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Synthesis and characterization of a novel ^{99m}Tc analogue of *I-*m*IBG showing affinity for nor-epinephrine transport positive tumors

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Radio-iodine (^{123}I/^{131}I) labeled *meta*-iodobenzylguanidine (*m*IBG) is a proven radiopharmaceutical used for diagnosis of neuroendocrine tumors (NET) related to neural crest origin. Here, a ^{99m}Tc analogue of *I-*m*IBG using ^{99m}Tc-4+1 labeling approach has been synthesized and evaluated for its potential in NET imaging. This involved preparation of benzylguanidine precursor (mono-dentate donor) which was isolated in a five step synthetic procedure. The precursor was designed such that, in presence of a tetra-dentate NS_3 co-ligand, $[^{99m}TcO_4]^-$ and Sn^{2+} reducing agent, it formed the desired ^{99m}Tc-4+1 complex. Complex formation was identified by radio-HPLC and structural details were affirmed by characterizing its rhenium analogue. Bio-evaluation studies of the complex were carried, both *in vitro* and *in vivo*, and the results obtained were compared with no-carrier added-^{125}I-*m*IBG (nca-^{125}I-*m*IBG). *In vitro* studies, in SK-N-SH neuroblastoma cell line showed affinity of the tracer towards nor-epinephrine transporters. Although the absolute uptake was lower compared to nca-^{125}I-*m*IBG, its specificity was similar (~90%) as the uptake reduced on inhibition with specific transport blocker, desmethylimipramine (DMI). Biodistribution studies in normal Wistar rats, pre-treated with *m*IBG and DMI, respectively, showed reduced myocardial uptake than its control. The results thus obtained merits high potential of synthesized ^{99m}Tc-4+1 complex for NET imaging.

Introduction

Neuroendocrine tumors (NETs) are a group of neoplasms, either of epithelial or neural origin, comprising mostly well-differentiated and slow growing tumors, with small population being poorly differentiated and malignant with an aggressive behavior. NETs are also known to express different transporters and hormone receptors (*e.g.* somatostatin receptors) on their cell surfaces, which is responsible for concentrating different hormones inside the cell. These cell parameters form the basis for designing specific radiolabeled ligands for NET imaging.[1–6] In this category, several radiolabeled nor-epinephrine and peptide analogs based on PET/SPECT are in clinical use. Peptide radiopharmaceuticals based on ^{111}In, ^{99m}Tc and ^{68}Ga have gained wide acceptance for NET imaging related to carcinoid tumors,[6–10] however, for tumors related to neural origin such as phaeochromocytoma, paraganglioma and neuroblastoma radiolabeled nor-epinephrine analogues have shown superior distribution behavior.[11–13]

$^{123/131}$I-*meta*-iodobenzylguanidine (*m*IBG), a structural analogue of nor-epinephrine, is one such SPECT based radiopharmaceutical that has been the mainstay for the diagnosis of these types of tumors. It enters the cell membrane of sympathomedullary tissues by an active, sodium- and energy-dependent amine uptake mechanism and gets stored into the intracellular catecholamine storing granules.[14,15] Although the uptake and retention of this radiopharmaceutical is ideal for favorable imaging, availability of ^{123}I radionuclide through high energy beam cyclotron limits its widespread use. ^{131}I, a theranostic radionuclide, widely available through neutron activation and fission pathways, is employed as a rational substitute and labeled ^{131}I-*m*IBG has now been in the clinics for nearly three decades, since its introduction by Donald M. Wieland in the 1980s.[14] Although ^{131}I-*m*IBG is satisfying the needs of the medical fraternity, the sub-optimal nuclear characteristics of ^{131}I for diagnostic applications demands a SPECT substitute, preferably ^{99m}Tc based, for the aforementioned application. This is due to the optimal nuclear characteristics of ^{99m}Tc, its ease of availability through a commercial ^{99}Mo–^{99m}Tc generator and ready formulation of the radiopharmaceutical through 'cold kits', ensuring the availability of radiopharmaceutical preparation in clinics round the clock. Limited efforts have been put forward by researchers in this direction to develop ^{99m}Tc labeled guanidine derivatives as an analogue of *I-labeled

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*m*IBG and although ^{99m}Tc-radiotracers evaluated exhibited affinity in preclinical animal models, the relatively low target uptake values limit their application for NET imaging in clinics.[16,17]

Over the past two decades, several new strategies for incorporating the ^{99m}Tc metal into the carrier molecule have emerged.[18] These employ several ^{99m}Tc-metal cores/fragments bonded through different bi-functional chelates, which affect the pharmacokinetics *in vivo*, leading to diverse bio-distribution characteristics with common targeting vectors or carrier molecules. For designing a ^{99m}Tc analogue of *I-*m*IBG, the selection of ^{99m}Tc incorporation approach should be such that the final ^{99m}Tc building block is kept to a minimum. In the present work, ^{99m}Tc-4+1 chemistry approach has been utilized, which is known to contribute little to the overall molecular framework of the complex. Also, the biomolecule attached to the mono-dentate donor leads to minimum distortion and no special care in respect of stereochemistry has to be taken into account.[18] The ^{99m}Tc-4+1 approach stabilizes the metal in +3 oxidation state by coordinating through two different sets of donor ligands, a tetra-dentate (NS_3) and a mono-dentate (isonitrile/phosphine) ligand. The carrier molecule can either be linked to a functionalized tetra-dentate ligand (*e.g.*, NS_3–COOH) or can be a part of the mono-dentate ligand for getting attached to the ^{99m}Tc(III) centre.[19,20] In the present approach, the active benzylguanidine functionality has been introduced *via* a mono-dentate donor with the standard NS_3 ligand as tetra-dentate co-ligand. The newly synthesized ^{99m}Tc-complex has been evaluated *in vitro* for its efficacy in neuroblastoma cell line and the results obtained have been compared with that of nca-^{125}I-*m*IBG. Further, the complex was evaluated *in vivo* in normal rats to evaluate its efficacy towards sympathetically innervated myocardium.

Experimental

General

The compounds *m*-xylylenediamine, *N*,*N*′-di-Boc-*S*-methyl isothiourea, ethyl formate, *p*-toluene sulphonic acid, mannitol and stannous chloride were obtained from Aldrich, USA. Ammonium perrhenate and dimethylphenyl phosphine were acquired from Alfa Aesar, Germany. Ethylenediamine tetraacetic acid disodium salt and trifluoroacetic acid (TFA) were procured from Merck, India. *p*-Toluene sulphonyl chloride and pyridine were obtained from S.D. Fine chemicals limited, India. 2,2′,2″-Nitrilotris(ethanethiol) (NS_3) was synthesized following a reported procedure.[21] Cuprous chloride was prepared fresh following a standard procedure from cupric chloride. All other reagents used were of analytical grade and were used without further purification. Sodium pertechnetate ($Na^{99m}TcO_4$) was eluted with normal saline prior to use from a ^{99}Mo–^{99m}Tc column generator, supplied by Board of Radiation and Isotope Technology (BRIT), India. nca-^{125}I-*m*IBG was prepared from *m*-trimethylsilylbenzylguanidine following a reported procedure.[22] Silica gel plates (Silica Gel 60 F_{254}) were obtained from Merck, India. The HPLC of the prepared complex was carried out on a JASCO PU 2080 Plus dual pump HPLC system, Japan, with a JASCO 2075 Plus tunable absorption detector and Gina Star radiometric detector system, using a C18 reversed phase HiQ Sil (5 μm, 4 × 250 mm) column. FT-IR spectra were recorded on JASCO FT/IR-460 Spectrophotometer, Japan. The ^{1}H-NMR spectra were recorded on a 500 MHz Varian spectrophotometer, USA. Low resolution mass spectra were recorded on Advion Mass Spectrometer, USA using electrospray ionization (ESI) in both positive and negative modes.

Synthesis

Synthesis of copper(I) complex of 3-(isocyanomethyl)benzylguanidine (mono-dentate donor)

N,*N*′-*Di-Boc-3-(aminomethyl)benzyl guanidine (**1**).* A mixture of *m*-xylelene diamine (300 mg, 2.2 mmol) and *N*,*N*′-di-Boc-*S*-methyl isothiourea (580 mg, 2.0 mmol) in THF (15 mL) and water (0.2 mL) was stirred overnight at room temperature. Upon completion of the reaction (*cf.* TLC), the reaction mixture was concentrated *in vacuo* and the crude product obtained was further purified by silica gel column chromatography to yield the desired product (650 mg, 86%).

R_f(CHCl$_3$/MeOH 9 : 1 v/v) = 0.3.

δ_H (500 MHz; $CDCl_3$; Me_4Si): 7.18–7.32 (4H, m, Ph); 4.59–4.67 (2H, m, CH_2–NH–C); 3.80–3.85 (2H, m, –CH_2NH$_2$); 1.45–1.67 (18H, br s, (CH_3)$_3$C–O–CO–N= and (CH_3)$_3$C–O–CO–NH–).

δ_C (125 MHz; $CDCl_3$; Me_4Si): 163.6 (=N–*C*OO); 156.1 (NH–*C*OO); 153.2 (BocN=*C*–NH); 138.2 (phenyl HC=C–*C*H$_2$–NH–C=); 137.2 (phenyl HC=C–*C*H$_2$–NH$_2$); 129.1, 128.8, 127.5, 126.5 (phenyl HC=*C*H–CH); 83.1 ((CH$_3$)$_3$*C*–C=O(N=)); 80.3 ((CH$_3$)$_3$*C*–C=O(NH)); 50.3 (Ar*C*H$_2$NH$_2$); 44.5 (Ar*C*H$_2$NHCO–); 29.6, 29.3, 28.9, 28.3, 28.2, 28.0 ((*C*H$_3$)$_3$C).

ESI-MS (+ve mode): mass (calculated) $C_{19}H_{30}N_4O_4$ 378.2; *m*/*z* (observed) 379.

N,*N*′-*Di-Boc-3-(formamide N-methyl)benzyl guanidine (**2**).* Ethyl formate (10 mL) was added slowly to a stirred ice cooled mixture of compound **1** (300 mg, 0.8 mmol) containing catalytic amount of *p*-toluene sulphonic acid. After addition is complete, the reaction mixture was brought to room temperature and then refluxed overnight. Upon completion of the reaction, excess ethyl formate was removed *in vacuo* to give the crude product which on purification by silica gel column yielded the pure compound **2** (225 mg, 70%).

R_f(EtOAc) = 0.6.

δ_H (500 MHz; $CDCl_3$; Me_4Si): 8.23 (1H, s, NH–C*H*O); 7.20–7.31 (4H, m, Ph); 4.59 (2H, d, J = 3 Hz, CH_2–NHC=N); 4.46 (2H, d, J = 6 Hz, CH_2–NHCHO); 3.70 (1H, s, –N*H*CHO); 1.45–1.50 (14H, br s, (CH_3)$_3$C–O–CO–N= and (CH_3)$_3$C–O–CO–NH–); 1.25–1.32 (4H, br s, (CH_3)$_3$C–O–CO–N= and (CH_3)$_3$C–O–CO–NH–).

δ_C (125 MHz; $CDCl_3$; Me_4Si): 164.2 (–NH*C*HO); 163.6 (tautomer –N=*C*HOH); 160.9 (=N–*C*OO); 156.2 (NH–*C*OO); 153.1 (BocN=*C*–NH); 138.1 (phenyl HC=*C*–CH$_2$–NH–C=); 137.3 (phenyl HC=C–*C*H$_2$–NH$_2$); 129.3, 127.1, 126.5, 126.2 (phenyl HC=*C*–CH$_2$); 83.4 ((CH$_3$)$_3$*C*–C=O(N=)); 79.5 ((CH$_3$)$_3$*C*–C=O(NH)); 61.3 (tautomer *C*H$_2$–N=CHOH); 44.6 (Ar*C*H$_2$-NHCHO); 41.9 (Ar*C*H$_2$NHC=NBoc); 29.6 29.3, 28.3, 28.2, 28.1, 28.0 ((*C*H$_3$)$_3$C).

ESI-MS (+ve mode): mass (calculated) $C_{20}H_{30}N_4O_5$ 406.2; *m*/*z* (observed) 406.7.

*N,N′-Di-Boc-3-(isocyanomethyl)benzyl guanidine (**3**).* To a cooled solution of compound **2** (200 mg, 0.5 mmol) in pyridine (5 mL), *p*-toluene sulphonyl chloride (190 mg, 1.0 mmol) was added and the mixture stirred at 0 °C for 15 min. The reaction mixture was then brought to room temperature and stirred for another 2 h. Upon completion of the reaction (*cf.* TLC), the reaction mixture was poured into water and extracted with chloroform (3 × 10 mL). The combined chloroform extracts were dried over anhydrous sodium sulphate and removed under *vacuo* to yield the crude product which was purified using silica gel column chromatography to give **3** (87 mg, 45%).

R_f(EtOAc) = 0.5.

IR (neat, ν_{max}/cm^{-1}): 3053 (Ar-H str., m); 2923 (C–H str., s); 2853 (C–H str., m); 2152 (N≡C str., s); 1665 (C=O str., bs); 1529 (m); 1440 (m); 1384 (m); 1241 (m); 1064 (m); 788 (m).

δ_H (500 MHz; $CDCl_3$; Me_4Si): 7.0–7.4 (4H, m, Ph); 4.59 (2H, s, CH_2–NHC=N); 4.49 (2H, s, CH_2–NC); 1.35–1.69 (14H, br s, (CH_3)$_3$C–O–CO–N= and (CH_3)$_3$C–O–CO–NH–); 1.22–1.33 (4H, br s, (CH_3)$_3$C–O–CO–N= and (CH_3)$_3$C–O–CO–NH–).

*Cu(I) complex of N,N′-di-Boc-3-(isocyanomethyl)benzyl guanidine (**4**).* Compound **3** (78 mg, 0.4 mmol) was heated with CuCl (10 mg, 0.1 mmol) in anhydrous EtOH (2 mL) at 90 °C for 1 h. The reaction mixture was then allowed to attain ambient temperature and was filtered through a 0.22 μm membrane filter (Millipore). The solvent ethanol was then removed under *vacuo* to obtain the desired compound **4** (130 mg, 80%).

IR (neat, ν_{max}/cm^{-1}): 3055 (Ar-H str., m); 2923 (C–H str., s); 2853 (C–H str., m); 2185 (N≡C str., s); 1667 (C=O str., bs); 1530 (m); 1440 (m); 1384 (m); 1244 (m); 1055 (m); 792 (m).

δ_H (500 MHz; $CDCl_3$; Me_4Si): 7.81 (1H, s, Ph); 7.51–7.62 (1H, m, Ph); 7.26–7.42 (1H, m, Ph); 7.07–7.13 (1H, m, Ph); 4.68 (2H, s, CH_2–NC); 4.59 (2H, s, CH_2–NHC=N); 1.08–1.38 (14H, m, (CH_3)$_3$C–O–CO–N= and (CH_3)$_3$C–O–CO–NH–); 0.84–0.92 (4H, m, (CH_3)$_3$C–O–CO–N= and (CH_3)$_3$C–O–CO–NH–).

δ_C (125 MHz; $CDCl_3$; Me_4Si): 161.3 (–N≡*C*); 161.0 (=N–*C*OO); 156.2 (NH–*C*OO); 153.1 (BocN=*C*–NH); 133.7 (phenyl HC=C–*C*H$_2$–NH–C=); 131.3 (phenyl HC=C–*C*H$_2$–NH$_2$); 129.4, 128.3, 126.3, 125.6 (phenyl HC=*C*H–CH); 83.4 ((CH$_3$)$_3$*C*–C=O(N=)); 79.5 ((CH$_3$)$_3$*C*–C=O(NH)); 48.7 (Ar*C*H$_2$NC); 41.5 (Ar*C*H$_2$NHCO–); 29.7, 29.5, 29.3, 28.9 ((*C*H$_3$)$_3$C).

*Cu(I) complex of 3-(isocyanomethyl)benzyl guanidine (**5**).* Compound **4** (100 mg, 0.06 mmol) was directly treated with trifluoroacetic acid (TFA) (2 mL) and the solution stirred for 2 h at room temperature. Thereafter, TFA was removed under vacuum to obtain compound **5** (50 mg, quantitative) and was analyzed as such without further purification.

IR (neat, ν_{max}/cm^{-1}): 3055 (Ar-H str., m); 2923 (C–H str., s); 2853 (C–H str., m); 2185 (N≡C str., s); 1530 (m); 1440 (m); 1384 (m); 1244 (m); 1055 (m); 792 (m).

δ_H (500 MHz; $CDCl_3$; Me_4Si): 7.2–7.7 (1H, m, Ph); 4.68 (2H, s, CH_2–NC); 4.55 (2H, s, CH_2–NHC=NH).

Radiolabeling

^{99m}Tc(III)–EDTA complex (6). The synthesis of the reactive intermediate complex was carried as per the reported procedure.[20] Briefly, freshly eluted [^{99m}TcO$_4$]$^-$ (30 mCi/1.1 GBq, 1.0 mL) was added to a vial containing EDTA (5.0 mg), mannitol (5.0 mg) and $SnCl_2 \cdot 2H_2O$ (100 μg) in 0.1 N HCl (100 μL) and incubated at room temperature for 30 min. The formation of the intermediate complex was analyzed by TLC (>99%) (R_f = 0, intermediate EDTA complex; R_f = 1, [^{99m}TcO$_4$]$^-$; solvent: acetone).

^{99m}Tc-(4+1) complex (7). Intermediate complex (**6**) (0.5 mL) was added to a vial containing a mixture of compound **5** (1–2 mg) and NS_3 ligand (1 mg) in methanol (0.5 mL). The vial was sealed and the reaction mixture heated in a boiling water bath for 30 min. After cooling the vial to room temperature, the complex **7** was analyzed by TLC (EtOH: $R_f \sim 0.9$ and $CHCl_3$: $R_f \sim 0.2$) and HPLC. To resolve the identity of the final complex **7** from the secondary intermediate ^{99m}Tc–NS_3 (only **4** intermediate complex, without mono-dentate donor), a fresh reaction was carried following the same procedure without addition of compound **5** and the reaction mixture was analyzed by HPLC.

Synthesis of Re-4+1 complex (8)

[Re(Me$_2$PPh)NS$_3$] intermediate complex. This intermediate was prepared following a reported procedure.[21]

δ_H (500 MHz; $CDCl_3$; Me_4Si): 7.1–7.9 (5H, m, Ph); 2.5–3.1 (12H, br s, –CH_2–S– and –CH_2N–); 2.18 (3H, s, –PCH_3); 2.05 (3H, s, –PCH_3).

ESI-MS (+ve mode): mass (calculated) $C_{14}H_{23}NPReS_3$ 516.7, 518.7; *m*/*z* (observed) 517.1, 519.1.

[ReNS$_3$ (3)]. A mixture of [Re(Me$_2$PPh)NS$_3$] (50 mg, 0.09 mmol) and compound **3** (37 mg, 0.09 mmol) in chloroform was refluxed overnight. Thereafter, the reaction mixture was cooled and solvent was removed under *vacuo* to give the crude product. The pure product (18 mg, 25%) was obtained using silica gel column chromatography with chloroform as the eluting solvent.

R_f(chloroform) = 0.5.

IR (neat, ν_{max}/cm^{-1}): 3331 (N–H str., m); 2924 (C–H str., s); 2854 (C–H str., m); 1972 (N≡C str., bs); 1725, 1641 (C=O str., s); 1441 (m); 1154 (m).

δ_H (500 MHz; $CDCl_3$; Me_4Si): 7.51 (1H, d, J = 6 Hz, Ph); 7.45 (1H, d, J = 8 Hz, Ph); 7.34–7.37 (1H, m, Ph); 7.23–7.26 (1H, m, Ph); 6.04 (2H, s, CH_2NC); 4.66–4.68 (2H, m, CH_2–NH–C); 2.8–3.2 (12H, br s, CH_2–S– and –CH_2N–); 1.45–1.56 (14H, m, (CH_3)$_3$C–O–CO–N= and (CH_3)$_3$C–O–CO–NH–); 1.21–1.33 (4H, m, (CH_3)$_3$C–O–CO–N= and (CH_3)$_3$C–O–CO–NH–).

ESI-MS (+ve mode): mass (calculated) $C_{26}H_{40}N_5O_4ReS_3$ 767.2, 769.2; *m*/*z* (observed) 767.4, 769.4.

Re-4+1 complex (8). The final complex was obtained on treatment of above Re-complex (10 mg) with TFA (1 mL) for 3 h. No further purification was carried and pure product (13 mg, quantitative) was obtained after TFA removal.

IR (neat, ν_{max}/cm^{-1}): 3333, 3198 (N–H str., m); 2926 (C–H str., s); 2854 (C–H str., m); 1966 (N≡C str., bs); 1781, 1739, 1681 (C=N str., bs); 1445 (m); 1203 (bm); 1154 (m); 777 (m).

δ_H (500 MHz; $CDCl_3$; Me_4Si): 7.23–7.52 (4H, m, Ph); 6.04 (2H, s, CH_2NC); 4.66–4.68 (2H, m, CH_2–NH–C); 2.8–3.2 (12H, br s, CH_2–S– and –CH_2N–).

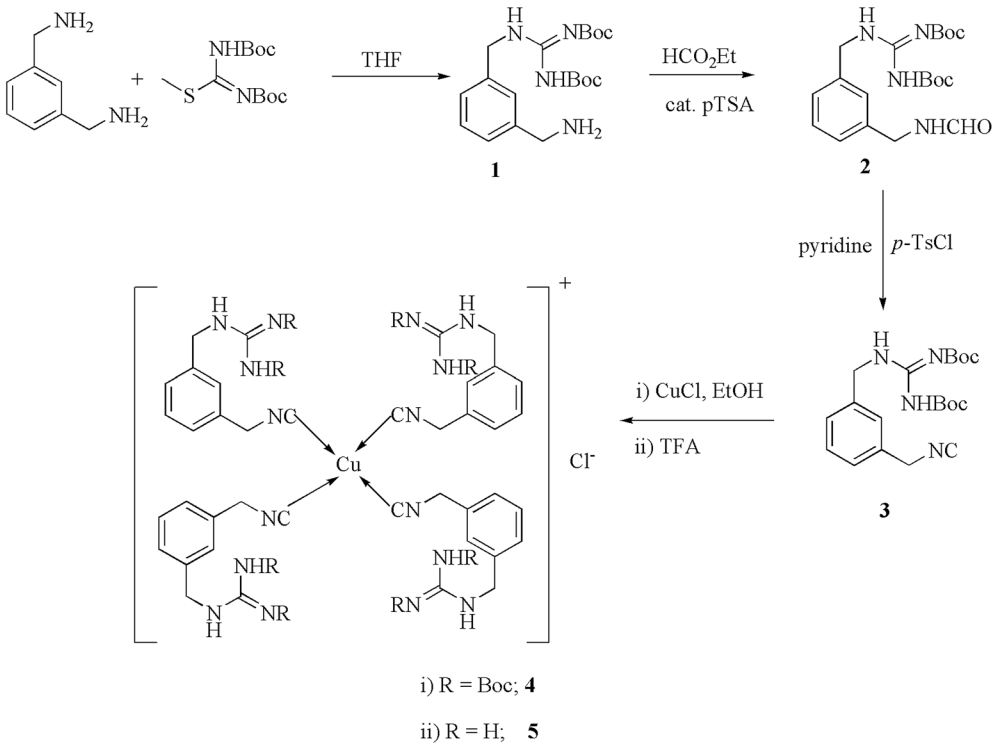

Fig. 1 Scheme for the synthesis of Cu(I) complex of (3-(isocyanomethyl)benzyl)guanidine.

ESI-MS (+ve mode): mass (calculated) $C_{16}H_{25}N_5ReS_3$ 567.1, 569.1; *m*/*z* (observed) 567.7, 569.7.

Quality control

HPLC. The radiochemical purity (RCP) of the ^{99m}Tc-4+1 complex (7) was determined by reversed phase HPLC. Gradient elution program was followed using water (solvent A) and acetonitrile (solvent B) as the mobile phase (0 min 90% A, 20 min 0% A, 30 min 0% A). Flow rate of the solvent was maintained at 1 mL min^{-1}. Test solution (20 μL) was injected into the column using a micro-syringe and elution was monitored by observing the radioactivity profile. The same C18 reversed phase analytical column was used for the purification of the complex 7. Fraction containing the complex 7 was collected, subjected to *vacuo* and reconstituted in 5% ethanol. Around 18.5 MBq (500 μCi) of pure radiolabeled complex was obtained by this method which was subsequently used for *in vitro* and *in vivo* studies.

The formation of Re-4+1 complex **8** too was confirmed using the same gradient elution system wherein UV profile of the complex was monitored to establish its identity in conformity with ^{99m}Tc-4+1 analogue.

Partition coefficient (log $P_{o/w}$). The HPLC purified complex 7 (0.1 mL, 185 KBq/5 μCi) was mixed with double distilled water (0.9 mL) and *n*-octanol (1 mL) and vortexed for 3 min.

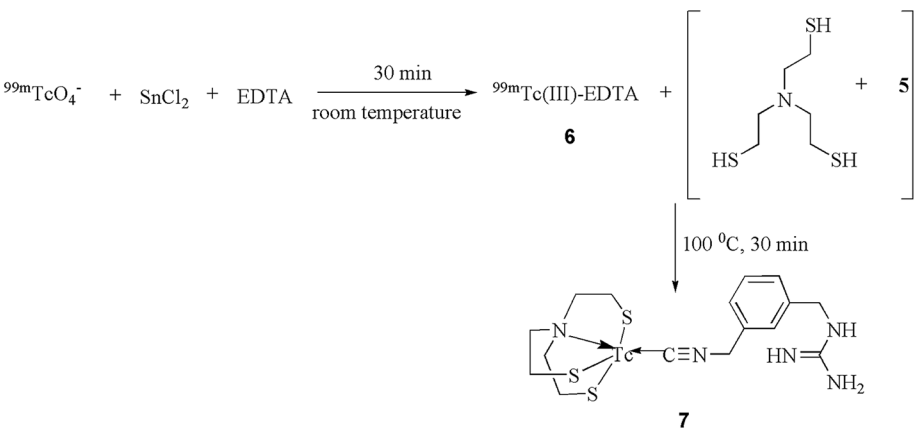

Fig. 2 Scheme for the preparation of ^{99m}Tc-4+1 complex.

The mixture was then centrifuged at 3500 *g* for 3 min to effect clear separation of the two layers. Equal aliquots from the two layers were withdrawn in triplicate and measured for associated radioactivity. The readings thus obtained were used to calculate the log $P_{o/w}$ value (octanol–water partition) of the complex.

Serum stability and protein association. The purified ^{99m}Tc-4+1 complex **7** (50 μL, 370 KBq/10 μCi) was incubated in human serum (450 μL) at 37 °C for 1 h and 2 h, respectively. Thereafter, the serum proteins were precipitated by addition of EtOH (500 μL). The solution was centrifuged and the supernatant was analyzed by TLC to ascertain the stability of the complex in serum. The precipitate was washed twice with ethanol and the activity associated with precipitate was expressed as a percent of the initial activity.

Cell uptake experiments

SK-N-SH neuroblastoma cell line[23,24] was used for the uptake studies. The cell line was obtained from National Centre for Cell Science, Pune, India and grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum, glutamine (2 mmol), penicillin–streptomycin (100 IU mL^{-1}) and amphotericin B (2.5 μg mL^{-1}). All other media and supplements used were purchased from Himedia, India. Cells were seeded in six well plates at an initial density of 5×10^5 per well per 3 mL of medium and were cultured as monolayers for 2–3 days at 37 °C in 5% CO_2 until semi-confluent. Triplicate wells in each plate containing monolayers were pre-incubated with desmethylimipramine (0.45 mg, 1.5 μmol) for 4 h. Subsequently, HPLC purified ^{99m}Tc complex **7** was introduced in wells and incubated for a specific time period. After incubation, media was removed and monolayers were washed twice with phosphate buffered saline. The cell bound radioactivity was then extracted with 10% (w/v) trichloroacetic acid (2 × 0.5 mL). The activity of the combined extracts was measured in a NaI(Tl) well counter with suitable energy window for ^{99m}Tc (140 keV ± 10%). The uptake *via* specific active transport pathway was deduced by calculating the difference between the uptakes observed for ^{99m}Tc complex in SK-N-SH cells to the uptake seen in same cells with desmethylimipramine blocking. The results obtained were compared with nca-^{125}I-*m*IBG evaluated following the identical procedure.

Bio-distribution studies

All procedures performed herein were in accordance with the national laws pertaining to the conduct of animal experiments. Normal Wistar rats (150–200 g body weight) were used for the *in vivo* distribution studies. The HPLC purified radiolabeled preparation **7** (100 μL, 740 KBq/20 μCi) was administered intravenously through tail vein of each rat. The animal distribution experiment was conducted in three different groups. The first study utilized animals (3 × 3) for normal distribution of ^{99m}Tc complex at three different time points (2 min, 30 min and 1 h). Another category of animals (2 × 3) were pre-treated with desmethylimipramine (10 mg kg^{-1} intra-peritoneal injection), 30 min prior to the activity injection and distribution of ^{99m}Tc complex seen at two different time-points (2 min and 1 h). Third set of animal (2 × 3) distribution involved co-injection of ^{99m}Tc complex **7** along with cold *m*IBG at two different time points (2 min and 1 h). At the end of each time point, respective set of animals were sacrificed and the relevant organs and tissue were excised for the measurement of associated activity. The organs were weighed and the activity associated with each organ was measured in a flat-bed type NaI(Tl) counter with suitable energy window for ^{99m}Tc (140 keV ± 10%). The activity associated with each organ/tissue was expressed as a percent injected dose per gram (%ID per g).

Results

$^{123/131}$I-labeled *m*IBG is a widely used diagnostic agent for neuroendocrine tumors of neural origin. The present work explores the possibility of synthesizing a ^{99m}Tc analogue of this agent for NET imaging. The schematic involved synthesizing a suitable guanidine precursor and attaching it to ^{99m}Tc radiometal *via* ^{99m}Tc-4+1 strategy. The active pharmacophore benzylguanidine was suitably modified at *meta*-position, wherein an isonitrile functionality was introduced to link it to ^{99m}Tc metal *via* mono-dentate linkage in presence of the tetra-dentate co-ligand (NS_3).[21] The scheme for the synthesis of Cu(I) complex of benzylguanidine derivative (**5**) is shown in Fig. 1. The synthetic procedure involved five steps starting from *m*-xylelene diamine, wherein monoguanylation at single amine functionality with *N*,*N*′-di-Boc-*S*-methyl isothiourea yielded **1** at room

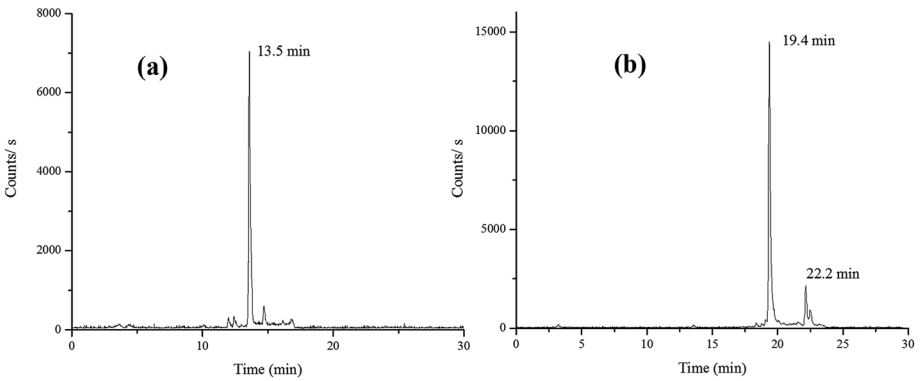

Fig. 3 HPLC profile of (a) ^{99m}Tc–NS_3 (b) complex **7**.

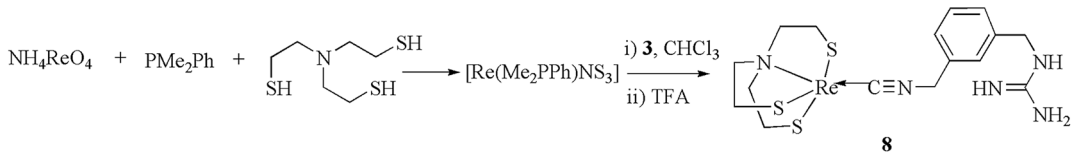

Fig. 4 Scheme for the synthesis of Re-4+1 complex.

temperature. The monoguanylated product **1** was then formylated in the presence of ethyl formate to give **2**, which on subsequent dehydration in the presence of *p*-toluene sulphonyl chloride and pyridine resulted in di-Boc protected isonitrile ligand **3**. Since the isonitriles are prone to degradation, a stable species in the form of Cu(I) complex **4** was prepared by treatment of the free isonitrile with freshly prepared CuCl. Finally, the target Cu(I) complex **5** was obtained on Boc de-protection using TFA. All the intermediates and target ligand were characterized by various spectroscopic methods. The radiolabeling scheme of **5** with ^{99m}Tc is shown in Fig. 2. The procedure involves generation of ^{99m}Tc(III)–EDTA intermediate **6** in the presence of a reducing agent and EDTA ligand. This intermediate was produced in near quantitative yield (characterized by TLC) which on *trans*-chelation with tetradentate NS_3 ligand (4 donor) and monodentate Cu(I) complex of (3-(isocyanomethyl) benzyl)guanidine donor yields the desired ^{99m}Tc-4+1 complex **7** in more than 80% radiochemical purity as characterized by HPLC ($R_t \sim 19.4$ min, Fig. 3). The product was purified by HPLC and used for bio-evaluation studies. The structural characterization of complex **7** was carried by preparing its Re-analogue **8** in millimolar amounts and characterizing the same using HPLC, IR, NMR and MS techniques. Fig. 4 shows the synthetic scheme followed for the preparation of Re-analogue **8**. The analogue **8** was prepared following a reported protocol,[21] wherein the intermediate [Re(Me_2PPh)NS_3] is first synthesized. HPLC elution profile of this labile rhenium intermediate showed a single sharp peak at 25.6 min which on further reaction with **3** followed by TFA treatment yielded the desired complex **8** (Fig. 5). The reproducibility in retention times (19.4 min) of **8** to that of ^{99m}Tc-4+1 derivative **7** (Fig. 3) confirm the identity and spectroscopic data substantiate the structure as proposed in Fig. 4.

The log $P_{o/w}$ of the complex **7** was found to be 1.9 which shows high lipophilic nature of the complex. The stability of HPLC purified ^{99m}Tc-4+1 complex in human serum, after protein precipitation, showed insignificant degradation of the complex (>90%, Fig. 6) up to a period of 2 h. Around 30% of the activity was found to be associated with the serum proteins.

In vitro cell uptake studies

*I-*m*IBG enters tumor cells by an active transport mechanism *via* nor-epinephrine transporters (NET) and gets stored in the

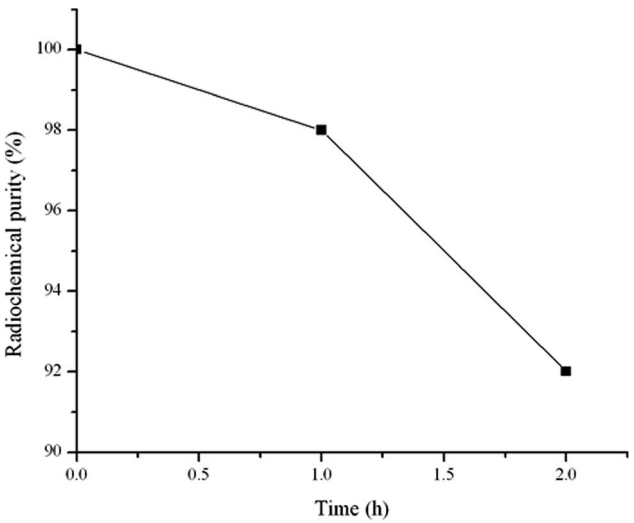

Fig. 6 Serum stability of ^{99m}Tc-4+1 complex **7**.

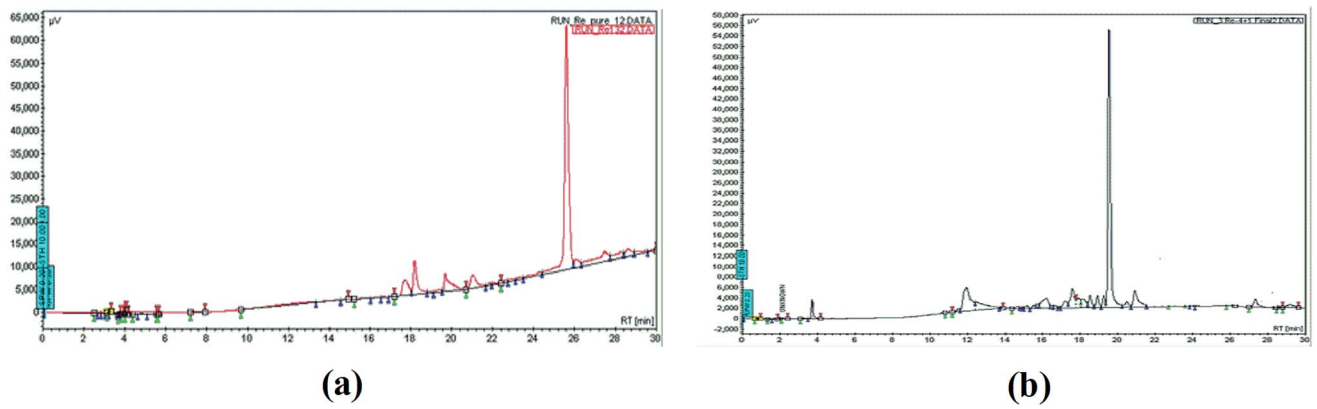

Fig. 5 HPLC UV profile of (a) [Re(NS_3)PMe_2Ph] (b) complex **8**.

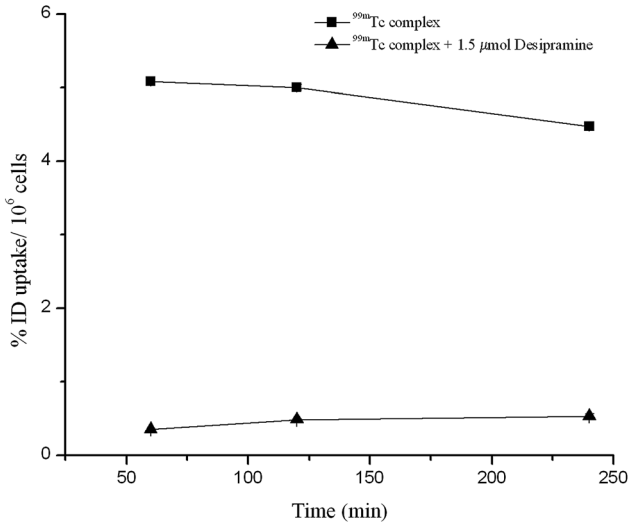

Fig. 7 Uptake studies of ^{99m}Tc-4+1 complex **7** in SK-N-SH cell line at different time points. The data shown is from a set of representative experiments.

cells. SK-N-SH is a neuroblastoma cell line with NET positive expression known to accumulate nor-epinephrine analogues. Uptake of synthesized ^{99m}Tc radiotracer 7 in SK-N-SH cells was carried out to evaluate the equivalence of the synthesized complex with respect to nca-^{125}I-*m*IBG. Fig. 7 represents the uptake of ^{99m}Tc complex in SK-N-SH cells at different incubation times. The initial uptake value of ^{99m}Tc complex at 1 h was significant (5.08 ± 0.02% ID per 10^6 cells) but was lower than nca-^{125}I-*m*IBG (12.2 ± 0.08% ID per 10^6 cells). The uptake of ^{99m}Tc tracer in neuroblastoma cells was found to be specific *via* NET transporter as the uptake reduced significantly (~90%) on inhibition with DMI, which is a known inhibitor of uptake-1 pathway responsible for nor-epinephrine transport. The initial uptake in the cells of ^{99m}Tc complex was found to be retained up to a study period of 4 h. A small percentage of non-specific uptake for complex 7 (~0.48 ± 0.01% ID per 10^6 cells) was seen in the cells which may be associated to other mechanistic pathways such as uptake-2 mechanism. Fig. 8 shows the uptake of ^{99m}Tc tracer in SK-N-SH cells at three different complex 7 concentrations (activity in cpm) each incubated for fixed period of 2 h. The percentage of specific binding with the cells increased from 4.2 to 5.2% ID per 10^6 cells with increase in tracer concentrations. The non-specific bound component exhibited a constant fraction of the total uptake activity and was observed to be less than 10%, similar to the behavior observed for nca-^{125}I-*m*IBG.

The bio-distribution results carried in normal Wistar rats are shown in Table 1. Fig. 9 shows the myocardial uptake of complex 7 and nca-^{125}I-*m*IBG at two different time points. Complex 7 showed initial uptake of 1.69 ± 0.42% ID per g in the myocardium at 2 min p.i., however it was significantly lower than nca-^{125}I-*m*IBG (4.36 ± 0.68% ID per g). Unlike nca-^{125}I-*m*IBG, the initial uptake of 7 in the myocardium was not retained and cleared with time over 60 min p.i. (0.58 ± 0.03%). The latter behavior indicates that the attachment of ^{99m}Tc-chelate modifies the basic benzyl guanidine moiety significantly, which is non-recognizable by the innervated sympathetic organ. Hence, the uptake observed may be non-specific in nature, where other uptake mechanisms such as passive diffusion contribute to the myocardial uptake. To ascertain the uptake pathway, *in vivo* NET transport blocking using *m*IBG and DMI were carried, respectively. Fig. 9 includes the tracer distribution in the myocardium, co-injected along with cold *m*IBG and 30 min DMI pre-treated animals. Significant difference in myocardial uptake was seen at 60 min injection ($p > 0.05$) where *m*IBG/DMI presence affects the tracer distribution, thereby favoring the uptake-1 pathway. The uptake of 7 in other non-target organs such as blood, lungs and liver was low and that too clears over a period. These factors favor high heart/non-target ratios, but the effect gets minimized as the complex 7 is not significantly retained in the myocardium.

Discussion

Development of ^{99m}Tc-agent for NET tumors of neural crest origin is of high significance as the use of ^{99m}Tc radioisotope allows ready availability of the tracer in the clinics. Further, even if the tracer uptake in the target is low, the injected ^{99m}Tc activity content can be increased to obtain high resolution images without delivering extensive radiation load to the

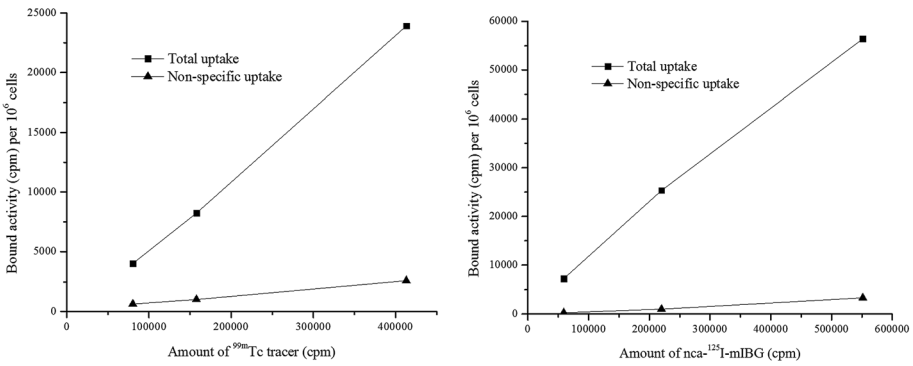

Fig. 8 Cell uptake studies of ^{99m}Tc-4+1 complex (**7**) and nca-^{125}I-*m*IBG in SK-N-SH human neuroblastoma cells as a function of tracer concentration. The data shown is from a set of representative experiments.

Table 1 Bio-distribution studies of ^{99m}Tc-4+1 complex (**7**) in normal Wistar rats in comparison with nca-^{125}I-*m*IBG[a]

	2 min			30 min	60 min		
Organ	Complex **7**	**7** + *m*IBG	**7** + DMI	Complex **7**	Complex **7**	**7** + *m*IBG	**7** + DMI
Liver	**1.21 (0.05)**	**1.39 (0.05)**	**1.35 (0.11)**	**1.13 (0.09)**	**1.28 (0.31)**	**1.25 (0.03)**	**1.30 (0.05)**
Int + GB	0.31 (0.03)	0.37 (0.06)	0.34 (0.03)	1.03 (0.15)	1.35 (0.33)	1.23 (0.33)	1.54 (0.21)
Stomach	0.34 (0.17)	0.30 (0.13)	0.39 (0.13)	3.76 (0.65)	2.19 (0.06)	3.86 (0.59)	1.11 (0.11)
Kidney	2.12 (0.01)	2.32 (0.27)	2.27 (0.30)	1.53 (0.06)	1.69 (0.01)	1.66 (0.10)	1.85 (0.14)
Heart	**1.69 (0.42)**	**2.28 (0.29)**	**1.9 (0.31)**	**0.75 (0.13)**	**0.58 (0.03)**	**0.31 (0.03)**	**0.39 (0.09)**
Lungs	1.95 (0.24)	2.87 (0.37)	2.21 (0.35)	0.82 (0.13)	0.62 (0.07)	0.54 (0.04)	0.60 (0.18)
Spleen	0.25 (0.12)	0.91 (0.13)	0.77 (0.26)	0.89 (0.13)	0.75 (0.16)	0.84 (0.12)	0.68 (0.32)
Muscle	0.37 (0.05)	0.33 (0.21)	0.4 (0.05)	0.25 (0.01)	0.18 (0.07)	0.13 (0.05)	0.07 (0.05)
Blood	**2.55 (0.27)**	**3.13 (0.37)**	**3.16 (0.41)**	**1.18 (0.11)**	**1.21 (0.08)**	**0.62 (0.14)**	**0.84 (0.18)**
Bone	0.22 (0.21)	0.25 (0.18)	0.29 (0.21)	0.09 (0.05)	0.03 (0.02)	0.14 (0.08)	0.12 (0.08)
H/Blood	0.65 (0.14)	0.73 (0.02)	0.6 (0.06)	0.63 (0.13)	0.48 (0.05)	0.54 (0.21)	0.46 (0.08)
H/Liver	1.42 (0.55)	1.64 (0.36)	1.41 (0.32)	0.66 (0.14)	0.47 (0.09)	0.24 (0.03)	0.29 (0.08)
H/Lung	0.85 (0.15)	0.79 (0.04)	0.86 (0.06)	0.91 (0.03)	0.94 (0.06)	0.57 (0.1)	0.66 (0.15)

[a] All results are expressed as %ID per g ± SD, 3 animals were used for each time point, DMI = desmethylimipramine; *m*IBG = *meta*-iodobenzylguanidine.

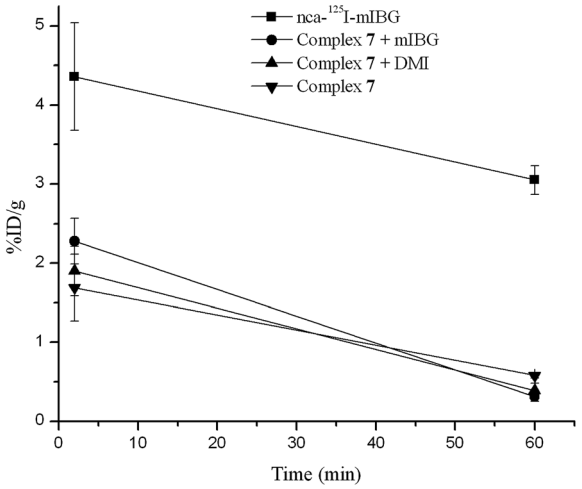

Fig. 9 Myocardial uptake (%ID per g) of complex **7** in comparison with nca-^{125}I-*m*IBG in normal Wistar rats.

patients. Also, the superior nuclear imaging characteristics of ^{99m}Tc over ^{131}I will further improve the resolution of the images. Considering these aspects, designing a ^{99m}Tc analogue of *I-*m*IBG is of high relevance but this has met with limited success until now. Diverse complexation chemistry of the ^{99m}Tc metal opens up a number of synthetic strategies for attaching the vector molecule to the same metal through different labeling methods. In the present work, ^{99m}Tc-4+1 labeling approach has been followed for introducing the benzylguanidine derivative. The latter moiety has been modified to introduce an isonitrile motif to be suitable for complexation through this approach. Since isonitriles are reactive intermediates and are prone to degradation, the isonitrile ligand synthesized was transformed into a stable Cu(I) isonitrile complex **5**. The formation of Cu(I) complex could be easily deduced from the IR spectrum which shows a shift in the frequency position of isonitrile group (N≡C) from 2152 cm^{-1} for the free isonitrile ligand to a higher frequency at 2185 cm^{-1} in the Cu(I) complex. The labeling of the latter ligand was achieved in high yield and the labeled product was purified using analytical HPLC, to remove excess cold ligand before carrying out biological studies.

SK-N-SH neuroblastoma cell line was used for assessing the affinity of the synthesized ^{99m}Tc tracer towards nor-epinephrine transport expression. The specific uptake of the present ^{99m}Tc-4+1 radiotracer (complex **7**) in SK-N-SH neuroblastoma cells, when compared with that of the previously reported ^{99m}Tc-**2** (N_2S_2 complex),[16] showed significantly improved results (90% *vs.* 60%) with non-specific uptake values being similar to nca-^{125}I-*m*IBG (~10%). The specific uptake values were also high for the complex **7** (50% of nca-^{125}I-*m*IBG *vs.* 10% of nca-^{125}I-*m*IBG) in comparison to the previously reported complex.

For evaluating the potential of the new tracers towards nor-epinephrine transporter *in vivo*, myocardium is taken as the organ of interest. This is because the heart, being a sympathetically innervated organ, requires transport of nor-epinephrine hormone for its functioning. ^{123}I-*m*IBG is a radiopharmaceutical which is used in sympathetic myocardial imaging and its uptake is found to correlate with myocardial defect. Though, the ^{99m}Tc tracer under study showed specific avidity (as established by *in vivo* inhibition using DMI/*m*IBG) towards myocardium, the uptake in the myocardium was observed to be lower. Nevertheless, the efficacy *in vivo* needs further introspection by carrying out bio-distribution in tumor bearing athymic mice.

Conclusions

A ^{99m}Tc analogue of *I-*m*IBG was synthesized *via* ^{99m}Tc-4+1 strategy and was biologically evaluated. This involved synthesis of a suitable Cu(I) complex of benzylguanidine precursor with isonitrile functionality at the *meta*-position in moderate yields. This complexed efficiently with ^{99m}Tc, in the presence of tetradentate NS_3 ligand, in high yield and purity. The chemical structure of ^{99m}Tc-4+1 complex was ascertained by preparing its

rhenium analogue. *In vitro* cell uptake studies of the complex in SK-N-SH neuroblastoma cell line showed significant specific uptake in the tumor cells. Similarly, normal biodistribution studies of the ^{99m}Tc-complex in Wistar rats showed specific myocardial uptake as it diminished on inhibition with *m*IBG and desmethylimipramine. Thus the present ^{99m}Tc-radiotracer holds high potential for NET imaging as a substitute for *I-*m*IBG *in vivo*.

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