Radiolabeling of Substance P with Lutetium-177 and biodistribution study in AR42J pancreatic tumor xenografted *Nude* mice

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Abstract. Pancreatic tumor (PT) is a neuroendocrine neoplasm that usually origin metastases in the respiratory and gastrointestinal tract. In recent years, new developments in targeted therapies have emerged and the presence of peptide receptors at the cell membrane of PT constitutes the basis of the clinical use of specific radiolabeled ligands. Substance P, an 11-amino acid peptide which has an important role in modulating pain transmission trough neurokinin 1 and 2 receptors (NKr), may play a role in the pathogenesis of PT, because approximately 10% of these tumors overexpress NKr. 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. ^{177}Lu (half-life 6.7 days) has both β and γ-emissions suitable for radiotherapy and imaging respectively. Substance P was successfully labeled with high yield (>99%) at optimized conditions and kept stable for more than 72 hours at 4º C and 24 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.

1. 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 the adrenal medulla, the pituitary, and the parathyroids, as well as endocrine islets within the thyroid or the pancreas and dispersed endocrine cells in the respiratory and gastrointestinal tract [1].

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. 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 [2]. 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 (MTC), non-small cell lung cancer and carcinoma of pancreas, but the incidence is low in the latter two. In addition, SP receptors were also found on peritumoral vessels associated with those tumors [3].

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 ^{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 possible [6].

The goal of this 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.

2. Materials and Methods

2.1. Reagents

DOTA-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (DOTA-Substance P - piChem) was provided from 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..

2.2. 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 ~100 MBq of $^{177}LuCl_3$ (in 0.05N HCl, specific activity 920GBq/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.

2.3. Radiochemical purity determination

Instant thin layer chromatography (ITLC) 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) [9]. Radiochemical purity was also determined by HPLC (Shimatzu) using RP C_{18} columns (Waters, 4.0 x 150 mm, 5 μm) with radioactivity (Shell) 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.

2.4. Stability of radiolabeled SP

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

2.5. Cell culture

AR42J rat pancreatic tumor cells were maintained in RPMI1640 supplemented 10% FBS and 1% antibiotics. Cell culture was incubated at 37°C in an atmosphere containing 5% CO_2. The cells were subcultured weekly.

2.6 In vivo studies

2.6.1. 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.

2.6.2. Biodistribution study

The radioactive substance P (0.185 MBq/100μL/mouse) was injected in the lateral tail vein. After 1 hour post injection the animals were sacrificed the blood was collected. Then, the mice were dissected and both vital organs and tumor were isolated, 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 dose per gram of organ and tumor (%ID/g).

2.8. Data analysis

Statistical analysis were performed using Prism 3.0 software. Results were subjected to Student´s *t*-test and expressed as mean ± SD.

3. Results

3.1 Radiolabeling of SP with Lutetium-177

Different Substance P mass (0.5 – 10 μg) were radiolabeled using 100 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 reduced to 2.5 μg the radiochemical purity decreased to 39.13 ± 3.2 and no radiolabeling reaction occurred using 1 and 0.5 μg of the peptide.

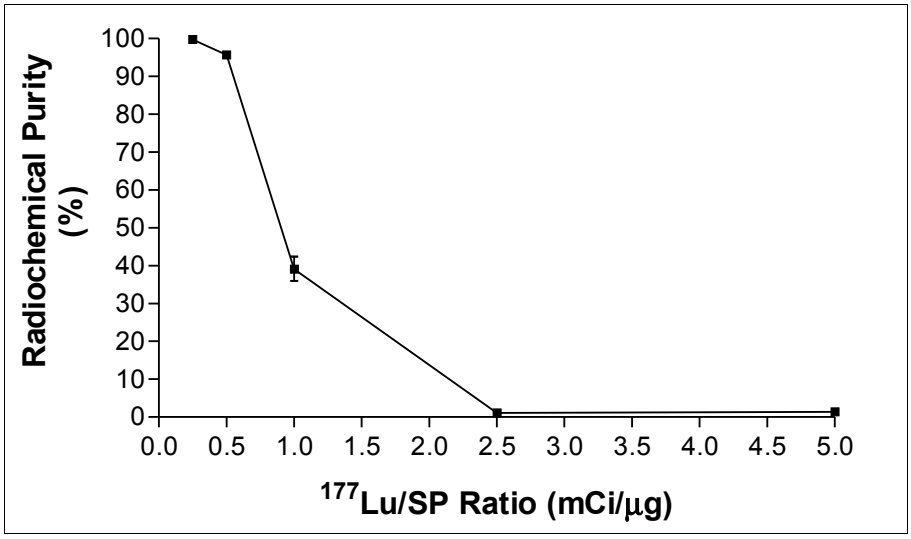

FIG. 1. Radiochemical purity of labeling conditions using different ^{177}Lu/SP ratios (mCi/μg). The reactions were performed at 90°C for 30 minutes. At 0.25 mCi / μg a radiochemical purity of 99.81 ± 0.1% was obtained.

The tables I and II show the effects of the radiolabeling time and temperature on the radiochemical purity of labeled SP. The reactions were performed at 90°C using 0.25 mCi / μg for different times. Labeling yield determined by ITLC was satisfactory in all times and temperature analysed.

Table I. Effect of the incubation time on radiochemical purity of ^{177}Lu-DOTA-SP.

	Radiolabeling time			
	15 minutes	20 minutes	25 minutes	30 minutes
Radiochemical Purity (%)	99.15±0.8	98.17±1.5	98.13±0.1	99.81±0.1

Table II. Effect of the temperature on radiochemical purity of ^{177}Lu-DOTA-SP.

	Radiolabeling temperature		
	70°C	80°C	90°C
Radiochemical Purity (%)	95.33±3.2	99.73±0.1	99.81±0.1

The FIG. 2 shows a typical radioactive HPLC profile of both lutetium-177 and ^{177}Lu-DOTA-SP. The labeled peptide (Rt = 7.02 minutes) can be clearly separated from free lutetium (Rt = 1.15 minutes).

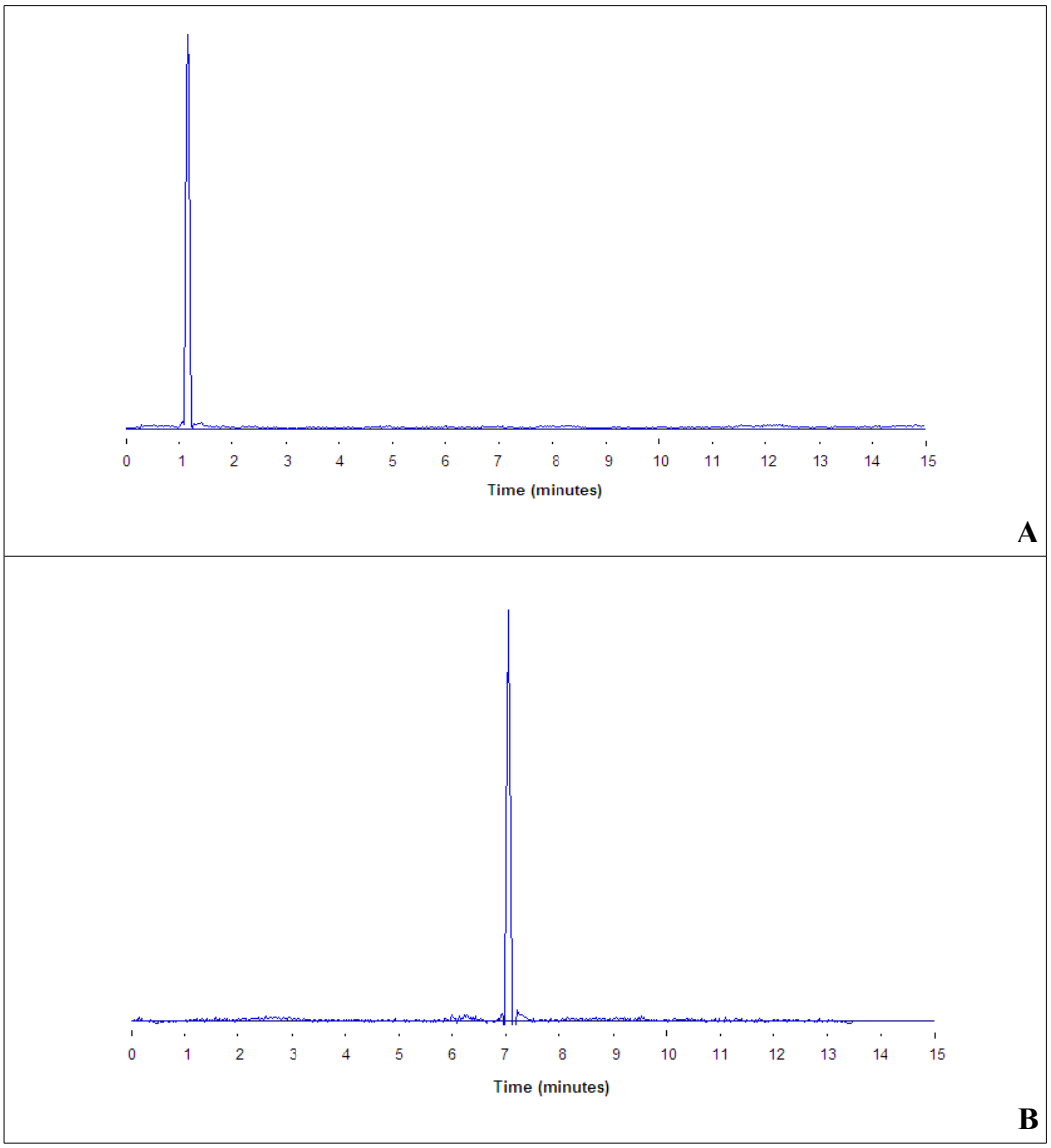

FIG. 2. HPLC profiles of (A) free lutetium-177 and (B) radiolabeled substance P.

3.2. Stability of ^{177}Lu-DOTA-SP

The stability of labeled peptide was evaluated by instant thin layer chromatography after storage at 4°C and incubation at 37°C in human plasma. The Table III shows the results obtained from the samples stored at 4°C at different times. ^{177}Lu-DOTA-SP remain stable at this temperature and the radiochemical purity were higher than 90% for more than 216 hours of storage.

Table III. *In vitro* stability of radiolabeled substance P after storing at 4°C for different times.

	Storage time at 4°C				
	Immediately	48 hours	72 hours	168 hours	216 hours
Radiochemical Purity (%)	99.81 ± 0.1	98.36 ± 0.3	97.90 ± 0.3	95.58 ± 0.1	94.94 ± 0.4

After incubation of the radiolabeled peptide with fresh plasma, no differences in the ITLC chromatogram were detected in all times analyzed, suggesting a metabolic stability of ^{177}Lu-DOTA-SP (Table IV).

Table IV. *In vitro* stability of radiolabed substance P in human serum at 37°C.

	Storage time in human serum (37ºC)			
	Immediately	1 hour	4 hours	24 hours
Radiochemical Purity (%)	99.81±0.1	98.39±0.7	99.24±0.5	99.41±0.2

3.3. In vivo studies

Preliminary results from biodistribution studies using the ^{177}Lu-labeled substance P were performed with *Nude* mice bearing AR42J tumor and are presented in Table V as the percentage of injected dose per gram of tissue (%ID/g). Appreciable radioactivity could be detected in the kidneys and intestine and stomach, indicating peptide excretion by renal pathway and SP binding to its 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 NK receptors in AR42J pancreatic tumor cells.

Table V. Biodistribution of ^{177}Lu-DOTA-SP in AR42J tumor mice 1 hour post intravenous injection.

Organs	Biodistribution of ^{177}Lu-DOTA-SP 1 hour p.i. %ID/g
Tumor	1.27 ± 0.3
Blood	1.18 ± 0.29
Lungs	1.58 ± 0.47
Heart	1.06 ± 0.03
Liver	0.93 ± 0.26
Kidneys	17.76 ± 6.99
Pancreas	0.79 ± 0.1
Spleen	1.13 ± 0.44
Small Intestine	3.14 ± 1.3
Large Intestine	1.35 ± 0.51
Stomach	1.04 ± 0.27
Skeletal Muscle	0.68 ± 0.42

4. 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 now 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 4ºC and 24 hours in human plasma. 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 [9] and that´s why we also purposed to study the presence of neurokinin receptors in AR42J pancreatic tumor. Our preliminary results showed a favorable 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 constitutes the target organ for dosimetric considerations. In addition, ^{177}Lu-DOTA-SP showed an higher uptake by tumor (1.27 $\pm$ 0.3 %ID/g) than pancreas (0.70 $\pm$ 0.1% ID/g), probably indicating the usefulness of this radiolabeled peptide in NK receptors detection. Further investigations are in development to predict the therapeutical potencial of this radiopharmaceutical evaluating the tumor uptake after times longer than 4 hours.

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6. References

[1] Rufini, V., Calcagni, M.L., Baum, R.P., Imaging of neuroendocrine tumors. Seminars in Nuclear Medicine 36:228-247 (2006)
[2] van Hagen, P.M., Breeman, W.A.P., Reubi, J.C., Postema, P.T.E., van den Anker-Lugtenburg, P.J., Kwekkeboom, D.J., Laissue, J., Waser, B., Lamberts, S.W.J., Visser, T.J., Kreening, E.P., Visualization of the thymus by substance P receptor scintigraphy in man. European Journal of Nuclear Medicine 23(11):1508-1513 (2005)
[3] Stangelberger, A., Schally, A.V., Varga, J.L., Zarandi, M., Szepeshazi, K., Armatis, P., Halmos, G., Inhibitory effect of antagonists of bombesin and growth hormone-releasing hormone on orthotopic and intraosseous invasiveness of PC-3 human prostate cancer in Nude mice. Clinical Cancer Research 11:49-57 (2005)
[4] Knight, L.C. Radiolabeled peptides for tumor imaging. In: Welch, M.J.; Redvanly, C.S. Handbook of Radiopharmaceuticals: radiochemistry and applications. Chichester: Wiley, 2003. Cap. 23.
[5] Oyen, W.J.G., Bodei, L., Giammarile, F., Maecke, H.R., Tenvall, J., Luster, M., Brans, B., Targeted therapy in nuclear medicine – current status and future prospects. Annals of Oncology April 13, 2007
[6] Ehlers, R.A., Kim, S., Zhang, Y., Ethridge, R.T., Murrilo, C., Hellmich, M.R., Evans, D.B., Townsend, C.M., Evers, B.M., Gut peptide receptor expression in human pancreatic cancers. Annals of Surgery 231 (6):838-848, 2000
[7] Zalutsky, M.R., Radionuclide Therapy. In: Vértes, A., Nagy, S., Zoltán K, Handbook of Nuclear Chemistry. Netherlands: Kluwer Academic Publishers, 2003. v.4.
[8] Guha, S., Eibl, G., Kisfalvi, K., Fan, R.S., Burdick, M., Reber, H., Hines, O.J., Strieter, R., Rozengurt, E., Broad-spectrum G protein-coupled receptor antagonist, [D-Arg^{1},D-Trp^{5,7,9},Leu^{11}]SP: a dual inhibitor of growth and angiogenesis in pancreatic cancer. Cancer Research 65(7):2738-2745, 2005
[9] Breeman, W.A.P., VanHagen, M.P., Visser-Wisselaar, H.A., van der Pluijim, M.E., Koper, J.W., Setyono-Han, B., Bakker, W.H., Kwekkeboom, D.J., Hazenberg, M.P., Lamberts, S.W.J., Visser, T.J., Kreening, E.P., In vitro and in vivo studies of substance P receptor expression in rats with the new analog [Indium-111-DTPA-Arg^{1}]Substance P. The Journal of Nuclear Medicine 37(1): 108-117, 1996