Chapter 4
Bivalent Ligand Approach to Synthesise MPP based SPECT Imaging Agent

Over the past few decades the understanding of GPCR structure and function has revealed that GPCRs are able to form homo- and hetero-oligomeric complexes. The “dimerization–oligomerization” concept for G protein-coupled receptors (GPCRs) was widely accepted by the end of 1990s based on the research on GABAB receptor from several groups, and was further supported by the X-ray crystal structures of some others later on [1-2]. The dimerization–oligomerization of GPCRs poses a differentiated pharmacology from the monomers [3-7]. The existence of homo- and heterodimers has been demonstrated for several classes of receptors including opioid receptors [8-15], adrenergic receptors [16], somatostatin receptors [17-18], dopaminergic receptors [19-21], muscarinic receptors [22,23], and the histamine receptor subtypes [24–31]. Moreover, receptor dimerization is often essential for receptor function and can also modulate ligand pharmacology. According to very recent findings, homodimers of serotonin receptors might be of particular importance in the pathophysiology of schizophrenia and, thus, serve as promising targets for the discovery of atypical antipsychotics [32]. Investigating postmortem striatal sections from schizophrenia patients and striatal tissue of animal models of schizophrenia, the expression of serotonin dimers proved to be significantly enhanced, while expression of dopamine monomers was decreased. A highly attractive approach to investigate and control GPCR dimerization may be provided by the exploration and characterization of bivalent ligands, which can act as molecular probes simultaneously binding two adjacent binding sites of a dimer.

The term bivalent ligand is widely used and refers to molecules containing two sets of pharmacophoric entities linked through a spacer [33–35]. There are two general classes of bivalent ligands: homobivalent ligands, containing two identical pharmacophores, and heterobivalent ligands, wherein the two pharmacophores are different. It is assumed that duplication of the pharmacophoric groups according to the bivalent ligand approach leads to a supra-additive increase in potency compared to the corresponding monovalent ligand. The bivalent ligand approach in the design of ligands targeting
GPCRs has proven to be promising to improve not only potency and selectivity but also the pharmacokinetic profile of compounds [36]. The rationale for employing the bivalent ligand approach stems from the possibility that dimeric structures may be capable of bridging independent recognition sites (i.e., two recognition sites on a receptor dimer or one receptor and an accessory site) resulting in a thermodynamically more favorable binding interaction than a monovalent binding of two molecules, thus giving enhanced activity. [37-39]. Portoghese and coworkers applied first this approach in the field of opioid research, obtaining excellent results in terms of affinity and selectivity among opioid receptor subtypes. This concept has been applied also to 5-HT1B/1D agonists, 5-HT4 ligands, serotonin reuptake inhibitors, muscarinic agonists, and melatonergic ligands [40-45].

Several other studies have also previously reported that dimerization can result in an increase of potency/ selectivity and, interestingly enough, can also improve resistance to degradation in the case of peptide agonists or antagonists [46-51]. In the field of monoamine neurotransmitters, however, only a few examples of bivalent ligands have been documented to date.

Cindy Horwedel et al have generated homodimers of artesunic acid molecules and heterohybrids of artesunic acid and betulin. The activity of these substances has been analyzed in a human leukemia cell line (CCRF-CEM) and its multidrug-resistant subline, CEM/ ADR5000, and compared to doxorubicin [52].
Serge Halazy et al have reported serotonin dimers 2 in which two serotonin moieties are linked together through their 5-hydroxyl residue. The dimer proved to be very potent ligand at 5-HT1B/1D receptors with increased binding and selectivity [53].

![Diagram of serotonin dimer 2]

Julia Kuhhorn et al have synthesized bivalent dopamine D2 receptor ligands 3 by incorporating the privileged structure of 1,4-disubstituted aromatic piperidines/piperazines (1,4-DAPs) and triazolyl-linked spacer elements. They have also reported radioligand binding studies and performed comparative analysis with respective monomers and unsymmetrically substituted analogues indicating a bivalent binding mode with a simultaneous occupancy of two adjacent binding sites [54].

![Diagram of dopamine D2 receptor ligands 3]

Daniela Huber et al have synthesized ligand 4 as bivalent ligands for D2 receptors. Merging two arylamidoalkyl substituted phenylpiperazines as prototypical recognition elements for dopamine D2-like receptors by oligoethylene glycol linkers led to a series of bivalent ligands. These dimers were investigated in comparison to their monomeric analogues for their dopamine D2long, D2short, D3 and D4 receptor binding. Radioligand binding experiments revealed strong bivalent effects for some para-substituted benzamide derivatives [55].
Tobias Birnkammer et al have utilized bivalent ligand approach by connecting pharmacophoric 3-(2-amino-4-methylthiazol-5-yl)-, 3-(2-aminothiazol-5-yl)-, 3-(imidazol-4-yl)-, or 3-(1,2,4-triazol-5-yl)propylguanidine moieties by NGacylation with alkanedioic acids of various chain lengths to develop highly potent and selective acylguanidine-type histamine H2 receptor agonists 5. They investigated the compounds for H2R agonism in GTPase and [35S]GTPγS binding assays at guinea pig (gp) and human (h) H2R-GsαS fusion proteins including various H2R mutants, at the isolated gp right atrium, and in GTPase assays for activity on recombinant H1, H3, and H4 receptors [56].

Yunyun Yuan have developed bivalent ligand with 21-atom spacer as a molecular probe to study the biological and pharmacological mechanisms of the putative heterodimerization between the mu opioid receptor and the chemokine receptor CCR5. The preliminary biological data from the calcium mobilization assay and MOR–CHO binding assay showed that the bivalent ligand 6 retained the characteristics of its pharmacophores, antagonizing MOR and/or CCR5 respectively [57].
4.2. Mechanism Supporting the Approach

Several theories have been proposed to rationalize bivalent ligand activity and selectivity. Among them, two potential bridging mechanisms have been proposed from the extensive studies of bivalent ligands in the opioid receptors field. First, if the spacer is of sufficient length, it may be possible for both pharmacophores in a bivalent ligand to occupy distinct but very similar or identical neighboring recognition sites; a second possible mechanism involves the bridging of the second pharmacophore of a bivalent ligand to an adjacent accessory site which is unique to the receptor system. Thus, the ligand can simultaneously interact with two primary (orthosteric) binding sites of two neighboring protomers. Alternatively, the bivalent ligand may address a primary binding site and a secondary (allosteric) binding site located in close proximity of an adjacent GPCR or at the identical protomer leading to a bitopic or dualsteric binding mode [58]. Bivalent ligands binding neighboring binding sites of two physically interacting GPCRs may serve as pharmacological tools to study the quaternary structure of receptor dimers and to gain insights into the role of GPCR oligomerization on biosynthesis, receptor function and internalization. On the other hand, bivalent ligands with a dualsteric binding mode facilitate the generation of subtype-selective agonists or antagonists because allosteric regions are frequently less conserved than the orthosteric binding pocket, which is usually very similar for all subtypes of a receptor family.

In this chapter, we describe the synthesis of homodimeric serotonin receptor ligands for SPECT imaging using arylpiperazines.

Arylpiperazines have high affinity for the 5-HT1A receptor. The lead compound known as WAY 100635 (1) is a potent and selective 5-HT1A receptor antagonist [59-60]. Aromatic ring system and basic nitrogen containing piperazine makes it primary recognition scaffold for 5HT1A receptors binding site. The presence of basic nitrogen in the piperazine moiety can be exploited for functionalisation with long chain linker. Long chain alkylation enhances the affinity towards these receptors. WAY-type molecules have been labelled with positron emitters $^{11}$C and $^{18}$F as a way to study the role of this receptor in various diseases and several major neuropsychiatric disorders such as depression, eating disorders and anxiety [61-67]. The majority of agents used to examine 5-HT1A are PET tracers including $[^{11}$C]WAY100635 and $p-[^{18}$F]MPPF. Advances in SPECT research resulted in the discovery of $p-[^{123}$I]MPPI (Figure 4.1).
Although promising, PET compounds, particularly $^{11}\text{C}$ compounds, are generally limited to those facilities with a cyclotron and the high cost of $^{123}\text{I}$ hinders commercialization of viable agents which is driving the need to develop $^{99m}\text{Tc}$-based agents that can target 5-HT1A. Progress in receptor pharmacology with PET radiotracers has led to the design of numerous technetium-99m candidate complexes. Fragments of WAY100635, (i.e. arylpiperazine), have been combined with various bifunctional chelates consisting of a ligand that binds to technetium-99m and a linker that joins to the targeting vector [68-73]. For example, Johannsen et al. prepared a WAY-based complex using an $N_2S_2$ chelate to bind to $^{99m}\text{Tc}$. Other similar derivatives of arylpiperazine linked to other technetium(V) chelates ($N_xS_y$ or $N_xP_y$, $x = 1-4$, $y = 4-x$) have also been developed. Although their IC$_{50}$ and Ki values appear promising, these agents have low or negligible brain uptake [74-82].

To date, no $^{99m}\text{Tc}$ labelled compound has been developed which can image the 5-HT1A receptor in vivo. The failure is due to insufficient brain uptake and the inability of compounds to achieve high receptor binding affinity and selectivity.

$^{99m}\text{Tc}$ is the most desirable radionuclide for diagnostic nuclear medicine. On average over 80% of the radiopharmaceuticals used in clinics are labeled with $^{99m}\text{Tc}$, the
metastable nuclear isomer of the long-lived $^{99m}\text{Tc}$ in the ground state which is obtained in weighable amounts from the fission of uranium-235 in nuclear reactors. It possesses almost optimal nuclear properties and is conveniently available from commercial generator columns. The physical half-life of $^{99m}\text{Tc}$ of only 6 h and the absence of tissue-damaging corpuscular radiation allows the injection of activities of more than 30 mCi with low radiation exposure of the patient. The half-life is however, long enough to carry out the labeling syntheses and the scintigraphic measurements without significant losses in activity. On the one hand the energy of the emitted photons of 140 keV is sufficient to study the organs lying deep in the body. Technetium is a second row transition metal, so it needs to be bound to a metal-binding chelator. Considering the above facts we have selected diethylene triamine pentaacetic acid as the chelator. It provides two fold advantage. The chemical method of incorporating $^{99m}\text{Tc}$ influences the radiochemistry as well as the biological properties of the agent. Acyclic polyaninopolycarboxylates such as DTPA can be used as bifunctional chelating (BFC) agents of rhenium and technetium for labeling bioactive ligands. A number of compounds of DTPA conjugated with antibodies are showing excellent results in clinical trials [83-86]. Choosing the right BFC can greatly affect the radiolabeling kinetics and conditions, increase the resulting compound’s stability, and influence, to some degree, the pharmacokinetics. DTPA derivatives have favourable renal route of excretion. In addition, the anhydride derivatives of DTPA are generally easier to synthesize owing to the ease of commercial availability of DTPA. In this study, we have synthesized bis derivative of DTPA using long chain arylpiperazine which ensures formation of thermodynamically stable and kinetically inert complex with $^{99m}\text{Tc}$. This robust $^{99m}\text{Tc}$ labeling strategy would be a substantial method for preparation of $^{99m}\text{Tc}$ complexes. Two methoxyphenylpiperazine moiety can easily be conjugated to yield homodimeric bivalent ligand for 5HT1A receptors.

4.3. Objectives

1-(2-methoxyphenyl)piperazine constitute the prime structural component of potent and selective ligands designed for serotonin receptors. Thus, there has been a progressing interest in developing 1-(2-methoxyphenyl)piperazine based ligands that can target specific receptors involved in various neuropsychiatric conditions.
We present an efficient strategy utilizing the reliable and efficient synthesis of bivalent ligand of 1-(2-methoxyphenyl)piperazine DTPA-bis(MPBA) in good yield. The design of the ligand has been made on the basis of structure activity relationship studies reported in the literature for enhanced affinities towards the target receptors.

The synthesis includes conjugation of two amino derivatised MPP to DTPA bis anhydride to yield bivalent ligand DTPA-bis(MPBA) (Figure 4.2). All intermediates and final compounds have been characterized by spectroscopic techniques, namely, $^1$H, $^{13}$C NMR and MS. Radiocomplexation has been performed with $^{99m}$Tc to analyze the biodistribution of the ligand. Cytotoxicity and cell uptake studies have been performed in HEK cell line.

4.4. Experimental

4.4.1. Synthesis

Figure 4.2: Synthesis of 5,8-bis(carboxymethyl)-15-(4-(2-methoxyphenyl)piperazine-1-yl)-2-(2-(4-(4-(2-methoxyphenyl)piperazine-1-yl)butylamino)-2-oxoethyl)-10-oxo-2,5,8,11-tetraazapentadecane-1-carboxylic acid.
Synthesis of 5,8-bis(carboxymethyl)-15-(4-(2-methoxyphenyl)piperazine-1-yl)-2-(2-(4-(2-methoxyphenyl)piperazine-1-yl)butylamino)-2-oxoethyl)-10-oxo-2,5,8,11-tetraazapentadecane-1-carboxylic acid (1)

To a stirring solution of DTPA anhydride (338 mg, 0.9 mmol) in 10 mL anhydrous DMF and 5 mmol of triethylamine was added dropwise. The reaction temperature was allowed to reach 55°C. This was followed by dropwise addition of 4-(4-(2-methoxyphenyl)piperazine-1-yl)butan-1-amine (500 mg, 1.89 mmol). The reaction completion was confirmed by running TLC in ammonium hydroxide and methanol (3:2). Triethylamine and DMF were evaporated under reduced pressure using a rotavaporator. The final product was precipitated using acetone and was well characterized by $^1$H, $^{13}$C NMR and MS. $^1$HNMR (400 MHz, 25°C, CDCl$_3$), $\delta$ (ppm): 1.49 (m, 2H, CH$_2$), 1.608 (m, 2H, CH$_2$), 2.73-3.74 (m, 22H, NCH$_2$CH$_2$N), 3.75 (s, 3H, OCH$_3$), 6.90-7.07 (m, 4H, ArOCH$_3$); $^{13}$CNMR (100 MHz, 25°C, CDCl$_3$), $\delta$(ppm): 22.02 (CH$_2$CH$_2$), 27.26 (CH$_2$CH$_2$), 41.80 (NHCH$_2$), 48.64 (NCH$_2$CH$_2$N), 51.96 (NCH$_2$CH$_2$N), 55.27 (CH$_2$CH$_2$), 58.53 (OCH$_3$), 109.85 (ArCH), 116.44 (ArCH), 120.92 (ArCH), 122.84 (ArCH), 141.68 (ArC-O), 152.17 (ArC-N); MS (ESI$^+$) m/z calcd for C$_{44}$H$_{69}$N$_9$O$_{10}$ 884.07, found [M - H]$^+$ 883.8.

4.4.2. Cytotoxicity Studies of DTPA-bis (MPBA)

4.4.2.1. Viability Assay: Trypan Blue Exclusion Assay

Cell suspension of monolayer culture of U-87MG 1x10$^6$ cells were prepared and 1:1 dilution of the suspension using a 0.4% trypan blue solution was done after 2h treatment with the compound. 10 µL of cell suspension was loaded on a hemocytometer. The number of stained cells and total number of cells were counted and percentage viability was calculated.

4.4.2.2. Macrocolony Assay

Monolayer culture of U-87MG cell line was trypsinized and 100 to 1000 cells were plated depending upon the concentrations of the agent in 60-mm petridishes and incubated at 37°C in 5% CO$_2$ humidified atmosphere for 8 days. Colonies were fixed in methanol and stained with 1% crystal violet. More than 50 Colonies were counted.
4.4.2.3. MTT Assay

To test the cytotoxic effect of DTPA-bis(MPBA) exposure on cells, MTT assays were performed. Briefly, exponentially growing cells were plated in a 96 well microtitre plate at a cell density of 4000 cells/well 24 h before treatment. Cells were treated with the varying concentrations of the drug at different time intervals 24 h, 48 h, 72 h and MTT assays were done. At the end of treatment, both the treated cells and negative control were incubated with MTT at a final concentration of 0.05 mg/ml for 2 h at 37°C and the medium was removed. Triplicate wells from each treatment were lysed and the formazan crystals were dissolved using 150µl of DMSO. Optical density on 150 µl of extracts at 570 nm was measured (reference filter: 630 nm). Surviving fraction at (0.00001-10 mM) concentration range was plotted against concentration for DTPA-bis(MPBA).

4.4.3. Radiolabeling of DTPA-bis(MPBA) \((^{99m}\text{Tc}\text{ DTPA-bis(MPBA)})\)

2 mg of an aqueous solution (100 uL) of DTPA-bis(MPBA) was added to100 µL of stannous chloride (100 µg; 1 mg dissolved in 1 ml 10% acetic acid).Using 0.1 M sodium carbonate solution the pH of the reaction mixture was adjusted to 7. This was followed by addition of 80 MBq of freshly eluted (< 1h old) \(^{99m}\text{Tc}\text{ pertechnetate saline solution and the mixture was maintained at 25°C for 15 min. Before purification the radiochemical purity was 88– 95% determined by HPLC (Nucleosil ODS [250 x 4 mm], at a flow rate of 1 mL/min, isocratic gradient mixture of 80% methanol and 20% 0.01M phosphate buffer (pH 7.4; Rt). During radiocomplexation of the chelate standard safety methods were employed.}

4.4.4. Radiochemical Purity of \(^{99m}\text{Tc DTPA-bis(MPBA) Conjugate}\)

Instant thin layer chromatography on ITLC-SG (Paul German, USA) using 100% acetone, ternary mixture of pyridine: acetic acid: water (3:5:1.5) and 0.9% saline as the mobile phase was used to determine the radiolabeling efficiency. The TLC was cut in 0.5 cm fragments and counts of each segment were taken. The percentage of free Na\(^{99m}\text{TcO}_4\) and complexed \(^{99m}\text{Tc}\) was calculated. \(^{99m}\text{Tc}\) DTPA-bis MPBA conjugate remained at the origin while free \(^{99m}\text{Tc}\) travelled with the solvent front in acetone. The
radiochemical purity was assessed by omniscan EZ-TLC scanner and then the radiolabeled conjugate was purified using a C-18 reversed phase extraction cartridge which was preconditioned with 20 mL methanol and subsequently activated with 30% methanol. The cartridge was successively rinsed with 5 mL distilled water and radiolabeled conjugate was eluted in 5 mL of 4% ethanol. The $^{99m}\text{Tc-DTPA}$-bis(MPBA) was reconstituted in saline, and filtered through a sterile 0.22 µm Millipore (Milford, MA) Millex-GV® disposable syringe filter. Radiotracer purity and stability were monitored by radio-HPLC. In each HPLC analysis, the eluate was collected and counted along with a standard prepared from the injectate, to observe that the observed radiochromatogram reflected all the injected radioactivity.

4.4.5. Determination of the Partition Coefficient for the Complex

The partition coefficient of the complex was determined by measuring the activity that partitioned between the 1-octanol and aqueous phosphate buffer (0.025 mol/L, pH 7.4) under strict equilibrium conditions. 2mL 1-octanol and 2mL $^{99m}\text{Tc DTPA-bis(MPBA)}$ phosphate buffer were mixed in a centrifuge tube. The mixture was vortexed at room temperature for 5 min and then centrifuged at 5000 rpm/min for 5 min. The counts in 0.1 mL samples of both organic and inorganic layers were determined by a well type gamma counter. The measurement was repeated three times. The partition coefficient (P) was calculated using the following equation

$$P = \frac{\text{cpm in octanol-cpm in background}}{\text{cpm in buffer-cpm in background}}$$

The final partition coefficient value was expressed as log P.

4.4.6. Human Serum Stability Evaluation

Human serum was prepared by allowing blood collected from healthy volunteers to clot for 1 h at 37 °C in a humidified incubator maintained at 5% carbon dioxide/95% air. Then, the samples were centrifuged at 400 g, and the serum was filtered through 0.22 µm syringe filter into sterile plastic culture tubes. The radiolabeled DTPA-bis(MPBA) was immediately placed in a CO$_2$ chamber incubated at 37 °C and then analyzed to check for any dissociation of the complex. Percentage of free pertechnetate
at a particular timepoint that was estimated using acetone and pyridine, acetic acid, and water (PAW) (3:5:1.5) as mobile phase represented percentage dissociation of the complex at that particular time point in serum.

### 4.4.7. In vitro Receptor Binding of $^{99m}$Tc-DTPA-bis(MPBA)

$^{99m}$Tc-DTPA-bis(MPBA) was tested for in vitro affinity for serotonin 5-HT1A, 5-HT2A and D2 receptors by radioligand binding assays. The following specific radioligands and tissue sources were used: (a) serotonin 5-HT1A receptor, Serotonin, rat brain cortex; (b) serotonin 5-HT2A receptor, ketanserin, rat brain cortex; (c) D2 receptor, spiperone, rat brain cortex; (d) HTT, paroxetine. Non-specific binding was determined as described in the experimental section, and specific binding as the difference between total and non-specific binding. Blank experiments were carried out to determine the effect of 5% DMSO on the binding and no effects were observed. $K_d$, receptor dissociation constant ($K_d$ values, obtained by Scatchard analysis, were calculated for each labeled ligand).

### 4.4.8. Measurement of $^{99m}$Tc-DTPA-bis(MPBA) Uptake in Hippocampal Primary Cultures

Primary cultured cortical neuronal cells were obtained from the hippocampus of fetal Sprague Dawley rats at 17–20 days of gestation, according to previously described procedures [87]. In brief, single cells dissociated from the hippocampus of fetal rats were sited on the poly-L-Lysine coated culture slide at 3 x 10$^5$ cells/ well. Culture was incubated in DMEM (F-12) medium supplemented with 10% heat inactivated fetal bovine serum for 1–7 days after plating or with 10% heat inactivated horse serum for 8–15 days after plating, in each case together with glutamine 2.5 mM, glucose 17.5 mM, and NaHCO$_3$ 14.3 mM. The cultures were subsequently maintained at 37 °C in a humidified 5% CO$_2$ atmosphere. Three days subsequent to plating, non-neuronal cells were removed by addition of cytosine arabinoside at 10 µM. Only mature cultures surviving 15–18 days in vitro were used for the experiments. In the cell culture, the ratio of nerve to glial was found to be 80 to 20 approximately. After 18 days of culture in vitro, successful cultured neurons were selected for binding studies which were
carried out as described previously. The amount of radioactivity (CPM) in cell lysates was determined by gamma scintillation counting. Uptake was then calculated and expressed in units of pmoles µg protein$^{-1}$ min$^{-1}$.

Competitive binding of $^{99m}$Tc-DTPA-bis(MPBA) with unlabeled Serotonin was assessed and the dissociation constant was calculated. Final concentrations in the well were 0.01–10000 nM. Hippocampal cultures were washed with HBSS and were then incubated for 20 min in HBSS at 37 °C prior to the experiment. Binding experiments was conducted at 37°C. Cells were incubated for 30 min with increasing concentrations (0.01 nM–10 µM) of $^{99m}$Tc-DTPA-bis(MPBA) in the absence and presence of the 100 folds excess unlabeled serotonin to estimate the total binding and non-specific binding respectively. Specific binding was obtained by subtracting non-specific binding from total binding. At the end of each experiment, the cells were washed with cold PBS and 0.9% saline four times. The cell-associated radioactivity was determined by gamma scintillation counting.

4.4.8.1. 5-HT1A Receptor Binding Assay

The hippocampus of rat brain was homogenized in 10 volumes of ice-cold buffer (50 mM Tris-HCl pH 7.6) using an Ultra Turrax T10 (IKA). The homogenate was centrifuged at 20,000 g for 10 min. The resulting pellet was resuspended with the Ultra-Turrax and centrifuged again at 20,000 g for 10 min. The same procedure was repeated again. Then the pellet was resuspended in 10 volumes of buffer and stored at –80°C until used in binding studies.

The binding assay was carried out in a final volume of 2.5 mL Tris-HCl buffer (50 mM, pH 7.4, 0.1% ascorbic acid, 2 mM CaCl$_2$) containing membrane homogenate (about 0.5 mg/mL protein) and various concentrations of the $^{99m}$Tc-DTPA-bis(MPBA) complex. Nonspecific binding was defined as the amount of $^{99m}$Tc-DTPA-bis(MPBA) bound in the presence of 10 mM serotonin. The binding assay was performed in triplicates at 20°C for 120 min. The incubation was terminated by rapid filtration through GF/B glass fiber filters. The filters were rapidly washed with 4 mL portions of ice cold buffer, transferred into 4 mL scintillation cocktail and analyzed for radioactivity by scintillation counting.
4.4.8.2. 5-HT2A Receptor Binding Assay

The cortex of rat brain was homogenized and prepared analogously to the procedure described above and stored at –80°C until used in binding studies. The assay was performed in a volume of 5.0 mL Tris-HCl buffer (pH 7.6) containing 100 folds excess of ketanserin tartrate, membrane homogenate (about 0.9 mg/mL protein) and various concentrations of $^{99m}$Tc-DTPA-bis(MPBA). Triplicates of the samples were incubated at 20°C for 60 min. Filtration and counting of the samples were the same as described above.

4.4.8.3. D2 Receptor Binding Assay

The striatum of rat brain was homogenized and prepared analogously to the procedure described above and stored at –80°C until used in binding studies. The final volume of the binding assay was 5.0 mL Tris-HCl buffer (pH 7.4) containing 10 mM MgCl$_2$, 1 mM EDTA, membrane homogenate (about 0.2 mg/mL protein) and various concentrations of the $^{99m}$Tc-DTPA-bis(MPBA) complex, which were dissolved and diluted with buffer as described above. Non specific binding was determined by 100 fold excess of spiperone.

4.4.8.4. 5-HT Transporter Binding Assay

The caudate nucleus of rat brain was homogenized and prepared analogously to the procedure described above and stored at –80°C until used in binding studies. The final volume of the binding assay was 5.0 mL Tris-HCl buffer (pH 7.4) containing 120 mM NaCl, 5mM KCl, membrane homogenate (about 1.2 mg/mL protein) and various concentrations of the $^{99m}$Tc-DTPA-bis(MPBA) presence and absence of 100 fold excess of proxetine. Filtration and counting of the samples were the same as described above.

4.4.9. Blood Kinetics

In normal rabbit, 300 µL of the $^{99m}$Tc-DTPA-bis(MPBA) (11.1 MBq activity) was injected intravenously through the dorsal ear vein. Blood was withdrawn from the other ear vein at different time intervals starting from 5 min to 24 h. Persistence of activity in the circulation was calculated, assuming total blood volume as 7% of the body weight.
4.4.10. SPECT Acquisition

Non-invasively dynamic study was performed in Newzealand Rabbit (3.1Kg) at a time interval of 1 min upto 30 min and Semiquantitative analysis was carried out by generating ROI on brain and soft tissue. Tumor imaging was performed in PC3 cell line implanted tumor bearing athymic mice administering 100 µL of the labeled conjugate (5 µmol/Kg, 3.2 MBq activity). Images were taken using planar gamma camera HAWKEYE. Images were obtained at different time intervals starting from 1, 2 and 4h after post injection.

Additional SPECT acquisitions were done on GE TRIUMPH trimodality system. SPECT reconstruction was done on FLEX_SPECT TM version 1.0.7 in reconstruction mode OSEM (Ordered Subset Expectation Maximization). CT-reconstruction was straightforward analytical. All images were fused and analysed with VIVID (Amira®, San Diego, USA).

4.4.11. Biodistribution

In biodistribution study, intravenous injection of $^{99m}$Tc-DTPA-bis(MPBA) conjugate in a volume of 100 µL (5 µmol/kg, 3.7 MBq activity) was injected through the tail vein of each mice. At 10 min, 1, 2, 4, and 24 h post injection mice were dissected to take out different tissues which were weighed and counted in a gamma counter calibrated for $^{99m}$Tc energy. Uptake of the radiotracer in each tissue was calculated and expressed as percentage injected dose per gram of the tissue (%ID/g) (Table 1). Animal protocols have been approved by Institutional Animal Ethics Committee.

4.5. Result and Discussion

4.5.1. Synthesis of DTPA-bis(MPBA)
‘Bivalent ligand’ approach is employed as the dimeric structures are capable of bridging independent recognition sites resulting in a thermodynamically more favorable binding interaction than a monovalent binding for enhancing the affinity towards the receptor. We have designed the ligands according to the bivalent ligand approach with the aim to increase the affinity towards 5-HT1A receptors. Two molecules of 4-(4-(2-methoxyphenyl)piperazine-1-yl)butan-1-amine were successfully conjugated to DTPA bis anhydride yielding bis derivative of MPP [DTPA-bis(MPBA)] shown in Figure 4.2. All intermediates and the final compound were successfully characterized by spectroscopic techniques such as $^1$H, $^{13}$CNMR and MSThe conjugation of DTPA to 4-(4-(2-methoxyphenyl)piperazine-1-yl)butan-1-amine is confirmed by the presence of multiplet at δ 2.73-3.74 integrating for 22 protons in addition to two methylene signals at 1.47-1.61, four aromatic protons and an upfield singlet at 3.75 integrating for three protons of methoxy proton in $^1$HNMR spectrum of 1. In $^{13}$CNMR spectrum, three carbonyl peaks at 164.7, 176.6 and 178.8 supported similar observations. Peak obtained at 883.8 [M-H]$^+$ in MS (ESI) of 1 validates the synthesis of bis MPP derivative DTPA-bis(MPBA).

According to the recently described QSAR of bivalent GPCRs ligands, the biological characteristics are defined by the presence of aromatic piperidine/piperazine group which simulates the endogenous biogenic amine and a spacer unit between the dimers to be 25Å [88]. By conjugation with DTPA as a linker long chain alkyl (n=4) spacer arm was created for enhancing the affinity towards 5HT1A receptors.

4.5.2. Cell Viability Assay

Cytotoxicity assay was performed in order to define the biological profile of the synthesized compound. We observed that this derivative was not able to induce cytotoxicity in normal human embryonic kidney cell line at lower concentrations. At 1mM concentration 52% of the cells were killed at 2h treatment and the cells were found to regain proliferation at 24 h with surviving fraction of 0.796 ± 0.080. Surviving fraction of 0.469 ± 0.035 was seen at 10 mM concentration of the compound (Figure 4.3). With continuous treatment upto 48h at 10mM concentration, 0.55 ± 0.044 cell survival fraction was observed. Dose dependent and time dependent growth inhibition
of HEK cells with IC$_{50}$ values ranging between 1 and 10 mM were obtained by clonogenic assay. A direct correlation was seen in MTT and clonogenic assay. The above observations exhibits its useful imaging applications using nuclear medicine technique.

**Figure 4.3:** Antiproliferative effect of DTPA-bis(MPBA) in HEK cell line. Standard deviation (SD) was always less than 10%.

### 4.5.3. Radiolabeling and Quality Control of $^{99m}$Tc-DTPA-bis(MPBA)

The conjugate was labeled with $^{99m}$Tc, evaluated at in vitro and in vivo conditions for its competence. Radiochemical analysis depicted that the drug is specifically coordinating with $^{99m}$Tc radionuclide. The labeling yield was found to be greater than 95 %, as determined by paper chromatography using different solvent systems. The reaction mixture was kept in saline for various time intervals and in vitro stability studies were carried out. Percentage radiolabeling was calculated for 0, 2, 4, 6, and 24 h. Even up to 24 h, labeling efficiency was found to be 96.58%, implying that the labeled DTPA-bis(MPBA) conjugate was quite stable.

The radiochemical purity of $^{99m}$Tc-DTPA-bis(MPBA) exceeded 98% as determined by radio HPLC. Further the radiolabeled conjugate was analysed on Omniscan EZ-TLC scanner which showed peak at Rf= 0.74 for free technetium and Rf= 0.19 for $^{99m}$Tc-DTPA-bis(MPBA). After cartridge purification of $^{99m}$Tc DTPA-bis(MPBA) presence of free $^{99m}$Tc was not seen. Radio HPLC analysis of the purified product showed 99 %
radiochemical yield and presence of only one peak ($R_t = 7.8$ min) confirming the formation of only one species of the complex (Figure 4.4). Typically, 95% or more of the activity injected onto the HPLC (for analysis) was recovered in the eluate, ensuring that the radio HPLC results quantitatively reflected the radiochemical purity of the sample.

4.5.4. Human Serum Stability Evaluation

In vitro human serum stability studies under physiological conditions suggested that there was least transcomplexation of the labeled drug. Freshly separated human serum was incubated with the purified $^{99m}$Tc-DTPA-bis(MPBA) under physiological conditions. Radiolabeled conjugates were challenged with the proteins (albumin and transferrin) present in the serum to estimate the stability of the radiolabeled $^{99m}$Tc-DTPA-bis(MPBA). $^{99m}$Tc-DTPA-bis(MPBA) exhibited 1.6% transcomplexation of $^{99m}$Tc in serum. After 2 h challenge at 37 °C approximately more than 94% of the radioactivity remains associated with the conjugate (Figure 4.5).
4.5.5. Glutathione Challenge

To test the stability of the $^{99m}$Tc-DTPA-bis(MPBA) conjugate, it was challenged with 25-100 mM concentration of glutathione. $^{99m}$Tc-DTPA-bis(MPBA) showed 5.9 % transcomplexation of $^{99m}$Tc to 25 mM glutathione. 92 % of the radioactivity remained associated with the conjugate after 2 h challenge at 37 °C with glutathione challenge.

4.5.6. Determination of Lipophilicity, Log P

In order to develop a tracer for a receptor in the central nervous system, it needs to have high affinity and selectivity for the target receptor. Lipophilicity of the tracer is relevant for good blood-brain barrier (BBB) permeability but the lipophilicity should not be too high in order to avoid nonspecific binding to protein and lipids. An optimal lipophilicity (log P near 2) of drugs is desirable to cross the BBB. Therefore, it appears that there is an optimal range of lipophilicity for brain radioligands, wherein brain uptake is high and nonspecific binding is comparatively low. Radiolabeling of a potent ligand may lead to a new chemical entity with a different pharmacological profile as compared to the original compound. Same has been observed in the DTPA-bis(MPBA) conjugate and its complex with $^{99m}$Tc. Log P value increased on radiolabeling with $^{99m}$Tc. The radiolabeled conjugate revealed log P value of 2.445 whereas the DTPA-bis(MPBA) conjugate revealed a log P value of -1.04. There was a significant difference in log P values of unlabeled and complexed compound within experimental error.
4.5.7. Radioligand Binding Assay

Primary Hippocampal cells were cultured as reported in literature. The specificity of the methoxyphenylpiperazine functionalized compound to bind to the cell surface receptors on primary hippocampal culture cells was examined by cell uptake assays using $^{99m}$Tc-DTPA-bis(MPBA) as the labeled ligand. Non specific binding was obtained by using 100 fold excess of unlabeled serotonin. Analysis of the binding curve exhibited saturable binding of the radioconjugate in subnanomolar range. Scatchard plot analysis revealed that the labeled compound exhibited high affinity on hippocampal cultures with a $K_d$ value of 0.069±0.001nm (Figure 4.6).

![Figure 4.6: Scatchard plot of the specific binding data to the ratio of bound to free (B/F).](image)

To corroborate a receptor-chelating binding mode, Hill coefficient ($n_H$) was calculated, competition curve revealed steepening leading to Hill slopes between 1.9 and 2.3 which was suggestive of bivalent binding in which there is a release of two equivalents of radioligand and substantial steepening of the competition curve. Radioligand binding experiments normally lead to competitive binding curves that follow the law of mass action and, thus, show a Hill slope of one [89]. For bivalent ligands, a steepening of the competition curves is observed, compared to the respective monomer, resulting in Hill slopes that reach the value of 2.0. This indicates of a positive cooperative binding [90]. Bivalent ligands addressing two adjacent binding sites of receptor dimers will induce such cooperativity because binding of the second pharmacophore is significantly accelerated due to the vicinity of the ligand and the, thus, facilitated enrichment of local
concentration. In this case, bivalent binding leads to the liberation of two equivalents of radioligand and a substantial steepening of the competition curve (Figure 4.7).

![Representative binding curve of \(^{99m}\text{Tc-DTPA-bis(MPBA)}\) resulting in hill slope (absolute value of nH) in range of 1.9-2.3 indicative of bivalent binding mode.](image)

Figure 4.7: Representative binding curve of \(^{99m}\text{Tc-DTPA-bis(MPBA)}\) resulting in hill slope (absolute value of nH) in range of 1.9-2.3 indicative of bivalent binding mode.

The 5HT2A and D2 receptor affinities of \(^{99m}\text{Tc-DTPA-bis(MPBA)}\) were found lower than those observed for 5HT1A receptors in brain homogenates. A subnanomolar affinity for the 5-HT1A was obtained in in vitro binding assay which is sufficiently high to carry out imaging. The affinity for the 5-HT2A receptor was observed lower. The selectivity of the complex for 5-HT1A receptors is more than 1,000 times as high as it is for 5-HT2A receptors (Table 4.1). 5HTT also showed high affinity with a value of 46 ±0.9 nM.

Table 4.1: Dissociation constants of \(^{99m}\text{Tc-DTPA- bis(MPBA)}\) for 5-HT1A, 5-HT2A, D2, 5-HTT receptors

<table>
<thead>
<tr>
<th>Kd (nM)</th>
<th>5HT1A</th>
<th>5HT2A</th>
<th>D2</th>
<th>5HTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^{99m}\text{Tc-DTPA-bis(MPBA)})</td>
<td>0.27±0.08</td>
<td>128 ±3.2</td>
<td>1.2±0.47</td>
<td>46 ±0.9</td>
</tr>
</tbody>
</table>

*Non specific binding was determined by serotonin, ketanserin, spiperone and peroxetin.

4.5.8. Blood Clearance Studies

The blood clearance studies of the radioconjugate DTPA-bis(MPBA) depicted a rapid clearance from the circulation as 82.72 % of injected activity cleared from the circulation,
at 30 min post administration. The maximum percentage of radioactivity in the blood was found to be 21.84% at 15 min post injection. After 4 h, the percentage radioactivity in the blood decreased further to 6.12±0.15% and at 24 h approximately 0.6±0.06% activity remained in the blood (Figure 4.8). Hence, the blood clearance followed a biphasic trend with a rapidly clearing initial phase followed by slow phase. The biological half-life obtained was $t_{1/2}$ (Fast): 10 min; $t_{1/2}$ (Slow): 6h and 6 min. Plasma clearance expresses the overall ability of the body to eliminate a drug by the drug elimination rate (amount per time) by the corresponding plasma concentration level. Clearance rate ($C_R$) was revealed in plasma clearance with a value of 4.86 µg.ml.min$^{-1}$ (Figure 4.9).

**Figure 4.8:** Blood clearance of $^{99m}$Tc-DTPA-bis(MPBA) (11.1 MBq activity) administered through ear vein in normal rabbit

**Figure 4.9:** Semi-logarithmic plot of Plasma concentration, (µg/mL), against time in hour after administration of (110µg/kg) in normal Rabbit, and blood samples were taken at 5, 15, 30, 60, 120, 240, and 1440 min post injection.
4.5.9. Scintigraphy in Animal Models

Significant accumulation of radioconjugate was observed in the Rabbit brain in dynamic lateral planar images indicating the role of MPP functionalized compound further characterizing and elucidating the physiological roles of 5-HT1A receptors. The images were acquired on HAWKEYE gamma camera for 20 minutes. The early phase showed high uptake in the whole brain and wash out of the activity was observed in 30 min. (Figure 4.10).

Figure 4.10: Summed dynamic lateral planar images of a normal rabbit upto 30 min post injection of $^{99m}$Tc-DTPA-bis(MPBA). The early phase shows high uptake in the whole brain.

Time activity curve for brain and blood in rabbit from the data of dynamic SPECT is shown in Figure 4.11. High accumulation in brain was observed at 10 min with 10% uptake.
Bioimaging in PC3 grafted athymic mouse was done as 5HTRs expression has been identified in PC3 cells [23]. Semiquantitative analysis was generated from Region Of Interest (ROI) placed over areas counting average counts per pixels with maximum radiotracer uptake on the tumor was found to be $16.45 \pm 2.09$ and compared with muscle ROI ($0.25 \pm 0.34$) at 1 h. The tumor-to-contralateral muscle tissue ratio of $^{99m}$Tc-DTPA-bis(MPBA) was found to be $65.8 \pm 3.3$ at 1 h (Figure 4.12). In the mice that received coadministration of 500 µg of blocking dose of serotonin revealed the uptake in the tumor as $0.78 \pm 0.41$ %ID/g which was significantly lower at 1h post injection attributed to specific binding.

Additional SPECT acquisitions were performed using GE Triumph Trimodality imaging system as non-invasive technology. N5F75A10 multipinhole collimator, mouse style with 1mm aperture was used to acquire the image in PC3 tumor grafted athymic mice. Tomo with pre-defined time frame, a series of circular tomographic images were acquired. Begining of accumulation of radioactive DTPA-bis(MPBA) at tumor site was observed as early at 15 min. At 1h post administration the imaging was done which revealed substantial amount of $^{99m}$Tc labeled DTPA-bis(MPBA) concentration (Figure 4.13).
Figure 4.12: Whole-body γ image of female athymic mice with subcutaneous PC3 tumor in the right hind leg at 1 h and 2h

Figure 4.13: Reconstructed Coregistered Anterior and Posterior SPECT/CT images of $^{99m}$Tc-DTPA-bis(MPBA) in PC3 xenografted athymic mice
This may serve as the important parameter to guide in vivo evaluation of radiolabeled MPP derivatives as SPECT biomarkers in tumor models.

### 4.5.10. Biodistribution

The in vivo biodistribution of $^{99m}$Tc-DTPA-bis(MPBA) was studied in athymic mice at different time intervals and is illustrated in Table 4.2. Rapid accumulation was seen in brain in 10 min in mice with $2.07\pm0.76$ % ID/g. It was observed that the radiolabeled complex rapidly cleared from the blood stream with only $0.86 \pm 0.13$ %ID/g remaining in the blood after 24 h in comparison to $3.88\pm 0.61$ % ID/g at 2 h. Major accumulation of the labeled compound was observed in kidneys ($15.03 \pm 2.73$ % ID/g) followed by liver ($5.82 \pm 1.06$ % ID/g) at 1 h showing that the complex is excreted mainly by renal routes.

**Table 4.2:** Biodistribution studies of $^{99m}$Tc-DTPA-bis(MPBA) in athymic mice

<table>
<thead>
<tr>
<th>Organ</th>
<th>10 min</th>
<th>1h</th>
<th>2h</th>
<th>4h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>3.76±0.62</td>
<td>3.88±0.61</td>
<td>3.05±0.54</td>
<td>2.42±0.38</td>
<td>0.86±0.13</td>
</tr>
<tr>
<td>Brain</td>
<td>2.07±0.76</td>
<td>0.15±0.03</td>
<td>0.11±0.02</td>
<td>0.09±0.02</td>
<td>0.04±0.01</td>
</tr>
<tr>
<td>Heart</td>
<td>1.24±0.30</td>
<td>1.23±0.31</td>
<td>0.98±0.29</td>
<td>0.67±0.15</td>
<td>0.29±0.06</td>
</tr>
<tr>
<td>Lungs</td>
<td>1.62±0.44</td>
<td>2.86±0.47</td>
<td>1.14±0.45</td>
<td>0.83±0.16</td>
<td>0.42±0.11</td>
</tr>
<tr>
<td>Liver</td>
<td>0.39±0.08</td>
<td>5.82±1.06</td>
<td>4.93±0.98</td>
<td>2.8±0.76</td>
<td>1.2±0.24</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.86±0.23</td>
<td>1.61±0.43</td>
<td>1.3±0.27</td>
<td>0.78±0.13</td>
<td>0.26±0.04</td>
</tr>
<tr>
<td>Kidneys</td>
<td>13.2±0.99</td>
<td>15.03±2.73</td>
<td>7.2±1.26</td>
<td>4.32±1.39</td>
<td>2.14±0.52</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.22±0.06</td>
<td>0.76±0.21</td>
<td>0.58±0.15</td>
<td>0.5±0.13</td>
<td>0.3±0.05</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.12±0.03</td>
<td>1.2±0.38</td>
<td>0.91±0.21</td>
<td>0.82±0.23</td>
<td>0.46±0.12</td>
</tr>
<tr>
<td>Bone</td>
<td>0.52±0.14</td>
<td>0.17±0.05</td>
<td>0.13±0.03</td>
<td>0.1±0.02</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.12±0.04</td>
<td>0.48±0.11</td>
<td>0.32±0.08</td>
<td>0.25±0.07</td>
<td>0.04±0.01</td>
</tr>
<tr>
<td>Tumor</td>
<td>1.89±0.09</td>
<td>5.24±0.5</td>
<td>5.98±0.28</td>
<td>4.23±0.66</td>
<td>2.04±0.44</td>
</tr>
</tbody>
</table>

Retention of radioactivity in the non target organs (lungs, spleen, intestine etc.) was low and very less background activity was observed after 24 h. Biodistribution studies in athymic mice xenografted with PC3 prostate carcinoma cell lines have shown that tumor uptake of $^{99m}$Tc-DTPA-bis(MPBA) was found to be $5.24 \pm 0.5$ % ID/ g at 1 h. Localization of the radioactivity in the liver, stomach, and small intestine was low, less than 5% ID/g at 4 h for $^{99m}$Tc-DTPA-bis(MPBA). Rapid clearance of the radiolabeled conjugate was also observed from these organs (Table 4.2).
4.5.11. Regional Brain Distribution in Rat

Whole brain section of rat showed rapid accumulation in 10 min attaining 2.81 % ID/g of the total injected dose. In post-mortem rat brain hippocampus and cerebral cortex showed highest uptake followed by thalamus and caudate putamen and a weaker uptake in cerebellum at 10 min and olfactory bulb. High uptake hippocampus and cerebral cortex accredited to the 5-HT1A receptor rich regions (Figure 4.14). Binding of $^{99m}$Tc-DTPA-bis(MPBA) to cerebellum was observed at 30 min which might be attributed to the tissue heterogeneity. Persistence of activity for 30 min was observed and the decline of the radioactivity was seen 60 min post injection.

![Figure 4.14: Regional brain distribution of $^{99m}$Tc DTPA-bis(MPBA) in rat brain at 10, 30 and 60 min](image)

WBS = Whole Brain Section; OB = Olfactory Bulb; CPu = Caudate Putamen; Hip = Hippocampus; Th = Thalamus; Ctx = Cortex; Cb = Cerebellum

4.6. Conclusion

Taking advantage of bivalent approach, a homodimeric system was designed and developed with reasonable pharmacokinetics for 5HT1A receptor imaging. We have synthesised a bivalent radioligand DTPA-bis(MPBA) for imaging 5HT1A receptors which could also be utilized in the determination of 5HTRs expression in refractory prostate cancer tissues. Radioligand binding assays demonstrated that the labeled DTPA-bis(MPBA), a bivalent ligand exhibited a distinct binding profile with steepening of the competitive binding curve for 5HT1A receptors in primary hippocampal cultures. These finding suggest that $^{99m}$Tc-DTPA-bis(MPBA) holds promise for clinical application in nuclear medicine.
4.7. Characterization Data

Spectrum 1

$^1$HNMR spectrum of 5,8-bis(carboxymethyl)-15-(4-(2-methoxyphenyl)piperazine-1-yl)-2-(2-(4-(4-(2-methoxyphenyl)piperazine-1-yl)butylamino)-2-oxoethyl)-10-oxo-2,5,8,11-tetraazapentadecane-1-carboxylic acid (I)

Spectrum 2

$^{13}$CNMR spectrum of 5,8-bis(carboxymethyl)-15-(4-(2-methoxyphenyl)piperazine-1-yl)-2-(2-(4-(4-(2-methoxyphenyl)piperazine-1-yl)butylamino)-2-oxoethyl)-10-oxo-2,5,8,11-tetraazapentadecane-1-carboxylic acid (I)
Spectrum 3

Mass spectrum of 5,8-bis(carboxymethyl)-15-(4-(2-methoxyphenyl)piperazine-1-yl)-2-(2-(4-(4-(2-methoxyphenyl)piperazine-1-yl)butylamino)-2-oxoethyl)-10-oxo-2,5,8,11-tetraazapentadecane-1-carboxylic acid (1)

![Mass spectrum image](image-url)
4.8. References


[54] Yunyun Yuan, Christopher K. Arnatt, Guo Li, Kendra M. Haney, Derong Ding, Joanna C. Jacob, Dana E. Selleyb and Yan Zhang. Design and synthesis of a bivalent ligand to explore the putative heterodimerization of the mu opioid receptor and the chemokine receptor CCR5. Org Biomol Chem, 2012, 10, 2633


