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Nuclear Medicine and Biology 32 (2005) 263–268

NUCLEAR MEDICINE — AND — BIOLOGY

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Biological properties of 2′-[^{18}F]fluoroflumazenil for central benzodiazepine receptor imaging

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Received 2 September 2004; received in revised form 23 December 2004; accepted 23 December 2004

Abstract

A novel positron emitting agent, 2′-[^{18}F]fluoroflumazenil (fluoroethyl 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-benzo-[*f*]imidazo [1,5-*a*][1,4]diazepine-3-carboxylate, FFMZ), has been reported for benzodiazepine imaging. In the present study, biological properties of [^{18}F]FFMZ were investigated. Stability tests of [^{18}F]FFMZ in human and rat sera were performed. Biodistribution was investigated in mice and phosphorimages of brains were obtained from rats. A receptor binding assay was performed using rat brain (mixture of cortex and cerebellum) homogenate. A static positron emission tomography (PET) image was obtained from a normal human volunteer. Although [^{18}F]FFMZ was stable in human serum, it was rapidly hydrolyzed in rat serum. The hydrolysis was 39%, 63% and 92% at 10, 30 and 60 min, respectively. According to the biodistribution study in mice, somewhat even distribution (between 2~3% ID/g) was observed in most organs. Intestinal uptake increased up to 6% ID/g at 1 h due to biliary excretion. Bone uptake slowly increased from 1.5% to 3.5% ID/g at 1 h. High uptakes in the cortex, thalamus and cerebellum, which could be completely blocked by coinjection of cold FMZ, were observed by phosphorimaging study using rats. Determination of K_d value and B_{max} using rat brain tissue was performed by Scatchard plotting and found 1.45±0.26 nM and 1.08±0.03 pmol/mg protein, respectively. The PET image of the normal human volunteer showed high uptake in the following decreasing order: frontal cortex, temporal cortex, occipital cortex, cerebellum, parietal cortex and thalamus. In conclusion, the new FMZ derivative, [^{18}F]FFMZ appears to be a promising PET agent for central benzodiazepine receptor imaging with a convenient labeling procedure and a specific binding property.

Keywords: Benzodiazepine receptor; FFMZ; PET; Flumazenil; Fluoroflumazenil

1. Introduction

Central benzodiazepine receptors have been visualized and quantified in vivo by positron emission tomography (PET) or single photon emission computed tomography (SPECT). The potent benzodiazepine antagonist flumazenil (Ro 15-1788, ethyl 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-benzo-[*f*]imidazo[1,5-*a*][1,4]diazepine-3-carboxylate) has been labeled with ^{11}C and ^{18}F for PET [1–4], and ^{123}I labeled Ro 16-0154 [^{123}I]iomazenil was described shortly thereafter for SPECT [5–8]. The above compounds all show high regional specific binding that corresponds to the known distribution of cerebral benzodiazepine receptors.

[^{11}C]Flumazenil is the most commonly used agent for imaging central benzodiazepine receptor using PET [9–11]. However, the short half-life of ^{11}C (20 min) limits its use. To overcome this problem, 5-(2′-[^{18}F]-fluoroethyl)flumazenil ([^{18}F]FEFMZ), a flumazenil analogue labeled with ^{18}F, has been developed using a two-step synthesis procedure [12]. However, its preparation was time-consuming and inefficient because of the two-step reaction. A further improvement in the synthetic procedure was achieved by synthesizing a new derivative 2′-[^{18}F]fluoroflumazenil(FFMZ) (scheme 1) that employs a tosylated precursor for one-step labeling reaction [13]. A two-step reaction procedure has also been applied to label [^{18}F]FFMZ later [14].

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doi:10.1016/j.nucmedbio.2004.12.004

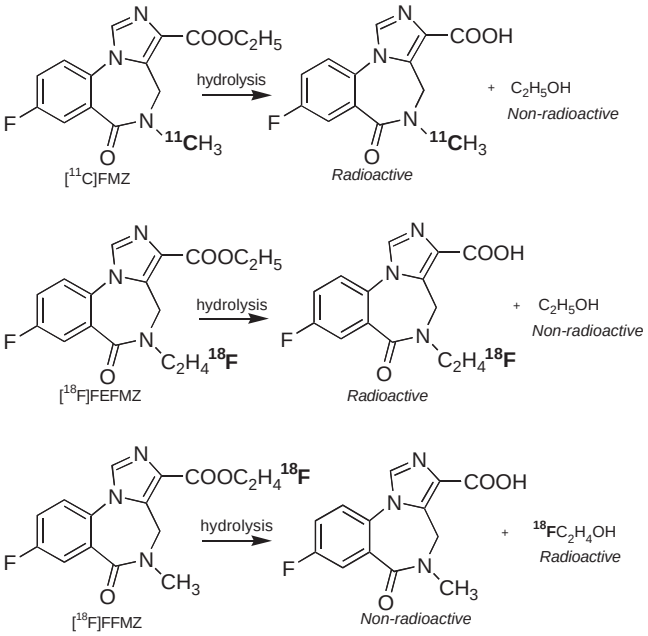

Fig. 1. Hydrolysis of [^{11}C]FMZ, [^{18}F]FEFMZ and [^{18}F]FFMZ. In cases of [^{11}C]FMZ and [^{18}F]FEFMZ, radioactivity remains in hydrophilic metabolites, that is, [^{11}C]FMZ acid and [^{18}F]FEFMZ acid, respectively. However, radioactivity remains to [^{18}F]fluoroethanol in the case of [^{18}F]FFMZ.

Although some biological evaluations of [^{18}F]FFMZ, such as the biodistribution in rats and the IC_{50} value of displacing [^{3}H]FMZ, have been reported [15], many other biological properties have not been reported. In the present study, we investigated the binding affinity of [^{18}F]FFMZ to mouse brain tissue, stability in serum, biodistribution in mice, phosphorimages of rat brain and static PET image of a normal human volunteer.

In addition, we tested the possibility that hydrolysis of the ester bond of [^{18}F]FFMZ would produce [^{18}F]fluoroethanol. On the other hand, [^{11}C]FMZ and [^{18}]FEFMZ would produce hydrophilic nondiffusible [^{11}C]FMZ acid and [^{18}F]FEFMZ acid, respectively (Fig. 1).

2. Materials and methods

2.1. Preparation of [^{18}F]FFMZ

[^{18}F]Fluoride was produced using a TR13 cyclotron (Ebco Technologies) by bombarding ^{18}O-enriched water (97%) with an accelerated proton beam. The synthesis of 2′-tosyloxyflumazenil and its labeling with ^{18}F were performed as previously published method [13]. The [^{18}F]fluoride produced was captured by an AG1-X8 microcolumn and eluted with a 1-ml solution of Kryptofix 2.2.2. (19.26 mg, 0.05 mmol)/K_2CO_3 (6.43 mg, 0.0465 mmol) in MeCN/H_2O (86/14) (v/v). In a reaction vessel located in an oil bath at 105°C, the eluted [^{18}F]fluoride was dried by purging with helium gas (flow rate, ~20 ml/min). After drying the azeotropic mixture, 1 ml of MeCN was added and the mixture was dried two more times to remove water thoroughly. To the dried [^{18}F]fluoride, 10 mg of TFMZ or MFMZ in 2.5 ml in MeCN was added, and the mixture was heated at 85°C for 12 min under continuous purging with helium for mixing. The reaction mixture was purified by a preparative HPLC (column: C_{18}, 300 mm×7.8 mm, 10 mm, Waters), and [^{18}F]FFMZ was eluted at 24.4 min by gradient elution (100% water, 7 min; 0% to 40% MeCN in water, 10 min; and continuous 40% MeCN in water) at a flow rate of 5 ml/min. The radiochemical purity of the [^{18}F]FFMZ was higher than 98% when checked by TLC (ethyl acetate/ethanol/hexane=1/1/2, AR2000, Bioscan, Washington, DC, USA) and an analytical HPLC (column: Bio-Rad RSiL C_{18}HL, 250×4.6 mm, 10 μm, MeCN/water=3/7, retention time=12.4 min). The specific activity of [^{18}F]FFMZ was $4.5\times10^9\pm1.6\times10^9$ MBq/mol as determined by an analytical HPLC. All other reagents used were of analytical grade and were obtained commercially.

2.2. Preparation of the rat brain membranes

Cerebral membranes for binding assay were prepared as previously described with some modifications [16]. Male Sprague–Dawley rats, weighing 270–290 g, were killed by decapitation and brains were rapidly removed. The cerebral cortex and the cerebellum were separated from the brain stem and immediately frozen in liquid nitrogen, and then stored in a deep freezer (−70°C) until use. The frozen cerebral cortex and the cerebellum (about 1 g) were placed in 1.4 ml of ice-cold 50 mM Tris–citrate buffer (pH 7.5) containing protease inhibitor cocktail (Sigma, St. Louis, USA), homogenized for 30 s using a polytron homogenizer (Kinematica, Westbury, Canada) on setting 7 and centrifuged at 48,000×g for 30 min at 4°C. The supernatant was then decanted and the pellet obtained was suspended in 1.4 ml of the same buffer, rehomogenized and recentrifuged at 48,000×g for 30 min at 4°C. The crude membranes were then suspended in 1 ml of the same buffer and frozen in liquid nitrogen. After freezing, the membranes were thawed and centrifuged at 48,000×g for 15 min at 4°C. These freezing and thawing steps were then repeated twice. The resulting pellet was suspended in 1 ml of 50 mM Tris–citrate buffer (pH 7.5) without protease inhibitor cocktail and stored at −70°C until required for binding studies. Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, USA).

2.3. Binding assay

[^{18}F]FFMZ binding assays were carried out by incubating aliquots of membranes suspension (0.1~1.6 mg) for 60 min at 0°C in 0.4 ml of 50 mM Tris–citrate buffer (pH 7.5) containing 0.76 nM [^{18}F]FFMZ. Nonspecific binding was determined in the presence of 10 μM cold FMZ. Binding reactions were stopped by rapid filtration through Whatman GF/B glass fiber filters presoaked with buffer under reduced pressure. Filters were washed twice with 5 ml of ice-cold 50 mM Tris–citrate buffer and

Table 1
Biodistribution of [^{18}F]FFMZ in ICR mice after intravenous injection through the tail vein

Organ	10 min	30 min	60 min
Blood	3.7±0.4	3.3±0.1	3.3±0.1
Muscle	2.4±0.3	2.0±0.1	1.9±0.1
Fat	2.2±0.1	1.8±0.1	1.6±0.0
Heart	2.7±0.5	2.4±0.3	2.8±0.4
Lung	2.8±0.4	2.6±0.1	2.6±0.1
Liver	3.1±0.3	2.5±0.2	2.4±0.1
Spleen	2.7±0.2	2.4±0.1	2.3±0.1
Stomach	4.0±1.3	3.0±0.7	2.5±0.1
Intestine	4.1±0.5	4.6±0.2	5.7±0.3
Kidney	3.0±0.4	2.4±0.1	2.3±0.1
Brain	2.5±0.4	2.2±0.3	2.1±0.1
Bone	1.7±0.6	2.0±0.2	3.6±0.1

Values denote % ID/g±S.D.
$n=3$ for each time point.

counted using a gamma counter. For saturation studies, membranes were incubated in 0.4 ml of ice-cold 50 mM Tris–citrate buffer (pH 7.5) consisting of six different concentrations of [^{18}F]FFMZ ranging from 0.42 to 13.32 nM. Nonspecific binding was determined in the presence of 10 μM cold FMZ.

2.4. Stability test in serum

To check its in vitro stability in serum, [^{18}F]FFMZ (25 μl) was incubated with human or rat sera (325 μL) for 1 h in CO_2 incubator at 37°C. Absolute ethanol (775 μl) was added to the reaction mixture to extract metabolites and the mixture was centrifuged (3000 rpm) for 5 min. The supernatant was analyzed by analytical HPLC as described above.

2.5. Biodistribution in mice

Male ICR mice ($n=3$/group, 36±2 g) were injected with 0.37 MBq (100 μL) [^{18}F]FFMZ through a tail vein. Subsequently, mice were sacrificed by cervical dislocation 10, 30 and 60 min later. Blood and other organs were rapidly removed, weighed and counted with a gamma counter (Packard, Albertville, USA). Results are expressed as percentage of injected dose per gram of tissue (% ID/g).

2.6. Phosphorimaging of rat brain

The distribution of [^{18}F]FFMZ in the rat brain was determined by phosphorimaging. [^{18}F]FFMZ (74 MBq, 0.2 ml) was injected intravenously into male Sprague–Dawley rats, weighing 350–380 g with or without cold FMZ (0.1 mg, 0.3 ml) through the tail vein. Animals were sacrificed by decapitation 5, 10 or 20 min after administration. Brains were quickly removed and frozen at −20°C. Radioactivities and weights of the brains were measured using a dose calibrator and an electric balance. The frozen brains were placed in a cryostat microtome (Leica CM 1800, Leica, Germany), and 20-μm thickness coronal sections were obtained and mounted onto the microscopic slide glasses. Imaging plates (Fujifilm, Japan) were exposed to the slide glasses overnight. The exposed imaging plates were analyzed using a BAS-2000 (Fujifilm FLA-2000, Fujifilm, Japan).

2.7. Positron emission tomography imaging of a normal human volunteer

Positron emission tomography PET study was performed on a healthy male volunteer (age 47) who was not taking any medications at the time of the study using an ECAT–EXACT 47 PET scanner (CTI/Siemens, Knoxville, TN, USA). A bolus of approximately 370 MBq of [^{18}F]FFMZ was injected intravenously, and a static PET image acquisition fraction was obtained from 20- to 60-min period.

3. Results

3.1. Binding assay

The specific binding of [^{18}F]FFMZ increased with increasing protein concentrations in the range of 0.1~1.6 mg protein. K_d and B_{max} values were determined by Scatchard plotting and found 1.46±0.26 nM and 1.08±0.03 pmol/mg protein, respectively.

3.2. Stability test in serum

Stability in human and rat sera at 37°C was analyzed by HPLC. Retention times of [^{18}F]fluoroethanol and

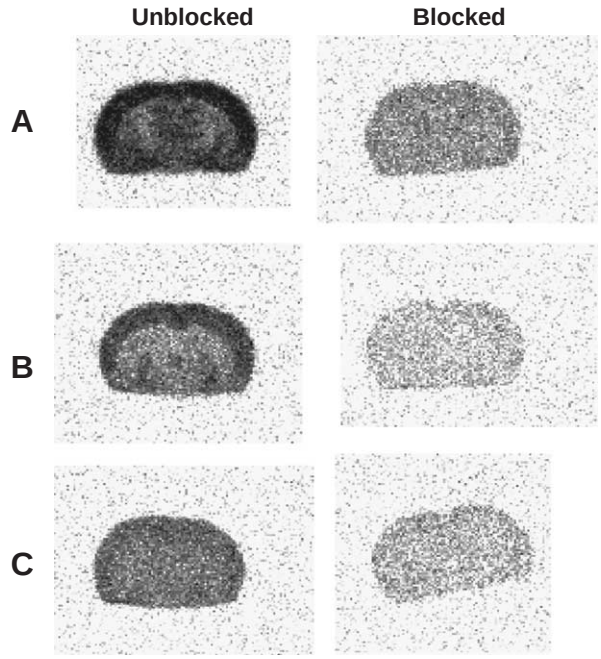

Fig. 2. Phosphorimages of rat brain 5 min (A), 10 min (B) and 20 min (C) after injecting [^{18}F]FFMZ (74 MBq, 0.2 ml) through the tail vein without a cold FMZ preinjection (left column) and with precold FMZ (0.1 mg, 0.3 ml) preinjection designed to block central benzodiazepine receptor (right column). High specific uptakes in the cortex, thalamus and cerebellum were observed.

[^{18}F]FFMZ were 3.8 min and 12.4 min, respectively. [^{18}F]FFMZ was stable for at least 1 h in human serum at 37°C. However, [^{18}F]FFMZ was rapidly hydrolyzed in rat serum. Thirty-nine percent of [^{18}F]FFMZ was degraded to [^{18}F]fluoroethanol at 10 min and only 8% remained intact at 60 min. No other metabolite was detected by HPLC in this experiment.

3.3. Biodistribution in mice

Biodistribution study showed relatively even distribution (between 2% and 3% ID/g) at all time points in the majority of examined organs; for example, muscle, heart, lung, spleen, kidney and brain (Table 1). Intestinal uptake increased up to 5.7% ID/g at 1 h due to biliary excretion, and bone uptake increased up to 3.6% ID/g at 1 h (Table 1).

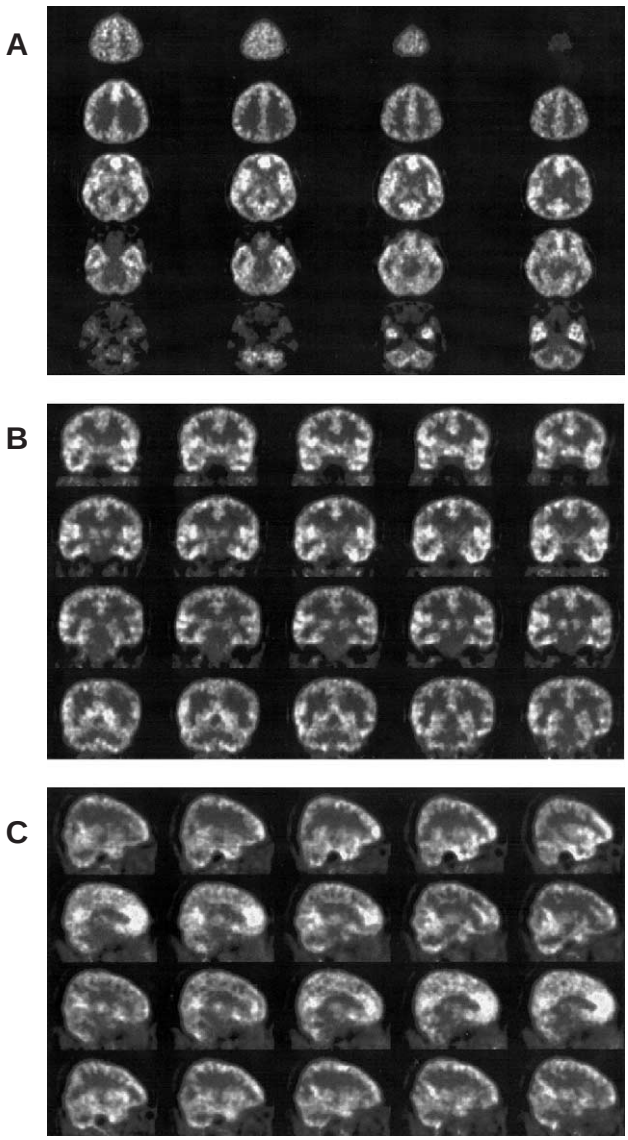

Fig. 3. PET study of [^{18}F]FFMZ in a healthy human volunteer (47-year-old man). A bolus of 370 MBq of [^{18}F]FFMZ was injected intravenously and a static PET image acquisition fraction was obtained from 20- to 60-min period (A, transaxial view; B, coronal view; C, sagittal view).

3.4. Phosphorimaging of rat brain

In the rat brain phosphorimaging experiment, high uptakes were observed in the cortex, thalamus and cerebellum (Fig. 2). All brain uptakes could be blocked by coinjection of cold FMZ (Fig. 2). Brain uptakes were 0.8±0.1% ID/g, 0.5±0.0% ID/g and 0.2±0.1% ID/g at 5, 10 and 20 min, respectively.

3.5. PET imaging of a normal human volunteer

The static PET image of the healthy human volunteer showed high uptakes in the decreasing order of frontal cortex, temporal cortex, occipital cortex, cerebellum, parietal cortex and thalamus (Fig. 3). The brain stem showed low uptake (Fig. 3).

4. Discussion

[^{18}F]FFMZ is convenient to use because of the previously reported simple labeling procedure [13]. However, increased cost of synthesis due to a consumption of expensive ^{18}O-water and an extended irradiation time might be a disadvantage over [^{11}C]FMZ.

[^{11}C]FMZ, [^{18}F]FEFMZ and [^{123}I]iomazenil have been used for quantitative imaging of the central benzodiazepine receptor by PET and SPECT [17–20]. According to a comparative study of [^{3}H]FMZ and [^{18}F]FEFMZ, [^{3}H]FMZ shows a higher accumulation in mouse brain [12]. [^{11}C]FMZ has several advantages over [^{18}F]FEFMZ, such as higher affinity, slower metabolism and slower kinetics; however, it has the disadvantage of a shorter half-life of ^{11}C (20 min) vs. ^{18}F (110 min). Despite its disadvantages, it has been proven that [^{18}F]FEFMZ is a suitable PET agent for the quantitative assessment of central benzodiazepine receptors [21]. The lower affinity of [^{18}F]FEFMZ for the receptor is due to the structural change of an *N*-methyl group to an *N*-fluoroethyl group. The higher structural similarity of FFMZ to FMZ than FEFMZ would make less decrease in affinity. In the present study, the affinity of [^{18}F]FFMZ to the benzodiazepine receptor of mouse showed a similar affinity with [^{3}H]FMZ in rat brain (K_d=0.6 nM) reported in the literature [22,23]. Along with the high affinity and a long half-life, it is highly predicted that [^{18}F]FFMZ would be a promising agent for imaging central benzodiazepine receptor.

The major metabolites of [^{11}C]FMZ are hydrophilic [^{11}C]FMZ acid and ethanol, which are formed by the hydrolysis of ester bond [10,11,24]. In a similar way, the major metabolites of [^{18}F]FEFMZ are [^{18}F]FEFMZ acid and ethanol [21]. In this study, we found that [^{18}F]FFMZ is hydrolyzed to FFMZ acid and [^{18}F]fluoroethanol in rat serum. The difference between the metabolites formed is that the radioactive metabolites of [^{11}C]FMZ and [^{18}F]FEFMZ are [^{11}C]FMZ acid and [^{18}F]FEFMZ acid, respectively, whereas [^{18}F]FFMZ forms [^{18}F]fluoroethanol (Fig. 1). It is predicted that the hydrophilic metabolites such as [^{11}C]FMZ acid and [^{18}F]FEFMZ acid would be more difficult to diffuse out of the brain cells than [^{18}F]fluoroethanol. These results

suggest that nonspecific accumulation of [^{18}F]FFMZ in the brain would be lower than the other previously reported agents such as [^{11}C]FMZ and [^{18}F]FEFMZ.

In this experiment, an interesting species difference was found in metabolism rate. [^{18}F]FFMZ was hydrolyzed more rapidly in rat serum than in human serum. Similarly, the metabolism of [^{18}F]FEFMZ has been reported to be more rapid in rat than in human microsomes [21].

The result suggests that [^{18}F]FFMZ would represent more accurate distribution of central benzodiazepine receptor than other agents such as [^{11}C]FMZ or [^{18}F]FEFMZ. Because, the radioactive metabolite of [^{18}F]FFMZ, [^{18}F]fluoroethanol, in the brain would rapidly diffuse out of the brain, while metabolites of the other agents would remain in the brain (Fig. 1).

The even distribution observed in most mouse organs during our biodistribution study was attributed to the lipophilic nature of [^{18}F]FFMZ, and the slightly increased bone activity at 1 h represented the mild defluorination of [^{18}F]FFMZ.

The specific binding of [^{18}F]FFMZ was proven by phosphorimaging of rat brain. The binding of [^{18}F]FFMZ was blocked by cold FMZ, and the specific uptake of [^{18}F]FFMZ decreased with time. Major uptake sites were the cortex, thalamus and cerebellum. This is consistent with previously reported central benzodiazepine receptor-rich sites in human brain [25,26].

In terms of normal human PET imaging, [^{18}F]FEFMZ distribution in the human brain matched known relative benzodiazepine receptor densities. However, slightly higher uptakes were observed in the frontal and temporal lobes compared to results obtained using [^{11}C]FMZ [25,26]. It is unknown whether this difference is due to affinity or metabolic differences of the PET agents.

With these results, we concluded that a new FMZ derivative, [^{18}F]FFMZ, is a promising PET agent for central benzodiazepine receptor imaging, as it can be conveniently labeled and it can specifically bind to the central benzodiazepine receptor.

Acknowledgments

This study was supported by grants from the Ministry of Science and Technology and from the Cancer Research Institute of the Seoul National University College of Medicine.

All animal studies were carried out in compliance with regulations of Seoul National University Hospital Clinical Research Center, and a human study was approved by Seoul National University Hospital Clinical Research Review Board and Radiation Safety Office.

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