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RADIOLABELING OF SUBSTANCE P WITH LUTETIUM-177 AND BIODISTRIBUTION STUDY IN RAT PANCREATIC TUMOR XENOGRAFTED *NUDE* MICE

E. B. DE ARAÚJO, P. B. PUJATTI AND J. MENGATTI

Directory of Radiopharmacy – Nuclear and Energy Research Institute (IPEN/CNEN)
Av. Prof. Lineu Prestes, 2242 – Cidade Universitária USP – Butantã – São Paulo – SP – Brazil
Tel: 55 11 31339547, Fax: 55 11 31338956, E-mail:ebaraujo@ipen.br

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Abstract – Pancreatic tumor (PT) is a neuroendocrine neoplasm that usually origin metastases in the respiratory and gastrointestinal tract. The presence of peptide receptors at the cell membrane of PT constitutes the basis of the clinical use of specific radiolabeled ligands for its diagnosis and targeted therapy. Substance P (SP), an 11-amino acid peptide which has an important role in modulating pain transmission trough neurokinin type 1 (NK1r) and 2 receptors (NK2r), may play a role in the pathogenesis of PT, because approximately 10% of these tumors overexpress NK1r. The aim of the present work was to produce a pure and stable SP analog (DOTA-SP) radiolabeled with lutetium-177 (^{177}Lu), and to evaluate its *in vivo* target to AR42J pancreatic tumor cells in *Nude* mice, in other to verify if SP can be used in this pancreatic tumor detection and treatment. Substance P was successfully labeled with high yield (>99%) at optimized conditions and kept stable for more than 72 hours at 2-8º C and 4 hours in human plasma. Biodistribution studies showed that SP excretion was mainly performed by renal pathway. In addition, ^{177}Lu-DOTA-SP showed higher uptake by tumor than normal pancreas, indicating the presence of NK receptors in AR42J pancreatic tumor.

Key words: Substance P, lutetium-177, radiolabeling, pancreatic tumor.

INTRODUCTION

Neuroendocrine tumors (NETs) are a heterogeneous group of neoplasms originating from endocrine cells, which are characterized by the presence of secretory granules as well as the ability to produce biogenic amines and polypeptide hormones. These tumors originate from endocrine glands, such as adrenal medulla, pituitary and parathyroids, as well as endocrine islets within the thyroid or pancreas and dispersed endocrine cells in the respiratory and gastrointestinal tract (6).

Abbreviations: DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; **DOTA-SP,** substance P targeted to DOTA; **FBS,** fetal bovine serum; **HPLC,** high performance liquid chromatography; **IAEA,** International Atomic Energy Agency; **ITLC-SG,** instant thin layer chromatography; **NETs,** neuroendocrine tumors; **NK1r,** neurokinin type 1 receptor; **NK2r,** neurokinin type 2 receptor; **PC,** pancreatic cancer; **PT,** pancreatic tumor; **SP,** Substance P; **^{177}Lu,** lutetium-177 isotope; **%IA/g,** percentage uptake of injected activity per gram of organ

Substance P (SP) is an 11-amino acid neuropeptide which is known as a powerful member of a family of tachykinins, characterized by the C-terminal sequence Phe-X-Gly-Leu-Met-NH_2, where X represents either phenylalanine, isoleucine or valine. It has been well established that SP plays an important role in modulating pain transmission from peripheral and central primary afferents through neurokinin 1 and 2 receptors and this peptide may be also involved in the pathogenesis of inflammatory diseases (8). SP receptors are also found in brain, lymphoid tissues, vessels, gut smooth muscle, airway glands and bronchiolar walls. In receptor autoradiography of tumor specimens *ex vivo*, SP receptors were found to be more abundant than somatostatin receptors on glioblastoma, medullary thyroid cancer, non-small cell lung cancer and pancreatic carcinoma, but the incidence is low in the last two. In addition, SP receptors were also found on peritumoral vessels associated with those tumors (7).

In recent years, a number of new developments in targeted therapies have emerged (4) and the presence of peptide receptors and transporters at the cell membrane of several

NETs constitutes the basis of the clinical use of specific radiolabeled ligands. Because around 27% of human pancreatic tumors express SP receptors (5), the introduction of radiolabeled SP analogs for peptide receptor imaging and radiotherapy can be a focus of interest to characterize and treat those tumors. Several radionuclides have been applied to label peptides for radionuclide therapy and 6.7 day half-life lutetium-177 (^{177}Lu) has emerged as a promising short-range β emitter for this purpose. The mean range of lutetium-177 β^- particles ($E\beta_{max}$ = 497 keV) is 670μm, making this radionuclide ideal for treating micro-metastatic disease. Because it also emits γ rays (208keV, 11% abundance), imaging of ^{177}Lu-labeled endoradiotherapeutic agents is also possible (2).

The goal of the present work was to produce a pure and stable substance P analog (DOTA-SP) radiolabeled with Lutetium-177 (^{177}Lu), and to evaluate its *in vivo* target to AR42J pancreatic tumor cells in *Nude* mice, in other to verify if SP can be used in this pancreatic tumor detection and treatment.

MATERIAL AND METHODS

Reagents

DOTA-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (DOTA-SP, piChem, Austria) was provided from International Atomic Energy Agency (IAEA) and $^{177}LuCl_3$ was obtained from NRG (Netherlands). All other chemicals and reagents required for experiments were of analytical grade and purchased from Sigma Aldrich Chemical Co.

Study of radiolabeling conditions

Several studies were done to establish the ideal labeling conditions for obtaining the highest yield of labeled substance P. DOTA-SP (0.5 - 10 μg), 0.4 mol/L sodium acetate buffer (0.2 mL, pH 4.5) and 92.5 MBq of $^{177}LuCl_3$ (in 0.05N HCl, specific activity 800-920 GBq/mg) were heated at different temperatures (70 – 90° C) for different times (15 – 30 minutes). All reagents of these experiments were prepared with Chelex 100 treated free metal water.

Radiochemical purity determination

Instant thin layer chromatography (ITLC-SG) was applied to determine free lutetium, with citrate/citric acid buffer pH 5.0 as solvent (Rf of labeled peptide was 0.1-0.3 and Rf of free lutetium was 0.9-1.0) (1). Radiochemical purity was also determined by high performance liquid chromatography (HPLC, Shimatzu, Japan) using RP C_{18} columns (Waters, 4.0 x 150 mm, 5 μm) with radioactivity (Shell, EUA) detection, flow rate of 1.5 mL/minute with a linear gradient of 10-90% (v/v) 0.1% TFA / acetonitrile in 0.1% TFA / H_2O for 15 minutes and the composition was maintained for another 10 minutes.

Stability of radiolabeled SP

To determine the *in vitro* stability of ^{177}Lu-DOTA-SP the preparation was stored at 2-8° C for different times (1 to 9 days) or in fresh human serum samples were spiked with ^{177}Lu-DOTA-SP (37 MBq/mL) and incubated for 1, 4 and 24 hours, followed by ITLC-SG analysis. All experiments were performed in triplicate.

Cell culture

AR42J rat pancreatic tumor cells were maintained in RPMI 1640 supplemented 10% fetal bovine serum (FBS) and 1% antibiotics. Cell culture was incubated at 37° C in an atmosphere containing 5% CO_2. The cells were subcultured weekly.

In vivo studies

Animals

Animal studies were performed in accordance with United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations as well as institutional guidelines. The subjects were 4- to 6-week old male *Nude* mice xenografted with rat AR42J pancreatic tumor cells (2 x 10^6 / mouse) in 0.1 mL phosphate-buffered saline. Biodistribution studies were performed in AR42J tumor mice with tumors averaging 0.5 - 1.0 g.

Biodistribution study

The radioactive substance P (1.85 MBq/100μL/mouse) was injected in the mice lateral tail vein. After 1, 4 or 24 hours post injection the animals were sacrificed the blood was collected. Then, the mice were dissected and both vital organs and tumor were isolated, washed, weighed and their respective radioactivity was measured in an automatic gamma counter (Packard). The biodistribution of labeled SP was calculated as percentage uptake of injected activity per gram of organ and tumor (%IA/g).

Data analysis

Statistical analyses were performed using Prism 3.0 software. Results were subjected to Student´s *t*-test and expressed as mean $\pm$ SD.

RESULTS

Radiolabeling of SP with Lutetium-177

Different Substance P mass (0.5 – 10 μg) were radiolabeled using 92.5 MBq (2.5 mCi) of radionuclide and the results are shown in Fig. 1. High radiolabeling yields (> 95%) were achieved when 5 and 10 μg of DOTA-SP reacted with lutetium-177. When the mass of DOTA-SP was reduced to 2.5 μg the radiochemical purity decreased to 39.13 $\pm$ 3.2 and no radiolabeling reaction occurred using 1 and 0.5 μg of the peptide.

The table I shows the effects of the radiolabeling time and temperature on the radiochemical purity of labeled SP. The reactions were performed using 0.25 mCi/μg (1.85MBq/μg) of ^{177}Lu-DOTA-SP. Labeling yield determined by ITLC was satisfactory in all times and temperatures analysed.

The Fig. 2 shows a typical radioactive HPLC profile of both 177-lutetium and ^{177}Lu-

DOTA-SP. The labeled peptide (Rt = 7.02 minutes) could be clearly separated from free lutetium (Rt = 1.15 minutes).

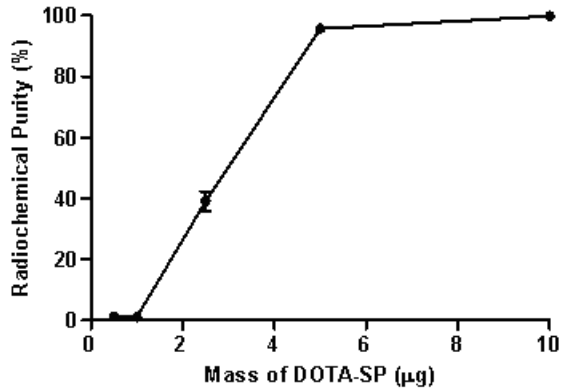

Figure 1. Radiochemical purity of labeling conditions using different mass of DOTA-SP. The reactions were performed at 90° C for 30 minutes. A radiochemical purity of 99.81 ± 0.1% was obtained when 10 µg of DOTA-SP was used in the reactions (n = 2).

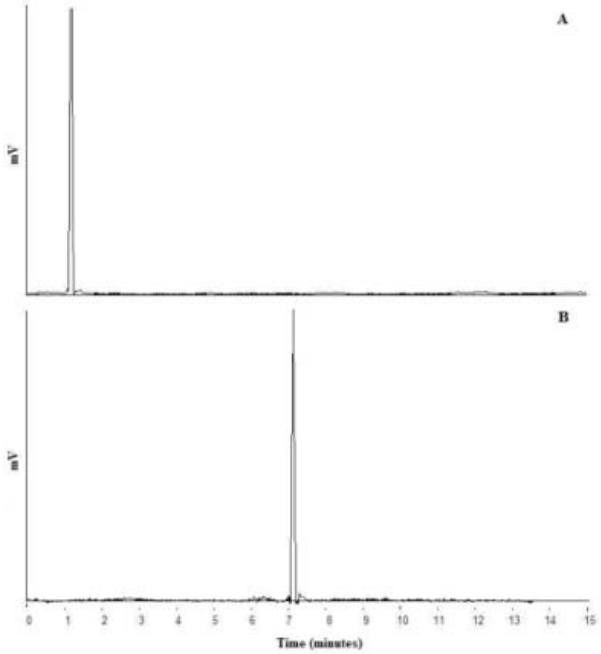

Figure 2. HPLC profiles of (A) free 177-lutetium and (B) radiolabeled Substance P (9.25 MBq/µg).

Stability of ^{177}Lu-DOTA-SP

The stability of labeled peptide was evaluated by instant thin layer chromatography after storage at 2-8° C and incubation at 37° C in fresh human serum. The Table II shows the results obtained from the samples stored at 2-8° C for different times. ^{177}Lu-DOTA-SP remained stable at this temperature and the radiochemical purity were higher than 90% for more than 216 hours of storage.

After incubation of the radiolabeled peptide with fresh serum, no differences in the ITLC-SG chromatogram were detected until 4 hours of incubation, suggesting a metabolic stability of ^{177}Lu-DOTA-SP (Table III).

In vivo studies

Results from biodistribution studies using the ^{177}Lu-DOTA-SP were performed in *Nude* mice bearing AR42J tumor and are presented in Table IV as %IA/g. Appreciable radioactivity could be detected in the kidneys in all times analysed, indicating peptide excretion by renal pathway. The uptake on intestines and stomach is probably related to the binding to specific receptors, mainly found in the gastrointestinal tract. In addition, it could be observed high tumor uptake when compared to normal pancreas, probably indicating the presence of NK1 receptors in AR42J pancreatic tumor cells.

DISCUSSION

Pancreatic cancer (PC) is the most fatal gastrointestinal malignancy, with only 3% to 5% overall 5-year survival rate. PC is mostly refractory to current therapeutic regimens, rendering it nearly 100% letal, and making it the fourth leading cause of cancer death in both men and woman. Thus, novel therapeutic strategies are urgently required, and these most likely arise from a better understanding of the biochemistry of pancreatic tumor cells (8).

In this work we reported the preparation of an analog of substance P – DOTA-SP – radiolabeled with lutetium-177. The physical properties of the 177-lutetium are particularly attractive to irradiate small tumor mass and the presence of a gamma emission of low energy allow to the acquisition of scintigraphic images before and after therapy. Substance P was successfully labeled with this radionuclide (>99% yield) at optimized conditions and kept stable for more than 72 hours at 2-8º C and 4 hours in human serum. These results indicate that ^{177}Lu-DOTA-SP can be an useful tool for *in vivo* studies because of its easy preparation and high stability.

Involvement of SP in the carcinoid syndrome has been suggested (1) and that´s why we also purposed to study the presence of neurokinin receptors in AR42J pancreatic tumor. Our preliminary results showed a favorable

Table 1. Effects of the reaction's time and temperature on the radiochemical purity of ^{177}Lu-DOTA-SP. The reactions were performed with 10 μg of DOTA-SP, 92.5 MBq of $^{177}LuCl_3$ and at pH 4.5 (n = 2).

Temperature of the reaction (° C)	**Time of the reaction (minutes)**	**Radiochemical Purity (%)**
70	30	95.3 ± 3.3
80	30	99.7 ± 0.1
90	30	99.8 ± 0.1
90	25	99.1 ± 0.8
90	20	98.1 ± 1.5
90	15	98.1 ± 0.1

Table 2. *In vitro* stability of radiolabeled substance P after storing at 2-8° C for different times (n = 3).

	Time of storage at 2-8° C				
Radiochemical	Immediately	24 hours	48 hours	72 hours	168 hours
Purity (%)	99.81 ± 0.1	98.36 ± 0.3	97.90 ± 0.3	95.58 ± 0.1	94.94 ± 0.4

Table 3. *In vitro* stability of ^{177}Lu-DOTA-Substance P (9.25 MBq/μg) in human serum at 37° C (n= 6).

Storage time in human serum (37ºC)	**Radiochemical Purity (%)**
Immediately	99.8 ± 0.1
1 hour	98.39 ± 0.7
4 hours	94.61 ± 3.3
24 hours	67.45 ± 0.2

Table I4. Biodistribution of ^{177}Lu-DOTA-SP in AR42J tumor mice 1, 4 and 24 hours post intravenous injection (n = 4).

	% IA/g of organ or mL of blood		
Organs	**1 hour**	**4 hours**	**24 hours**
Tumor	**1.27 ± 0.3**	**1.18 ± 0.3**	**0.70 ± 0.1**
Blood	1.79 + 0.2	1.18 ± 0.3	0.01 ± 0.01
Lungs	1.58 ± 0.4	1.72 ± 0.4	0.29 ± 0.1
Heart	1.06 ± 0.1	0.95 ± 0.1	0.19 ± 0.1
Liver	0.93 ± 0.2	0.82 ± 0.2	0.69 ± 0.1
Kidneys	17.76 ± 6.9	14.89 ± 6.58	6.53 ± 1.3
Pancreas	0.79 ± 0.1	0.64 ± 0.1	0.18 ± 0.1
Spleen	1.13 ± 0.4	0.98 ± 0.3	0.14 ± 0.1
Small Intestine	3.14 ± 1.2	2.63 ± 0.86	2.13 ± 0.3
Large Intestine	1.36 ± 0.5	1.12 ± 0.4	0.82 ± 0.2
Stomach	1.04 ± 0.2	0.99 ± 0.3	0.73 ± 0.1
Skeletal Muscle	0.67 ± 0.4	0.59 ± 0.2	0.06 ± 0.01
Brain	0.13 ± 0.01	0.12 ± 0.04	0.02 ± 0.01
Thyroid*	1.06 ± 0.3	0.10 ± 0.1	0.02 ± 0.01

* % IA per organ.

biodistribution kinetic of the compound, that presents fast blood clearance, resulting in rapid and effective uptake in the tumor. The peptide is mainly excreted by the kidneys, which may constitute the target organ for dosimetric considerations. In addition, ^{177}Lu-DOTA-SP showed an higher uptake by tumor than pancreas (0.70 ± 0.1% ID/g), probably indicating the usefulness of this radiolabeled peptide in NK receptors detection. In addition, this radiopeptide present a therapeutical potencial because of its retention by tumor cells, indicated by significative radioactivity in tumor 4 and 24 hours p.i. Further investigations are in development in order to make a formulation to produce a high activity ^{177}Lu-DOTA-SP radiopharmaceutical and to evaluate its target to other tumor cells *in vivo* and *in vitro*.

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Other articles in this theme issue include references (10-17).

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