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Original Article

PRELIMINARY EVALUATION OF AN ANTHRAQUINONE CONJUGATED DOTA DERIVATIVE AS SPECT AGENT

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ABSTRACT

Objective: An anthraquinone derivative, DO3A-Act-AQ having DO3A (1, 4, 7, 10-tetraazacyclododecane-1, 4, 7-trisacetic acid) scaffold is radio labeled with ^{99m}Tc radioisotope and evaluated as a SPECT imaging agent for tumor.

Methods: Preliminary in-vivo evaluation of ^{99m}Tc-DO3A-Act-AQ radioconjugate including blood kinetics, biodistribution and gamma scintigraphic imaging is performed on BMG-1 tumor xenografted mice after successful optimization of the radiolabeling condition.

Results: The radiotracer, ^{99m}Tc-DO3A-Act-AQ was produced in high radiochemical yield of>96% and specific activity of 3.62 MBq/nmol at pH 7.5 and 150 µg stannous chloride. Radioconjugate displayed excellent in-vitro and in-vivo stability with only ~2% transchelation of radiometal at 24 h p. i and rapid blood clearance from the system with $t_{1/2}$(F) = 38.04±0.35 min and $t_{1/2}$(S) = 5 h 30 min±0.67. Significant tumor-to-muscle ratio of>7 at 2 h p. i. in biodistribution and SPECT imaging studies in BMG-1 tumor xenografted mice suggested the tumor specificity of the radioconjugate.

Conclusion: Stable radiocomplex formation of ^{99m}Tc-DO3A-Act-AQ and its significant tumor specificity demonstrated its future application as a promising SPECT radioligand for tumor imaging.

Keywords: Anthraquinone, Tumor, Single Photon Emission Computed Tomography.

INTRODUCTION

Deoxyribonucleic acid (DNA) has a great biological importance and is the primary intracellular target for various anticancer drugs like doxorubicin, cisplatin, mitoxantrone, tallimustine and brostallicin [1-2]. Anthracyclines or anthraquinones are among the most widely used DNA targeting chemotherapeutic agents that are effective against the broad spectrum of tumors [3]. The tricyclic system of anthraquinone intercalate with the DNA base pairs leading to interference in the transcription and replication processes of the cancer cells resulting cellular death [4-5]. Many anthraquinone derivatives are reported but the promising clinical activity with 1, 4-bis ((aminoalkyl) amino) anthracene-9, 10-diones-substituted anthraquinone; mitoxantrone and ametantrone led to numerous pharmacological studies [6-12]. Tricyclic core comprising agents like 1, 3-dihydroxy-9, 10-anthraquinone (DHA), 1-hydroxy-3-(3-alkylaminopropoxy)-9, 10-anthraquinone (MHA) and 3-(3-alkylaminopropoxy)-9, 10-anthraquinone (NHA), pyrrolo [2, 1-c] [1, 4] benzodiazepine-anthraquinone conjugates are potent antitumor agents, which lead to tumor cell apoptosis [13-18].

Substituted anthraquinone derivatives also represent as potential anticancer drugs that target G-quadruplex structures of DNA through its stabilization [19-23]. Anthraquinone derivatives like cationic porphyrin–anthraquinone dyads target the G quadruplex DNA by π-π interactions whereas the cationic side chains interact with the negatively charged phosphate backbones present in G-quadruplex. Derivatives of anthraquinone are also stated as effective inhibitors of c-Met Kinase (product of the MET proto-oncogene) pathway which could be useful for cancer therapy [24]. Macrocyclic complexes that are linked with DNA binding agents are stated to demonstrate sequence selective interaction with DNA depending on the nature of the agent, linker and metal ion used, eg., N-Substituted cyclam-amino acid conjugates interacts with DNA in a highly selective manner [25-26].

In nuclear imaging, stable complexes of bifunctional chelators with radiometals are crucial factor for the development of metal based imaging and therapeutic agent. The polyaza polycarboxylic macrocycles with seven to eight donor atoms such as DOTA (1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid) are recognized for their strong complexation stability, extraordinary rigid and symmetrical structure with various metal ions in comparison with non-cyclic ligands such as DTPA.

In our previous studies, we reported the theranostic application of DO3A-Act-AQ, whereby ^{68}Ga metal ion was utilized for PET [27]. The demand of non cyclotron based radiopharmaceutical prompted us to label the developed ligand with ^{99m}Tc to further explore its utility as a SPECT agent. ^{99m}Tc was the radiometal of choice for SPECT due to the favorable imaging characteristics, low cost and easy availability. Moreover, various ^{99m}Tc labeled DOTA derivatives having application in tumor imaging are reported for SPECT [28-29].

Here, we report the preliminary preclinical evaluation of ^{99m}Tc-DO3A-Act-AQ; radiolabeling, quality control, serum stability profile, blood kinetics, bio distribution profile and tumor imaging after standardization of radiolabeling procedure with $^{99m}TcO_4^-$intended for its future application in SPECT.

MATERIAL AND METHODS

Chemicals

All the chemicals and solvents were purchased from Sigma-Aldrich. ^{99m}Tc was obtained in the form of its sodium salt, Na[$^{99m}TcO_4^-$], eluted from a ^{99}Mo/ ^{99m}Tc generator by solvent extraction method, as supplied by the Regional Center for Radiopharmaceuticals (northern region), Board of Radiation and Isotope Technology (BRIT) (unit of BARC), Department of Atomic Energy. Instant thin layer chromatography (ITLC-SG) used were purchased from Gelman Sciences, Ann Arbor, MI.

Instrumentation

Radioactivity counts were measured in a well-type gamma-ray counter (type CRS23C; ECIL). Radio imaging studies were carried out using a planar gamma camera fitted with parallel collimator (ECIL).

Animal models

All animal experiments were performed according to guidelines of Institute's Animal Ethics Committee (Reg. No. 8/GO/a/99/CPCSEA). Healthy, albino New Zealand rabbits with body weight between 2.5–3 kg were utilized for blood kinetics studies. Female athymic nude mice (weighing between 25–30 g), with no prior drug treatment, were employed for biodistribution and scintigraphic imaging studies. Animals were housed under controlled temperature of 22–25 °C with a 12 h day-night cycle and reared on laboratory chow pallets, fed ad libitum and had free access to food and water at all the time. All possible measures were taken to minimize the animals suffering during experiment. Athymic mice were inoculated subcutaneously with 100 µl of BMG-1cell suspension (5×10^6) in the right hind leg under sterilized conditions and were used when the tumor volume reached ~400 mm^3 (after 3–4 weeks of inoculation).

Experimental

Radiolabeling of DO3A-Act-AQ with ^{99m}Technitium

Direct radio labeling of DO3A-Act-AQ was performed using stannous chloride dihydrate ($SnCl_2.2H_2O$) as reducing agent. Briefly, to an aqueous solution of DO3A-Act-AQ (2 µg/100 µl) was added stannous chloride (30 µL; 1 µg/µl in 10 % acetic acid). pH of the reaction mixture was adjusted to 7.5 using 0.1 M sodium carbonate solution followed by addition of freshly eluted (<1 h) $^{99m}TcO_4^-$(80 MBq; 200 µL). The reaction mixture vial was allowed to stand for 15 minutes at room temperature (25 ˚C).

Radiolabeling efficiency and quality control

The radiolabeling efficiency of the ^{99m}Tc-DO3A-Act-AQ was measured using ascending instant thin layer chromatography (ITLC) on silica gel (SG) strips using 100% acetone as developing solvent and simultaneously in pyridine: acetic acid: water (PAW) (3: 5: 1.5). Each TLC was cut in equal segments of 0.5 cm and counts were measured in each segment using a gamma counter. Percentage of free Na $^{99m}TcO_4^-$, reduced/hydrolysed ^{99m}Tc and the radioconjugate formation was calculated by using this method where free ^{99m}Tc moved with the solvent front in acetone while ^{99m}Tc-DO3A-Act-AQ remained at the point of application on ITLC-SG. Whereas in PAW solvent system, technetium colloids remained at origin (base spot) and radioconjugate moved with the solvent. In similar manner, radiochemical purity of ^{99m}Tc-DO3A-Act-AQ was assessed utilizing ascending instant thin layer chromatography (ITLC-SG) in citrate buffer where the radiolabeled complex remained at the origin (point of application). The radiolabeled conjugate was purified using a C-18 reversed phase extraction cartridge (preconditioned with methanol and water (10 mL). The cartridge was sequentially washed with water and ethanol (5 mL each) and radiolabeled conjugate was eluted using 5% ethanol. The ^{99m}Tc–DO3A-Act-AQ was reconstituted in saline and filtered through a sterile 0.22 mm Millipore filter and radiotracer purity was monitored by ITLC.

Human serum stability

Human serum is extracted from healthy volunteer's blood by clotting the blood for 50 min at 37 ˚C in a humidified incubator at 95% air / 5% CO_2. The incubated samples were centrifuged at 400 rpm and serum was filtered through 0.22 µm syringe filter. 100 µL of ^{99m}Tc-DO3A-Act-AQ was incubated with 1 mL of human serum in test tube. The stability of the complex was determined at different time interval of 1, 2, 4, 6 and 24 h post incubation using ITLC with pyridine/ acetic acid/ water (3:5:1.5) as mobile phase. Percentage of free pertechnetate at each time point was estimated which indicates the percentage dissociation of the complex at that particular time point in serum.

Blood kinetics studies

For the blood kinetics studies, ^{99m}Tc-DO3A-Act-AQ was intravenously administered in healthy rabbits. Briefly, 0.3 mL of ^{99m}Tc-DO3A-Act-AQ was administrated through the dorsal ear vein of the rabbit. The blood sample from the other ear vein was taken at different time intervals (0.25, 0.5, 1, 2, 4 and 24 h) and collected in heparinised tubes. The blood samples were accurately weighed to the second place of decimal in terms of g and number of counts was measured using gamma well counter. Data is expressed as percent administrated dose present in the whole body blood considering whole body blood as 7% of the body.

With the same blood samples, plasma was separated out by centrifugation to measure the protein binding of the radioconjugate. The plasma proteins were precipitated by addition of 10% trichloroacetic acid (TCA). The radioactivity of the precipitate and supernatant was measured in a well-type gamma counter.

Ex-vivo biodistribution studies

In-vivo tissue distribution of ^{99m}Tc-DO3A-Act-AQ was examined in female athymic nude mice xenografted with BMG-1 tumor. Radiolabeled complex (100 µl, 3.7 MBq) was administered in a group of 10 mice through their tail vein. The mice were humanely sacrificed, 2 at a time, at 1, 2, 4, 6 and 24 h post-injection. Blood was collected by cardiac puncture and all the different organs, namely the heart, liver, kidneys, stomach, intestines, muscle and tumor were removed and washed with normal saline to clear the organs of surface blood and debris. The blood and organs were collected in pre-weighed tubes and residual radioactivity in each organ was measured using a gamma counter (calibrated for ^{99m}Tc energy). Uptake of the radiotracer in each organ was calculated and expressed as percentage of injected dose per gram of the organ (% ID/g).

Scintigraphic imaging

The scintigraphic imaging was performed in athymic mice xenografted with BMG-1 tumor on right hind leg, using γ-camera. 100 µl of ^{99m}Tc-DO3A-Act-AQ (2.5 MBq) was injected intravenously through the tail vein of the animal and imaging of the mice was carried out at different intervals of time starting from 0.5–6 h post injection of probe. Semi quantitative analysis was carried by generating ROI on tumor and soft tissues.

Statistical analysis

Results are depicted as the mean±standard error of the mean (SEM). Statistical analysis was performed using a student's t test. $P<0.05$ was considered significant.

RESULTS

Radiolabeling, quality control and stability studies of DO3A-Act-AQ

Radiochemical yield of ^{99m}Tc-DO3A-Act-AQ was found to be >94% with a specific activity of 3.62 MBq/nmol. As observed by ITLC radiochromatogram in fig. 1, radiochemical purity for the complex was observed to be >96%. The optimum pH for radiolabeling was found to be 7.5 which decreased at lower and higher pH; 85% and 91% at pH 4 and 9 respectively, as shown in fig. 2. Variation in the stannous chloride concentration also affected the radiolabeling yield and was found to be highest at 150 µg $SnCl_2$. The stability of ^{99m}Tc-DO3A-Act-AQ in human serum was >97% till 24 h post incubation with the radiocomplex. The radiocomplex was found to be adequately stable in-vitro and in-vivo as only ~6% of the radiolabeled complex dissociated in after 24 h post incubation (fig.3). Plasma protein binding of the radioconjugate was only 61%.

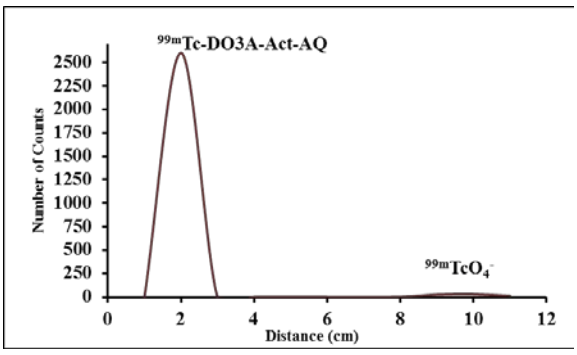

Fig. 1: ITLC radiochromatogram of ^{99m}Tc-DO3A-Act-AQ in phosphate buffer saline (PBS)

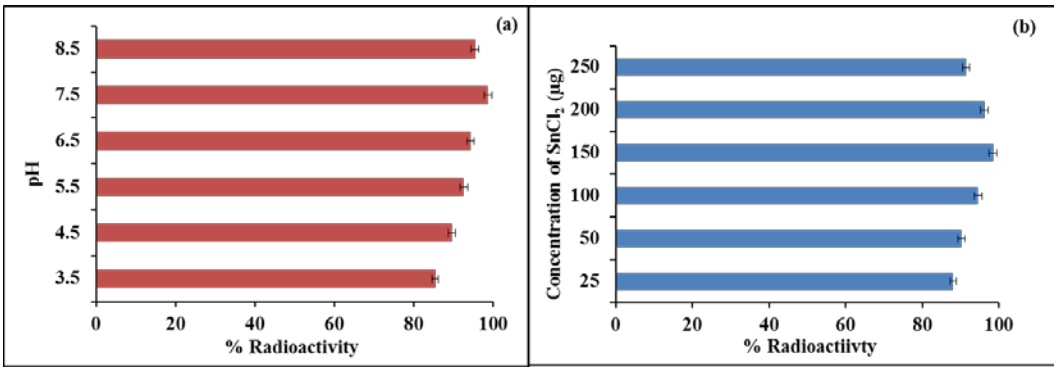

Fig. 2: Effect of (a) pH and (b) stannous chloride ($SnCl_2$) concentration on the labeling efficiency of ^{99m}Tc-DO3A-Act-AQ

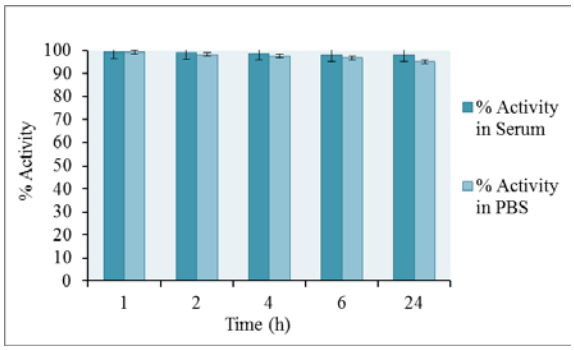

Fig. 3: Transchelation of ^{99m}Tc radiometal from ^{99m}Tc-DO3A-Act-AQ in human serum and phosphate buffered saline (PBS)

Blood kinetics studies

To determine the pharmacokinetics behavior and stability of ^{99m}Tc-DO3A-Act-AQ in human serum, blood kinetics studies were performed in normal rabbits. The complex displayed 41% of the residual activity present in blood at 15 min which reduced to 33% after 30 min post injection. The activity clearance exhibited a slow clearance in initial 2 h, but after 4 h most of the activity washed out from blood circulation. The biological half-life was found to be $t_{1/2}$(F) = 38.04±0.35 min and $t_{1/2}$(S) = 5 h 30 min±0.67, fig.4.

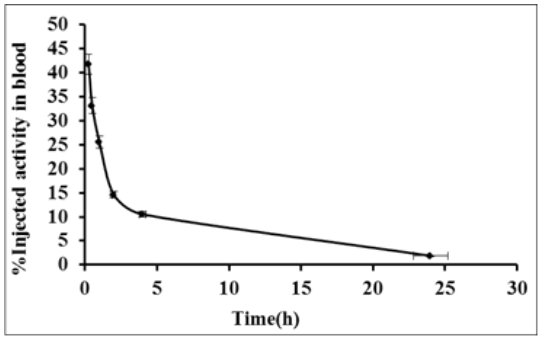

Fig. 4: Blood kinetics pattern of ^{99m}Tc-DO3A-Act-AQ administered intravenously through the ear vein in normal healthy rabbits

Biodistribution and imaging studies

Biodistribution profile of ^{99m}Tc-DO3A-Act-AQ was discerned in athymic mice bearing BMG-1 tumor at different time intervals between 1 to 24 h. The maximum uptake was observed in kidneys with 12.54±1.02% ID/g at 1 h p. i which reduced to half; 6.17±0.98% ID/g after 6 h of intravenous injection. The accumulation of ^{99m}Tc-DO3A-Act-AQ at tumor site was found to be 0.66±0.12% ID/g at 1 h post injection which increased to a maximum of 1.82±0.17 % ID/g after 2 h. It was observed that radiolabeled DO3A-Act-AQ rapidly cleared within 2 h of injection from the blood stream with 0.84±0.3% ID/g residual activity remaining in the blood. In the intestine, the uptake was less than 1.5% ID per g at 1 h, fig.5. The retention of ^{99m}Tc-DO3A-Act-AQ in various parts (lungs, heart, spleen, stomach and liver) of the body after 4 h injection was found to be low. Imaging was performed at different time intervals after administering labeled compound intravenously. The imaging studies provide strong support to the tumor targeting ability of the labeled probe and displayed maximum activity accumulation in tumor at 2 h p. i. Semi-quantitative analysis was performed during scintigraphy to estimate the tumor uptake. The tumor-to-muscle tissue ratio was found to be 10 while the mice that received co-administration of 200 µg blocking dose of 1-amino anthraquinone exhibited lower tumor uptake with tumor-to-muscle of ~0.68 at 2 h p. i, fig. 6.

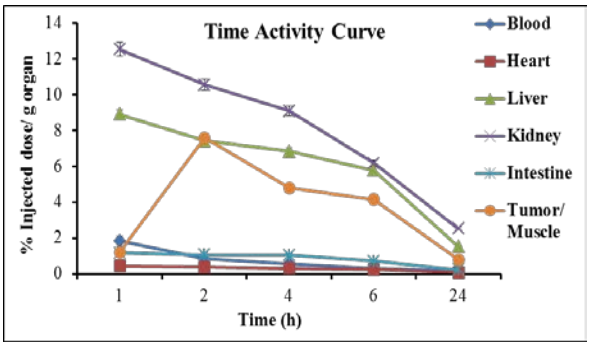

Fig. 5: Time-activity curve of ^{99m}Tc-DO3A-Act-AQ for selected organs of BMG-1 xenografted athymic nude mice (data presented from group of five male mice and expressed as mean % ID/ g±S. D)

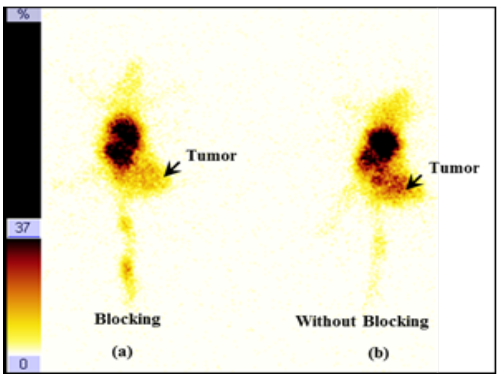

Fig. 6: Whole-body γ image of BMG-1 tumor xenografted athymic nude male mice at 2 h p. i with and without co-administration of blocking dose of DO3A-Act-AQ (200 µg)

DISCUSSION

DNA is a famous intracellular target for development of various anticancer drugs where they interact through diverse ways like intercalation, groove binding, cleavage, electrostatic interactions etc. Besides exhibiting luminescence property anthraquinones and its derivatives epitomize an important class of anticancer agents which commonly binds to DNA by insertion and stacking between the base pairs of the duplex DNA [30-33]. Research for the development of anticancer drugs with anthraquinone core having anticancer potential is going on for long but its utility in tumor diagnostic purpose is an unexplored area. For tumor diagnosis, SPECT radiopharmaceuticals with ^{99m}Tc are regularly being utilized due to their various advantages of nuclear properties; sufficient half-life of 6.02 h, γ emitter with energy 140 KeV and convenient availability from the ^{99}Mo/ ^{99m}Tc generator. 1, 4, 7, 10-Tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid (DOTA) are known to form the stable complex with ^{99m}Tc. We have reported in our previous work an anthraquinone conjugated macrocyclic chelating agent, 2, 2', 2''-(10-(2-(9, 10-dioxo-9, 10-dihydroanthracen-1-ylamino)-2-oxoethyl)-1, 4, 7, 10-tetraazacyclododecane-1, 4, 7-triyl)triacetic acid or DO3A-Act-AQ obtained by reacting tri-substituted cyclen (DO3A) with 2-chloro-N-(9, 10-dioxo-9, 10-dihydro-anthracen-1-yl)-acetamide for application in PET imaging utilizing ^{68}Ga radionuclide. The success of ^{99m}Tc in nuclear medicine and high half-life than [68]Ga prompted us to radiolabel DO3A-Act-AQ with ^{99m}Tc and assess its future application as SPECT agent for tumor imaging. DO3A-Act-AQ was radiolabeled with $^{99m}TcO_4^-$ in high radiochemical purity (>96%) and radiochemical yield (>94%) with a specific activity of 3.62 MBq/nmol. As pH plays a key role in ^{99m}Tc radiolabeling therefore the optimum pH was calculated and ideal complexation yield for ^{99m}Tc with DO3A-Act-AQ was realized in the pH range of 7.5 while at low and high pH radiochemical yield was observed to be low. The ideal pH range could be corroborated to the presence of carboxylic acid groups in DO3A-Act-AQ structure responsible for the formation of an intact coordinate bond with the radiometal. To make $^{99m}TcO_4^-$ a chemically reactive species, prior reduction of ^{99m}Tc is required. Therefore stannous chloride ($SnCl_2$) concentration as a reducing agent has an important role on the radiolabeling yield. The effect of stannous chloride concentration on radiolabeling keeping other parameters constant was assessed and was found maximum at 150 µg of $SnCl_2$ as indicated by ITLC-SG. In-vitro stability of radioconjugate was checked in phosphate buffered saline (PBS) and human serum which showed non-significant transchelation of radiometal from the radiocomplex with only 2-5% transchelation of ^{99m}Tc after 24 h of incubation. Low transchelation of ^{99m}Tc from the radiocomplex in PBS and serum indicates its high stability which is a prerequisite condition for good diagnostic agent and attributable to the presence of large number of electron donor sites in DO3A-Act-AQ. The protein binding of radiocomplex affects its tissue distribution and its uptake by the targeted organ, strong serum protein binding delay the blood clearance of radioconjugate which leads to low target-to-background ratio therefore low binding with human serum protein is a necessary requirement for the application of the probe in tumor imaging. Therefore the extent of protein binding of ^{99m}Tc-DO3A-Act-AQ was evaluated and found to be 61%. To discern the pharmacokinetic behavior of ^{99m}Tc-DO3A-Act-AQ, blood kinetics study was accomplished in normal rabbits which showed rapid blood clearance and its biological half-life was found to be $t_{1/2}$(F) = 38.04±0.3 min and $t_{1/2}$(S) = 5 h 30 min±0.6. The biological distribution of ^{99m}Tc-DO3A-Act-AQ performed on BMG-1 tumor xenografted athymic mice suggested a prominent uptake of the probe in kidneys at all measured time points signifying its renal excretion route which well correlated with other reported anthraquinone derivatives. It was observed that radiolabeled DO3A-Act-AQ rapidly cleared from the blood stream with only 50% injected activity remaining in the blood pool at 2 h p. i. The high activity in the liver reveals the lipophilic nature of the complex which further implicates predominant hepatobiliary route of excretion of the administered radioactivity. The activity accumulation in other non target organs was found to be quite low; <1% ID/g after 1 h p. i. The uptake of ^{99m}Tc-DO3A-Act-AQ at the target site; tumor is seen maximum at an initial time point of 2 h with tumor-to-muscle reaching to>7 which decreased with time and reached to ~4 at 6 h p. i. The significant tumor uptake established the prospective of this hybrid compound as a tumor-seeking SPECT agent. The scintigraphic imaging studies of ^{99m}Tc-DO3A-Act-AQ in BMG-1 xenografted nude mice further provide strong support to the tumor targeting ability of the developed probe. Blocking experiments with unlabeled DO3A-Act-AQ confirmed its specific uptake. Semi-quantitative analysis showed maximum radiotracer uptake with tumor-to-contralateral muscle tissue ratio ~10 while mice that received co-administration of 200 µg blocking dose of DO3A-Act-AQ, the ratio was 0.68 at 2 h significantly lower at 1 h post injection. We have successfully developed a new imaging agent ^{99m}Tc-DO3A-Act-AQ showing considerable possibilities for tumor scintigraphy, as significant accumulation is observed in athymic mice bearing the subcutaneous BMG-1 cell line with high specificity using ^{99m}Tc which is a cost effective radioisotope having advantage of non requirement of on-site cyclotron.

CONCLUSION

A DOTA based polyazamacrocyclic anthraquinone derivative, DO3A-Act-AQ has been successfully radiolabeled with a gamma emitting generator based radionuclide ^{99m}Tc. The preliminary preclinical investigation shows that the DO3A-Act-AQ ligand is proficiently forming stable ^{99m}Tc-complex that is exhibited by its high radiochemical yield and in-vivo stability. The positive features in in-vivo results of the radiocomplex such as fast renal clearance and low abdominal uptake will minimize the probability of radiation burden to the body. The promising characteristics including simple radiolabeling procedure and significant uptake at tumor to non target organs lays the basis for the future prospective of ^{99m}Tc-DO3A-Act-AQ conjugate as a potential SPECT radiopharmaceutical for visualizing tumors.

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CONFLICT OF INTERESTS

There is no conflict of interest between all the authors of this work.

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