Development of Radioactively Labelled Cancer Seeking Biomolecules For Targeted Radiotherapy

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INTRODUCTION

Within the framework of the above project we are studying the labelling of biomolecules, peptides and antibodies, with radionuclides emitting β^- and γ radiation. More specifically, for the time being, we have investigated the labelling of peptides with Re-188 and of antibodies with Sm-153 and Re-188. The radiolabelled derivatives are further evaluated *in vivo* for possible application in Oncology. For these radiobiological studies we are trying to apply ectopic and orthotopic tumour animal models and to develop, in collaboration with other national and foreign institutes, proper imaging devices for small animal imaging.

^{188}Re-Labelled Peptides

INTRODUCTION

We have studied the preparation and evaluation of two Rhenium-188 labelled peptides. The first was Lanreotide, a peptide, which, labelled with In-111 and/or Y-90, is currently investigated for the detection of a variety of human tumours. The second one was a decapeptide, RGD derivative, which labelled with Tc-99m, has been already evaluated for the localization of metastatic melanoma lesions in man and thrombi in experimental animals. In the present study we are evaluating this peptide labelled also with Re-188. A brief description of the studies performed till now is presented here.

Structure of the Peptides Studied

Lanreotide: β-(2-Naphthyl)-Ala-Cys-Tyr-Trp-Lys-Val-Cys-Thr-amide

RGD-derivative: Arg-Gly-Asp-Ser-Cys-Arg-Gly-Asp-Ser-Tyr-OH

Arg-Gly-Asp: RGD moiety

Experimental

Chemistry: Decapeptide-Rhenium Complex Formation (RGD derivative)

Initially the complexion of the above decapeptide with non radioactive Rhenium was studied by reacting, at room temperature, a methanolic solution of the peptide with sodium perrhenate, at a molar ratio 1:1, for three days. After evaporation of the solvent, the supernatant was purified by HPLC. The HPLC fraction, corresponding to the Re-RGD derivative was isolated and studied. The structure of the complex formed was investigated by ^{1}H-NMR and electron mass spectrometry.

^{1}H-NMR Identification

1D and 2D (TOCSY, ROESY) NMR spectra were recorded in 90% H_2O/10% D_2O at 25 °C on a Bruker Avance DRX-400 spectrometer. Suppression of the water peak was achieved by employing the Bruker Watergate pulse sequence. DSS was used as an external reference.

Electrospray Ionization Mass Spectrometry (ESI-MS)

A solution of 20 ul of the peptide in water of analytical grade was inserted into the electrospray source. The temperature was arranged at 70°C. A solution of 5% of acetic acid in acetonitrile was used for the elution. Positive electrospray conditions were applied, with 3.8 KVolts capillary voltage and 0.38 Kvolts lens voltage. The counter electrode potential was set at 150 Volts, suitable for peptide detection.Both the synthetic peptide and its respective Renium complex were identified by ESI-MS.

^{188}Re-Labelling of the Peptides

Na $^{188}ReO_4$ Reduction: Initially, perrhenate, obtained by a $^{188}W/^{188}Re$ generator, was reduced to a lower oxidation state in order to get bound to the peptide, using an aqueous solution of stannous chloride, ascorbic acid and citric acid. The reduction time was evaluated from 15 to 120 min.

Peptides Labelling Procedure

Complexation of the reduced Rhenium to the peptide was studied according to a method applied for the preparation of Technetium-peptide complexes. It comprises the following steps:

- An intermediate labile Rhenium-188 complex was initially created by the addition of the reduced Rhenium to a mixture of sodium gluconate, stannous chloride and sodium bicarbonate. The ^{188}Re-gluconate formation was followed by chromatographic methods.
- A quantity of 0.4 ml of the above Rhenium complex, containing 2.0-10.0 mCi of Re-188 was added to 0.5 ml of an aqueous peptide solution, containing 1.0 mg/ml of the biomolecule.
- The mixture was left to react for a period, ranging from 15-60 min, at Room Temperature and at 90^{0}C. Peptide labelling was studied by HPLC.

According to the above method, no previous reduction of the peptide S-S bonds is necessary, for Rhenium labelling.

Radiochemical Study

Evaluation of the Labelling System

- $Na^{188}ReO_4$ reduction was studied by Instant Thin Layer Chromatography on Silica Gel (ITLC-SG) in acetone and in methylethylketone.
- The formation of the intermediate ^{188}Re-gluconate complex was investigated by ITLC in methylethylketone and by Reverse Phase HPLC using a gradient system with solvent A: 0.5% trifluoroacetic acid (TFA) in water and B:0.5% TFA in acetonitrile. We used an μ-Bondapack C_{18} Reverse Phase column, 300 x 3.9 mm I.D. The separation was tested by both UV and radioactivity detectors, coupled in line.
- ^{188}Re-peptide complexes formation was studied by HPLC analysis, with the solvent system referred above.

In-Vitro Stability Investigation

The *in-vitro* stability of the radiolabelled species was studied at different temperatures, for diverse time intervals.

Radiobiological Evaluation of the ^{188}Re-Labelled Peptides

Biodistribution Study

Evaluation of the *in-vivo* behaviour of the radiolabelled peptides was performed, conventionally, by biodistribution in normal Swiss mice (average weight 25 g) after i.v. injection in the tail vein. *In-vivo* studies were performed in compliance with the European legislation. Animal protocols have been approved by the Hellenic authorities.

***In-Vitro* Cell Binding Study**

Cell binding was studied according to well-established methods. For ^{188}Re-Lanreotide an epithelial-like Colo 205 cell line (CCL 222 of the American Type Culture Collection) isolated from a human colon adenocarcinoma was used. For ^{188}Re-RGD derivative an ARJ4 cell line was used.

In-Vivo Tumour Uptake

Tumour uptake was evaluated in female BALB/c nude mice, 5 weeks old. Scintigraphic studies were performed with a Sopha γ-camera, using a pin-hole collimator. Images were obtained up to five hours, starting at one hour p.i. The animals were previously anaesthetized by an intramuscular injection of diazepam (50.0 mg/kg). After imaging, the animals were sacrificed by an injection of a lethal dose of diazepam. Biodistribution of the radiolabelled peptide in the scintigraphed tumour-bearing animals was studied in the same manner as that described for normal mice. The tumour was excised along with a muscle sample from a corresponding point on the opposite side and the tumour to normal tissue ratio was calculated.

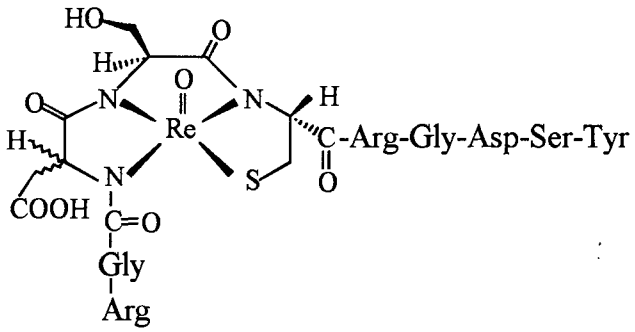
Results

Chemistry: Decapeptide-Rhenium Complex Formation (RGD-derivative)

The structure of the complex of the decapeptide with oxorhenium was elucidated with NMR spectroscopy with the use of two-dimensional techniques (publication in preparation). Spectra were obtained at 25 °C in 90% H_2O-10% D_2O. Only one isomer was present in solution and it remained stable for a period of weeks. Analysis of the NMR peaks, especially in the amide proton region, revealed that the peptide coordinates to the oxorhenium core through the N_{amide} of aspartic acid (Asp-3), the N_{amide} of serine (Ser-4), the N_{amide} and $S_{thiolate}$ atoms of cysteine (Cys-5) to form a complex of the $ReO[N_3S]$ type (*Fig.1).*

The proposed structure based on the ^{1}H-NMR is in agreement with the mass spectral data on the complex that gives a MH^+ peak of m/z 1312.2, consistent with the formation of a mononuclear, monoligand complex.

Fig.1: Proposed Rhenium-Decapeptide Structure



By comparison of the NMR chemical shifts to existing data in the literature on related complexes, it is proposed that the configuration of the side chain of serine is *syn* with respect to the oxygen of the oxorhenium core while the configuration of the peptide chain Arg-Gly-Asp-Ser-Tyr is *anti.*

Radiochemical Study of ^{188}Re-Labelled Peptides

Both in aceton and in methylethylcetone reduced Re-188 remained at the origin, while perrhenate had an Rf=0.9-1.0. Rhenium reduction was completed within 90 min.

In ITLC the gluconate complex had an Rf=0.00, while HPLC studies showed the formation of a species with Rt= 2.7 min. Quantitative formation of the ^{188}Re-gluconate complex was achieved within 15.0 min.

Chromatographic (HPLC) studies of ^{188}Re-Lanreotide indicated the formation of a single derivative with Rt=17.0-17.5. It was found that its formation was influenced by the experimental conditions applied, namely by the reaction time and the temperature. Fifteen minutes after the addition of ^{188}Re-gluconate to the peptide solution, a radioactive species with Rt=17.2 min starts to form, in a yield of about 15%. The yield increased to 80% twenty hours later. Complex formation is accelerated by increasing the temperature. Thus a 92% yield is obtained by heating the reaction mixture at 90^0 for 60 min. The stability study showed that ^{188}Re-Lanreotide remained practically stable at 4^0, for at least 48 hrs.

Similar studies of the ^{188}Re-RGD derivative showed also the formation of a single derivative with Rt=10.5-11.0. Also in this case it was found that the experimental conditions influenced the formation of ^{188}Re-complex. Highest yield of labelling was obtained by warming the reaction mixture at 80°C, for 60 min. The ^{188}Re-RGD complex was stable at 4°C, for 64 hrs.

Radiobiological Evaluation Of The ^{188}Re-Labelled Peptides Biodistribution Study

Results of the *in-vivo* evaluation of ^{188}Re-Lanreotide are presented in the following Table I.

Table I

	% DOSE PER ORGAN			
	1 Hour	**2 Hours**	**4 Hours**	**24 Hours**
Blood	3.90±0.7	2.08±0.6	0.88±0.2	0.35±0.5
Liver	17.80±5,5	17.50±4.6	2.39±1.2	0.60±0.2
Lungs	0.71±0.3	0.45±0.2	0.21±0.0	0.02±0.0
Stomach	1.33±0.5	1.40±0.4	1.51±0.5	1.97±0.4
Kidneys	0.96±0.6	1.00±0.3	0.54±0.0	0.22±0.0
Intestine	74.10±23.6	63.30±19.8	46.90±9.0	42.90±14.4
Muscles	17.60±12.3	3.43±1.8	1.83±0.9	0.0

As it can be observed the radiolabelled biomolecule presents fast blood clearance and, practically, absolute hepatobiliary elimination. Thus, blood value ranges from 3.90±0.7, 60 min p.i. to 0.35 ±0.5 at twenty four hours, liver uptake is 17.8±5.5 in one hour and becomes 0.6±0.2 in twenty four hours p.i., while intestine values range from 74.1±23.6 in one hour p.i. to 42.9±14.4 at twenty four hours. Kidney uptake was found less than 1.0 even one hr p.i. Urine elimination was negligible.

The biodistribution study of ^{188}Re-RGD derivative is presented in Table II.

Table II

	% DOSE PER ORGAN			
	15 min	**30 min**	**60 min**	**120 min**
Blood	3.13 ± 1.0	1.71 ± 0.6	0.56 ± 0.2	0.19 ± 0.1
Liver	0.62 ± 0.2	0.59 ± 0.1	0.34 ± 0.1	0.12 ± 0.1
Lungs	0.15 ± 0.0	0.08 ± 0.0	0.06 ± 0.0	0.07 ± 0.0
Stomach	0.16 ± 0.0	1.07 ± 0.4	0.55 ± 0.3	0.55 ± 0.4
Kidneys	2.94 ± 1.3	1.64 ± 0.7	0.98 ± 0.5	0.19 ± 0.0
Intestines	0.71 ± 0.2	1.09 ± 0.5	0.96 ± 0.4	1.54 ± 0.8
Muscle	5.47 ± 1.8	2.67 ± 0.7	2.80 ± 1.7	0.42 ± 0.3
Urine	13.09 ± 4.3	19.08 ± 8.1	31.09 ± 11.3	38.31 ± 11.0

It can be observed that this ^{188}Renium-labelled decapeptide presents fast blood clearance, since only 0.19% of the injected dose remains in the circulation within 120 min p.i. There is not any gastrointestinal accumulation and practically the radiolabelled biomolecule is completely eliminated by the urinary tract. This *in-vivo* behaviour is in perfect accordance with the biodistribution of the ^{99m}Tc-labelled species.

In-Vitro Cell Binding Assay

Preliminary *in vitro* cell binding studies did not show specificity of ^{188}Re-Lanreotide for the cell line tested. Further experimentation is in progress, for both ^{188}Re-labelled peptides.

In-Vivo Tumour Uptake

Tumour uptake of the ^{188}Re-labelled Lanreotide is presented in Picture 1. The experimentally induced gastrointestinal tumour is clearly delinated, two hours after the intravenous injection of the radiolabelled peptide, although Lanreotide is not known to be specific for this particular type of cancer. Tumour to normal tissue ratio was found equal to 4.3.

Picture 1

Conclusions-Future Steps

Lanreotide has been succesfully labelled with Re-188, with the methodology reported. The radioactive species was found stable in refrigerator. Biodistribution studies showed fast blood clearance and high gastrointestinal uptake. Cancer cell binding showed no specificity of ^{188}Re-Lanreotide for the Colo 205 cell line. However colon cancer, created in experimental animals by this cell line, was clearly depicted 2.0 hr after the i.v. injection of ^{188}Re-labelled Lanreotide.

The other peptide under study, the decapeptide-RGD derivative, has been also succesfully labelled with Re-188, with the above referred methodology, yielding a single, stable radiolabelled species. Biodistribution studies showed very fast blood clearance and complete urine elimination. Cancer cell binding studies are in progress.

Our future plans are:

- To evaluate in normal mice and rats possible receptor competition of ^{188}Re-Lanreotide to cold peptide. This will be performed as soon as we receive a new batch of the peptide.
- To investigate the *in-vitro* binding in other types of cancer cells and the *in-vivo* tumour uptake of ^{188}Re-labelled Lanreotide in other types of gastroenteropancreatic (GEP) and breast tumours.
- To expand stability studies to incubation of ^{188}Re-Lanreotide with human serum and to the cystein challenge test.
- To study further the *in-vitro* binding of ^{188}Re-Lanreotide to several, proper cancer cell lines.
- Similar *in-vitro* and *in vivo* studies as those referred for ^{188}Re-Lanreotide are planned for the ^{188}Re-RGD derivative.

^{153}Sm-Labelled Monoclonal Antibodies

Introduction

The radiolanthanide Samarium-153 has physical characteristics, which render it proper for Radioimmunotherapy, so it has been proposed for MoAbs labelling. It is a beta-emitter [Emax=640(30%), 710 (50%) and 810 (20%) KeV], emitting also gamma rays of 103.2 KeV and has a half-life of 1.95 days. In literature MoAbs have been labelled with Sm-153 using aliphatic or cyclic DTPA derivatives as bifunctional chelates to bind the radionuclide onto the antibody. In our study till now we have investigated MoAbs labelling, by using DOTA for the conjugation of the radiolanthanide onto the biomolecule.

Radionuclide Preparation: Samarium-153 was produced in the reactor by irradiation of enriched $^{152}SmO_3$, by a neutron flux of 4.81 x 10^{13} neutrons/cm^{-2}. sec, for five hours. The radionuclide produced was converted to the respective chloride salt by the addition of a 5.0 N HCl solution.

MoAbs Investigated: We have performed our initial labelling studies using the commercially available polyclonal antibody "Sandoglobuline". Further on, we plan to work with the MoAb antiCEA, kindly offered by Cuba.

Steps for the labelling of MoAbs with Sm-153:

Synthesis of 1,4,7,10-tetraazacyclododecano-1,4,7,10 tetraacetic acid (DOTA):

DOTA was synthesized by the reaction of chloracetic acid on the sulphuric salt of 1,4,7,10-tetraazocyclododecane, at 5°C, at pH=9-10. The reaction was completed in 24 hr and after adjusting the pH=2-3 a precipitate was obtained at a final yield of 80-85%.The product was purified by recrystallization and isolated by filtration.

Study of the labelling of DOTA with Sm-153

Labelling of DOTA with radioactive Samarium was initially investigated for comparison. In an aqueous solution of the ligand (15mg/ml) at pH=7.0, $^{153}SmCl_3$ (0.1-1.0 mCi) was added. The reaction mixture was incubated at 80°C for one hour and the pH of the final solution was adjusted to 7.0 by Phosphate Buffer. Labelling was studied by ITLC-SG, in NH_4OH : H_2O.

Conjugation of DOTA to the Antibodies

For the complexation of DOTA with the antibody one carboxylic group was activated through N-succinic ester. The ester was formed in 24 h, by reacting DOTA to N-succinic ester at room temperature and at a ratio of 1:1.

A quantity of the above solution was added to an antibody solution (3mg/ml) in phosphate buffer pH:8.0, so that the molar ratio of Ab:DOTA is 1:15. The mixture was stirred at room temperature for two hours and was purified from unreacted DOTA by gel filtration (on a Bio-Gel P30 column), using 0.9% NaCl with 0.02% NaN_3, for the elution. Spectroscopic examination of all the eluted fractions at 280nm permitted antibody monitoring. For further elimination from any DOTA excess, the fractions containing the antibody were mixed together and dialysed for 24 hr at 4°C against 3 changes of 0.9% NaCl with 0.02% NaN_3 and further for other 24 hr against sodium citrate buffer, pH:5.0. The final solution, containing congugated DOTA-Ab was aliquoted to 1.0 mg fractions and freezed.

Samarium-153 labelling of theConjugate DOTA-Ab

To a fraction of DOTA-Ab a solution of $^{153}SmCl_3$ (5-20 mCi) was added. pH was adjusted to 7.0 and the mixture was left to react at room temperature for a period from 3.0 to 48 hours, in order to find out the optimum labelling conditions. Purification of the ^{153}Sm-labelled conjugate was performed by gel filtration, on a Bio Gel 30 column, eluted with NaCl 0.9%. One ml fractions were collected and counted in a gamma counter. By this purification procedure the % yield in labelled antibody can be calculated as well.

In-vivo Evaluation

Initial, comparative biodistribution experiments were performed in normal, female Swiss mice weighting 25-30 gr. In three different animal groups, of five mice each, ^{153}Sm-DOTA, ^{153}Sm-DOTA-Ab and $^{153}SmCl_3$ were injected intraveniously in the tail vein. Animals were sacrificed four hours p.i.The study was performed as reported above for the ^{188}Re-labelled peptides.

Results

Synthesis of DOTA

The ligand was synthesized with an overall yield 80-85%. The product synthesized was identified by its melting point (280-282°C), as well as by spectroscopic methods, as follows:

Infra Red Spectroscopy (KBr): Absorption at: 3429.1, 1709.8, 1154.3, 619.5 cm-1
^{1}H-NMR (D2O): 3.72, 3.25 ppm

Study of theLabeling of DOTA with Sm-153

Labelling of DOTA was evaluated by Instant Thin Layer Chromatography on Silica Gel (ITLC-SG) using NH_4OH :H_2O, 1:25, as solvent system. Rf values were 0.0 for $^{153}SmCl_3$ and 0.9-1.0 for ^{153}Sm-DOTA.

Conjugation of DOTA to the Antibodies

Gel filtration lead to isolation of the conjugate Ab-DOTA from any excess of the ligand, as presented in Fig.2.

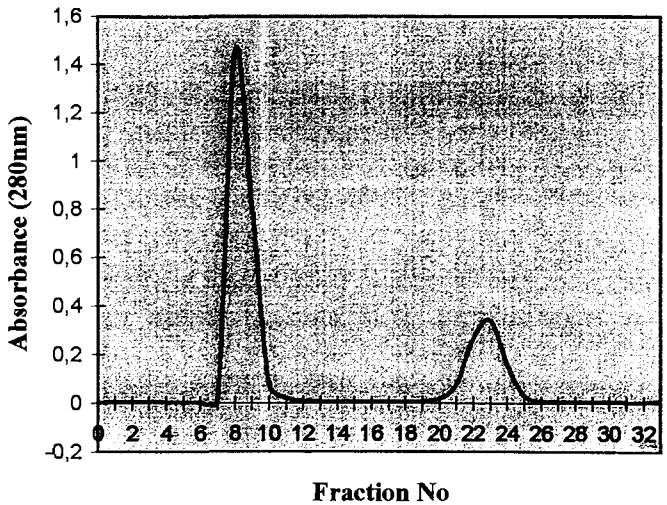


Fig 2: Ab-DOTA Elution Profile

Samarium-153 labelling of The Conjugate DOTA-Ab

Studying the labelling conditions we have found that the pH of the final solution plays an important role to the yield of labeling. At pH values lower than 5.0 we have no labelling. Also, the incubation period of the conjugate of DOTA-Ab with ^{153}Sm is an important factor for good labelling. Thus, a labelling yield of 40% was obtained three hours after the addition of the radionuclide to the conjugate. This value increased to 60%, for the time interval of 24 hours to reach the 75-85% at 48 hours. Column purification was very efficient, as presented in Fig.3. So even at the 24 hours period the ^{153}Sm-DOTA-Ab, more than 95% pure, can be isolated and used.

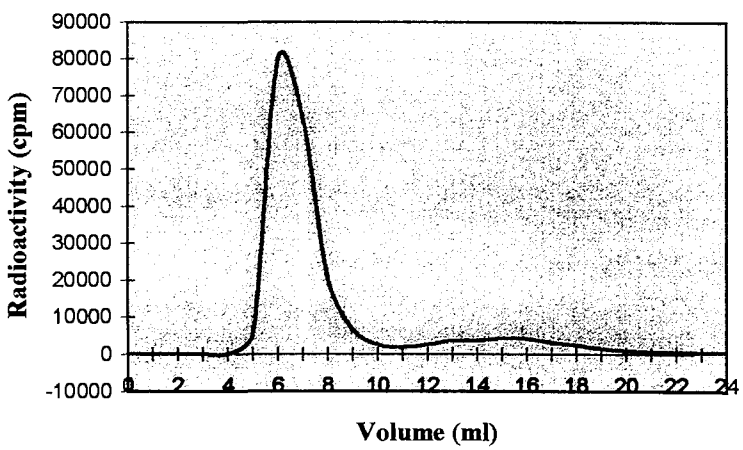
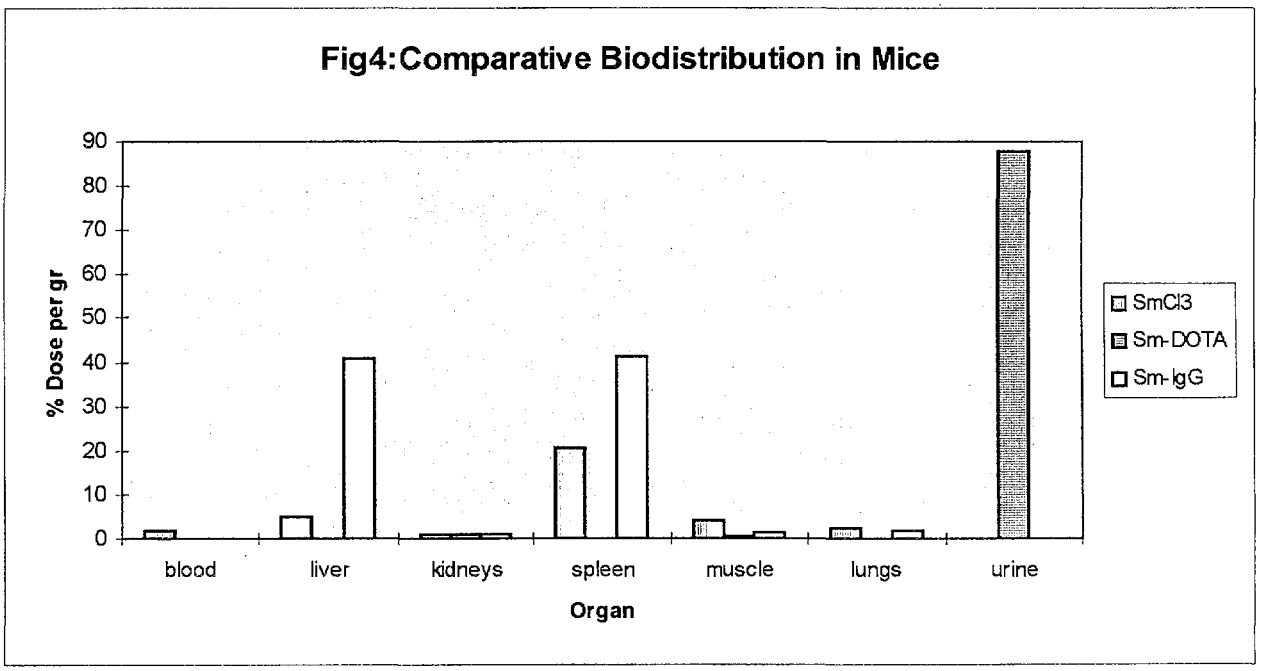


Fig. 3: ^{153}Sm-DOTA-Ab Elution Profile

Instant Thin Layer Chromatography (ITLC), by the system referred before, showed that $^{153}SmCl_3$ and the conjugate^{153}Sm-DOTA-Ab remain at the origin of the chromatogram, while ^{153}Sm-DOTA has Rf=1.0. Non unconjugated ^{153}Sm-DOTA was detected in the final solution, even before column purification.

Preliminary results of the comparative *in-vivo* evaluation study are presented in Fig. 4, for $^{153}SmCl_3$, ^{153}Sm-DOTA and ^{153}Sm-DOTA-IgG.

As it can be observed[153]Sm-DOTA is mainly eliminated by the renal system, while the radiolabelled antibody presents liver accumulation. We are trying to evaluate if this high reticuloendothelial (RES) uptake is the normal antibody behaviour or it is due to *in vivo* formation of Sm colloids. Other investigators, having observed similar *in-vivo* behaviour of [153]Sm-labelled antibodies, consider it as a combination of both antibody binding and *in-vivo* dissociation effects.



Conclusions-Future Steps

The method applied and presented above seems to give satisfactory results for the radiolabelling of antibodies with Samarium-153. However some points have still to be clarified. So the following experiments are planned:

1. To examine by Polyacrylamide Gel Electrophoresis (PAGE) the protein, for possible damage due to the labelling conditions.
2. To identify the obtained radioactive species and examine the purity of the final product also by High Performance Liquid Chromatography.
3. To check the stability of the ^{153}Sm-labelled IgG at different temperatures, as well as in the presence of human serum and of cystein.
4. To study, in a similar way, the preparation of ^{153}Sm-antiCEA or of any other MoAb available.
5. To investigate the biodistribution of ^{153}Sm-antiCEA in tumour bearing nude mice, in comparison to ^{99m}Tc-antiCEA, already studied for radiodiagnosis.

We also plan to study comparatively ^{153}Sm-labelling of antibodies using DTPA as indermediate exchange ligand.

^{188}Re-Labelled Monoclonal Antibodies

Introduction

For the labelling of antibodies with Re-188 we have worked till now with IgG commercially available. Both perrhenate and the antibody were reduced, according to literature procedures.

Experimental

Na $^{188}ReO_4$ Reduction: Metal reduction was performed as reported for the peptides labelling and it was complete within 90 min.

Antibody Reduction and Labelling: For the partial reduction of MoAbs and the formation of some sulphydryl groups the antibody was reduced by ascorbic acid, as follows:

- To one ml of an antibody solution (1.0 mg/ml) an ascorbic acid (AA) solution was added, containing 4.0 mg AA in 100 μl water. pH=3.0-3.5.
- Incubation at Room Temperature for 60 min.
- To the reduced antibody 1.0 ml of the reduced perrhenate was added.
- Incubation for 3 to 5 hrs at 37^{0}C.
- Staying overnight at Room Temperature.

Radiochemical Study: Antibody labelling was followed by ITLC-SG in 2M urea.

Results

The above reported procedure resulted in high yield (> 93%) of ^{188}Re-labelled IgG. In the chromatographic system tried ^{188}Re-IgG had Rf=0.0 while for ^{188}Re-perhenate the Rf value was 0.9-1.0. Increasing the incubation period from 3 to 5 hours, at 37^{0}C, did not increase further the yield.

Future Steps

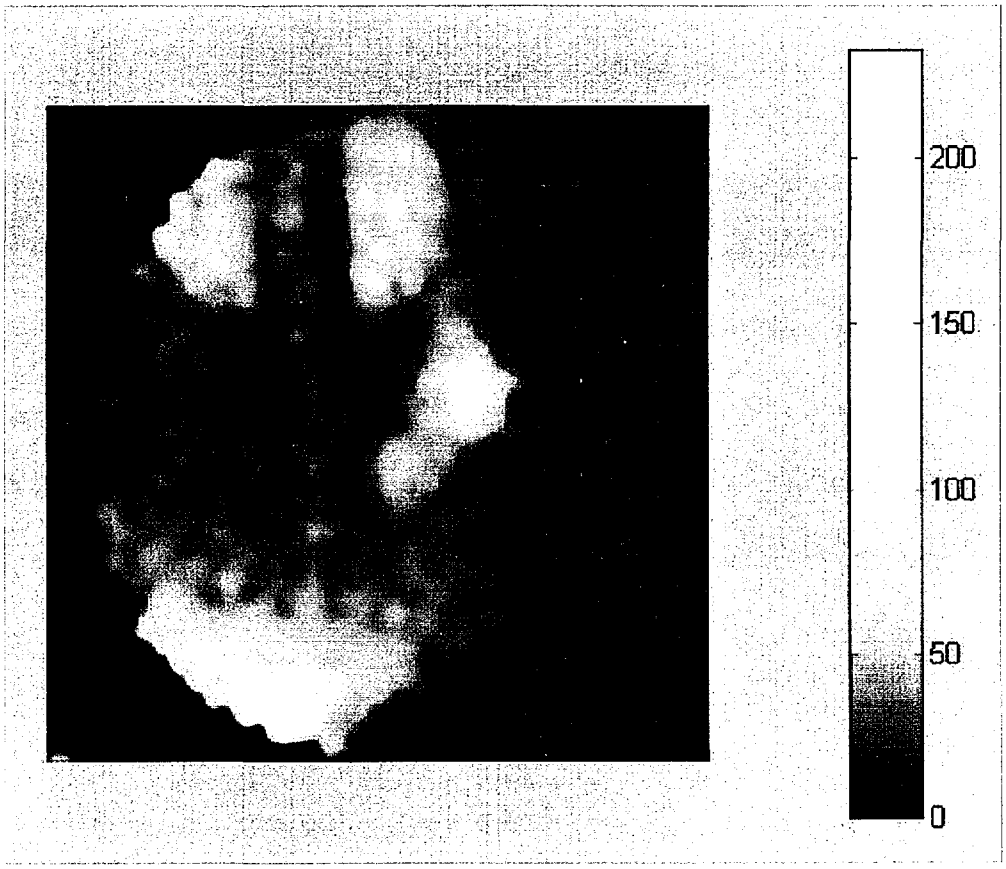
- Chromatographic (HPLC) study of ^{188}Re-IgG, to evaluate further the labelling procedure.
- Preparation of ^{188}Re-antiCEA.
- PAGE studies of the ^{188}Re-labelled antibodies, to evaluate possible protein damage, by the labelling system.
- Biodistribution evaluation of the ^{188}Re-labelled antibodies
- Investigation of tumour uptake of ^{188}Re-antiCEA in tumour-bearing animals.

Animal Models- Small Animals Imaging Devices

For bringing forward our studies, we have started to evaluate the radiolabelled derivatives to be used in Oncology, in tumour-bearing animals. Although similar studies are widespread abroad, it is the first time that they take place in Greece. Thus, making use of an already existing colony of nude mice, we have created ectopic animal models for colon cancer and small cell lung carcinoma as well as for epithelial cancers. In these models we have evaluated, successfully, ^{99m}Tc- and ^{188}Re-labelled peptides and antibodies. Presently, we are trying to develop and evaluate, comparatively, orthotopic tumour models as well.

For the time being our scintigraphic studies are performed using the pinhole collimator of the Sopha gamma camera of "Alexandra" University Hospital. At the same time and in

collaboration with the Accelerators Institute of Athens University, we are trying to develop an experimental gamma camera, for small animals imaging. In Picture 2 we report, for comparison with Picture 1, the image of the same nude mouse injected with ^{188}Re-Lanreotide. This image was obtained in the experimental gamma camera of Athens University.



Picture 2

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