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Research Article

[^{68}Ga]Ga-AntiCAD1: Radiosynthesis and First Imaging Study on Rats

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Abstract

Cadherins are cell adhesion and cell signaling molecules that provide the molecular link between each adjacent cells and have critical importance for the initiation and continuation of adhesion mechanism. N-cadherin expression provides a correlation between upregulation of N-cadherin and inflammation of the lesions. In this paper, we concentrated on the radiolabeling and evaluation of [^{68}Ga]Ga-AntiCAD1 agent as a potential candidate for *in vivo* PET/CT imaging of adhesions. The synthetic N-Ac-CHAVC-NH_2 cyclic peptide sequence designed as (ADH-1)c containing the selective binding His-Ala-Val (HAV) motif based on the chimeric antigen receptor sequence acts as the N-cadherin antagonist. In our previous study, AntiCAD1 conjugate has been studied in detail which is in the process of publication. In this study, the conjugate was radiolabelled with the [^{68}Ga]Ga radionuclide eluted from the ^{68}Ge/^{68}Ga generator (IDB Holland). Radiochemical purity of [^{68}Ga]Ga-AntiCAD1 agent was analysed with TLC methods. The 'shake-flask' method was applied to determine lipophilicity of the agent by calculating the P distribution coefficient (logP=-2.69±0.54). The biodistribution of the agent was investigated using PET/CT on Wistar Albino rats. Significant uptake was found in liver, kidneys, spleen, salivary gland and targeted region with $SUV_{max-mean}$ of 1.36, 1.96, 1.38, 1.16 and 2.14 respectively. The Pearson Factorial method is used to test the relationship between the targeted region and other body tissues, to measure the degree of this relationship (R=0.73). Radiolabelled agent was demonstrated to react specifically with N-cadherin in targeting of rat tissues.

Keywords: *AntiCAD1 agent, Peptide radiopharmaceuticals, PET/CT imaging, N-cadherin expression*

[^{68}Ga]Ga-AntiCAD1: Radyosentez ve Sıçanlar Üzerinde İlk Görüntüleme Çalışması

Öz

Kadherinler, bitişik hücreler arasındaki moleküler bağlantıyı sağlayan, yapışma mekanizmasının başlatılması ve devamı için kritik öneme sahip olan hücre adezyon ve hücre sinyal molekülleridir. N-kadherin ekspresyonu, N-kadherinin artışı ve lezyonların inflamasyonu arasında bir korelasyon sağlar. Bu makalede, adezyonların *in vivo* PET/CT görüntülenmesi için potansiyel bir aday olarak [^{68}Ga]Ga-AntiCAD1 ajanının radyoişaretlenmesi ve değerlendirmesine odaklanılmıştır. Kimerik antijen reseptörü dizisine dayanan seçici bağlayıcı His-Ala-Val (HAV) motifini içeren (ADH-1)c olarak tasarlanan sentetik N-Ac-CHAVC-NH_2 siklik peptid dizisi, N-kadherin antagonisti olarak işlev görür. Yayın aşamasındaki önceki çalışmamızda AntiCAD1 konjugatı ayrıntılı olarak incelenmiştir. Bu çalışmamızda, konjugat, ^{68}Ge/^{68}Ga jeneratöründen (IDB Holland) elde edilen [^{68}Ga]Ga radyonüklidi ile radyoaktif olarak işaretlendi. [^{68}Ga]Ga-AntiCAD1 ajanının radyokimyasal saflığı TLC metodları ile analiz edildi. Ajanın lipofilisitesini belirlemek için P dağılım katsayısı hesaplanarak 'çalkalama şişesi' yöntemi uygulandı (logP=-2.69±0.54). Ajanın biyodağılımı, Wistar Albino sıçanları üzerinde PET/CT kullanılarak araştırıldı. Karaciğer, böbrekler, dalak, tükürük bezi ve hedef bölgede sırasıyla ortalama 1.36, 1.96, 1.38, 1.16 ve 2.14 SUV_{max} değerleri ile kayda değer oranda tutulum tespit edildi. Hedeflenen bölge ile diğer vücut dokuları arasında olan ilişkini test etmek ve bu ilişkinin derecesini ölçmek için istatistiksel Pearson Faktörü hesaplama yöntemi kullanılmıştır (R=0.73). Sıçan dokularının hedeflenmesinde radyoişaretli ajanın N-kadherin ile spesifik olarak etkileşime girdiği gösterildi.

Anahtar sözcükler: *AntiCAD1 ajanı, Peptid radyofarmasötikleri, PET/CT görüntüleme, N-kadherin ekspresyonu*

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INTRODUCTION

Cadherins are cell surface glycoproteins responsible for morphogenesis in the embryo, selective cell recognition in the adult organism, and lifelong normal tissue architecture. Adherens junctions which are essential for the formation and maintenance of functional cellular junctions are composed of cadherins [1]. Cadherins are cell adhesion and cell signaling molecules that provide the molecular link between adjacent cells and have critical importance for the initiation and continuation of adhesion mechanism. It differs from the other members of the cadherin group compounds with a large extracellular N-terminal consisting of repeating domains and important Ca^{++} binding, and a single transmembrane region linked to the cytoplasmic region. N (Neural)-cadherin is presented in mesenchymal tissues, in endothelial cells and found in adhesive complexes that cover them between endothelial cells and pericytes [2-4]. N-cadherin antagonists which are expressed in some tumor cells and blood vessels target N-cadherin. The re-expression of N-cadherin in endothelial cells is accompanied by neovascularization. There was a correlation between upregulation of N-cadherin and inflammation events by indicating it in the enlarged blood vessels of the inflamed tissue of the lesions [5]. It has been shown that the synthetic N-Ac-CHAVC-NH_2 cyclic peptide sequence designed as ADH-1 which contains the selective binding HAV (His-Ala-Val) motif based on the CAR(Chimeric Antigen Receptors)sequence acts as the N-cadherin antagonist [6]. Cyclic (ADH-1)c peptide analogue has the ability to disrupt many processes through N-cadherin, as well as the linear peptide [7]. There are studies on targeting of N-cadherin expression in order to understand the mechanism of many different cancer types and the functions of proximal renal tubular epithelial cells [8-10]. In this study, AntiCAD1 agent was targeted to the N-cadherin receptor in epithelial tissue adhesion. The data based on the positron emission tomographic imaging-computed tomography (PET/CT) which is the 'gold standard' in imaging was obtained by the combination of metabolic chemical activity of the agent and anatomic information in rat organism. PET imaging provides detailed information about the results of metabolic activities and cellular functions. Peptides labelled with a positron-emitting radionuclide can be used to make an accurate diagnosis, determine staging and quantify the radiation dose to tumors and critical organs, thus allowing dose planning and dose monitoring for successful radiotherapy, and to follow target response to therapy, thus providing personalized patient management [11-13]. The compatibility of [^{68}Ga]Ga with peptides for targeted imaging of such receptors as somatostatin, bombesin, human epidermal growth factor, integrin, vascular endothelial growth factor, cholecystokinin-2, gastrin-releasing peptide, melanocyte stimulation hormone, glucagon-like peptide 1, gonadotropin-releasing hormone, folate, neurotensin, and neuropeptide Y receptors has been demonstrated [12,13]. The corresponding ligands commonly have satisfactory pharmacokinetics with fast blood clearance, relatively low hepatobiliary excretion, and mostly renal elimination as well as excellent tissue penetration, minimal side-effects, and no or low antigenicity.Imaging of vascular endothelial growth factor (VEGF) receptors overexpressed in tumors, adhesive and violated tissues(by injury or surgical intervention) that have extensive formation of new capillaries is an important means not only for diagnosis and monitoring response therapy but also for antiangiogenic drug development.

MATERIAL and METHODS

The cyclic (ADH-1)c peptide analogue was obtained in our laboratory with using CEM Liberty microwave assisted automatic peptide synthesis system and applying cyclization procedure, respectively. EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride], EDTA (Ethylenediaminetetraacetic acid) and all other chemicals purchased from Sigma Aldrich Chemicals (St. Louis), USA. Ultra-pure water was obtained from Millipore Milli-Q system.

Koc University National Ethics Committee for Animal Experiments gave approval for the animal experiments (Protocol no: 2016.HADYEK.018, 02/06/2016 dated).

Preparation of Cyclic (ADH-1)c Peptide-EDTA Conjugate

Peptide synthesis and conjugation with EDTA have been studied in detail in our previous study, which is in the process of publication. Briefly, the linear ADH-1 peptide sequence (Ac-Cys-His-Ala-Val-Cys-$CONH_2$) was synthesized using the Fmoc chemistry in the CEM Liberty Microwave Assisted Solid Phase Peptide Synthesis System. The peptide precipitated as a white powder was stored at -18°C for cyclization [14-16]. The cyclization process is based on the procedure described by Dubey et al.[17]. A disulfide bridge was formed between the two cysteines by oxidation of the thiol groups of the cysteine residues.After the process was complete, the cyclic peptide was lyophilized [17]. Conjugate of the cyclic (ADH-1)c peptide with EDTA chelator was synthesized by the water-soluble carbodiimide procedure in the presence of the EDC crosslinker [18-20]. LC-MS m/z for cyclic(ADH-1)c peptide$[M+H^+]^+$=572.85 and EDTA-(ADH-1) c conjugate (AntiCAD1 conjugate) $[M+H^+]^+$=867.

Elution of ^{68}Ge/^{68}Ga Generator

The ^{68}Ga radionuclide ($t_{1/2}$ = 67.7 min., β^+ = 89%, E β^+maks, 1.9 MeV, EC: 11%, Eγmaks: 4.0 MeV) was manually eluted from the SnO_2-based ^{68}Ge/^{68}Ga generator (IDB Holland iThemba Lab. Generator, 30mCi) using a disposable syringe with 15 mL of 0.6 M HCI acid and collected into a vial containing 1 mL of9.5 MHCI acid. The resulting ^{68}Ga solution was subjected to cationic pre-purification to bring it to levels that would allow labeling with

peptides. Approximately 1.5 mL of 15 mCi [^{68}Ga]Ga was obtained.

Radiolabelling of AntiCAD1 Conjugatewith [^{68}Ga]Ga Radionuclide

To carry out the labeling with the [^{68}Ga]Ga radionuclide eluted from the ^{68}Ge/^{68}Ga generator, after transferring the 30 µL AntiCAD1 conjugate to the reaction vial, 1.5 µM HEPES buffer was added and pHadjusted to 4. Immediately thereafter, 1.5 mL of eluted [^{68}Ga]Ga was added and the reaction was carried out in a microwave oven under nitrogen and dry air atmosphere at 125 degrees centigrade for 6 min. At the end of the reaction, the radiopharmaceutical labeling rate of the product *(Fig. 1)* was evaluated by adding 15 mL of PBS buffer to the reaction vial.

TLC for Radiochemical Purity Analysis of [^{68}Ga]Ga-AntiCAD1 Agent

Product purity was analyzed by TLC on iTLC-SG plates: Labeling rate of [^{68}Ga]Ga-AntiCAD1 agent were studied with three different mobile phase solutions [Solution A; Isopropil alcohol:n-butanol:0.2 N amonnium hydroksit (2:1:1 (v:v:v)), Solution B; n-butanol:water:acetic acid(4:2:1 (v:v:v)) and Solution C; TFA 0.1%:MeOH (30:70 (v:v))]. Solution A, B and C were added to the chromatography tanks. Ten µL of the synthesized product was dropped onto the sample spot in the start line of each chromatographic strip. The solutions were run to solvent line. Each strip was allowed to dry at room temperature. The drying strips were cut to equal lengths. The activity value of each piece was read in a gamma counter (Biodex, Atom Lab 500).

Rf and dose values were calculated for each parts. Quantitative analysis of radioactive spots were done to determine the amount of radiolabelled agent and un-chelated radiometal [21,22].

Lipophilicity

Lipophilicity to drug development is an important early indicator of potential *in vivo* pharmacokinetic and dynamic behavior. Lipophilicity measurements provide information on non-specific binding, metabolic stability, drug distribution and excretion [23,24]. Because of this diversity, lipophilicity is one of the most important physicochemical properties and most frequently analyzed and published parameters [23,25]. The'shake-flask' method is the 'gold standard' for determining the logP value [25-28]. While lipophilic peptides are excreted through the liver and intestines (hepatobiliary excretion), hydrophilic peptides are primarily excreted through the kidneys (renal excretion). Radiolabeled peptides are usually highly hydrophilic and therefore do not show significant accumulation in the hepatobiliary system. However, peptide lipophilicity is increased by labeling through prosthetic groups [29]. In our study, the shake-flask method was applied to determine lipophilicity. Lipophilicity values were determined by calculating the P distribution coefficient ($[A]_o/[A]_w$). One hundred µL of the radiolabelled compound was added to the tube containing 3 mL of n-octanol and 3 mL of water. The mixture was stirred with magnetic stirrer for 1 h. It was centrifuged at 3000 rpm for 5 min to completely separate the phases. Activity values were measured in the gama counter by pipetting 500 µL from both phases. LogPvalue was calculated by the logarithm of the ratio between the radioactive counts in the octanol and water fractions.

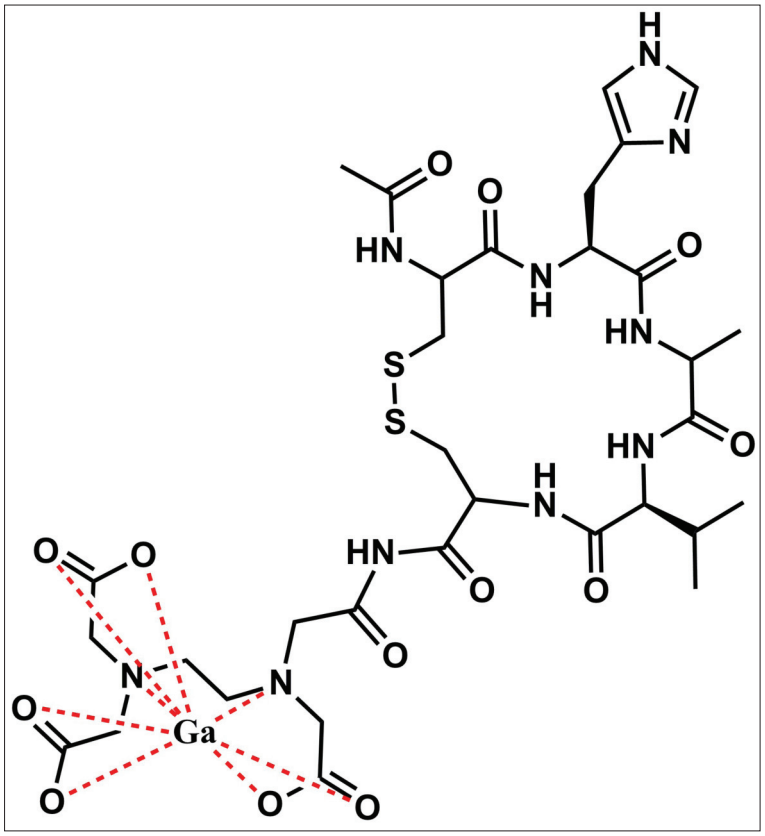


Fig 1. Molecular structure of [^{68}Ga]Ga-AntiCAD1 agent

Animal Studies

Metabolic functions of the agent applied on Wistar Albino rats were studied withPET/CT (General Electric Healthcare/Discovery 710) and Gama Kamera/SPECT(Siemens) equipped with a low energy, high-resolution collimator under a protocol approved by Koç University Animal Experiments Local Ethics Committee. Ratswerefasted for four hours before the each intervention. All interventional and imaging studies were performed under anesthetize by using isoflurane without a vaporizer. The liquid anesthetic was applied to cotton which was

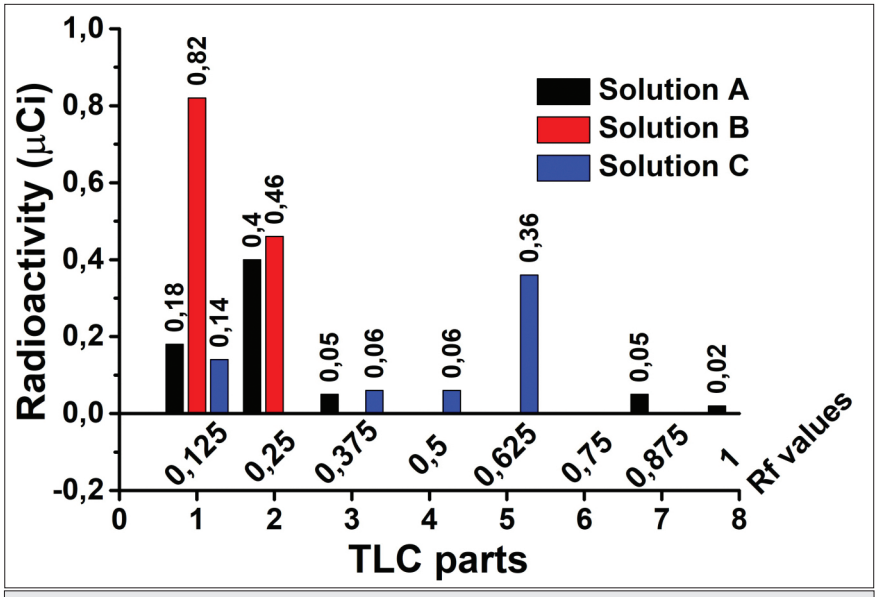


Fig 2. Rf values of relevant complex region

Table 1. *The binding ratios of the radioactive compounds on the TLC*

Substance	Solvent A	Solvent B	Solvent C
[^{68}Ga]Ga-AntiCAD1	57.14%	64.06%	58.06%
[^{68}Ga]Ga-compounds	25.71%	35.94%	22.58%

placed in a conical tube for maintenance of anesthesia. Because of the high risk of waste anesthetic gas exposure, this method was performed in a special biosafety cabinet with a carbon filter.

RESULTS

Radionuclide Purity Assay with Radio-TLC

Product purity was analyzed by iTLC-SG plates using TLC with three different mobile phases.The Rf values calculated for each activity value read in the gamma counter are shown in *Fig. 2*. The un-chelated metalalso seen in the first part (Rf=0.125) for the three solution types, the second part for solution A and solution B, the fifth part for the solution C and a higher Rf value. The impurities in the solution are migrated to the upper region due to the chemical structure (solubility and polarity) of solution A. The relatively high radioactivity value of the first part in Solution B is due to the fact that solution B has activity in the same part as the un-chelated metal without dragging impurities. The un-chelated metal Rf values and doses measured in the first part in solution A and solution C support each other. The ratios of labelled analytes and unchelated metals are shown in *Table 1*.

Scintigraphic and Tomographic Studies

[^{99}Tc^{m}]pertechnetate and[^{177}Lu]Lu-PSMAPre-Imaging Studies with Gamma Camera: Pre-imaging study was carried out for the purpose of reference imaging. The studies were performed on Wistar Albino rat by using agents which are routinely administered in Nuclear Medicine and Molecular Imaging Department except our new agent. Biodistribution of the new agent in rat tissues and organs can be investigated more sensitively and accurate by using reference imaging. In addition, the amount of dose to be administered to the Wistar Albino rat is determined in advance so that the animal is prevented from overdosing.

Scintigraphic images were obtained from Siemens Gamma Camera/ SPECT. Prior to the injection of the radio-pharmaceutical agent, [^{99}Tc^{m}] pertechnetate and [^{177}Lu]Lu-PSMA injections were performed at physiological pH at different times. Many preimages were obtained by the application of intravenous injection of radiolabeled agentsto the Wistar Albinoratwhichuptakeandaccumulation regions in physiological tissue is previously known.

Gamma camera images were examined at 5th, 10th, 15th, 30th, 60th, 120th, 180th, 240th min after intraperitonal injection by using 0.1 mCi pertechnetate. In the first 10 min, stomachuptake, that is the injection site and thyroid uptake, a selective target for the pertechnetate were displayed. At the 15th min, the [^{99}Tc^{m}] pertechnetate accumulation in bladder started and after the 120 min the intestinal accumulation was viewed. In this way, thyroid, intestine, stomach and bladder regions were determined in the ratas shown in *Fig. 3*.

Gamma camera images were investigated at 10th, 60th, 120th, 180th min and 24th h after intravenous injection from the tail vein with 0.1 mCi [^{177}Lu]Lu-PSMA at physiological pH. *Fig. 4* indicates that as the injection region tail vein and epididymis-testis region accumulation, right-left kidney uptake and bladder-prostate region accumulation were displayed at the 10th min imaging. It is understood from the first hour image that the accumulation in the injection site decreases while uptake increases in the other regions. At the second and third hour's of imaging, left-right kidney uptake which is selective to the prostate-specific membrane agent, and bladder-prostate area accumulation were displayed. At the 24th h, right and left kidney uptake was displayed intensely. After all; right-left kidney, urethral continence zone-prostate, testis-epididymis and tail vein regions were determined with [^{177}Lu]Lu-PSMA injection.

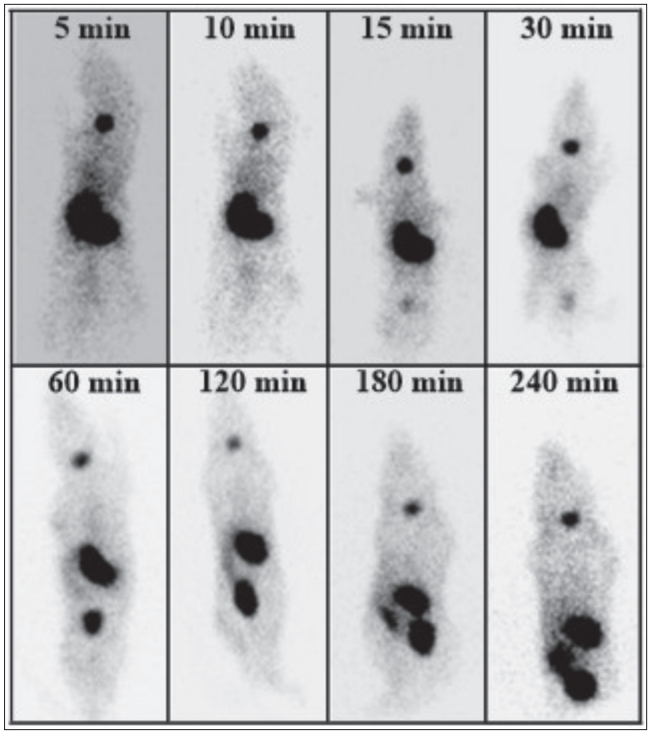


Fig 3. Gamma camera images at 5th, 10th, 15th, 30th, 60th, 120th, 180th, 240th min after intraperitoneal injection of rat with using[$^{99}Tc^{m}$] pertechnetate

PET/CT Adhesion Imaging Studies of [^{68}Ga]Ga-AntiCAD1 Agent: [^{68}Ga]Ga-AntiCAD1 consisting of five amino acids was applied to display adhesion molecules in violated tissue via specific binding of bioconjugate to the N-cadherin. The distribution of this agent was studied inthe Wistar Albino rat organism by using PET/CT. Tomographic images given in *Fig. 5* were obtained from GE Healthcare/Discovery 710 PET/CT. In the study with left hand injuried rat as shown in *Fig. 6*, administration of 0.1 mCi[^{68}Ga]Ga-AntiCAD1 at physiological pH was performed by intravenous tailinjection. Increased uptake of the [^{68}Ga] Ga-AntiCAD1agent in the injuried adhesive left hand was detected. When PET/CT images of the 1st and 3rd h were examined besides the intense kidney and bladder uptake as well as synthesized radiopharmaceutical agent there was an expected selective uptake in the targeted region.

Correlation of PET/CT Standardized Uptake Mesaurement Values: The binding specificity was detected with the increased uptake of the [^{68}Ga]Ga-AntiCAD1 agent in the injuried adhesive left hand.In other tissues the uptake of the agent was low. The statistical method of Pearson Factor was used to test the relationship betweenthe targeted region and other body tissues, to measure the degree of this relationship and to predict similar outcomes. In this way, the degree of linear relationship for all body uptake of two continuous or parametric variables of uptake regions can be calculated. Mean standardized uptake values $(SUV)_{max\text{-}mean}$ in liver, kidneys, spleen, salivary gland and targeted region were found 1.36, 1.96, 1.38, 1.16 and 2.14 respectively. The R value was calculated to be 0.7331. This value is in the range of 0.70-0.89, indicating a strong positive correlation.

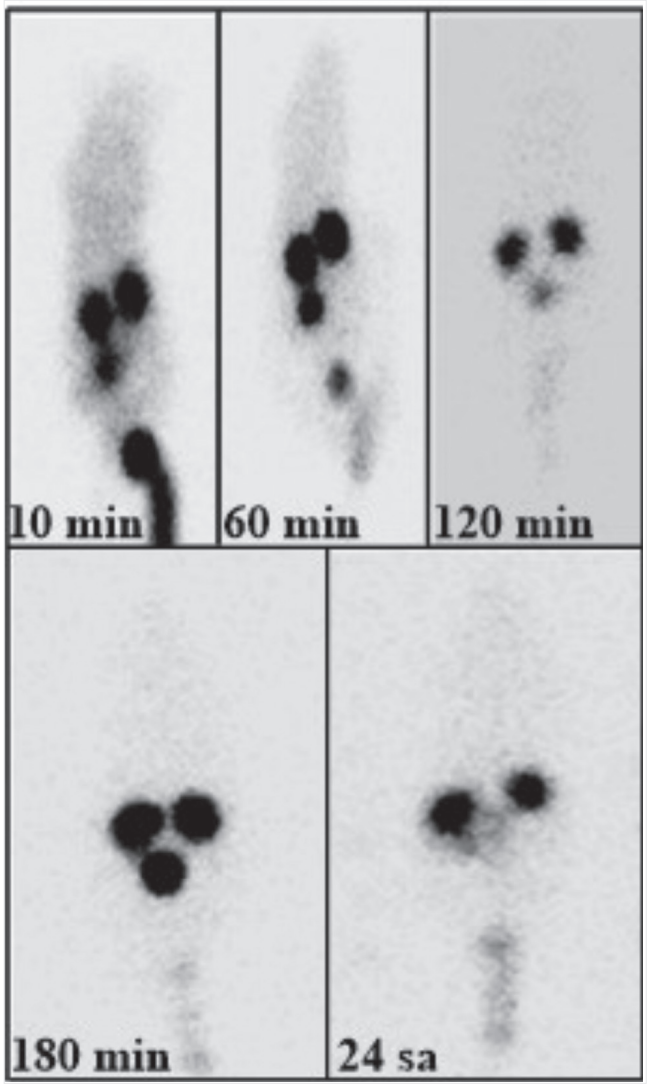


Fig 4. Gamma camera images at 10th, 60th, 120th, 180th minutes and 24 h after intravenous injection of rat with using[^{177}Lu]Lu-PSMA

Determination of Lipophilicity

The measured lipophilicity value (log P) of [^{68}Ga]Ga-AntiCAD1was calculated at pH 7 as -2.69±0.54 (n=3) by the 'shake-flask method'.Minus logP values indicate that the concentration of the labelled compounds in water is higher than its concentration in octanol. This value indicates that the targeted agent is primarily excreted by renal excretion [21,22,30].

DISCUSSION

The synthesized conjugate was labeled with [^{68}Ga] Ga radionuclide whichwas eluted from the ^{68}Ge/^{68}Ga generator and the product purity was checked by TLC on iTLC-SG plates. The results were found appropriate for

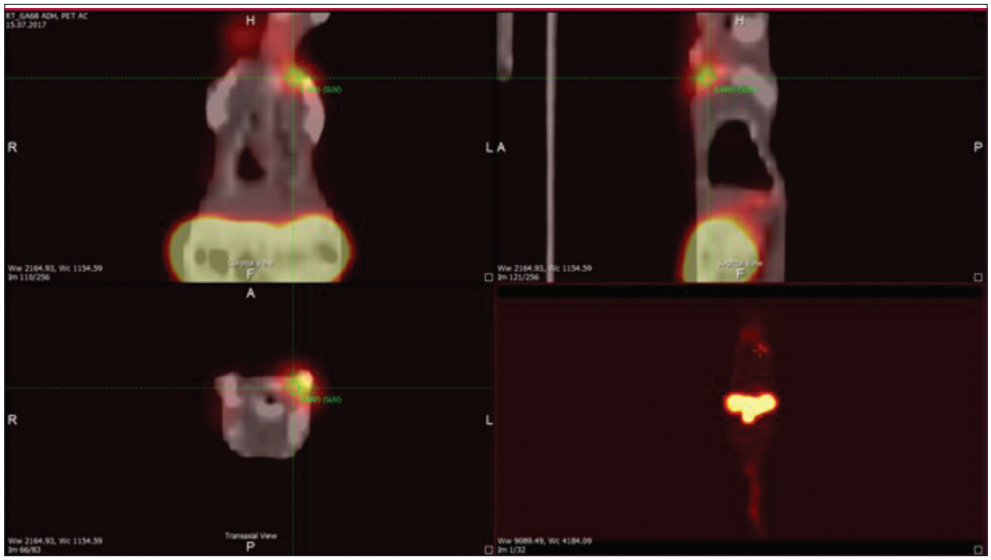
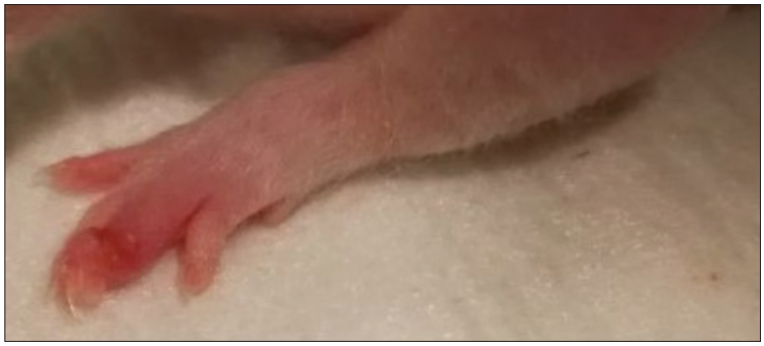


Fig 5. PET/CT adhesion imageat 60th min after intravenous tail injection of rat with using [68Ga]Ga-AntiCAD1 agent

Fig 6. Left hand injuried ratmodel



animal studies, but the labeling yield could be increased by developing different methods.

Lipophilicity study was performed for *in vitro* evaluation of radiolabelled agent. logP values emphasize that excretion is primarily eliminated through the renal clearance. Because of the low lipophilicity of the radiolabelled agent, SUV_{max} value of the kidney uptakesis higher in comparison with liver, spleen, and salivary gland uptakes. As a result, theoretical lipophilicity value (-2.69±0.54) was supported with SUV_{max} values [31,32].

Determination of the anatomical location of organs which is essential for animal imaging studies in nuclear medicine is very important in terms of evaluation of the images. The accumulation regions of [$^{99}Tc^{m}$] and [^{177}Lu]Lu-PSMA are thyroid, intestine, stomach, bladder and salivary gland, kidneys, prostate and liver respectively. The critical organ locations of the animal were fully identified by the uptake of these regions by scintigraphy studies.

PET/CT images were obtained via tail injection of the radiolabelled AntiCAD1 agent. The first and third hours of PET/CT images were analyzed bycalculating SUV_{max}within the scope of tomographic study. In this study, we investigated injury-induced disruption of the N-cadherin in adhesive tissue and it was sufficient to maintenance of functional cellular junctions. The uptakes of the [68Ga]Ga-AntiCAD1 agent in the liver, spleen, kidney, salivary gland and adhesive tissue were compared on the basis of SUV_{max} values and the uptake in the targeted region was found to be higher. This result is important for the protection of non-target healthy organs. Radiolabeled agent was demonstrated to react specifically with N-cadherin in targeting of rat tissues or selectively targeted in the injured adhesive tissue. The degree of the linear relationship between the uptake regions and all body uptake was calculated by the Pearson factorial method. The R value (0.7331) indicates that the uptake in the target region correlates with organ uptakes.

Although N-cadherin expression and targeting have been studied, there is no information available about the metabolic chemical activity that results from the specific targeting of this receptor.In this context, PET/CT provides detailed information on metabolic activity and the results of cellular functions. In addition, at this moment injured tissues and focus of infection can not be detected with PET/CT and there is not any common specific PET agent used in the imaging of all solid tumor types. The targeted agent may be a new strong candidate as radiopharmaceutical agent because of its less hepatic uptake than prostate-specific new generation peptidic

agent DKFZ-1007-PSMA, less urine excretion than DCFPyL and through protection feature of healthy tissues [31]. As a result, radiolabeled agent derived from the cyclic (ADH-1)c peptide analogue which is an adhesion molecule from cancer epithelial mesenchymal transformation markers may be a potential compound that can be used to discrimination of damaged mesenchymal tissues, determination of metastatic solid tumors anddetection of focus of infection by using PET/CT imaging technique.

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Conflicts of Interest

The authorsdeclare that they have no conflict of interest.

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