Supporting Information

Design, Physico-chemical and Pre-clinical evaluation of homo-bivalent $^{99m}$Tc-(BTZ)$_2$DTPA radio-ligand for dimeric 5-HT$_{1A}$/5-HT$_7$ Receptors

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Figure 1: $^1$H-NMR Spectrum of Compound 1.

Figure 2: $^{13}$C-NMR Spectrum of Compound 1.
SI 2: The ESI-LC-MS Spectrum of 3-(3-aminopropyl)benzo[d]thiazol-2(3H)-one (1)

![Figure 3: LC-MS Spectrum of Compound 1.](image)

SI 3: The HPLC Profile of 3-(3-aminopropyl)benzo[d]thiazol-2(3H)-one (1)

![Figure 4: The HPLC Profile of Compound 1.](image)
SI 4: The $^1$H-NMR characterization of 6,9-bis(carboxymethyl)-11-oxo-3-(2-oxo-2-((3-(2-oxobenzod[b]thiazol-3(2H)-yl)propyl)amino)ethyl)-15-(2-oxobenzod[b]thiazol-3(2H)-yl)-3,6,9,12-tetraazapentadecan-1-oic acid (2)

Figure 5: $^1$H-NMR Spectrum of Compound 2.
SI 5: $^{13}$C-NMR characterization of 6,9-bis(carboxymethyl)-11-oxo-3-(2-oxo-2-((3-(2-oxobenzo[d]thiazol-3(2H)-yl)propyl)amino)ethyl)-15-(2-oxobenzo[d]thiazol-3(2H)-yl)-3,6,9,12-tetraazapentadecan-1-oic acid (2)

Figure 6: $^{13}$C-NMR Spectrum of compound 2.
SI 6: The HR-MS spectrum and HPLC profile of 6,9-bis(carboxymethyl)-11-oxo-3-(2-oxo-2-((3-(2-oxo-2H)-[1H]-[2]benzo[d]thiazol-3(2H)-yl)propyl)amino)ethyl)-15-(2-oxo-2H-[1H]-[2]benzo[d]thiazol-3(2H)-yl)-3,6,9,12-tetraazapentadecan-1-oic acid (2)

Figure 7: HR-MS spectrum of Compound 2.

Figure 8: HPLC profile of Compound 2.
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SI 7: Physicochemical characterization of unlabelled-(BTZ)₂DTPA ligand.

**UV-Vis Spectroscopy:** The UV-Vis absorption spectrum for bivalent (BTZ)₂DTPA (Figure 18) showed absorption bands at $\lambda_{\text{max}1} \approx 280$ nm and $\lambda_{\text{max}2} \approx 240\text{-}260$ nm respectively, when excited in the wavelength range of 230-350 nm. The appearance of an intense band around 240-260 nm, corresponds to the amidic $\pi-\pi^*$ transition, whereas the absorption at 280 nm was observed due to the $\pi-\pi^*$ transition of the benzothiazolone moiety. It can be inferred that the appearance of two emission bands (due to amide bond and phenyl ring of BTZ moiety) in the UV-Vis spectrum of the synthesized ligand, confirms a successful bis-conjugation of primary amine intermediate with bifunctional DTPA chelator.¹

![Figure 9: Ultraviolet absorbance spectrum of (BTZ)₂-DTPA.](image)

**Potentiometric Titration:** In order to evaluate the protonation constants, potentiometric titrations were carried for the ligand (BTZ)₂DTPA at 298 K. The obtained lower $pK_{a1}$, $pK_{a2}$, $pK_{a3}$ and $pK_{a4}$ values of the ligand as compared to DTPA² indicated fast ionization of (BTZ)₂DTPA ligand, facilitating a quick release of its protons in the solution for an efficient metal complexation as shown in table below.

**Table 1:** Protonation constant of free ligand:

<table>
<thead>
<tr>
<th>$pK_a$</th>
<th>DTPA${}^6$</th>
<th>(BTZ)₂DTPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$pK_{a1}$</td>
<td>10.4</td>
<td>8.9</td>
</tr>
<tr>
<td>$pK_{a2}$</td>
<td>8.5</td>
<td>6.8</td>
</tr>
<tr>
<td>$pK_{a3}$</td>
<td>4.3</td>
<td>3.7</td>
</tr>
<tr>
<td>$pK_{a4}$</td>
<td>2.7</td>
<td>2.6</td>
</tr>
<tr>
<td>$pK_{a5}$</td>
<td>2.0</td>
<td>-</td>
</tr>
</tbody>
</table>
SI 8: Optimization of radiolabeling parameters.

Figure 10: Radiolabeling parameters optimization: (a) effect of stannous chloride concentration, (b) effect of incubation time and (c) effect of pH on the radiolabelling efficiency of the radioligand ($^{99m}$Tc-(BTZ)$_2$DTPA).
SI 9: Partition coefficient of unlabelled-(BTZ)$_2$DTPA ligand.

The log $P_{o/w}$ value calculated from the Schrödinger software was -1.66, whereas -1.70 was obtained from MolInspiration. The obtained log $P_{o/w}$ value for (BTZ)$_2$DTPA was 0.23 at pH 7.4. As a result of bifunctional-conjugation and the hydrophobic nature of two BTZ rings, the overall lipophilicity of the developed ligand was enhanced, and thus a positive log $P$ value was obtained.

**Method:** The partition coefficient of the unlabelled (BTZ)$_2$DTPA ligand was determined by UV-Vis spectrophotometry method. Similar protocol was followed as done for the $^{99m}$Tc-labeled complex, except the radioactivity part. The optical density (OD) of 50 µL volume of both phases were measured on Biotek Synergy H4 hybrid multiplate reader. The log $P$ was determined by taking the ratio of OD values of octanol fraction to that of the water fraction. The experiment was performed in triplicates and reported the final value as the average of the three experiments.

SI 10: The SPECT imaging of $^{99m}$Tc-(BTZ)$_2$DTPA in New Zealand Rabbit.

![Figure 11](image_url): The anterior view dynamic SPECT scan of $^{99m}$Tc-(BTZ)$_2$DTPA in normal New Zealand rabbit.
SI 11: Biodistribution results of $^{99m}$Tc-(BTZ)$_2$DTPA in female Balb/c mice.

**Table 2:** Biodistribution of $^{99m}$Tc-(BTZ)$_2$DTPA in female Balb/c mice at the defined p.i. time points.

<table>
<thead>
<tr>
<th>Organ</th>
<th>2 min p.i.</th>
<th>5 min p.i.</th>
<th>10 min p.i.</th>
<th>15 min p.i.</th>
<th>30 min p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>4.11±0.16</td>
<td>3.78±0.15</td>
<td>3.12±0.12</td>
<td>2.71±0.11</td>
<td>2.05±0.08</td>
</tr>
<tr>
<td>Brain</td>
<td>1.36±0.05</td>
<td>1.78±0.07</td>
<td>2.08±0.08</td>
<td>1.37±0.05</td>
<td>1.12±0.04</td>
</tr>
<tr>
<td>Heart</td>
<td>2.56±0.10</td>
<td>2.11±0.08</td>
<td>2.08±0.08</td>
<td>1.76±0.07</td>
<td>1.12±0.04</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.85±0.03</td>
<td>0.94±0.04</td>
<td>1.04±0.04</td>
<td>0.98±0.04</td>
<td>0.83±0.03</td>
</tr>
<tr>
<td>Liver</td>
<td>7.56±0.29</td>
<td>6.57±0.26</td>
<td>6.85±0.27</td>
<td>6.08±0.24</td>
<td>5.78±0.23</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.91±0.04</td>
<td>1.09±0.04</td>
<td>1.28±0.05</td>
<td>1.66±0.06</td>
<td>1.96±0.08</td>
</tr>
<tr>
<td>Kidney</td>
<td>9.89±0.39</td>
<td>12.45±0.49</td>
<td>15.12±0.59</td>
<td>17.26±0.67</td>
<td>18.24±0.71</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.06±0.00</td>
<td>0.13±0.01</td>
<td>0.22±0.01</td>
<td>0.25±0.01</td>
<td>0.45±0.02</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.78±0.03</td>
<td>0.84±0.03</td>
<td>1.03±0.04</td>
<td>1.13±0.04</td>
<td>1.56±0.06</td>
</tr>
</tbody>
</table>

SI 12: In vitro receptor binding affinity experiments.

**Figure 12:** Saturation curve obtained from radioligand binding affinity experiment of (a) hippocampus and (b) cortex membrane homogenates of rat brain. Non-specific binding was determined with 100-fold concentration of methoxyphenylpiperazine, where $K_d$ of 0.33 nM and 0.29 nM were obtained for 5-HT$_{1A}$ and 5-HT$_7$ receptors respectively.
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Chemicals and reagents
2-Hydroxybenzothiazole, potassium carbonate, trimethylamine, N’, N’-dimethylformamide, diethylenetriaminepentaacetic dianhydride, TritonX-100, L-cysteine, MTT, sodium bicarbonate, phosphate buffered saline (PBS, pH 7.4) and tetrabutylammonium hydroxide solution (TBAOH, 1.0 M in methanol) were purchased from Sigma-Aldrich. Acetone, acetonitrile (HPLC grade), water (HPLC grade), methanol, concentrated hydrochloric acid and diethyl ether were obtained from E. Merck Ltd., India. The reactions requiring anhydrous conditions or involving moisture sensitive reactants were carried out under an atmosphere of continuous supply of dry nitrogen and using an oven-dried (80-85 °C) glassware. All the reported reaction-temperatures, indicate the temperature of the paraffin oil of the oil-bath in contact with the reaction vessel. The thin layer chromatography (TLC) was run on the silica coated aluminium sheets (Silica Gel 60 F$_{254}$, Merck, Germany) and subsequently visualized in either iodine impregnated silica or UV-Vis. Instant thin layer chromatography (ITLC) was used to determine the radio complexation of ligands with $^{99m}$Tc and the chemical purity of radiolabelled compounds.

Instrumentation
$^1$H and $^{13}$C-NMR spectra were recorded on a Bruker DPX-300 MHz spectrometer ($^1$H: 300 MHz, $^{13}$C: 75 MHz), using D$_2$O as the solvent with tetramethylsilane (TMS) as the internal standard at room temperature. Chemical shifts are given in δ (ppm) relative to TMS. The coupling constants (J) are given in Hz. LC/MS ESI-MS (in positive and/or negative ion mode) was performed on in-house Agilent 6310 system ion trap at INMAS. The HRMS was performed on high resolution mass instrument using quadruple-TOF mass analyser. UV-vis studies were performed on a Biotek Synergy H4 hybrid multiplate reader. Lyophilisation was performed using a Labconco system. The HPLC analyses were performed on Atlantis T3 C-18 reverse phase column (5 µm, 4.6 mm x 250 mm) using Agilent1620 Infinity Analytical-Scale Purification system. The mobile phase was 0.05% TFA in water (solvent A) and acetonitrile (solvent B) with a flow rate of 0.8 mL/min. UV detection were performed at 250 nm and 280 nm using UV-Vis detector. The injection volume was 20 µL and the HPLC profiles (retention time) are provided in supporting information. The potentiometric measurements were carried on an automatic titration system consisting of Metrohm 713 pH meter equipped with a Metrohm A.60260.100 electrode and 800 Dosino autoburette. The MTT and haemolysis assays were read at 570 nm and 540 nm using 630 nm as a reference wavelength on an ELISA reader (BioTek instruemnts, Vinooski, VT, USA). The radiolabelling, biodistribution and
scintigraphic imaging studies were conducted using a γ-scintillation counter GRS230, ECIL (India) and a planar gamma camera Symbia True-Point from Siemens. The Scintigraphs were obtained using a rectangular large field of view gamma camera (Symbia True-Point Dual Head) with a low energy all-purpose collimator.

**UV-Vis characterization and potentiometric titrations**

The product formation was assured by analysing the shifts in the excitation wavelength of the ligands upon conjugation with DTPA. The $\lambda_{\text{max}}$ of ligands were determined on a Biotek Synergy H4 hybrid multiplate reader. The protonation constant were determined by using potentiometric titrations, carried out on an automatic titration system consisting of Metrohm 713 pH meter equipped with Metrohm A.60262.100 electrode, 800 Dosino autoburet. The pH meter electrodes were precalibrated using standard buffer solutions. The $pK_a$ were determined by titrating 1 mM of DTPA-ligands with 10 mM tetrabutylammonium hydroxide (TBAOH) in the pH range of 3-12. Titrations were performed at 25 °C to maintain the constant ionic strength. The experiments were performed in triplicates. The protonation constants of the ligands were calculated from the titration data using the program Tiamo 2.0.

**References:**
