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Abstracts


A homodimeric bivalent radioligand derived from 1-(2-methoxyphenyl)piperazine with high affinity for in vivo 5-HT1A receptor imaging†

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The bivalent ligand approach is very promising and can give rise to molecular probes with enhanced conformational flexibility for tissue selective modulation. In this study we describe the synthesis of a homodimeric 5-HT1A receptor ligand by incorporating two identical pharmacophores, 1-(2-methoxyphenyl)piperazine, linked through DTPA. This bivalent derivative was efficiently synthesized and characterized by 1H, 13C NMR and mass spectroscopy. 99mTc-labeling was performed with a high radiolabeling yield (>95%) and radiochemical purity (>98%) using very low ligand concentration. In vitro binding assays in rat hippocampal cultures demonstrated the high affinity of the complex for 5-HT1A receptors. Further studies include in vivo organ distribution and gamma scintigraphy carried out in rat and rabbit. Plasma clearance rate (Ck) revealed a value of 4.86 µg mL⁻¹ min⁻¹ in normal rabbit. Dynamic imaging performed in rabbit showed the beginning of radiolabeled uptake in brain as early as 2 min. The tumor-to-contralateral muscle tissue ratio of 99mTc–DTPA–bis(MPBA) in athymic mice with PC3 xenograft was found to be 65 ± 3.3 at 1 h. Significant accumulation was seen in mice and rat brain at 10 min with 2.07 ± 0.76% ID g⁻¹ and 2.81% ID g⁻¹ respectively. The high uptake in hippocampus and cerebral cortex was accredited to the 5-HT1A receptor rich regions in post mortem rat brain. This imaging agent holds a promising future in imaging 5-HT1A receptors for the effective diagnosis of neuropathological disorders.

Introduction

5-HT receptors are a group of G-protein coupled receptors (GPCRs) found in the central and peripheral nervous systems.1 One of the serotonin receptor subtypes 5-HT1A, is highly implicated in Alzheimer’s disease, schizophrenia, anxiety, depression, hallucinogenic behavior, motion sickness and eating disorders.2 GPCRs can cross-react and form homo or heterodimers or higher order oligomers which are often essential for modulation/regulation of receptor function. In this paper we describe the synthesis of homodimeric 5-HT1A receptor ligand by incorporating two identical pharmacophores linked through a spacer or a chelator for coordinating metal ion for imaging. Being capable of bridging independent recognition sites, the dimeric structure would express thermodynamically more favorable ligand interactions than a monovalent binding thus resulting in enhanced activity by binding simultaneously to two binding sites of a dimer. This approach was first employed by Portoghese and co-workers in the field of opioid research and excellent results were obtained in terms of affinity and selectivity among opioid receptor subtypes. It has also been applied to 5-HT1B/1D agonists, 5-HT4 ligands, muscarinic agonists, serotonin reuptake inhibitors and melanergic ligands.3–9

For diagnostic applications there is a great interest in the development of highly selective and high affinity radioligands for this receptor as brain imaging agents. For studying the location and density of 5-HT1A receptor there are several 11C- and 18F-labeled ligands for positron emission tomography (PET) imaging.10–12 Despite having higher resolution, their low adoption is attributed to the dependency of this imaging modality on a nearby cyclotron for 11C and 18F production and the more complex chemistry needed to incorporate these molecules into...
the radiopharmaceutical. The wide use of single photon emission computed tomography (SPECT) imaging can be credited to the convenience of the $^{99m}$Tc generator, simplicity of the kit chemistry and longer half-life of $^{99m}$Tc which broadens the observational time window.

 Arylpiperazines constitute the most important classes of the 5-HT1A receptor ligands. The most commonly studied agents are buspirone, gepirone, NAN-190, flesinoxan, WAY-100135, and WAY-100635. WAY-100635 (N-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-N-(2-pyridyl)-cyclohexane carboxamide) is reported in the literature as one of the first potent, silent and selective antagonists for serotonin 5-HT1A receptors. The moiety present in WAY-100635, 1-(2-methoxyphenyl)piperazine is established to have high affinity for the 5-HT1A receptors and provides a good starting lead for the development of potential radiotracers. The aromatic ring system and basic nitrogen containing piperazine makes it a primary recognition scaffold for 5-HT1A receptor binding sites. The presence of basic nitrogen in the piperazine moiety can be exploited for functionalisation with a long chain linker. Long chain alkylation enhances the affinity towards these receptors.13

For the development of $^{99m}$Tc complexes, considerable progress has been made in the last few years, primarily based on WAY-100635, in performing SPECT imaging and in vitro studies of 5-HT1A receptors. Various approaches for attaching the technetium to the pharmacophore moiety of WAY-100635 are reported in literature. These include the tetradentate N$_2$S$_2$ chelate approach, mixed ligand complex approach and the tricarbonyl approach.14–18 The chemical method of incorporating $^{99m}$Tc influences the radiochemistry as well as the biological properties of the agent. Acyclic polyanilinopoly-carboxylates 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 have shown excellent results in clinical trials.19–21 Choosing the right BFC can greatly affect the radiolabeling kinetics and conditions and can 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 a bis-derivative of MPP using long chain arylpiperazine which ensures formation of a thermodynamically stable and kinetically inert complex with $^{99m}$Tc. This robust $^{99m}$Tc labeling strategy would be a substantial method for preparation of $^{99m}$Tc complexes conjugated with two molecules forming bivalent ligands to image 5-HT1A receptors.

We hereby report the synthesis, characterization, and radiochemistry of a $^{99m}$Tc labeled DTPA–bis(MPBA) conjugate and its biological evaluation. Attachment of a strong chelating agent, DTPA, provides binding sites to which $^{99m}$Tc is strongly bound. Radiolabeling of the DTPA–bis(MPBA) conjugate with $^{99m}$Tc, stability studies in human serum under physiological conditions, and blood kinetics in rabbits were carried out. MTT and clonogenic assays were used to determine cytotoxicity. The in vitro efficiency of $^{99m}$Tc–DTPA–bis(MPBA) was assessed by cell uptake studies. In vivo studies included biodistribution studies and scintigraphy in athymic mice and rabbits respectively.

**Results and discussion**

**Synthesis of DTPA–bis(MPBA)**

The ‘bivalent ligand’ approach was 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. The synthetic scheme involves alkylation of 1-(2-methoxyphenyl)piperazine (with the aim to prepare MPBA) with 4-bromobutylphthalimide in DMF at 70 °C in the presence of potassium carbonate to yield phthalimide-protected butyl-MPP in excellent yield (90%). This was followed by cleavage of phthalimide group in presence of hydrazine hydrate to yield pure amionobutyl functionalised MMP (MPBA). Two molecules of MPBA were then successfully conjugated to DTPA bis anhydride yielding the bis derivative of MMP (DTPA–bis(MPBA)) shown in Fig. 1. All intermediates and the final compound were successfully characterized by spectroscopic techniques such as 1H, 13C NMR and MS. The conjugation of DTPA to the aminobutyl derivative of MPB is confirmed by the presence of a 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 the methoxy group in the 1H NMR spectrum of 3. In the 13C NMR spectrum, three carbonyl peaks at 164.7, 176.6 and 178.8 supported similar observations (see ESI†). The peak obtained at 883.8 [M – H] in MS (ESI+) of 3 validates the synthesis of bis MMP derivative DTPA–bis(MPBA) (Fig. 2). According to the recently described QSAR of bivalent GPCR ligands, the biological characteristics are defined by the presence of an aromatic piperidine/piperazine group which simulates the endogenous biogenic amine and a spacer unit between the dimers of 25 A.22 By conjugation with DTPA as a linker a long chain alkyl (n = 4) spacer arm was created, enhancing the affinity towards 5-HT1A receptors.

**Cell viability assay**

A 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 a normal human embryonic kidney cell line at lower concentrations. At 1 mM concentration 52% of the cells were killed at 2 h treatment and the cells were found to regain proliferation at 24 h with a surviving fraction of 0.796 ± 0.080. A surviving fraction of 0.469 ± 0.035 was seen at 10 mM concentration of the compound (Fig. 3). With continuous treatment up to 48 h at 10 mM concentration, a 0.55 ± 0.044 cell survival fraction was observed.
Dose dependent and time dependent growth inhibition of HEK cells with IC\textsubscript{50} values ranging between 1 and 10 mM was obtained by clonogenic assay. A direct correlation was seen in MTT and clonogenic assay. The above observations suggest useful imaging applications using nuclear medicine technique.

Radiolabeling and quality control of \(^{99m}\text{Tc}\)-DTPA–bis(MPBA)

The conjugate was labeled with \(^{99m}\text{Tc}\) and evaluated using \textit{in vitro} and \textit{in vivo} conditions for its competence. Radiochemical analysis depicted that the drug is specifically coordinating with \(^{99m}\text{Tc}\) 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 \textit{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.

Fig. 2 Representative mass spectrum of DTPA–bis(MPBA) demonstrating single peak at 883.8 in negative mode.

Human serum stability evaluation

\textit{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 \(^{99m}\text{Tc}\)-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 \(^{99m}\text{Tc}\)-DTPA–bis(MPBA). \(^{99m}\text{Tc}\)-DTPA–bis(MPBA) exhibited 1.6\% transcomplexation of \(^{99m}\text{Tc}\) in serum. After 2 h challenge at 37°C approximately more than 94\% of the radioactivity remains associated with the conjugate.

Glutathione challenge

To test the stability of the \(^{99m}\text{Tc}\)-DTPA–bis(MPBA) conjugate, it was challenged with 25–100 mM concentration of glutathione.
**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 non-specific 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 non-specific 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. This has been observed in the DTPA–bis(MPBA) conjugate and its complex with $^{99m}$Tc. The log $P$ value increased on radiolabeling with $^{99m}$Tc. The radiolabeled conjugate revealed a 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.

**Radioligand binding assay**

Primary hippocampal cells were cultured as reported in the 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 the subnanomolar range. Scatchard plot analysis revealed that the labeled compound exhibited high affinity on hippocampal cultures with a $K_d$ value of 0.069 ± 0.001 nm (Fig. 4). To corroborate a receptor-chelating binding mode, the 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.

The 5-HT2A and D2 receptor affinities of $^{99m}$Tc–DTPA–bis(MPBA) were found to be lower than those observed for 5-HT1A receptors in brain homogenates. In an in vitro binding assay a subnanomolar affinity for 5-HT1A was obtained, 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 1000 times as high as it is for 5-HT2A receptors (Table 1). 5-HTT also showed high affinity with a value of 46 ± 0.9 nM.

**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 (Fig. 5). 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): 6 h 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$^{-1}$ (Fig. 5).

**Table 1** Dissociation constants of $^{99m}$Tc–DTPA–bis(MPBA) for 5-HT1A, 5-HT2A, D2 and 5-HTT receptors$^a$

<table>
<thead>
<tr>
<th>Complex</th>
<th>5-HT1A</th>
<th>5-HT2A</th>
<th>D2</th>
<th>5-HTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{99m}$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>

$^a$ Non specific binding was determined by serotonin, ketanserin, spiperone and paroxetine.
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 30 minutes. The early phase showed high uptake in the whole brain and wash out of the activity was observed in 30 min (Fig. 6A). Time–activity curve for brain and blood from the data of dynamic SPECT is shown in Fig. 6B. High accumulation in brain was observed at 10 min with 10% uptake. Bioimaging in PC3 grafted athymic mouse was done as 5-HTRs expression has been identified in PC3 cells.33 Semiquantitative analysis was generated from Region Of Interest (ROI) placed over areas counting average counts per pixels, with maximum radiotracer uptake on the tumor found to be 16.45 ± 2.09 and compared with muscle ROI (0.25 ± 0.34) at 1 h. The tumor-to-contralateral muscle tissue ratio of $^{99m}$Tc–DTPA–bis(MPBA) was found to be 65.8 ± 3.3 at 1 h (Fig. 7). In the mice that received co-administration of 500 μg of blocking dose of serotonin the uptake in the tumor was revealed as 0.78 ± 0.41%ID g⁻¹ which was significantly lower at 1 h 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 1 mm 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. The beginning of accumulation of radioactive DTPA–bis(MPBA) at the tumor site

**Fig. 5** (A) Blood clearance of $^{99m}$Tc–DTPA–bis(MPBA) (11.1 MBq activity) administered through ear vein in normal rabbit. (B) Semi-logarithmic plot of plasma concentration, (μg mL⁻¹), against time in hours after administration (of 110 μg kg⁻¹) in normal rabbit; blood samples were taken at 5, 15, 30, 60, 120, 240, and 1440 min post injection.

**Fig. 6** (A) Summed dynamic lateral planar images of a normal rabbit up to 30 min post injection of $^{99m}$Tc–DTPA–bis(MPBA). The early phase shows high uptake in the whole brain. (B) Time–activity curve of brain and blood derived from the semiquantitative analysis of Dynamic SPECT.

**Fig. 7** Whole-body γ image of female athymic mice with subcutaneous PC3 tumor in the right hind leg at 1 h and 2 h.
was observed as early at 15 min. At 1 h post administration the imaging was performed which revealed a substantial amount of ⁹⁹mTc labeled DTPA–bis(MPBA) concentration (Fig. 8). This may serve as an important parameter to guide in vivo evaluation of radiolabeled MPP derivatives as SPECT biomarkers in tumor models.

**Biodistribution**

The in vivo biodistribution of ⁹⁹mTc–DTPA–bis(MPBA) was studied in athymic mice at different time intervals and is illustrated in Table 2. Rapid accumulation was seen in the brain in 10 min in mice with 2.07 ± 0.76% ID g⁻¹. It was observed that the radiolabeled complex rapidly cleared from the blood stream with only 0.86 ± 0.13% ID g⁻¹ remaining in the blood after 24 h in comparison to 3.88 ± 0.61% ID g⁻¹ at 2 h. Major accumulation of the labeled compound was observed in the kidneys (15.03 ± 2.73% ID g⁻¹) followed by liver (5.82 ± 1.06% ID g⁻¹) at 1 h, showing that the complex is excreted mainly by renal routes. Retention of radioactivity in the non target organs (lungs, spleen, intestine etc.) was low and very much 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 ⁹⁹mTc–DTPA–bis(MPBA) was found to be 5.24 ± 0.5% ID g⁻¹ at 1 h. Localization of the radioactivity in the liver, stomach, and small intestine was low, less than 5% ID g⁻¹ at 4 h for ⁹⁹mTc–DTPA–bis(MPBA). Rapid clearance of the radiolabeled conjugate was also observed from these organs (Table 2).

**Regional brain distribution in rat**

Whole brain section of rat showed rapid accumulation in 10 min attaining 2.81% ID g⁻¹ of the total injected dose. In post-mortem rat brain hippocampus and cerebral cortex the highest uptake was shown, followed by thalamus and caudate putamen, with a weaker uptake in cerebellum at 10 min and olfactory bulb. The high uptake in the hippocampus and cerebral cortex was accredited to the 5-HT1A receptor rich regions (Fig. 9). Binding of ⁹⁹mTc–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 a decline of the radioactivity was seen 60 min post injection.

**Table 2 Biodistribution of ⁹⁹mTc–DTPA–bis(MPBA) in PC3 grafted athymic Balb/c mice following intravenous (i.v.) injection**

<table>
<thead>
<tr>
<th>Organ</th>
<th>10 min</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
<th>24 h</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>

*Athymic mice body weight = 18–22 g. Data from groups of five mice are expressed as mean % ID g⁻¹ ± S.D. Based on estimates assuming: blood volume = 7% of the body weight.*

**Fig. 8** Reconstructed coregistered anterior and posterior SPECT/CT image of ⁹⁹mTc–DTPA–bis(MPBA) in PC3 xenografted athymic mice.

**Fig. 9** Regional distribution of ⁹⁹mTc–DTPA–bis(MPBA) in rat brain. WBS = whole brain section, Hip = hippocampus, Th = thalamus, Ctx = frontal and parietal cortex, Cpu = caudate putamen, Cb = cerebellum at 10, 30 and 60 min post injection.
Conclusion

Taking advantage of a bivalent approach, a homodimeric system with reasonable pharmacokinetics for 5-HT1A receptor imaging was designed and developed. We have synthesised a bivalent radioligand DTPA–bis(MPBA) for imaging 5-HT1A receptors which could also be utilized in the determination of 5-HT1A 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 5-HT1A receptors in primary hippocampal cultures. These finding suggest that $^{99m}$Tc–DTPA–bis(MPBA) holds promise for clinical application in nuclear medicine.

Experimental

Materials

Chemicals. All reactions requiring anhydrous conditions or involving moisture sensitive reactants were performed under an atmosphere of dry nitrogen using oven dried (80 °C) glassware. All reaction temperatures reported indicate the temperature of the bath in contact with the reaction vessel. Methoxyphenylpiperazine, 4-bromobutylphthalimide, hydrazine hydrate and DTPA bis anhydride, triethylamine, ketanserine tartrate, serotonin hydrochloride, spiperone, and paroxetine hydrochloride and DTPA bis anhydride, triethylamine, ketanserine tartrate, serotonin hydrochloride, spiperone, and paroxetine were purchased from Sigma-Aldrich and Merck. All solvents used were of analytical grades. TLC was run on silica gel coated aluminum sheets (Silica gel 60 F254, Merck, Germany) and visualized in UV light 254 nm. Radiocomplexation and radiochemical purity was checked by instant thin layer chromatography.

Instrumentation. 'H and 13C NMR spectra were recorded on a Bruker Avance II 400 MHz system (Ultra shield). Mass spectra (ESI-MS in positive and negative ion mode) were performed on a Bruker micro-ESI-FTICR mass spectrometer (Bruker Avance II 400 MHz system (Ultra shield)). Mass spectra (ESI-MS in positive and negative ion mode) were performed on a Bruker micro-ESI-FTICR mass spectrometer (Bruker Avance II 400 MHz system (Ultra shield)).

Synthesis of 2-(2-(4-(2-methoxyphenyl)piperazine-1-yl)isoindoline-1,3-dione (1). To a solution of methoxyphenylpiperazine (1 g, 5.2 mmol) in dimethylformamide (50 mL) was added potassium carbonate (1.07 g, 7.8 mmol) under nitrogen. The reaction was stirred at 70 °C and was followed by the dropwise addition of 4-bromobutylphthalimide (1.75 g, 7.24 mmol). The reaction was stirred at 70 °C for 8 hours. The reaction mixture was cooled to room temperature, filtered, and the filtrate was evaporated under reduced pressure and then extracted with dichloromethane (3 × 50 mL). The combined organic extracts were dried over anhydrous Na2SO4 and evaporated to dryness under reduced pressure to obtain the crude product which was purified by column chromatography on silica gel (eluents: dichloromethane–methanol, 9 : 1) to afford the desired product as a yellow solid (90%). The final product was well characterized by 1H, 13C NMR and HRMS. 1H NMR (400 MHz, 25 °C, CDCl3) δ (ppm): 1.57 (t, 2H, CH2), 1.61 (t, 2H, CH2), 2.44 (t, 2H, CH2), 2.64 (s, 4H, NCH2CH2N), 3.08 (s, 4H, NCH2CH2N), 3.7 (t, 2H, CH2), 3.86 (s, 3H, OCH3), 6.68–7.02 (m, 4H, phenyl–OCH3), 7.24 (m, 2H, phenyl–CH–O), 7.30 (m, 2H, phenyl–CH–C=O), 7.70 (m, 2H, phenyl–CH–C=O); 13C NMR (100 MHz, 25 °C, CDCl3) δ (ppm): 24.15 (CH2CH3), 26.61 (CH2CH3), 37.7 (NCH3), 49.12 (NCH2CH2N), 52.74 (NCH2CH2N), 55.27 (CH2CH3), 78.08 (OCH3), 111.09 (ArCH), 118.16 (ArCH), 120.94 (ArCH), 123.37 (ArCH), 132.03 (ArC=O), 133.88 (ArCH), 141.28 (ArC=O), 152.2 (ArC=O), 160.32 (ArC=O), 238.16 (ArC=O). MS (ESI) m/z calculated for C23H16N2O3, 394.48, found [M + H]+ 394.6.

Synthesis of 4-(4-(2-methoxyphenyl)piperazine-1-yl)butan-1amine (2). To a stirring solution of 1 (1 g, 2.5 mmol) in ethanol was added hydrazine hydrate (200 µL). The solution was allowed to reflux for 1 h. The reaction mixture was then cooled and adjusted to pH 1 using concentrated hydrochloric acid. The white precipitate formed was filtered. The pH of filtrate was adjusted to pH 12 using 5 mmol L–1 NaOH. After further filtration, the solution was extracted three times with dichloromethane (20 mL for each extraction). The organic phase was dried overnight with anhydrous Na2SO4. After removal of excess reagents under vacuum, the product was obtained as a colourless oil (85%). The final product was well characterized by 1H, 13C NMR and MS.

Animal models. All animal experiments were performed in accordance with guidelines of INMAS animal ethics committee. New Zealand rabbits, athymic Balb/c mice were used for blood clearance, imaging, and biodistribution. Mice and rabbits were housed under conditions of controlled temperature of 22.2 °C and normal diet. Athymic mice were inoculated subcutaneously with 0.1 mL of cell suspension (5 × 106 PC3) in the right hind leg under sterilized conditions.

Data analysis. The competition curve of the receptor binding experiments was analyzed by nonlinear regression using algorithms in GraphPad PRISM 5.0 (San Diego, CA). Competitive curve was fitted to a sigmoid curve in which KD and Hill coefficient were free parameters. Biodistribution data is reported as mean ± standard deviation (S.D.).

Methods

Synthesis

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NMR (400 MHz, 25 °C, CDCl3), δ (ppm): 1.39–1.54 (m, 6H, CH3), 2.35 (t, 2H, CH2), 2.59–2.65 (m, 6H, NCH2CH2N), 2.96–3.04 (s, 4H, NCH2CH2N), 3.78 (s, 3H, OCH3), 6.78–6.95 (m, 4H, ArCH2CH2N); 13C NMR (100 MHz, 25 °C, CDCl3), δ (ppm): 22.02 (CH2CH2), 27.26 (CH2CH2), 41.80 (NCH2CH2N), 48.64 (NCH2CH2N), 51.96 (OCH2CH2N), 55.27 (CH2CH2), 58.53 (OCH3), 109.85 (ArCH), 116.44 (ArCH), 120.92 (ArCH), 122.84 (ArCH), 141.68 (ArC–O), 152.17 (ArC–N); MS (ESI) m/z calc for C15H26N2O3 263.4, found [M + H]+ 264.5.

Synthesis of 5,8-bis(carboxymethyl)-15-(4-(2-methoxyphenyl)piperazine-1-yl)-2-(2-(4-(4-(2-methoxyphenyl)piperazine-1-yl)butylamino)-2-oxo-2,5,8,11-tetraazapentadecane-1-carboxylic acid (5). To a stirring solution of DTPA anhydride (339 mg, 0.9 mmol) in 10 mL anhydrous DMF was added 5 mmol of triethylamine dropwise. The reaction completion was confirmed by running TLC in ammonium hexahydroxy and methanol (3:2). Triethyamine and DMF were evaporated under reduced pressure using a rotary evaporator. The final product was precipitated using acetone and was well characterized by 1H NMR and MS. 1H NMR (400 MHz, 25 °C, CDCl3), δ (ppm): 1.49 (m, 2H, CH2), 1.608 (m, 2H, CH2), 2.73–3.74 (m, 22H, NCH2CH2N), 3.75 (s, 3H, OCH3), 6.90–7.07 (m, 4H, ArCH2), 13C NMR (100 MHz, 25 °C, CDCl3), δ (ppm): 22.02 (CH2CH2), 27.26 (CH2CH2), 41.80 (NCH2CH2N), 48.64 (NCH2CH2N), 51.96 (NCH2CH2N), 55.27 (CH2CH2), 58.53 (OCH3), 109.85 (ArCH), 116.44 (ArCH), 120.92 (ArCH), 122.84 (ArCH), 141.68 (ArC–O), 152.17 (ArC–N); MS (ESI) m/z calc for C44H66N6O12 883.8.

Cytoxicity studies of DTPA–bis(MPBA)

Viability assay: trypan blue exclusion assay. Cell suspension of monolayer culture of U-87MG cell line (500 mg, 1.89 mmol). The reaction was carried out after 2 h treatment with the compound. 10 mm of stannous chloride (100 mg) was added. The final product was precipitated using acetone and was well characterized by 1H NMR and MS. The final product was precipitated using acetone and was well characterized by 1H NMR and MS. 13C NMR (100 MHz, 25 °C, CDCl3), δ (ppm): 22.02 (CH2CH2), 27.26 (CH2CH2), 41.80 (NCH2CH2N), 48.64 (NCH2CH2N), 51.96 (NCH2CH2N), 55.27 (CH2CH2), 58.53 (OCH3), 109.85 (ArCH), 116.44 (ArCH), 120.92 (ArCH), 122.84 (ArCH), 141.68 (ArC–O), 152.17 (ArC–N); MS (ESI) m/z calc for C44H66N6O12 883.8.

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Radiolabeling of DTPA–bis(MPBA) (99mTc–DTPA–bis(MPBA))

2 mg of an aqueous solution (100 μL) of DTPA–bis(MPBA) was added to 100 mL 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 (<1 h old) 99mTechnetium pertechnetate saline solution and the mixture was maintained at 25 °C for 15 min. Before purification the radiolabeling efficiency was 88–95% determined by HPLC (Nucleosil ODS (250 × 4 mm), at a flow rate of 1 mL min−1, isocratic gradient mixture of 80% methanol and 20% 0.01 M phosphate buffer (pH 7.4, R1). During radiochromatography of the eluted standard safety methods were employed.

Radiochemical purity of 99mTc–DTPA–bis(MPBA) conjugate.

Instant thin layer chromatography on ITLC-SG (Paul German) 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 into 0.5 cm fragments and counts of each segment were taken. The percentage of free Na99mTcO4 and complexed 99mTc was calculated. 99mTc–DTPA–bis(MPBA) conjugate remained at the origin while free 99mTc 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 99mTc–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.

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−1, pH 7.4) under strict equilibrium conditions. 2 mL 1-octanol and 2 mL 99mTc–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−1 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

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

The final partition coefficient value was expressed as log P.
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 CO₂ chamber incubated at 37 °C and then analyzed to check for any dissociation of the complex. Percentage of free pertechnetate at a particular time point 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.

In vitro receptor binding of ⁹⁹mTc–DTPA–bis(MPBA). ⁹⁹mTc–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. Kᵦ, receptor dissociation constant (Kᵦ values, obtained by Scatchard analysis, were calculated for each labeled ligand).

Measurement of ⁹⁹mTc–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.³² In brief, single cells dissociated from the hippocampus of fetal rats were sited on the poly-l-lysine coated culture slide at 3 × 10⁵ cells per well. Culture was incubated in DMEM (F-12) supplemented with 10% heat inactivated fetal bovine serum at 17–20 days of gestation. Three days subsequent to plating, non-neuronal cells were removed by addition of cytosine arabinoside at 10 μM. Cells were then incubated 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 NaHCO₃ 14.3 mM. The cultures were subsequently maintained at 37 °C in a humidified 5% CO₂ 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 observed.

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 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 CaCl₂) containing membrane homogenate (about 0.5 mg mL⁻¹ protein) and various concentrations of the ⁹⁹mTc–DTPA–bis(MPBA) complex. Non specific binding was defined as the amount of ⁹⁹mTc–DTPA–bis(MPBA) bound in the presence of 10 mM serotonin. The binding assay was performed in triplicate 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.

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⁻¹ protein) and various concentrations of the ⁹⁹mTc–DTPA–bis(MPBA) complex. Triplicates of the samples were incubated at 20 °C for 60 min. Filtration and counting of the samples were the same as described above.

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 MgCl₂, 1 mM EDTA, membrane homogenate (about 0.2 mg mL⁻¹ protein) and various concentrations of the ⁹⁹mTc–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.

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, 5 mM KCl, membrane homogenate (about 1.2 mg mL⁻¹ protein) and various concentrations of the ⁹⁹mTc–DTPA–bis(MPBA) presence and absence of 100 fold excess of paroxetine. Filtration and counting of the samples were the same as described above.

Blood kinetics. In normal rabbit, 300 μL of the ⁹⁹mTc–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.

**SPECT acquisition.** Non-invasively dynamic study was performed in New Zealand rabbit (3.1 kg) at a time interval of 1 min up to 30 min and Semiquantiative 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⁻¹, 3.2 MBq activity). Images were taken using planar gamma camera HAWKEYE. Images were obtained at different time intervals starting from 1, 2 and 4 h 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 single reconstruction mode OSEM (Ordered Subset Expectation Maximization). CT-reconstruction was straightforward analytical. All images were fused and analysed with VIVID (Amira®, San Diego, USA).

**Biodistribution.** In biodistribution study, intravenous injection of ⁹⁹mTc–DTPA–bis(MPBA) conjugate in a volume of 100 µL (5 µmol kg⁻¹, 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 ⁹⁹mTc energy. Uptake of the radiotracer in each tissue was calculated and expressed as a percentage injected dose per gram of the tissue (% ID g⁻¹) (Table 1). Animal protocols have been approved by Institutional Animal Ethics Committee.

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**Notes and references**