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Session 4, Wednesday, June 17, 2009

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Abstract: The use of enzymes for the preparation of deuterium and tritium labelled biogenic amines was detailed. In addition, the direct labelling of Anti-VEGF-MAB with ^{188}Re was discussed.

Keywords: Radiolabeled antibody; VEGF; Dopamine; Histamine

INVESTIGATIONS OF DIRECT LABELING OF ANTI-VEGF-MAB WITH RE-188

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Abstract: Our aim was to establish a reproducible method for labeling an antiVEGF-Mab (anti vascular endothelial growth factor monoclonal antibody) with ^{188}Re and obtaining *in house* the radiotherapeutic agent ^{188}Re-antiVEGF-Mab ready to use for targeted therapy of cancer. We have developed a simple and efficient procedure for labelling antiVEGF-Mab with ^{188}Re at tracer levels. The method is simple, efficient and reproducible and requires basic facilities that usually exist in nuclear medicine units. This paper presents preliminary in vitro and in vivo testing of ^{188}Re-anti-VEGF aiming to check the specific uptake, the saturation binding concentration, to determine the critical organs and also the therapeutically efficient doses to be administered.

Keywords: RIT; radiolabeled antibody; VEGF

Introduction: Receptor-mediated radiotherapy relies on the use of a receptor specific ligand to transport a radionuclide to tumor cells that overexpress the target receptors. The basic premise of radioimmunotherapy (RIT) is that the monoclonal antibodies (mAbs) directed against a tumor-associated antigen can be used to target radionuclides to cancer cells for in situ radiation therapy.[1,2] RIT is dependent on 3 principal interdependent factors: the antibody, the radionuclide, and the target tumor and host.

The selection of the antibody is based on its ability to selectively target cancer cells binding to the specific antigens expressed on the tumor cell surface, resulting in a very stable antigen-antibody complex. The therapeutic radiation is delivered to tumors while minimizing radiation exposure to normal tissues. The efficacy of the radiation is in turn influenced by target tumor location, size, morphology, physiology, and radiosensitivity, and by the kinetics of the antibody.[3] Angiogenesis is a fundamental process of development of new vascular tissue. All cells require oxygen and blood supply for growing and the formation of new vessels is influenced by many factors. Tumors also utilize this same process to enhance their own blood supply in order to nourish their aberrant growth, the density of the blood vessels determine whether the cancer can become metastatic and to what extent. Vascular endothelial growth factor (VEGF) is a key player in a broad variety of biological processes, including embryonic development, reproductive functions, growth of the long bones in the body, and the generation of blood cells. More relevant for this study, VEGF is a major regulator of vascular functions including angiogenesis. VEGF is released from many cancer cell types stimulating new vessel formation. Recent studies in experimental animals have shown that combining antiangiogenic and radiation therapies, the tumor response can be enhanced.[4,5]

A second major factor is the radionuclide, where the nonpenetrating emissions are relevant for therapy. The suitability of a radionuclide for RIT depends on its physical and chemical properties, its fate after antibody metabolism in vivo, and the nature of the radiation, such as low or high linear energy transfer (LET) emission. Whether the best choice is a low-energy, high LET, Auger-emitter or a traditional ß-emitter is a basic question that is still open and needs to be considered in the context of the tumor type and size, and the disease state.

The availability of simple, efficient, and reproducible clinical-scale radiolabeling procedures is also essential for creating commercial products.

Aim: VEGF is a key mediator of tumor angiogenesis and the use of a neutralizing antibody can reduce tumor growth. We tried to demonstrate that the specific delivery of beta radiation to tumor cells, using ^{188}Re-anti-VEGF can enhance the therapeutic efficacy.

The study is focused on the following aspects: the radiolabelling of the VEGF monoclonal antibody with ^{188}Re and evaluation of the pharmacokinetic aspects and the therapeutic effects of ^{188}Re-anti-VEGF on tumor bearing animal models.

Ex vivo biodistribution study together with the saturation binding experiments, evaluation of the induced cytotoxicity of the radioantibody in tumor cells by inhibition of the VEGF receptor functions and delivery of β radiation doses lead to the conclusion that ^{188}Re-anti-VEGF could be an effective agent for radioimmunotherapy of cancer.

Experimental: *The labeling of anti-VEGF-Mab with Re-188*

A direct labelling method of mAb VEGF was employed, using 2-mercaptoethanol as a reducing agent of the–S-S- cysteine bounds (2-mercaptoethanol:ethanol 1:10 v/v). The amounts of sodium glucoheptonate, ascorbic acid and stannous chloride were varied to achieve the optimum labeling yield. We also studied the influence of the pH, temperature and incubation time.

Quality control of sodium perrhenate eluted from the ^{188}W/^{188}Re generator was performed by paper chromatography (PC), using saline solution as the solvent. The radiochemical purity (RCP) of the radiolabelled antibody was determined by ascending instant thin-layer chromatography (ITLC), using silicagel-coated fiberglass sheets 20 cm length (Polygram SIL G, Macherey-Nagel, Germany), MEK and saline as solvents and by HPLC.

Evaluation of the saturation binding: Antigen expression was determined by indirect immuno-fluorescence (IIF) and flowcitometry FACScan (Becton Dickinson) and WinMdi 2.8.

Samples containing 4×10^5 HeLa cells and 0.3; 0.6; 1.0; 5.0 and 10.0 μg anti-VEGF respectively in 0.5 mL culture medium were prepared for evaluation of the saturation binding experiment. To each sample ^{188}Re-anti-VEGF, 20000 cpm was added. Ependorf tubes were centrifugated at 3000 rpm; the cells were washed with phosphate buffer, the supernatant was discharged. Probes were incubated 3 h at 37°C and 5% CO_2. The radioactivity of each sample was than measured.

Evaluation of the induced radiotoxicity: The induced radiotoxicity of the ^{188}Re-antiVEGF was evaluated by the MTT method using MDA-MB-231 cell line. Probes containing 10^6 cells were incubated with increasing radioactive concentration (25, 50, 100, 300 μCi) of radiolabelled antibody. Spectrophtometric measurement of the optical density were done at 570 nm and 650 nm, using as a blank, a mixture of MTT 1 mg/mL in phosphate buffer and acidified isopropanol 1:1 v/v. The absorbances of the samples were compared with those of a control sample. The data are presented as percent viability.

Biodistribution studies: The experimental model used were tumour bearing (RS1 hepatoma and B12 melanoma) young male rats from Wistar line (*rattus norvegicus* albinos variety, rodentia, mammalia), 200–250 g. The animals were kept in cages under ambiental temperature and humidity, receiving commercial ration and water *ad libitum*.

Studies regarding biodistribution of ^{188}Re-antiVEGF were done using 3 animals/each time point. The animals were anesthetized with a mixture (0.2 mL/animal) containing 0.15 mL ketamine 10% and 0.05 mL acepromazine (Calmivet), injection using an insuline type syringe and needle in the peritoneal cavity. They received ^{188}Re-antiVEGF by i.v. injection in the tail vein and then they were sacrificed at 2 h, 4 h, 24 h, 48 h, 72 h p.i., expressive tissues were removed and their radioactivity were measured. The results are expressed as %ID/organ.

Evaluation of the therapeutic efficacy: Animals from 2 lots (5 animals each) were injected i.v. with 0.7 and respectively 1.4 mCi ^{188}Re-anti-VEGF. 9 days after, samples of blood, liver, bone and tumor were analyzed for evaluation of DNA ploidy by flow-cytometry and comet assay.

Results and Discussion: Synthesis of ^{188}Re-antiVEGF

We have developed a simple and efficient procedure for labelling anti-VEGF-Mab with ^{188}Re at tracer level, under sterile and pyrogen-free conditions. This method was based on our preliminary research regarding the direct labeling method. The 2-mercaptoethanol (1;10 v/v 2-mercaptoethanol:ethanol) was used as a reducing agent of–S-S- cysteine bounds. ^{188}Re-anti-VEGF was composed from two vials A and B. Vial A contained 100 μg anti-VEGF-Mab in reduced form, 0.4 mg $SnCl_2.2H_2O$ and 20.0 mg sodium oxalate under a nitrogen atmosphere. Vial B content, 0.1 ml glacial acetic acid and 0.9 ml generator-eluted [$^{188}ReO_4$] (activity 0.1–12.0 GBq), was added to vial A. The resulting solution was kept at 70°C (water bath) for 30 min.

The ^{188}Re-anti-VEGF complex was synthesized in high yield; the RCP of ^{188}Re-antiVEGF was 95–96% (Figure 1) and the specific activity was 100 mCi/mg. The ^{188}Re-labelling yield was directly correlated with the antibody to 2-mercaptoethanol molar ratio.

Evaluation of the saturation binding and induced radiotoxicity: Tumor antigen expression of HeLa and MCF-7 are presented in Figure 2. HeLa and MCF-7 were characterized as heterogenous tumor cells with VEGF receptors mainly. The maximum concentration of the anti-VEGF corresponds to the maximum activity of ^{188}Re-antiVEGF, as a result of stable bond ^{188}Re-antiVEGF–VEGFR, was assayed. At 0.3 μg of antibody, there is maximum binding of ^{188}Re-VEGF to the expressive tumor cell receptors (Figure 3).

The spectrophotometric measurements gave the radioactivity level for which the induced radiotoxicity of the ^{188}Re-antiVEGF to the MDA-MB-231 cells was maximum. A fast decreasing of the cell viability was registered in the range 50–100 μCi ^{188}Re-antiVEGF, while the maximum induced radiotoxicity (90–100%) was observed at radioactivity levels higher than 100 μCi (Figure 4).

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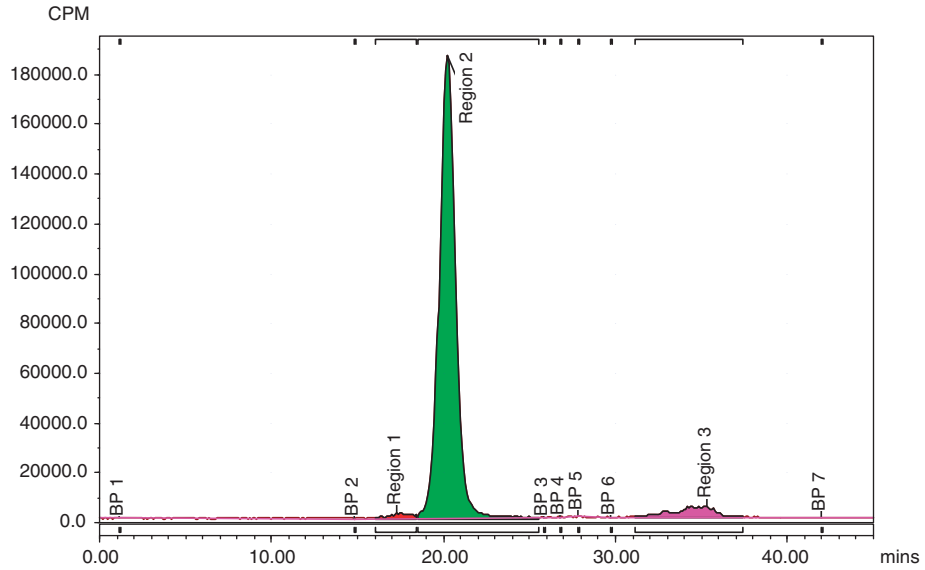

Figure 1. Radiochromatogram of ^{188}Re-antiVEGF.

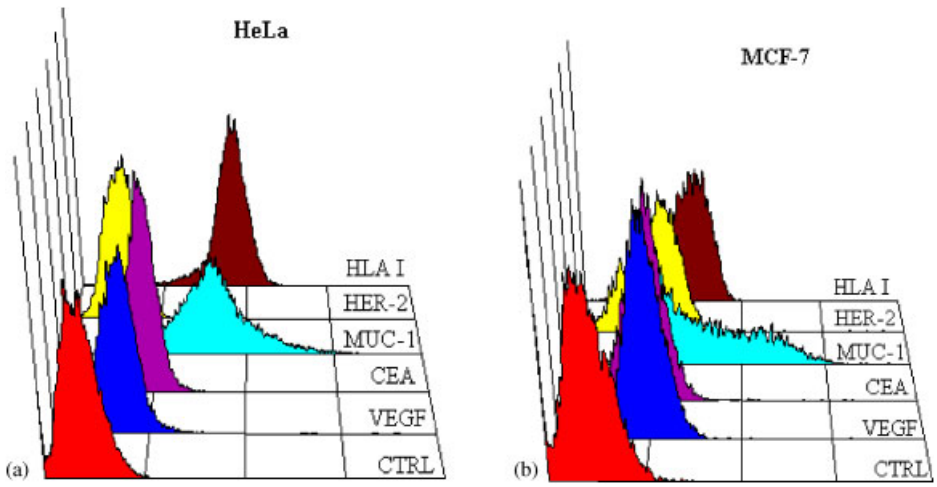

Figure 2. Tumor-associated antigens histograms of: (a) HeLa cell line and (b) MCF-7 cell line.

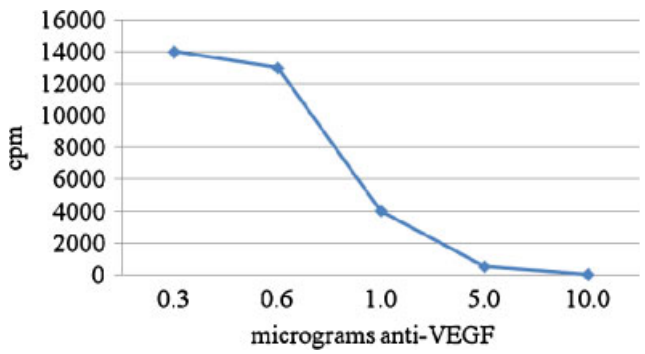

Figure 3. Saturation binding curve of ^{188}Re-antiVEGF to VEGFR.

Biodistribution of ^{188}Re-antiVEGF: The ex-vivo biodistribution data are presented in the Figures 5 and 6 showing the percent of the injected dose per organ (%ID/organ) in the case of the hepatoma model and the melanoma model respectively.

In the case of hepatoma, we observed a good and stable accumulation of the injected radioactivity in the tumor (12% ID) but a high retention in blood. The kidneys critically received 25 to 57% ID in the first 24 h p.i.

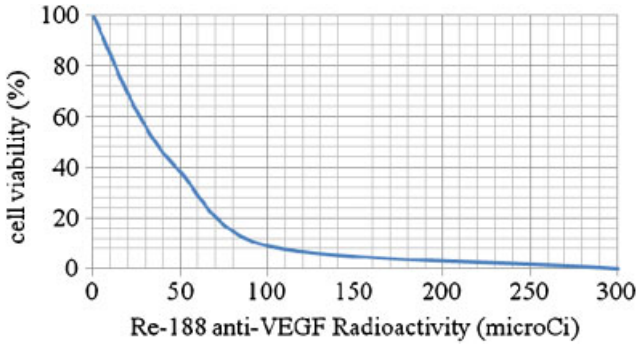

Figure 4. Induced radiotoxicity on MDA-MB-231 cell line.

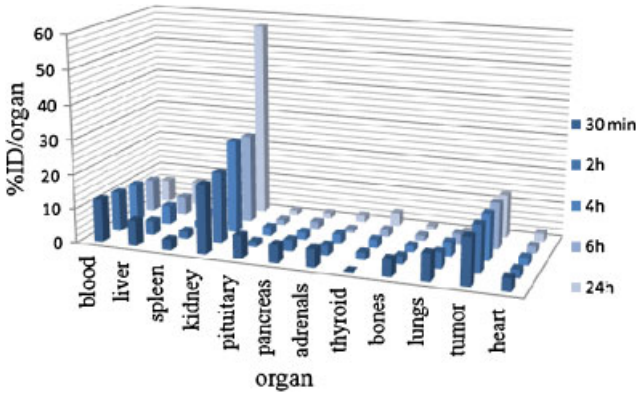

Figure 5. Biodistribution of ^{188}Re-antiVEGF in hepatoma-bearing rats.

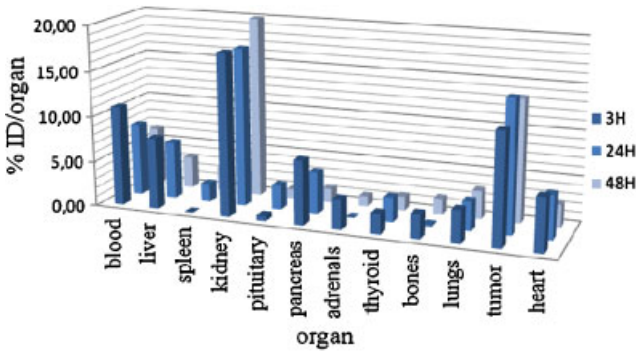

Figure 6. Biodistribution of ^{188}Re-antiVEGF in melanoma-bearing rats.

Melanoma, a high vascularized type of tumor, retained 14.5% ID at 24 h p.i. and 13.5 % ID still remain there at 48 h p.i. A slow but constant blood clearance was observed and the radioactivity in the blood decreased from 11% ID to 6.5% ID in the first 48 h p.i. The unprotected kidneys are also critical organs, as the main elimination route is renal. The biodistribution patterns in all the organs suggest a stable in vivo radiolabelled antibody.

Evaluation of the therapeutic efficacy: Figures 7 and 8 show the cell genotoxicity measured by the comet assay, after ^{188}Re-antiVEGF administration, expressed by tail intensity tail length in blood and bone marrow cells respectively. The results show a high induced cytotoxicity in tumor cells as a result of delivery of the beta radiation doses. The induced apoptotic process is directly correlated with injected radiation doses (0.7 mCi and 1.4 mCi respectively), as can be observed in Figure 9.

Acknowledgements: The research was supported by the National Authority for Scientific Research (ANCS) under contracts PNII 71-073 and Eureka E!3832.

The experiments comply with the Romanian current laws for animal studies.

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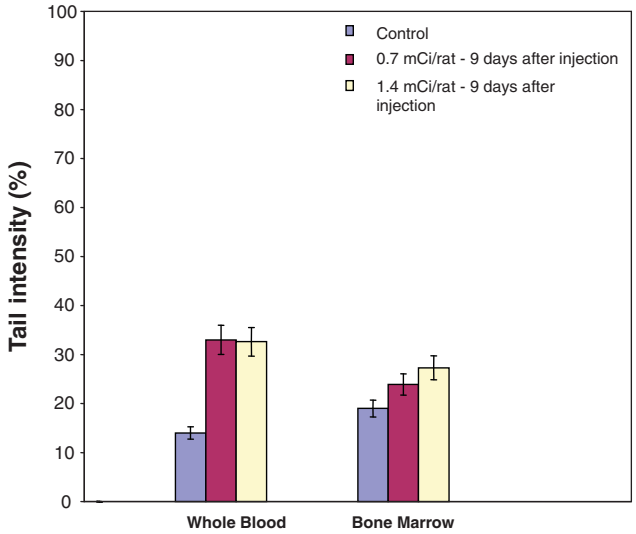

Figure 7. Cell genotoxicity after ^{188}Re-antiVEGF administration expressed by tail intensity.

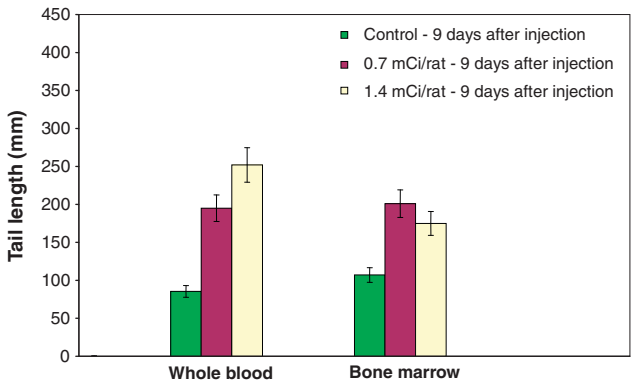

Figure 8. Cell genotoxicity after ^{188}Re-antiVEGF administration expressed by tail length.

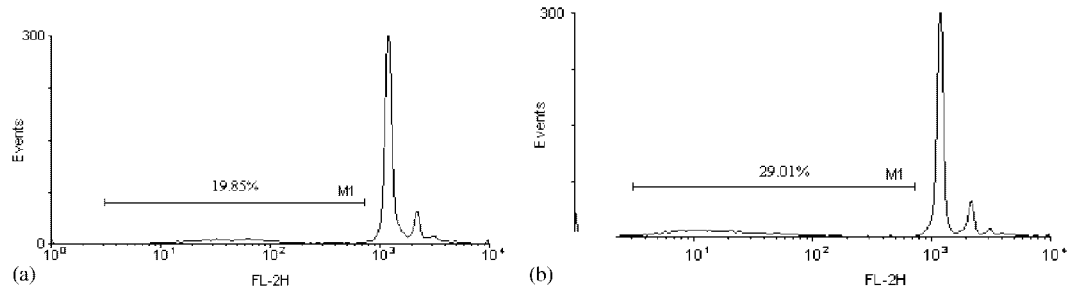

Figure 9. Flow cytometric assay of induced apoptosis in tumor cells after ^{188}Re-antiVEGF administration doses: (a) 0.7 mCi and (b) 1.4 mCi.

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ENZYMATIC SYNTHESIS OF ^{2}H-, AND ^{3}H-LABELED BIOGENIC AMINES

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Abstract: The isotopomers of tyramine, dopamine and histamine labeled with deuterium and tritium have been obtained by combining chemical and enzymatic routes. Deuteriated and tritiated water have been used as a sources of stable and radioactive label.

Keywords: dopamine; enzyme; histamine; labeling; tyramine

Introduction: Biogenic aromatic amines such as tyramine (TA), histamine (HA) and dopamine (DA) plays very important roles in many physiological processes. They are neurotransmitters in the central nervous system of human and disturbances in their production and metabolism leads to many pathologies such as allergies, Alzheimer's and Parkinson's diseases, and many other diseases. Tyramine is also responsible for generating melanin in human and plant cells. In living cells biogenic amines are produced by decarboxylation of corresponding amino acids (L-tyrosine, L-histidine and L-DOPA). Therefore, their labeled isotopomers have become a subject of growing interest in the study of mechanism of action of enzymes responsible for production and further metabolism of biogenic amines. For these purposes we have made numerous attempts to develop the methods of synthesis of isotopomers of biogenic amines specifically labeled with deuterium and tritium. Some of them were published by us earlier[1-4] and other are presented on this report.

Results and discussion: Synthesis of isotopomers of tyramine (TA). *Isotopomers of TA labeled in the position (1S).*

For synthesis of[(*1S*)-^{2}H]-,**1**,[(*1S*)-^{3}H]-, **2**, and[(*1S*)-^{2}H/^{3}H]-TA, **3**, two enzymes are used, Figure 1. First, tryptophanase (EC 4.1.99.1) introduces the label into the 2-position of L-tyrosine (L-Tyr), and second, tyrosine decarboxylase (EC 4.1.1.25) catalyses the decarboxylation of the labeled L-Tyr to TA.

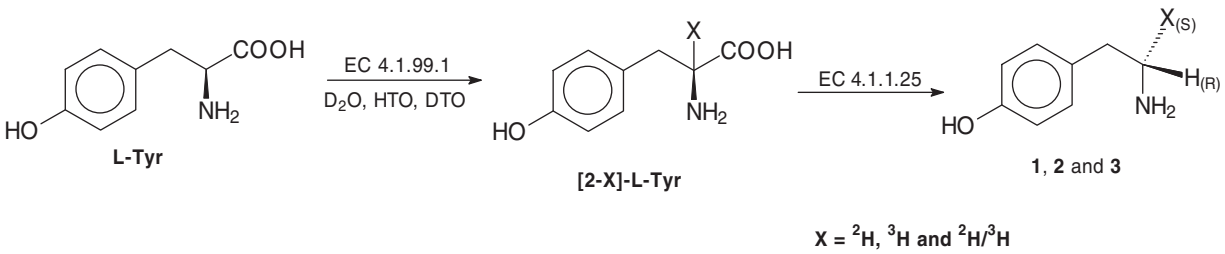

Figure 1. Enzymatic synthesis of (1S) isotopologues of TA labeled with deuterium and tritium.

In the course of the decarboxylation of L-Tyr a solvent proton replaces the carboxyl group with retention of configuration[5] and the isotopomers of TA retain the label at the (*1S*)-position.The intermediates [2-X]-L-Tyr were obtained using fully deuteriated, tritiated and mixed (DTO) media, respectively, for [2-^{2}H]- [2-^{3}H]- and [2-^{2}H/^{3}H]-L-Tyr.

*Isotopomers of TA labeled in the position (1R). Isotopomers:[(*1R*)-^{2}H]-,* ***4****,[(*1R*)-^{3}H]-,* ***5****, and[(*1R*)-^{2}H/^{3}H]-TA,* ***6****, were obtained as a result of decarboxylation of L-Tyr carried out in fully deuteriated, tritiated and DTO media, respectively, Figure 2.*

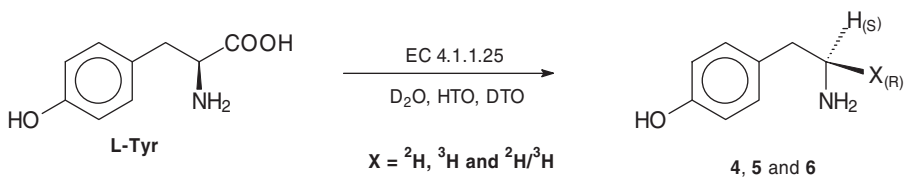

Figure 2. Enzymatic synthesis of (1R) isotopologues of TA labeled with deuterium and tritium.

Ring labeled isotopomers of TA. The isotopomers of TA labeled with deuterium or tritium were synthesized *via*two different routes. In the first, H/D or H/T acid catalyzed isotope exchange between isotopic water and L-Tyr gave isotopomers of L-Tyr labeled exclusively *ortho*to the hydroxyl group respectively. In this case, the *p*-position is blocked by a side chain.[6] The second step involves enzymatic decarboxylation of labeled L-Tyr giving [3′, 5′-^{2}H$_{2}$]-, **7**, [3′, 5′-^{3}H$_{2}$]-, **8**, and [3′, 5′-^{2}H/^{3}H]-TA, **9**, Figure 3.

Similarly, [2′,6′-^{2}H$_{2}$]-TA, **10**, was obtained as a result of enzymatic decarboxylation of commercial [2′,6′-^{2}H$_{2}$]-L-Tyr.

The second route of synthesis of **7, 8** and **9** involves the direct deuteriation or tritiation of authentic TA under acid catalyzed conditions, Figure 4. Also in this case the deuterium or tritium label is incorporated exclusively in the 3 and 5 ring positions of TA.

Journal of Labelled Compounds and Radiopharmaceuticals

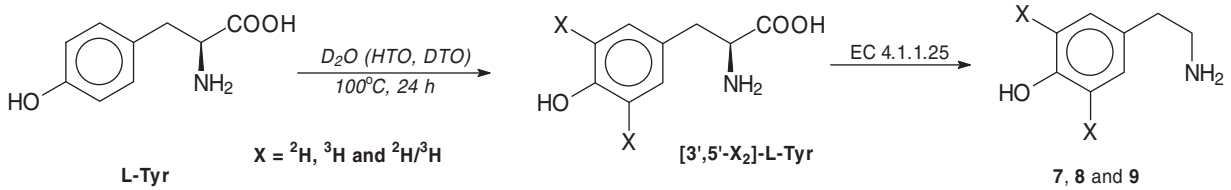

Figure 3. Combined chemical and enzymatic synthesis of ring labeled TA.

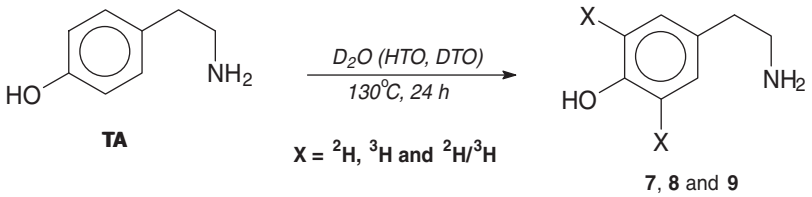

Figure 4. Acid catalyzed isotope exchange between isotopic water and tyramine.

Synthesis of isotopomers of histamine and its derivatives: *Isotopomers of histamine* (*HA*) *labeled in the position* (*αS*). These compounds labeled with deuterium and tritium are obtained in a two step synthesis. In the first step the appropriate isotopomer of L-histidine, L-His, is synthesized by H/D or H/T isotope exchange which is then subjected to the enzymatic decarboxylation catalyzed by the enzyme histidine decarboxylase (HDC, EC 4.1.1.22), yielding selectively labeled HA. As a result of acid catalyzed isotope exchange between D_2O or HTO and L-His the [(*βS*)-^{2}H]-, or [(*βS*)-^{3}H]-L-His isotopomers were obtained, from which [(*αS*)-^{2}H]-, **11**, and [(*αS*)-^{3}H]-HA, **12**, were afforded by decarboxylation in aqueous medium in the presence of enzyme HDC. Under base catalyzed H/T exchange racemic [2,5,*β*-$^3H_3$]-DL-His was obtained which was subjected to enzymatic decarboxylation yielding [2,5,(*αS*)-$^3H_3$]-HA, **13**, leaving [2,5,*β*-$^3H_3$]-D-His intact, Figure 5.

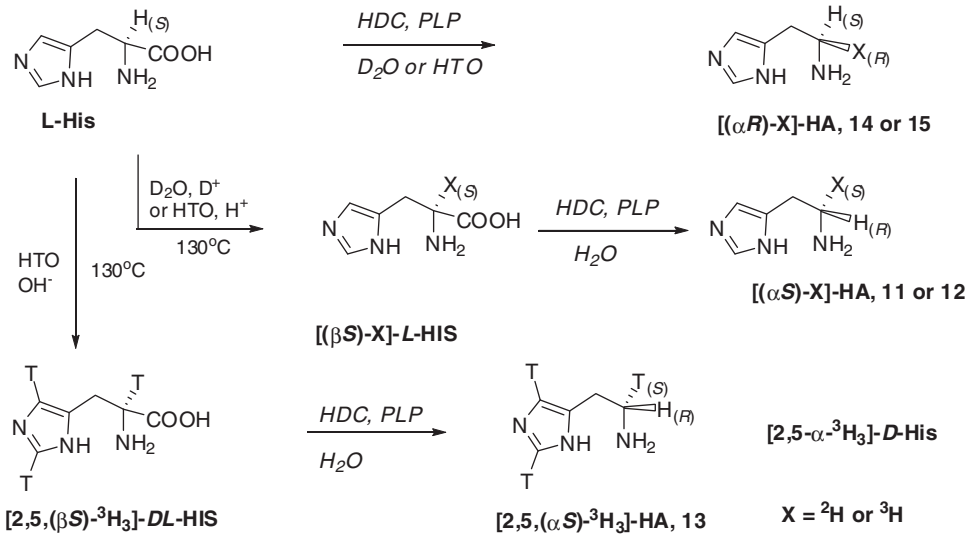

Figure 5. The combined chemical and enzymatic synthesis of isotopologues of histamine.

Isotopomers of HA labeled in the position (αR). Enzymatic decarboxylation of L-His carried out in a fully deuteriated or tritiated medium leads to the formation of isotopomers in configuration (*αR*), i. e., [(*αR*)-^{2}H]-, **14**, and [(*αR*)-^{3}H]-HA, **15**, Figure 5.

Isotopomers of N$^\pi$-and N$^\tau$-methylhistamines labeled in the position (αR). By enzymatic decarboxylation of N$^\pi$-and N$^\tau$-methyl-L-histidines, respectively, catalyzed by the enzyme HDC, the selectively deuterium labeled [(*αR*)-^{2}H]-N$^\pi$-, **16,** and [(*αR*)-^{2}H]-N$^\tau$-CH$_3$]-HA, **17** and tritium [(*αR*)-^{3}H]-N$^\pi$-, **18,** and [(*αR*)-^{3}H]-N$^\tau$-CH$_3$]-HA, **19** isotopomers were afforded. Numeration and denoting of C and N atoms in imidazole compounds are accorded with UPACP recommendation.[7] Figure 6 presents biotransformation of N$^\tau$-methyl-L-histidine into **17** and **19**. Similarly, by decarboxylation of N$^\pi$-methyl-L-histidine **16** and **18** were obtained.

Synthesis of isotopomers of dopamine (DA): *Isotopomers of DA labeled in the position (1R) and (1S)*. Synthesis of [(*1S*)-^{2}H]-, **20**, [(*1S*)-^{3}H]-, **21**, and [(*1S*)-^{2}H/^{3}H]-DA, **22**, is consistent with the biotransformation of the earlier obtained isotopomers of [2-X]-L-Tyr (where *X* = ^{2}H, ^{3}H and ^{2}H/^{3}H), Figure 1, in appropriate isotopomers of [2-X]-L-DOPA using the enzyme tyrosinase (EC 1.14.18.1). Next, these intermediates were converted into (*1S*)-isotopomers of DA using the enzyme tyrosine decarboxylase, Figure 7. The enzymatic

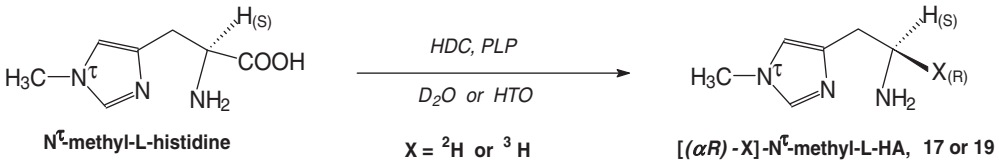

Figure 6. Synthesis of (αR) isotopologues of N^{τ}-methylhistamine.

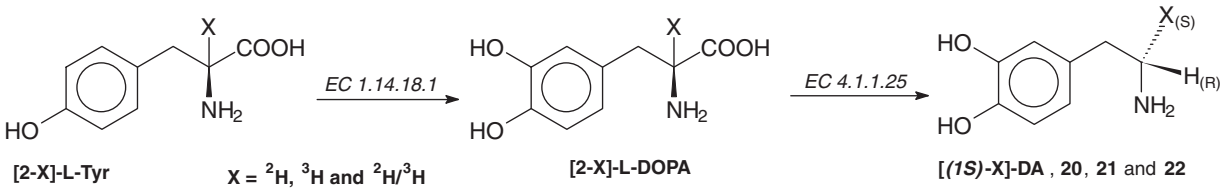

Figure 7. Enzymatic biotransformation of L-tyrosine into dopamine.

decarboxylation of authentic L-DOPA carried out in deuteriated or tritiated incubation medium leads to the formation of [(*1R*)-X]-isotopomers of dopamine, i.e., [(*1R*)-^{2}H]-, **23**, [(*1R*)-^{3}H]-, **24**, and [(*1R*)-^{2}H/^{3}H]-DA, **25**.

Ring labeled isotopomers of DA. [2′,5′,6′-$^{2}H_3$]-, **26**, and [2′,5′,6′-$^{2}H_3$]-DA, **27** were obtained by isotope exchange between isotopic water and authentic dopamine carried out at 50°C under acid catalyzed conditions (similarly, as in the case of **7**, **8** and **9**). The second route consisted of the deuteriation or tritiation of L-DOPA and its subsequent decarboxylation to **26** and **27**, respectively. The isotopomers labeled in the 5′-position, i. e., [5′-^{2}H]-, **28**, [5′-^{3}H]-, **29**, and [5′-^{2}H/^{3}H]-DA, **30**, were obtained by enzymatic hydroxylation of **7**, **8** and **9**, respectively, using the enzyme tyrosinase (EC 1.14.18.1) which exclusively introduces the hydroxyl group into the 3′-position, Figure 8. Similarly, [2′,6′-$^{2}H_2$]-DA, **31** was obtained by hydroxylation of [2′,6′-$^{2}H_2$]-TA, **10.**

TLC and column chromatography were used for the separation and purification of the obtained compounds. All deuteriation procedures were monitored by ^{1}H NMR. The radioactivity of samples were measured using liquid scintillation counting.

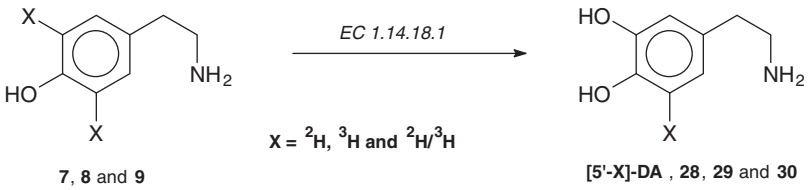

Figure 8. Enzymatic hydroxylation of tyramine.

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