Development of a selective and high affinity radioligand,

$[^3]H]VU6013720$, for the $M_4$ muscarinic receptor

Aidong Qi, Haley E. Kling, Natasha Billard, Alice L. Rodriguez, Li Peng, Jonathan W. Dickerson, Julie L. Engers, Aaron M. Bender, Mark S. Moehle, Craig W. Lindsley, Jerri M. Rook, and Colleen M. Niswender

Department of Pharmacology and Warren Center for Neuroscience Drug Discovery, Vanderbilt University, Nashville, TN 37232, USA (AQ, HEK, NB, ALR, LP, JWD, JLE, AMB, CWL, JMR, CMN)

Department of Chemistry, Vanderbilt University, Nashville, TN 37232, USA (CWL)

Vanderbilt Kennedy Center, Vanderbilt University Medical Center, Nashville, TN 37232, USA (CMN)

Vanderbilt Brain Institute, Vanderbilt University School of Medicine, Nashville, TN 37232, USA (CMN)

Vanderbilt Institute of Chemical Biology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA (CWL, CMN)

Current address: Department of Pharmacology and Therapeutics and Center for Translational Research in Neurodegeneration, University of Florida, Gainesville, FL 32610 (MSM)
Abbreviations:

\( B_{\text{max}} \)  maximal binding
BCA  bicinchoninic acid
CHO-K1  Chinese Hamster Ovary K1
\( \text{CO}_2 \)  carbon dioxide
CNS  central nervous system
FBS  fetal bovine serum
HEPES  4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
\( \text{IC}_{50} \)  inhibitory concentration 50
\( K_d \)  equilibrium dissociation constant
\( K_i \)  equilibrium inhibitory constant
\( K_{\text{off}} \)  dissociation off rate
\( K_{\text{on}} \)  association on rate
nM  nanomolar
NMS  N-methyl scopolamine
PAM  positive allosteric modulator
PET  Positron Emission Tomography


Abstract

M₄ muscarinic receptors are highly expressed in the striatum and cortex, brain regions that are involved in diseases such as Parkinson’s disease, schizophrenia, and dystonia. Despite potential therapeutic advantages of specifically targeting the M₄ receptor, it has been historically challenging to develop highly selective ligands, resulting in undesired off-target activity at other members of the muscarinic receptor family. Recently, we have reported first-in-class, potent, and selective M₄ receptor antagonists. As an extension of that work, we now report the development and characterization of a radiolabeled M₄ receptor antagonist, [³H]VU6013720, with high affinity (pK₉ of 9.5 ± 0.2 at rat M₄, 9.7 at mouse M₄, and 10 ± 0.1 at human M₄ with atropine to define nonspecific binding) and selectivity (80-fold over M₂ and >1,000 fold over M₁, M₃ and M₅) for the M₄ receptor versus the other muscarinic subtypes. Binding assays using this radioligand in rodent brain tissues demonstrate loss of specific binding in Chrm4 knockout animals. Dissociation kinetics experiments with various muscarinic ligands show differential effects on the dissociation of [³H]VU6013720 from M₄ receptors, suggesting a binding site that is overlapping but may be distinct from the orthosteric site. Overall, these results demonstrate that [³H]VU6013720 is the first highly selective antagonist radioligand for the M₄ receptor, representing a useful tool for studying the basic biology of M₄ as well for the support of M₄ receptor-based drug discovery.
Significance statement: This manuscript describes the development and characterization of a novel muscarinic acetylcholine subtype 4 receptor antagonist radioligand, [³H]VU6013720. This ligand binds to or overlaps with the acetylcholine binding site, providing a highly selective radioligand for the M₄ receptor that can be used to quantify M₄ protein expression in vivo and probe the selective interactions of acetylcholine with M₄ versus the other members of the muscarinic receptor family.
Introduction

The five subtypes of muscarinic (M) acetylcholine receptors mediate a wide variety of physiological functions and pathophysiological processes. The M₁, M₃, and M₅ subtypes are preferentially coupled to G_{q/11} G proteins and activate phospholipase C, which catalyzes the phosphatidylinositol trisphosphate cascade and leads to activation of protein kinase C and intracellular Ca^{2+} mobilization. In contrast, the M₂ and M₄ subtypes are coupled to G_{i/o} and inhibit adenylyl cyclase activity and reduce cyclic AMP production upon activation; additionally, signaling downstream of these receptors also modulates ion channels (Ashkenazi et al., 1988, Peralta et al., 1988, Offermanns et al., 1994, Ishii et al., 2006, Lebois et al., 2018). Muscarinic acetylcholine receptor subtypes are distributed in both the central nervous system (CNS) and periphery. Although non-selective muscarinic receptor antagonists have shown therapeutic potential for the treatment of certain neurological disorders, usage has been limited due to intolerable side effects resulting from their broad inhibition of all the muscarinic receptor subtypes (Fahn et al., 1990, Cloud et al., 2010). This has led to the development of subtype-selective ligands to improve efficacy while reducing side effects.

Of the muscarinic receptors, the M₄ subtype is most highly expressed in striatum, cortex, and some subcortical regions such as the thalamus (Brann et al., 1988, Buckley et al., 1988, Weiner et al., 1990). It has been reported that M₄ is the primary muscarinic receptor subtype responsible for regulating dopamine signaling and release in the basal ganglia (Gomeza et al., 1999, Jeon et al., 2010, Foster et al., 2016, Moehle et al., 2017, Moehle et al., 2019) and M₄ has been implicated in several neurological disorders such
as Parkinson’s disease, schizophrenia, and dystonia (Katzenschlager et al., 2003, Moehle et al., 2019, Moran et al., 2019).

Despite these biological observations regarding the therapeutic potential of M₄ modulation, development of highly selective M₄ receptor orthosteric and allosteric modulators has been challenging, and, by extension, the development of radioligands specifically targeting M₄ has historically been difficult. Recently, two M₄ receptor positive allosteric modulators (PAMs), [¹¹C]-VU0467485/AZ13713945 (Deng et al., 2019) and [¹⁸F]-M4R-1911 (Deng et al., 2020) have been radiolabeled and demonstrated to specifically bind to M₄ receptors. Another M₄ PAM radioligand, [¹⁸F]12, has also been shown to display species’ differences in terms of its binding specificity and selectivity (Haider et al., 2023). Encouragingly, the M₄ receptor PAM radioligand [¹¹C]MK-6884 has been recently reported to specifically bind to the M₄ receptor and penetrate into the brain and has been used clinically as a Positron Emission Tomography (PET) ligand (Tong et al., 2020, Li et al., 2022). In this report, we complement these selective M₄ PAM radioligands with [³H]VU6013720, a highly selective M₄ antagonist radioligand.

Recently, our group successfully synthesized and characterized several highly selective M₄ receptor antagonists, VU6013720, VU6021302, and VU6021625 (Moehle et al., 2021), VU6028418 (Spock et al., 2021), and VU6015241 (Bender et al., 2022). Of these compounds, VU6013720 exhibited the best in vitro functional potency (rat M₄ IC₅₀ = 20 nM, human M₄ IC₅₀ = 0.6 nM from calcium mobilization assays) as well as a good selectivity profile among the muscarinic receptor family, with IC₅₀ values of >10,000 nM at rM₃ and rM₅. At rM₁, VU6013720 exhibited a functional IC₅₀ of 1700 nM, displaying ~85-fold selectivity, while VU6013720 had an IC₅₀ of 670 nM at rM₂, displaying ~34-fold
selectivity. Additionally, the compound exhibited unbound percentages of 24% and 31% in rat and mouse plasma, as well as 5% in both rat and mouse brain tissue.

In the current report, we generated a tritiated form of this compound, \([^{3}H]VU6013720\). As shown here, \([^{3}H]VU6013720\) selectively binds to M4 receptors with little to no binding to the M1, M2, M3, and M5 receptor subtypes. Binding assays with brain tissue homogenates reveal that \([^{3}H]VU6013720\) exhibits specific binding in extracts from cortex and striatum. As other existing radioligands bind to allosteric sites, it is anticipated that a selective antagonist radioligand that interacts with the acetylcholine binding site should provide a valuable tool to explore selective binding of acetylcholine to the M4 receptor in native tissues, in vivo, in physiological and pathophysiological disease states and for selective M4 receptor drug development.

**Materials and Methods**

**Cell culture and membrane preparation.** Chinese Hamster Ovary (CHO-K1) cells were purchased from the American Tissue Culture Collection (ATCC, Manassas, Virginia). CHO-K1 cells stably express rat, human, or mouse muscarinic receptors (M1–M5); for M2 and M4, cells were co-transfected with Gq15. Cells were cultured in F12 medium containing 10% fetal bovine serum, 20 mM HEPES, and 1X Antibiotic/Antimycotic (contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Gibco Amphotericin B; Thermo Fisher Scientific, Waltham, MA) at 37°C in the presence of 5% CO2. Cells were monitored by periodical PCR detection using a LookOut Mycoplasma PCR Detection Kit (Sigma-Aldrich, St
Louis, MO) to eliminate potential mycoplasma infection and cells were not mycoplasma positive for the studies described here.

Cell membranes were made as previously reported (O’Brien et al., 2018, Moehle et al., 2021). CHO-K1 cells stably expressing different muscarinic receptor subtype were seeded in 150-mm dishes and allowed to grow to approximately 80%–90% confluence. Cells were washed once with ice-cold phosphate-buffered saline, scraped from the dishes, and collected using a 5-minute centrifugation at 1000g. The supernatant was removed, and the cell pellet was stored at -80°C. To prepare membrane protein, cell pellets were thawed, re-suspended in membrane binding buffer (20 mM HEPES with 10 mM MgCl₂ and 10 mM NaCl, pH 7.4), and homogenized using two 15-second bursts from a TR-10 polytron (Tekmar, Vernon, BC, Canada). Cell lysates were then centrifuged at 1000g for 10 minutes at 4°C, and the pellet was discarded. The resultant supernatant was centrifuged at 20,000g for 30 minutes at 4°C and the supernatant was discarded. The remaining pellet was re-suspended in membrane binding buffer. The protein concentration was determined by BCA assay (Thermo Scientific, Rockford, IL). Membranes were then stored at -80°C until use.

**Radiochemistry.** The synthesis of VU6013720 has been reported previously (Moehle et al., 2021). Cold VU6013720 compound was radiolabeled by RC TriTec (Teufen, Switzerland) with photocatalytic H/T exchange (Figure 1), with the [³H] label most likely in the alpha position of the tertiary amine. The final radioligand, [³H]VU6013720, had a purity > 98% with a specific activity 23.6 Ci/mmol (233.8 GBq/mmol) and a concentration of 1 mCi/ml (37 MBq/ml). [³H]NMS was manufactured
on 03/08/2018, Specific activity: 80.0 Ci/mmol, Dilution: 1mCi/ml (PerkinElmer, Boston, MA).

[^3H]VU6013720 saturation binding assays. Saturation binding experiments were performed to determine the specificity of[^3H]VU6013720 binding to the muscarinic receptor subtypes. Briefly, 10 µg CHO-K1 cell membranes expressing each receptor were incubated with[^3H]VU6013720 in deep 96-well plates (Corning, New York) on a shaker at room temperature for 3 hours in a reaction volume of 500 µL. Saturation binding experiments were performed in triplicate or duplicate, and nonspecific binding was assessed by addition of 10 µM atropine or VU6013719 (VU6013720 structural analogue, Supp. Fig. 1). The reactions were terminated by vacuum filtration onto Unifilter-96 GF/B plate through a 96-well harvester. The filters were washed three times with cold membrane harvesting buffer (50 mM Tris-HCl and 0.9% NaCl, pH 7.4). Plates were then dried overnight and 40 µl of MicroScint 20 (PerkinElmer) was added to each well. The membrane-bound radioactivity was determined using a TopCount NXT microplate scintillation and luminescence counter (PerkinElmer). Calculation of specific binding versus total counts revealed that radioligand depletion was not a factor in these studies using this assay protocol.

Competition binding assays with[^3H]NMS and[^3H]VU6013720. Radioligand competition binding assays were performed as previously described (Moehle et al., 2021) using concentrations of radioligand that were within two-fold of the calculated Kd. Briefly, M4 antagonists were 1:3 serially diluted into membrane binding buffer and added to each well of a 96-well plate, along with 10 µg/well cell membrane and approximately 100 pM[^3H]-N-methylscopolamine ([^3H]NMS, PerkinElmer, Boston, MA)
or 100 - 400 pM [³H]VU6013720 in a reaction volume of 500 µL. Following a 3-hour incubation period on a shaker at room temperature, the membrane-bound ligand was separated from free ligand by filtration through glass fiber 96-well filter plates (Unifilter-96, GF/B; PerkinElmer, Boston, MA) using a 96-well Brandel harvester. The filters were washed three times with cold membrane harvesting buffer (50 mM Tris-HCl and 0.9% NaCl, pH 7.4). Plates were then dried overnight and 40 µl of MicroScint 20 (PerkinElmer) was added to each well. The membrane-bound radioactivity was determined using a TopCount NXT microplate scintillation and luminescence counter (PerkinElmer). Nonspecific binding was determined using 10 µM atropine.

**Kinetic studies.** Kinetic studies of [³H]VU6013720 association/dissociation from M₄ receptors were performed at room temperature. For association assays, membranes were incubated with [³H]VU6013720 at concentrations 2-fold over the K_d value for 0, 2, 5, 10, 20, 30, 45, 60, 90, 120, 150, and 180 minutes. For dissociation assays, membranes and [³H]VU6013720 were allowed to equilibrate for 120 minutes and then 10 µM of cold competitor was added at 120, 90, 60, 45, 30, 20, 15, 10, 5, 2, and 1 minute before termination of the assay. Following these pretreatment times, membranes were immediately harvested by quick filtration to a GF/B plate using ice-cold harvesting buffer and a 96-well Brandel harvester. Buffer and temperature conditions for all binding studies were the same.

**Animals.** Male and female wildtype (2 male and 2 female) or Chrm4 knockout (2 male and 2 female) mice (bred in-house) and male Sprague Dawley (n=15) rats (Envigo, Indianapolis, IN) were used for this study. They were housed in the animal care facility certified by the American Association for the Accreditation of Laboratory Animal
Care (AALAC) under a 12-hour light/dark cycle (lights on: 7 a.m.; lights off: 7 p.m.). The experimental protocols performed during the light cycle were approved by the Institutional Animals Care and Use Committee of Vanderbilt University and conformed to the guidelines established by the National Research Council Guide for the Care and Use of Laboratory Animals.

**Brain tissue membrane preparation and in vitro saturation binding.** Rats and mice (wild-type and Chrm4 knockout) were anesthetized by continuous isoflurane (5%) and killed by decapitation, and striatum and cortex were dissected on ice. The dissected brain regions were weighed and homogenized at a concentration of 1 mg of tissue per 25 volumes of membrane binding buffer with a polytron for two 20-second bursts. The homogenates were centrifuged at 20,000g for 20 minutes at 4°C, and the supernatant was discarded. The pellet was homogenized in membrane binding buffer and centrifuged under the same conditions as above. The final homogenate was re-suspended in membrane binding buffer and incubated at 37°C for 10 minutes and centrifuged as above. The resultant pellet was re-suspended in membrane binding buffer, and the protein concentration was determined by BCA assay. The brain homogenate was stored at -80°C until use. To determine the specific binding of [³H]VU6013720 using brain tissue membranes, saturation assays were performed as above with CHO cell membranes but using 150 µg of brain homogenate protein per well.

**Data analysis.** All experiments were carried out in duplicate or triplicate and were performed at least three times (unless stated otherwise), which aligns with our historical and standard assay protocol. Studies presented in this manuscript are
exploratory in nature and were performed to characterize a new radioligand of general interest to scientists interested in the biology of the muscarinic receptors. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA) with the following equations and were not constrained at the top or bottom.

**Saturation binding:** nonspecific binding at each radioligand concentration was subtracted from total binding and the data were fit to the following equation:

\[
\text{Specific Binding} = \frac{B_{\text{max}}*[L]}{K_d+[L]}, \text{ where } [L] \text{ is the concentration of free radioligand in nM.}
\]

**Competition binding:** Data were analyzed using the Cheng Prusoff equation:

\[
K_i=\frac{IC_{50}}{1+[\text{radioligand}]/K_d}.
\]

**Association kinetics:** \(Y=Y_0 + (\text{Plateau}-Y_0)*(1-\exp(-K*x))\), where \(Y_0\) is the \(Y\) value when time \(X\) is 0, expressed as % \(^{3}\text{H}\)VU6013720 binding; plateau is the \(Y\) value at infinite time, expressed as % \(^{3}\text{H}\)VU6013720 binding; \(K\) is the rate constant, expressed as inverse minutes; \(\text{Tau}\) is the time constant, expressed in minutes; half-time is calculated as \(\ln(2)/K\), expressed as minutes, and span is the difference between \(Y_0\) and Plateau, expressed as % \(^{3}\text{H}\)VU6013720 binding.

**Dissociation kinetics:** One-phase or two-phase exponential decay

One phase: \(Y=(Y_0-\text{NS})*\exp(-K*X) + \text{NS}, \) where \(Y_0\) is the binding at time zero, expressed as % \(^{3}\text{H}\)VU6013720 binding; \(\text{NS}\) is nonspecific binding at infinite time,
expressed as % [³H]VU6013720 binding; K is the rate constant in inverse minutes; half-time equals \( \ln(2) \) divided by K.

Two phase:  

\[
\begin{align*}
\text{Span}_{\text{Fast}} &= (Y_0 - \text{Plateau}) \times \text{Percent}_{\text{Fast}} \times .01 \\
\text{Span}_{\text{Slow}} &= (Y_0 - \text{Plateau}) \times (100 - \text{Percent}_{\text{Fast}}) \times .01 \\
Y &= \text{Plateau} + \text{Span}_{\text{Fast}} \exp(-K_{\text{Fast}} \times X) + \text{Span}_{\text{Slow}} \exp(-K_{\text{Slow}} \times X)
\end{align*}
\]

where \( Y_0 \) is the Y value when X (time) is zero, expressed as % [³H]VU6013720 binding; plateau is the Y value at infinite time, expressed in minutes; \( K_{\text{fast}} \) and \( K_{\text{slow}} \) are the rate constants, expressed as inverse minutes; time units; \( \text{Tau}_{\text{Fast}} \) and \( \text{Tau}_{\text{Slow}} \) are the two time constants, expressed as time; Half-life (fast) and Half-life (slow) are computed as \( \ln(2)/K \) and expressed as minutes; \text{Percent}_{\text{Fast}} \) is the fraction of the span (from \( Y_0 \) to \( \text{Plateau} \)) accounted for by the faster of the two components.

Data from individual experiments were fit independently and then presented as the pIC\(_{50}\) or pK\(_i/pK_d\) as Mean ± Standard Deviation. Data shown in the figures represents either combined data from multiple experiments or, in some cases, as a data set from a typical experiment as noted in the various figure legends.
Results

**VU6013720 is a selective M₄ antagonist.** VU6013720 (Figure 1) binding affinities and subtype selectivity at the five rat muscarinic receptor subtypes were assessed using binding competition against the non-selective orthosteric radioligand [³H]N-methylscopolamine (NMS) in membranes made from CHO-K1 cells expressing each receptor subtype (Table 1 and Figure 2). VU6013720 fully displaced [³H]NMS binding from rat M₄ receptors with a pKᵢ of 8.8 ± 0.1 (n=4), while pKᵢ values were 6.9 ± 0.2 (n=4) for M₂ and affinity values were above a micromolar for M₁, M₃, and M₅. The ratio of the nanomolar Kᵢ value at the receptor with the closest binding affinity, M₂, versus M₄ was 80-fold. These competition binding data, together with IC₅₀ values from calcium mobilization assays (Moehle et al., 2021), demonstrate that VU6013720 is a highly selective M₄ antagonist with single-digit nanomolar affinity, and possesses the characteristics needed to move to further characterization as a radioligand.

[³H]VU6013720 binds specifically to M₄ receptors with high affinity. We radiolabeled VU6013720 and determined the interaction of this novel radioligand across the muscarinic receptor family using saturation binding assays performed with cell membranes expressing the various receptor subtypes. Nonspecific binding was determined in the presence of a structurally related antagonist, VU6013719 (Supp. Fig. 1; synthesis provided in Supp. Methods), or the nonselective muscarinic antagonist, atropine. [³H]VU6013720 bound specifically to the rat M₄ receptor with a pKᵦ of 9.7 ± 0.2 (Figure 3A and Table 2) and a Bₘₐₓ of 4800 ± 500 fmol/mg using VU6013719 as the nonspecific binding control (n=4) and a pKᵦ of 9.5 ± 0.2 and a Bₘₐₓ of 4100 ± 500 fmol/mg using atropine (Figure 3B and Table 2, n=3). These values were slightly different when compared to the pKᵢ determined with [³H]NMS (8.8 versus 9.7/9.5), which
may suggest differential interaction of $[^3]$H$\text{VU6013720}$ and $[^3]$H$\text{NMS}$ with the receptor. In contrast, $[^3]$H$\text{VU6013720}$ exhibited no specific binding at the rat $M_1$, $M_2$, $M_3$, and $M_5$ receptor subtypes (Figure 3C-F, n=3 per receptor). These data suggest that $[^3]$H$\text{VU6013720}$ binds specifically to the $M_4$ receptor with high affinity. We performed a comparison of the pK$_i$ value of atropine with both $[^3]$H$\text{NMS}$ and $[^3]$H$\text{VU6013720}$ and found the pK$_i$ to be statistically different between the two radioligands (8.7 ± 0.1 with $[^3]$H$\text{NMS}$ and 9.0 ± 0.1 with $[^3]$H$\text{VU6013720}$, Mean ± S.D., **p=0.009). As these two radioligands are of different structure, this would suggest that the binding sites of the two, or the binding pose adopted by atropine in the presence of each radioligand, are not exactly the same. We also compared B$_{\text{max}}$ values for saturation binding studies of $[^3]$H$\text{NMS}$ and $[^3]$H$\text{VU6013720}$ and determined B$_{\text{max}}$ values of 4500 ± 700 and 4100 ± 500 fmol/mg (Supp. Fig. 2, paired Student’s t-test, p=0.219, n=3), respectively, suggesting that the two radioligands label a similar number of binding sites on the receptor. To assess cross-species binding of $[^3]$H$\text{VU6013720}$, saturation binding was also assayed with membranes expressing the human and mouse $M_4$ receptors. Similar to the rat $M_4$ receptor, $[^3]$H$\text{VU6013720}$ bound to these receptors with high affinity, with pK$_d$ values of 10 ± 0.1 (n=3) at the human $M_4$ receptor and 9.7 at the mouse $M_4$ receptor, respectively (n=1, Supp. Fig. 3 and Table 2).

**Kinetic evaluation of $[^3]$H$\text{VU6013720}$.** To fully characterize this novel radioligand, kinetic binding studies examining association and dissociation were next conducted using a concentration of radioligand calculated to be within two-fold of the K$_d$ in each experiment. Association experiments demonstrated that $[^3]$H$\text{VU6013720}$ binding reached equilibrium within 10 minutes (Figure 4A) with an association on-rate ($K_{\text{on}}$) of
1.7 ± 0.1 nM⁻¹min⁻¹ (n=3). [³H]VU6013720 dissociated completely by adding 10 µM VU6013719 or atropine, although with distinct time courses (Figure 4B; Table 3, n=3). The data for both of these compounds was better fit to a two-site decay model versus a single site. We observed faster dissociation with the structural analog VU6013719 than atropine (Table 3), suggesting that [³H]VU6013720 and VU6013719 may share a binding site that is distinct or overlapping that of atropine. We also examined dissociation kinetics induced by the positive allosteric modulator, VU0467154 (Bubser et al., 2014). VU0467154 induced partial but saturable displacement of [³H]VU6013720, similar to what we had previously observed with [³H]NMS (Bubser et al., 2014). This suggests that VU0467154 is binding to an allosteric site but induces negative cooperativity with [³H]VU6013720 by partially displacing the ligand.

[³H]VU6013720 specifically binds to M₄ in the rodent brain. One of the main advantages of a highly specific radioligand is sensitivity for detection of receptors in native tissues. To determine the utility of [³H]VU6013720 for detecting M₄ receptors in brain tissue, membranes were prepared from the striatum and cortex of rats. As shown in Figure 5, saturation binding assays detected specific binding of [³H]VU6013720 to homogenates from rat striatum and cortex with high affinities (pKᵩ = 9.0 ± 0.2 in cortex, (n=3, A)) and 9.3 ± 0.2 in striatum, (n=3, B)). Bₘᵦ values of 260 ± 110 (cortex) and 350 ± 60 (striatum) fmol/mg protein were also determined (n=3). We would note that, when the pKᵩ values for rat M₄ cells, cortex, and striatum were compared using a one-way ANOVA, there was a significant difference (p<0.05) between rat M₄ cells and the cortex, but not the striatum. This may reflect distinct microenvironments between an in vitro cell system versus the brain.
To further confirm that these binding sites were indeed M₄ receptors, saturation binding was also performed with membranes made from both wildtype (WT) and Chrm4 knockout mouse cortex. As shown in Figure 5C and D, [³H]VU6013720 specifically bound to WT mouse cortex and exhibited essentially no binding to cortical membranes from Chrm4 knockout mice. These data demonstrate that [³H]VU6013720 is a useful tool for quantification of M₄ receptors in brain tissue.

**Discussion**

M₄ muscarinic receptors are highly expressed in the brain (e.g., striatum, cortex, thalamus) and have been demonstrated to be involved in several CNS diseases (Pancani et al., 2015, Felder et al., 2018, Moehle et al., 2019, Teal et al., 2019, Dean et al., 2020, Foster et al., 2021, Moehle et al., 2021). Lack of selective muscarinic receptor antagonists has prevented the utilization of anti-muscarinic therapy due to intolerable adverse effects, and tools that assist with drug discovery efforts, such as highly specific radioligands, may open new avenues for exploration. Here, we demonstrate that [³H]VU6013720 is a novel, high-affinity, highly specific antagonist radioligand for the M₄ receptor. [³H]VU6013720 selectively binds to the M₄ receptor with pKᵢ values of 9.5 at rat, 10 at human, and 9.7 at mouse M₄ receptors. Based on this profile, we used this high-affinity radioligand to quantify M₄ receptors in brain tissue by saturation binding with brain tissue homogenates.

There are some interesting, subtle findings from the current studies. While the majority of our data point to an orthosteric or overlapping binding site for VU6013720, the pKᵢ for cold VU6013720 is 8.8 at rat M₄ using [³H]NMS binding while the pKᵢ was
calculated to be 9.5 with atropine and 9.7 with VU6013719. Additionally, competition binding studies using VU6013720 itself revealed a pKi of 9.5 (data not shown), which is close to the Kd value. When considered in the context of the significant shift of the pKi of atropine between [3H]NMS and [3H]VU6013720, it suggests that these two radioligands might make distinct contacts within similar, but not identical, binding sites. This is further supported by the dissociation kinetics experiments in which the dissociation of [3H]VU6013720 differs in the presence of atropine versus VU6013719. Other factors may impact these results, such as differences in residence time. The discrepancy between pKis for atropine with the two radioligands at M4 also suggested the possibility that, due to the high sequence conservation, a similar leftward affinity shift might exist for M2 which might have resulted in specific binding at M2; however, we did not observe any saturable binding to this receptor subtype.

We previously reported a 30-fold selectivity difference for the human and rat M4 receptors for VU6013720 in a chimeric G protein assay that allows the M4 receptor to couple to calcium mobilization (Moehle et al., 2021). In contrast, our data here show that there is only a three-fold difference in affinity between the human and rat M4 receptors. We currently do not have an explanation for this difference, although there are several possibilities. First, the functional assays include acetylcholine whereas the binding assays do not. If VU6013720 is truly orthosteric, this should not be a confound; however, if ACh/NMS and VU6013720 have subtly different binding sites and these binding sites are slightly different between the two species, this could contribute to these discrepancies. Additionally, if VU6013720 is actually an inverse agonist, its functional potency may be dissociated from its binding affinity due to G protein
interactions or other protein/protein interactions that are induced in the cells when the receptor is functionally assessed. We also do not currently know if there is a species difference in terms of contact with the chimeric G protein used in these studies, Gqi5, and whether Gqi5 impacts receptor conformation in binding studies. Future experiments around this issue could include assessment of VU6013720 effects in an assay in which constitutive activity of the receptor might be observed, such as inositol phosphate accumulation or cAMP generation, to determine if there is a distinction in inverse agonist activity, and further exploration of the effects of various G proteins or signaling partners.

To our knowledge, this is the first development of a broadly useful, highly specific antagonist radioligand with high affinity for M4 receptors, and the development of [3H]VU6013720 is anticipated to be a powerful tool for M4 receptor studies. Previous approaches to investigate the tissue distribution of the M4 receptor include RNA detection techniques, or by antibody, with confirmation using knockout tissue (Brann et al., 1988, Buckley et al., 1988, Weiner et al., 1990, Levey et al., 1991, Levey 1993, Vilaro et al., 1993, Wolfe et al., 1995, Tice et al., 1996, Gomez et al., 1999). Although these studies provide important insight into relative distribution, detected RNA levels are not always consistent with protein expression levels, and antibodies do not provide the same level of quantitation that is possible using specific radioligand binding. As we have shown in this report, our studies using [3H]VU6013720 were able to confirm and quantify the M4 receptor protein expression pattern and receptor density levels in native brain tissues. For this reason, we anticipate that [3H]VU6013720 will be an important tool for investigating the M4 receptor distribution in native tissues and may also be
useful for interrogating potential differences that may occur in different disease states or models.

In the past several years, several M₄ receptor radioligands have been reported, the M₂/M₄ PAM [³H]LY2119620 (Schober et al., 2014), and the selective M₄ PAMs [¹¹C]-VU0467485/AZ13713945 (Deng et al., 2019), [¹⁸F]-M4R-1911 (Deng et al., 2020), [¹¹C]MK-6884 (Tong et al., 2020), and [¹⁸F]12 (Haider et al., 2023). However, these radioligands have been developed from M₄ positive allosteric modulator (PAM) scaffolds and bind to allosteric sites on the M₄ receptor. [³H]VU6013720 is the first reported selective radiolabeled M₄ receptor antagonist. Therefore, it is a unique tool that could be paired with PAM radioligands to study compound binding sites as well as for the development of future M₄ antagonists and modulators. Additionally, the compound could potentially be used to map acetylcholine levels in the brain relevant for M₄ function and be useful for in vitro screening of M₄ antagonists for therapeutic treatment of movement disorders and may serve as a starting point for development of antagonists with higher affinity and favorable drug metabolism and pharmacokinetic properties to eventually lead to PET ligands.

In conclusion, we have developed the first highly selective M₄ receptor antagonist radioligand with high affinity for the rodent and human M₄ receptors, [³H]VU6013720. A reliable tool will lead to a better understanding of the physiology and pathophysiology associated with this important signaling protein and facilitate the support of drug development for neurological diseases for which M₄ modulators are predicted to exhibit efficacy, such as Parkinson’s disease, essential tremor, and schizophrenia.
Acknowledgments

The authors thank Dr. Jeff Conn and members of the Warren Center for Neuroscience Drug Discovery for stimulating and formative discussions. The authors also thank the William K. Warren Foundation for endowing the WCNDD.

Data Availability Statement

The authors declare that all processed data supporting the findings of this study are available within the paper and its Supplemental Data. Raw data are available on request from the corresponding author.

Author Contributions

Participated in research design: Qi, Rodriguez, Dickerson, Bender, Moehle, Lindsley, Rook, Niswender

Conducted experiments: Qi, Kling, Billard, Peng, Dickerson, Bender

Contributed new reagents or analytic tools: Bender, Engers, Lindsley

Performed data analysis: Qi, Rodriguez, Dickerson, Rook, Niswender

Wrote or contributed to the writing of the manuscript: Qi, Rodriguez, Niswender with input from all authors
References


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Footnotes

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Figure Legends

Figure 1. Structure of VU6013720. Cold VU6013720 was labeled using nonspecific hydrogen/tritium exchange with 99% tritium gas (RC Tritec, Switzerland) with a specific activity of 23.6 Ci/mmol.

Figure 2. Binding selectivity of VU6013720 at muscarinic acetylcholine receptor subtypes using [3H]NMS. VU6013720 and atropine competition binding curves at various muscarinic acetylcholine receptor subtypes were performed using the orthosteric muscarinic radioligand, [3H]-N-methylscopolamine. Data are the Mean ± SD from a representative of four independent experiments performed in triplicate.

Figure 3. Among the five muscarinic receptors, [3H]VU6013720 binds specifically to the rat M4 receptor. Total and specific binding for [3H]VU6013720 was determined using cells expressing each of the rat muscarinic receptors. [3H]VU6013720 bound specifically to the rat M4 receptor in a saturable manner using either VU6013719 (A) or atropine (B) as nonspecific binding controls, whereas no detectable binding was observed at rat M1, M2, M3, or M5 using atropine to determine nonspecific binding (C-F). Data shown are the Mean ± SD of a representative of at least three independent experiments performed in triplicate.

Figure 4. Kinetic characterization of the [3H]VU6013720 radioligand. Time course experiments defining association and dissociation of [3H]VU6013720 at rat M4 membranes were performed at room temperature. A. Association was initiated by addition of [3H]VU6013720 to membranes at the indicated time points before filtration. B. Dissociation experiments were performed by allowing [3H]VU6013720 to equilibrate with membranes for 2 hours; at this point, a 10 μM final concentration of VU6013719
(white), atropine (black), or VU0467154 (red) was added at designated times before terminating the reaction by filtration. Data are the Mean ± SD of three independent experiments performed in triplicate.

**Figure 5.** $[^3\text{H}]$VU6013720 binds specifically to rat brain cortical and striatal tissue as well as to cortical membranes from WT but not from Chrm4 knockout mice. Rat cortical (A) and striatal (B) homogenates were incubated with $[^3\text{H}]$VU6013720 in the presence and absence of 10 μM atropine to determine total and nonspecific binding. $[^3\text{H}]$VU6013720 bound to rat cortical membranes with a pK$_d$ of 9.0 ± 0.2 and a B$_{max}$ of 260 ± 110 fmol/mg of protein and to rat striatal membranes with a pK$_d$ of 9.3 ± 0.2 and a B$_{max}$ of 350 ± 60 fmol/mg of protein. Data are Mean ± SD and a representative of three independent experiments performed in triplicate. Cortical homogenates from WT (C) and Chrm4 knockout mice (D) were incubated with $[^3\text{H}]$VU6013720 in the presence and absence of 10 μM atropine to determine total and nonspecific binding. $[^3\text{H}]$VU6013720 binds to cortical membranes from WT mice with a pK$_d$ of 9.0 ± 0.4 and a B$_{max}$ of 240 ± 90 fmol/mg of protein, whereas little specific $[^3\text{H}]$VU6013720 binding was detected in cortical homogenates from Chrm4 knockout mice. Data are the Mean ± SD and a representative of three independent experiments performed in duplicate or triplicate.
Tables

<table>
<thead>
<tr>
<th></th>
<th>pKᵢ (Mean ± SD)</th>
<th>Kᵢ (nM)</th>
<th>Selectivity (fold over M₄ Kᵢ)</th>
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<tr>
<td>M₄</td>
<td>8.8±0.0</td>
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<tr>
<td>M₁</td>
<td>5.7±0.1</td>
<td>2000</td>
<td>1300</td>
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<tr>
<td>M₂</td>
<td>6.9±0.2</td>
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<td>80</td>
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<tr>
<td>M₃</td>
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<td>1600</td>
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<td>M₅</td>
<td>5.5±0.1</td>
<td>3100</td>
<td>2000</td>
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</tbody>
</table>

Table 1. Characterization of the binding specificity of VU6013720 using the orthosteric muscarinic antagonist radioligand [³H]-N-methylscopolamine. pKᵢ values were obtained from [³H]-NMS competition binding assays with CHO-K1 cell membranes expressing different rat muscarinic receptor subtypes. Data represent the Mean ± SD of four independent experiments performed in triplicate and refer to the curve fits in Figure 2.

[³H]-VU6013720 | Human M₄# | Mouse M₄# | Rat M₄# | Rat M₄@ |
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>pKᵢ</td>
<td>10±0.1</td>
<td>9.7</td>
<td>9.7±0.2</td>
<td>9.5±0.2</td>
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<tr>
<td>Bₘₐₓ (fmol/mg)</td>
<td>1200±40</td>
<td>1400</td>
<td>4800±500</td>
<td>4100±500</td>
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</table>

Table 2. Summary of binding properties of [³H]VU6013720 at multiple species of M₄ receptor. Data represent Mean ± SD from at least 4 experiments performed in quadruplicate (human and rat) and 1 experiment (mouse) performed in triplicate. # and @ denote the use of VU6013719 and atropine, respectively, for non-specific binding. Data refer to saturation binding curves shown in Figure 3 and Supplemental Figure 3.
<table>
<thead>
<tr>
<th></th>
<th>% Fast</th>
<th>$K_{off}$ fast (min⁻¹)</th>
<th>$K_{off}$ slow (min⁻¹)</th>
<th>Half-life fast (min)</th>
<th>Half-life slow (min)</th>
<th>Plateau</th>
<th>$R^2$</th>
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</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>51±5</td>
<td>0.56±0.39</td>
<td>0.05±0.01</td>
<td>1.6±0.9</td>
<td>15±3</td>
<td>1.0±0.7</td>
<td>0.98±0.02</td>
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<tr>
<td>VU6013719</td>
<td>44±8</td>
<td>2.0±0.5*</td>
<td>0.17±0.02***</td>
<td>0.40±0.10</td>
<td>4.2±0.5**</td>
<td>-2.2±3.2</td>
<td>0.98±0.01</td>
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<tr>
<td>VU0467154</td>
<td></td>
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Dissociation was performed by adding 10 μM of atropine, VU6013719, or VU0467154 at designated times. Data were fit with both one- and two-phase decay curves. For atropine and VU6013719, two-site fits were the preferred model; for VU0467154, a one-site model was preferred. *p<0.05, **p<0.01, ***p<0.001 between atropine and VU6013719.
Figure 1
Figure 2
Figure 4

(A) Association

(B) Dissociation

[|H|VU6013720 Binding (\%)|

Time (min)

0 2 4 6 8 10 12 60 120 180

0 25 50 75 100 125

Atropine

VU6013719

VU0467154

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 5
Supplemental Data

Development of a selective and high affinity radioligand, 

$[^3]H]VU6013720$, for the $M_4$ muscarinic receptor

Aidong Qi, Haley E. Kling, Natasha Billard, Alice L. Rodriguez, Li Peng, Jonathan W. Dickerson, Julie L. Engers, Aaron M. Bender, Mark S. Moehle, Craig W. Lindsley, Jerri M. Rook, and Colleen M. Niswender

Supplemental Figures

Supplemental Figure 1. VU6013719 fully competes for binding of $[^3]H]VU6013720$. Increasing concentrations of VU6013719 were added with $[^3]H]VU6013720$ to rat $M_4$-expressing cells. Data are the Mean ± SD from a representative assay of three independent experiments performed in triplicate.
Supplemental Figure 2. $[^3H]$VU6013720 and $[^3H]$NMS label a similar number of sites in $M_4$-expressing cells. Increasing concentrations of $[^3H]$VU6013720 (white) or $[^3H]$NMS (black) were added to membranes from rat $M_4$ cells and specific binding was assessed. $B_{\text{max}}$ values were $4500 \pm 700$ with $[^3H]$NMS and $4100 \pm 500$ fmol/mg with $[^3H]$VU6013720, and $pK_d$ values were $10 \pm 0.1$ with $[^3H]$NMS and $9.6 \pm 0.3$ with $[^3H]$VU6013720, respectively. Data represent Mean $\pm$ SD performed three times in triplicate; data shown are representative of a single experiment.
Supplemental Figure 3. $[^3]H$VU6013720 specifically binds to the human and mouse M$_4$ receptors. Specific binding of $[^3]H$VU6013720 to human (A) and mouse (B) M$_4$ receptors was evaluated using saturation binding assays, as described in Methods and Figure 3. $[^3]H$VU6013720 binds to human and mouse M$_4$ receptors with high affinity and in a saturable manner (refer to Table 2 for affinities). Data are the Mean ± SD from four experiments performed in quadruplicate (human) and 1 assay (mouse) performed in triplicate. Atropine was used as the nonspecific binding control.
Supplemental Methods

Synthesis of VU6013719.

tert-butyl (3aR,5s,6aS)-5-((6-chloropyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (3) was prepared in a similar manner as Intermediate 3 from (Moehle, Bender et al. 2021). tert-butyl (3aR,5s,6aS)-5-aminohexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (1, 5.0 g, 22.1 mmol, 1 eq) and 3,6-dichloropyridazine (2, 9.87 g, 66.3 mmol, 3 eq) were combined in tert-butanol (40 mL) and DIPEA (11.5 mL, 66.3 mmol, 3 eq) was added. The resulting solution was heated to 150 °C under microwave irradiation for 2 h, after which time the reaction mixture was concentrated under reduced pressure, and crude residue was purified by normal phase
column chromatography on silica gel (3-100% EtOAc in hexanes) to give the title compound as a tan solid (4.82 g, 64%). \(^1^H\)-NMR (400 MHz, MeOD) \(\delta\) 7.27 (d, \(J = 9.4\) Hz, 1H), 6.87 (d, \(J = 9.4\) Hz, 1H), 4.41 (p, \(J = 6.3\) Hz, 1H), 3.55 (dd, \(J = 11.1, 8.0\) Hz, 2H), 3.19 (dd, \(J = 11.4, 3.8\) Hz, 2H), 2.90 – 2.80 (m, 2H), 1.90 – 1.92 (m, 2H), 1.89 – 1.81 (m, 2H), 1.46 (s, 9H). \(^1^C\)-NMR (101 MHz, MeOD) \(\delta\) 159.3, 156.3, 146.8, 130.3, 120.5, 80.8, 53.7, 53.2 (signal broadening is observed), 42.3 (signal broadening is observed), 39.5, 28.8; ES-MS [M+H-t-butyl]^+ = 283.2.

\[
\text{tert-butyl (3aR,5s,6aS)-5-)((6-(2-chloro-5-fluorophenyl)pyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (5).} \\
\text{tert-butyl (3aR,5s,6aS)-5-((6-chloropyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (3, 102 mg, 0.3 mmol, 1.0 eq), 2-chloro-5-fluorophenylboronic acid (4, 68.0 mg, 0.39 mmol, 1.3 eq), potassium carbonate (126 mg, 0.9 mmol, 3.0 eq) and BrettPhos-Pd-G3 (27.2 mg, 0.03 mmol, 0.1 eq) were combined in a flask, and 1,4-dioxane (2.4 mL) and water (0.6 mL) were added. The resulting mixture was evacuated and purged with nitrogen (3x). The resulting mixture was stirred under an inert atmosphere at 100 °C. After 2 h, the reaction mixture was diluted with EtOAc, filtered through a pad of Celite which was rinsed thoroughly with EtOAc, and the filtrate was concentrated under reduced pressure. The crude residue was purified using normal phase column chromatography on silica gel (0-100% EtOAc in hexanes) to provide the title compound as a pale-yellow powder (84 mg, 64%).} \\
\text{\(^1^H\) NMR (400 MHz, DMSO) \(\delta\) 7.61 (dd, \(J = 8.9, 5.2\) Hz, 1H), 7.54 (d, \(J = 9.3\) Hz, 1H), 7.44 (dd, \(J = 9.3, 3.1\) Hz, 1H), 7.33 (ddd, \(J = 8.8, 8.1, 3.2\) Hz, 1H), 7.17 (d, \(J = 6.7\) Hz, 1H), 6.86 (d, \(J = 9.3\) Hz, 1H), 4.45 (h, \(J = 6.6\) Hz, 1H), 3.49 (dd, \(J = 11.1, 8.2\) Hz, 2H), 3.30 (s, 2H),}
Molecular Pharmacology [MOLPHARM-AR-2022-000643]

3.09 (dd, J = 11.5, 3.7 Hz, 2H), 1.90 (ddd, J = 12.7, 6.3, 3.3 Hz, 2H), 1.83 – 1.72 (m, 2H), 1.40 (s, 9H); \( ^{13}C \) NMR (101 MHz, DMSO) \( \delta \) 160.8 (d, \( J = 246.4 \) Hz), 157.7, 153.5, 149.0 (d, \( J = 2.0 \) Hz), 138.9 (d, \( J = 8.1 \) Hz), 131.7 (d, \( J = 9.1 \) Hz), 128.3, 126.7 (d, \( J = 2.0 \) Hz), 117.8 (d, \( J = 24.2 \) Hz), 116.9 (d, \( J = 22.2 \) Hz), 113.5, 78.3, 52.0 (signal broadening is observed), 51.7, 38.3, 28.2; ES-MS [M+H]\(^+\) = 433.0.

\((3aR,5s,6aS)\)-N-(6-(2-chloro-5-fluorophenyl)pyridazin-3-yl)-2-(cyclohexylmethyl)octahydrocyclopenta[c]pyrrol-5-amine (VU6013719). tert-butyl (3aR,5s,6aS)-5-((6-(2-chloro-5-fluorophenyl)pyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (5, 83.6 mg, 0.19 mmol, 1.0 eq) was dissolved in 1,4-dioxane (1.9 mL). A solution of HCl in 1,4-dioxane (4M, 0.72 mL, 2.96 mmol, 15 eq) was added dropwise. After 2 h at r.t., the reaction mixture was concentrated under reduced pressure to give the HCl salt, which was dried under vacuum and carried forward without additional purification (71 mg). The HCl salt was suspended in DCE (2.0 mL) and THF (0.5 mL) and cyclohexanecarbaldehyde (64.7 mg, 0.58 mmol, 3.0 eq) was added. The resulting mixture was stirred at r.t. for 15 min and sodium triacetoxyborohydride (122 mg, 0.58 mmol, 3.0 eq.) was added. After 1 h at 50 °C, sat. NaHCO\(_3\) solution was added to the reaction mixture, and the aqueous layer was extracted with DCM (3x). The combined organic extracts were washed with brine, dried over Na\(_2\)SO\(_4\), filtered and concentrated. Purification using normal phase chromatography on silica gel (0-10% MeOH in DCM) provided the title compound (56 mg, 68% over 2 steps); \(^1\)H NMR (400 MHz, DMSO) \( \delta \) 7.61 (dd, \( J = 8.9, 5.2 \) Hz, 1H), 7.53 (d, \( J = 9.3 \) Hz,
1H), 7.44 (dd, J = 9.3, 3.1 Hz, 1H), 7.32 (ddd, J = 8.8, 8.1, 3.2 Hz, 1H), 7.04 (d, J = 6.9 Hz, 1H), 6.83 (d, J = 9.3 Hz, 1H), 4.49 – 4.41 (m, 1H), 2.67 – 2.61 (m, 1H), 2.50 – 2.46 (m, 2H), 2.31 – 2.23 (m, 2H), 2.15 (d, J = 7.2 Hz, 2H), 1.86 – 1.75 (m, 4H), 1.70 – 1.57 (m, 5H), 1.46 – 1.35 (m, 1H), 1.28 – 1.08 (m, 3H), 0.89 – 0.79 (m, 2H); $^{13}$C NMR (101 MHz, DMSO) δ 160.8 (d, J = 246.4 Hz), 157.9, 148.9 (d, J = 2.0 Hz), 138.9 (d, J = 8.1 Hz), 131.6 (d, J = 9.1 Hz), 128.3, 126.6 (d, J = 2.0 Hz), 117.8 (d, J = 24.2 Hz), 116.8 (d, J = 22.2 Hz), 113.4, 62.4, 61.9, 52.0, 36.4, 31.4, 26.4, 25.6; ES-MS [M+H]$^+$ = 429.1.