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Comparison of BTSE-RGD with DOTA-RGD as Potential Imaging agents for Tumor

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Abstract

RGD and its analogues are very important compounds and can be used as a potential tumor targeting agents. Bisthiosemicarbazone conjugated RGD (BTSE-RGD) and DOTA-RGD have been prepared by using chemical strategy based on peptide synthesis and chemoselective ligations. BTSE-RGD comprises two domains, first as a tumour selective' domain and the other a chelating vehicle for conjugation of radioisotope. Both the compound were synthesized and labelled with ^{99m}Tc and radiochemically analysed by HPLC method. The stability of radiconjugate was checked in the presence of human serum at 37°C up to 8 h. Labeling yield of 96.8 ± 0.32% was obtained corresponding to a specific activity in the range of 36-89 MBq/µmol for BTSE-RGD. The BTSE-RGD conjugate was examined *in vitro* for its ability to bind with αvβ3 receptor. The functionalized BTSE-RGD displayed a binding affinity toward αvβ3 integrin (31.9 ± 6.8 nM) many fold better than the DOTA-RGD. BTSE-RGD showed slower distribution half life (T1/2α) and elimination half-life (T1/2β) (65 ± 0.001 min and 21h 15 min ± 0.001 min) in comparison to 99mTc- DTPA-RGD (T1/2α 18 ± 0.001 min and T1/2β 9h 10 min ± 0.005 min).Biodistribution study showed better tumor to muscle ratio which reaches maximum around 3.5 (% ID) for BTSE-RGD in 2h while for DTPA-RGD maxima was 13.60 at 24h.

Keywords- Bisthiosemicarbazone, SPECT, RGD, Tumor and Biodistribution

Introduction

Integrin are very important family of transmembrane glycoproteins made from 19 α- and 8 β-subunits. These integrins are expressed in various α/β heterodimeric combinations on the cell surface and are important during tumor invasion, angiogenesis, immune dysfunction and infection by pathogenic microorganisms.(1-5) The most extensively used integrin is $\alpha_v\beta_3$, a receptor for extracellular matrix proteins with the arginine-glycine-aspartic (RGD) tripeptide sequence.(6, 7) Integrin $\alpha_v\beta_3$ is highly expressed in glioblastomas, melanomas, osteosarcomas, and carcinomas of lung and breast.(8-10)

Thiosemicarbazones are versatile bifunctional ligands that can coordinate as neutral or deprotonated form. They are flexible spacers with potential multiple binding sites that can be used to construct coordination polymers with various topologies and multiple dimensions .They can act as mono- or bidentate ligands (11-12) and may used for tumor or other specific purpose when conjugated with specific peptides (13-14).

In last decade, a series of RGD conjugates was analysed for the non-invasive imaging of tumours by targeting $\alpha_V\beta_3$ integrin at molecular level (15-17). Further structural modification for improving tumor-targeted imaging and elucidating its mechanism is currently underway. All these should facilitate the discovery and development of novel tumor-targeted imaging and therapeutic agents.All four techniques were used including magnetic resonance imaging (MRI), single photon emission tomography (SPECT), positron emission tomography (PET), and optical imaging with the help of bifunctional vehicle. In this line DTPA, DOTA and DO3A has been already exploited, therefore it was worthfull to use BTSC as a vehicle with RGD to see the efficacy for imaging.

The work presented here describes initial efforts towards the synthesis of optimum condition for radiolabeling of conjugate with ^{99m}Tc using (BTSE-RGD) as coligands. In addition, we studied *in vitro* binding affinity and stability in human serum and *ex vivo* tumor uptake and tissue biodistribution of radiolabeled compound and finally compare with our previous macrocyclic conjugate DTPA-RGD(18-19).

Results and Discussion

The complete synthetic process is mentioned in scheme 1 and 2. For bifunctional bis(thiosemicarbazone) proligands, the synthesis proceeds *via* isolation of the corresponding Monoketo-thiosemicarbazone species, which facilitates the construction of unsymmetrical systems. Compound **1** was synthesised in 70% yield as a white microcrystalline solid by using standard procedures. The product was prepared from the aqueous reaction mixture at 0 °C using 4 equiv of 2,3-butadione, 4-*N*-methyl-3-thiosemicarbazide and 2 mL of concentrated HCl (36%) to catalyze imine condensation. Excess amount of diketone was required to limit the formation of symmetric bis- (thiosemicarbazone) by products. Further purification was achieved by repeated recrystallization from hot aqueous ethanol.

The RGD peptide derivative, **3** was synthesised by the solid phase method on a rink amide resin, using Fmoc chemistry. The tripeptide Arg-Gly-Asp was synthesised in high yield (>90%). The bis(thiosemicarbazone)-tripeptide (RGD) was obtained in yield of 65-70% (Scheme 2) after HPLC purification. Peptides were characterized by their retention times by analytical RP-HPLC. RGD retention time was R_t = 3.8.The progress of the amino acid coupling was monitored by the colour change of ninhydrin in the Kaiser test. The RGD-succinic anhydride was synthesised by anchoring the RGD by adding succinic anhydride in DMF and its mass was confirmed using

ESI-MS analysis. Then RGD-succinic anhydride conjugate hence obtained was further coupled with bis(thiosemicarbazone) to achieve the desired conjugate for further analysis and application. After synthesis compound was radiolabeled with ^{99m}Tc using $SnCl_2$ as reducing agent. The radiolabeled purity was estimated chromatographically using instant thin-layer chromatography (ITLC- SG) strips as the stationary phase and acetone as the mobile phase. The radiolabeling yield 96.8 ± 0.32% was obtained corresponding to a specific activity in the range of 36-89 MBq/μmol (n = 5) as shown in supplementry table 1 .

Binding nature towards plasma protein was analysed by size exclusion chromatography. In general, low protein binding is must in order to ensure adequate pharmacokinetics of the radiopharmaceuticals. Relatively low protein binding of 18.1± 4.1% was obtained for ^{99m}TcBTSE-RGD correlating with the high in vitro stability and low lipophilicity of this complex. This value was well comparable to ^{99m}Tc DOTA-RGD.

The BTSE-RGD conjugate was examined *in vitro* for its ability to bind with αvβ3 receptor. The functionalized BTSE-RGD displayed a binding affinity toward αvβ3 integrin (31.9 ± 6.8 nM) comparable to that of the reference ligand DB58 (20.2 ± 1.9 nM) indicating that the conjugation of BTSE to the RGD did not substantially change the affinity towards integrin αvβ3. This value seems many fold better than the DOTA-RGD.

The rapid clearance of BTSE-RGD from the blood stream was reflected in the low blood activity, indicated its high stability against exchange reactions with blood proteins and no long-term retention in organs or tissues. Approximately 78% of activity was cleared within 1h and more than 95% in 4h. The biological distribution half-life ($T_{1/2\alpha}$) and elimination half-life ($T_{1/2\beta}$) of ^{99m}TcBTSE-RGD was found as 65 ± 0.001 min and 21h 15 min ± 0.001. (Figure 2)

The biodistribution data for ^{99m}TcBTSE-RGD in tumor bearing mice were shown in Figure 2. The percentage distribution of drug in various organs of mice is shown as percentage dose per gram (%ID/g) at different time interval. Their organs were harvested and weighed, and their radioactivity was measured in a gamma-counter and counted to determine the percentage of activity incorporated into the organ. ^{99m}TcBTSE-RGD excreted through both renal and hepatobiliary pathways which is indicated by high uptake in both liver and kidneys. High and persistent activity in the kidneys may be attributed to the electrostatic interaction due to overall charge of ^{99m}TcBTSE-RGD with negatively charged renal proximal tubular cells, which is responsible for re-absorption of glomerularly filtered peptide into renal cells (20).Possibily such type of retention may be reduced by modifying the overall charge on the ligand. The liver, kidneys and spleen were the only organs to have higher uptake of ^{99m}TcBTSE-RGD than the tumor. The little accumulation in heart and stomach was observed and negligible accumulation was observed in the brain. The radioactivity uptake in the non target organs was comparatively low. Relatively low background activity was observed after 24 h and ^{99m}TcBTSE-RGD showed maximum tumor uptake at 2 h.

Conclusion

In this study, we have presented a stable BTSE-RGD analogue with intact high affinity for the $\alpha_v\beta_3$ receptor. The phrmacokinetic profile of this compound has been studied through labelling with ^{99m}Tc. The result showed promising aspect under in vitro and in vivo conditions and gives a promsing prospects for visualisation and radionuclide therapy of major human cancers. The comparative studies with DTPA-RGD showed different pharmacokinetics for imaging applications.

Experimental

***Synthesis of DOTA*-RGD**. The synthesis was performed as per our previous work [18]. The coupling of bromoacetylated RGD with trisubstituted cyclen (25 mg) K_2CO_3 (40 mg) in the presence of acetonitrile at 70°C for 7 h in anhydrous condition provide the desired DOTA-RGD-tripeptide. The mixture was dried, washed again with 50% MeOH/H2O (3x), MeOH (3x) and DCM (3x) dried in vacuum.

Deprotection of DOTA*-RGD *from the rink amide resin. Deprotection and cleavage from the resin were carried out using a trifluoroacetic acid mixture (TFA/TIS/H2O/EDA (9.5/0.2/0.2/0.1 v/v/v/v/v/)) for 2 h at room temperature . After deprotection, the solutions were concentrated and the peptides were precipitated by cold diethyl ether. The white precipitate of the peptides was washed with cold diethyl ether. Finally the peptides were purified and separated by preparative RP-HPLC, lyophilized and stored at 4°C.

Synthesis of Diacetyl-2-(4-*N*-methyl-3-thiosemicarbazone) (1)

An aqueous solution (10 mL) of 4-methyl-3-thiosemicarbazide (0.50 g, 4.75 mmol) was treated with 2mL of conc. HCl (36%). The solution was cooled to 10 ˚C. A cooled solution (10 ˚C) of 2,3-butanedione (0.52 mL, 5.92 mmol) in water (10 mL) was added. The mixture was stirred in an ice-bath and a white solid immediately settled out of the solution. The solid was collected by filtration and washed with ice-cold water (5 x 25 mL) and a small amount of diethyl ether to give **1** as cream/white solid. The cream/white solid was recrystallized from warm DMSO/water (0.57 g, 70%). ^{1}H NMR (DMSO-*d6,*400 MHz,): δ (ppm) , 2.00 (3H, s, CH_3C=N). 2.44 (3H, s, CH_3C=O), 3.18 (3H, s, CH_3-NH),3.31(1H,NH), ^{13}CNMR (DMSO-*d6,*100 MHz,) : δ (ppm) , 10.48 (*C*H_3C=N), 25.22 (*C*H_3C=O), 31.66 (*C*H_3NH), 145.97 (C=N), 178.90 (C=S),197.45

(C=O), 197.97(CO). *m/z* (ESI) found: 174.1 ($M+H^+$), 196.0 ($M+Na^+$). $C_6H_{11}N_3OS$ calculated 173.2.

Synthesis of Diacetyl-2-(4-*N*-methyl-3-thiosemicarbazone)-3-(4-*N*-amino-3-thiosemicarbazone) (2)

Thiocarbohydrazide (0.37g, 3.46 mmol) was taken in 80 mL of ethanol and the suspension was stirred at 50 °C. The 4-N-substituted monoketothiosemicarbazone 3a (0.50g, 2.89 mmol) was added in portions over 30 min followed by 5 drops of conc. HCl (36%) and heated to refluxed for 4 h. The mixture was allowed to cool to room temperature, and the cream precipitate was collected by filtration, washed with ethanol (4 x 25 mL) and copious amounts of diethyl ether (5x 25 ml), then dried *in vacuo* to give the corresponding unsymmetrical bis(thiosemicarbazone) proligand in good yield (0.44 g, 80%). ^{1}H NMR (DMSO-*d*6, 400 MHz): δ (ppm) = 2.16 (6H, s, C*H*$_3$C=N), 3.31 (3H, s, C*H*$_3$NH-). ^{13}CNMR (DMSO-*d*6, 100 MHz): δ (ppm) , 12.23 (*C*H$_3$C=N), 12.78 (*C*H$_3$C=N), 31.72 (*C*H$_3$NH), 147.80 (*C*=N), 152.23 (*C*=N), 177.66 (NH$_2$NH*C*=S), 178.96 (CH$_3$NH*C*=S). *m/z* (ESI) found 262.4 (M+H). $C_7H_{15}N_7S_2$ calculated 261.3.

Synthesis of Arg-Gly-Asp (RGD)tripeptide (3)

The resin (200 mg) was swelled in DCM (4 mL) for 60 min, and drained. The Fmoc group was deprotected using a 20% (v/v) solution of piperidine in DMF for 20 min. A mixture of 4.0 equiv of the Fmoc-Asp (otbu)- OH (relative to resin capacity), 4 equiv of HOBt and DIC (relative to the amino acid) and 2 mL of dry DMF was added to the resin, and the mixture was stirred for 2 h. The mixture was washed again with DMF (3x 25mL), MeOH (3x 25mL) and DCM (3x 25mL) and

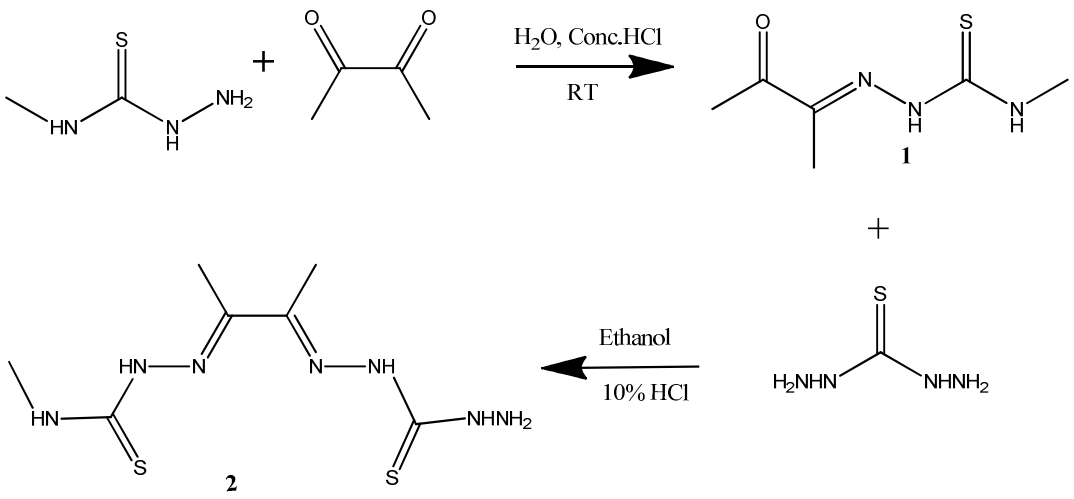
dried *in vacuo*. A mixture of 4 equiv (relative to resin capacity) of the Fmoc-Gly-OH amino acid, 4 equiv of HOBt (relative to the amino acid), 4 equiv of DIC (relative to the amino acid), and 2 mL of DMF was added to the Rink amide resin with the corresponding *N*-deprotected peptide (NH_2-Asp(Otbu)-CONH-Rink amide. The mixture was stirred at room temperature for 2 h. The mixture was dried, washed again with DMF (3x 25mL), MeOH (3x 25mL) and DCM (3x 25mL) and dried *in vacuum*. Finally Fmoc-Arg(Pbf)-OH was coupled to (NH_2-Gly-Asp(Otbu)-CONH-Rink amide by the above mentioned protocol. The mixture was dried, washed again with DMF (3x 30mL), MeOH (3x 30mL) and DCM (3x 30mL) dried in vacuum. The synthesize RGD peptide 3c was characterized by mass spectrometry: RGD; *m/z* = found 346.4 [M^+]; $C_{12}H_{22}N_6O_6$ calculated 346.3. The progress of the amino acid coupling and deprotection of Fmoc group was monitored by the colour change of ninhydrin in the Kaiser test: A few beads of the resin were washed with DCM and mixed two drops of ninhydrin (5 g in 100 mL of EtOH), phenol (80 g in 20 mL of EtOH) and KCN (2 mL, 1 mmol/L in H_2O, pyridine 98 mL). The resulting mixture was heated to 120 ºC for 4-6 min. If primary amino groups are present on the support the beads turn blue (positive test), resin and solution colourless to light yellow, negative test.

Synthesis of RGD succinic anhydride (4)

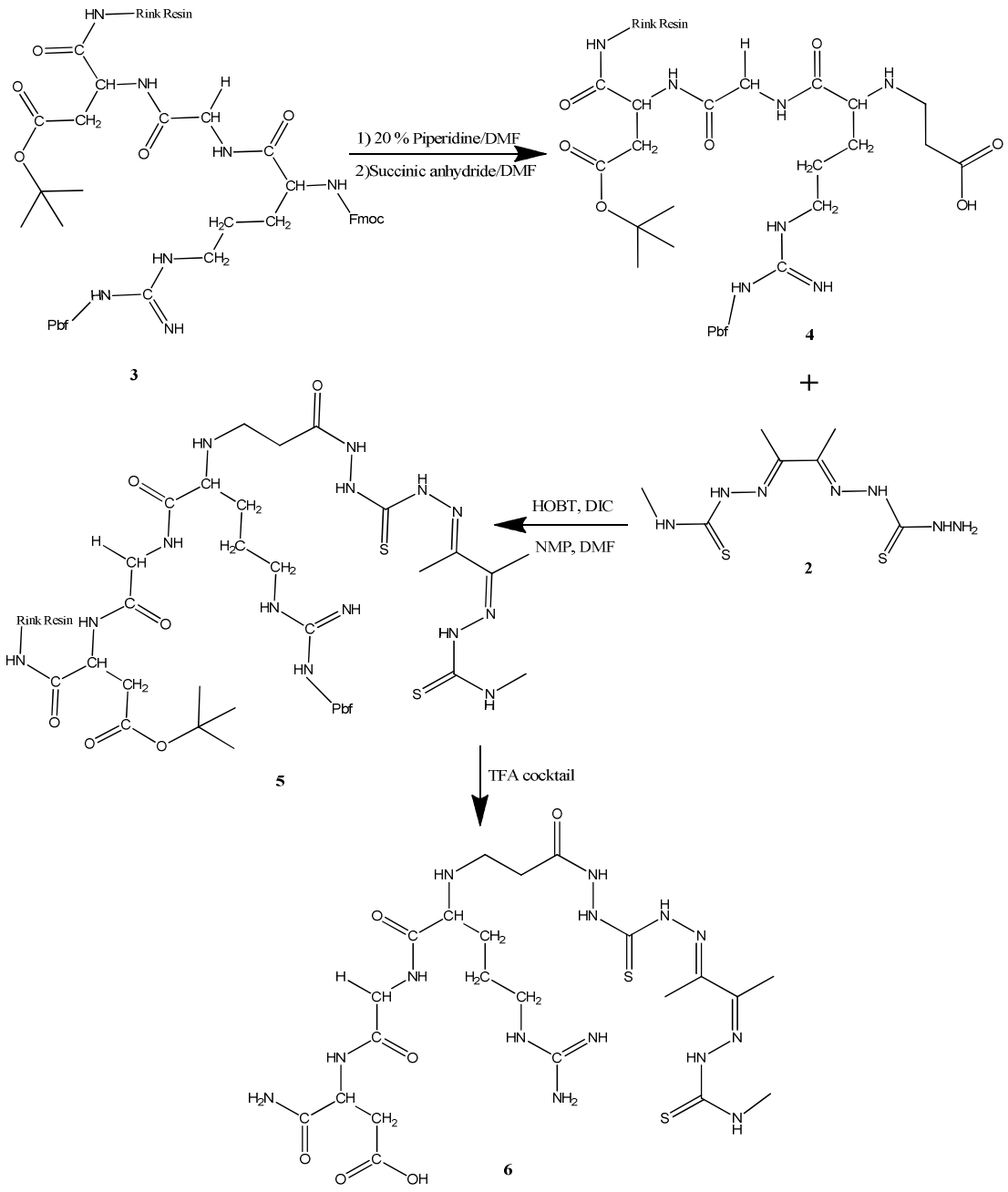
N-terminal Fmoc group was removed from previously synthesised RGD by using 20% (v/v) solution of piperidine in DMF. The RGD-Succinic anhydride was synthesised by anchoring the RGD by adding a mixture of succinic anhydride (5 equiv.) in DMF and stirred for 6h. Deprotection and cleavage from the resin were carried out using a mixture of trifluoroacetic acid (TFA/TIS/H_2O/EDA (9.5/0.2/0.2/0.1/v/v/v/v/) at room temperature. The pure conjugate was obtained as a light yellow solid following the stated protocol as for the RGD peptide. *m/z* (ESI) found 446.6 ($M+2H^+$) . $C_{16}H_{26}N_6O_9$ calculated 444.4.

Conjugation of Bis(thiosemicarbazone) with RGDsuccinic anhydride (6)

The RGD succinic anhydride conjugate (3d) hence obtained was further coupled with diacetyl-2-(4-*N*-methyl-3-thiosemicarbazone (**2**) to achieve the desired ligand **6**. The conjugation was performed by the coupling of the synthesised RGD-succinic anhydride and diacetyl-2-(4-*N*-methyl-3-thiosemicarbazone (5 equiv.) in 1:1 solution of NMP and DMF for 24 h at room temperature. The product anchored on the solid support was obtained by deprotection using a mixture of (TFA/TIS/H_2O/EDA (9.5/0.2/0.2/0.1/v/v/v/v/) at room temperature to obtain a yellow solid 3f after addition of cold diethyl ether. The mass of the pure conjugate was confirmed using ESI-MS analysis. *m/z* (ESI) found 689.4(M^+) . $C_{23}H_{39}N_{13}O_8S_2$ calculated 689.7.



Scheme 1



Scheme 2

Radioabeling with ^{99m}Tc

The DOTA-RGD-tripeptide was labeled with ^{99m}Tc using a volume of 99mTc-ertechnetate-generator eluent to provide 890 MBq/mmol of the peptide in citrate buffer to provide a final concentration of 5.0–5.5 mg/mL.

BTSE-RGD (10.0 μmol) was dissolved in water in a shielded vial and stannous chloride (1.0 μmol, dissolved in N_2 purged 10% acetic acid) was added. The pH of the resulting solution was adjusted to 6.5-7.0 with 0.5 M $NaHCO_3$ solution. The mixture was passed through a 0.22 μm Millipore filters into a sterile vial. Freshly eluted $^{99m}TcO_4^-$ (3.7 MBq) was added, and the complex was incubated for 30 min at room temperature for optimum labeling yield. The labeling efficiency was estimated chromatographically using ITLC-SG strips as the stationary phase and 100% acetone as mobile phase. The paper was cut into 15×120 mm strips. An aliquot (5 μl) of the labeled compound was spotted 1.5 cm from the bottom of the strip. The strip was then placed in a solvent that allowed free ^{99m}Tc to migrate along the solvent front while ^{99m}Tc-labelled complex remained at the origin. After being developed and dried, the strip was cut at one third the distance between the origin and the solvent front. Each part was counted separately in the γ-counter and the first part was considered as ^{99m}Tc- complex. The paper chromatography demonstrated an absence of unbounded $Na^{99m}TcO_4$. The radio colloids were detected in the product by using a cocktail of pyridine/acetic acid/water (3:5:1.5) as developing solvent.

Biological Studies

Animal

All animals were maintained and handled in accordance with recommendations of the institutional guidelines of the Institute of Nuclear medicine and allied sciences (INMAS). These experiments

conducted in INMAS were approved by the Animal Ethics Committee of INMAS. The animals were housed under a 12/12-h dark/ light cycle under optimal conditions. In addition human serums were collected from healthy people with informed consent.

In-vitro Studies

Serum Stability of Complexes

The above freshly prepared technetium radio complex (0.3 Ci) was incubated in 0.9 % saline solution (1 mL) at 37 oC and stability of the complex was monitored by ITLC-SG at different time intervals. The complexes as stable in dilute saline solution. Similarly, 99mTc complex of RGD was incubated in fresh human serum at physiological conditions *i.e.* at 37 ^{o}C at a concentration of 100 nM/mL.

For serum-binding assessment, SPE-purified complexes were incubated at a concentration of 20–100 pmol/ml in fresh human plasma at 37°C and analyzed by size exclusion chromatography up to 8 h. Serum binding of the ^{99m}Tc complex was determined by measuring columns and eluates in a γ counter.

Cytotoxicity Study

Cytotoxicity was determined using the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay. Brain homogenate was treated with varying concentrations of BTSE-RGD(μM–mM range) for 2 h and MTT assays were performed at 24, 48, 72 and 96 hpost treatment. 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^{o}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: 690 nm). Mitochondrial activity was expressed as percentage of viability compared to negative control (mean +/- SD of triplicate cultures). % of viability = [OD (570 nm - 630 nm) test product / OD (570 nm - 630 nm) negative control] x 100 %.

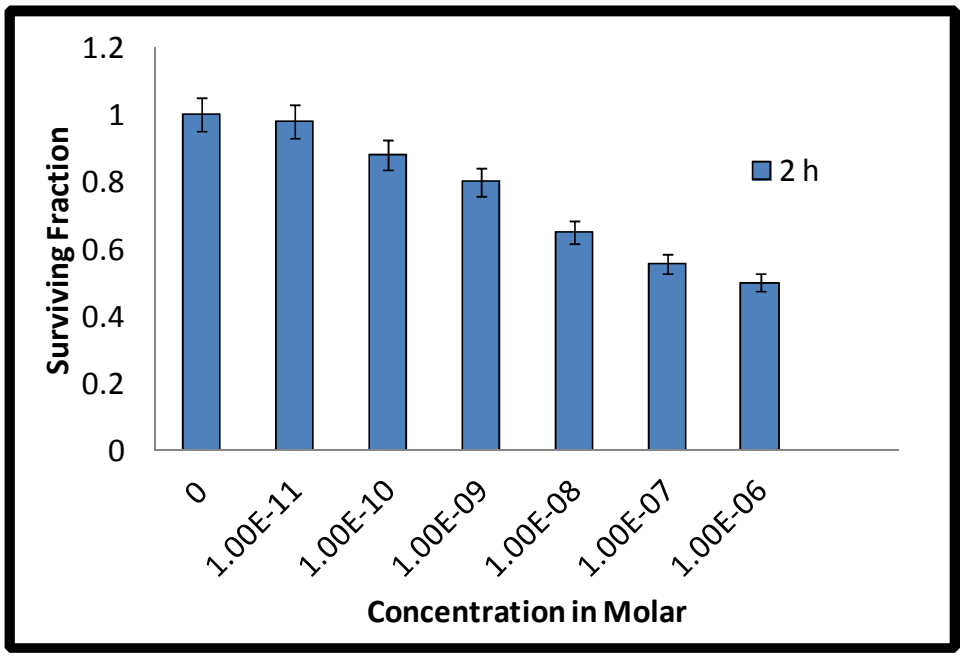


Figure 1 Cytotoxicity study on brain cells

***In-vivo* Studies**

Kinetic Studies

A Blood clearance study with above ^{99m}Tc complex was performed in adult New Zealand Rabbits. The 0.6 Ci of complexes solution in saline was injected intravenously and blood samples were collected at different time intervals. The amount of activity present in the blood was calculated by comparison with standard counts injected (Figure 2).

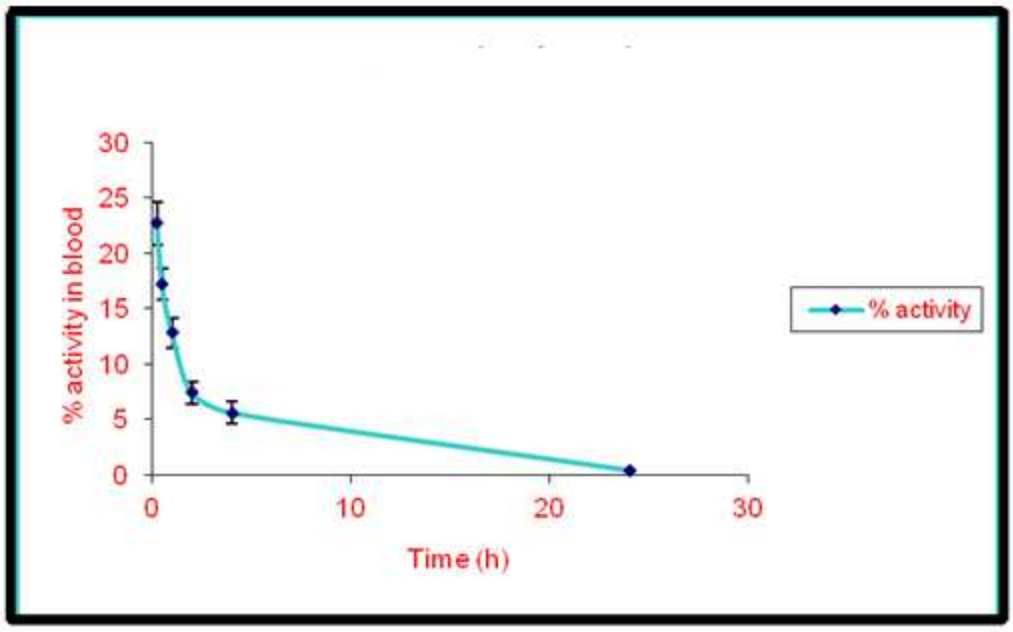


Figure 2 Blood kinetic Profile of ^{99m}TcBTSE-RGD

Biodistribution of ^{99m}Tc Complexes

The tissue distribution studies were conducted in adult BALB/c male mice (twelve in numbers). The tumors developed 12 days post-implantation, and the animals received 10 μCi of the labeled complex in 100 μL of a saline/ ethanol mixture (ethanol <5%) *via* lateral tail-vein injection. The animals were dissected at desired time intervals (1, 2, 4, and 24 hrs*)* and the organs were collected (blood, lung, liver, spleen, kidney, heart, brain, bone, tumor, and intestines). Once the tissues and organs of interest were removed, they were weighed and the radioactivity was measured in a γ-counter. The percent dose per gram (%ID/g) in organs has been shown in figure 3.

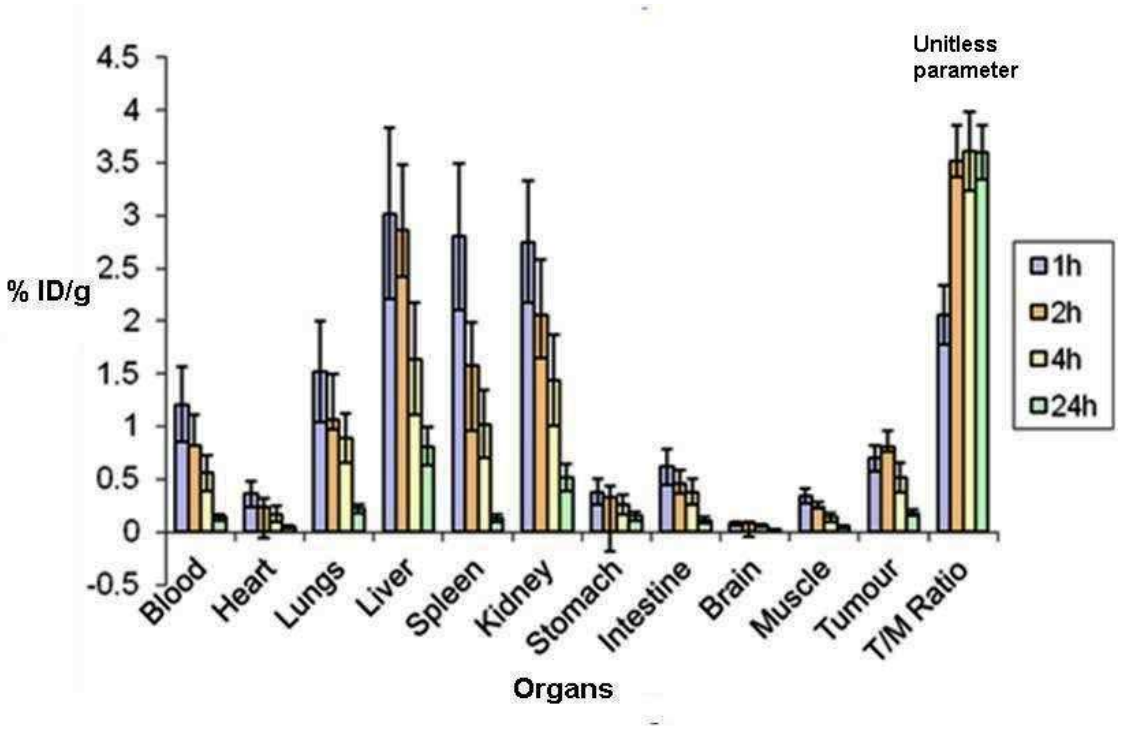


Figure 3 Biodistribution studies of ^{99m}TcBTSE-RGD in tumor bearing mice

While biodistribution of ^{99m}Tc- DTPA-RGD showed hepatobiliary and renal route of excretion as radioactive peptide conjugate persisted in stomach and intestine up to 4 h. Major route of clearance was renal with >6% radioactivity remaining in kidneys at 24 h.

Comparison with DTPA-RGD

All the studies for DTPA-RGD were performed as per the protocol mentioned in our paper (18).BTSE-RGD showed different protfolio regarding in vitro pharmacokinetics as it showed slower distribution half-life ($T_{1/2\alpha}$) and elimination half-life ($T_{1/2\beta}$) of ^{99m}Tc- BTSE-RGD were 65 ± 0.001 min and 21h 15 min ± 0.001 min, in comparison to ^{99m}Tc- DTPA-RGD(distribution half-life ($T_{1/2\alpha}$) and elimination half-life ($T_{1/2\beta}$) 18 ± 0.001 min and 9h 10 min ± 0.005 min.In biodistribution both showed better tumor to muscle ratio which reaches maximum around 3.5 (%

ID) for BTSE-RGD in 2h. while for DTPA-RGD it showed maxima on 13.60 at 24h (15).The differences in pharmacokinetics gives warrant for different application for both the ligands

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