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PAPER

Design, synthesis and biological evaluation of choline based SPECT imaging agent: Ga(III)-DO3A-EA-Choline†

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The enhanced choline uptake and phosphorylation in tumor cells has motivated the development of radiolabeled choline derivatives as diagnostic markers for imaging cell membrane proliferation and noninvasive detection of prostate, brain and breast tumors. In the present work, we report a facile strategy for the synthesis of choline functionalized macrocyclic chelating agent (DO3A-EA-choline) and its radiocomplexation with ^{67}Ga for potential tumor imaging applications. The synthesis of the desired compound featured quaternization of *N*,*N*-dimethylaminoethanol with 1,2-dibromoethane followed by subsequent alkylation with trisubstituted cyclen (DO3A). All intermediates and final compounds have been fully characterized by spectroscopic techniques, namely, ^{1}H, ^{13}C NMR and mass spectroscopy. The compound has been successively labeled with ^{67}Ga-citrate in ammonium acetate buffer (pH 6.5) at 80 °C. MTT assays have been performed on the HEK cell line to determine the cytotoxicity of the compound. Cell uptake studies carried out on the U-87 MG cell line exhibited saturable binding of the radioconjugate in picomolar range with a K_d value of 0.528 pM. The in vivo biodistribution and blood kinetics studies exhibited rapid clearance of the radiolabeled complex and excretion through the renal and hepatobiliary route. The present studies demonstrate the potential applications of ^{67}Ga-DO3A-EA-choline as a radiopharmaceutical for molecular imaging using ($^{67/68}$Ga) SPECT and PET modalities.

Introduction

The most important aspect in radiopharmaceutical development is the design and synthesis of imaging agents that target specific receptors and transporters involved in pathological conditions and hence, substantially advance the sensitivity and specificity of imaging techniques by improving cancer detection and characterization.

In the present work, we have directed our efforts towards the design, synthesis and biological evaluation of a Ga(III) labeled choline based radiopharmaceutical as a potential tumor imaging agent. Ga(III) ion has been classified as a hard Lewis acid because of its high charge density and small ionic radius (0.62 Å). As a result, its chelate chemistry is dominated by strong binding to highly ionic hard Lewis bases such as nitrogen and oxygen donor atoms. Thus, polyamino-polycarboxylate types of ligands form thermodynamically stable complexes with Ga(III) and further neutralize the metal ion charge by negatively charged groups present in the pendant arms.[1] Among various macrocyclic ligands, NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid), DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), NODASA (1,4,7-triazacyclononane-*N*-succinic acid-*N*′,*N*″-diacetic acid) and NODAGA (1,4,7-triazacyclononane-*N*-glutamic acid-*N*′,*N*″-diacetic acid) have been employed for ^{67}Ga chelation.[2]

To impart enhanced tumor targeting properties to the compound, we focused on its conjugation with a biomolecule that accumulates with high specificity in the tumor with high target/background signal. Tumor cells are often characterized by their uncontrolled growth and increased synthesis of phospholipid membranes (phosphatidylcholine) which leads to substantial up-regulation of the choline transport and choline-kinase (ChoK) enzyme overexpression. ChoK is the first enzyme in the Kennedy pathway, responsible for the generation of basic lipid components of cell membranes, phosphocholine and phosphatidylcholine from choline.[3,4,5]

Due to an increased uptake and phosphorylation of choline to form choline phosphate, the intracellular concentration of phosphocholine is very high in tumor cells.[6,7] Therefore, differentiation of malignant cancer tissue from neighbouring non malignant tissues can be achieved by exploiting changes in choline processing and its metabolites that occur in response to metabolic, genetic or microstructural changes in the malignant cells.[8] These facts encouraged us to develop macrocyclic chelating agents conjugated

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to choline as promising radiopharmaceuticals in the field of oncologic imaging.

Progressing interest in the synthesis and applications of choline based radiotracers is ascribed to its utmost biological significance within living systems. The capacity to take up and secrete high levels of choline and its metabolites is a central function of mammary epithelial cells.[9] It has been observed that choline and its metabolites serve three main physiological purposes: (i) structural integrity and signalling roles for cell membranes, (ii) cholinergic neurotransmission (acetylcholine synthesis), and (iii) major source of methyl groups *via* its metabolite trimethylglycine (betaine) that participates in the *S*-adenosylmethionine synthetic pathways.

Choline deficiency affects the expression of genes involved in cell proliferation, differentiation and apoptosis and it has also been associated with liver dysfunction and cancer. Abnormal choline transport and metabolism have been implicated in a number of neurodegenerative disorders such as Alzheimer's and Parkinson's disease.[10] These facts demonstrate the utility of choline based tracers to be used as biochemical indicators of tumor progression and therapy.[11]

The first approach towards synthesis of labeled choline derivatives was reported by DeGrado *et al.*[12] Haeffner and coworkers[13] have also investigated choline transport in Ehrlich ascites tumor cells using labeled choline derivatives such as ^{3}H-choline and ^{14}C-choline.

^{18}F ($t_{1/2}$ = 110 min) and ^{11}C ($t_{1/2}$ = 20 min) labeled choline analogs have been developed as oncologic PET tracers for evaluating brain, breast and prostate carcinoma.[12,13,14] However, the relatively short half-life of these radioisotopes necessitates an onsite cyclotron and requires the use of the radiotracer soon after preparation, making the routine and long time clinical use of labeled choline derivatives difficult. Hence, use of longer living radioisotope such as ^{67}Ga ($t_{1/2}$ = 78.3 h) is desirable for practical and widespread diagnostic imaging applications. Considering the above facts, we herein report the synthesis of a choline based macrocyclic chelating agent (DO3A-EA-choline), its radiocomplexation with ^{67}Ga and biological evaluation.[15]

Results and discussion

Synthesis

The synthesis of ^{67}Ga-DO3A-EA-choline is shown in Scheme 1. All intermediates and the final compound were successfully characterized and analyzed by spectroscopic techniques such as ^{1}H, ^{13}C NMR and mass spectroscopy. The high resolution ^{1}H NMR spectrum of DO3A-EA-choline is shown in Fig. 1.

Radiolabeling: quality control of ^{67}Ga-DO3A-EA-choline

Radiolabeling of the ligand **7** with Ga(III) was carried out in double distilled water under nitrogen atmosphere. Gallium citrate (^{67}Ga, 74 MBq) was added to the vial containing the ligand and the temperature was raised to 80 °C. The pH of the reaction mixture was adjusted to 6.5 with ammonium acetate solution (0.5 M) to get optimum radiolabeling yield. After 4 h, the reaction mixture was analysed using thin layer chromatography in 10% ammonium acetate : methanol (1 : 1) solvent system. TLC analysis demonstrated high radiochemical purity (above 96%) for Ga(III)-DO3A-EA-choline. The formation of Ga(III) complex of ligand DO3A-EA-choline was ascertained by high resolution mass spectroscopy showing the isotopic pattern of Ga(III) centred at *m*/*z*: 554.3 $[M + Na]^{+}$ as shown in Fig. 2.

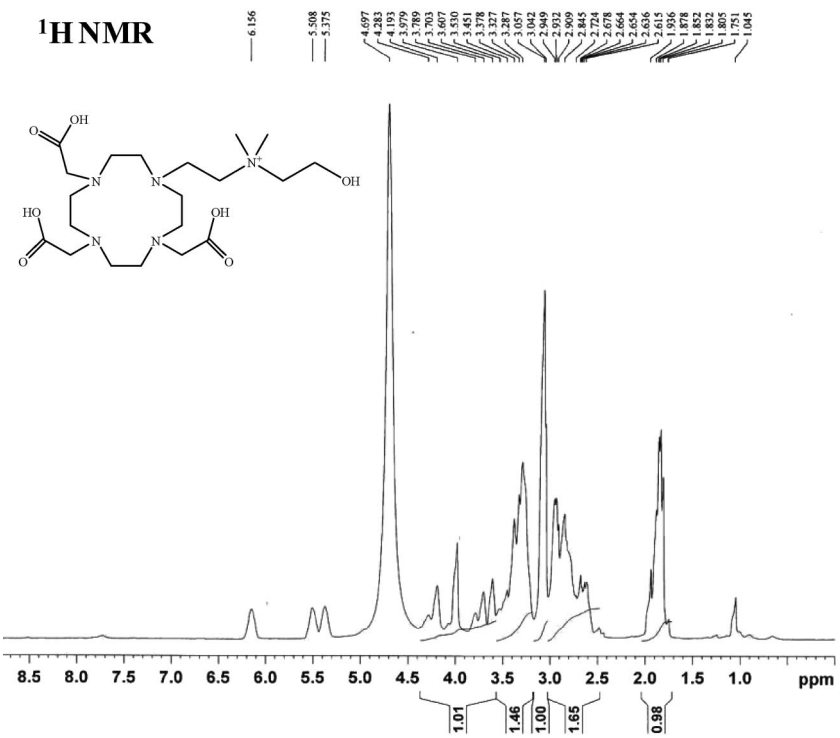
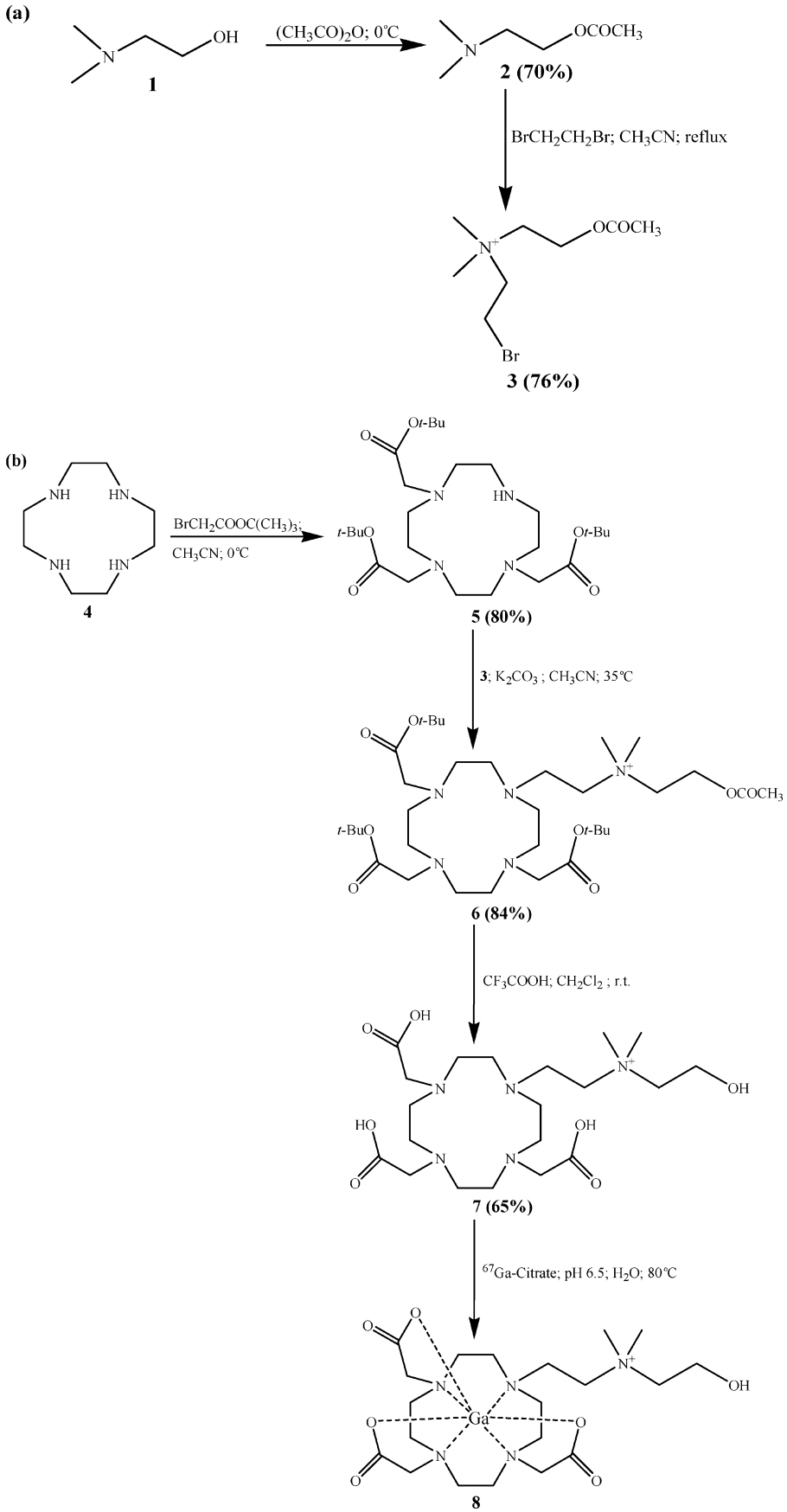


Fig. 1 High resolution ^{1}H NMR spectrum (D_2O) of DO3A-EA-choline.



Scheme 1 Synthesis of (2-hydroxy-ethyl)-dimethyl-[2-(4,7,10-tris-carboxymethyl-1,4,7,10-tetraaza-cyclododec-1-yl)-ethyl]-ammonium.

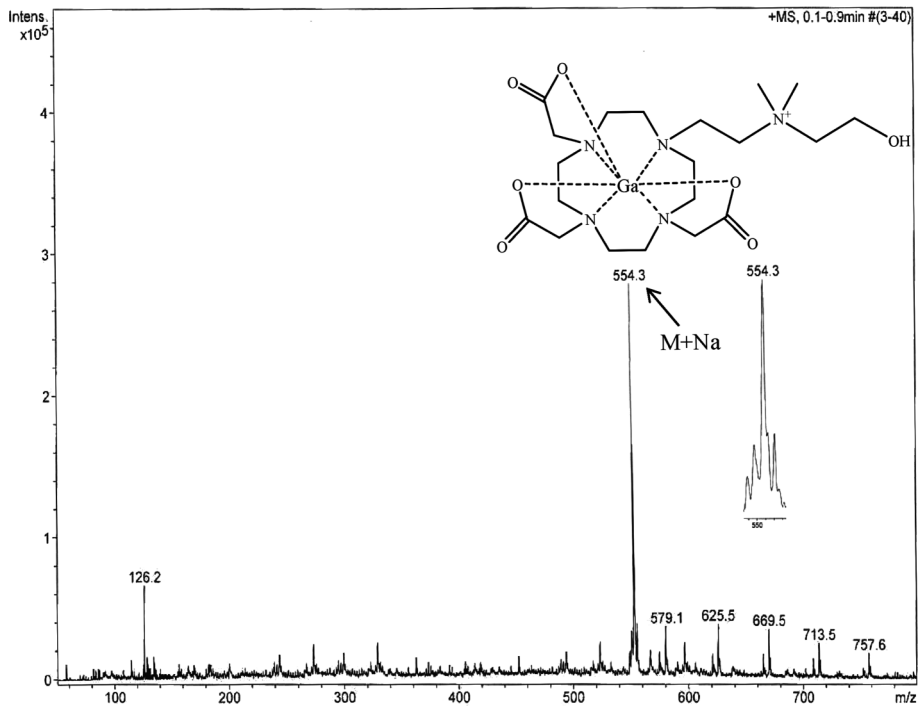


Fig. 2 Mass spectrum showing the characteristic peak at *m/z*: 554.3 $[M + Na]^+$ along with the isotopic pattern of Ga(III) in the Ga(III) complex of ligand DO3A-EA-choline.

The stability of the Ga(III) labeled complex was ascertained by mixing 10 μL of the above reaction mixture with 2 mL of healthy human serum. Comparative stability studies were performed using ^{67}Ga labeled diethylenetriaminepentaacetic acid (Fig. 3).

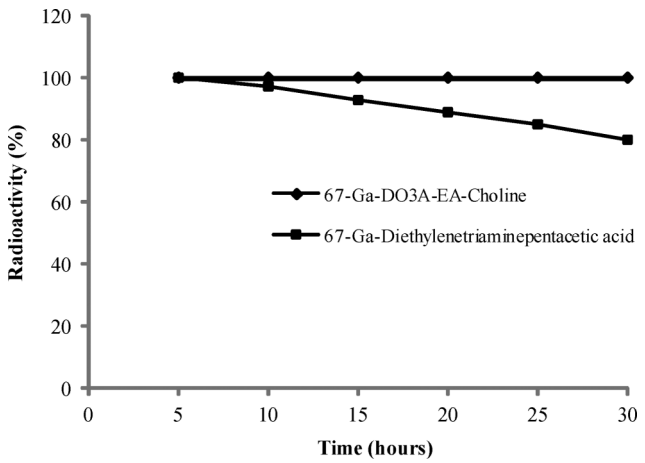


Fig. 3 Serum stability studies of ^{67}Ga-DO3A-EA-choline.

HEK cells. At lower concentrations the conjugate did not show any adverse effect on the cell survival as only 12% of the cell death was observed when 1 μM of the DO3A-EA-choline was incubated with HEK cell line for 2 h. Subsequently, 55% of cell lyses was observed after 48 h exposure at 10 mM concentration of the compound. The IC_{50} value for the compound was obtained to be 10 ± 2 mM (Fig. 4).

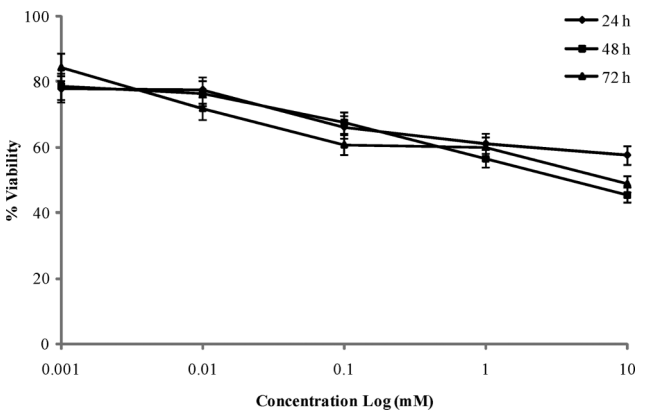


Fig. 4 Colorimetric estimation of the mitochondrial activity for cytotoxicity of DO3A-EA-choline (MTT assay) in HEK cell line (0.001–10 mM concentration range).

Biological evaluation

Cytotoxicity studies of DO3A-EA-choline

Cellular toxicities were determined by MTT assay carried out on HEK cells treated with varying concentrations (0.001–10 mM) of DO3A-EA-choline at time intervals of 24, 48 and 72 h. The data revealed 43% reduction in the MTT assay (43% cell death) when 10 mM concentration of compound was incubated for 24 h on

Cell uptake studies

The ability of DO3A-EA-choline to bind to the U-87 MG tumor cell line was determined by saturation binding assay using ^{67}Ga-DO3A-EA-choline as the labeled ligand. Non specific binding was obtained by using 100 fold excess of unlabeled choline.

Analysis of the binding curve exhibited saturable binding of the radioconjugate in the picomolar range. Scatchard plot analysis revealed that the labeled compound exhibited high affinity on the U-87 MG cell line with a K_d value of 0.528 pM (Fig. 5)

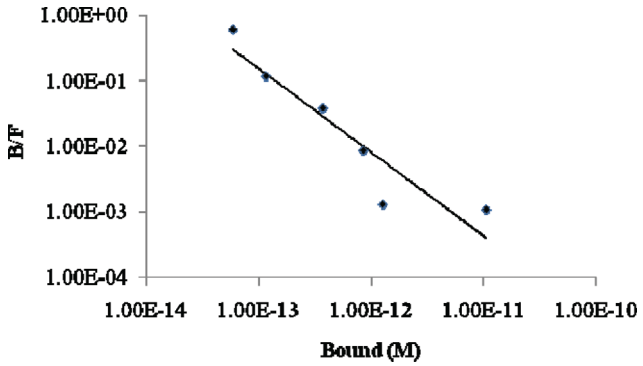


Fig. 5 Scatchard plot of the specific binding data to the ratio of bound to free (B/F) for the U-87 MG cell line for DO3A-EA-choline.

Blood kinetics

Blood kinetics studies in rabbits showed that there was a rapid clearance of the conjugate **8** from the body as only 13% of injected activity persisted in circulation at 1 h. After 2 h, the clearance followed a slow pattern and at 24 h approximately 0.79% activity remained in the blood (Fig. 6). The biological half-life was obtained to be $t_{1/2}$ (fast): 70 min; $t_{1/2}$ (slow): 16 h and 10 min.

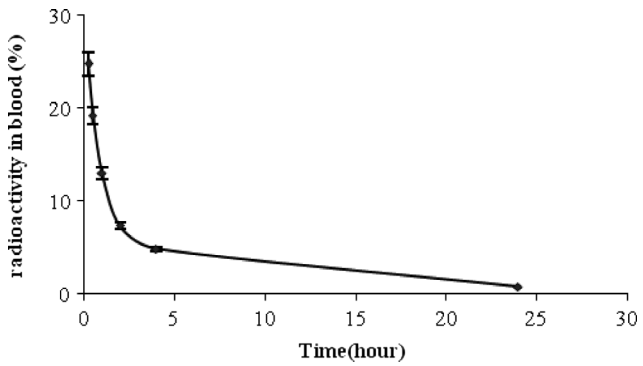


Fig. 6 Blood clearance of ^{67}Ga-DO3A-EA-choline (9.8 MBq activity) administered through the ear vein in a normal rabbit.

SPECT imaging in a normal rabbit

SPECT imaging in a normal rabbit was conducted to visualize the biodistribution at earlier time point *viz.*, 10 min, 30 min following intravenous administration of DO3A-EA-choline. It showed accumulation of activity in liver, kidney and bladder illustrating the hepatobiliary and renal route of excretion of the radioconjugate (Fig. 7). Use of a diuretic agent such as furosemide decreased the absorbed dose of the bladder wall. Image quality in the pelvis adjacent to the bladder improved, because the concentration of tracer in the bladder was markedly reduced when oral administration of furosemide (1 mg kg^{-1}) was given 30 min prior to ^{67}Ga-DO3A-EA-choline injection. The image clearly demonstrated the decrease in the radioactivity in the bladder

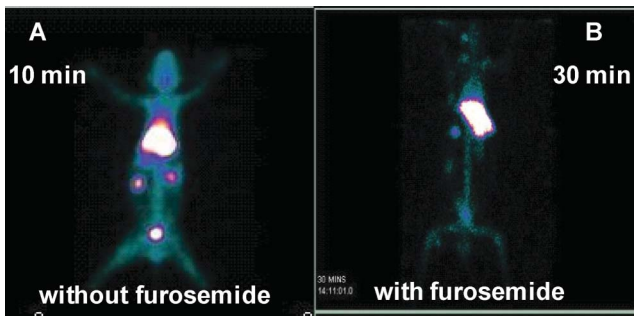


Fig. 7 Whole body γ image of a normal rabbit at 10 min (**A**, without furosemide) and 30 min (**B**, with furosemide) following intravenous administration of ^{67}Ga-DO3A-EA-choline (2.96 MBq activity).

thereby enhancing the sensitivity of the compound to be used in prostate cancer imaging.

Tumor imaging

Imaging of animals was performed at different time intervals following intravenous administration of the radiolabeled conjugate to five mice. The mice illustrated the beginning of accumulation of activity in the tumor at 30 min, which reached a maximum at 1 h (Fig. 8) and remained stable for 4 h. Semiquantitative analysis was generated from the region of interest (ROI) placed over areas counting average counts per pixels with maximum radiotracer uptake at the tumor site and compared to symmetric counterparts with ROI in soft tissues. The target to non target (T/NT) ratio at different time intervals was calculated.

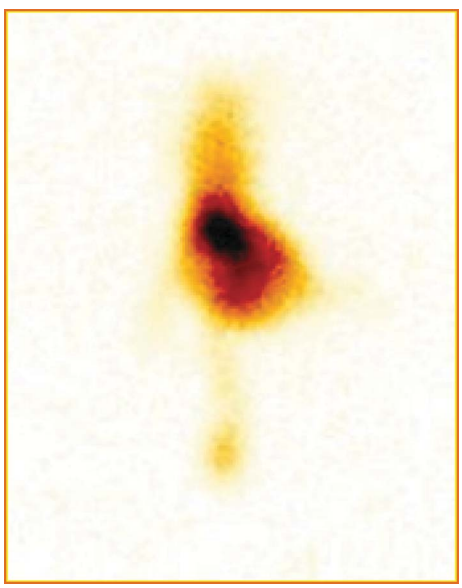


Fig. 8 Whole body γ image of female athymic mice with U-87 MG tumor in right thigh at 1 h following intravenous administration of ^{67}Ga-DO3A-EA-choline (2.96 MBq activity).

The ratio of tumor to soft tissue at 1 h was found to be 18 ± 1.8 which then increased to 46 ± 3.6 at 4 h. The radiotracer uptake was decreased at 24 h but the ratio was increased due to the clearance of the labeled compound from the body.

Table 1 Biodistribution of ^{67}Ga-DO3A-EA-Choline in Balb/c mice following intravenous injection

Organ	%ID/g[a] 0.5 h	%ID/g[a] 1 h	%ID/g[a] 2 h	%ID/g[a] 4 h	%ID/g[a] 24 h
Blood	1.87 ± 0.31	1.06 ± 0.26	0.83 ± 0.18	0.61 ± 0.13	0.3 ± 011
Heart	0.48 ± 0.12	0.35 ± 0.13	0.23 ± 011	0.17 ± 0.09	0.07 ± 0.02
Lungs	1.72 ± 0.41	1.13 ± 0.33	1.01 ± 0.29	0.93 ± 0.17	0.31 ± 0.12
Liver	4.6 ± 1.2	4.5 ± 1.1	3.9 ± 0.8	3.5 ± 0.8	1.43 ± 0.9
Spleen	2.15 ± 0.52	1.83 ± 0.41	1.04 ± 0.37	0.76 ± 0.14	0.15 ± 0.03
Kidney	6.2 ±1.3	5.5 ± 1.33	3.12 ± 1.21	2.73 ± 1.19	1.8 ± 0.42
Stomach	0.78 ± 0.16	0.81 ± 0.16	0.53 ± 012	0.4 ± 0.11	0.31 ± 0.1
Intestine	3.5 ± 1.23	2.2 ± 1.17	1.63 ± 0.65	0.97 ± 0.23	0.19 ± 0.03
Brain	0.07 ± 0.01	0.08 ± 0.02	0.06 ± 0.01	0.07 ± 0.02	0.02 ± 0.001

[a] Percentage of injected ^{67}Ga-DO3A-EA-Choline dose per gram following intravenous administration, values shown represent the mean ± standard deviation of data from five animals.

Biodistribution

The biodistribution of ^{67}Ga-DO3A-EA-choline was studied in Balb/c mice at different time intervals. The biodistribution of the radioconjugate is illustrated in Table 1. It was observed that the radiolabeled complex rapidly cleared from the blood stream with only 0.69 ± 0.11% ID/g remaining in the blood after 24 h in comparison to 1.87 ± 0.31% ID/g at 0.5 h. Major accumulation of the labeled compound was observed in kidneys (6.2 ± 1.3% ID/g) followed by liver (4.6 ± 1.2% ID/g) at 0.5 h showing that the complex is excreted both by renal as well as hepatobiliary routes.

These observations may serve as important parameters to guide in vivo evaluation of radiolabeled choline derivatives as SPECT biomarkers in tumor models. Retention of radioactivity in the non target organs (lungs, spleen, intestine *etc.*) was low and very much less background activity was observed after 24 h.

Discussion

Choline is an essential nutrient for cells because it plays a vital role in the synthesis of the membrane phospholipid components of the cell membranes, as a methyl group donor in methionine metabolism as well as in the synthesis of the neurotransmitter acetylcholine. Therefore, choline and its derivatives have been emerging as effective diagnostic biomarkers for imaging and differentiating the metabolic differences between normal and malignant cells. ^{11}C and ^{18}F choline analogues have been developed as PET tracers for detection of cancer and evaluation of brain, lung, oesophageal, colon, bladder and prostate cancer. Due to the short half-life of these radioisotopes and limited practical applications, we attempted to design an expedient strategy for the synthesis of ^{67}Ga labeled choline based macrocyclic chelating agent (DO3A-EA-choline) to be used as a tumor seeking tracer for noninvasive detection of cancer.[16]

The synthesis of the choline functionalized macrocyclic agent was a convenient process utilizing readily available starting materials and simple reaction conditions. The key step in the synthesis of the desired compound **7** was the alkylation of *N*,*N*-dimethylaminoethanol with 1,2-dibromoethane affording (2-acetoxy-ethyl)-(2-bromo-ethyl)-dimethyl-ammonium in good yield. For the synthesis of the above linker, *N*,*N*-dimethylaminoethanol was reacted with 1,2-dibromoethane at 90 °C for 4 h.[17,18] This reaction suffered from the drawback of the formation of the major cyclic by-product 4,4-dimethylmorpholinium along with the desired product (2-acetoxy-ethyl)-(2-bromo-ethyl)-dimethyl-ammonium.

To circumvent the by-product formation the hydroxyl group of *N*,*N*-dimethylaminoethanol was protected using acetic anhydride at 0 °C for 2 h. The acetylation of this substrate was a feasible process and did not require any harsh conditions for protection and subsequent deprotection and resulted in increased overall yields of the final product.

The resulting *N*,*N*-dimethylaminoethyl ester was subsequently reacted with 1,2-dibromoethane for the synthesis of linker **3**. The trisubstituted cyclen was alkylated with (2-acetoxy-ethyl)-(2-bromo-ethyl)-dimethyl-ammonium in presence of potassium carbonate at 35 °C. This reaction was performed at low temperature as high temperature resulted in the decomposition of positively charged linker **3**. The final step involved deprotection of the *t*-butyl groups and acetate group with trifluoroacetic acid at 0 °C yielding the desired compound DO3A-EA-choline.

The compound was radiolabeled with ^{67}Ga in aqueous solution at pH 6.5 (ammonium acetate buffer). Radiochemical analysis by TLC demonstrated that the chelate formed stable electron donor complexes with ^{67}Ga with high reproducible labeling efficiency.

The rate of decomplexation of the radiolabeled complex was studied in serum under physiological conditions at different time intervals of 5, 10, 15, 20, 25 and 30 h. The studies revealed no measurable loss of metal ion from the macrocyclic core and high stability of the ^{67}Ga complex as compared to the acyclic chelate diethylenetriaminepentaacetic acid.

The most prevalent oxidation state of gallium in aqueous solution is +3. Due to its high charge density, Ga(III) prefers hard donor atoms, such as amine-nitrogen and carboxylate-oxygen atoms for complexation. A significant challenge faced during radiolabeling of ^{67}Ga is the hydrolysis of Ga(III) to insoluble $Ga(OH)_3$ in the pH range of 3–7, however, at high pH > 7, gallium hydroxide redissolves as $[Ga(OH)_4]^-$. Hydrolysis and formation of insoluble gallium hydroxide can be avoided by ligand exchange reaction in the presence of weak, stabilizing ligands such as citrate, acetate or oxalate.[19] Another critical aspect to be kept in consideration after ^{67}Ga radiopharmaceuticals are injected into a biological system is the ligand exchange with transferrin as Ga(III) bears similarity with Fe(III) with respect to its coordination chemistry and biological properties.

To assess the in vitro cellular toxicity of compound, DO3A-EA-choline, MTT assays were carried out on the HEK cell line. Cell survival (percentage relative to untreated controls) in the

range of 50–60% was observed after 24 and 48 h incubation of 10 mM concentration of the compound. Significant toxicity could not be observed at lower concentrations. The specificity of the choline functionalized compound to bind to cell surface receptors on tumor cells was examined by cell uptake assays on the U-87 MG cell line. The saturation binding assay revealed that ^{67}Ga-DO3A-EA-choline has a high affinity on the U-87 MG cell line with a K_d value of 0.528 pM. The blood clearance studies of the radiolabeled compound **8** followed a biphasic trend with a rapidly clearing initial phase followed by a slow phase and showed its high target uptake with the diagnostically useful target/non target ratio in a short period of time. It exhibited rapid clearance from blood circulation thereby resulting in the reduction of the background activity.

SPECT images in normal rabbits were acquired to visualize the biodistribution of the compound at early time points with and without use of a diuretic agent (furosemide). The image illustrated a distinct decrease in the radioactivity in the bladder thereby suggesting the potential for the compound to be used as an effective radiotracer in prostate cancer imaging. The onset of action after oral administration is within 1 h and the diuresis lasts about 6–8 h.[20] This protocol (application of furosemide, static emission scan at 30 min after injection) is similar to the protocol used for ^{18}F-choline PET in our institution, which is well tolerated by most of our patients. The high specificity of the compound results in selective uptake and distribution of the radiolabeled ligand that can be visualized in the high quality images, obtained 1 h after administration in athymic mice bearing U-87 MG tumor which offers the advantage of comparing in vitro receptor binding studies and tumor bearing animal studies.

The biodistribution studies illustrated that ^{67}Ga-DO3A-EA-choline persisted in kidney and liver up to 4 h and is cleared through renal and hepatobiliary routes. The radioactivity uptake in the non target organs was comparatively low and very low background activity was observed after 24 h. SPECT imaging of ^{67}Ga-DO3A-EA-choline in tumor bearing nude mice directly correlated with the biodistribution studies.

To summarize, we have developed ^{67}Ga-DO3A-EA-choline, a radiometal labeled choline analog which can be used for SPECT imaging. It exhibits superior characteristics as compared to other radiogallium derivatives reported in the literature. It shows not only significant tumor uptake but also rapid blood clearance and low accumulation of the radioactivity in the non target tissues thereby resulting in high and increased target/non target ratio and less radiation burden to the patient. Numerous applications of choline based organic PET radiopharmaceuticals (^{18}F-choline and ^{11}C-choline) are known. However, their synthesis requires multiple steps and extensive purification. The former requires elaborate apparatus whereas the latter requires a skilled radiochemist in addition to an airtight working environment. Also, the short half-life of PET radiotracers limits their shipping and subsequent use for long term study of the tumor model.

Hence, ^{67}Ga-DO3A-EA-choline is a better candidate for tumor imaging as its synthesis need not be performed within or in the vicinity of the cyclotron. Further, the interesting physical properties and easy availability of ^{67}Ga make it an interesting nuclide for SPECT radiopharmaceutical research. The development of this ^{67}Ga radiotracer offers new opportunities for researchers to focus on the production and use of future PET gallium homologs.

Experimental

General methods and materials

Acetic anhydride, *N*,*N*-dimethylaminoethanol, 1,2-dibromoethane and *t*-butylbromoacetate were purchased from Sigma-Aldrich and Merck. All solvents used were of analytical grades. For reactions to be performed under dry conditions, solvents were dried by the usual reported laboratory procedures. 1,4,7,10-tetraazacyclododecane-1,4,7-tris(*t*-butyl-acetate) was synthesized in our laboratory using the method of Li and co-workers (2002).[21] TLC was run on silica gel coated aluminium sheets (Silica gel 60 F_{254}, Merck, Germany) and visualized in UV light 254 nm. Radiocomplexation and radiochemical purity were checked by thin layer chromatography. ^{67}Ga was procured from Amersham-GE Healthcare.

Cell Culture. Monolayer cultures of U-87 MG (obtained from NIMHANS, Bangalore), were maintained at 37 °C in a humidified CO_2 incubator (5% CO_2, 95% air) in DMEM (Sigma, USA) supplemented with 10% foetal calf serum (Biological industries, Israel), 50 U mL^{-1} penicillin, 50 μg mL^{-1} streptomycin sulfate and 2 μg mL^{-1} nystatin. Cells were routinely subcultured twice a week using 0.05% Trypsin (Sigma, USA) in 0.02% EDTA.

Instrumental analysis. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II 400 MHz system (Ultra shield). Mass spectra (ESI-MS in positive and negative ion mode) were performed on Agilent 6310 system ion trap. The receptor binding data were analyzed using iterative curve-fitting program EQUILIBRATE software from graph pad. Radioimaging was performed using HAWKEYE gamma camera and single well type capintec γ-scintillation counter respectively.

Animal Models. Animal protocols have been approved by Institutional Ethics Committee. Mice were housed under conditions of controlled temperature 22 ± 2 °C and normal diet.

Synthesis of *N*,*N*-dimethylaminoethylacetate 2. A mixture of *N*,*N*-dimethylaminoethanol (1.12 ml, 11.21 mmol) and acetic anhydride (1.58 ml, 16.82 mmol) was stirred for 1 h at 0 °C in an ice bath. The pH of the reaction mixture was adjusted to 7 by the addition of 0.1 M sodium bicarbonate solution and it was extracted with dichloromethane (3 × 50 mL). The combined organic extracts were dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure to obtain the crude product which was purified by column chromatography on a silica gel (eluent: dichloromethane–methanol: 8.5/1.5) to afford the desired product as a colorless oil (70%). The final product was well characterized by ^{1}H, ^{13}C NMR and MS. ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H ppm: 1.92 (s, 3H, -CH_3), 2.54 (s, 6H, -CH_3), 2.98 (t, 2H, -CH_2), 4.23 (t, 2H, -CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C ppm: 21.87, 43.37, 55.77, 59.43, 176.27; MS(ESI$^+$) *m*/*z* calcd. for $C_6H_{13}NO_2$ 131.17, found $[M + H]^+$ 132.1

Synthesis of (2-acetoxy-ethyl)-(2-bromo-ethyl)-dimethyl-ammonium 3. To a solution of *N*,*N*-dimethyl-2-aminoethyl-acetate (0.5 mL, 5.0 mmol) in acetonitrile (30 mL) was added 1,2-dibromoethane (1.41 g, 7.51 mmol) under nitrogen and the reaction contents were refluxed for 2 h. The reaction mixture was cooled to room temperature and evaporated to dryness under reduced pressure to obtain the crude product which was

recrystallized from acetonitrile and diethyl ether under cold conditions. The pure product was obtained as a colorless oil (76%). The final product was well characterized by ^{1}H, ^{13}C NMR and MS. ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H ppm: 1.99 (s, 3H, -CH_3), 2.02–2.12 (m, 8H, -CH_2), 2.73 (s, 6H, -CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C ppm: 20.93, 43.65, 57.95, 58.94, 170.59; MS(ESI$^+$) m/z calcd. for $C_{18}H_{17}BrNO_2^+$ 239.13, found $[M + H]^+$: $[M + 2H]^+$ 237 : 239

Synthesis of (4,7-bis-*t*-butoxycarbonylmethyl-1,4,7,10-tetraaza-cyclododec-1-yl)-acetic acid *t*-butyl ester 5. To a solution of 1,4,7,10-tetraazacyclododecane (0.5 g, 2.90 mmol) in acetonitrile (50 mL) was added sodium bicarbonate (0.73 g, 8.71 mmol) at 0 °C under nitrogen. This was followed by the dropwise addition of *t*-butylbromoacetate (1.7 g, 8.71 mmol). The reaction was allowed to warm to room temperature and stirred for 12 h. The reaction mixture was filtered and the filtrate was evaporated under reduced pressure to obtain the crude product. It was purified by column chromatography on a silica gel (eluent: dichloromethane–methanol: 9.5/0.5) to afford the desired product as a white solid (80%). The final product was well characterized by ^{1}H, ^{13}C NMR and MS. ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H ppm: 1.29 (s, 27H, -CH_3), 2.77–2.99 (m, 16H, -CH_2), 3.18–3.28 (m, 6H, -CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C ppm: 28.09, 47.40, 48.73, 49.05, 51.19, 58.00, 81.51, 169.54, 170.43; MS(ESI$^+$) m/z calcd. for $C_{26}H_{50}N_4O_6$ 514.70, found $[M + H]^+$ 515.4

Synthesis of (2-acetoxy-ethyl)-dimethyl-[2-(4,7,10-tris-*t*-butoxycarbonylmethyl-1,4,7,10-tetraaza-cyclododec-1-yl)-ethyl]-ammonium 6. To a solution of 1,4,7,10-tetraazacyclododecane-1,4,7-tris(*t*-butyl acetate) (0.5 g, 0.97 mmol) in acetonitrile (50 mL) was added potassium carbonate (1.34 g, 9.72 mmol) under nitrogen. The reaction temperature was raised to 35 °C followed by the dropwise addition of **2** (0.45 g, 1.94 mmol). The reaction was stirred at 35 °C for 12 h. The reaction mixture was cooled to room temperature, filtered and the filtrate was evaporated under reduced pressure to obtain the crude product. It was recrystallized from dichloromethane and diethyl ether under cold conditions to afford the pure compound as a brown oily product (84%). The final product was well characterized by ^{1}H, ^{13}C NMR and MS. ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H ppm: 1.46 (s, 30H, -CH_3), 2.15 (m, 6H, -CH_2), 2.75–2.95 (m, 12H, -CH_2), 3.10 (m, 4H, -CH_2), 3.47 (m, 6H, -CH_3), 3.61 (m, 8H, -CH_2), 3.81 (s, 2H, -CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C ppm: 28.18, 49.61, 50.49, 51.47, 52.91, 57.84, 60.42, 66.09, 81.58, 170.14, 171.28; MS(ESI$^+$) m/z calcd. for $C_{34}H_{66}N_5O_8^+$ 674.50, found $[M + 3H]^+$ 677.5

Synthesis of (2-hydroxy-ethyl)-dimethyl-[2-(4,7,10-tris-carboxymethyl-1,4,7,10-tetraaza-cyclododec-1-yl)-ethyl]-ammonium 7. To a solution of **6** in anhydrous dichloromethane was added trifluoroacetic acid (4 mL) and the reaction was stirred for 24 h at room temperature. The reaction progress was monitored by TLC. The solvent was evaporated to dryness under reduced pressure to obtain the crude product. The product was washed well with chloroform (3 × 50 mL). Addition of cold diethyl ether led to precipitation of pure product as a pale white solid (65%). The final product was well characterized by ^{1}H, ^{13}C NMR and MS. ^{1}H NMR (D_2O, 400 MHz) δ_H ppm: 1.93 (s, 6H, -CH_3), 2.61–3.05 (m, 16H, -CH_2), 3.28–3.70 (m, 8H, -CH_2), 3.97 (s, 6H, -CH_2), 5.50 (brs, -OH), 6.15 (brs, -OH); ^{13}C NMR (D_2O, 100 MHz) δ_C ppm: 44.53, 49.94, 51.55, 54.58, 55.31, 56.15, 56.64, 57.97, 60.60, 176.61; MS(ESI$^+$) m/z calcd. for $C_{20}H_{40}N_5O_7^+$ 462.56, found $[M - 2H]^-$ 460.2

Synthesis of Ga(III) complex of (2-hydroxy-ethyl)-dimethyl-[2-(4,7,10-tris-carboxymethyl-1,4,7,10-tetraaza-cyclododec-1-yl)-ethyl]-ammonium 8. Radiolabeling of the ligand **7** with ^{67}Ga was carried out in double distilled water under nitrogen atmosphere. Gallium citrate (^{67}Ga, 74 MBq) was added to the vial containing the ligand and the temperature was raised to 80 °C. The pH of the reaction mixture was adjusted to 6.5 with ammonium acetate solution (0.5 M) to get optimum radiolabeling yield. After 4 h, the reaction mixture was analysed using thin layer chromatography in 10% ammonium acetate : methanol (1 : 1) solvent system. TLC analysis demonstrated high radiochemical purity (above 96%) for ^{67}Ga–DO3A-EA-choline.

Cytotoxicity of DO3A-EA-choline

Cytotoxicity was determined using the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay. Exponentially growing cells were plated in a 96 well microtitre plate at a uniform cell density of 4,000 cells/well 24 h before treatment. Cells were treated with varying concentrations of the compound for various time intervals, 24 h, 48 h, 72 h and MTT assays were performed. At the end of treatment, negative control and treated cells were incubated with MTT at a final concentration of 0.05 mg mL^{-1} for 2 h at 37 °C and the medium was removed. The cells were lysed and the formazan crystals were dissolved using 150 μL of dimethylsulfoxide. Optical density was measured on 150 μL of extracts at 570 nm (reference filter: 630 nm). Mitochondrial activity was expressed as percentage of viability compared to negative control (mean ± SD of triplicate cultures). Percentage of viability = [OD (570 nm – 630 nm) test product/OD (570 nm – 630 nm) negative control] × 100%. Percentage viability of HEK cells was plotted against (0.001–10 mM) concentration range for DO3A-EA-choline.

Cell uptake assay

Cell uptake studies were performed using radiolabeled ^{67}Ga-DO3A-EA-choline. The specificity of choline conjugates to bind to cell surface receptors on tumor cells was examined by receptor binding assays on U-87 MG cell line grown in normal DMEM (10% serum). Monolayer cultures of the cell lines were washed with HBSS and were then incubated for 2 h in HBSS at 37 °C prior to the experiment. Binding experiments were conducted at 37 °C. The cell line cultures were then incubated for 40 min with ^{67}Ga-DO3A-EA-choline at 37 °C in HBSS containing various concentrations (0.00001 μM–1 μM) in the absence and presence of 100 fold excess unlabeled choline for estimation of total binding and non specific binding respectively. Specific binding was obtained by subtracting non specific binding from total binding. At the end of each experiment, the cells were washed with cold PBS and 0.9% saline four times. The cell associated radioactivity was determined by gamma scintillation counting. Scatchard plot analysis was done using EQUILIBRATE software from graph pad.

Biodistribution

An intravenous injection of ^{67}Ga-DO3A-EA-choline conjugate (100 μL; 3.7 MBq activity) was injected through the tail vein of each mouse. Mice were dissected at 1 h, 4 h and 24 h post injection; different tissues were isolated, weighed and counted in a gamma counter calibrated for ^{67}Ga energy. Uptake of the radiotracer in each tissue was calculated and expressed as percentage injected dose per gram of the tissue (%ID/g).

Conclusion

We have developed choline conjugated DO3A employing a novel synthetic approach and radiolabeled it with ^{67}Ga for SPECT applications. The above strategy circumvents the drawbacks of using the ^{18}F and ^{11}C labeled choline derivatives developed which are too short lived to perform elaborate investigations and report the tumor status for a longer period of time. The biological evaluation results of DO3A-EA-choline provide valuable information for the further development of $^{67/68}$Ga labeled radiopharmaceuticals for molecular imaging applications using SPECT and PET.

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