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^{99m}Tc LABELING OF ANNEXIN FRAGMENT BY TWO DIFFERENT TECHNIQUES FOR DETECTION OF APOPTOSIS IN CANCER

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ABSTRACT

Introduction: Apoptosis or programmed cell death is an active, energy-dependent mechanism for elimination of cells that have been injured, infected, or immunologically recognized as harmful or superfluous. Apoptosis may be used in diagnosis of malignant tumors as it often occurs in hypoxic zones adjoining areas of necrosis, and it is endpoint of most forms of anticancer therapy. The N-terminal sequence of Annexin V, consisting of 13 amino acids (Anx13), is considered a useful marker in this context because of interaction with other molecules such as phosphatidyl-serine, the compound expressed on the surface of the apoptotic cells. *Aim*: The scope of this study was the labeling of Anx13 fragment with the derivatization of it for nitrido and tricarbonyl technetium approach, and their biological evaluation. *Methods*: For nitrido technetium labeling, a readily-available lyophilized kit (Schering CIS Bio International) was used. $Na^{99m}TcO_4$ was added to this kit as well absolute ethanol. The reaction vial was left standing at room temperature for a period of 30 min. Then, a 0.9mM aqueous solution of Cys-Anx13 and diphosphine (PNP6), was added. This reaction was carried out for one hour at 100 ^{o}C. Preparation of the ^{99m}Tc-carbonyl consisted in flushing the mixture of 4.4 mg of sodium carbonate, 5.5 mg of sodium borohydride and 20 mg of sodium-potassium tartrate tetrahydrate with CO gas during 30 min. Pertechnetate was added and the vial was heated for 35 min at 75°C. The reaction was stopped in ice bath, and pH was adjusted to 7. Then 100μL of the precursor was added to 1.1 mM (50 μg) of His-Anx13 and heated at 75°C for 60 min. The reaction was stopped in ice bath. Radiochemical analysis of both complexes ^{99m}TcN(PNP6)-Cys-Anex13 and $^{99m}Tc(CO)_3$-His-Anx13 was performed by thin layer chromatography as well as by HPLC. Biodistribution studies were carried out in healthy animals, 4 hours post injection of the ^{99m}Tc-complexes. An apoptosis animal model was developed by using an alkylating agent (cyclophosphamide) in mice bearing lung tumor cells (A549). *Results*: Radiochemical purity of ^{99m}TcN(PNP6)-Cys-Anex13 and $^{99m}Tc(CO)_3$-His-Anx13 was 92.4 ± 1.2% and 88.6 ± 1.7%, respectively. Comparing biodistribution results of both complexes in healthy Swiss mice, 4 hours post-injection, it was observed that with Carbonyl-Anx excretion by the urinary system was higher than with Nitrido-Anx complex. Nevertheless, the hepatobiliary route was also substantially activated, so that renal and hepatointestinal elimination had similar importance. No difference in tumor uptake was observed in animals that underwent apoptosis-inducing treatment. *Conclusions*: 1) The different labelling techniques were successfully conducted, and biodistribution showed comparable results for both compounds; 2) The procedure employed for enhancing uptake via apoptosis failed, thus additional studies in this area are required.

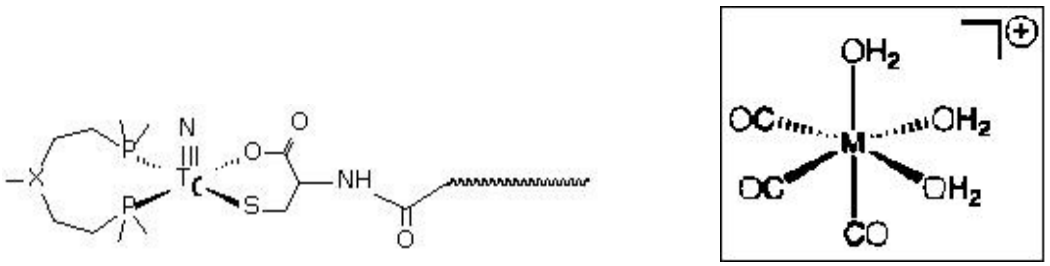
1. INTRODUCTION

Imaging of the programmed cell death, i.e. apoptosis is one of the most interesting questions of radiopharmaceutical chemistry and this modality is of high importance in oncology, in cardiology and in the studies of atherosclerosis [1-3].
At the same time it is known from the literature that phosphatidyl-serine is expressed on the surface of apoptotic cells [1,4,5]. The large protein, Annexin V, consisting of 320 amino acids, is considered such a tracer [1,3,6], but it has many disadvantages from the point of view of pharmaceutical preparation (i.e. kit formulation).

On the other hand, it is known that Annexin type proteins generally possess their biospecific sequences on the N-terminal, and in case of Annexin-V, the phosphatidyl-serine specific sequence might be attributed to a chain on the N-terminal [1,7], consisting of 13 amino acids.

H_2N-Ala-Glu-Val-Leu-Arg-Gly-Thr-Val-Thr-Asp-Phe-Pro-Gly-OH, (Anx13)

Recent advent of the new, low valent $[Tc(CO)_3]^+$ metal core and of the $[Tc(N)]^{2+}$ intermediate (Fig. 1) have introduced new avenues for ^{99m}Tc labeling of biologically active compounds with high specific activity [8,9,10].



(A) **(B)**

Figure 1. (A) ^{99m}Tc – nitrido and (B) ^{99m}Tc-carbonyl cores

For the labeling of the anexin analogs to the right precursor is need to bind the peptide sequence with a derivative. Cysteine annexin (Cys-Anx 13) and histidine annexin (His-Anx13), the synthesized derivatives of annexin 13 fragments, were prepared for labeling with ^{99m}TcN intermediate and ^{99m}Tc carbonyl precursor respectively.

H_2N-Cys-Ala-Glu-Val-Leu-Arg-Gly-Thr-Val-Thr-Asp-Phe-Pro-Gly-OH, Cys-(Anx13)

H_2N-Hys-Ala-Glu-Val-Leu-Arg-Gly-Thr-Val-Thr-Asp-Phe-Pro-Gly-OH, His-(Anx13)

The aim of this study was the labeling of Anx13 fragment with the derivatization of it for nitrido and carbonyl technetium approach, and their biological evaluation.

2. MATERIAL AND METHODS

2.1. Material

- Peptides were provided by Izotop (Institute of Isotopes Co. Ltd, Budapest, Hungary)
- $^{99}Mo/^{99m}Tc$ generator – Institute of Energetic and Nuclear Research (IPEN-CNEN/SP)-Brazil
- Nitrido kit – Schering CIS Bio International, France.
- Diphosphine- provided by Dr. A. Duatti, University of Ferrara, Italy.
- Monoxide carbon gas – White Martins Gases Industriais AS, Sao Paulo, Brazil.

2.2. Methods

2.2.1. Preparation of ^{99m}Tc-nitrido and labeling of Cys-annexin

To a readily-available lyophilized kit (Schering CIS Bio International), it was added 0.5 mL of aqueous $Na^{99m}TcO_4$ and 0.5 mL of absolute ethanol. The reaction vial was left standing at room temperature for a period of 30 min for completing the process.

^{99m}TcN(PNP6)-Cys-Annexin was prepared by addition of 50 μL of aqueous solution of Cys-Annexin and diphosphine (PNP6), to the precursor vial. This reaction was carried out for one hour at 100°C. The same procedure was carried.

2.2.2. Preparation of ^{99m}Tc-carbonyl and labeling of His-annexin

The organometallic precursor was prepared according to the procedure published by Alberto et al. 1998 [8] with minor modifications. Briefly, 4.4 mg Na_2CO_3, 20 mg Na/K-tartrate.H_2O and 5.5 mg $NaBH_4$ were purged for 1 hour with monoxide carbon gas. Then 1 mL of $Na^{99m}TcO_4$ (3700 – 5550 MBq) was added to the vial containing the mixture of salts purged with CO gas. Then the vial was heated at 75-80 °C for 35 min. The reaction was stopped in an ice bath. Then the pH was adjusted to 7 using 0.2 mL of 1M HCl / 0.1 M Phosphate buffer solution (2:1).

In the vial containing 1.1 mM (50 μg) of the molecule it was added first 100 μL of $^{99m}Tc(CO)_3$. The vials were heated at 75°C for 60 minutes. The reaction was stopped in an ice bath.

2.2.3. Quality control for ^{99m}Tc-carbonyl-annexin and for ^{99m}Tc-N(PNP)-annexin

The quality control for ^{99m}Tc-carbonyl-annexin was done using paper and thin layer chromatography with Whatman n.1 and TLC-Aluminium as support and the the mixture of methanol/HCl 6M(99.5:0.5) used as mobile phase.
Radiochemical evaluation of the ^{99m}TcN kit and ^{99m}TcN(PNP6)-peptide was done using ITLC-SG with a solvent system Ethanol/Chloroform/Toluene/Ammonium Acetate 0.5M (E/C/T/AA, 5; 3; 3; 0.5) and ethyl acetate to detect $^{99m}TcO_4$ and ^{99m}TcN, along with ethanol/water (1:1) for $^{99m}TcO_2$.

2.2.4. Biodistribution studies in healthy animals

The studies were approved by the local Animal Welfare Committee.

^{99m}Tc-labeled preparations were purified in a C18 SepPak cartridge and then (0.1 mL) were i.v. administrated via the tail vein in *Swiss* male mice (body mass 20-25 g) in a radioactive concentration of 185-370 MBq/mL. Mice were sacrificed at 4 h post injection. Organs and tissues (blood, heart, lung, spleen, kidneys, liver, pancreas, stomach, large and small intestines, muscle, bone) were excised, weighed and the radioactivity was determined by γ-counting. Results were expressed as percentage of injected dose per gram (% ID/g) of tissue.

2.2.5. Animal model for annexin studies

An apoptosis animal model was developed by using an alkylating agent (cyclophosphamide) in mice bearing lung tumor cells (A549). Cyclophosphamide was injected 24 hours before the administration of $^{99m}Tc(CO)_3$-His-Anx.

3. RESULTS AND DISCUSSION

Radiochemical purity of ^{99m}TcN(PNP6)-Cys-Anx and $^{99m}Tc(CO)_3$-His-Anx was 92.4 ± 1.2% and 88.6 ± 1.7%, respectively.

Biodistribution in healthy animals can be observed in Table 1.

Table 1. Biodistribution studies of ^{99m}TcN(PNP6)-Cys-Anx and ^{99m}Tc(CO)$_3$-His-Anx in healthy *Swiss* mice (%ID/g)

Organ	^{99m}TcN(PNP6)-Cys-Anx	^{99m}Tc(CO)$_3$-Hist-Anx
Blood	0.11 ± 0.02	0.97 ± 0.11
Heart	0.51 ± 0.03	0.25 ± 0.05
Lung	0.53 ± 0.14	0.70 ± 0.08
Kidneys	2.27 ± 0.16	13.90 ± 1.23
Spleen	0.34 ± 0.03	0.71 ± 0.07
Stomach	0.58 ± 0.13	1.90 ± 0.17
Pancreas	0.30 ± 0.04	0.34 ± 0.09
Liver	4.61 ± 0.78	15.12 ± 1.10
Large Intestine	12.66 ± 2.20	7.88 ± 0.35
Small Intestine	6.46 ± 1.19	7.62 ± 0.68
Muscle	0.14 ± 0.03	0.11 ± 0.03
Bone	0.39 ± 0.08	0.24 ± 0.07

(N=6)

The tracers cleared the bloodstream fast.

Comparing the biodistribution of radiolabeled Nitrido-Anx and Carbonyl-Anx in healthy animals we can see that in the last the excretion was higher in kidneys. Nevertheless, the hepatobiliary route was also substantially activated, so that renal and hepatointestinal elimination of the drug had similar importance.

A pilot study to evaluate the use of cyclophosphamide as an inducer apoptosis agent was done in two animals bearing lung tumor. In one of them intraperitonial cyclophosphamide was injected 24h before the administration of ^{99m}Tc(CO)$_3$-His-Anx. No difference in tumor uptake was observed. This study must be done in more animals, maybe with some modification of the model used.

4.CONCLUSIONS

1) The different labelling techniques were successfully conducted, and biodistribution showed comparable results for both compounds; 2) The procedure employed for enhancing uptake via apoptosis failed, thus additional studies in this area are required.

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