeled DTPA-CB86 (300 μg) through the tail vein. At 30, 90, and 180 min after injection, the mice were anesthetized with 2% isoflurane and placed on the SPECT bed (ventral side down). SPECT acquiring parameters were as follows: a 140 keV energy peak for ⁹⁹mTc, window width of 20%, a matrix of 256 × 256, medium zoom, and time frame 30 s. Whole-body static images (200 000 counts) were acquired with a matrix of 218 × 218, and a zoom of 2.0. CT data were acquired using an X-ray voltage biased to 50 kVp with a 670 μA anode current, and the projections were 720°. Regions of interest (ROI) were drawn over the left inflammatory ankle and normal muscle, and then the ratios of the left inflammatory ankle to muscle (LIA/M) were calculated.

**Statistical Methods.** The experimental data were analyzed by SPSS 18.0 (SPSS Company, Chicago, IL). Statistical analysis was performed using a two-tailed Student’s *t* test for unpaired data. Data are expressed as mean ± standard deviation and *P* < 0.05 was considered to indicate a statistically significant difference.

### ASSOCIATED CONTENT

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c04066.

Details of the chemical characterization of DTPA-CB86 and synthesis and characterization of coumarin-CB86 (PDF)

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**Notes**

The authors declare no competing financial interest.
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ABBREVIATIONS USED

RA, rheumatoid arthritis; TSPO, translocator protein, 18 kDa; PET, positron emission tomography; SPECT, single-photon emission computed tomography; CB266, [2-(4-chlorophenyl)-8-amino- imidazo[1,2-a]pyridin-3-yl]-N,N-di-n-propylacetamide; CB256, 2-[(2-[(3-pyridin-2-yl)methyl]ami no)-acetalimido]-2-(4-chlorophenyl)H-imidazo[1,2-a]pyridin-3-yl-N,N-di-n-propylacetamide; 99mTc, technetium-99m; Mtb, mycobacterium tuberculosis H37 Ra; IFA, incomplete Freund's adjuvant; DTPA, diethyltriaminepentaacetic acid; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; RAW264.7; mouse macrophage cell lines; 4T1, mouse breast cancer cell lines; PBS, phosphate-buffered saline; LIA/M, the ratio of the left inflammatory ankle to muscle; LIA/B, the ratio of the left inflammatory ankle to blood; ROI, regions of interest; % ID/g, percentage of the injected radioactivity per gram of tissue.

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