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Experimental study of ^{99m}Tc-depreotide preparation and its affinity with A549 cell

Wei Xia[1,2], Xiaoping Zhang[1], Fei Yu[1], Peijun Wang[3], Haidong Cai[1], Renhua Hou[1], Guoyu Wang[2], Xueyu Yuan[1], Zhongwei LV[1]

[1]Department of Nuclear Medicine, 10th People's Hospital, Tongji University of Medcine School, Shanghai 200072, China, [2]Department of Nuclear Medicine, Shanghai Putuo Distict People's Hsopital, Shanghai 200060,China, [3]Department of Radiology, Tongji Hospital,Tongji University,Shanghai 200071, China

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1. ABSTRACT

The ^{99m}Tc-labeled agent, [^{99m}TcO]depreotide, has received regulatory approval in the United States and Europe for use in the detection of cancer. It is essential to establish a simple and reliable method of direct radiolabeling of ^{99m}Tc-depreotide and to investigate its specific receptor binding properties with human non small cell lung cancer (NSCLC) A549 cell *in vitro*. So we made some researches as follow: Depreotide was labeled with ^{99m}Tc using $SnCl_2$ as a reductant. Labeling efficiencies at different pH values and temperatures were compared. Radioreceptor assay was used to observe the uptake kinetics, stagnation and retention half time of ^{99m}Tc-depreotide in A549 cells. As the results of the investigation ,many facts is shown below: The labeling rate of pH 6.0 group was higher than that of pH 5.0 and pH7.0 groups. The labeling rate decreased when temperature increased from 15 °C to 50 °C. The uptake rate increased with rising temperature, and the maximum uptake was observed at 60min at 37 °C. The cleaning curves were similar at different temperatures, and the half cleaning time at 37 °C was 48min. The results showed that the optimal conditions for labeling depreotide with 99mTc was found to be below 15 °C at a pH lower than 6.0. Furthermore, at 37 °C, ^{99m}Tc-depreotid may have the potential as an ideal imaging agent for somatostatin receptors.

2. INTRODUCTION

Depreotide (cyclo-[(N-Me)Phe-Tyr-D-Trp-Lys-Val-Hcy]CH_2-CO.â-Dap-Lys-Cys-Lys.amide, P829) is a SSTR binding peptide and has been developed as a tumor-imaging radiopharmaceutical due to its optimal biodistribution, *in vivo* half-life, and somatostain receptor (SSTR) binding affinity[1]. The ^{99m}Tc-labeled agent, [^{99m}TcO]depreotide, has received regulatory approval in the United States and Europe for use in the detection of malignant lung cancer. [^{99m}TcO]depreotide binds to a wider range of somatostain receptor subtypes (SSTR2, SSTR3, and SSTR5)[2] than octreotide and its radiolabeled derivatives (mainly those binding to SSTR2) and, therefore, may be useful in diagnosing a broader range of tumor types. [^{99m}TcO]depreotide has shown promise in the detection of a variety of tumors, including lung cancer[3], lymphoma[4], breast cancer[5], thyroid carcinoma[6], colorectal carcinoma[7], and melanoma[8]. However, the labeling rate of ^{99m}Tc depreotide under different conditions has not been defined clearly, and the mechanism of receptor binding with the tumor cell at different temperatures has not been clarified.

The overall goal of the current project was to unambiguously determine the optimum conditions of radiolabelling depreotide (which was synthesized using

Fmoc solid-phase peptide synthesis (SPPS) as described previously[1,9]) with ^{99m}Tc, and to evaluate the biological behavior of ^{99m}Tc-depreotide while interacting with non small-cell lung cancer cell line A549.

3. MATERIALS AND METHODS

3.1. reagents and instruments

Symbols and abbreviations for amino acids generally followed IUPAC-IUB recommendations. Fmoc-protected L-amino acids, Rink amide MBHA resin, 2-Cl-TRT resin and most chemicals and solvents (including dimethyl sulfoxide (DMSO), 4-dimethylaminopyridine (DMAP), N,N'-diisopropylcarbodiimide (DIC), DMF, N,N-Diisopropylethylamine (DIEA), 1,2-ethanedithiol (EDT), trifluoroacetic acid (TFA), 1-Hydroxy-7-azabenzotriazole (HOAt), 1-Hydroxybenzotrizole (HOBt), 2-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate(HATU), O-(Benzotriazol-1-yl)-N,N,N',N'-tetra methyluronium tetrafluoroborate(TBTU), methanol and thioanisole) were purchased from GL BioChem (Shanghai, China) Ltd. Sephadex (G-25) was obtained from Shellgene bio. (Shanghai, China) Co.. Ethylenediamine tetraacetic acid (EDTA), ammonium acetate and ethanedithiol were purchased from Sigma-Aldrich (Shanghai,China) Trading Co., Ltd. Culture medium RPMI1640, fetal bovine serum (FBS), trypsinase and Cell Lysis Solution were purchased from HyClone, Fisher Scientific company. ^{99m}Tc-pertechnetate (^{99}Mo/^{99m}Tc generator) and $SnCl_2.H_2O$ were presented by Shanghai (China) GMS Pharmaceutical Co., Ltd. and Jiangsu Institute of Nuclear Medicine(China).

Preparation of phosphate buffer solution (PBS) was as follows. 36.14g $NaH_2PO_4{\cdot}2H_2O$ was dissolved in 1000 L H_2O to make the solution (A) of NaH_2PO_4 at 0.2mol/L, and 71.7g $Na_2HPO_4{\cdot}12H_2O$ was also dissolved in 1000 L H_2O to make the solution(B) of Na_2HPO_4 at 0.2mol/L. PBS of different pH values(pH6, pH7 and Ph7.4) were prepared. In addition, PBS (pH5) was prepared by adding 0.2mol/L NaOH to a specified volume of A.

For both analytical and preparative work of HPLC , the mobile phase consisted of (A) 0.1% TFA in H_2O and (B) 100% acetonitrile, or consisted of (A) 0.1% TFA in H_2O and (C)0.085% acetonitrile. For analytical HPLC, three methods were used: Method 1 - Column: Agillent1100 (Agillent Technologies Co.,USA) 5μ C18 100 Å, 150×4.6mm; mobile phase gradient: 30-90% B over 30 min; flow rate: 1.0 mL/min; Method 2 - Analytical HPLC utilized (Method 1) the same HPLC column but a different mobile phase: (A) 0.1% TFA in H_2O and (C)0.085% acetonitrile, mobile phase gradient: 5-65% C over 30 min; flow rate: 1.0 mL/min; Method 3 - Analytical HPLC utilized (Method 2) the same HPLC column and mobile phase but a different mobile phase gradient: 35-95% C over 30 min; flow rate: 1.0 mL/min. For preparative HPLC, three methods were used: Method 4 - Column: Dalian Elite (Dalian Elite Analytical Instruments Co., Ltd.,China), 10μ C18 300 Å, 250×50mm; mobile phase gradient: 40% B for 30 min, 30%-90%B for 30-90min and 90%-40%B for 90-120min; flow rate: 1.0 mL/min; Method 5 consisted of Waters DeltaPak 5μ C18 300 Å, 19.0 ×250mm column; mobile phase gradient: 20% B for 10 min,20%-50%B for 10-70min and 50%-90%B for 70-90min; flow rate: 1.0 mL/min; Method 6 - preparative HPLC utilized (Method 5) the same HPLC column and mobile phase but a different mobile phase gradient: 15% B for 10min,5%-45%B for 10-70min and 45%-95% B for 70-90min; flow rate: 1.0 mL/min.

3.2. Synthesis of depreotide

3.2.1. cyclopeptide, cyclo-[(N-Me)Phe-Tyr-D-Trp-Lys-Val-Hcy]

Cyclopeptide, cyclo-[(N-Me) Phe-Tyr-D-Trp-Lys-Val-Hcy] containing a SSTR binding sequence, was synthesized as follows (Figure 1). 2-Cl-TRT resin (1.0g) was soaked in DCM and dehydrated by anhydrous K_2CO_3 for 30 min. When the resin was swelling sufficiently, DCM was extracted. 65mg Fmoc-Hcy(trt)-OH and 0.5ml DIEA were dissolved in 10ml anhydrous DCM , and the mixed solution was poured in a vial in which the resin was loaded with a slow sparge of nitrogen for 1 h. Then the resin was washed three times with the mixed solution of DCM/methanol/DIEA(v/v/v=17/2/1,) to seal surplus chloridion. After being washed by DCM, DMF, methanol and DCM in turn, the resin was reacted with 20% pyridina and DMF for 1 min and 20 min, respectively. Finally, the resin was washed with DMF, DCM and DMF in sequence. So the first amino acid was linked with the resin and the From-protecting group was removed by confirmation with ninhydrin test.

The acibenzolar of amino acid, which was created by Fmoc-val-OH,HOBt,DIC with 4-time equivalent weight, was added in the vial containing the Hcy-linked resin for 2 h at room temperature. When the resin turned pale yellow as indicated by ninhydrin detection, condensation reaction of the amino acids was completed. The resin was then washed with DMF, DCM and DMF in sequence. When the resin turned dark blue as indicated by ninhydrin detection, the Fmoc-protecting group was removed. Fmoc-Lys(boc)-OH, Fmoc-D-Trp-OH, Fmoc-Tyr(tbu)-OH and Fmoc-(N-Me)-Phe-OH were added in the resin in sequence according to the aforementioned procedure, and these amino acids were joined as expected. 20ml lysate containing 1%TFA/DCM was added in the reaction vial, and 1 h later the resin was washed with the lysate. Then, the resin containing the lysate and the peptide synthesized were transferred into a round flask. In succession, the mixed solution was cooled in ice water and neutralized by DIEA. The mixed solution contained the precursor of cyclopeptide (N-Me)Phe-Tyr(tbu)-D-Trp-Lys(boc)-Val-Hcy(trt)as expected .

The solution containing the precursor of cyclopeptide was diluted with DMF to 500ml, in which HATU, HOAt and DIEA were dissolved. The flask was left overnight at room temperature until the reaction was completed. The solution was condensed to 100ml at 45 °C and then added with 100ml methanol and 100ml deionized water. The mixed solution was then filtered with a 0.45μm organic filtration membrane and purified by prepared

Preparation of 99mtc-depreotide

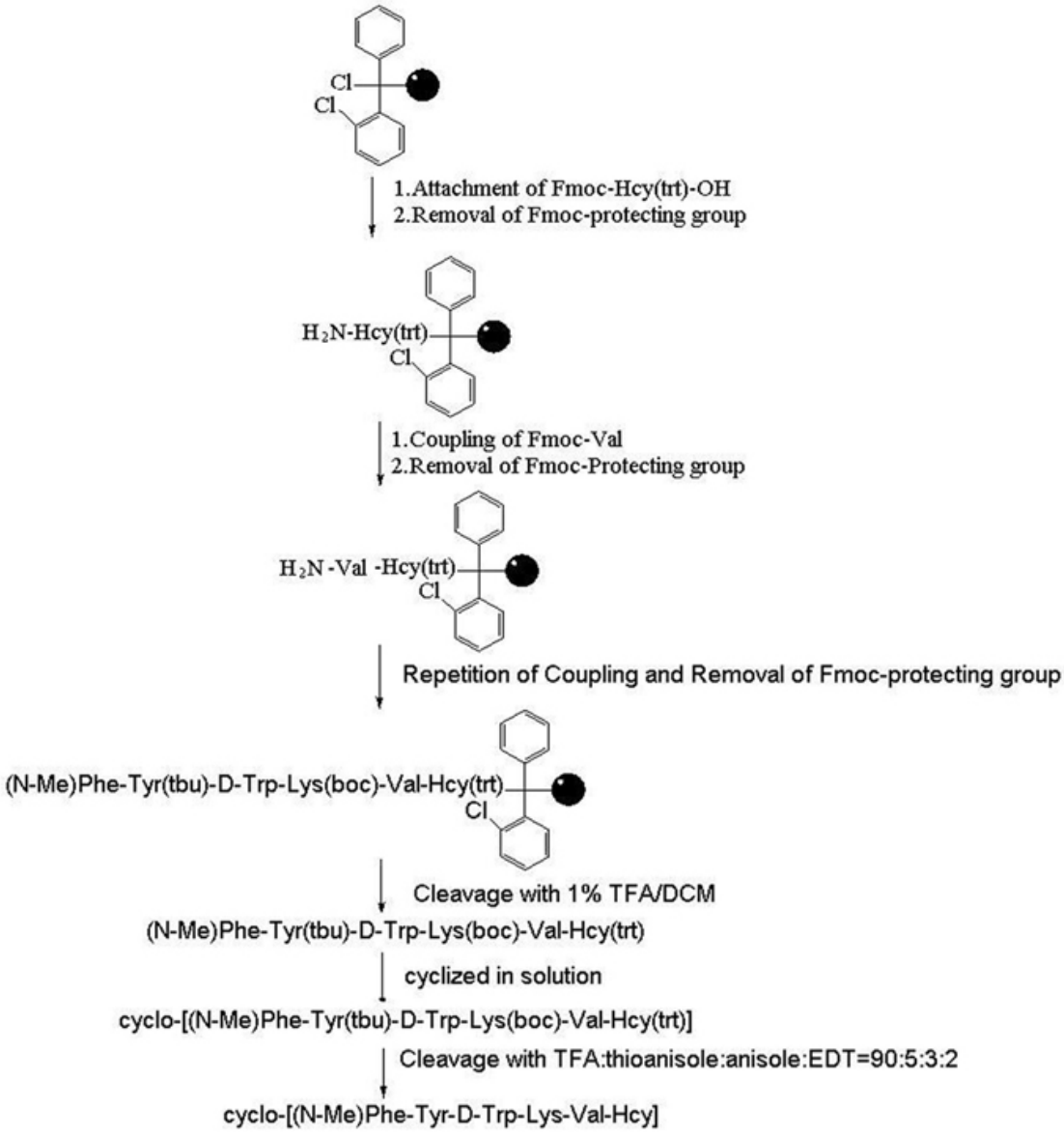

Figure 1. Procedure of cyclopeptide 2 synthesis.

HPLC (method 4). The fraction was analyzed by HPLC (method 1) to confirm that it was cyclopeptide 1, cyclo-[(N-Me)Phe-Tyr(tbu)-D-Trp-Lys(boc)-Val-Hcy(trt)].

120mg cyclopeptide 1 was added to the lysate solution containing TFA, thioanisole, anisole and EDT(V_{TFA}:$V_{thioanisole}$:$V_{anisole}$:V_{EDT}=90:5:3:2), and which would preserve stirred for 2 h with magnetic stirring. After addition of 100ml anhydrous ether, the mixed solution sample was placed in a refrigerator at 20 °C below zero for 1 h. The precipitate of the mixed solution was collected by centrifugation and dissolved in methanol after washes with anhydrous ether. Then the sample solution was filtered with a 0.45μm organic filtration membrane and purified by prepared HPLC (Method 5, vide supra). The product was analyzed by HPLC (method 2) to confirm that it was cyclopeptide 2, cyclo-[(N-Me)Phe-Tyr-D-Trp-Lys-Val-Hcy]. The result was also verified by MALDI-TOF mass spectrography.

3.2.2. linear peptide $ClCH_2$-CO.β-Dap(boc)-Lys(boc)-Cys(trt)-Lys(boc).amide

The linear peptide was designed to form a coordination complex with technetium at the Dap-Lys-Cys sequence (Dap) β-diaminopropioninc acid) (Figure 2). Acibenzolar of amino acid created by Fmoc-Lys(boc)-OH,HOBt,DIC with 4-time equivalent weight was added to the vial containing the resin and left for 2 h at room temperature. After three washes with CMF, DCM and DMF, the Lys-linked resin was processed by 20% pyridine for 5 min and DMF for 15 min to remove the Fmoc-protecting group. Then the mixed solution containing the sample was washed with DMF, DCM and DMF 3 times.

Preparation of 99mtc-depreotide

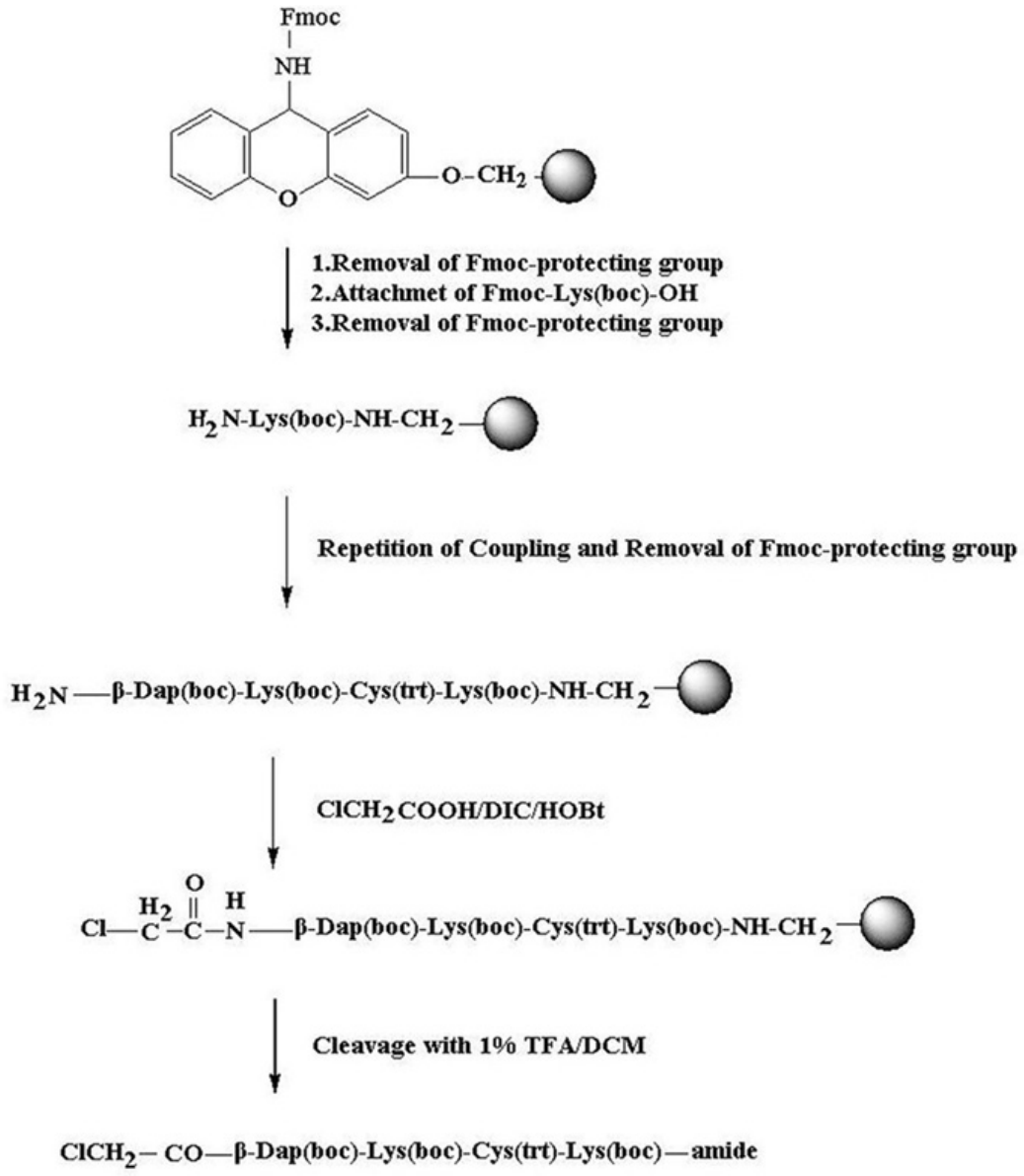

Figure 2. Procedure of linear peptide synthesis.

The reaction was confirmed by ninhydrin detection as expected.

Fmoc-Cys(trt)-OH, Fmoc-β-Dap(boc)-OH and $ClCH_2COOH$ were added to the resin in sequence according to the aforementioned procedure, and these amino acids were joined as expected. The resin was then washed with DMF, DCM and methanol 3 times, and dried at room temperature. In addtion, the fraction was collected after the resin was washed with 20ml lysate containing 1%TFA and DCM, and condensed to be an oleaginous sample. The sample was added to the anhydrous ether and centrifugated. The precipitate was collected by centrifugation, added with 50% acetonitrile in water, and acetonitriled to obtain the solid matter. The solid matter was analyse by analytical HPLC (method 3).

3.2.3. depreotide

Cyclopeptide 2 and linear peptide were dissolved in DMF under the condition of nitrogen protectiion, and the $NaHCO_3$ (Ph 10) solution was then added dropwise. The mixed solution was stirred with a magnetic stirrer and monitored by HPLC. After the reaction was completed, the mixed solution was diluted with 0.1% TFA and 50% acetonitrile in water, and filtered with a 0.45μm organic filtration membrane. The filtrate was re-dissolved in 0.1% TFA, and purified by prepared HPLC (method 6). The eluent was rotarily concentrated to make up crystals, and the crystals were added in the cutting fluids containing TFA/water/EDT/TIS(94:2.5:2.5:1) to react for 2 h at room temperature (Figure 3). Then the mixed solution was added suitable anhydrous ether, and was placed in a refrigerator

Preparation of 99mtc-depreotide

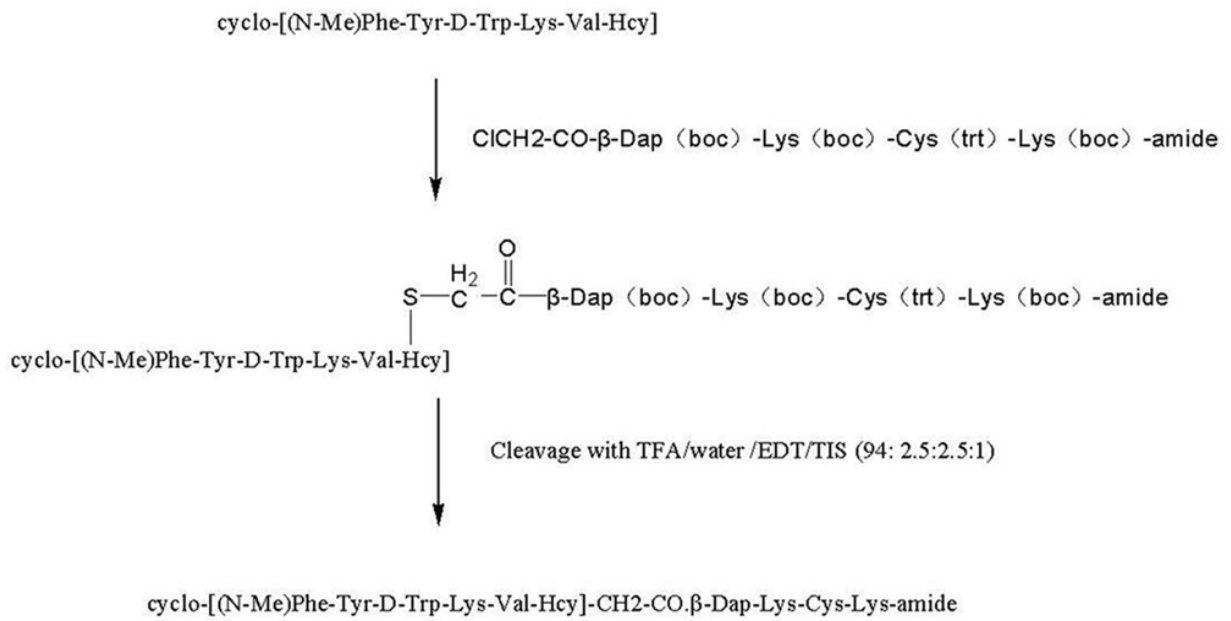

Figure 3. Procedure of depreotide synthesis.

at 4 °C for 1 h. The precipitate was collected by centrifugation, dissolved in 50% acetonitrile water, and cryodesiccated to depreotide crystals. Finally, the crystals were re-dissolved in 50% acetonitrile water and analyzed by analytical HPLC (method 2) and MALDI-TOF mass spectrography.

3.3. Preparation and Purification of ^{99m}Tc-depreotide

Depreotide was radiolabeled with ^{99m}Tc indirectly in 9 groups at different conditions (different pH values and temperatures, Table 1). Depreotide (20ug/each group) was dissolved in 2ml 0.2N PBS solution (pH5.0, 6.0 and 7.0). Sodium [^{99m}Tc] pertechnetate (1ml, ca.1500-2000MBq) containing stannous chloride as a reducing agent was added in the vial at different temperatures (15 °C, 37 °C and 50 °C) for 30 min.

The labeling rate of ^{99m}Tc-depreotide prepared under different conditions was determined with the two paper chromatographic systems: the first system used Xinhua No.1 chromatographic paper as the stationary phase and normal saline (SAS) as the mobile phase, and the second system used Xinhua No. 1 chromatographic paper as the stationary phase and methanol/ ammonium acetate (MAM) as the mobile phase (Figure 4).The sample from each vial was dropped on the origin point of the chromatographic paper. The developed strip was cut into two pieces, and radioactivity was measured separately. The bound ^{99m}Tc-depreotide and ^{99m}Tc non-mobiles stayed at the zone that was close to the origin(Rf=0-0.4), whereas the free ^{99m}Tc migrated to the top (Rf=0.8-1.0) in the first system. However, the bound ^{99m}Tc-depreotide and the free ^{99m}Tc migrated to the top (Rf=0.7-1.0), whereas the ^{99m}Tc non-mobiles stayed at the zone close to the origin (Rf=0-0.3) in the second system. The labeling rate of ^{99m}Tc-depreotide was calculated according to the equation stated in figure 4. Labeling values of ^{99m}Tc-depreotide prepared under different pH values and temperatures were compared.

The sample solution containing ^{99m}Tc-depreotide was purified by self-made SephadexG25 column, using the mixed solution containing 0.2mol/L PBS (pH 5.0) and 10% ethanol as the mobile phrase. After the eluent was collected into the cuvettes, radioactivity of each cuvette was measured. In addition, the chemical purity of ^{99m}Tc-depreotide was studied by UV spectrophotometry. The radiochemical purity (RCP) of ^{99m}Tc-depreotide purified was analyzed using the same protocol as that of determination of the labeling rate (Figure 4, vide supra). The stability of ^{99m}Tc-depreotide was observed by measuring the RCP of ^{99m}Tc-depreotide after standing at room temperature for 4 h.

3.4. SSTR receptor-binding assay

3.4.1. Cell culture

A549 cells were cultured in RPMI medium (RPMI1640, Invitrogen) with 10% FBS, 2mM glutamine, 50units/mL penicillin, and 50μg/mL streptomycin in a humidified 5% CO_2 atmosphere at 37 °C in T-flasks. Cells were routinely passed by treatment with trypsin (0.05%)/EDTA. Cells were passed every 3-4 days for several passages and reseeded from frozen stocks after reaching passage number 20.

3.4.2. The uptake rate

Cells were seeded in 24-well tissue culture plates, maintained overnight in RPMI medium, and then incubated with 100μl ^{99m}Tc-depreotide(37MBq/L) for 20 min to 2 h at different temperatures (4°C, 25°C and 37°C) (Table 2). After removing the medium, cells were washed twice with PBS (pH 7.4), digested with 0.25% trypsin, and

Preparation of 99mtc-depreotide

Table 1. The condition of radiolabeling depreotide with ^{99m}Tc

Groups	1	2	3	4	5	6	7	8	9
pH of PBS	5.0	5.0	5.0	6.0	6.0	6.0	7.0	7.0	7.0
T(°C)	15	37	50	15	37	50	15	37	50

Table 2. The labeling rate of depreotide with ^{99m}Tc under different conditions $\bar{X}$

pH, T	15 °C	25 °C	37 °C	50 °C
pH 5	64.28±1.38	62.31±1.25	61.05±1.87	58.02±2.00
pH 6	70.95±0.84	68.32±1.53	66.19±2.06	59.11±1.65
pH 7	59.22±2.62	57.98±2.55	57.09±2.55	54.61±0.80

T: °C

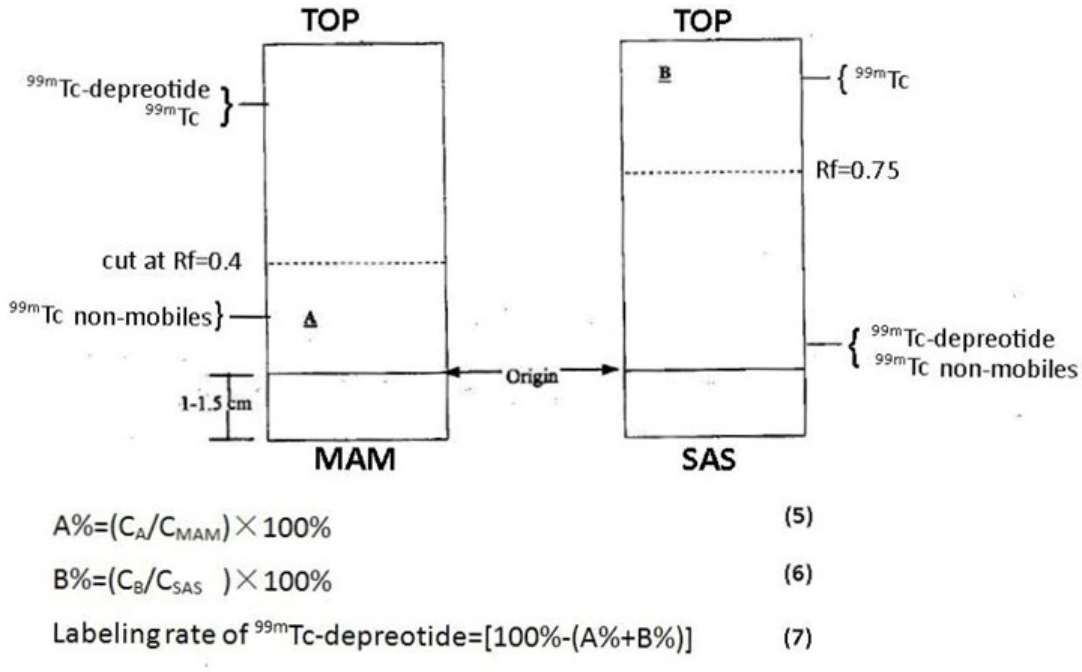

Figure 4. Paper chromatography of determining the labeling rate. Note:CA:radiocounting of A segment. CMAM: total radiocounting of the chromatographic paper of the MAM system. CB:radiocounting of B segement.CSAS: total radiocounting of the chromatographic paper of the SAS system.

collected by centrifugation at 2400 rpm (800g) for 5 min for measurement of radiocounting with a GC 1200γ radioactive counter (USTC Chuangxin Co., Ltd. Zonkia Branch). The uptake rate of the cells under different conditions was calculated using Equation 1: uptake rate (%) = (radiocounting of cells/the total radiocounting)×100%. (1).

In addition, cell retention of ^{99m}Tc-depreotide was observed. A549 cells, grown in 24-well plates, were incubated with ^{99m}Tc-depreotide for 2 h at 4°C, 25°C and 37°C. After removal of the medium, cells were washed twice with PBS (pH 7.4). The medium without ^{99m}Tc-depreotide was then added to the plates and incubated from 0 min to 90 min at 4°C,25 °C and 37°C. Cells were collected by centrifugation and measured for radiocounting (Cn). The cell retention rate under different conditions was calculated using Equation 2: The retention rate(%) = (C_n/C_0) × 100% (2). (note:C_n: radiocounting of the cells incubated with the medium for different time; C_0: radiocounting of the cells incubated with the medium at the right beginning (zero time)).

Furthermore, the internalization efficiency, time needed for ^{99m}Tc-depreotide to internalize into A549 cells, was observed. A549 cells were incubated with ^{99m}Tc-depreotide from 5 min to 120 min at 37°C. The medium was removed rapidly at 5 min, 15 min, 30 min, 60 min, 90 min and 120 min. The plates were first washed twice with PBS (pH7.4) at 4°C, and then with the same PBS after centrifugation. The radiocounting of cells was measured as the total radiocounting of the cells (C_t). The cells were then left in acidic PBS (pH 2.5) for 10 min to remove ^{99m}Tc-depreotide bond to the surface of cytomembrane, and the radiocounting of the acidic PBS was measured as the radiocounting of the cell surface binding ^{99m}Tc-depreotide (Cs). Finally, the cells were washed with neutral PBS (pH 7.4) twice, and measured for radiocounting as the internalized radiocounting (C_i) of cells. The percentage of internalization (% int) was expressed as Equation 3. The

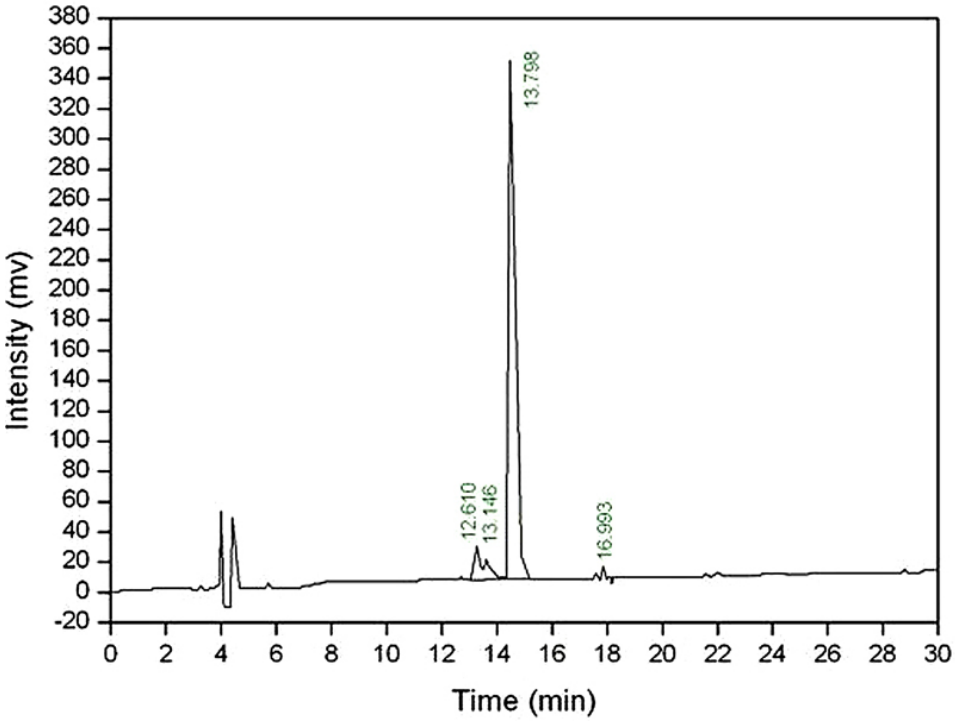

Figure 5. HPLC of cyclopeptide: the main peak in the chromatogram is cyclo-[(N-Me)Phe-Tyr-D-Trp-Lys-Val-Hcy] and its isolated purity is 91.52%.

binding rate of cell surface(B_{sur}%) with ^{99m}Tc-depreotide was represented by Equation 4: % int =Ci/Ct×100% (3) %B_{sur}=Cs/Ct×100% (4)

4. RESULTS

4.1. Synthesis and HPLC, MS Characterization of cyclopeptide, linear peptide and depreotide

The synthetic protocol for the preparation of the depreotiide described herein was designed such that pharmacophore (cyclopeptide) and chelator moieties (linear peptide) were prepared separately and subsequently conjugated via a sulfhydryl alkylation reaction involving a homocysteine residue or N-methylhomocysteine on the pharmacophore peptide and an N-terminal chloroacetyl on the chelator peptide. Purity of cyclopeptide is an important issue for binding to SSTR. This study used analytic HPLC to show purity of the cyclopeptide as 91.52% by HPLC peak analysis (Figure 5). The cyclopeptide was analyzed by MALDI-TOF mass spectrometry (Figure 6). The most abundant peak (m/z =854.7) was assigned to $M+H^+$ (cyclo-[(N-Me)Phe-Tyr-D-Trp-Lys-Val-Hcy]+H^+), and the second abundant peak (m/z=877.6) was assigned to $M+Na^+$ (cyclo-[(N-Me)Phe-Tyr-D-Trp-Lys-Val-Hcy]+ Na^+). HPLC peak analysis showed that purity of the linear peptide as 86.44% (Figure 7). Depreotide was synthesized by linking the cyclopeptide and the linear peptide (vide supra) and its purity was 95.29% as shown by analytic HPLC. The observed mass of the most abundant peak (m/z=1369.6) was assigned to $M+H^+$ (depreotide+H^+) (Figure 8). Within the error of the experiments, both analyses are consistent with the formulation shown in figure 9, with the formula $C_{65}H_{94}N_{16}O_{13}S_2$; $MW_{average}$=1370.

4.2. The labeling rate and RCP of ^{99m}Tc-depreotide

Each chromatographic paper was cut into two at the broken line, and radiocounting of each segment was measured. The labeling rate was calculated according to the equations stated in figure 4. Table 1 summarizes the labeling values of ^{99m}Tc-depreotide under different conditions. Figure 10 is a histogram designed according to table 2, indicating that the labeling rates were significantly different between the different temperatures and pH values. The labeling rate of pH 6.0 group was higher than that of pH 5 and pH 7 at the same temperature, and the labeling rate of all the three groups reduced when temperature increased from 15 °C to 50°C in this study.

4.3. Purity and stability of ^{99m}Tc-depreotide

^{99m}Tc-depreotide was purified by self-made SephadexG25 column (vide supra). The eluate was collected in the cuvette at 0.5ml per cuvette in turn and the total number of the cuvettes was 26. The cuvettes were measured for radiocounting by a GC 1200 γ radioactive counter and analyzed by a UV spectrophotometer at 280nm. The results were showed in figure 11 and figure 12. Two peaks were observed in figure 11 during measurement. The result of UV spectrophotometry indicated the first peak corresponded to ^{99m}Tc-depreotide, whereas the second peak corresponded to free $^{99m}TcO_4^-$. RCP of ^{99m}Tc-depreotide purified was analyzed (vide supra) and the result was 90.21% . ^{99m}Tc-depreotide was kept at room temperature for 4 h, and RCP was also analyzed and the result was 87.31%.

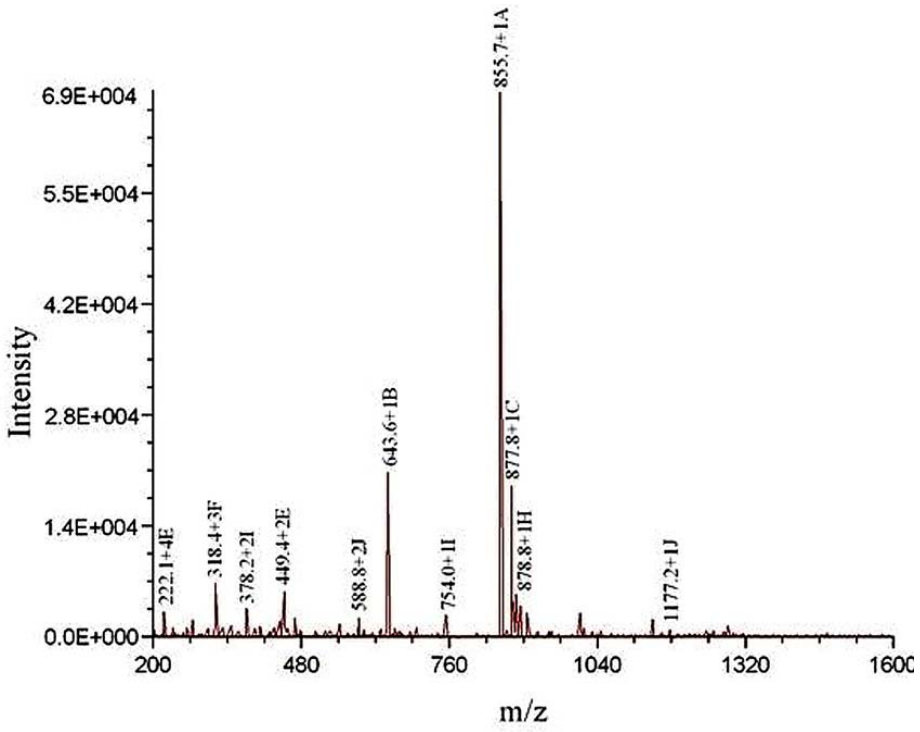

Figure 6. The mass spectrum of cyclopeptide: the molecular weight is 854 (M+H=855) according to the main peak of the spectrum.

4.4. SSTR Receptor-binding

4.4.1. The cellular uptake rate of ^{99m}Tc-depreotide

The uptake rate of ^{99m}Tc-depreotide by A549 cells at the different conditions was recorded as shown in table 3, which indicated that the uptake rate increased steadily with the time at 4°C or 25°C, and reached the peak at 60 min at 37°C. In addition, the uptake rate rose with temperature increasing from 4 °C to 37°C at the same time points.

4.4.2. The cell retention rate of ^{99m}Tc-depreotide

The cell retention rate of ^{99m}Tc-depreotide in A549 cells at different temperatures was recorded as shown in table 4, which showed that the retention rate was slightly higher at 37°C than that at 4°C. The mean retention rate was 62.70% and 63.58% at 4 °C and 37°C at 15 min, respectively, though the difference was not significant ($P>0.05$). But there was significant difference in the retention rate between the three groups at 4°C and at 37°C from 30 min to 90 min ($0.01<$all values of $p<0.05$), indicating that the retention rate was independent of temperature in the initial stage, but subsequently, it was likely to be dependent on temperature to a certain degree.

The percentage of internalization (%int) and the binding rate of cell surface (%B_{sur}) are listed in table 5. The data showed that %int increased with time, implying that the receptor that bond with ^{99m}Tc-depreotide had good activity at 37 °C. But there was no significant change in %B sur, indicating that there was abundant SSTR expression on the A549 cell surface and might bind steadily with ^{99m}Tc-depreotide.

5. DISCUSSION

Peptides are compounds containing amino acids joined by peptide bonds. They regulate many bodily functions, acting as chemical messengers, neurotransmitters, and are highly active stimulators or inhibitors. The term *small peptide* is used to refer to peptides of less than 30 amino acids in size, or 3500 Daltons in molecular weight[10–11]. In recent decades, they have been used for diagnostic and therapeutic purposes with regard to their small sizes, ease in preparation , and ability to attach bifunctional agents from their -C and -N terminals in nuclear medicine. Various receptors are overexpressed in particular tumor types, and peptides binding to these receptors can be used to visualize tumor lesions scintigraphically. Thus, radiolabeled peptides have the potential to be used as carriers for delivery of radionuclides to tumors, infarcts, and infected tissues for diagnostic imaging and radiotherapy[11].

Somatostatin, a cyclic peptide initially isolated from the hypothalamus, has been shown to have an inhibitory effect on secretion of many hormones including growth hormone. Since the initial discovery, several related somatostatin peptides have been identified, including tetradecapeptide compound 1 (Figure 9), which was designated as somatostatin 14 [1]. These peptides are widely distributed throughout the body. They can be found in the gut, various exocrine and endocrine glands, and most organs. However, the observation that tumors expressed a higher density of somatostatin receptors (SSTR) than did normal tissues[12] has generated a great deal of interest in

Table 3. The uptake ratio of ^{99m}Tc-depreotide by A549 cell at different conditions

	n	4 °C	25 °C	37 °C
20min	5	7.247±0.216	14.811±0.183	15.493±0.170
40min	5	11.393±0.550	15.403±0.238	16.03±0.091
60min	5	13.393±0.335	16.383±0.115	20.75±0.481
120min	5	14.65±0.457	18.463±0.352	18.917±0.093

Table 4. The cell retention rate of ^{99m}Tc-depreotide in A549 cell at different temperatures

	n	4 °C	25 °C	37 °C
0min	5	100	100	100
15min	5	62.70±1.97	63.24±2.21	63.58±2.19
30min	5	52.94±2.89	53.47±2.12	58.13±2.35
60min	5	41.99±2.97	44.20±2.85	46.76±2.72
90min	5	26.53±1.97	28.91±2.35	31.83±2.92

Table 5. The percentage of internalization and cell surface binding

	5 min	15 min	3 0min	60 min	90 min	120 min
% int	19±1.2	21.7±1.0	22.5±0.5	27.1±1.1	33.9±1.0	84.4±2.4
%B_{sur}	18±1.5	15.8±0.9	14.1±0.5	15.4±2.6	17.6±0.4	16.3±0.3

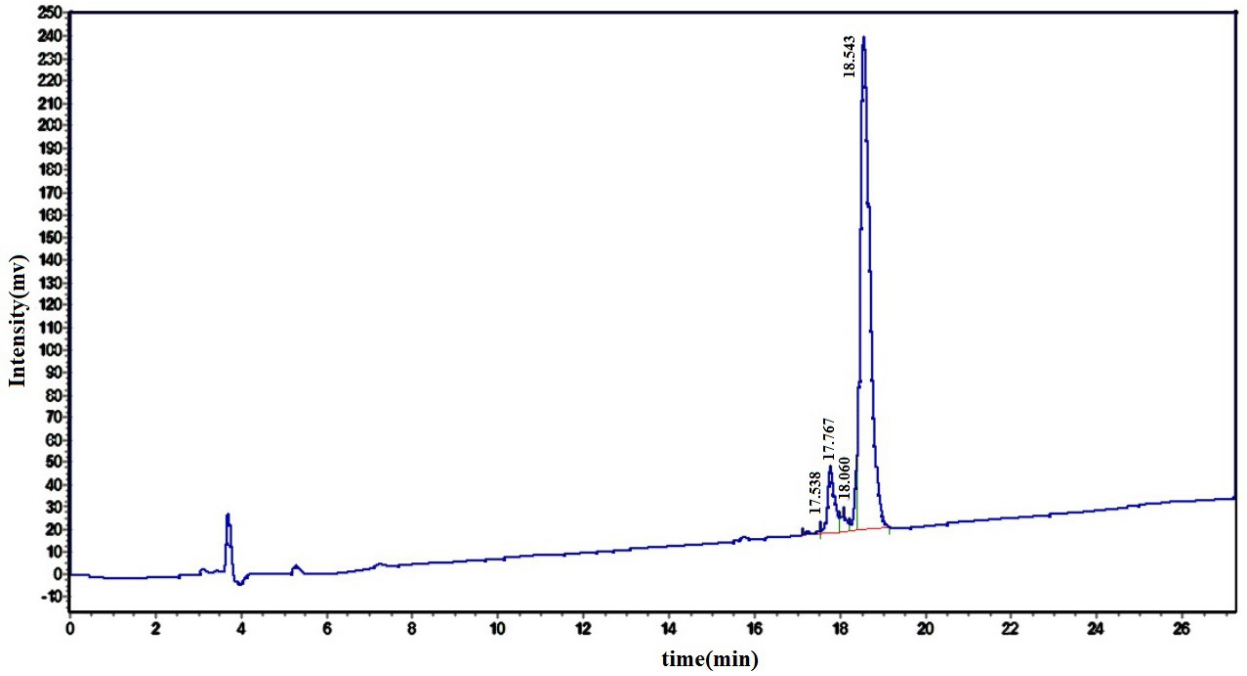

Figure 7. HPLC chromatogram of the linear peptide: the main peak in the chromatogram is [$ClCH_2$-CO.beta-Dap(boc)-Lys(boc)-Cys(trt)-Lys(boc).amide] and its isolated purity is 86.44%.

radiolabeled somatostatin analogs for tumor imaging[13-17]. The first peptide-based receptor binding radiopharmaceutical was 111In-DTPA-octreotide[18]. Octreotide (D-phenylalanyl-1-cysteinyl-1-phenylalaninyl-D-tryptophyl-1-lysyl-1-threonyl-n-(2-hydroxy-1-(hydroxymethyl)propyl)-1-cysteinamide cyclic (2-7)-disulfide) is a shortened peptide analog of somatostatin, which is a cyclic disulfide-containing peptide hormone of 14 amino acids present throughout the central nervous system.

The success of 111In DTPA-octreotide stimulated the search for receptor-specific imaging agents based on 99mTc peptides. A somatostatin derivative radiolabeled with 99mTc would presumably offer advantages over an 111In product, because the 99mTc radionuclide has a more favorable photon abundance for gamma imaging and a more convenient half-life (6 h vs 60 h). In addition, it is more readily available.

The radiolabeled product ^{99m}Tc-depreotide has been confirmed to be a promising radiodiagnostic agent of SSTR expressing tumors. This paper aimed to uncover the simple and reliable method of directly radiolabeling ^{99m}Tc-depreotide. Fisrtly, the cyclic hexapeptide (the SSTR binding sequence of depreotide) and the linear tetrapeptide (chelator moieties of depreotide) were synthesized by solid-

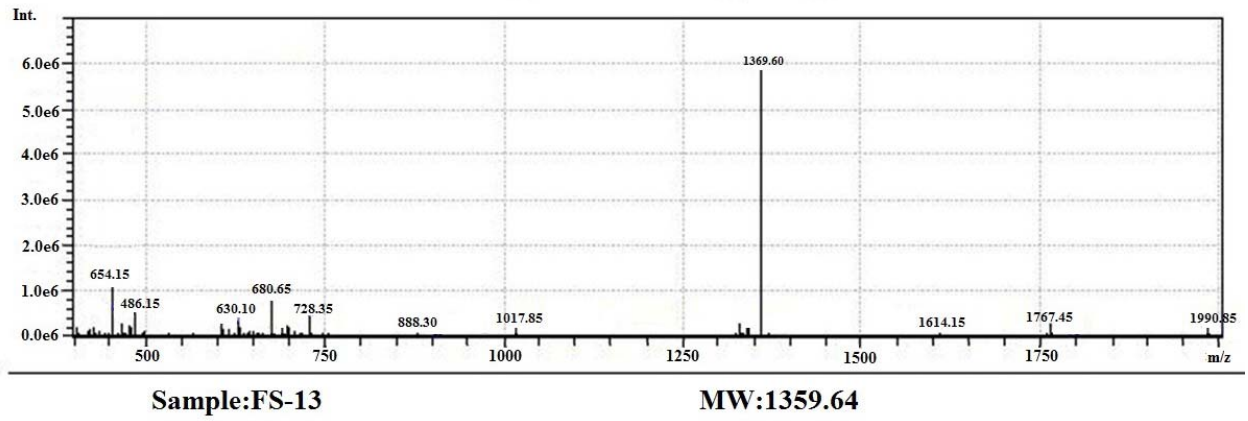

Figure 8. The MALDI-TOF mass spectrum of depreotide: the molecular weight is 1368(M+H=1369) according to the main peak of the spectrum.

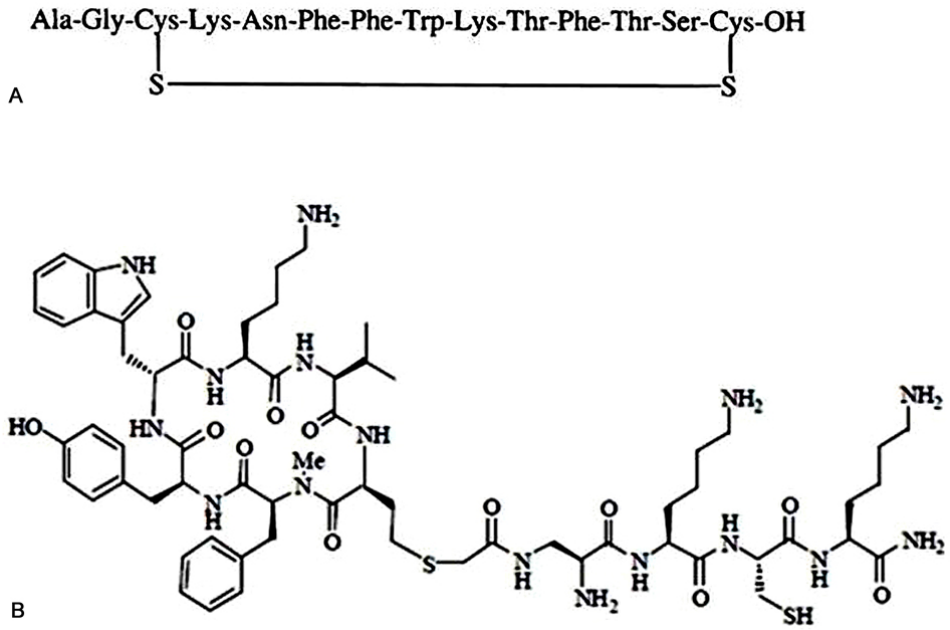

Figure 9. A. tetradecapeptide compound formulation. B. Depreotide formulation.

phase combinatorial synthesis separately, and subsequently both were conjugated by solution-phase combinatorial synthesis and depreotide obtained was of high purity(95.29%). Subsequently, the depreotide was radiolabeled with ^{99m}Tc directly at different conditions, and the labeling rate was highest at 15 °C and pH 6 in this study. The result indicated that the condition of ^{99m}Tc-depreotide should be subacidic and not at a high temperature. Furthermore, the high RCP of the ^{99m}Tc-depreotide was attainable and keep for over 4 h, from which it was deemed that ^{99m}Tc-depreotide was highly stable.

Lung cancer is one of the most prevalent cancers and the leading cause of cancer mortality worldwide. There is a clear correlation between tumor stage and survival. Thus, patients with small tumors without nodal or distant metastasis (stage 1A) have a 60–80% 5-year survival rate after surgery, whereas patients with metastatic disease have less than a 5% 5-year survival rate[19]. Tumor stage is one of the decisive prognostic parameters and crucial in choosing an appropriate therapeutic option. In anatomical imaging such as helical computed tomography (CT), the nodal (N-) status is based entirely on nodal size, which correlates unsatisfactorily with tumor involvement [20]. Other molecular/anatomical functional imaging techniques have therefore attracted increasing attention during the past decade.

Positron emission tomography (PET) with ^{18}F-2-fluoro-2-deoxy-D-glucose (FDG), which is based on the recognition of altered metabolism, has been used for tumor staging in countries with access to PET cameras. metaanalysis of use of this imaging technique in the

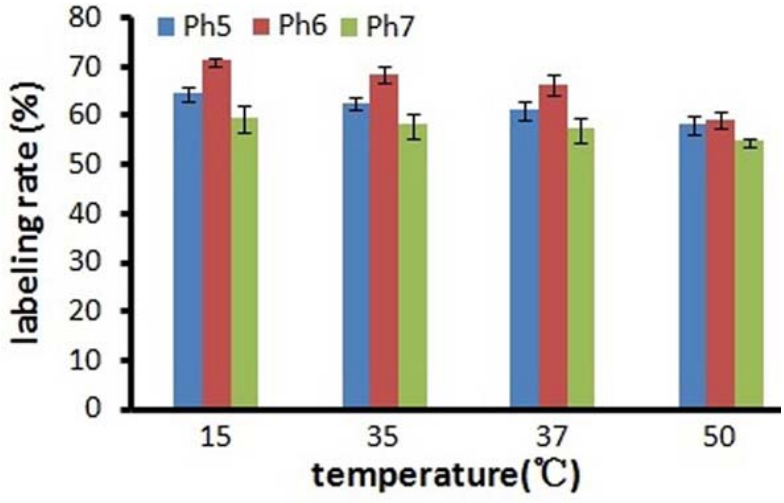

Figure 10. The labeling rate of depreotide with ^{99m}Tc under different conditions. The labeling rates were significantly different between the different temperatures and pH values,and labeling rate is higher in the condition of 15 °C and pH 6 than other condition.

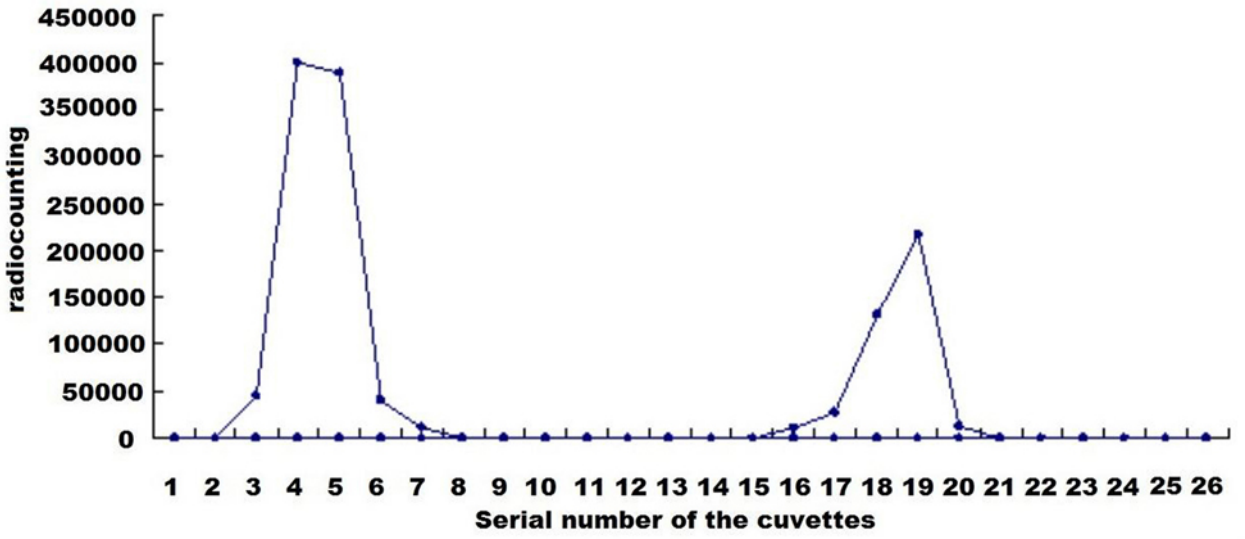

Figure 11. Radiocounting of serial cuvettes containing the eluate during the procedure of purification of ^{99m}Tc- depreotide. The first peak correspond to ^{99m}Tc-depreotide, whereas the second peak correspond to free $^{99m}TcO_4^-$.

detection of nodal spread revealed a sensitivity of 83% and a specificity of 89%[21]. Several other tracers, such as ^{67}Ga citrate and ^{99m}Tc-sestamibi, have previously been studied in the imaging of lung cancer, but these tracers are not in routine use owing to their low accuracy. Recently, a radiolabelled somatostatin analogue, ^{99m}Tc-depreotide, was approved by The National Swedish Board of Health and Welfare for evaluation of pulmonary nodules. A multicenter trial including 114 patients with indeterminate lung nodules showed a high sensitivity (96.6%) of somatostatin receptor scintigraphy with ^{99m}Tc-depreotide with respect to lung cancer [22].

The biological behavior of ^{99m}Tc-depreotide together with non small-cell lung cancer cell line A549 was evaluated in this study. The result confirmed that the uptake and internalization of ^{99m}Tc-depreotide by A549 cell was dependent greatly on temperature and time, while the retention rate was only dependent slightly on temperature. In addition, ^{99m}Tc-depreotide had good biological activity at 37°C, a suitable temperature for diagnostic application in humans. Binding affinity of ^{99m}Tc-depreotide was optimized *in vitro* to A549 human lung carcinoma cell.

A simple and reliable method of directly radiolabeling ^{99m}Tc-depreotide was established, and it was confirmed that finer affinity of specific receptor binding properties of ^{99m}Tc-depreotide with human non small cell lung cancer (NSCLC) A549 cell *in vitro* in this study.

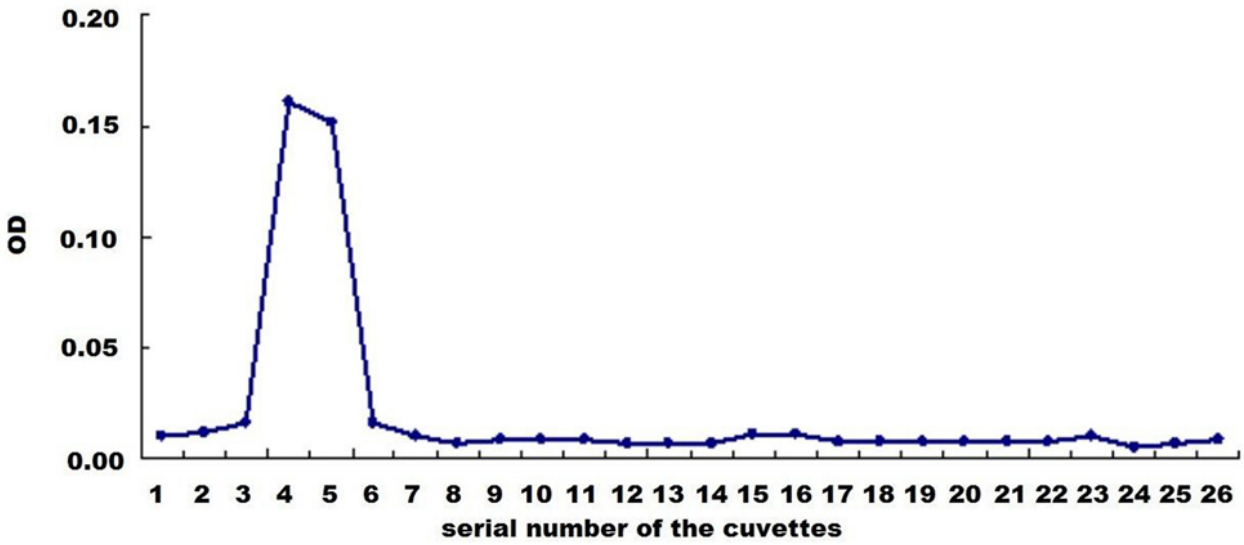

Figure 12. The measured value the eluate of time serial cuvettes during the procedure of purification of ^{99m}Tc- depreotide by UV spectrophotometer testing. The peak correspond to ^{99m}Tc-depreotide.

6. CONCLUSION

The highly purified ^{99m}Tc-depreotide was obtained by direct radiolabeling method. The results from current study showed that ^{99m}Tc-depreotide can be applied as a potential somatostatin receptor imaging agent for NSCLC.

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Abbreviations: NSCLC: non small cell lung cancer; SSTR: somatostain receptor; DMSO: dimethyl sulfoxide; DMAP: 4-dimethylaminopyridine; DIC: N,N'-diisopropylcarbodiimide; FBS: fetal bovine serum; PET: Positron emission tomography; FDG: ^{18}F-2-fluoro-2-deoxy-D-glucose; NSCLC: non small cell lung cancer

Key Words: Somatostatin, ^{99m}Tc, Depreotide, Radionuclide

Send correspondence to: Zhongwei LV, Department of Nuclear Medicine, Shanghai 10th People's Hospital, Tongji University School of Medicine, 301 Yanchang Road, Shanghai 200072, China, Tel: 086-21-66301009, Fax: 086-21-66301051, E-mail: lzw_doctor@yeah.net