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Evaluation of Methionine and Tryptophan derivatised vehicles: Met-ac-TE3A/Trp-ac-TE3A for tumor imaging

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Abstract

Two novel amino acid (methionine and tryptophan) appended 1, 4, 8, 11 tetraazacyclotetradecane triacetate (TE3A) compounds **Met-ac-TE3A** and **Trp-ac-TE3A** were synthesized and evaluated for imaging application. The pharmacokinetics of these compounds was analyzed by ^{99m}Tc labelled tracer methods.

In vitro human serum stability of ^{99m}Tc labelled Met-ac-TE3A/Trp-ac-TE3A was found to be 96.5% and 96.0% after 24 h respectively. **Blood kinetics of both the labeled probes on normal rabbits showed biphasic clearance.** The tumor (EAT cell line) grafted in balb/c mice were readily identifiable in the gamma images. Biodistribution revealed significant tumor uptake and good contrast in the EAT tumor bearing mice and **also showed high tumor/muscles ratio which is a requisite condition to work as SPECT-radiopharmaceutical for tumor imaging**. To look its futute applicability for therapy using M^{+2} and M^{+3} metal ions, we performed thermodynamic stability constants of complexes derived from Met-ac-TE3A and Trp-ac-TE3A with Cu^{II} and Ln^{III} metal ions.

Key Word: Amino Acid, Imaging, Kinetics, Macrocycles, Biodistribution.

1. Introduction

Radiotracer based imaging probes are essential for the diagnosis of disease, monitoring and targeted therapy in the field of neuroimaging, oncology, neurooncology and infections using positron emission tomography (PET) and single photon emission computed tomography (SPECT). Radiolabeled amino acids has been proved a diverse and useful class of PET and SPECT tracers that records amino acid transportation and uptake exhibited by many tumor cells i.e. brain, neuroendocrine, prostate cancer [1- 4].Increase rate for amino acid uptake is one of the earliest and most important events associated with proliferation [5].

Recent studies have shown that of IDO (indoleamine 2, 3-dioxygenase) is overexpressed in a variety of human tumors, including lung tumors resulting increase of abnormal tryptophan metabolism *via* the kynurenine pathway. L-tryptophan is a substrate of IDO [6-8]. Intracellular IDO activity may result in the trapping of polar tryptophan (or its derivative) metabolites or indirect accumulation *via* intracellular L-tryptophan depletion. There are many evidences that show fast growing progression of tumors because of a failure of the immune system to maintain control over budding tumors. **Various studies indicate that the consumption of tryptophan is critical factor in progressive tumor** [9-10]. **The PET radiotracer, [^{11}C] methyl- L-tryptophan (AMT)** is well suited for such studies; AMT is not a substrate for protein synthesis [11] but can be metabolized by IDO because of the low substrate specificity of this enzyme [12]. Similarly the major metabolic function of methionine is in protein synthesis and conversion to *S*-adenosylmethionine (Adomet), which is required in multiple metabolic pathways. Methionine (Met) dependence has been shown *in vitro* in a number of human cell lines of different cellular origin, and the dependence may reflect the overall imbalance in the transmethylation. Metabolic

defects in cancer cells often manifest in the inability to grow in media where Met has been replaced by its precursor homocysteine.

It was therefore considered valuable to focus our attention on the development of specific amino acid based imaging system **that can be utilized for imaging techniques viz. PET, SPECT and magnetic resonance imaging (MRI)** for tumour targeting by conjugation of amino acid with a suitable chelate. Currently a variety of cyclic and acyclic polyamino-polypcarboxylate is being evaluated as vehicles in variety of radiopharmaceuticals and MR agents. These polyamino-polycarboxylates have capability to adopt an organized conformation in the complex formation with metal ions. Two of the most important chelators studied were DOTA and TETA [13]. In continuation of our work for design of novel agent having these vehicles, we previously synthesised DTPA-bis(methionine)[14] and DO3A-Act-Met[15] for imaging applications.

Here, we report the synthesis of two new ligand, synthesis of (11-((3-(1H-indol-3-yl)-1-methoxy-oxopropan-2-yl)amino)-2-oxoethyl)-1,4,8,11-tetraazacyclo tetradecane-1,4,8-triyl)triacetic acid, **Trp-ac-TETA** and 2,2,2,-(11-((3-(1H-indol-3-yl)-1-methoxy-oxopropan-2-yl)amino)-2-oxoethyl)-1,4,8,11-tetraazacyclotetradecane-1,4,8-triyl)triacetic acid, **Met-ac-TETA** . After wet synthesis and spectroscopic characterisation both the molecules were evaluated *in vitro* and *in vivo* after coordinating with suitable metal ion of biomedical interest.

2. Experimental

2.1. Ligand synthesis and characterization

2.1.1. General Procedure for Chloroacetylation

The methyl ester of L-methionine/L-tryptophan was dissolved in water (50 mL). The chloroacetyl chloride (1.2 eq.) solution in dichloromethane (50 mL) and K_2CO_3 solution in water (1.2 eq.) were added slowly to the stirring solution at 0°C using a pressure equalized dropping funnel. The resulting reaction mixture was stirred at room temperature overnight. After completion of the reaction, the pale yellow viscous oil was washed with H_2O (2 x 20 mL), 0.1M HCl (2 x 20 mL) and brine (2 x 20 mL). The organic layer was dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure to yield pure chloroacetylated product.

2.1.2. General Procedure for Amino Acid Conjugation

The tris(carbobutoxymethyl)-1,4,8,11-tetraaza-cyclotetradecane **1** was dissolved along with K_2CO_3 (3 eq.) in 60 mL of dry CH_3CN under nitrogen atmosphere. Chloroacetylated amino acid (3 eq.) was added drop wise to the above reaction mixture at 0°C. The mixture was stirred for 10 min and afterwards refluxed at 70°C for 3 days under nitrogen. After completion of the reaction (monitored by TLC, 9:1, chloroform: methanol), the reaction mixture was cooled, filtered, and evaporated under reduced pressure to give crude oily residue. The compound was purified by column chromatography (methanol: chloroform) to give the compounds.

2.1.3. General Procedure of Deprotection

The compound was dissolved in trifluoroacetic acid (3 mL) and stirred at room temperature for another 16 h. The solvent was evaporated and residue was dissolved in 2 mL of MeOH, followed by addition of 50 mL of diethyl ether drop wise and stirred for 1 h at room temperature. The

compound was dried, dissolved in water, and neutralized to pH 7 by the addition of 1 M NaOH. The crude product was purified by preparative HPLC to give final compound.

2.1.4. Synthesis of ***1, 4, 7-tri(tert-butoxymethane)-1,4,8,11-tetraazacyclotetradecane*** **(1)**

The 1, 4,8,11 tetraazacyclotetradecane (1.00 g, 5.00 mmol) was dissolved in dry acetonitrile (25 mL) under nitrogen atmosphere at RT. After stirring the solution at 0^0C for 10 min, $NaHCO_3$ (1.26 g, 15.00 mmol) was added drop wise to the solution and stirred for 30 min. *Tert*-butylbromoacetate (2.92 g, 15.00 mmol) was added slowly in 3 h at 0^0C. The reaction was stirred at room temperature for 48 h and then refluxed at 65-70^0C for 8 h. The completion of the reaction was checked by TLC (9:1; dichloromethane: methanol). After the completion of the reaction, the reaction mixture was filtered and evaporated to dryness. The crude compound was purified by column chromatography (silica gel, 9:1:chloroform:methanol) to give **1** (*t*-Bu-TE3A) as white powder (2.03 g, 75%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS) : δ_H = 1.45 (3s, 27H; $C(CH_3)_3$), 1.62-1.63 (m, 4H), 1.99 (brs, 1H), 2.55-3.40 (m, 22H) ; ^{13}C-NMR (100 MHz, $CDCl_3$) : δ_C = 23.52, 24.37 (**C**H_2), 29.01, 29.07, 29.12, (C(**C**H_3), 47.06, 47.53, 49.86, 50.87, 51.88, 53.38, 55.59, 56.70, 56.89, 81.72, 82.15, 82.40 (**C**$(CH_3)_3$), 171.53, 172.47, 172.62 (**C**O). *m/z* (ESI) found 543.9 $[M+H]^+$, $C_{28}H_{54}N_4O_6$, calculated 542.7.

2.1.5. Synthesis of ***methyl-2-(2-chloroacetamido)-4-(methylthio) butanoate*** **(2)**

According to the general procedure methyl ester of methionine (1.00 g, 6.13 mmol), chloroacetyl chloride (0.82 g, 7.36 mmol) and K_2CO_3 (1.03 g, 7.36 mmol) to afford **1** (0.69g, 80%) as a colorless oil. ^{1}HNMR (400 MHz, $CDCl_3$, TMS) : δ_H = 2.11 (s, 3H; CH_3-S-), 2.18-2.20 (m, 2H), 2.51-2.55 (m, 2H), 3.79 (s, 3H; CH_3-O), 4.08 (s, 2H; CH_2-Cl), 4.73-4.75 (m, 1H; -CH-

NH-); ^{13}CNMR (100 MHz, $CDCl_3$,) : δ_C = 15.48 (CH_3-S-), 29.83 (CH_2-S), 31.29 (CH_2), 42.43 (CH_3-O), 51.82 (CH_2), 52.76 (CH_2-NH-), 165.93 and 171.72 (CO). *m/z* (ESI) found 262.5 ($M+Na^+$), $C_8H_{14}ClNO_3S$, calculated 238.5.

2.1.6. Synthesis of ***methyl-2-(2-chloroacetamido)-3-(1H-indol-3-yl) propanoate*** **(3)**

By using general procedure the hydrochloride of methyl ester of tryptophan (1.00 g, 3.93 mmol), chloroacetyl chloride (0.53 g, 4.71 mmol) and K_2CO_3 (0.65g, 4.71 mmol) as reactant to afford **3** (1.05 g , 78%) as a colorless oil. ^{1}HNMR (400 MHz, $CDCl_3$, TMS) : δ_H = 3.38 (d, 2H, *J* = 5.6 Hz), 3.69 (s, 3H, -OC**H**$_3$), 3.96 (s, 2H), 4.92-4.97 (m, 1H), 6.99 (s, 1H), 7.14-7.28 (m, 2H), 7.34 (d, 1H, *J* = 8.0 Hz), 7.58 (d, 1H, *J* = 7.6 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$) : δ_C = 27.52, 42.47, 52.58, 53.17, 109.59, 111.32, 118.53, 119.80, 122.41, 122.78, 127.42, 136.12, 165.72 (CO), 171.68 (CO), *m/z* (ESI) found 296.0 ($M+ 2H^+$), $C_{14}H_{15}ClN_2O_3$, calculated 294.08.

2.1.7. Synthesis of ***tri-tert-butyl-2,2,2-(11-(3-((1-methoxy-4-(methylthio)-)-1-oxobutan-2yl)amino)-2-oxoethyl)-1,4,8,11-tetraazacyclotetradecane-1,4,8-triyl)triacetate*** **(4)**

According to the general procedure the tris(carbobutoxymethyl)-1,4,8,11-tetraaza-cyclotetradecane **1** (0.40 g, 0.70 mmol), K_2CO_3 (0.51 g, 3.60 mmol) and methyl-2-(2-chloroacetamido)-4-(methylthio)butanoate (0.52g, 2.2 mmol) to give crude oily residue. The compound was purified by column chromatography (silica gel, 95:5: chloroform: methanol) to give the **4** (0.41g, 75%) as a brown solid..^{1}HNMR (400 MHz, $CDCl_3$, TMS) : δ_H = 1.39 (s, 27H; C(**C**H_3)$_3$), 1.58-1.59 (m, 4H), 2.04-2.15 (m, 5H, SCH_3 and methionine proton), 2.46-2.69 (m,

19H), 3.18 (s, 4H), 3.25 (s, 2H), 3.74 (s, 2H), 3.68 (s, 3H, OCH_3), 4.58-4.63 (m, 1H) ; ^{13}C-NMR (100 MHz, $CDCl_3$) : δ_C = 15.38 (CH_3), 25.27, 25.65, 28.15 ($C(\mathbf{C}H_3)_3$), 30.24, 31.20, 42.38, 50.12, 50.20, 50.52, 51.10, 51.47, 51.76, 52.25, 52.38, 53.00, 56.18, 56.31, 56.36, 58.55, 80.60, 80.65, 80.78 ($\mathbf{C}(CH_3)_3$), 170.50, 170.83, 171.03, 172.10, 172.54, (CO) . *m/z* (ESI) found 747.8 ($M+H^+$), 769.7 ($M+ Na^+$), $C_{36}H_{67}N_5O_9S$, calculated 746.0.

2.1.8. Synthesis of ***tri-tert-butyl-2,2,2-(11-((3-(1H-indol-3-yl)-1-methoxy-1-oxopropan-2yl)amino)-2-oxoethyl)-1,4,8,11-tetraazacyclotetradecane-1,4,8-triyl)triacetate*** **(5)**

The general procedure described above was followed using tris(carbobutoxymethyl)-1,4,8,11-tetraaza-cyclotetradecane **1** (0.50 g, 0.92 mmol), K_2CO_3 (0.38 g, 2.76 mmol) and methyl-2-(2-chloroacetamido)-3-(1H-indol-3-yl)propanoate **3** (0.81 g, 2.76 mmol) as a reactant to give crude oily residue. The compound was purified by column chromatography (silica gel, 95:5:chloroform:methanol) to give the compound as a brown solid (0.56g, 75%).^{1}HNMR (400 MHz, $CDCl_3$, TMS) : δ_H =1.44-1.47 (m, 31H), 2.42–2.65 (m, 15H), 2.99-3.24 (m, 11H), 3.68 (s, 3H; CH_3-O-), 4.82-4.87 (m, 1H; CH-NH-), 7.08–7.52 (m, 5H; indolylprotons); ^{13}CNMR, (100 MHz, $CDCl_3$) : δ_C = 25.12, 25.38, 28.20 $(CH_3)_3$, 42.41(OCH_3), 50.81, 51.11, 51.17, 51.53, 51.71, 51.97, 52.23, 52.26, 52.49, 52.61, 52.75, 53.29, 55.84, 56.41, 80.75, 80.87, 80.96, ($C(CH_3)_3$), 109.38, 111.57, 118.38, 119.39, 121.98, 123.29, 127.44, 136.43, 165.97, 170.76 (CO), 171.14 (CO), 171.19 (CO), 172.38 (CO). *m/z* (ESI) found 802 ($M+ 2H^+$), $C_{42}H_{68}N_6O_9$, calculated 800.0

2.1.9. Synthesis of ***2,2,2-(11-(3-((1-methoxy-4-(methylthio)-)-1-oxobutan-2yl)amino)-2-oxoethyl)-1,4,8,11-tetraazacyclotetradecane-1,4,8-triyl)triacetic acid*** **(6)**

The general procedure described above was followed using compound **4** (0.40 g, 0.53 mmol). The compound was dried, dissolved in water, and neutralized to pH 7 by the addition of 1 M NaOH. The crude product was purified by preparative HPLC to give final compound **6** (0.22 g, 70%.). ^{1}HNMR (400 MHz, D_2O, TMS) : δ_H = 1.79-2.07 (m, 9H, S-CH_3; 4 H Cyclam ring and 2 H Methionine), 2.85-3.16 (m, 16H), 3.16-3.96 (m, 11H; Cyclam, OCH_3), 4.44-4.47 (m, 1H; CH); ^{13}C-NMR (100 MHz, D_2O) : δ_C = 13.88 (s, CH_3), 21.82, 24.18, 29.17, 48.72, 50.51, 50.99, 51.50, 51.84, 52.01, 52.34, 52.59, 52.95, 53.09, 55.21, 54.20, 54.59, 56.28, 56.38, 162.59, 171.72, 172.81, 173.60, 174.93 (CO). **High resolution mass spectrometer (HRMS) (FAB) calculated for $C_{24}H_{43}N_5O_9S$, 577.6521; Observed: 577.5511.**

Chemical purity: > 99% by HPLC: Capcell Pack UG80 C18 column, 4.6 mm i.d. X 250 mm; MeCN/H2O/Et_3N, 75/25/0.01 (v/v/v); flow rate, 1.0 mL/min; λ_{uv}, 254 nm); retention time (t_R), 9.7 min. (**Supplementary figure 1**).

2.1.10. Synthesis of ***(11-((3-(1H-indol-3-yl)-1-methoxy-oxopropan-2-yl)amino)-2-oxoethyl)-1,4,8,11-tetraazacyclo tetradecane-1,4,8-triyl)triacetic acid*** **(7)**

According to the general procedure compounds **5** (0.50 g, 0.62 mmol) was deprotected with and 3 mL of TFA. The compound was dried, dissolved in water, and neutralized to pH 7 by the addition of 1 M NaOH. The crude product was purified by preparative HPLC to give final compound. ^{1}HNMR (400 MHz, D_2O, TMS) : δ_H = 1.64-1.73 (m, 4H), 2.50-3.98 (m, 30H), 6.99-7.50 (m, 5H; indolyl protons); ^{13}CNMR (100 MHz, D_2O) : δ_C = 21.50, 26.88, 35.21, 44.47,

50.58, 51.72, 51.96, 52.29, 52.41, 52.62, 53.02, 53.42, 54.10, 54.36,55.79, 56.20, 109.32, 111.94, 118.42, 119.52, 122.07, 124.58, 126.90, 135.93, 162.73(CO), 163.08(CO), 173.27(CO).

High resolution mass spectrometer (HRMS) (FAB) calculated for $C_{30}H_{44}N_6O_9$, 632.4132; Observed: 632.3190.

Chemical purity: > 99% by HPLC: Capcell Pack UG80 C18 column, 4.6 mm i.d. X 250 mm; MeCN/H_2O/Et_3N, 80/20/0.01 (v/v/v); flow rate, 1.0 mL/min; λ_{uv}, 254 nm); retention time (t_R), 8.1 min. (**Supplementary figure 2**).

2.2. Determination of Protonation and Stability Constant

The protonation constant of [Trp-ac-TE3A] and [Met-ac-TE3A] and stability constants of the complexes formed with Eu^{III} and Cu^{II} have been determined by pH potentiometric titrations (Table 1). Potentiometric measurements were carried out with an automatic titration system consisting of Metrohm 713 pH meter equipped with a Metrohm A.60262.100 electrode 800 Dosino autoburet. The acidity constants of both the compound were determined potentiometrically by titrating 25.0 mL of aqueous 2.5 mM HCl in the presence of 0.01 M both substituted TE3A with 0.1 M NMe_4OH.

2.3. Radiolabeling of Met-ac-TE3A/Trp-ac-TE3A with ^{99m}Tc

Met-ac-TE3A/Trp-ac-TE3A (1 mg) was dissolved in a shielded vial and stannous chloride (300 μL; 1 mg dissolved in N2 purged 1 mL of 10% acetic acid) was added followed by addition of freshly eluted (<1 h) ^{99m}Technetium pertechnetate (82 MBq; 200 μL).The pH of the reaction mixture was adjusted to 7 with 0.1 M Na_2CO_3 and purged with N_2, and shaken to mix. The vial was allowed to stand for 15 min at room temperature (25 °C).

2.4. Radiochemical Purity

The number of ligand molecules involved in complexation with ^{99m}Tc was determined by ascending instant thin layer chromatography on ITLC-SG (Paul Gelman,USA) strips using 100% acetone as developing solvent and simultaneously in pyridine/acetic acid/water (PAW) (3:5:1.5) and saline. Each TLC was cut in 0.5 cm segments and counts of each segment were taken. By using this method percentage of free $Na^{99m}TcO^{4-}$, reduced/hydrolysed ^{99m}Tc, and the complex formed between ^{99m}Tc and Met-ac-TE3A/Trp-ac-TE3A conjugate could be calculated. Met-ac-TE3A/Trp-ac-TE3A conjugate remained at the origin, and free technetium traveled with the solvent front in acetone.

The radiolabeled conjugate was then purified using a C-18 reversed-phase extraction cartridge, which was preconditioned with 10 mL methanol and subsequently activated with 30% methanol to make prepare it more appropriate in terms of purity. The cartridge was successively rinsed with 5 mL distilled water, and radiolabeled conjugate was eluted in 5 mL of 5% ethanol.

2.5. In Vitro Studies

2.5.1. Human Serum Stability Assay

The above freshly prepared technetium radiocomplexes (300 μCi of radioactivity) were incubated in 0.9 % saline solution (1 mL) at 37 ^{0}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. Complexes were stable in dilute saline solution. The ^{99m}Tc complexes of amino acid analogues were added to 425 μL of male AB type human serum and incubated at 37^{0}C for 48 h then analyzed by ITLC-SG to assess any dissociation based on the R_f. Percentage of free pertechnetate at a particular time was

determined using saline and acetone as mobile phase, represents percentage dissociation of the complex at particular time in serum.

In tumor cell both the ligands were found intact even after 6 hrs and no other polar metabolite compound was found in hplc chromatograms.

2.5.2. Cell Binding Studies

The specificity of ^{99m}Tc[Met-ac-TE3A] to bind with tumor cells was examined by receptor binding assays. U-87MG was grown in normal DMEM, 10% and 5% FBS, respectively. Monolayer cultures of the cell lines were washed with HBSS and were then incubated for 2 h at 37 ^{0}C prior to the experiment. Binding experiments was conducted at 37^{0}C. Further these cells were incubated for 30 min with increasing concentrations (0.01nM-10 μM) of ^{99m}Tc[Met-ac-TE3A] in the absence and presence (100-fold excess) of unlabeled methionine to estimate the total binding and nonspecific binding respectively. At the end of each experiment, the cells were washed with cold PBS four times. The cell-associated radioactivity was determined by gamma scintillation counting. Scatchard plot analysis was done. The same procedure was applied for other ligand.

2.5.3. MTT Assay

MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay was used to determine the cytotoxicity. Exponentially growing MCF-7 and U-87MG cells were plated in a 96-well microtitre plate at a uniform cell density of 4000 cells/well, 24 h before treatment. Cells were treated with varying concentrations of drug (μM–mM range) for 2 h and MTT assays were performed at 24, 48, 72 and 96 h post treatment. **After the treatment, negative control and**

treated cells were incubated with MTT at a final concentration of 0.05 mg/mL for 2 h at 37^{o}C and the medium was removed. After cell lyses, formazan crystals were dissolved using 150 μL of DMSO. Optical density of the extracts (150 μL) was measured at 570 nm (reference filter: 690 nm). Percentage of cell viability compared to negative control (mean +/- SD of triplicate cultures) indicated the mitochondrial activity. % of viability = [OD (570 nm - 630 nm) test product / OD (570 nm - 630 nm) negative control] x 100 %

2.6. In Vivo Studies

2.6.1. Blood Kinetics

The blood clearance study was performed in albino New-Zealand rabbits weighing approximately 2.5-3.0 kg after administration of 10 MBq of the ^{99m}Tc labeled compounds in 0.3 ml *via* the ear vein. At different time intervals about 0.5 ml blood samples were withdrawn from the dorsal vein of other ear starting from 5 min to 24 h and radioactivity in the circulation was calculated, assuming total blood volume as 7% of the body weight.

2.6.2. Scintigraphy

Tumor imaging was performed in EAT (Ehrlich ascites tumor) cell line implanted tumor bearing balb/c mice after receiving Intravenous *i.v.* (**tail vein) injections of 100 µL of the labeled conjugate (0.1 mCi activity) of each ^{99m}Tc[Met-ac-TE3A] and ^{99m}Tc[Trp-ac-TE3A]** . SPECT scans were acquired 4, 24, and 48 h post injection.

2.6.3. Biodistribution

The *in vivo* biodistribution was assessed by female balb/c mice (n=3 per group, 20-22g) implanted with EAT tumor in hind leg. Each group received i.v. (tail vein) injections of approximately 100 µCi **(0.1 mCi activity)** of ^{99m}Tc labeled Met-ac-TE3A/Trp-ac-TE3A.

Animals were euthanized at the designated times and selected tissues were removed, weighed, and counted on a gamma counter to determine ^{99m}Tc-complex distribution. Uptake of the radiotracer in each tissue was calculated and expressed as percentage injected dose per gram of the tissue (%ID/g).

3. Result and Discussion

3.1. Synthesis and radiochemistry of Met-ac-TE3A/Trp-ac-TE3A

In an attempt to develop target specific macrocyclic vehicle, two novel ligands Trp-ac-TETA, **6** and Met-ac-TE3A,**7** were synthesized as Scheme-1. The cyclam was first converted into **1** by the reaction of *tert*-butylbromoacetate on 1,4,8,11-tetraazacyclotetradecane in 75% yield using a modified procedure reported in literature.[16] The slow addition of *tert*-butylbromoacetate at 0^{o}C and purification by column chromatography led to appreciable increase in the final yield. **Methyl-2-(2-chloroacetamido)-4-(methylthio) butanoate (2) was prepared by the reaction of methyl ester of methionine and chloroacetyl chloride in presence of base K_2CO_3 .Similarly methyl-2-(2-chloroacetamido)-3-(1H-indol-3-yl) propionate (3) was also obtained as a colorless oil. These two intermediates were trearted with 1 in presence of base to give tri-tert-butyl-2,2,2-(11-(3-((1-methoxy-4-(methylthio)-)-1-oxobutan-2yl)amino)-2-oxoethyl)-1,4,8,11-tetraaza-cyclotetradecane-1,4,8-triyl)triacetate (4) and tri-tert-butyl-2,2,2-(11-((3-(1H-indol-3-yl)-1-methoxy-1-oxopropan-2yl)amino)-2-oxoethyl)-1,4,8,11-tetraaza-cyclotetradecane-1,4,8-triyl)triacetate (5) ,which were further deprotected to get final product (6) and (7).** ^{1}H NMR spectra of **6** was showing α-H at 4.82-4.87 ppm and heterocyclic ring of tryptophan between 7.08 to 7.52 ppm while for **7** characteristic multiplet of amino acid backbone α-H at 4.58-4.63 ppm and S-CH_3 at 2.11ppm. In the ^{13}C spectrum, the peak at 165

ppm represented the carbonyl carbon of COOH in both **6** and **7** and peak at 80 ppm tertiary carbon disappeared after deprotection of COOH group.

The compounds were radiolabeled with ^{99m}Tc using $SnCl_2$ as reducing agent. All the labeling parameters such as pH, concentration of reducing agent etc were standardized, to achieve the maximum labeling efficiency. *In vitro* and *in vivo* stability of the labeled complexes was checked and the complexes were found to be stable for 24h under physiological conditions. The labeling yield was found to be greater than 95%, as determined by different solvent systems chromatographically. The reaction mixture was kept in saline for various time intervals carried out in *in vitro* stability studies. Percentage radiolabeling was calculated for 0, 2, 4, 6, and 24 h. The metal binding assay confirmed the radiochemical purity to be >99%. Radiochromatogram showed a single peak for both the ligands which was found intact over 6 hrs.

3.2. pH-Potentiometric Studies

The metal complexes of macrocyclic ligands must be chemically stable under physiological conditions to ensure a desired biodistribution of the conjugates. This is also important to avoid the release of potentially toxic metal ions and transchelation of endogenous metal ions. Thus, such complexes should show high thermodynamic stability as well as strong kinetic inertness to dissociation, and the latter is generally favored in macrocyclic ligand. The prerequisite condition for synthesized ligands **6** and **7** to be used as Cu based PET radiopharmaceuticals and possible agents for lanthanide complexes in MR and other optical uses; it should form a highly stable complex with these metals *in vitro*. The values of the overall protonation constants and stability

constants of their copper and europium complexes are presented in (Table 1 and 2) respectively and compared with other known vehicles DTPA and DOTA.

As anticipated, first and second protonation constants of the ligand were high, *viz*. log K_1 and log K_2 were 10.40 and 8.1 for Trp-ac-TE3A and 10.25 and 8.80 for Met-ac-TE3A. In general these two protonation constant are assigned to two trans group of macrocyclic backbone. Only mononuclear complexes could be found with copper, something that is understandable in view of the metal-to ligand ratio of 1:1 that was used. The metal ligand ratios were 2:1, 1:1 and 1:2. Titration for each ratio was carried out at least three times. It was likely that the presence of an excess of metal ions would lead to the formation of dinuclear complexes, but low stability constants of such species diminishes such possibility.

The values of the stability constants with copper was high for both the ligands and differs from those determined for Zn^{2+} and Eu^{3+} ions (Table 2). The values are comparable with those found for other similar ligands and are mainly determined by overall basicity of the nitrogen atoms (pK_1+pK_2). The detail natures of species are also represented in species distribution curve (**Supplementary figure 3 and 4**).

3.3.In vitro studies

3.3.1. In vitro human serum stability assay

The stability of the amino acid conjugates were assessed in human serum with ^{99m}Tc labelled molecules. ^{99m}Tc methionine was showing major transcomlexation, 66% while 58% transcomplexation was observed in ^{99m}Tc tryptophan after 1h. ^{99m}Tc[Met-ac-TE3A] showed 2.5% transcomplexation in serum at 1h. It was stable up to 24 h, as only 3.5% of the radiolabeled

drug dissociated in serum at 24 h. ^{99m}Tc [Trp-ac-TE3A] showed only 2.7% transcomplexation at 1h. ^{99m}Tc [Trp-ac-TE3A] was also found to stable up to 24 h, as only 4% radiolabeled drug dissociated in serum at 24 h similar to ^{99m}Tc[DTPA-bis(methionine)][14]. These results validated the use of these ligands for further analysis as below 5-6 % transcomplexation is found permissible for quantification studies.

3.3.2 Cell Binding Studies

The ability of both the conjugates ^{99m}Tc[Trp-ac-TE3A] and ^{99m}Tc[Met-ac-TE3A] to bind LAT1 transporters on the surface of tumor cell line U-87MG was examined by competitive binding assay (Figure 1). Nonspecific binding was determined by using 100-fold excess of unlabeled methionine/tryptophan. Scatchard analysis showed relatively good binding affinity for ^{99m}Tc[Met-ac-TE3A] (K_d= 0.106 nm) in comparison to ^{99m}Tc[Trp-ac-TE3A] (K_d= 0.145 nm). K_d value were find below 0.1 nM for Met-ac-TE3A(0.09nM) and was slightly higher for Trp-ac-TE3A(0.12nM).Though the binding data were find very appropriate for both ligand but in comparison Met-ac-TE3A was found better for application.

3.3.3. MTT Assay

The cell viability was determined by using MTT assays on MCF-7 breast adenocarcinoma cells and U87 glioma cells at different concentrations (μM to mM range).**The range of concentrations were determined on the basis of our previous studies for such vehicle based ligand system for SPECT [14-16].** Significant toxicity was not observed at lower

concentrations for Met-ac-TE3A , which showed concentration-dependent cytotoxicity. It was observed that at a concentration of 0.1 mM Met-ac-TE3A at 2 h treatment resulted in lyses of 40% of U-87MG cells. Vice versa 10 μM of the same when incubated with MCF-7 cell line for only 2 h showed 16% cells death (**Supplementary figure 5**). In case of Trp-ac-TE3A, 7% and 25% cells were killed when incubated with U87 and MCF-7 cell line respectively at 0.1mM concentration. Trp-ac-TE3A was not showing any significant toxicity at lower concentrate against both the cell lines (**Supplementary figure 6**).

3.4. In Vivo Studies

3.4.1. Blood Kinetics

The blood clearance profile of both ^{99m}Tc [Met-ac-TE3A] and ^{99m}Tc [Trp-ac-TE3A] showed high uptake initially with the diagnostically useful target-to-nontarget ratio. The blood clearance of both the radiocomplex in rabbits followed biphasic trend with a rapid clearance at initial phase and a slow clearance in second phase. The biological half-life of ^{99m}Tc [Met-ac-TE3A] was obtained to be $t_{1/2}$ (fast): 2 h; $t_{1/2}$ (slow): 14 h and 30 min. Almost similar trend was exhibited by ^{99m}Tc [Trp-ac-TE3A]. Only 16% activity persisted in circulation after 1h post administration which reduced to 0.82 % by 24h. The biological half life ^{99m}Tc[Trp-ac-TE3A] was found to be $t_{1/2}$ (fast) 1h 15 min and $t_{1/2}$ (slow) 22 h 15 min (Figure 2).This showed relatively more retention initially when compared with acyclic system ^{99m}Tc [DTPA-bis(methionine)][14],which showed $t_{1/2}$ (fast) 36 min. This reflects the additional use of this system in those tumors where tracer take time to reach because it gives more time to quantify activity initially compare to acyclic system.

3.4.2. Scintigraphy

The in vivo scintigraphy studies were carried out in EAT (Ehrlich ascites tumor) cell line implanted tumor bearing balb/c mice by imaging the animals at 4 h p.i. The mice injected with ^{99m}Tc [Met-ac-TE3A] exhibited uptake in tumor with a gradually increasing trend reaching maximum in 4 h while it was relatively low for ^{99m}Tc[Trp-ac-TE3A] (Figure 3). The possible explanation is that methionine derivative has very high affinity as compared to tryptophan derivative. Blocking studies was also performed to see the selectivity of these vehicles by using unlabeled methionine and tryptophan (1mg/kg and 3 mg/kg) for respective ligands. Figure 4 showed that on blocking the uptake decrease by 60 % by 1mg/kg unlabeled methionine and decreases around 75 % with the use of 3mg/kg for ^{99m}Tc [Met-ac-TE3A], **which demonstrate also the stability of this compound also because the metabolized and subpart of this molecules are not able to show such amount of selectivity.** In case of ^{99m}Tc[Trp-ac-TE3A] it decrease only 25 %,which itself express that trotophan analogue has low selectivity for tumor cells. **This blocking study explains the difference for in *vitro* and *in vivo* results of these two compounds that the affinity may be the similar for both but the change in selectivity may play major role for uptake after a substantial time period after injection.**

3.4.3. Biodistribution

Biodistribution of ^{99m}Tc [Met-ac-TE3A] revealed high tumor uptake in the EAT tumor bearing mice as compared to ^{99m}Tc [Trp-ac-TE3A]; tumor to muscle ratio reached maximum at 4 h post injection in the both the cases. The concentration for both the radiolabeled compound was

rapidly cleared from blood confirmed by the data of blood kinetic study. Biodistribution data of both the compounds showed low accumulation of radioactivity in the stomach which precludes the presence of free pertechnetate, indicative of the *in vivo* stability of radiotracers. However both the compound showed very high accumulation in liver, related to the positive net charge of the complex. **Significant accumulation of radioactivity was found in the kidney (6.1 ± 0.25 %ID/g at 1 h and 1.3 ± 0.12 %ID/g at 24 h after injection),which indicated renal route as a possible way for excretion**. ^{99m}Tc [Met-ac-TE3A] and ^{99m}Tc [Trp-ac-TE3A] showed maximum tumor uptake at 4h (Figure 5 and 6). **The modest accumulation of radioactivity was also observed in non target organ *i.e.* heart, spleen and stomach.**

4. Conclusion

We have designed and developed possible biocompatible probes which can diagnose the tumor non-invasively and also provide a platform for the further development of multimodal imaging tools. Two novel amino acid based ligands Met-ac-TE3A and Trp-ac-TE3A were successfully synthesized and well characterized through spectroscopic techniques. In the preliminary studies they have shown good prospect as SPECT agent with ^{99m}Tc , which may extrapolated for ^{64}Cu and ^{67}Cu. The high stability of the compound with metal ions Eu^{III} and Cu^{II} further expands its possible application from diagnosis to therapy as well as MR agent.

5. Acknowledgement

We thank Dr. R. P. Tripathi, Director INMAS, for providing necessary facilities. The work was supported by Defence Research and Development Organization, Ministry of Defence, under R&D project INM -311.

6. References

1. Huang C and McConathy J (2013) J. Nucl. Med. 54: 1007-10

2. Isselbacher KJN (1972) Engl. J. Med. 286: 929–33.

3. Isselbacher KJN (1972) Proc. Natl. Acad. Sci. U.S.A, 69: 585–89.

4. Miyagawa T, Oku T and Uehara H (1998) J. Cereb, Blood Flow Metab, 18: 500–09

5. Comar D, Cartron JC, Maziere M, Marazano C, (1976) Eur. J. Nucl. Med. 1: 11–14.

6. La°ngstrom B, Antoni G, Gulberg P, (1987) J. Nucl. Med. 28: 1037–40.

7. Ishiwata K, Vaalburg W, Elsinga PH, Paans AMJ, Woldring MG, (1988) J. Nucl. Med. 29, 1419–27.

8. Chung JK, Kim K, Kim SK, Lee YJ, Paek S, Yeo JS, Jeong JM, Lee DS, Jung HW, Lee MC, (2002) Eur. J. Nucl. Med. Mol. Imaging. 29: 176–82.

9. Singhal T, Narayanan TK, Jain V, Mukherjee J, Mantil J (2008) Mol. Imaging Biol. 10: 1–18.

10. Higashi K, Clavo AC, Wahl RL (1993) J. Nucl. Med. 34: 773–79.

11. Munn DH, Mellor AL (2004) Trends Mol. Med. 10: 15–18.

12. Uyttenhove C, Pilotte L, Theate I, (2003) J. Benoit, Nat. Med. 9: 1269–74.

13. Mishra A, Pfeuffer J, Mishra R, Engelmann J, Mishra AK, Ugurbil K, Logothetis NK(2006) Bioconjug Chem. 17:773-80.

14. Hazari PP, Shukla G, Goel V, Chuttani K, Kumar N, Sharma R, Mishra AK, (2010) Bioconjugate Chem. 21: 229–39.

15. Kadiyala KG, Datta A, Tanwar J, Adhikari A, Kumar BS, Chuttani K, Thirumal M, Mishra AK (2014) Pharm Res. DOI 10.1007/s11095-014-1509.

16. Uppal JK, Hazari PP, Raunak, Chuttani K, Allard M, Kaushik NK, Mishra AK.(2011) Org Biomol Chem. 9:1591-9.

Figure Legend

Fig 1: Scatchard plot for Met-ac-TE3A and Trp-ac-TE3A

Fig 2: Blood clearance profile of ^{99m}Tc [Met-ac-TE3A] and ^{99m}Tc [Trp-ac-TE3A] administered through ear vein in normal rabbit

Fig 3: Whole-body γ image of balb/c mice with subcutaneous EAT tumor above the right hind leg at 4h after *i.v.* injection of ^{99m}Tc [Met-ac-TE3A] and ^{99m}Tc [Trp-ac-TE3A].

Fig 4: Blocking studies for Met-ac-TE3A and Trp-ac-TE3A

Fig 5: Tissue distribution data (% ID/g Organ) of ^{99m}Tc [Met-ac-TE3A] in at 1h, 2 h, 4h and 24 h after *i.v.* administration in tumor bearing mice (n = 3).

Fig 6: Tissue distribution data (% ID/g Organ) of ^{99m}Tc [Trp-ac-TE3A] in at 1h, 2 h, 4h and 24 h after *i.v.* administration in tumor bearing mice (n = 3).

Tables legends

Table 1: Protonation constants (log β ± SD, n = 3) of the ligands DTPA, DOTA and Trp-ac-TE3A/ Met-ac-TE3A at 25 °C

Table 2: Stability constants (log β ± SD, n = 3) of the ligands DOTA and Trp-ac-TE3A and Met-ac-TE3A with the metal ions Eu^{III} and Zn^{II} and Cu^{II} (0.10 M KCl, at 25 °C).

Scheme-1 Synthesis of Met-ac-TE3A/Trp-ac-TE3A

Table 1

Protonation constants (log β ± SD, n = 3) of the ligands DTPA, DOTA and Trp-ac-TE3A/ Met-ac-TE3A at 25 °C.

Protonation constants	**DTPA**[30a]	**DOTA**[30a]	**Trp-ac-TE3A**	**Met-ac-TE3A**
	0.1 M KCl	0.1 M KCl	0.1 M KCl	0.1 M KCl
$\log K_1^H$	10.48	11.14	10.40	10.25
$\log K_2^H$	8.60	9.69	8.10	8.80
$\log K_3^H$	4.28	4.84	4.10	4.00
$\log K_4^H$	2.6	3.9	2.65	2.52
$\log K_5^H$	2.0	-		–

Table 2

Stability constants (log β ± SD, n = 3) of the ligands DOTA and Trp-ac-TE3A and Met-ac-TE3A with the metal ions Eu^{III} and Zn^{II} and Cu^{II} (0.10 M KCl, at 25 °C).

Stability Constant	**DOTA**[30b] 0.1 M KCl	**Trp-ac-TE3A** 0.1 M KCl	**Met-ac-TE3A** 0.1 M KCl
$\log \beta_{EuL}$	24.5	18.9	17.6
$\log \beta_{ZnL}$	18.1	16.8	15.3
$\log \beta_{CuL}$	-	20.4	19.2
$\log \beta_{Cu2L}$	22.2	6.2	5.1

*Calculated in separate reaction condition

Fig 1: Scatchard plot for Met-ac-TE3A and Trp-ac-TE3A

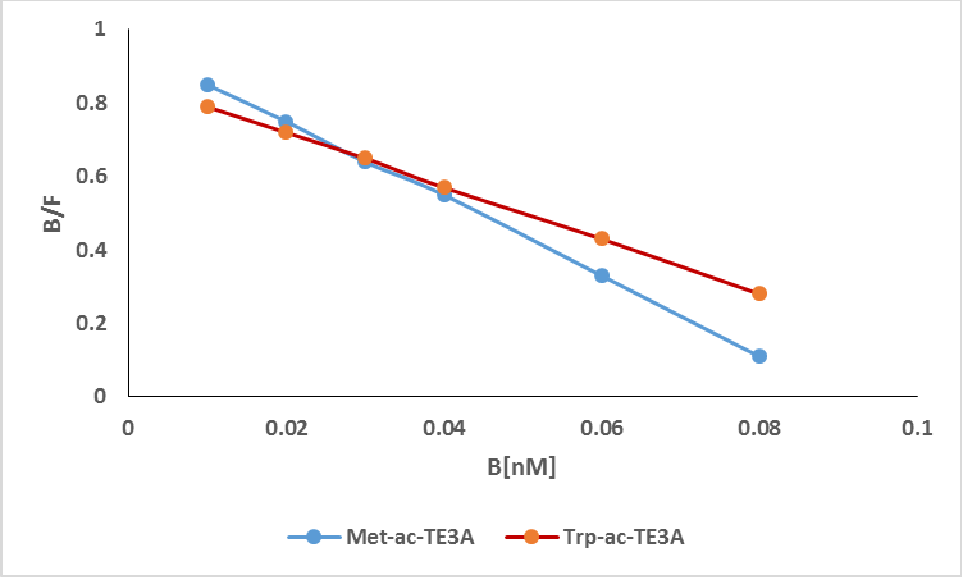


Fig. 2 Blood clearance profile of ^{99m}Tc [Met-ac-TE3A] and ^{99m}Tc [Trp-ac-TE3A] administered through ear vein in normal rabbit.

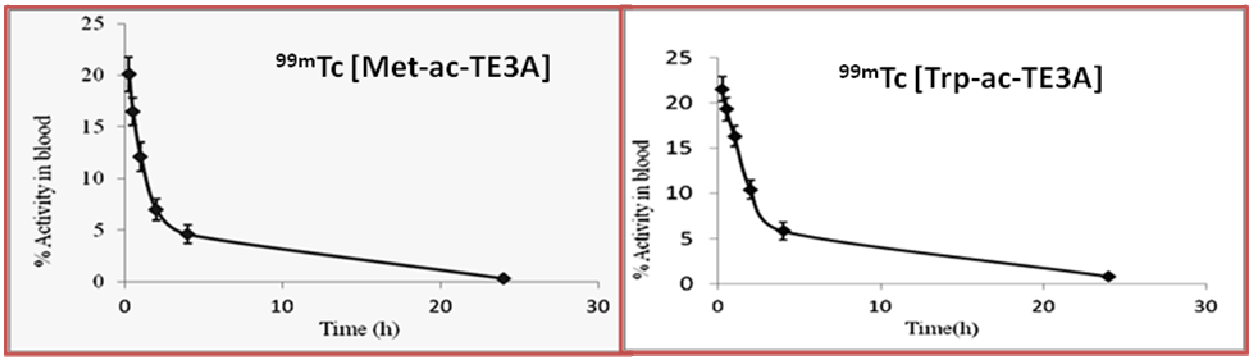
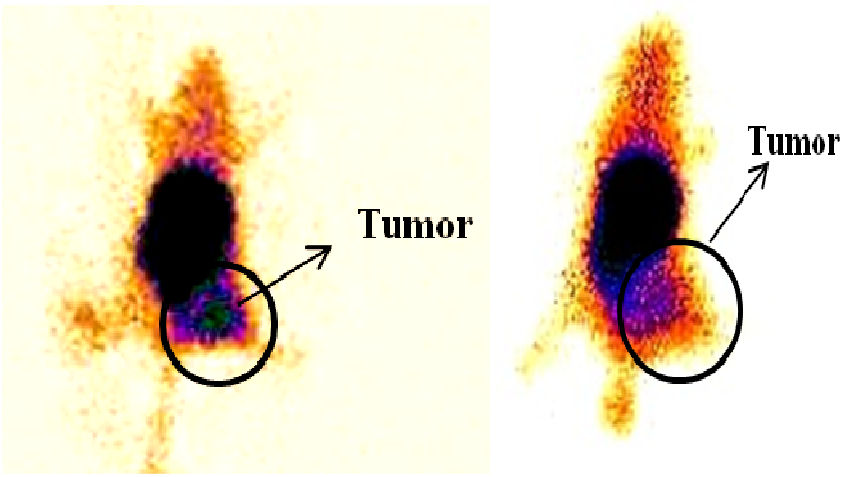


Fig.3 Whole-body γ image of balb/c mice with subcutaneous EAT tumor above the right hind leg at 4h after *i.v.* injection of ^{99m}Tc [Met-ac-TE3A] and ^{99m}Tc [Trp-ac-TE3A].



^{99m}Tc [Met-ac-TE3A] ^{99m}Tc [Trp-ac-TE3A]

Fig 4: Blocking studies for Met-ac-TE3A and Trp-ac-TE3A

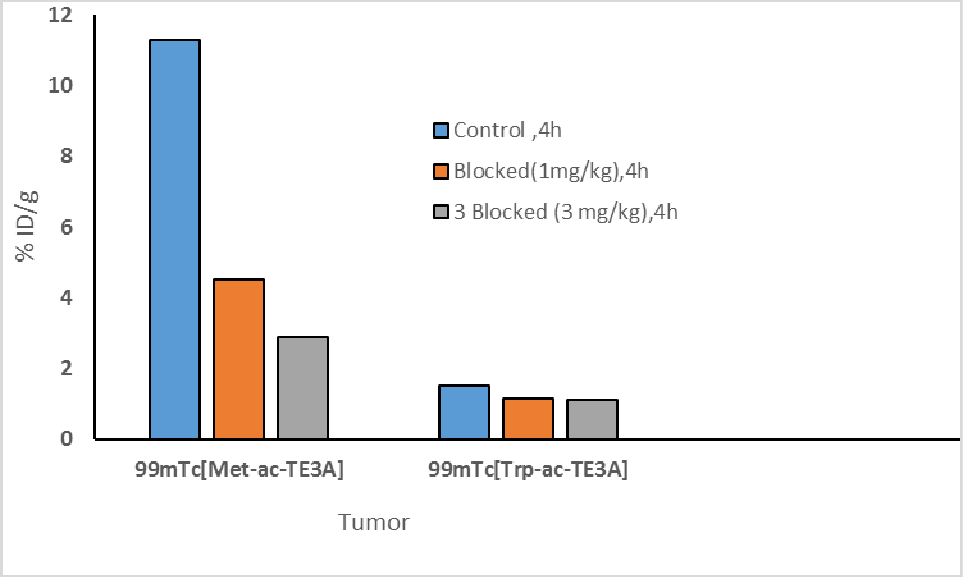


Fig. 5 Tissue distribution data (% ID/g Organ) of ^{99m}Tc [Met-ac-TE3A] in at 1h, 2 h, 4h and 24 h after *i.v.* administration in tumor bearing mice (n = 3).

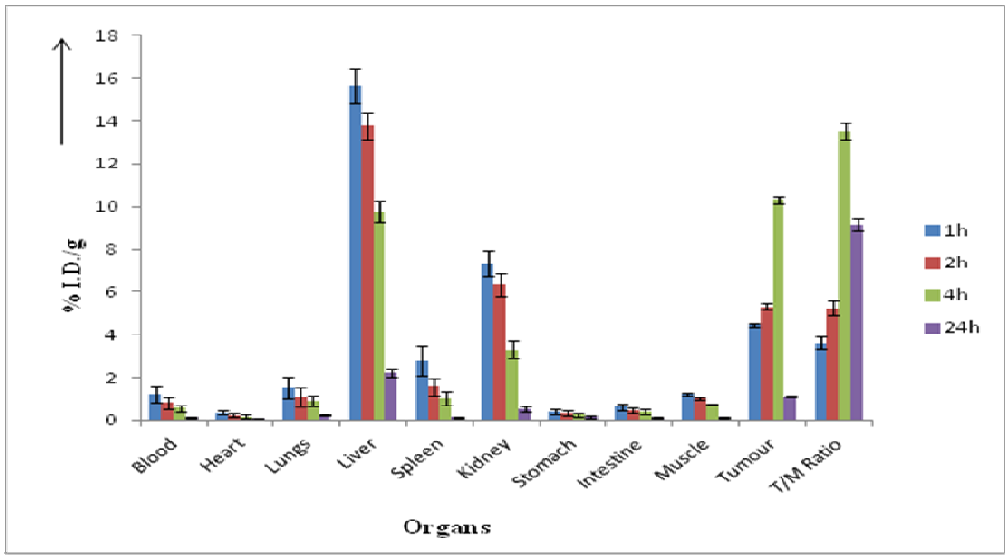
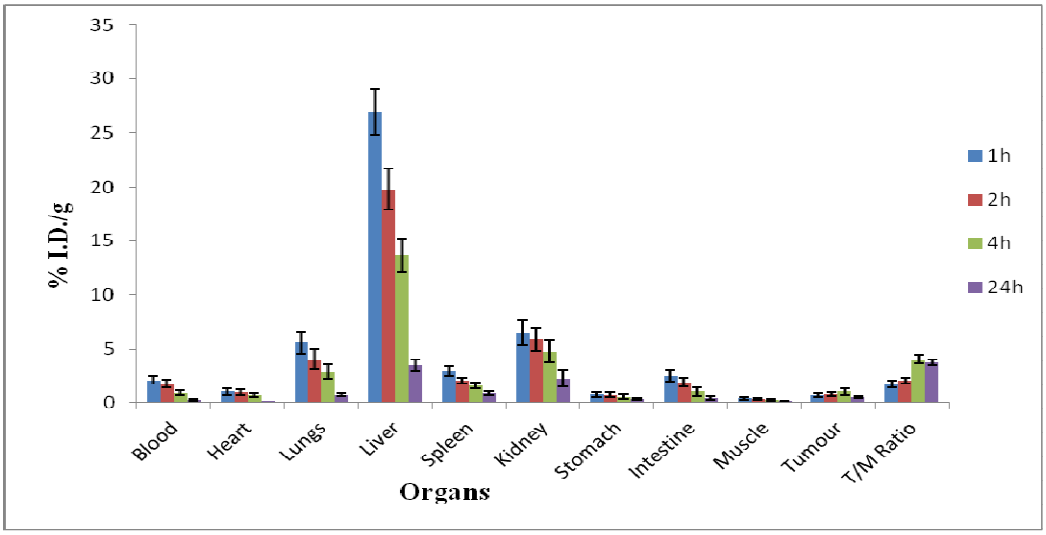
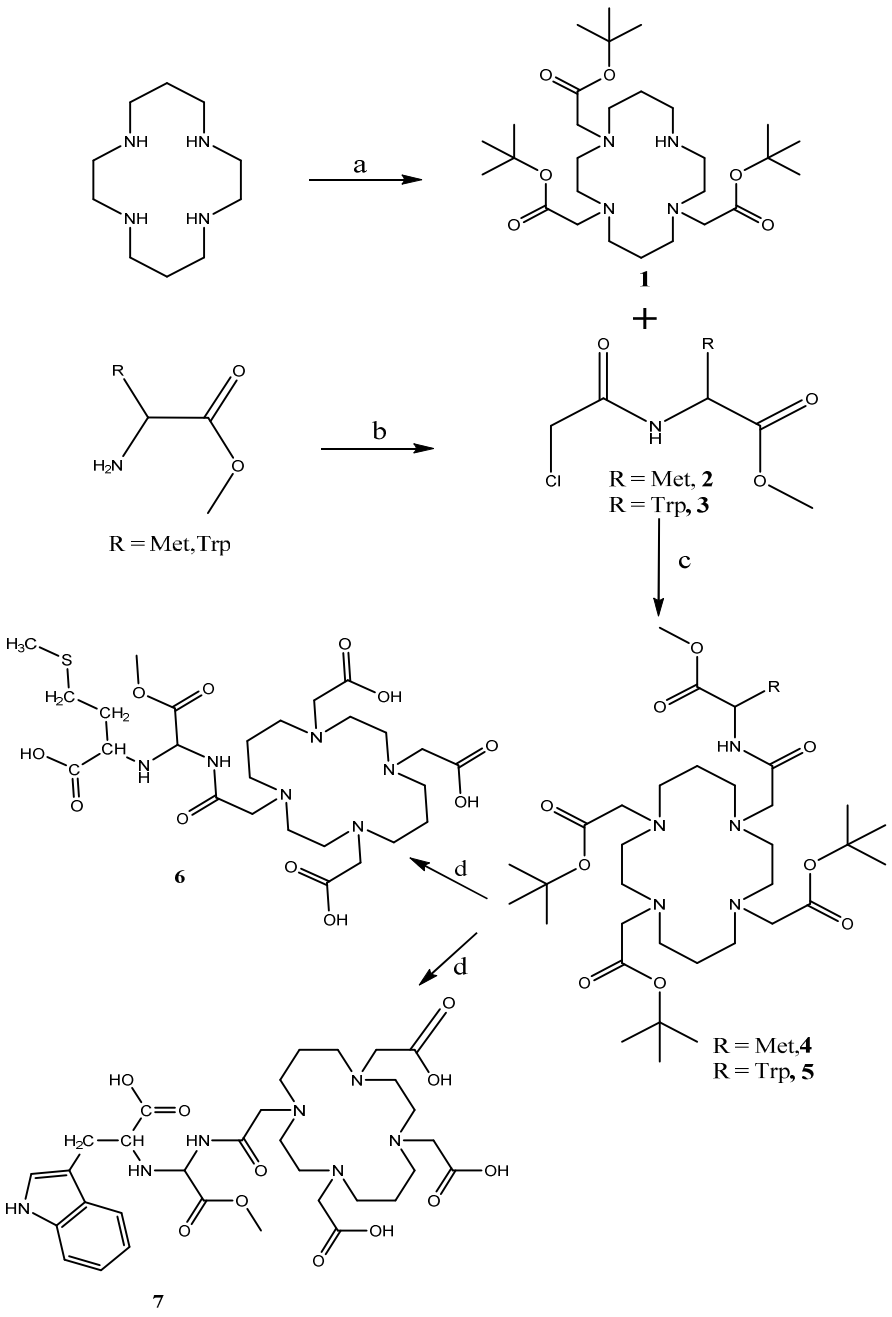


Fig. 6 Tissue distribution data (% ID/g Organ) of ^{99m}Tc [Trp-ac-TE3A] in at 1h, 2 h, 4h and 24 h after *i.v.* administration in tumor bearing mice (n = 3).





Scheme-1 Synthesis of Met-ac-TE3A/Trp-ac-TE3A

Reagents and conditions: General synthetic pathway of Met-ac-TE3A and Trp-ac-TE3A. a) K_2CO_3, tBu-bromoacetate,ACN, 65-70^{0}C b) chloroacetyl chloride, K_2CO_3, CH_2Cl_2:H_2O, RT c) K_2CO_3, ACN, 65-70^{0}C d) TFA, RT.

Supplementary Figures

Evaluation of Methionine and Tryptophan derivatised vehicles: Met-ac-TE3A/Trp-ac-TE3A for tumor imaging

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Supplementary Fig. 1 Analytical HPLC chart of **6**.

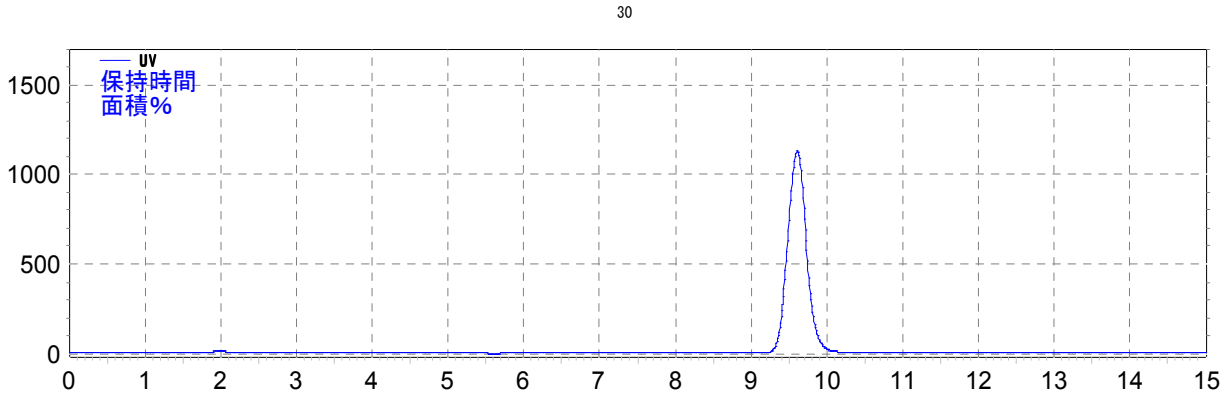
Supplementary Fig. 2 Analytical HPLC chart of **7**.

Supplementary Fig. 3 Species distribution diagram for the Cu^{II} complex of Met-ac-TE3A at $C_M=C_L=1\times10^{-3}$ M.

Supplementary Fig. 4 Species distribution diagram for the Cu^{II} complex of Trp-ac-TE3A at $C_M=C_L=1\times10^{-3}$ M

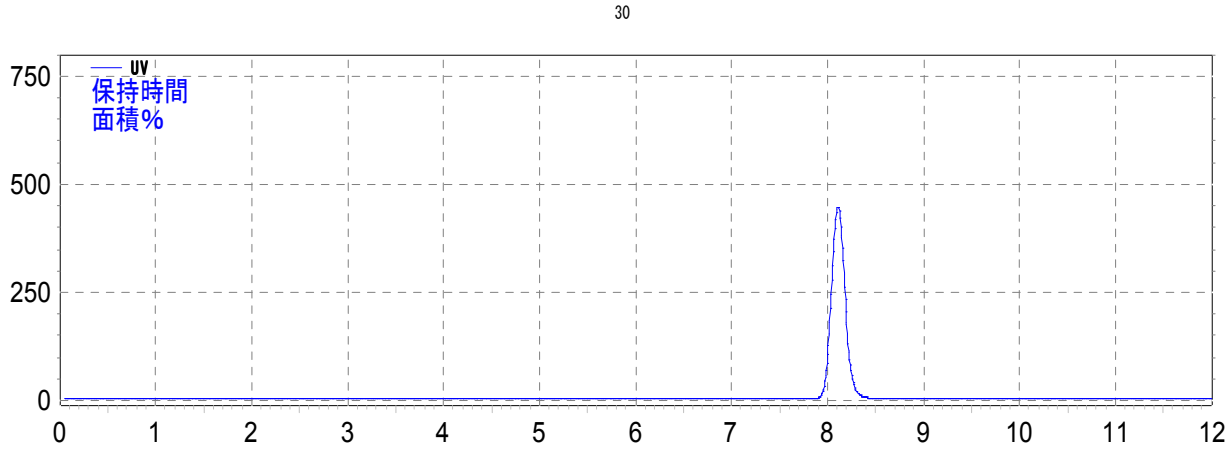
Supplementary Fig. 5 Cytotoxicity of Met-ac-TE3A conjugate in U-87MG and MCF-7 cell lines.

Supplementary Fig. 6 Cytotoxicity of Trp-ac-TE3A conjugate in U-87MG and MCF-7 cell lines.



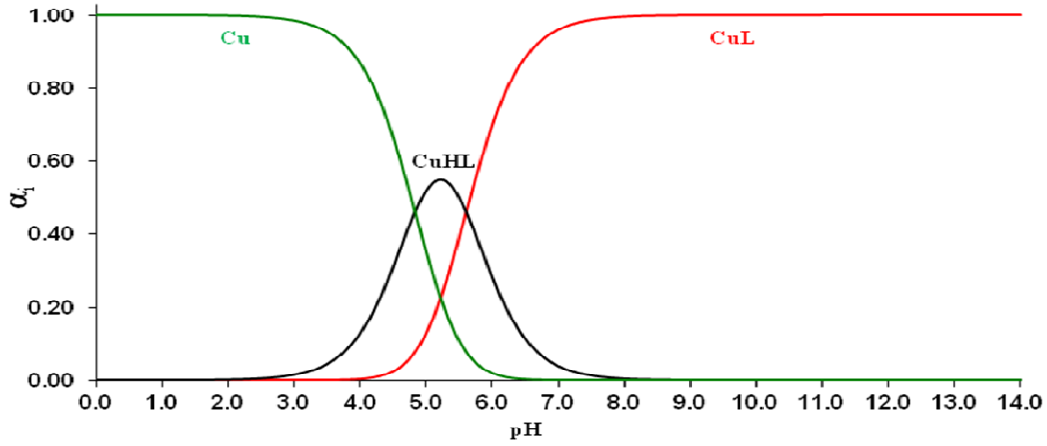
Supplementary Fig. 1 Analytical HPLC chart of **6**.

Chemical purity: > 99% by HPLC (Capcell Pack UG80 C_{18} column (4.6 mm i.d. × 250 mm). MeCN/H_2O/ Et_3N, 75/25/0.01 (v/v/v), flow rate = 1.0 mL/min, λ_{uv}= 254 nm); retention time (t_R) = 9.7 min.

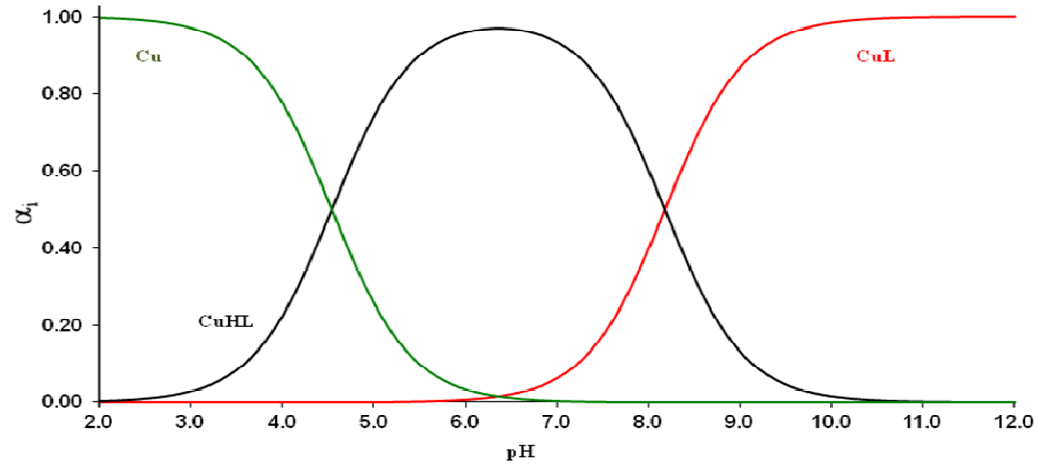


Supplementary Fig. 2 Analytical HPLC chart of **7**.

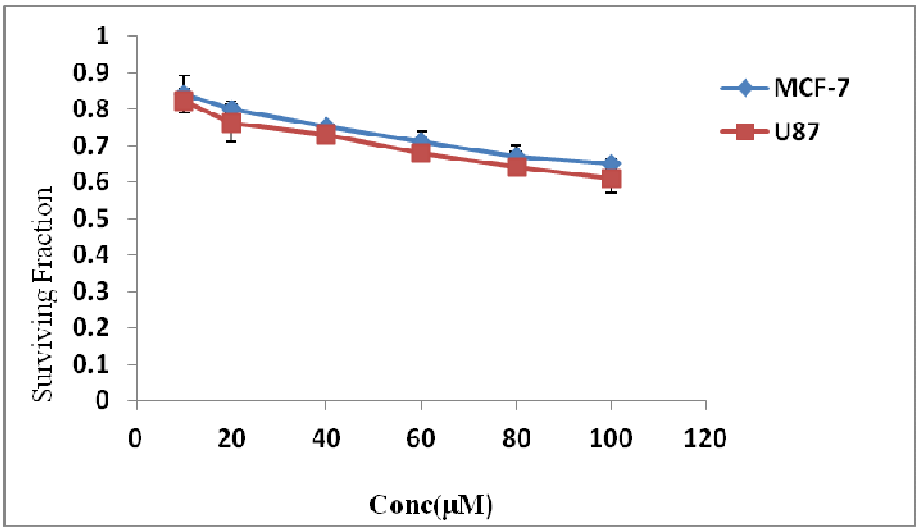
Chemical purity: > 99% by HPLC (Capcell Pack UG80 C_{18} column (4.6 mm i.d. × 250 mm). MeCN/H_2O/ Et_3N, 80/20/0.01 (v/v/v), flow rate = 1.0 mL/min, λ_{uv}= 254 nm); retention time (t_R) = 8.1 min.



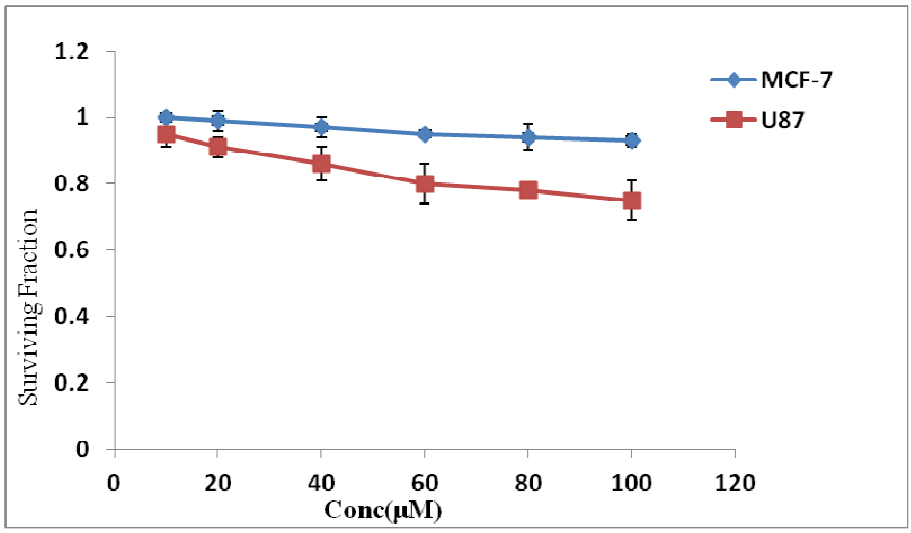
Supplementary Fig. 3 Species distribution diagram for the Cu^{II} complex of Met-ac-TE3A at $C_M=C_L=1\times10^{-3}$ M.



Supplementary Fig. 4 Species distribution diagram for the Cu^{II} complex of Trp-ac-TE3A at $C_M=C_L=1\times10^{-3}$ M.



Supplementary Fig. 5 Cytotoxicity of Met-ac-TE3A conjugate in U-87MG and MCF-7 cell lines.



Supplementary Fig. 6 Cytotoxicity of Trp-ac-TE3A conjugate in U-87MG and MCF-7 cell lines.

Chemicals

1,4,8,11-Tetraazacyclotetradecane (cyclam), 2-chloroacetylchloride, *tert*-butylbromoacetate, trifluoroacetic acid, methyl ester of L-methionine and L-tryptophan, stannous chloride, potassium carbonate, sodium sulphate, sodium chloride, acetonitrile, chloroform, dichloromethane, methanol and water were purchased from Sigma-Aldrich Co, USA. All solvents used were of analytical grades. For reactions to be performed under dry conditions, solvents were dried by the usual reported laboratory procedures. Column chromatography was carried out using silica MN60 (60-200 μm) and TLC was run on silica gel coated aluminium sheets (Silica gel 60 F_{254}, Merck, Germany) and visualized in UV light 254 . Radiocomplexation and radiochemical purity were checked by instant thin layer chromatography (ITLC).

Instrumentation

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II 400 MHz system (Ultra shield, Switzerland) using the solvent peak as internal reference. Mass spectra (ESI-MS in positive and negative ion mode) were performed on Agilent 6310 system ion trap (USA). The clinical scintigraphs were obtained using a rectangular large field of view gamma camera (HAWKEYE dual\head, GE medical systems, Milwaukee, Wisconsin, USA) with a low-energy all-purpose collimator. *In vivo* scintigraphic imaging was performed by γ-camera, Hawkeye Camera (USA), and ex-vivo studies were performed on γ-scintillation counter GRS230, ECIL (India).

HPLC analyses were performed on a Waters Chromatograph efficient with 600s coupled to a Waters 2487 photodiode array UV detector. Radioactive samples were counted using Capintech automated well-type counter.

Statistical Methods. Data is reported as mean standard deviation (S.D.). Data were analyzed by the two-tailed Student's *t* test for comparison with the control. Value of $P < 0.05$ was considered statistically significant.

Cell Culture

Monolayer cultures of human malignant glioma cells, U-87MG (obtained from NIMHANS, Bangalore), and MCF-7 breast adenocarcinoma cells were maintained at 37 °C in a humidified CO_2 incubator (5% CO_2, 95% air) in DMEM (Sigma, USA) supplemented with 10% fetal bovine serum (GIBCO), 50 U/mL penicillin, 50 µg/mL streptomycin sulfate, and 2 µg/mL nystatin. Cells were routinely subcultured twice a week using 0.05% Trypsin (Sigma, USA) in 0.02% EDTA.

Animal Models

In vivo procedures were performed according to the protocol approved by the Institutional Animal Ethics Committee (Regn. No: 8/GO/a/99/CPCSEA). New Zealand rabbits of 2–3 Kg were used for blood kinetics studies. Athymic nude mice (age 7–9 weeks, wt. 20–25 g) were used for imaging and biodistribution studies. Mice and rabbits were housed under conditions of controlled temperature of 22±2°C and normal diet. A U-87MG xenograft model was generated by subcutaneous (s.c.) injection of respective cells in the fore/ hind limb of athymic nude mice which were used three to four weeks after inoculation when the tumor volume was 100–400 mm^3.