Research Article

Journal of Labelled Compounds and Radiopharmaceuticals

Received 26 January 2009, Revised 16 October 2009, Accepted 21 October 2009 Published online 14 January 2010 in Wiley Interscience

(www.interscience.wiley.com) DOI: 10.1002/jlcr.1699

Radiosynthesis of ^{13}N-labeled thalidomide using no-carrier-added [^{13}N]NH_3

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Recent studies revealed that thalidomide (1) has unique and broad pharmacological effects on multi-targets although the application of 1 in therapy is still controversial. In this study, we synthesized nitrogen-13-labeled thalidomide ([^{13}N]1) as a potential positron emission tomography (PET) probe using no-carrier-added [^{13}N]NH_3 as a labeling agent. By use of an automated system, [^{13}N]1 was prepared by reacting *N*-phthaloylglutamic anhydride (2) with [^{13}N]NH_3, following by cyclization with carbonyldiimidazole in a radiochemical yield of 56 ± 12% (based on [^{11}N]NH_3, corrected for decay) and specific activity of 49 ± 24 GBq/μmol at the end of synthesis (EOS). At EOS, 570–780 MBq (n = 7) of [^{13}N]1 was obtained at a beam current of 15 μA after 15 min proton bombardment with a synthesis time of 14 min from the end of bombardment. Using a small animal PET scanner, preliminary biodistribution of [^{13}N]1 in mice was examined.

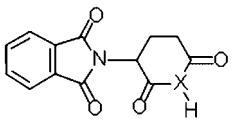
Keywords: nitrogen-13; positron emission tomography; [^{13}N]thalidomide; automated synthesis

Introduction

Thalidomide (2-(2,6-dioxo-3-piperidyl)isoindoline-2,3-dione, **1**; Scheme 1) was first developed in 1956 as a drug with sedative and hypnotic effects. However, due to its catastrophic effects on fetal malformations, this drug was withdrawn from the market in the 1960s. In recent years, interest in **1** has been increasing for the treatment of several diseases: rheumatoid arthritis,[1] AIDS,[2] leprosy,[3] Crohns disease,[4] cancer related to pathologic angiogenesis.[5,6] The unique and broad pharmacological effects of this agent have been approved in the USA for the treatment of erythema nodosum leprosum. Recent studies revealed that this drug is an agent acting on multi-targets and is still under study for other diseases.[7,8] However, because the precious mechanisms of the therapeutic and side effects are unknown, the application of **1** in therapy is still controversial.[9]

Positron emission tomography (PET) is a useful medical imaging method using radioactive probes labeled with positron emitting radioactive isotopes, such as ^{11}C, ^{18}F, and ^{13}N. PET can be used to provide pharmacokinetic and pharmacodynamic information about a drug in living bodies to determine the drug efficacy and potential biochemical mechanisms of drug action, including the side and toxic effects.[10] The effectiveness of PET prompted us to label **1** using a positron emitter to elucidate its action mechanism and to image new targets through *in vivo* evaluation in animals. So far, ^{18}F-labeled **1** has been synthesized; however, the introduction of ^{18}F into the small molecule changed the structure of **1**, which may also change its pharmacological profile, such as binding affinity, bioavailability and pharmacokinetics etc.[11] Labeling **1** with ^{11}C was also performed; however, no in vitro and in vivo evaluation in animals was reported.[12]

Herein, we labeled **1** with ^{13}N and determined the biodistribution of [^{13}N]**1** in mice as a novel PET probe (Scheme 1). The short half-life (9.965 min; 100% β^+ decay) of ^{13}N has the advantage that repeated studies can be performed on the same individual. Compared with other positron emitters such as ^{11}C and ^{18}F, PET scans using a [^{13}N]ligand cause relatively low radiation damage on the subject. [^{13}N]**1** does not change the pharmacological profiles of **1**, which could provide real and accurate information. On the other hand, in PET study with high specific activity, generally about 10 nmol of labeled compound is introduced into the living body, so the pharmacological effect induced by the amount of molecules on the subject is usually negligible. However, although there were a number of reports about the syntheses of [^{13}N]ligands using low specific activity [^{13}N]NH_3 or carrier-added [^{13}N]NH_3,[13–17] no [^{13}N]ligands with high specific activity except [^{13}N]NH_3 have been used for



Thalidomide (**1**): X = ^{14}N
[^{13}N]Thalidomide ([^{13}N]**1**): X = ^{13}N

Scheme 1. Chemical structures of thalidomide[1] and [^{13}N]thalidomide ([^{13}N]**1**).

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biological evaluation. Owing to the short half-life of ^{13}N and easy contamination by the nitrogen carrier, practical labeling technique using no-carrier-added [^{13}N]NH_3 has not been established well except our recent result.[18] About 10 years ago, Suzuki *et al*. developed an automated synthetic system to produce [^{13}N]NH_3 with ultra-high specific activity (1850 GBq/μmol).[19] Using this system, they have prepared *p*-nitrophenyl [^{13}N]carbamate by reacting the corresponding chloroformate with nca [^{13}N]NH_3.[20]

In this study, by use of an automated synthesis system, we synthesized [^{13}N]**1** using anhydrous [^{13}N]NH_3 gas as a labeling agent. Then, we performed a preliminary biodistribution study of [^{13}N]**1** on mice using a small animal PET scanner. This is the first report about the synthesis and preliminary distribution of a promising PET probe labeled with no-carrier-added [^{13}N]NH_3.

Results and discussion

Chemical synthesis simulating radiosynthesis

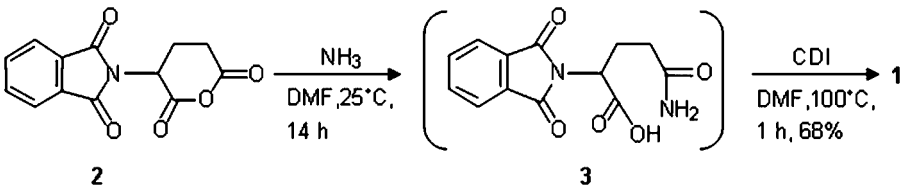
Prior to radiosynthesis, we determined a route for synthesizing **1** using unlabeled NH_3 (Scheme 2). In the previous synthesis of unlabeled **1**, urea, glutarimide or glutamine was used to introduce N into the final product, which required relatively long reaction times.[21–24] Obviously, the routes were not suitable for radiosynthesis with [^{13}N]NH_3, because common radiosynthesis must be accomplished within a short time, compatible with the half-life of ^{13}N, using only limited step sequences. In the present route, a solution of anhydrous NH_3 in DMF was reacted with *N*-phthaloylglutamic anhydride (**2**) to yield the amide intermediate **3**, which was cyclized with carbonyldiimidazole (CDI) to form **1** with a total chemical yield of 68%. To shorten the synthesis time while guaranteeing the reaction efficiency, two treatments were administered here: (1) heating the two-step reaction mixtures at 130°C for 2 min twice; (2) achieving one-pot synthesis without purifying **3**.

Radiosynthesis

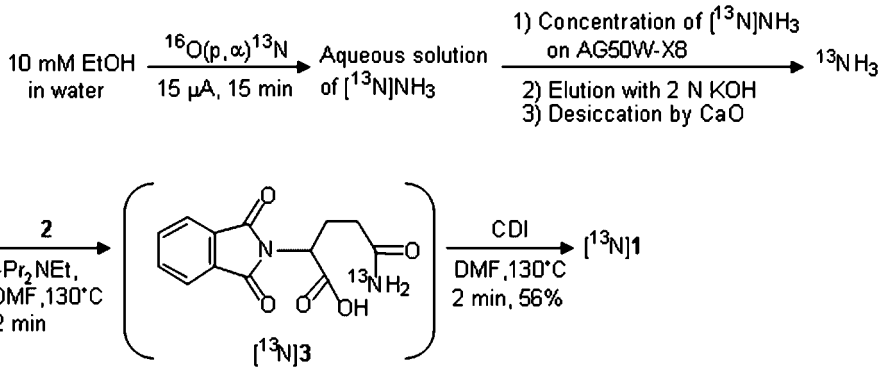
Anhydrous [^{13}N]NH_3 gas for labeling was produced according to the procedures reported previously with some modifications.[19] The nuclear $^{16}O(p, \alpha)^{13}N$ reaction in the target generated an aqueous [^{13}N]NH_3 solution that was quickly passed through a pre-conditioned cation exchange column to concentrate [^{13}N]NH_3 onto the column. Then, [^{13}N]NH_3 was released with aqueous KOH solution and passed through a hot column filled with CaO to give anhydrous [^{13}N]NH_3 gas, which was introduced into a cooled vessel containing **2**. From the end of bombardment (EOB), the preparation of anhydrous NH_3 took about 4 min. The specific activity of [^{13}N]NH_3 used for the experiment was 50–80 GBq/μmol at the end of [^{13}N]NH_3 synthesis.

Radiosynthesis of [^{13}N]**1** was performed according to Scheme 3. After [^{13}N]NH_3 (about 0.1 μmol) trapping into a solution of **2**, the reaction mixture was heated at 130°C for 2 min. The reaction of **2** with [^{13}N]NH_3 gave [^{13}N]**3** only with a radiochemical yield of 20% (corrected for decay). We assumed that trace mass of [^{13}N]NH_3 was trapped by the carboxylic acid moiety of [^{13}N]**3**. This was supported by the result that the addition of unlabeled NH_3 (100 μmol) improved [^{13}N]ammonolysis efficiency to 68%. However, the addition of NH_3 resulted in a marked decrease of specific activity (<10 MBq/μmol) of [^{13}N]**3**. A PET probe with too low specific activity could not give high-quality images of specific binding.[20] To liberate [^{13}N]NH_3 from [^{13}N]**3**, in place of unlabeled NH_3, *i*-Pr_2NEt was added to the reaction mixture before the [^{13}N]NH_3 trapping. This treatment increased [^{13}N]ammonolysis efficiency to 76%.

Following [^{13}N]ammonolysis, CDI was added to the mixture, which was continuously heated for 2 min to accomplish cyclization to form [^{13}N]**1**. The radioactive mixture was loaded onto a normal phase semi-preparative HPLC system. After HPLC purification, [^{13}N]**1** was obtained with a radiochemical purity of 98% and a specific activity of 49 ± 24 GBq/μmol ($n = 7$). Starting from 3–4.4 GBq (15 μA, 15 min proton bombardment) of [^{13}N]NH_3,



Scheme 2. Chemical synthesis of **1**.



Scheme 3. Radiosyntheis of [^{13}N]**1**.

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570–780 MBq ($n=7$) of [^{13}N]**1** was obtained at the end of synthesis (EOS) as an injectable solution. This amount of radioactivity was enough for animal experiments. The radiochemical yield and purity of [^{13}N]**1** was 56±12% based on [^{13}N]NH_3 (corrected for decay) and >97%. The average production time from the recovery of [^{13}N]NH_3 to the formulation of [^{13}N]**1** was about 14 min. The synthesis time was thus only slightly longer than the half life of ^{13}N. In the final product solution, no significant peak relative to CDI, **2** and their decomposition products was detected. Moreover, the radiochemical purity of [^{13}N]**1** remained \gt 95% after maintaining the preparation at room temperature for 60 min, indicating that this PET probe was radiochemically stable for the time compatible with that of a PET scan.

Biodistribution

The preliminary biodistribution of [^{13}N]**1** in mice ($n=3$) was determined using a small animal scanner (Figure 1). Owing to the relatively high specific activity of [^{13}N]**1**, the mass of [^{13}N]**1** injected into the living body of mice was less than 200 ng. Thus, the pharmacological effects of **1** on the mice may be decreased to a minimal extent. After the probe injection, radioactivity immediately appeared in the lung and heart but decreased rapidly. High reactivity in the liver, gall bladder and small intestine from the early time period suggested biliary excretion of this probe. In the blood, the radioactivity displayed a rapid initial decline and then remained at a steady level. In the brain, a high initial uptake was observed and radioactivity decreased slowly over time, suggesting that [^{13}N]**1** could pass through blood–brain barrier, a prerequisite as a promising PET probe for brain imaging. Now, we are evaluating [^{13}N]**1** using several animal models, including neuroinflammation and tumor. The data will be reported elsewhere.

Experimental

Materials and general methods

Melting points were uncorrected. Nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JNM-GX-270 spectrometer (JEOL, Tokyo) with tetramethylsilane as an internal standard. All chemical shifts (δ) were reported in parts per million downfield

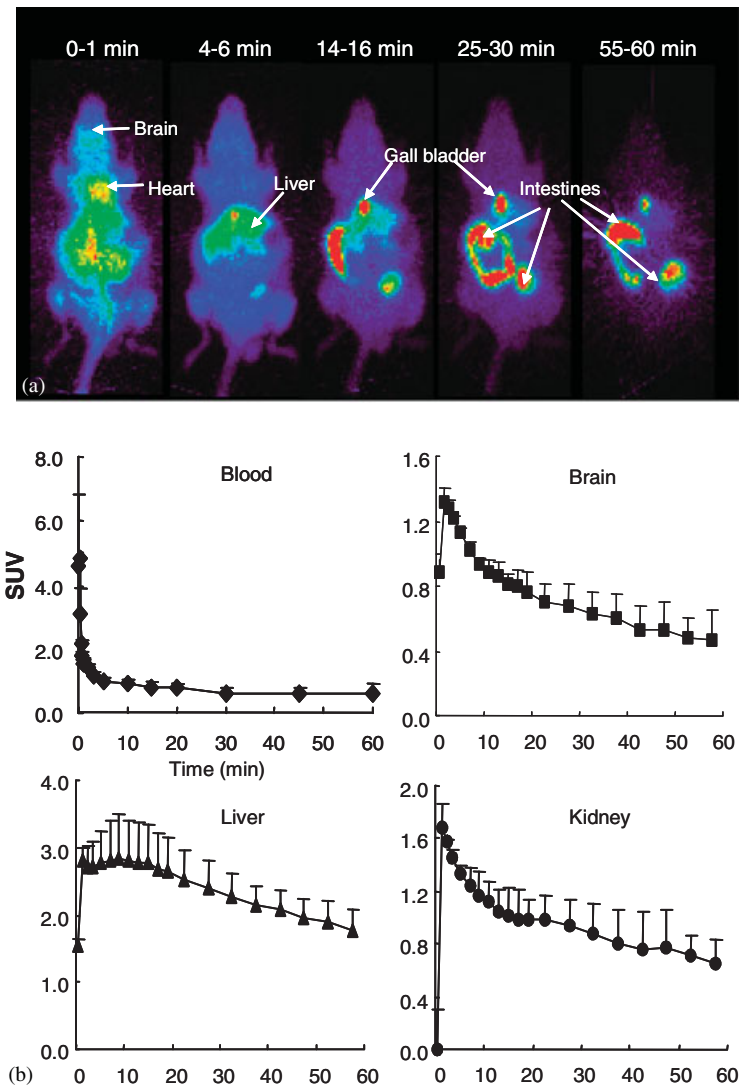

Figure 1. Biodistribution of [^{13}N]**1** in mice ($n=3$). A: Typical static micro PET images at different time points after tracer injection. B: Time–activity curves of [^{13}N]**1** in mouse brain, blood, liver and kidneys. Radioactivity was detected and expressed as SUV. The SUV value (mean±SD, $n=3$) was calculated according to the following formula: measured activity concentration (Bq/mL) × body weight (g)/injected activity (Bq).

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from the standard. Fast atom bombardment mass spectra (FAB-MS) were obtained on a JEOL NMS-SX102 spectrometer (JEOL, Tokyo). Column chromatography was performed on Merck Kieselgel gel 60 F_{254} (70–230 mesh). Nitrogen-13 (^{13}N) was produced by the $^{15}O(p, \alpha)^{13}N$ nuclear reaction using a CYPRIS HM-18 cyclotron (Sumitomo Heavy Industry, Tokyo). Radioactivity was measured with a dose calibrator (IGC-3R Curiemeter, Aloka, Tokyo). HPLC was performed using a JASCO HPLC system (JASCO, Tokyo): effluent radioactivity was monitored using a NaI (Tl) scintillation detector system. If not otherwise stated, chemicals were purchased from Aldrich Chemical (Milwaukee, WI) and Wako Pure Industries (Osaka) with the highest grade commercially available.

Chemical synthesis of thalidomide (1)

A mixture of *N*-phthaloylglutamic anhydride (**2**; 258 mg, 1 mmol) and anhydrous NH_3 in DMF (10%, 5 mL) was stirred on room temperature for 14 h. After removal of unreacted NH_3 by evaporation, a solution of carbonyldiimidazole (CDI; 486 mg, 3 mmol) was added and the reaction mixture was heated at 100°C for 1 h. The mixture was quenched with CH_2Cl_2 and water and the organic layer was washed with water and a saturated NaCl solution. The CH_2Cl_2 layer was dried over Na_2SO_4 and was removed to give a residue, which after recrystallization with ethanol gave pure **1** (177 mg, 68.3%) as a white solid; mp: 270°C (269–271°C).[25] ^{1}H-NMR (300 MHz, DMSO–d_6): 2.04–2.12 (1H, m), 2.49–2.63 (2H, m), 2.84–2.96 (1H, m), 5.17 (1H, dd, $J = 5.4$, 12.8 Hz), 7.87–7.97 (4H, m), 11.14 (1H, s). FAB-MS (*m/z*): 259.1 ($M^+ + 1$)

Radiosynthesis of [^{13}N]thalidomide ([^{13}N]1)

Production System

Radiosynthesis was performed using an automated system for rapid production of ^{13}N-labeled compounds with high specific activity using anhydrous [^{13}N]NH_3.[19] This system was designed to minimize the inner volume of the production line and the required amount of reagent, which decreased the radioactivity loss in the line to a low level. The system enables one to carry out all the procedures from production of [^{13}N]NH_3 to formulation of [^{13}N]**1**. The target chamber (21 mm $\phi \times 6$ mm) was made of SUS316 and two SUS316 foils (100 mm thick) were welded on both sides of the chamber by an electron beam in vacuo.

Production of anhydrous [^{13}N]NH_3 gas

Before irradiation, a solution of 10 mM ethanol in water was loaded into the target chamber. The target was irradiated at 15 μA for 15 min with 18 MeV protons (15.7 MeV on target) from the cyclotron. A small column (1 mm $\phi \times 40$ mm, Teflon) filled with 25 mg of cation exchange resin AG50W-X8 (100–200 mesh, Bio-Rad, CA) was preconditioned by successive washing with 2 N HCl, water, 2 N KOH and water. The irradiated solution was quickly passed through this exchange column and [^{13}N]NH_3 in the irradiated water was concentrated on this column. Then, [^{13}N]NH_3 was eluted with aqueous KOH (2 N) under a He gas flow and desiccated through a small column filled with 250 mg of CaO (3 mm $\phi \times 30$ mm, kept at 150°C), and introduced into a cooled solution containing a precursor for radiosynthesis.

Preparation of [^{13}N]thalidomide ([^{13}N]1)

Anhydrous [^{13}N]NH_3 gas was bubbled into a reaction vessel containing **2** (2.0 mg), *i*-Pr_2NEt (5 μL) and anhydrous DMF (1 mL) cooled to −15°C. After radioactivity reached a plateau, the reaction vessel was warmed to 130°C and maintained for 2 min. A solution of CDI (10 mg) in DMF (300 μL) was added to the mixture, which was heated for another 2 min. The radioactive mixture was loaded to the semi-preparative HPLC system. HPLC purification was completed on a Capcell Pak column (normal phase, AG-80; 10 mm $\phi \times 250$ mm; SHISEIDO, Tokyo) using a mobile phase of AcOEt at a flow rate of 5.0 mL/min. The retention time for [^{13}N]**1** was 4.1 min. The radioactive fraction corresponding to the desire product was collected in a sterile flask, evaporated to dryness, and re-dissolved in 2 mL of sterile normal saline for analysis and animal experiments. Total synthesis time was about 14 min from the EOB. At EOS, 570–780 MBq ($n = 7$) of [^{13}N]**1** was obtained as an *i.v.* injectable solution using a beam current of 15 μA and 15 min proton bombardment.

Radiochemical purity and specific activity determinations

Radiochemical purity of [^{11}N]**1** was assayed by analytical HPLC (Capcell Pak C_{18}, 4.6 mm $\phi \times 150$ mm; SHISEIDO, Tokyo). The retention time of [^{13}N]**1** was 5.2 min with a mobile phase of H_2O/CH_3CN (7/3) at a flow rate of 2.0 mL/min. Confirmation of the identity of [^{13}N]**1** was achieved by co-injection with authentic non-radioactive **1**. The same analytical HPLC system was used for the determination of specific activity of [^{13}N]**1**. The mass (μmol) of **1** with a known radioactivity (GBq) was determined by HPLC comparison of UV absorbance at 254 nm of [^{13}N]**1** with those of known concentrations of non-radioactive **1**.

Animal experiment

The animal experimental procedures were approved by the Animal Ethics Committee of the National Institute of Radiological Sciences. Male ddY mice (7 weeks old) were purchased from Japan SLC (Shizuoka, Japan). Before experiments, animals were housed under a 12 h dark–light cycle and were allowed free access to food pellets and water. PET scans were performed using small animal PET scanner Inveon™ (Siemens Medical Solutions USA; Knoxville, TN), which provides 159 transaxial slices 0.796 mm (center-to-center) apart, a 10 cm transaxial FOV, and a 12.7 cm axial FOV.[26] Combing the high light output of LSO with 1.6 mm × 1.6 mm detector pixel spacing, the Inveon delivers less than 1.4 mm FWHM spatial resolution in images reconstructed using the filtered back projection algorithm.[27] Prior to the scans, the rats were anesthetized with 5% (v/v) isoflurane, and maintained thereafter by 1–2% (v/v) isoflurane. After transmission scans for attenuation using a ^{57}Co point source, emission scans were acquired for 60 min after the intravenous injection of [^{13}N]**1** (average 40 MBq). During these PET scans, ten arterial blood samples (15–30 μL each) were drawn into heparin-treated syringes at 15 s intervals until 2 min, followed by 30 μL aliquots at 3, 5, 10, 20, 15, 30, 45, 60 min. Samples were measured for radioactivity and metabolite analysis was not performed.

All list-mode acquisition data were sorted into three-dimensional sinograms, which were then Fourier rebinned into two-dimensional sinograms (frames × min; 4 × 1, 8 × 2, 14 × 5). Dynamic images were reconstructed with filtered back-projection

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using a ramp's filter, a Nyquist cutoff of 0.5 cycle/pixel. Regions of interest were placed on the whole brain, lung, heart, kidney by using ASIPro VM^{TM} (Analysis Tools and System Setup/Diagnostics Tool; Siemens Medical Solutions USA) with reference to the MRI template. Regional uptake of radioactivity was decay corrected to injection time and expressed as the standardized uptake value (SUV), normalized for injected radioactivity and body weight. SUV = (radioactivity per cubic centimeter tissue/injected radioactivity) × gram body weight. Data are expressed as mean ± SD.

Conclusion

We synthesized [^{13}N]**1** for the first time as a promising PET probe. At the EOS, 570–780 MBq of [^{13}N]**1** was obtained at a specific activity of 49 ± 24 GBq/μmol using a beam current of 15 μA for 15 min. [^{13}N]**1** was obtained with a reproducible radiochemical yield of 56% (based on [^{13}N]NH_3, corrected for decay) and a radiochemical purity of 98%. [^{13}N]**1** may be a tool for elucidating the action mechanism, binding site and adverse effects of **1**. We also believe that the rapid radiosynthesis using no-carrier-added [^{13}N]NH_3 will contribute to the development of more ^{13}N-labeled probes for PET imaging.

Acknowledgement

The authors are grateful to the crew of the Cyclotron Operation Section and Department of Molecular Probes, Molecular Imaging Center of National Institute of Radiological Sciences for their support in the operation of the cyclotron and production of radioisotopes. This study was supported in part by Grant-in-Aid for the Molecular Imaging Program from the Ministry of Education, Culture, Sports, Science and Technology, Japanese Government.

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