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RESEARCH ARTICLE

In vitro and *in vivo* evaluation of ^{99m}Tc-DO3A-EA-Folate for receptor-mediated targeting of folate positive tumors

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Abstract

Background: The cell membrane folate receptor is a potential molecular target for tumor selective drug delivery via receptor-mediated endocytosis, including delivery of radiolabeled folate-chelate conjugates for diagnostic imaging.

Method: A new radiopharmaceutical, ^{99m}Tc-1,4,7-tris (carboxymethyl)-10-(4-aminoethyl)-1,4,7,10-tetraazacyclododecane (DO3A-EA)-Folate has been synthesized introducing DO3A-EA to the γ-carboxyl group of folic acid and was characterized by different spectroscopic techniques. Cytotoxicity was determined by macrocolony and MTT assay on three different cell lines. Cell uptake studies and receptor binding assay were performed using ^{99m}Tc-DO3A-EA-Folate. Tumor imaging was performed in KB cell line implanted tumor bearing nude mice, and uptake of the radiotracer was estimated.

Results: The synthesized conjugate binds with ^{99m}Tc at high efficiency at ambient temperature. The resulting conjugate is stable under physiological conditions for 24 h after radiocomplexation. Using an *in vitro* receptor binding assay, the conjugate showed K_d in μM range on human tumor cell lines (KB, U-87MG and OAW). The pharmacokinetic data revealed rapid wash out of the more than 75% activity within 5 min from the circulation with hepato-biliary clearance. Data from γ scintigraphic and biodistribution studies performed in KB tumor bearing nude mice revealed major accumulation of radiotracer at tumor site. High tumor uptake was shown in the tumor bearing mice; tumor to blood ratios reached 2.27 ± 0.32 and 6.05 ± 1.02 at 1 and 4 h after post injection, respectively.

Conclusion: These results suggest that ^{99m}Tc-DO3A-EA-Folate may be clinically useful as a noninvasive radiodiagnostic imaging agent for the detection of FR-positive human cancers.

Keywords: Folate receptor, ^{99m}Tc-Folate, tumor imaging, bifunctional chelating agent

Introduction

The human folate receptor-α is a glycophosphatidyli nositol-anchored membrane glycoprotein that binds physiological folates and mediates their intracellular transport via receptor mediated endocytosis (Antony, 1996; Toffoli et al., 1997). Meanwhile, normal tissue distribution of folate receptor is highly restricted which has prompted search for promising folate-conjugated radio metal chelates as potential imaging and therapeutic agents. A monoclonal antibody against the folate receptor (MOv-18) and its derivatives has been evaluated for the scintigraphy and tomography imaging, radiotherapy and immunotherapy of ovarian cancer in clinical studies (Weitman et al., 1992; Leamon and Reddy, 2004). There has been a lot of literature for folate and their conjugates for the application of the above studies. The reason behind being, that folic acid, a high-affinity ligand of the folate receptor retains its receptor binding property when covalently derivatized via its gamma-carboxyl. Folate conjugates have been shown to taken into receptor-bearing tumor cells via folate receptor-mediated endocytosis (Cho et al., 1997; Mathias et al., 2000; Siegel et al., 2003). Henceforth folate conjugate presents a useful method for

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receptor-mediated drug delivery into receptor-positive tumor cells.

Several folate receptor systems have been targeted with low molecular weight folate-chelate conjugates. However, only ^{111}In-DTPA-Folate derivatives are exploited for clinical application (Turek et al., 1993; Panwar et al., 2004). The field of radiodiagnostic imaging has been transitioning toward using ^{99m}Tc-based probes as it decays more rapidly and decreases the patient's exposure time to the radioactive agent. Moreover, ^{99m}Tc-based radiodiagnostic agents are less expensive, safer and produce high quality images (Reddy and Low, 1998). Consequently, several ^{99m}Tc-based folate conjugates have been developed such as ^{99m}Tc-ethylenedicysteine, ^{99m}Tc-6-hydrazinonicotinamido-hydrazide (HYNIC) and ^{99m}Tc-DTPA-Folate (Wang and Low, 1998; Guo et al., 1999; Ni et al., 2002; Hilgenbrink and Low, 2005).

The research has been extended using a bifunctional chelate conjugated to folate; as receptor specific radiopharmaceutical for imaging neoplastic tissues expressing folate-binding protein, we report the synthesis, characterization and radiolabeling of ^{99m}Tc with 1,4,7-tris (carboxymethyl)-10-(4-aminoethyl)-1,4,7,10-tetraazacyclododecane folate conjugate (^{99m}Tc-DO3A-EA-Folate).

Material and methods

Chemicals

Acetonitrile (high-performance liquid chromatography (HPLC) grade) was obtained from E. Merck Germany Ltd., Folic acid, N-hydroxysuccinimide, dicyclohexylcarbodiimide, 1,4,7-tris(carboxymethyl)-10-(4-aminoethyl)-1,4,7,10-tetraazacyclododecane (DO3A-EA), stannous chloride dihydrated ($SnCl_2.2H_2O$), trifluoroacetic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, Unites States) ^{99m}Tc was procured from Regional Center for Radiopharmaceuticals (Northern Region), Board of Radiation and Isotope Technology (BRIT), Department of Atomic Energy, India.

Instrumentation

The ^{1}H NMR spectra were determined by using Bruker Avance II 400. Mass spectrum (ES^+MS) was recorded on a mass spectrometer HPLC analyses were performed on a Waters Chromatograph efficient with 600s coupled to a Waters 2487 photodiode array UV detector. The C-18 RP Beckman column (5 μm, 1 mm × 1.25 cm) and Shimadzu ODS-AQ (5 μm, 4.6 mm × 250 mm) column were used applying elution system described in the text. Radioimaging and biodistribution studies were done using a planner gamma camera Hawk-Eye. The receptor binding data were analyzed using iterative curve-fitting program Equilibrate software from Graphpad.

Animal models

Animal protocols have been approved by Institutional Animal ethics Committee. New Zealand rabbits 2–3 kg and nude mice 18–24 g were used for blood clearance, and imaging and biodistribution studies. Rabbits were housed under conditions of controlled temperature of 22 ± 2°C and normal diet. Athymic mice were inoculated subcutaneous with 0.1 ml of cell suspension (1×10^6 KB cells) into the right thigh under sterilized conditions.

Cell culture

Monolayer cultures of human oral carcinoma KB cells ovarian carcinoma cells, OAW (obtained from Zoology Department, Delhi University, India) and human malignant glioma cells, U-87MG (obtained from NIMHANS, Bangalore, India), were maintained at 37°C in a humidified CO_2 incubator (5% CO_2, 95% air) in DMEM (Sigma, St. Louis, MO, Unites States) supplemented with 10% fetal calf serum (Gibco), 50 U/ml penicillin, 50 μg/ml streptomycin sulfate and 2 μg/ml nystatin. When the cells were grown up to 80–90% of cellular confluence, the fault culture cells were differentiated with trypsin-EDTA (Gibco, Grand Island, NY).

Chemical strategy

The synthesis of Folate-DO3A-EA is a multistep process, which is described under the following schematic steps (Figure 1).

Scheme 1: N-Boc-2-aminoethyl bromide

Di-t-butyl-dicarbonate (9.6 g, 1.0 equivalent) in dicholoromethane (100 ml) was added drop-wise over 20–30 min to a stirring of solution of 2-bromoethylaminehydrobromide (9.02 g, 1.0 equivalent) in water (50 ml) and sodium hydroxide (3.52 g, 2.0 equivalents, and 50 ml water). After 2 h, the layers were separated and the organic layer was washed with 1N HCl (25 ml) twice and a saturated sodium chloride (50 ml) once. The organic layer was then dried over sodium sulfate and concentrated in vacuum to colorless oil. Compound was purified by classical column chromatography using stationary phase silica gel 60 and mobile phase are 5% EtOAc in n-hexane. Purified compound N-Boc-2-aminoethyl bromide **1** was concentrated under reduced pressure at 50°C. Total yield was 8.26 g, 83.77% of N-Boc-2-aminoethyl bromide.

ESI-MS (+): calculated for $C_7H_{14}NO_2Br$: m/z 223.4, 225.4; found 245.9, 247.9 (M+Na). ESI-MS-MS of 247.9 ^{13}C NMR (CDCl3, 62.9 MHz) δ (ppm): 155.97 (C-2'), 80.0 (C-3'), 42.7 (C-1), 32.9(C-2) and, 28.7 (C-4'). ^{1}H NMR ($CDCl_3$), 250 MHz, δ (ppm): 5.1 (1H of NH), 3.45-3.39 (4H of C-1, C-2) 1.39 (9H of C-4')).

Scheme 2: Synthesis of DO3A-tri-tert-butyl-ester 2 (1,4,7-tris(carbobutoxymethyl)-1,4,7,10-tetraazacyclododecane)

2.20 equivalents of *tert*-butyl bromoacetate (4.0 g, 20.0 mmol) were added drop wise (in $CHCl_3$ ~250 ml) to a solution (in $CHCl_3$) of one equivalent of tetraazacyclododecane (1.58 g, 9.1 mmol) and the solution was stirred at room temperature for 72 h. This produced a mixture of the two products. The free protonated cyclen

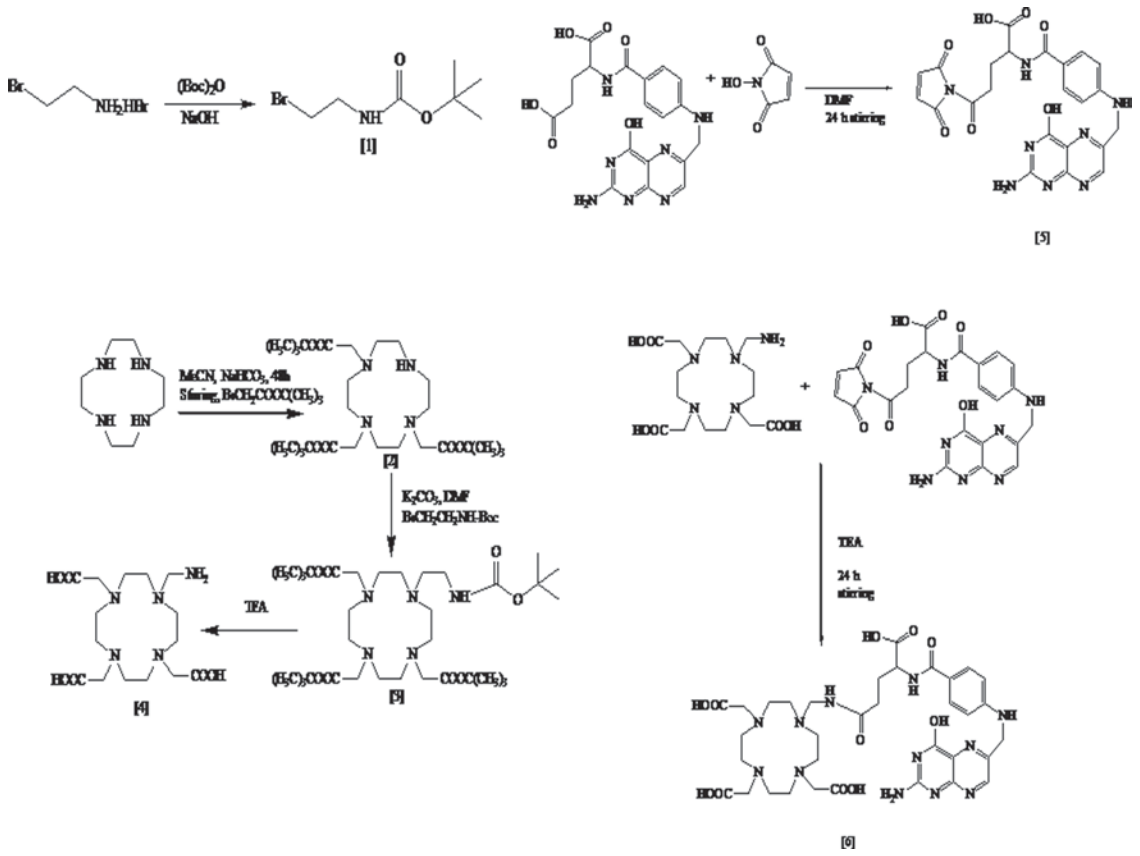

Figure 1. Chemical scheme for synthesis of Folate-DO3A-EA.

was then removed by filtration, and the resulting filtrate was washed with water to remove remaining protonated cyclen (which was in chloroform) and dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure yielding 80% of tri-N-substituted tetraazacyclododecane **2,** which was used for the next step without further purification.

ESI-MS (+): found: 515 $[M+H]^+$; calculated for $C_{26}H_{50}N_4O_6$: m/z 514. Rf = 0.64 ($CHCl_3$/MeOH:: 5:1). ESI-MS-MS of 515: (EI/intensity in %) (Supporting data) m/z = 459 (100%); 409 (88%), 347 (20%). ^{1}H NMR (250 MHz, $CDCl_3$): 1.45 (s, 27H): 2.17 to 2.81 (m, 16H): 3.33 (s, 6H): 10.01 (br, 1H). ^{13}C NMR ($CDCl_3$): 28.61 (C-16); 48.08 (C-9, C-11); 50.69 (C-2, C-6); 52.32 (C-8, C-12); 53.02 (C-3, C-5); 57.21 (C-13); 80.74 (C15); 171.25, 171.41 (C-14a, C-14c).

Scheme 3: 10-[N-Boc-2-aminoethyl]-1,4,7-tri (carbobutoxymethyl) -1, 4,7,10-tetraazacyclododecane (3)

A solution of 2 (1 g, 1 equivalent), sodium carbonate (2.6 g, 10 equivalents) and ter-butyl bromoacetate (1.64 ml, 3.5 equivalents) in acetonitrile (100 ml) was stirred at 60–70°C for 7–8 h. Reaction was monitored by TLC, after completion of reaction; the reaction mixture was filtered and was washed with DCM (20 ml, two times). The filtrate was then concentrated in vacuum to obtain pale yellow oil. Compound was purified by classical column chromatography using stationary phase silica gel 60 and mobile phase DCM: MeOH. Off white solid obtained after complete evaporation. Total yield was 1.79 g, 86.05% of 10-[N-Boc-2-aminoethyl]-1, 4,7-tri (carbobutoxymethyl)-1, 4,7,10-tetraazacyclododecane.

ESI-MS (+): calculated $C_{33}H_{63}N_5O_8$: m/z 657.5; found 658.5 $[M+H]^+$, 680.4 [M+Na]. ESI-MS-MS of 658.5. 13C NMR ($CDCl_3$, 62.9 MHz, δ(ppm) 165.13(C-1B,C-4B, C-7B), 159.85 (C-2'), 79.47 (C-3'), 77.12 (C-1C, C-4C, C-7C), 49.74 (C-1A, C-7A), 48.57 (C-8, C-9, C-11, C-12), 46.89(C-2, C-6), 45.98 (C-3, C-5), 44.05 (C-10A), 43.34 (C-10B), 23.34 (C-4') and 23.04 (C-1D, C-4D, C-7D). ^{1}H NMR ($CDCl_3$), 250MHz, δ (ppm): 5.5 (^{1}H of–NH-), 3.4-2.2 (^{16}H of C-2, C-3, C-5, C-6, C-8, C-9, C-11, C-12, and ^{8}H of C-1A, C-4A, C-7A, C-10A, ^{2}H C-10B), 1.6-1.4 (^{36}H of C-1D, C-4A, C-7A, C-4').

Scheme 4: Synthesis of 1, 4,7-tris (carboxymethyl)-10-(2-aminoethyl)-1, 4,7,10-tetraazacyclododecane 4: $C_{16}H_{31}N_5O_6$

The carbobutoxy derivative **3** (0.15 g, 0.19 mmol) was dissolved in trifluoroacetic acid (3 ml) with stirring at 0°C for 2 h followed by an additional 6 h at ambient temperature. The reaction mixture was monitored by LC-MS. After completion, the pH was adjusted to 6 using 0.1 M NH_4OAc and water was removed to dryness. The glassy solid was redissolved in one liter of water and the product was lyophilized. Yield was 80%. Reverse-phase C_{18} HPLC: solvent A, 0.1M ammonium acetate, pH 6; solvent B, MeOH; 15–65% B, 0–25 min, 65–100% B, 25–35 min; 100–15% B, 35–40 min; product peak 18 min. ESI-MS (+): found: 390 $[M+H]^+$; calculated for $C_{16}H_{31}N_5O_6$: m/z 389. ^{1}H NMR

(D_2O): 1.35 (t, 3H); 1.87 (mixed d and t, 2H); 2.41 (t, 2H); 2.51–3.29 (m, 22H), 4.07 (q, 2H). ^{13}C NMR (D_2O): 23.12 (C-18); 48.01 (C-17); 50.10 (C9, C-11); 52.4 to 52.7 (C-2 to C-6); 56.62 (C-8, C-12); 57.21 (C-13); 173.59 (C-14).

Conjugation of DO3A-EA to folate

A solution of **5** (0.38 g, 0.88 mmol), folic acid (0.2 g, 0.88 mmol), NMM (0.18 ml, 1.6 mmol) and HOBt (0.13 g, 0.97 mmol) in DMF (5 ml) was stirred at 0–5°C for 15 min and then EDC (0.19 g, 0.97 mmol) was added. The reaction mixture was stirred overnight at room temperature. The progress of reaction was monitored by thin layer chromatography. After completion, the reaction mixture was poured in water, extracted with DCM (3 × 100 ml), organic layer was dried over Na_2SO_4, filtered, filtrate was evaporated under reduced pressure and purified by column chromatography (silica gel, 10% MeOH in dichloromethane) to give 0.32 g (55%) of **6** as dark yellow viscous oil. ESI-HRMS (+): calcd $C_{29}H_{51}N_7O_8S$: m/z 658.35926 $(M+H)^+$; found 658.35913 $(M+H)^+$.

Radiolabeling and radiochemical purity

Radiolabeling was carried out as per the reference 24 and 25. 2 mg solution of the compounds dissolved in water was taken in a shielded vial and 100 µl of 1×10^{-2} M $SnCl_2.2H_2O$ (dissolved in N_2 purged 1 ml (10% acetic acid) was added followed by addition of (<1 h) freshly eluted saline solution of sodium pertchnetate ($NaTcO_4^-$) (82 MBq, 200 ml). The pH of the reaction mixture was adjusted to 6.5 with 0.1 M $NaHCO_3$ solution and shook to mix the contents. The vial was allowed to stand for 15 min at room temperature. Labeling of the compound, radiochemical purity as well as R_f of the ^{99m}Tc complexes was determined by ITLC-SG strips using 0.9% NaCl aqueous solution (saline) as developing solvent and simultaneously in acetone and PAW (Pyridine, acetic acid and water in 3:5:1.5 ratio) as mentioned in previous literature. Each TLC was cut in 0.1 cm segments and counts of each segment were taken. By using this method percentage of free $Na^{99m}TcO_4^-$, reduced ^{99m}Tc and the complex formed between ^{99m}Tc and folate conjugate could be calculated. ^{99m}Tc-DO3A-EA-Folate conjugate remained at the origin and free technetium migrated with the solvent front in acetone.

The radiolabeled drug was injected on HPLC using solvent gradient (eluant A, 10 mM ammonium acetate buffer at pH 8; eluant B, acetonitrile gradient, 0 min at 4% B, 10 min at 12% B and 15 min 15% B at a flow rate of 0.5 ml/min) and fractions were collected and radioactivity was counted on automated gamma counter.

In vitro serum stability assay

The fresh human serum was prepared by allowing blood collected from healthy volunteers to clot for 1 h at 37°C in a humidified incubator maintained at 5% carbon dioxide, 95% air. Then the sample was centrifuged at 400 rpm and the serum was filtered through 0.22 micron syringe filter into sterile plastic culture tubes. The above freshly prepared technetium radio complex were incubated in fresh human serum at physiological conditions, i.e. at 30°C at a concentration of 100 nM/ml and then analyzed by instant thin layer chromatography (ITLC-SG) at different time intervals to detect any dissociation of complex. Percentage of free pertchnetate at a particular time point that was estimated using saline and acetone as mobile phase, represented percentage dissociation of the complex at that particular time point in serum.

Receptor binding studies

Exponentially growing cells (KB, OAW and U-87MG) 0.1×10^6 cells/PD were plated at a uniform cell density and incubated overnight. Monolayer culture of the cell lines were washed twice for 2 min with ice cold binding buffer (25 mM HEPES, 10 mM $MgCl_2$ and 1% BSA). The cell line culture were then incubated for 40 min with labeled DO3A-EA-Folate (varying concentration) in the absence and presence of the 100-folds excess unlabeled DO3A-EA-Folate for estimation of total binding and nonspecific binding, respectively. Specific binding was obtained by subtracting nonspecific binding from total binding. At the end of each experiment, the cells were washed with ice cold binding buffer three times for 3 min. The cells were lysed with 200 µl of Lysis buffer. The cell-associated radioactivity was determined by gamma scintillation counting. Scatchard plot analysis was done using Equilibrate software from graph pad.

Cytotoxicity of folate and DO3A-EA-Folate

Cytotoxicity was determined using the MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide] assay. Exponentially growing cells were plated in a 96-well microtitre plate at a uniform cell density of 10,000 cells/well 24 h before treatment. Cells were treated with varying concentrations of conjugate (mM–µM range) for various time intervals viz., 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 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 DMSO. Optical density is 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). % of viability = [OD (570–630 nm) test product/OD (570–630 nm) negative control] × 100. Percentage viability at 1 µM and 10 µM concentrations was plotted against time for folic acid and DO3A-EA-Folate.

Macrocolony assay

Monolayer cultures of OAW and U-87MG cell lines were trypsinized and 100–1000 cells were plated depending upon the concentrations of the drug in 60-mm petridishes and incubated at 37°C in 5% CO_2 humidified atmosphere for 8 days. Colonies were fixed in methanol and stained with 1% crystal violet. Colonies containing more than 50 cells were counted.

Blood kinetic studies

In normal rabbit weighing about 2–2.5 kg, 11.1 MBq of the ^{99m}Tc-DO3A-EA-Folate was administered intravenously through the dorsal ear vein of the animal. At different time interval starting from 5 min to 24 h persistence of the activity in terms of percentage administered dose in samples at different time intervals was calculated using gamma counter.

Scintigraphy in tumor bearing nude mice

Tumor imaging was performed in KB cell line implanted tumor bearing nude mice administering 100 µl of the labeled conjugate (40 µg, 2.96 MBq activity). Images were taken using planar gamma camera equipped with pinhole collimator. Images were obtained at different time intervals starting from 15 min to 24 h after post injection. Semiquantitative analysis was done to accurately evaluate ^{99m}Tc-DO3A-EA-Folate uptake. Regions of interest (ROI) were drawn on the right thigh at tumor site. Using the INTEGRA system's ROI program, the same ROI could be easily moved to the contralateral muscle representing specific and nonspecific binding areas were drawn. The ratios were calculated from counts per pixel of the individual ROI for semiquantitative analysis.

Biodistribution studies in KB cell line implanted in nude mice

Human oral carcinoma KB cell line that over expresses the folate receptor was injected subcutaneous in the thigh of the right hind leg of nude mice. When the tumors were easily palpable and approximately of 0.17 g, mice were used for biodistribution study. An equal dose of 3.7 MBq of labeled DO3A-EA-Folate was injected in mice through tail vein of each animal. Mice were dissected at 1 h, 4 h and 24 h post injection; different tissues were taken out, weighed and counted in a γ-counter calibrated for ^{99m}Tc energy. The actual amount of radioactivity administered to each animal was calculated by subtracting the activity left in the tail from the activity injected. Radioactivity accumulated in each organ was expressed as percentage administered dose per gram of tissue. Total volume of the blood was calculated as 7% of the body weight.

Results

Synthesis

Alkylation reactions performed at room temperature in chloroform did not require drastic conditions or large excess of free cyclen to provide 70–80% of the trisubstituted product. The macrcocycle **4** was obtained from trisubstituted **2** (1, 4,7,10-tetrazacyclododecane-1, 4,7-triacetic acid tri-tert-butyl ester) reacted N-Boc-2-aminoethyl bromide in N, N-dimethylformamide at 80–90°C in the presence of potassium carbonate as the base, to form 3 as depicted in scheme. Finally, carboxylic tert-butyl ester groups were removed in neat triflouoroacetic acid, by adjusting the pH to 6.

Quality control of labeled folate conjugate

Radiochemical purity of ^{99m}Tc-DO3A-EA-Folate was estimated chromatographically using ITLC-SG (instant thin layer chromatography-silica gel) paper as the stationary phase and 100% acetone as the mobile phase. The complex remained at the point of spotting. The labeled complex remained at the point of spotting and $^{99m}TcO_4^-$ moved toward the solvent front in acetone. Thus the yield of free and complex ligand could be estimated. The labeling yield was found to be more than 97%. Percentage radiolabeling was calculated for 0, 2, 4, 6 and 24 h. Even up to 24 h, labeling efficiency was found to be 95.8% implying that the labeled folate conjugate was relatively more stable as compared to unmodified labeled folic acid.

Human serum stability evaluation

The folate conjugate was successfully labeled with ^{99m}Technetium with more than 97% labeling efficiency as determined by instant thin layer chromatography using different solvent systems. It was sufficiently stable up to 24 h as only 4.2% of the radiolabeled drug dissociated in serum at 24 h (Figure 2).

Receptor binding studies

KB, U-87MG and OAW cells were examined by saturation binding assay-using ^{99m}Tc-DO3A-EA-Folate for the ability of folate conjugate to bind folate receptors on the surface of these tumor cell lines (Figure 3). Nonspecific binding was determined using 100-fold excess of unlabeled folic acid. As compared, it was observed that the affinity of the conjugate was more than the free folate. This could be partly attributed to the receptor-mediated internalization or decreased accessibility of the folate receptor in the folate-replete medium. Scatchard plot analysis revealed affinity of the labeled drug on tumor cell lines. K_d was found to be 4.2 ± 0.8, 22.54 ± 0.002 and 16.68 ± 0.01 µM (mean ± S.E.) in KB, U-87MG and OAW, respectively (Figure 4).

Cytotoxicity studies of folic acid and folate conjugate

By analyzing the data of MTT assay done at different concentrations on KB cells line, it was found that after

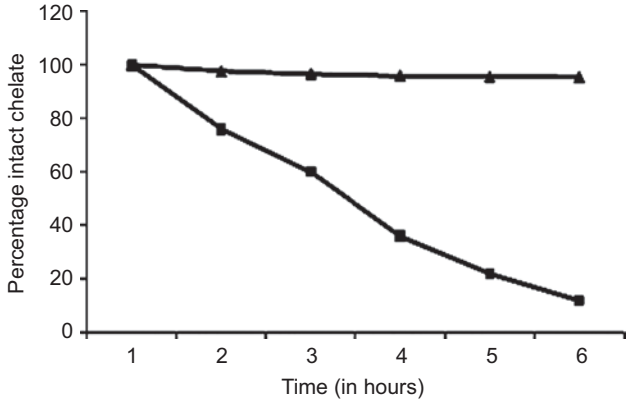

Figure 2. *In vitro* human serum stability study of ^{99m}Tc-DO3A-EA-Folate (▲) and ^{99m}Tc-Folate (■) under physiological conditions.

72 h exposure lyses was only 20% at the concentration of 1 mM of folic acid, whereas the conjugate showed only 5%. When KB cells were incubated with 10 μM of the conjugate for 72 h only 3% of the cell death was observed. For U-87MG cell line, at 72 h of exposure, resulted in 18% of the lyses, whereas the conjugate did not have any effect on the cell survival at 1 mM (Figure 5). It was observed that when 10 μM of conjugate was incubated for 72 h only 2% of the cells were killed.

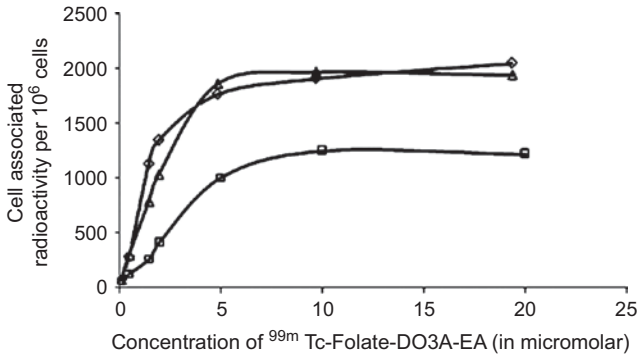

Figure 3. Cell-associated ^{99m}Tc radioactivity per 10^6 cells following incubation of KB (□), U-87MG (Δ) and OAW (◇) cells with increasing concentrations (0.1–20 μM) of ^{99m}Tc-DO3A-EA-Folate.

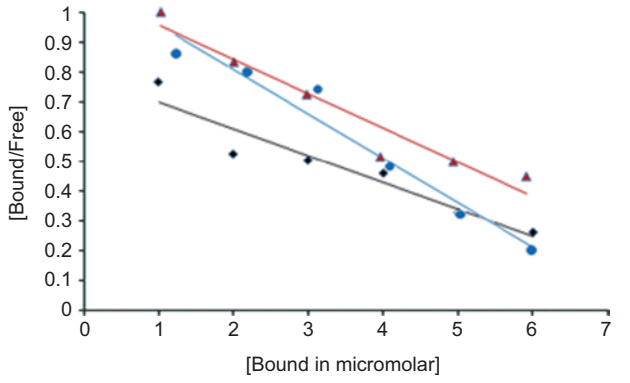

Figure 4. Scatchard plot of the specific binding data to the ratio of bound to free (B/F) for U-87MG (●), KB (◆) and OAW (▲) in (0.00001–1 μM).

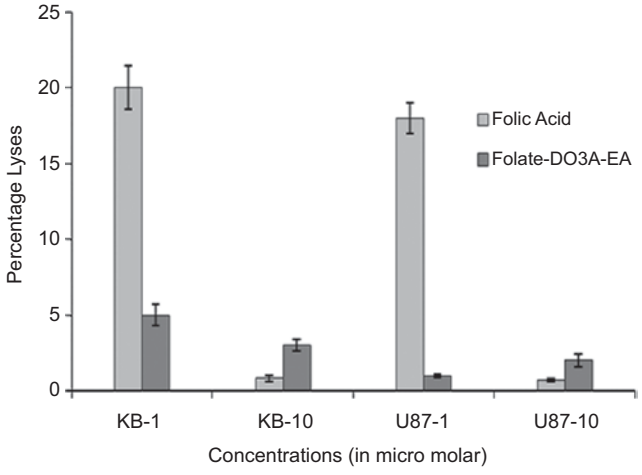

Figure 5. Colorimetric estimation of the mitochondrial activity for cytotoxicity of DO3A-EA-Folate and folic acid (MTT assay) in KB and U-87MG cell line after an incubation time of 72 h at 1 and 10 μM concentrations.

Macrocolony assay

The two cell lines U-87MG and OAW showed rather high survival values for DO3A-EA-Folate, of 1.65 ± 0.002 and 1.08 ± 0.01 at mM. Folic acid showed survival of 0.64 ± 0.02 and 0.60 ± 0.001 at 1 mM in the clonogenic assay. Macrocolony assay carried out in two cell lines U-87MG and OAW revealed that folate and conjugate at lower concentration (nM to mM range) were less toxic in both the cells lines. Higher (1 mM) concentration of folate is comparatively more toxic than DO3A-EA-Folate in these cells lines though the toxicity of folic acid was in significant.

Blood kinetics

Blood radioactivity counts showed that the radioligands were cleared from the circulation very rapidly. Figure 6 of blood kinetics, data reveal the biphasic pattern of clearance, biological half-life was found to be $t_{1/2}$ (fast), 12 min; $t_{1/2}$ (slow), 2 h and 20 min. Approximately, 15% of the injected activity at 30 min remains in the circulation for DO3A-EA-Folate.

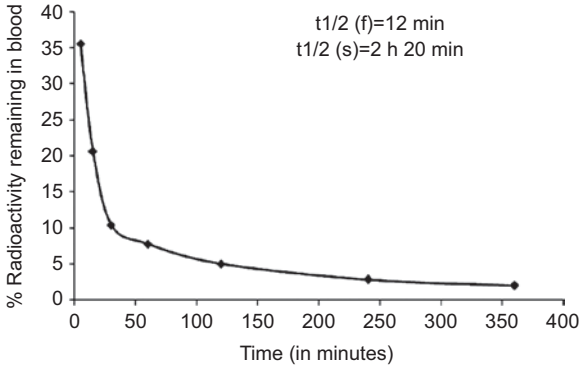

Figure 6. Blood clearance of ^{99m}Tc-DO3A-EA-Folate (11.1 MBq activity) administered through ear vein in normal rabbit.

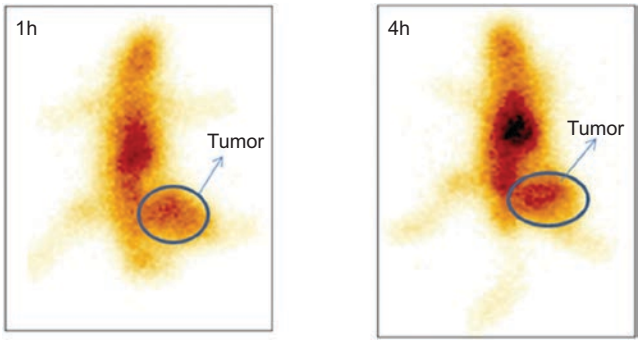

Figure 7. Whole body γ image of female nude mice with subcutaneous folate receptor-positive tumor in right thigh after 1 h and 4 h following intravenous administration of ^{99m}Tc-DO3A-EA-Folate (2.96 MBq activity).

Scintigraphy

Scintigraphic images of ^{99m}Tc-labeled DO3A-EA-Folate in tumor grafted nude mice showed rapid accumulation of radioactivity in tumor. Imaging of animals was carried out at different time intervals after administering labeled compound via tail vein. The mice depicted the beginning of accumulation of activity in tumor at 30 min, which reached to maximum at 1 h and remained almost stable for 4 h (Figure 7). Semiquantitative analysis revealed tumor to contralateral muscle ratio to be 11.8 ± 1.2 at 1 h and 19.7 ± 3.3 at 4 h which accounted for the clearance of the background activity and enhanced contrast at tumor site.

Biodistribution

Athymic nude mice implanted with human KB cell line exhibited major accumulation in liver (17.6%/g) followed by kidneys (15.0%/g) at 1 h showing that the complex is excreted both by hepato-biliary as well as renal routes (Figure 8). The high tumor accumulation of 4.06 ± 0.32 %ID/g at 1 h that remains high 6.26 ± 0.4 up to 4 h post injection was observed. In animal models with and without coadministration of high dose of cold folic acid (blocking dose) together with the radiolabeled reveals that the uptake was significantly blocked by (>80%) (Figure 9), which allows the advantage of comparing *in vitro* receptor binding, and imaging tumor bearing experimental model. As the background activity gradually cleared from the body, there was increase in target-to-nontarget ratios. High tumor uptake was shown in the tumor bearing mice; tumor-to-blood ratios reached 2.27 ± 0.32 and 6.05 ± 1.02 at 1 and 4 h after post injection, respectively.

Discussion

DO3A-EA (**4**) is a bifunctional ligand (Subramanian and Meares, 1990), bearing amine group that is readily reactive toward most electrophiles such as aldehydes, carboxylic acids and isothiocyanates to form different types of linkage with various biologically active molecules for the development of targeted imaging agents. Though DO3A-EA has already been part of several synthetic schemes (Mishra and Chatal, 2001; Mishra et al., 2006; Duimstra et al., 2005), we report here the application of DO3A-EA in a new context, as precursor for the synthesis of folate conjugate to develop SPECT pharmaceuticals for targeted imaging. In this synthetic route, our product (DO3A-EA) was obtained from cyclen in 50 % yield in 3 steps, by the reaction with tert-butyl bromoacetate followed by alkylation's reaction to get the DO3A-EA by cleaving the tert-butyl groups by the treatment of TFA at room temperature.

After the successful synthesis of precursors, the bioconjugate ligand (**6**) was synthesized in two-step reaction. Folate-conjugated DO3A-EA compound **6** was obtained by the coupling of folate with ligand **4** using EDCI/HOBt/NMM in DMF. The choice of such mild conditions for the conjugation of folate with DO3A-EA and amide bond formation was mandatory due to the sensitivity of folate toward the harsher conditions (temperature etc.). In the following step, folate conjugate was obtained in 77% yield by deprotection of tert-butyl groups with THF: MeOH: H_2O.

Recent efforts to develop tumor-specific imaging agents have focused on the use of targeting ligands that deliver attached radio-emitters/contrast agents to receptors that are over-expressed on cancer cells (e.g. EGFR, Vit.B_{12} etc.). With the use of SPECT and PET, well-defined tumor images have been achievable in nanomolar concentrations. (Lee and Low, 1994; Ilgan et al., 1998; Leamon et al., 2005; Leamon et al., 2007). A number of BFCs were synthesized and hooked with the folate using different conjugation chemistry as well as different radio metals (Gabizon et al., 2004; Müller et al., 2006). ^{99m}Technetium-labeled diethylenetriamine pentaacetic acid-polyethylene glycol-folate (DTPA-PEG-folate) tested as a radiopharmaceutical agent, which targeted the lymphatic system with metastatic tumor (Min et al., 2005). ^{111}In-DTPA-folate was advanced to phase I/II clinical trials for imaging of ovarian cancers. Mathias et al. (1999) showed rapid and specific ^{67}Ga-deferoxamine-folate uptake by

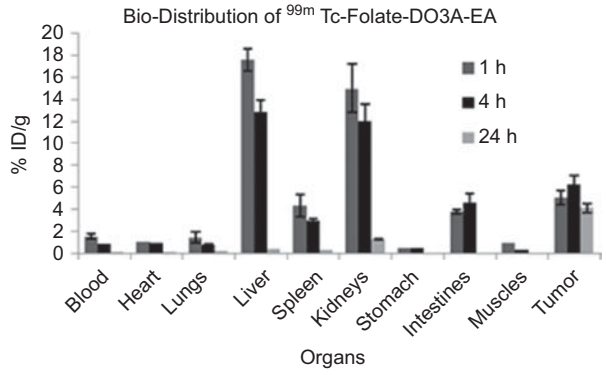

Figure 8. Biodistribution of ^{99m}Tc-DO3A-EA-Folate in KB cell tumor grafted in athymic mice injected with 3.7 MBq activity.

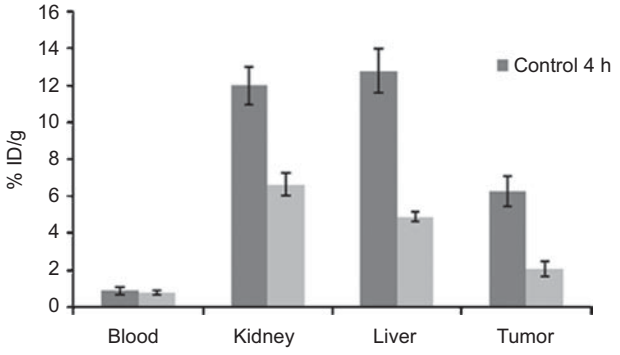

Figure 9. Blood, kidneys, liver and tumor activity of ^{99m}Tc-DO3A-EA-Folate administrated in athymic mice implanted with KB cell tumor with and without a coadministration of blocking dose at 4 h.

tumors (~5% injected dose/g tumor at 4 h post administration), with an excellent tumor:blood ratio of 400:1. ^{125}I and ^{67}Ga were limited, because of the long half-life and poor image quality, this imaging agent was rapidly replaced by candidate with greater clinical potential. Thus ^{99m}Tc-labeled folate conjugates proved better candidates as targeted radiopharmaceutical.

It has been demonstrated that only those folate conjugates containing the adduct attached at the γ-carboxyl of folic acid retain the ability to bind to cell surface folate receptors with the same affinity as free folic acid (Luhrs and Slomiany, 1989). Importantly, this unique region specific activation of the γ-carboxyl on the folic acid can potentially be applied in the preparation of many folate-targeted agents. The newly developed DO3A-EA derivative of folate shows promising characteristics for radiodiagnostic imaging of neoplastic tissues over expressing folate receptors. This BFC was synthesized taking lead from ^{99m}Tc-DTPA where they have used polyaminopolycarboxylate chelator. Similarly we introduced a strong chelating agent, 1, 4,7-tris(carboxymethyl)-10-(4-aminoethyl)-1,4,7,10-tetraazacyclododecane on the gamma-carboxyl group of folic acid providing chelators to bind with reduced ^{99m}Tc, which was characterized by different spectroscopic techniques. The folate conjugate have a better hand over the previously reported compounds as it forms stable electron donor complex with ^{99m}Tc with high yield and specific activity with simplified radiolabeling procedure.

In this compound, there was least transcomplexation of the labeled conjugate which was revealed by *in vitro* human serum stability studies under physiological conditions. The rate extent of drug absorption of the drug in drug delivery system depends upon the balance between the hydrophilic and lipophilic nature. The rate of transfer of the drug molecule across biological membranes and to the target site is very rapid as the conjugate enters the site by endocytosis of folate.

The labeled folate conjugate is in direct competition with the folate present in the medium, though the endogenous folate was removed by acidic wash, may bring about intriguing possibility of modulating the effectiveness of folate conjugate binding to folate receptor. The amount of receptor activity increases markedly when cells are depleted of folate through growth in folate-depleted medium (**7**). K_d was found to be 4.2 ± 0.8, 22.54 ± 0.002 and 16.68 ± 0.01 μM (mean ± S.E.) in KB, U-87MG and OAW, respectively (Figure 3). A direct correlation was found between MTT assay and clonogenic assay (Figure 4). The conjugate delivered was less active as a cytotoxic agent, with only 2–5% lyses (U-87MG and KB cell line) as compared to the folate alone where the toxicity was 18–20% in U-87MG and KB cell line.

The labeled conjugate showed rapid clearance with $t_{1/2}$ (fast) = 12 min (Figure 5) from the circulation and showed its high target uptake with the diagnostically useful target to nontarget ratio in a short period of time, which are known to contain a substantial concentration of the target receptor that can be visualized in the high quality images, obtained 1 h after administration (Figure 6). The radiotracer was found to substantially concentrate in the tumor, and excellent tumor to nontarget tissue contrast was observed at 4 h for all tissues except kidneys (tumor-to-blood = 6.05 ± 1.02, tumor-to-kidney = 0.25 ± 0.04).

Thus, the newly synthesized folate conjugate shows high selectivity, rapid clearance kinetics and substantial promise for tumor scintigraphy as there is considerable accumulation in athymic mice bearing subcutaneous folate receptor-positive tumor of human KB cell line, hence, making the conjugate an ideal candidate for development as imaging agents for the detection of FR-positive tumors.

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Declaration of interest

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