Characterization of a Novel M₁ Muscarinic Acetylcholine Receptor Positive Allosteric Modulator Radioligand, [³H]PT-1284

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ABSTRACT

Selective activation of the M₁ muscarinic acetylcholine receptor (mAChR) via a positive allosteric modulator (PAM) is a new approach for the treatment of the cognitive impairments associated with schizophrenia and Alzheimer’s disease. Herein, we describe the characterization of an M₁ PAM radioligand, 8-((1S,2S)-2-hydroxycyclohexyl)-5-((6-(methyl-tributylstannyl)pyridin-3-yl)methyl)-8,9-dihydro-7H-pyrrolo[3,4-h]quinolin-7-one (PT-1284), as a tool for characterizing the M₁ allosteric binding site, as well as profiling novel M₁ PAMs. 8-((1S,2S)-2-Hydroxycyclohexyl)-5-((6-(methyl)pyridin-3-yl)methyl)-8,9-dihydro-7H-pyrrrolo[3,4-hour]quinolin-7-one (PT-1284 (1)) was shown to potentiate acetylcholine (ACh) in an M₁ fluorometric imaging plate reader (FLIPR) functional assay (EC5₀, 36 nM) and carbachol in a hippocampal slice electrophysiology assay (EC5₀, 165 nM). PT-1284 (1) also reduced the concentration of ACh required to inhibit [³H]N-methylscopolamine ([³H]NMS) binding to M₁, left-shifting the ACh Ki approximately 19-fold at 10 μM. Saturation analysis of a human M₁ mAChR stable cell line showed that [³H]PT-1284 bound to M₁ mAChR in the presence of 1 mM ACh with Kd, 4.23 nM, and saturable binding capacity (Bmax), 6.38 pmol/mg protein. M₁ selective PAMs were shown to inhibit [³H]PT-1284 binding in a concentration-responsive manner, whereas M₁ allosteric and orthosteric agonists showed weak affinity (>30 μM). A strong positive correlation (R² = 0.86) was found to exist between affinity values generated for nineteen M₁ PAMs in the [³H]PT-1284 binding assay and the EC5₀ values of these ligands in a FLIPR functional potentiation assay. These data indicate that there is a strong positive correlation between M₁ PAM binding affinity and functional activity, and that [³H]PT-1284 can serve as a tool for pharmacological investigation of M₁ mAChR PAMs.

Introduction

Muscarinic acetylcholine receptors (mAChRs) are members of the superfamily of G protein-coupled receptors (GPCR). Five mammalian subtypes have been identified and are referred to as M₁–M₅, with distribution in the central nervous system as well as the periphery. mAChRs subtypes M₁, M₃, and M₅ couple to Gₛ₁ proteins, which results in IP₃ production and subsequent calcium mobilization, whereas subtypes M₂ and M₄ couple to Gₛₐ proteins, thereby inhibiting adenyl cyclase activity (Bonner et al., 1987; Caulfield 1993; Caulfield and Birdsell, 1998). The M₁ subtype is highly expressed in the hippocampus, striatum, and cerebral cortex (Wall et al., 1991; Levey 1996; Porter et al., 2002) and activation of these receptors in regions known for memory and cognitive function are expected to bestow a procognitive effect (Caufield et al., 1983; Hagan et al., 1987; Messer et al., 1990).

The orthosteric agonist xanomeline achieved proof of concept in human clinical trials for improvements in positive, negative, and cognitive symptoms associated with schizophrenia (Shekhari et al., 2008), as well as improvements in cognitive function associated with Alzheimer’s disease (Bodick et al., 1997). Although reported as an M₇/M₄ mAChR-preferring agonist, xanomeline caus ed peripherally mediated adverse effects such as nausea, vomiting, salivation, and gastrointestinal distress, which led to a high dropout rate in these trials.

M₁ mAChRs are G protein-coupled receptors (GPCRs) that couple to members of the superfamily of G protein-coupled receptors (GPCR). Five mammalian subtypes have been identified and are referred to as M₁–M₅. They are highly expressed in the hippocampus, striatum, and cerebral cortex (Wall et al., 1991; Levey 1996; Porter et al., 2002) and activation of these receptors is expected to bestow a procognitive effect (Caufield et al., 1983; Hagan et al., 1987; Messer et al., 1990).

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ABBREVIATIONS: ACh, acetylcholine; Bmax, saturable binding capacity; BQCA, 1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid, benzyl quinolone carboxylic acid; CHO, Chinese hamster ovary; CNS, central nervous system; compound 9, 8-((1S,2S)-2-hydroxycyclohexyl)-5-((6-(methyl)pyridin-3-yl)methyl)-8,9-dihydro-7H-pyrrrolo[3,4-hour]quinolin-7-one; compound 10, (2-((1S,2S)-2-hydroxycyclohexyl)-5-((6-(methyl)pyridin-3-yl)methyl)-1,2-dihydro-3H-beno[e]isoindol-3-one; compound 12, 5-((6-chloropyridin-3-yl)methyl)-8-((1S,2S)-2-hydroxycyclohexyl)-8,9-dihydro-7H-pyrolo[3,4-hour]quinolin-7-one; compound 13, 8-((1S,2S)-2-hydroxycyclohexyl)-5-((6-(tributylstannyl)pyridin-3-yl)methyl)-8,9-dihydro-7H-pyrrrolo[3,4-hour]quinolin-7-one; DMF, dimethylformamide; FBS, fetal bovine serum; FLIPR, fluorometric imaging plate reader; Fₛₐ, fraction unbound in brain; GPCR, G protein-coupled receptor; Hₘ, human M₁; HPLC, high-performance liquid chromatography; [³H]PT-1284, 8-((1S,2S)-2-hydroxycyclohexyl)-5-((6-(methyl)pyridin-3-yl)methyl)-8,9-dihydro-7H-pyrrrolo[3,4-hour]quinolin-7-one; mAChR, muscarinic acetylcholine receptor; MPO, multiparameter optimization; NMS, N-methylscopolamine; ¹H NMR, proton nuclear magnetic resonance; PAM, positive allosteric modulator; PET, positron emission tomography; POCA, 1-[[4-cyano-4-[(2-pyridinyl)-1-piperidinyl][methyl]-4-oxo-4H-quinolizine-3-carboxylic acid; PT-1284 (1), 8-((1S,2S)-2-hydroxycyclohexyl)-5-((6-(methyl)pyridin-3-yl)methyl)-8,9-dihydro-7H-pyrrrolo[3,4-hour]quinolin-7-one; ¹³C NMR, carbon-13 nuclear magnetic resonance.
clinical trials. Alleviating these adverse effects and preserving biologic efficacy by developing subtype-selective orthosteric agonists has proven to be challenging (Messer, 2002) owing to complete homology of the muscarinic receptors at the orthosteric acetylcholine (ACh) site (Kruse et al., 2013).

A new approach for achieving subtype selectivity is the development of allosteric modulators that bind at sites distinct from the orthosteric binding site (Kruse et al., 2013). Unlike the orthosteric binding site, allosteric binding sites display heterogeneity across mAChR subtypes, providing opportunities for achieving desired subtype selectivity (Ma et al., 2009; Kruse et al., 2013). Therefore, by targeting allosteric binding sites, selective positive allosteric modulators (PAMs) could be identified to enhance both the affinity and intrinsic activity of the endogenous agonist, ACh, at the M₁ mAChR without activation of other mAChR subtypes. This profile could potentially lead to therapeutics with improved safety and toleration profiles, compared with nonselective mAChR activators. In 2009, BQCA, a highly selective M₁ PAM, was disclosed by Merck (Kenilworth, NJ; Ma et al., 2009). As a follow-on to BQCA, the second generation M₁ PAM PQCA (Kuduk et al., 2011) was shown to improve cognition in animal models designed to measure recognition memory, spatial working memory, and...
7.25 (d, J = 8.03 Hz, 1 H), 4.91–5.05 (m, 2 H), 4.60 (s, 3 H), 4.03–4.14 (m, 1 H), 3.83–3.92 (m, 1 H), 2.50 (s, 3 H), 2.18–2.12 (m 1 H), 1.71–1.97 (m, 4 H), 1.54 – 1.41 (m, 3 H).

Membrane Preparation
Stably transfected CHO cells overexpressing the human M1 mAChR subtype (M1 WYE clone 200-101, Wyeth Research) were grown adherently in M1 Dulbecco’s Modified Eagle Medium growth medium containing 10% fetal bovine serum (FBS), 1% nonessential amino acid, 1% penicillin/streptomycin, and 500 μg/ml genetecin. The cells were harvested by gently scraping in a physiologic phosphate-buffered saline solution. The collected cells were homogenized on ice using a Dounce glass/Teflon homogenizer. The homogenate was centrifuged at 1000 g for 10 minutes and the pelleted fraction was collected and stored at –80°C in 50 mM Tris (pH 7.4) at a protein concentration of approximately 2 mg protein/ml, determined using the Pierce BCA protein assay kit. Rat, nonhuman primate, and dog brain membranes were also prepared as described earlier.

Fluorometric Imaging Plate Reader Functional Potentiation Assay
CHO cells expressing the hM1, hM3, and hM5 mAChR (HD Bioscience, Shanghai, China) were seeded at a density of 10K cells per well in black-wall, clear-bottomed 384-well plates in F12 nutrient media supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were grown overnight at 37°C in 50 mM Tris (pH 7.4) at a protein concentration of approximately 2 mg protein/ml, determined using the Pierce BCA protein assay kit. Rat, nonhuman primate, and dog brain membranes were also prepared as described earlier.

CNS PET MPO

Fluorometric Imaging Plate Reader Functional Potentiation Assay
CHO cells expressing the hM1, hM3, and hM5 mAChR (HD Bioscience, Shanghai, China) were seeded at a density of 10K cells per well in black-wall, clear-bottomed 384-well plates in F12 nutrient media supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were grown overnight at 37°C in the presence of 5% CO2. The cell media was subsequently removed and replaced with loading solution, containing 2 μM Fluo-8-AM dye, 2 mM probenecid, 1/2 acid red 1, in Hanks’ balanced salt solution, and the plate was incubated for 1 hour at 37°C in the dark. For PAM potentiation measurements, the cells were preincubated with test compound for 10 minutes before being stimulated with an EC20 concentration of ACh (the actual concentration was adjusted for each experiment, but was found to be between 1 and 3 nM). Ca2+ modulation was measured using a FLIPR Tetra fluorometric imaging plate reader (Molecular Devices, Sunnyvale, CA).

Fig. 1. Analysis of known literature M1 PAMs comparing CNS PET multiparameter optimization (MPO) with reported potency data. A database of 280 compounds were compiled from the literature and their potencies were correlated with CNS PET MPO. Initial leads were limited to compounds with CNS PET MPO > 3 and M1 FLIPR calcium mobilization EC50 < 20 nM.

Hippocampal Slice Electrophysiology
Acute hippocampal slices were prepared from 7–10 week old rats whose brains were removed and immersed in ice-cold cutting solution (206 mM sucrose, 3 mM KCl, 1.25 mM NaH2PO4, 7 mM MgCl2, 26 mM NaHCO3, 10 mM d-glucose, 0.5 mM CaCl2, 1 mM L-ascorbate, 1 mM sodium pyruvate, bubbled continuously with 95% O2/5% CO2). Coronal slices (300 μm), containing the dorsal hippocampus were cut poly-ε-lysine coated plates in Dulbecco’s Modified Eagle Medium media supplemented with 10% FBS, 1% penicillin streptomycin, 500 μg/ml genetecin, 200 μg/ml hygromycin B, and 1% Glutamax for 24 hours at 37°C with 5% CO2. The culture media was replaced with equilibration media (88% CO2-independent media, 10% FBS, and 2% GloSensor cAMP reagent stock) for 2 hours at room temperature away from light. Following the 2-hour incubation, a mixture was added containing ACh at its EC20 and isoproterenol at its EC50 (concentrations were adjusted for each experiment) to the cell plate, which was then incubated for 10 minutes at room temperature. The plate was then read by Envision (PerkinElmer).

Fig. 2. Selection of PT-1284 (1). Incorporating a heteroaryl-N at the naphthalene core led to a quinoline analog PT-1284 (1) with comparable M1 PAM potency, much reduced lipophilicity, and a 4-fold higher Fm0 than compound 10.
with a vibratome (VT1000S or 1200S; Leica, Buffalo Grove, IL) and allowed to recover in artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 1.3 mM MgCl2, 26 mM NaHCO3, 10 mM D-glucose, 2 mM CaCl2, 1 mM L-aspartate, 1 mM sodium pyruvate, bubbled continuously with 95% O2/5% CO2 at 32°C for a recovery period of at least 1 hour. After recovery, slices were transferred individually to MED-P515A 64-electrode arrays (AutoMate Scientific, Inc., Berkeley, CA) and positioned with the CA1 stratum pyramidale directly over one row of eight electrodes and perfused continuously with recirculating ACSF warmed to 32°C. Electrophysiological signals were high-pass filtered at 0.1 Hz and digitized at 20 kHz using MED64 Software Mobius QT (WitWerx, Inc., Tustin, CA). Extracellular action potentials were detected offline using custom-written Matlab scripts. CA1 neuron spiking activity was measured by analytical HPLC (Phenomenex Luna C-18 column, 5-micrometer particle size, 250 mm x 4.6 mm) at a flow rate of 1 ml/min and ultraviolet at 254 nm, using 85% of 1% triethylammonium acetate (pH 4) and 15% ethanol to 1 mCi/ml. The radiochemical purity and the identity were determined by radio-HPLC analysis. Approximately 90% of the radioactivity coeluted with the reference standard. Purification to a radiochemical purity of 97.8% was achieved using a Luna C-18 column, 5-μm, 250 x 10 mm (Phenomenex P/N 00G-4252-NO, S/N 529990-1). Fractions were collected, analyzed for radiochemical purity, pooled, rotary-evaporated to dryness, and dissolved in ethanol to 1 mCi/ml. The radiochemical purity and the identity were measured by analytical HPLC (Phenomenex Luna C-18 column, 5-μm, 250 x 4.6 mm) at a flow rate of 1 ml/min and ultraviolet at 254 nm, using 85% of 1% triethylammonium acetate (pH 4) and 15–40% CH3CN and a 20-minute linear gradient and then 10 minutes at 100% CH3CN. Identity was confirmed by coelution with the reference standard. Specific activity was calculated by mass spectral analysis to be 79.5 Ci/mmol.

Fig. 3. Synthesis of PT-1284 (1). Pd(t-Bu3Sn), palladium tris-tert-butylphosphine; (Bu3Sn)2, bis(tributyltin); Pd(PPh3)4, tetrakis(triphenylphosphine) palladium; Mel, iodomethane; Pd2dba3, tris(tribenzylideneacetone) palladium; CsF, cesium fluoride.

8-((1S,2S)-2-Hydroxy-cyclohexyl)-5-((6-((tributylstannyl)pyridin-3-yl)methyl)-8,9-dihydro-7H-pyrrolo[3,4-hour]quinolin-7-one (compound 13; MW 662.4, 60 mg, 0.09 mmol, yellow glue-like residue), tri(dibenzylideneacetone)-dipalladium (0; 5 mg, 0.00546 mmol), tri(o-tolyl)phosphine (2.76 mg, 0.009 mM), and cesium fluoride (50 mg, 0.329 mM) were weighed into a 1-ml microflask and chilled to ice-cold temperature. DMF (0.5 ml) was added and immediately followed by introduction of [3H]-methyl iodide in DMF (1000 μCi at 85 Ci/mmol, 0.0116 mM, 1 ml toluene). The reaction was stirred at room temperature for 2 hours. Unreacted [3H]-methyl iodide was removed under vacuum. The residue was filtered and concentrated under reduced pressure. The dark-green residue was analyzed by radio-high-performance liquid chromatography (HPLC) analysis. Approximately 90% of the radioactivity coeluted with the reference standard. Purification to a radiochemical purity of 97.8% was achieved using a Luna C-18 column, 5-μm, 250 x 10 mm (Phenomenex P/N 00G-4252-NO, S/N 529990-1). Fractions were collected, analyzed for radiochemical purity, pooled, rotary-evaporated to dryness, and dissolved in ethanol to 1 mCi/ml. The radiochemical purity and the identity were measured by analytical HPLC (Phenomenex Luna C-18 column, 5-μm, 250 x 4.6 mm) at a flow rate of 1 ml/min and ultraviolet at 254 nm, using 85% of 1% triethylammonium acetate (pH 4) and 15–40% CH3CN and a 20-minute linear gradient and then 10 minutes at 100% CH3CN. Identity was confirmed by coelution with the reference standard. Specific activity was calculated by mass spectral analysis to be 79.5 Ci/mmol.

Fig. 4. PAM functional concentration-response curves for hM1-M5 mAChR. Measurement of functional responses for PT-1284 (1) in the presence of an EC20 concentration of ACh. Data are expressed as percentage of functional effect, and the endpoints were generated using FLIPR for M1, M3, and M5 and cAMP assays for M2 and M4. Open circles for M1 data were not fitted to the curve; this “inverted U” effect occurred at concentrations at which direct agonist activity was observed using agonist mode assay conditions. Data shown are representative of two (M1, M3), or fourteen (M1) separate experiments and expressed as the mean ± S.E.M.
Saturation Binding Assay

[3H]PT-1284 saturation studies were conducted using 96-well microtiter plates in a total volume of 250 μl containing various concentrations of [3H]PT-1284 (0.4-50 nM) in the presence of ACh (800 nM or 1 mM). The binding assay was initiated by the addition of either 100 μg of CHO hM1 mAChR membranes or brain membranes in binding buffer (50 mM Tris, 2 mM MgCl2, pH 7.4) and incubated at room temperature for 60 minutes. Nonspecific binding was defined with 10 μM compound 9 (Kuduk et al., 2012). The reaction was terminated by rapid vacuum filtration through presoaked (0.5% polyethylenimine) Whatman GF/B filter sheets using the Brandel ML 96-well Harvester (Brandel, Gaithersburg, MD). The filters were washed with ice-cold 50 mM Tris, pH 7.4, and allowed to dry prior to addition of Ultima Gold MV scintillation cocktail. Filter-bound radioactivity was measured using the PerkinElmer Tri-Carb scintillation counter. Reported Ki values were all mean ± S.E.M.

Displacement Binding Assay

[3H]PT-1284 displacement studies were conducted in the presence of ACh (800 nM and 1 mM). A total volume of 250 μl containing either 30 or 5 nM [3H]PT-1284 (corresponding to studies run with 800 nM ACh or 1 mM ACh, respectively) with increasing concentrations of compound added to 96-well microtiter plates. The binding assay was initiated by the addition of approximately 100 μg of CHO hM1 membranes in binding buffer (50 mM Tris, 2 mM MgCl2, pH 7.4). Nonspecific binding was defined with 10 μM compound 9. The assay was incubated at room temperature for 60 minutes. The reaction was terminated by rapid vacuum filtration through presoaked (0.5% polyethylenimine) Whatman GF/B filter plates. Filters were washed with ice-cold 50 mM Tris, pH 7.4, and allowed to dry prior to addition of MicroScint-20 scintillation cocktail. Radioactivity was measured using the PerkinElmer TopCount NXT scintillation counter. Reported Kᵢ values are all mean ± S.E.M.

 Autoradiographic Study of [3H]PT-1284

Male Sprague Dawley rat brains were rapidly removed and immediately placed on dry ice to freeze and then stored at -80°C. The brains were mounted, and 20-μm cryostat-cut (Zeiss, Thornwood, NY) coronal sections were transferred onto gelatin-coated slides and stored at -80°C. Prior to the assay, the sections were thawed in a 37°C incubator. Sections were then incubated for 30 minutes at room temperature in assay buffer (50 mM Tris, 2 mM MgCl2, pH 7.4) with 5 nM [3H]PT-1284 in the presence of 1 mM ACh. Nonspecific binding was defined with 10 μM compound 9. Following the incubation period, the sections were washed (2 × 2 minutes) in ice-cold assay buffer followed by a 30-second wash in ice-cold deionized water. The sections were dried rapidly under a cool air stream and further dried in a desiccant-containing vacuum. Labeled sections were exposed to Kodak BioMax MR Film for 47 days before being scanned on a BioRad 800 densitometer. Sections were later analyzed using Quantity One 1-D Software (Bio-Rad, Hercules, CA).

Data Analysis

All data were analyzed using GraphPad Prism 6.03 (GraphPad Software, La Jolla, CA). Displacement binding curves were best fit to a one-site four-parameter model, where Kᵢ values were determined from the Cheng-Prusoff relationship such that $K_i = IC_{50}/(1 + [ligand]/K_d)$, where [ligand] is the concentration of the free radioligand used in the assay and Kᵢ is the dissociation constant of the radioligand for the receptor (Cheng and Prusoff, 1973). Saturation curves were fitted using the one-site binding hyperbola model, where the Kᵢ and B_max values are calculated.

**Fig. 5.** Hippocampal slice electrophysiology. PT-1284 (1) increased spontaneous firing of CA1 neurons in rat hippocampal slices. When the compound was applied alone (agonist mode), CA1 firing rates increased in a concentration-dependent manner (EC$_{50}$ = 1.1 ± 0.32 μM, max = 35 ± 6 Hz; n = 8). When the compound was applied in the continuous presence of an EC$_{50}$ concentration of carbachol (PAM mode), PT-1284 (1) increased spontaneous firing at much lower concentrations (EC$_{50}$ = 165 ± 44 nM, max = 96 ± 20 Hz; n = 9). Data are shown as mean ± S.E.M.

**Fig. 6.** [3H]NMS binding to hM1 cell membranes. (A) PT-1284 (1) competes at the [3H]NMS orthosteric binding site. Inhibition of [3H]NMS binding to hM1 mAChR membranes by PT-1284 (1). (B) Inhibition of [3H]NMS binding to hM1 mAChR membranes by ACh in the absence or presence of various concentrations of PT-1284 (1). Data are expressed as % inhibition and analyzed for Kᵢ values using the one-site heterologous with depletion model (Swillens 1995). Data shown are representative curves from three separate experiments performed in duplicate and expressed as the mean ± S.E.M.
were derived from the formula $Y = \left( B_{max} + X \right) \left( K_d + X \right)$, where $Y$ is the specific binding and $X$ is the concentration of the ligand. To account for ligand depletion in $[^3\text{H}]$NMS saturation binding analyses for the human $M_1$, $M_3$, and $M_4$ mACHR cell lines and in $[^3\text{H}]$PT-1284 saturation binding analysis for the human $M_1$ mACHR cell line, a global-fit model with parameter-sharing was used (Motulsy and Christopoulos, 2004). Also, owing to ligand depletion, $[^3\text{H}]$NMS displacement binding was best fit to a one-site heterologous binding with depletion model (Swillens, 1995). Fluorometric imaging plate reader (FLIPR) EC$_{50}$ data were fitted to the compound percent effect using four-parameter logistic fit models.

**Results**

For the development of $M_1$-selective PAM radioligands, we used a set of central nervous system (CNS) positron emission tomography (PET) ligand design-and-selection criteria that our group had previously published, considering the similar property requirements between a PET ligand and a $[^3\text{H}]$ radioligand (Zhang et al., 2013). Specifically, we targeted leads that possessed potent $M_1$ PAM activity and high selectivity against other mAChR subtypes, and, importantly, demonstrated low nonspecific binding levels. Toward this end, a database of approximately 280 patent compounds with reported $M_1$ PAM potency values were compiled, and their CNS PET multiparameter optimization (MPO) values were calculated. Upon filtering by potency ($M_1$ PAM EC$_{50} < 20$ nM) and physicochemical property criteria (CNS PET MPO $> 3$), 18 leads were identified (Fig. 1). Notably, eight out of these 18 leads shared the tricyclic lactam core exemplified by compound 10 (Kuduk et al., 2012), and only three had a synthetic handle for tritiation, among which compound 10 emerged as the most promising lead, with a reported $M_1$ PAM EC$_{50}$ of 18 nM and good physicochemical properties (CNS PET MPO desirability of 3.15).

One of the leading causes of radioligand failure is high nonspecific binding. Fraction unbound in brain ($F_{u,b}$; Di et al., 2011) has shown to be a useful predictor for nonspecific binding and, specifically, we targeted $F_{u,b} > 0.05$ to minimize this risk (Zhang et al., 2013). Toward this end, compound 10 was synthesized and its $F_{u,b}$ was measured to monitor nonspecific binding risk. The relatively low $F_{u,b}$ value of compound 10 (0.025) prompted us to synthesize and profile additional close-in analogs of compound 10 aiming for higher $F_{u,b}$, thus lowering the risk of high nonspecific binding, at the same time maintaining favorable $M_1$ PAM potency and selectivity. As shown in Fig. 2, incorporation of a heteroaryl-N at the naphthalene core led to a quinoline analog, PT-1284 (1), that had comparable $M_1$ PAM potency [PAM EC$_{50}$ of 36 nM ($n = 14$) versus 18 nM for compound 10] with reduced lipophilicity (ELogD 1.83 versus 2.90 for compound 10; Lombardo et al., 2001), which led to much improved $F_{u,b}$ (0.10 versus 0.025 for compound 10). The synthesis of PT-1284 (1) can be seen in Fig. 3. It is worth noting that PT-1284 (1) was expected to have low brain permeability (Feng et al., 2008) as it is a P-glycoprotein substrate (MDR BA/AB = 8.8; Johnson et al., 2014). $[^3\text{H}]$PT-1284 showed no specific binding in vivo when dosed up to 100 μCi. However, it is fit-for-purpose to enable in-vitro or ex-vivo tissue radioligand binding assays for which brain permeability is not required and, subsequently can be used to facilitate the development of novel $M_1$ PAM in vivo radio-tracers and PET ligands.

**FLIPR Functional Potentiation Activity.** The effect of PT-1284 (1) on functional activity was assessed for selectivity among all mAChRs. PT-1284 (1) increased $M_1$ calcium levels in a concentration-dependent manner whether applied alone (agonist mode) or in the presence of a low concentration (EC$_{50}$) of the nonselective agonist ACh (PAM mode). In the presence of ACh at its EC$_{50}$, signaling endpoints were generated using the FLIPR assay for $M_1$, $M_3$, and $M_5$, whereas the cAMP accumulation assay was used to generate data for $M_2$ and $M_4$ (Fig. 4). In the agonist mode of our FLIPR assay, PT-1284 (1) displayed transient partial agonist activity that ranged from 125 nM to >13 μM with variable efficacy. Though this activity was noted, its relevance was uncertain and we were unable to quantify it. In response to an EC$_{20}$ challenge with $M_1$ endogenous agonist ACh, free-base PT-1284 (1) displayed robust potentiation of ACh with an EC$_{50}$ of 36 nM ≤ 7 (mean ± S.E.M., $n = 14$). There were no functional PAM responses observed for PT-1284 (1) at the $M_2$–$M_5$ mAChR subtypes when tested up to 10 μM.

**Hippocampal Slice Electrophysiology.** $M_1$ receptors are expressed in hippocampal CA1 pyramidal neurons and interneurons, where they increase cell excitability (Langmead et al., 2008, Dasari and Gullidge, 2011). In acute hippocampal

![Fig. 7. ACh concentration-response curve in the $[^3\text{H}]$PT-1284 binding assay. $[^3\text{H}]$PT-1284 (5 nM) binding to hM$_1$ mACHR membranes by ACh. Data shown are representative of four separate experiments performed in duplicate and expressed as counts per minute (mean ± S.E.M.).](image-url)

<table>
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<th>Membrane</th>
<th>$K_d$ (nM)</th>
<th>$B_{max}$ (fmol/mg)</th>
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<tr>
<td>hM$_1$</td>
<td>29 ± 3.15</td>
<td>2479 ± 415</td>
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<tr>
<td>hM$_1$</td>
<td>4.23 ± 0.17</td>
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<td>Rat Frontal Cortex</td>
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<tr>
<td>Rat Cerebellum</td>
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<tr>
<td>NHP Cortex</td>
<td>2.28 ± 0.19</td>
<td>244 ± 36</td>
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<td>NHP Cerebellum</td>
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<tr>
<td>Dog Cortex</td>
<td>2.88 ± 0.69</td>
<td>118.5 ± 7.72</td>
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$^a$Saturation binding in the presence of 800 nM ACh.

$^b_n = 2$

$^c$No specific binding detected.
slices, this can be measured as an increase in the spontaneous-action-potential-firing rate of CA1 neurons (Langmead et al., 2008). Since acetylcholinesterase is present in rat brain slices and has the ability to catalyze the breakdown of the mAChR endogenous ligand ACh (Lockhart et al., 2001), carbachol was used as the cholinergic agonist to define PAM mode for this assay. In acute slices of dorsal hippocampus from adult rats, PT-1284 (1) increased spontaneous CA1 neuron firing in a concentration-dependent manner whether applied alone (agonist mode) or in the presence of a low concentration (∼EC10–EC30) of the nonselective cholinergic agonist carbachol (PAM mode). However, the potency and efficacy of the compound were both enhanced by the presence of carbachol, consistent with an agonist-PAM pharmacological profile. When the concentration-response data from each slice were fitted individually, the agonist EC50 was 1.1 ± 0.32 μM (mean ± S.E.M., n = 8 slices) and the maximal agonist effect was 35 ± 6 Hz, whereas the PAM EC50 was 165 ± 44 nM (mean ± S.E.M., n = 9 slices) and the maximal PAM effect was 96 ± 20 Hz (Fig. 5).

**[3H]NMS Displacement.** The effects of PT-1284 (1) on the affinity of ACh at the [3H]NMS orthosteric binding site were examined using CHO cells stably transfected with the hM1 mAChR. Data shown are a representative of three separate experiments performed in triplicate and expressed as the mean ± S.E.M.

![Fig. 8. [3H]PT-1284 saturation curves and Scatchard analyses (inset graphs) for (A) hM1 cell membranes (total binding = 46,204 dpm and nonspecific binding = 2714 dpm at approximately 3 nM [3H]PT-1284), (B) rat frontal cortex membranes (total binding = 4329 dpm and nonspecific binding = 2559 dpm at approximately 3 nM [3H]PT-1284), (C) nonhuman primate cortex membranes (total binding = 1594 dpm and nonspecific binding = 735 dpm at approximately 3 nM [3H]PT-1284), (D) dog cortex membranes (total binding = 706 dpm and nonspecific binding = 360 dpm at approximately 3 nM [3H]PT-1284). Saturation binding was measured in the presence of 1 nM ACh. Data shown are a representative of three separate experiments performed in triplicate and expressed as the mean ± S.E.M.

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mAChR subtype ($[^{3}H]$NMS: $K_d = 0.506 \text{ nM}$; $B_{\text{max}} = 3069 \text{ fmol/mg protein}$). Although PT-1284 (I) was shown to compete with the nonselective muscarinic receptor antagonist $[^{3}H]$NMS with weak affinity ($K_i = 7.84 \pm 1.42 \text{ nM}$; mean $\pm$ S.E.M., $n = 3$; Fig. 6A), there were no changes to the maximal response of ACh, indicating that PT-1284 (I) did not compete with ACh for binding to $M_1$ mAChR (Fig. 6B). PT-1284 (I) reduced the concentration of ACh required to inhibit $[^{3}H]$NMS binding to $M_1$ mAChR, left-shifting the $EC_{50}$ of ACh by approximately 19-fold at 10 $\mu$M (Fig. 6B).

**Optimization of $[^{3}H]$PT-1284 Binding Protocol.** Preliminary studies to ascertain assay conditions for $[^{3}H]$PT-1284 binding to CHO cells stably expressing the h$M_1$ mAChR were performed at radiolabel concentrations below PT-1284 (I)'s $EC_{50}$ of 36 nM. Binding experiments were initially performed in the absence of the orthosteric agonist ACh and no specific

![Chemical Structures](image)

**Fig. 9.** Structures of compounds used in $[^{3}H]$PT-1284 displacement binding assay. Structures include mAChR allosteric agonists, orthosteric agonists, and $M_1$ PAMs.
Kinetic studies showed the rate of association of \(^{3}H\)PT-1284 membranes and plateaued between 50 and 200. Specific binding increased with increasing concentrations of ACh, the percent specific binding of \(^{3}H\)PT-1284 to hM1 incubations were performed at room temperature for 60 minutes. Concentration of 5 nM, membrane concentration of 100 nM; 244 fmol/mg protein; 2.88 ± 0.69 nM; 118.5 ± 7.2 f mol/mg protein, respectively (mean ± S.E.M., n = 3). \(^{3}H\)PT-1284 bound to rat frontal cortex and hippocampus with similar affinity and saturable binding capacity [2.65 ± 0.34 nM and 415 ± 31 fmol/mg protein; 2.55 ± 0.57 nM and 410 ± 51 fmol/mg protein, respectively (mean ± S.E.M., n = 3)]. Scatchard plots for all saturation experiments could be fitted by a straight line, indicating that \(^{3}H\)PT-1284 bound to a single site in each case (Fig. 8, A–D). M1 mAChR specific binding was not detected in rat or nonhuman primate cerebellum when tested up to a \(^{3}H\)PT-1284 concentration of 50 nM (data not shown).

\(^{3}H\)PT-1284 Displacement Binding. \(^{3}H\)PT-1284 displacement studies were performed with a series of compounds previously determined to be PAMs, allosteric agonists, or orthosteric agonists at the M1 mAChR (data not shown). The rank order of potency was consistent with structural recognition of the \(^{3}H\)PT-1284 binding site, in that PAMs showed higher affinity than compounds known to be allosteric or orthosteric agonists (Table 2; Fig. 9). M1 mAChR PAM affinities were 10-fold weaker when the assay was run in the presence of 800 nM ACh. For example, the affinity of compound 3 for the \(^{3}H\)PT-1284 binding site decreased from 9.7 ± 0.23 nM to 84 ± 15 nM (mean ± S.E.M., n = 3; data not shown), respectively. As shown in Fig. 10, for a cohort of 19 M1 PAMs previously reported by our group (Davoren et al., 2016), a positive correlation (R² = 0.86) was found between the EC₅₀ values obtained using the M1 mAChR FLIPR PAM assay and the binding pKi values determined with the \(^{3}H\)PT-1284 binding displacement assay.

![Fig. 11. Autoradiographic analysis of representative rat brain coronal sections using \(^{3}H\)PT-1284. (A) Cortex, caudate, and nucleus accumbens and (B) hippocampus represent autoradiograms for total specific binding. (C) Inhibition of specific binding by 10 μM compound 9. All binding was done in the presence of 1 mM ACh. (D) Density scale in the range of 3.7–2000 μCl/g protein.](image-url)
**Autoradiographic Analysis for [3H]PT-1284 Binding to Rat Brain.** Autoradiographic distribution of [3H]PT-1284 binding to coronal sections of rat brain is shown in Fig. 11. In representative sections shown in (A) and (B), the highest distribution of M₁ mAChR [3H]PT-1284 binding can be seen in the cortex (CTX), caudate (CPu), nucleus accumbens (Abc), and the hippocampus (Hip). [3H]PT-1284 binding was displaced by 10 μM compound 9 (Fig. 11C).

**Discussion**

Although recent advancements have been made in the development of mAChR allosteric modulators to improve subtype selectivity, little has been reported on the development of radiolabeled modulators to study the actual allosteric binding pocket. With the characterization of the M₂/M₄ mACHr allosteric modulator radioligand [3H]LY2119620, great strides were taken to further the understanding of mAChR allosteric binding sites (Schober et al., 2014). As with other GPCR PAM radioligands such as the mGluR4 PAM, and [3H]PAM2 (Le Poul et al., 2012), these authors showed positive cooperativity between the orthosteric and allosteric binding sites and further showed that selectivity between M₂ and M₄ mAChR subtypes was dependent on the allosteric ligand used. This is the first report on the development and characterization of a novel M₁-selective mAChR PAM radioligand that possesses high M₁ PAM potency and broad spectrum selectivity, and demonstrated high M₁-specific binding in vitro.

Herein, we presented evidence from several in vitro assays to show the pharmacological profile of PT-1284 (1) as an agonist-PAM. PT-1284 (1) was shown to have PAM characteristics in a FLIPR assay by potentiating M₁ mAChR calcium mobilization when in the presence of an EC20 concentration of ACh. PT-1284 (1) also showed higher potency and induced a greater response in hippocampal CA1 neurons when applied in the presence of carbachol. In the past, the only way to assess greater response in hippocampal CA1 neurons when applied in the presence of carbachol. In the past, the only way to assess greater response in hippocampal CA1 neurons when applied

- Increased the affinity of [3H]PT-1284 for the receptor, revealing subnanomolar affinity at maximal concentrations of ACh. In this study, we showed that cooperativity between our PAM ligand and the orthosteric ligand ACh may be improved by increasing the number of G protein-bound receptors. In spite of the fact that differences in ACh levels have been reported across species (Fuji et al., 1995), [3H]PT-1284 bound with similar affinity to native rat, dog, and NHP tissues when enhanced with the addition of ACh, which is important in that cross-species translation can be used to predict occupancy of a clinical compound in humans from rat in vivo binding or NHP PET receptor occupancy studies (Shaffer et al., 2014). Autoradiographic localization studies showed distribution in the cortex, caudate, nucleus accumbens, and hippocampus of the rat brain, which correlates with the observed binding capacities likewise found in our saturation binding studies. Also, with [3H]PT-1284 displacement studies, we observed greater affinity of M₁ PAM compounds in the presence of higher concentrations of ACh. The rank order of affinities was consistent with structural recognition of the PT-1284 (1) binding site, in that PAMs showed higher affinity compared with compounds known to be allosteric or orthosteric agonists. Taken together, these data showed cooperation between the ACh orthosteric binding site and the PT-1284 (1) binding site. To demonstrate [3H]PT-1284 as a tool in characterizing novel M₁ mAChR PAMs, a direct correlation of binding pKᵣ and functional EC₅₀ values (R² = 0.86) was observed for a cohort of compounds.

In summary, we have demonstrated PT-1284 (1) to be a M₁-selective PAM agonist that acts as a PAM when [3H]PT-1284 binding is performed using 5 nM. With the characterization of the radioligand and a direct correlation with our calcium mobilization assay, we have shown [3H]PT-1284 to be a tool in profiling future M₁ mAChR PAMs. These findings could enable improvements in the treatment of symptoms of schizophrenia and cognitive deficits associated with Alzheimer’s disease.

**Authorship Contributions**

**Participated in research design:** Smith, Davoren, Lee, Zhang, Grimwood.

**Conducted experiments:** Smith, Edgerton, Lee, Neal.

**Performed data analysis:** Smith, Edgerton, Lazzaro, Neal.

**Wrote or contributed to the writing of the manuscript:** Smith, Davoren, Edgerton, Lee, Zhang, Grimwood.

**References**


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**References**


Cheng Y, Prassad BV, and Inoue M (1991) Inhibition of M1 and M2 muscarinic receptors by 1871697:480 and 23490 and 24471 (K1) and the concentration of inhibitor which causes 50 per cent inhibition (IC50) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108.


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