Molecular Pharmacology Fast Forward. Published on July 21, 2010 as doi: 10.1124/mol.110.065771

MOL #65771

The M1 Muscarinic Receptor Allosteric Agonists, AC-42 and TBPB Bind to a Unique Site Distinct From the Acetylcholine Orthosteric Site

Marlene A. Jacobson, Constantine Kreatsoulas, Danette M. Pascarella, Julie A. O'Brien, and Cyrille Sur

Departments of Schizophrenia Research (M.A.J.,D.M.P.,J.A.O), Molecular Systems (C.K) and Imaging (C.S.), Merck Research Labs, West Point, PA 19486 USA

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

MOL #65771

Running Title Page

AC-42 and TBPB Share a Similar Binding Pocket

Dr. Marlene A. Jacobson
Merck Research Labs
Schizophrenia Research
WP26-265
West Point, PA 19486
Tel: 215-652-7122
Fax: 215-652-2075
marlene_jacobson@merck.com

Text pages: 29 (excluding figure legends)

Tables: 3

Figures: 7

References: 38

Word count

Abstract 250

Introduction 745

Discussion 1509

Abbreviations: ACh, acetylcholine; CHONFAT, Chinese hamster ovary; NFAT, nuclear factor of activated T-cells; QNB, quinuclidinyl benzilate; FLIPR, fluorometric imaging plate reader; TM, transmembrane domain; AC-42, 4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride; GPCR, G-protein-coupled receptor; HEPES, 4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid; TBPB, 1-[1'-(2-methylbenzyl)-1,4'-bipiperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Activation of M1 muscarinic receptors occurs through orthosteric and allosteric binding sites. To identify critical residues, site directed mutagenesis and chimeric receptors were evaluated in functional calcium mobilization assays to compare orthosteric agonists, acetylcholine and xanomeline, M1 allosteric agonists AC-42 (4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride), TBPB (1-[1'-(2-methylbenzyl)-1,4'-bipiperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one) and the clozapine metabolite, N-desmethylclozapine. Previously, Spalding et al (Mol Pharmacol (2002) 61:1297-1302) defined a minimal epitope for AC-42 comprising the first 45 amino acids, the third extracellular loop and seventh transmembrane domain. Using chimeric M1 and M3 receptor constructs, the AC42 minimal epitope has been extended to also include transmembrane II. Phe 77 was identified as a critical residue for maintenance of AC-42 and TBPB agonist activity. In contrast, the functional activity of N-desmethlyclozapine did not require Phe 77. To further map the binding site of AC-42, TBPB and N-desmethylclozapine, point mutations previously reported to affect activities of M1 orthosteric agonists and antagonists were studied. Docking into a M1 receptor homology model revealed that AC-42 and TBPB share a similar binding pocket adjacent to the orthosteric binding site at the opposite face of tryptophan 101. In contrast, the activity of N-desmethylclozapine was generally unaffected by the point mutations studied and the docking indicated that N-desmethylclozapine bound to a site distinct from AC-42 and TBPB overlapping with the orthosteric site. These results suggest that structurally diverse allosteric agonists, AC-42, TBPB and N-desmethylclozapine may interact with different subsets of residues, supporting the hypothesis that M1 receptor activation can occur through at least three different binding domains.

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Cholinergic dysfunction has been associated with contributing to the pathophysiology of Alzheimer's disease and psychiatric disorders, including schizophrenia. Evidence from preclinical and clinical studies suggest that overcoming the deficits in the M1 muscarinic acetylcholine receptor with mechanisms leading to selective receptor activation may contribute to improving neuropsychiatric symptoms in patients with Alzheimer's disease and schizophrenia (Langmead et al., 2008; Caccamo et al., 2009). Currently, the use of acetlycholinesterase inhibitors is a first line treatment of symptoms resulting from the decline in cognitive function in Alzheimer's disease. The M1/M4 agonist, xanomeline has shown improvements in psychotic behaviors and cognitive abilities in Alzhemier disease patients (Bodick et al., 1997) and improving psychosis in schizophrenia patients (Shekhar et al., 2008). The M1 muscarinic receptor belongs to a family of G-protein coupled receptors comprised of five subtypes designated M1 through M5. M1, M3 and M5 subtypes couple preferentially through Gq and M2 and M4 receptors couple through Go/Gi. The M1 muscarinic receptor subtype is most abundantly expressed in prefrontal cortex and hippocampus, regions which are associated with involvement with cognitive functioning. Development of selective agonists of the muscarinic receptor subtypes has been challenging due to the high conservation of the orthosteric acetylcholine binding site among the five subtypes. Lack of subtype selectivity has prevented development of muscarinic agonists as therapeutics as activity on peripheral M2 and M3 muscarinic subtypes are associated with adverse side effects such as salivation, bradycardia and gastrointestinal disturbances (Bymaster et al., 2003; Wess et al., 2003).

Significant advancement in the identification of selective M1 muscarinic receptor agonists have been realized by focusing on the discovery of allosteric modulators of the M1 receptor (Spalding et al., 2002; Jones et al., 2008; Ma et al., 2009; Marlo et al., 2009). Two types of allosteric modulators of M1 muscarinic receptors have been reported – allosteric agonists and allosteric modulators. The pharmacological action of these molecules differ in that allosteric agonists can produce receptor activation in the absence of the orthosteric agonist, acetylcholine, whereas allosteric potentiators require the

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

presence of the orthosteric agonist and exert no pharmacological action alone. Several models of the orthosteric binding site have been proposed based on mutagenesis and molecular docking studies with the endogenous agonist, acetylcholine and the antagonist, N-methylscopolamine (Hulme at al., 2003; Peng et al., 2006). These studies have revealed that the binding site for acetylcholine is located within the transmembrane domains with contact residues contributed by TM3, TM4, TM6 and TM7. In contrast, both allosteric agonists and positive modulators are postulated to bind to sites distinct from the orthosteric site. The number of unique sites and molecular determinants of allosteric binding sites on M1 receptors and the proximity of allosteric sites to the orthosteric agonist site are unknown and it is unclear whether there may be overlap among these sites for binding these ligands.

AC-42 was the first allosteric agonist described for the M1 muscarinic receptor (Spalding et al., 2002; Langmead et al., 2006). Mutagenesis studies employing chimeric receptors and point mutations in the orthosteric acetylcholine binding site defined a minimum epitope comprised of the first 45 amino acids, the third extracellular loop and the seventh transmembrane domain. More recently, a novel structure, TBPB has also been described as a M1 selective allosteric agonist (Jones et al., 2008). Previously, we have shown that N-desmethlyclozapine, a major metabolite of the atypical antipsychotic, clozapine displayed partial agonist activity on M1 receptors (Sur et al., 2003). Furthermore, N-desmethlyclozapine was postulated to be an allosteric agonist of the M1 receptor on the basis of observing maintenance of functional activity on M1 receptors with a Y381A mutation in the orthosteric agonist site which significantly diminished the receptor's potency for acetylcholine. However, kinetic dissociation studies demonstrated that N-desmethylclozapine failed to modulate the binding of orthosteric ligands and therefore, did not meet the criteria for an allosteric modulator (Spalding et al 2006). The specific binding site for N-desmethylclozapine was not defined in these studies.

In this study, we utilized mutagenesis and chimeric receptor approaches to define amino acid residues required for the binding of agonists acetylcholine, xanomeline, the allosteric agonists, AC-42 and TBPB and N-desmethylclozapine. This study further

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

extends the model proposed for AC-42 binding (Spalding et al., 2002; Spalding et al., 2006; Lebon et al., 2009) and in addition, proposes a model for the newly described M1 allosteric agonist, TBPB. As a result, we define the structural relationship between orthosteric and allosteric binding sites and provide further support for M1 receptor activation occurring through at least three different binding domains.

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Ligands. Acetylcholine and atropine were from Sigma-Aldrich (St. Louis, MO), N-desmethylclozapine, clozapine and gallamine were purchased from Tocris (Ellisville, MO). AC-42 (4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride, TBPB (1-[1'-(2-methylbenzyl)-1,4'-bipiperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one) and xanomeline were synthesized at Merck Research Labs, Medicinal Chemistry. [^{3}H]quinuclidinyl benzilate (QNB) (30-60 Ci/mmol) and [^{3}H]-N-methylscopolamine (NMS) (81 Ci/mmol) were obtained from Perkin Elmer Life Sciences (Boston, MA).

DNA Constructs and Expression. Chimeric receptor cDNAs were constructed by overlapping PCR from human M1 and M3 muscarnic cDNA templates. NC3 M1M3 corresponds to M1 amino acids 1-104, M3: 148-513 and M1: 388-460. NC4M1M3 corresponds to M1 amino acids 1-45, M3: 89-513, M1: 388-419 and M3: 546-590. Single point mutations of M1 and NC4M1M3 were introduced with the Stratagene Quik-Change Kit (La Jolla, CA) and confirmed by sequencing. M1, M1Y381A, NC3M1M3 and NC4M1M3 were subcloned in pcDNA3.1 and transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) into CHONFATbla cells. Stable clones were selected in 1 mg/mL G418 and cells were cultured in growth media (D-MEM containing 10 % FBS, 25 mM HEPES, 1mM sodium pyruvate, 0.1 mM non-essential amino acids, 50 units/ml Pen/Strep, 2 mM glutamine, 250 μg/ml Zeocin) and grown in 5% CO2, 37^0 C. Point mutations of NC4M1M3 or M1 wild type were transiently transfected with Lipofectamine 2000 and analyzed in parallel with M1 wild type and non-mutated NC4M1M3 receptors, 48 h post transfection.

Membrane Preparations and Binding Studies Membranes from stably or transiently transfected CHONFAT cell lines were prepared by homogenization in 20 mM HEPES, 1 mm EDTA, pH 7.4. Membranes were resuspended in 20 mM

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

HEPES, 5 mM $MgCl_2$, pH 7.4. The expression level of wild-type, chimeric and mutated muscarinic receptors was determined by [^{3}H]QNB binding performed according to Ward et al (1999) in 20 mM HEPES, 100 mM NaCl, 1 mM $MgCl_2$ pH 7.4 at 30 ^oC, with 20 µg protein per tube, 500 µl final volume for 3 hours. Nonspecific binding was determined using 10 µM atropine. The assay was terminated by rapid filtration onto GFC filter plates pre-soaked in 0.3% PEI, followed by four washes with ice cold assay buffer using a Brandel 96 Harvester. Dried plates were counted in a Topcount with Microscint-20 (Perkin Elmer Life Sciences, Boston, MA). Binding data represents the mean of two determinations and data were analyzed by nonlinear regression analysis using PRISM software (GraphPad Software, Inc., San Diego, CA). Receptor expression levels for stable M1 wildtype and Y381A point mutant cell lines were 475 +/- 25 fmol/mg and 1050 +/- 50 fmol/mg, respectively. Receptor expression for stably expressed chimeric M1 M3 constructs, NC3M1M3 and NC4M1M3 were 3.1 +/- 0.5 pmol/mg and 3.3 +/- 0.2 pmol/mg, respectively. Receptor expression for transiently expressed M1 wildtype and M1 point mutants are reported in Table 3.

Kinetic off-rate experiments were performed with 200 pM [^{3}H]NMS and 10 µg/tube hM1 membranes in 20 mM HEPES, 100 mM NaCl, 5 mM MgCl2, pH = 7.4 buffer. Membranes plus [^{3}H]NMS in the presence or absence of 1 µM atropine were incubated at room temp for 1 hr to achieve equilibrium. At the end of the incubation, 1 µM atropine in the absence or presence of 100 µM TBPB, 10 µM clozapine, or 1 mM gallamine were added and the bound [^{3}H]NMS was captured at various timepoints on GF/C filtermats pre-soaked in 0.3% PEI by filtration on a Skatron and washing with ice cold assay buffer. The amount of specific [^{3}H]NMS bound was calculated and analyzed by GraphPad/Prism using the one phase exponential decay equation.

Flourescence Imaging Plate Reader (FLIPR) Measurements. Stable or transiently transfected CHONFAT cells were plated in clear-bottomed, poly-D-lysine coated 384-well plates (Becton-Dickinson, Franklin lakes, NJ) in growth

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

medium using a Labsystems Multidrop. The plated cells were grown overnight at 37 ^{o}C in the presence of 5% CO_2. The following day, the cells were washed with 3 x 100 µl assay buffer (Hanks Balanced Salt Solution containing 20 mM HEPES, 2.5 mM probenecid, and 0.1% bovine serum albumin) using a Skatron Embla cell washer. The cells were incubated with 1 µM Fluo-4AM (Invitrogen, Carlsbad, CA) for 1 h at 37 ^{o}C and 6% CO_2. The extracellular dye was removed by washing as described above. Calcium flux was measured using Molecular Devices $FLIPR_{384}$, fluorometric imaging plate reader. For dose response curves, cells were incubated for 4 minutes in assay buffer before addition of various concentrations of agonists and calcium flux measured for 4 minutes after agonist addition. Functional data represents the means of at least three determinations performed in triplicate and were analyzed using PRISM software (GraphPad Software, Inc., San Diego, CA).

M1 Receptor Homology Model Building The homology model of M1 was built using the rhodopsin x-ray crystal structure (Palczewski et al., 2000) as a template. Alignment of the seven transmembrane helices was accomplished using established conserved residues which are hallmarks of class A GPCRs (Fredriksson et al., 2003). Residues Leu225-Lys-353, comprising intracellular loop 3 (ic3) of M1 were deleted to facilitate model building. One thousand initial models were built using MOE 2005.06 (CCG, 2005) and the ten lowest energy models were taken into Maestro's Prime module for loop refinement (Jacobson et al., 2004; Schrodinger, 2006), while maintaining the disulfide bond between Cys98 (3.25) and the second extracellular loop Cys178. Amino acids in the model are referred to according to their M1 sequence position and supplemented by the nomenclature of Ballesteros and Weinstein (1995). Rotamer sampling using the software vendor's rotamer libraries was allowed during all phases of refinement.

Small Molecule Docking Protocol Initial ligand docking was accomplished using the induced fit protocol as implemented in the Maestro interface (Sherman et al., 2006) (Schrodinger, 2006) using GlideXP with enlarged inner and outer boxes and a distance dependant dielectric constant (ε=4). All ligands poses within 25 kcal/mol of the global

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

minima (irrespective of score) were kept and further flexibly refined using MMFF94s (Halgren, 1999) as implemented in our in-house MIX (modeling in UNIX) system. During this stage of docking, all protein sidechains within an 8 Å radius and the backbone of the extracellular loops were allowed mobility while maintaining the disulfide bond between Cys98 (3.25) and the second extracellular loop Cys178. After this round of refinement, ligand poses within the lowest 10 kcal/mol of their global *in vacuo* minima were inspected visually and the partner protein conformer from each pair was inspected for side-chain/backbone geometries which fell within the allowed Ramachandran space. Those with many Ramachandran violations were rejected. Individual sidechain rotamers were sampled using the rotamer library in PyMol (DeLano, 2002) and re-minimized using the above protocol.

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

Results

The M1 muscarinic receptor agonists profiled in these studies included the orthosteric agonist, acetylcholine, the muscarinic M1/M4 agonist xanomeline, the clozapine metabolite, N-desmethylclozapine, the M1 allosteric agonist AC42 and a newly described M1 allosteric agonist, TBPB (Figure 1). The functional activities of these ligands were first evaluated on stably expressed wild-type M1 and Y381A receptors in calcium mobilization assays measured by FLIPR. Mutation of the highly conserved tyrosine 381(6.51) in transmembrane VI (TMVI) has been previously shown to drastically reduce the ability of orthosteric ligands to bind and activate the M1 receptor (Ward et al., 1999). The Y381A point mutation was also utilized in the first in vitro characterization of AC-42 by Spalding et al., (2002) where in contrast to acetylcholine, functional agonist activity was observed to be maintained, suggesting that the binding site for AC-42 on M1 receptors may be different from the orthosteric agonist site. We have also previously shown that N-desmethlyclozapine, a major metabolite of the atypical antipsychotic clozapine, retained functional activity on the Y381A mutant and exhibited increased potency and efficacy (Sur et al., 2003). This is in contrast to a marked decreased activity observed with acetylcholine on the Y381A mutant. TBPB, an M1 allosteric agonist (Jones et al., 2008) was also found to exhibit an increased potency of ~ 10-fold and increased efficacy on the Y381A mutant compared to wild type M1 receptors (Table 1). In comparison to these observations with TBPB, AC-42 and N-desmethlyclozapine, the functional potency and maximal efficacy of xanomeline was found to be slightly reduced on the Y381A mutant.

To further investigate the allosteric interactions of TBPB at M1 receptors, kinetic experiments of [^{3}H]NMS dissociation were carried out on CHO-hM1 cell membranes. As reported in Figure 2, the orthosteric ligand atropine (1 μM) alone displaced bound [^{3}H]NMS with a k_{off} of 0.060 ± 0.004/min (mean ± s.e.m.) equivalent to a $t_{1/2}$ of 12 min. In contrast, the classical muscarinic allosteric compound gallamine drastically slowed the atropine-induced dissociation of [^{3}H]NMS as evidence by a $t_{1/2}$ of 97 min. and k_{off} of 0.0071 ± 0.0016/min. This result was expected and consistent what has been previously reported for gallamine (Langmead et al., 2006; Spalding et al., 2006). In comparison,

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

TBPB (100 μM) showed a significant retardation of [^{3}H]NMS dissociation, $t_{1/2}$ of 20 min. and k_{off} of 0.034 ± 0.002/min. This effect of TBPB was similar to that reported for AC-42, where a significant retardation of the [^{3}H]NMS dissociation rate was observed (Langmead et al., 2006; Spalding et al., 2006). In contrast to AC-42, Spalding et al., (2006) demonstrated that neither N-desmethlyclozapine nor clozapine produced retardation of the [^{3}H]NMS dissociation rate. Similarly to previous published reports, clozapine was found to lack a significant effect ($t_{1/2}$ = 12 min.; k_{off} = 0.057 ± 0.004/min (Figure 2).

The functional activity of the M1 muscarinic agonists were further profiled on chimeric receptors constructed between M1 and M3 muscarinic receptor subtypes in the attempt to determine binding epitopes for the various ligands. Previously, in a series of elegant experiments, Spalding et al., (2002) employed chimeric receptors composed of M1 and M5 muscarinic receptor sequences and defined a minimal binding epitope for AC-42 comprised of the first 45 amino acid residues of M1 corresponding to the amino terminus and TMI, the third extracellular loop and TMVII domains. In the present study, the M3 muscarinic receptor subtype was chosen to generate chimeric receptors with M1, as the M3 receptor exhibits less homology with M1 sequences in comparison to the M5 receptor subtype. Evaluation of M1-M3 chimeras could potentially identify additional critical residues for binding of AC-42 and other M1 agonists which may have been obscured by the high degree of homology between M1 and M5 receptor amino acid sequences. The chimeric constructs were designated NC3M1M3 and NC4M1M3. NC3M1M3 consisted of the M3 receptor with substitution the first 45 amino acids of the M1 sequence (amino terminus and TMI), the third extracellular loop and TMVII of M1. NC3M1M3 contained additional M1 sequence in comparison to NC4M1M3 and included the first intracellular loop and TMII of M1 and the carboxy terminal tail of M1. Stable clones of NC3M1M3 and NC4M1M3 were established and the functional activities of the M1 agonists were measured on the chimeric receptors in calcium mobilization assays measured by FLIPR and compared with stably expressed wild type M1 receptors (Table 2). Cell lines were selected for evaluation and comparison of functional activities on the basis of having comparable expression levels. The functional potency and maximum

response of acetylcholine, xanomeline and N-desmethylclozapine were found to be similar between wild type M1, NC3M1M3 and NC4M1M3 chimeric receptors (Table 2). In contrast to the profiles observed with acetylcholine, xanomeline and N-desmethylclozapine, a dramatic reduction in activity was determined for both AC-42 and TBPB (EC50 values of > 100 μM) on the NC4M1M3 chimeric receptor (Table 2). In comparison, a partial restoration of activity was observed for AC-42 and TBPB on the NC3M1M3 chimera. These results indicated a role for amino acid residues within TMII of the M1 receptors for maintaining functional potency and efficacy of AC-42 and TBPB and suggested that the binding sites for the allosteric agonists were differentiated from acetylcholine, xanomeline and N-desmethylclozapine.

To further elucidate the amino acid residues in TMII involved in binding AC-42 and TBPB, single point mutations were introduced into the NC4M1M3 construct in the attempt to generate a "gain of function" mutant and identify amino acid residues which may contribute restoration of activity with AC-42 and TBPB on the chimeric receptor. To guide selection of amino acids for mutagenesis, the sequences comprising TMII, intracellular loop 1 (i1) and extracellular loop 1 (o1) were aligned between M1, M3 and M5 receptor subtypes to locate differences between the sequences, specifically, those amino acids in M3 which differed from M1 and M5 (Figure 3). A comparison with M5 sequences was included as previously Spalding et al., (2002) had shown that AC-42 activity was retained in a corresponding chimera, NC4M1M5, comprised of M1 and M5 sequences. Therefore, amino acids for mutagenesis were selected on the basis of being conserved between M1 and M5 sequences in the designated region of the NC4 chimera, yet differed between M1 and M3 subtypes. Using this discrimination, three new chimeric constructs were generated to individually replace amino acids in NC4M1M3 originating from M3 sequence with corresponding residues derived from the M1 TMII sequence. Specifically, amino acids corresponding to M3 TM sequences, Ile 120 (2.56) and Asn 132 and Asn 138 in the extracellular loops were identified for mutagenesis, substituted with the analogous M1 amino acid sequence, Phe77 (2.56), Gly 89 and Thr95, respectively, in the NC4M1M3 chimeric construct and evaluated for the potential role in restoring activity of AC-42 and TBPB. The constructs were evaluated on transiently

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

expressed receptors with the functional calcium mobilization assay in FLIPR (Figure 4) Resultant expression levels were comparable for the three transiently expressed constructs (5.8 +/- 0.9 pmol/mg, 9.1 +/- 1.6 pmol/mg, 7.3 +/- 0.2 pmol/mg for Phe, Gly and Thr substitutions, respectively). The functional potency and maximum response of acetylcholine was similar for the mutated NC4M1M3 constructs; acetylcholine EC50 = 2.1 +/- 0.4 nM, 3.0 +/- 0.9 nM, 5.2 +/- 1.5 nM for Phe, Gly and Thr substitutions, respectively. Substitution of the extracellular loop residues, Asn 89 or Asn 95 with corresponding M1 residues glycine or threonine residues, respectively, had no effect on restoring potency or efficacy of AC-42 or TBPB on the NC4M1M3 chimera. In contrast, substitution of phenylalanine 77 in NC4M1M3 for isoleucine, resulted in an increase in both AC-42 and TBPB potencies and maximal response, however this substitution did not completely restore the agonist potencies to that observed on M1 wild type receptors. EC50 values were determined to be EC50 = 3602 $\pm$ 331 nM, % max = 75 $\pm$ 4 % for AC-42 and EC50 = 361 $\pm$ 80 nM, % max = 55 $\pm$ 6 % for TBPB and reflected a 4-fold and 16-fold lower potency for AC-42 and TBPB respectively, in comparison to values measured on wild type M1 receptors (Table 1). Additionally, maximal activity of AC-42 and TBPB was found to be improved with the introduction of the phenylalanine residue into the NC4M1M3 chimeric construct. Nonetheless, these results implicate the contribution of Phe 77 in TMII of the M1 receptor in supporting the functional activity of AC-42 and TBPB.

To further understand the molecular components of the binding pocket for M1 agonists and the relationship between orthosteric and allosteric agonist binding sites, a homology model of the M1 receptor was generated utilizing the bovine rhodopsin crystal structure as a template. In this model, the side chain of phenylalanine 77 (2.56) identified to be a critical residue for maintenance of AC-42 and TBPB functional potency was determined to be positioned facing inward into a pocket composed of hydrophobic residues contributed from TMIII, TMVI and TMVII (Figure 5). The orientation of phenylalanine 77 is similar to that observed in the analogous residue (F83, 2.56) of the squid rhodopsin x-ray crystal structure (Murakami et al., 2008). In contrast, glycine 89 and threonine 95, residues which were found to have no effect on restoring AC-42 and

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

TBPB activity in the NC4M1M3 chimera, were located in the first extracellular loop spatially distinct from the orthosteric site. The phenylalanine 77 residue in M1 was found to reside at the base of this hydrophobic channel of amino acid residues leading up to the aspartate 105 (3.32), previously identified by mutagenesis to be a critical residue for binding acetylcholine and postulated to participate in a salt bridge with the choline head group of the orthosteric agonist (Page et al., 1995; Hulme et al., 2003). Furthermore, the orthosteric binding pocket has been proposed to be comprised of a hydrophobic network of aromatic residues contributed by the exofacial domains of TMIV, TMVII, TMIII and TMVI. Site directed mutagenesis of the M1 muscarinic receptor was previously employed to evaluate the role of specific amino acids contributed by TMIII and TMVI in binding acetylcholine (Matsui et al., 1995; Lu and Hulme, 1999; Lu et al., 2001). From these studies, tryptophan 101 (3.28), tyrosine 404 (7.39) and tyrosine 408 (7.43) have been reported to impact on orthosteric agonist binding as mutagenesis of these residues resulted in decreases for acetylcholine binding affinity. We further explored the consequence of mutating these specific amino acids on the functional activity of AC-42, TBPB, xanomeline and N-desmethlyclozapine, and evaluated the role of hydrophobic side chains by substitution of either phenylalanine or alanine for the wild type aromatic residue to determine the functionality of the hydroxyl group separately from the phenyl ring. Using the M1 homology model, tyrosine 179 and phenylalanine 374 (6.44) were also identified as residues which could participate in the allosteric binding pocket and were included in the mutagenesis studies. It is noteworthy that a corresponding tyrosine epitope was identified on the M2 receptor, M2Y177 to be important for subtype selectivity of alkane-bisammonium-type and caracurine V-type allosteric modulators (Voigtlander et al., 2003).

The functional activities of the muscarinic agonists acetylcholine, xanomeline, AC-42, TBPB and N-desmethylclozapine on transiently expressed receptors containing the selected point mutations are summarized in Table 3. Since differences in receptor expression could impact on interpretation of the functional activity results measured for the point mutations and mutagenesis of specific amino acids in M1 has been previously reported to decrease M1 receptor expression (Hulme et al., 2003), the level of expression

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

was measured for all the mutant receptors by [^{3}H]-QNB binding and values are included in Table 3. The majority of the point mutants generated were found to express at levels (2-3 pmol/mg) comparable to levels measured for wild type M1 receptors. In contrast, the mutants F374A and Y408A were determined to have decreased levels of expression of less than 4-5% of wild type receptors. No expression of the D105A (3.32) mutant could be measured by [^{3}H]-QNB binding (data not shown) and therefore, no functional response with any of the muscarinic receptor ligands could be detected. The compromised activity measured for acetylcholine measured on mutants Y404F, Y408F and W101F and lack of activity with the D105A mutant was anticipated based on previous studies where these mutations, were shown to exhibit decreases in acetylcholine binding affinities (Lu et al., 2001). In comparison to the acetylcholine, the functional potencies of AC-42, TBPB and N-desmethlyclozapine were found to be less severely affected by the mutations studied and in addition, the potencies of AC-42, TBPB and N-desmethylclozapine were found to be increased on a number of mutants compared with that measured on wild type receptors.

Substitution of W101 (3.28) in TMIII with an alanine residue resulted in a dramatic decrease in acetylcholine functional potency. Replacement of W101 with phenylalanine was better tolerated with only a 2.6-fold reduction in potency measured compared to wild type M1 receptors. The potency for xanomeline was also observed to be decreased on the W101A mutant (26-fold), whereas the potency was found to be only slightly decreased on W101F mutant. In contrast to the decreased activity measured for acetylcholine and xanomeline on substitutions at W101, the affinities of both AC-42 and TBPB were found to be increased. The potencies of AC-42 and TBPB were increased by 77- and 5-fold respectively on W101A. Substitution of phenylalanine for tryptophan 101 resulted in less marked changes with only a 1.7 and 2.5-fold increase in potency measured for AC-42 and TBPB, respectively. In comparison, the affinity of N-desmethylclozapine was only slightly changed on W101A and W101F, where a 2-fold decrease and 1.25-fold increase in potency was measured, respectively.

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

Substitution of tyrosine 179 with either alanine or phenylalanine resulted in only minor changes in functional potencies for all the M1 agonists studied The affinity of acetylcholine was decreased by only 2.5- and 2-fold on Y179A and Y179F, respectively. The affinity for xanomeline was essentially unchanged on either mutant. The affinity for AC-42 and TBPB were increased on Y179A by 1.25- and 2.9-fold, respectively. In comparison, AC-42 exhibited a 2.8-fold decrease in affinity on Y179F, whereas the affinity for TBPB was unchanged compared with wild type M1 receptors.

The functional potencies for acetylcholine, xanomeline, AC-42 and N-desmethylclozapine were all reduced on F374A compared to wild type receptors. This mutant was found to be expressed at low levels (4% of wild type M1) and may account for the decreased potency determined in the functional assay. In comparison, TBPB exhibited a 12.5-fold increase in affinity on F374A, but this result may be confounded by the low mutant protein expression levels.

The reduction of acetylcholine binding affinity on Y404A and Y408A has been reported by Lu et al., (2001). The activity of the M1 agonists were evaluated in functional assays on these mutations and in addition, substitutions with phenylalanine (Y404F and Y408F) were generated to evaluate the contribution of the hydroxyl group separately from the phenyl ring in binding AC-42, TBPB, N-desmethylclozapine and xanomeline. For acetylcholine, large reductions in potency were measured on both Y404A and Y408A consistent with previous published reports. Substitution of the phenylalanine ring was better tolerated, however reductions of 162- and 35-fold were observed with acetylcholine on Y404F and Y408F, respectively. The potency of xanomeline was also reduced on Y404A and Y408A, 26- and 79-fold, respectively. Similar to acetylcholine, a smaller decrease in xanomeline potency was observed on Y404F (6-fold) compared with wild type receptors. In comparison to acetylcholine, the activity of xanomeline was found to be slightly increased on Y408F. In contrast to the reductions observed for acetylcholine and xanomeline on the Y404A and Y404F mutants, the potencies of both AC-42 and TBPB were observed to be increased. AC-42 exhibited a 59-fold increase on Y404A and a smaller 1.7-fold increase on Y404F. TBPB affinity was also found to be

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

increased on both Y404A (5-fold) and Y404F (2.5-fold), however, in comparison, the magnitude was not observed to be as large as that observed with AC-42. In contrast, only small decreases in potency were observed with N-desmethlyclozapine on either Y404A or Y404F mutants.

In contrast to the observed increase in AC-42 and TBPB potencies resulting from modifications of tyrosine 404, substitution of either alanine or phenylalanine at tyrosine 408 resulted in a reduction in AC-42 and TBPB activities. The Y408A mutant was found to be expressed at only 5% of the wild type receptor levels and a 1700-fold reduction in acetylcholine affinity was determined. The reduced receptor expression is consistent with what has previously been reported (Hulme et al., 2003). On this mutant, AC-42 activity could not be detected and in addition, the affinity of TBPB was reduced by 246-fold. Affinities for xanomeline and N-desmethylclozapine were less severely affected, yet showed reductions of 79- and 3-fold respectively. It is likely that the low level of receptor expression may have contributed to the reduction of agonist activity measured on this mutant. In contrast, the Y408F mutant was expressed at receptor levels similar to the wild type M1 receptor. The functional potencies of AC-42 and TBPB on Y408F were found to be decreased by 10.5 and 9-fold, respectively. In comparison, a small increase was observed with xanomeline (1.7-fold) and N-desmethylclozapine (2-fold).

To define the binding sites for the M1 agonists evaluated in this study, the ligands were docked into the M1 receptor homology model (Figure 5) using the mutagenesis results to support the orientation of the ligands. Multiple orientations of each ligand were docked into a variety of sites using flexible docking methods until a set on internally self-consistent sites were identified. Additional hand and computational refinement was then carried out for the final models presented in this work. The model supported the docking of acetylcholine consistent with previously published reports (Hulme et al., 2003; Peng et al., 2006). In comparison, xanomeline was also found to occupy a similar site as acetylcholine. The orthosteric agonists, acetylcholine and xanomeline interact with aspartate 105 to stabilize the anion (Figure 5). Both ligands were found to make specific interactions with the hydroxyl group of tyrosine 404 (7.39). Cation-π interactions

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

between acetylcholine and the aromatic residues tryptophan 101 (3.28), tyrosine 106 (3.33), tryptophan 378 (6.48), tyrosine 404 and tyrosine 408 (7.43) stabilize the ligand (Figure 5A). Xanomeline was found to make favorable hydrophobic contacts between its lipophilic tail and tyrosine 106, tryptophan 378 (6.48) and tyrosine 381 (6.51) (Figure 5B). In contrast to the orthosteric ligands, the allosteric agonists, AC-42 and TBPB were determined to occupy a binding site adjacent to the orthosteric site on the opposite face of tryptophan 101 (Figure 6). The binding pocket is analogous to the hydrophobic channel between TM1, III and VII with specific polar interactions contributed by threonine 83 (2.62) and van der Waals interactions contributed by the residues lining the channel. Phenylalanine 77 (2.56) lies at the base of the allosteric agonist binding site acting as a lid at the base of the channel.

The docking of N-desmethylclozapine was challenging as the functional activity was largely unaffected by the mutants explored in this study. The only truly discernible effects were observed for mutations to tyrosine 404. After repeated rounds of docking to plausible sites, it is hypothesized that N-desmethylclozapine is expected to bind proximal to the orthosteric site (Figure 7). The highly hydrophobic nature of this ligand drives the chloro group toward the hydrophobic pocket formed by isoleucine 180, threonine 189 (4.39), and tryptophan 400 (6.50). The phenyl group para to the chlorine is stabilized by an interaction with Cys 407 (7.42), a face contact with tyrosine 381 and an edge to face interaction with tryptophan 378. The polar piperizine makes contacts with aspartic acid 105, tyrosine 404 and tyrosine 408. As discussed above, this is the most speculative of the modeled small molecules due to the lack of mutagenesis data indicating a clear impact on binding and functional activity.

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

Selective targeting of M1 muscarinic receptors for the development of novel therapeutics has proven challenging due to the high degree of homology of the orthosteric agonist binding site between the five muscarinic receptor subtypes. Allosteric modulation of G protein-coupled receptors represents an attractive alternative for identifying subtype selective ligands as it is expected that allosteric binding sites, unlike the site for orthosteric agonists, are less or not conserved among subtypes. An increasing number of examples have been reported for G protein-coupled receptors, including M1 muscarinic receptors subtypes and have recently been reviewed (De Amici et al., 2010; Langmead and Christopoulos, 2006; May et al., 2007).

Activation of M1 muscarinic receptors have been shown to occur with orthosteric agonists and allosteric potentiators requiring the presence of orthosteric agonists to produce activation (Matsui et al., 1995; Lazareno, et al., 1998; Birdsall and Lazareno, 2005; Ma et al., 2009; Marlo et al., 2009). Futhermore, selective M1 receptor allosteric agonists, exemplified by AC42 (Spalding et al., 2002; Langmead et al., 2006) and TBPB (Jones et al., 2008), modulate receptor activation without requirement for orthosteric agonists. Mutagenesis studies have focused on the orthosteric binding site for acetylcholine and the antagonist, N-methylscopolamine to define residues comprising the binding sites. Several orthosteric binding site models have been proposed based on mutagenesis and molecular docking studies utilizing homology models constructed from the crystallography structure of bovine rhodopsin (Hulme et al., 2003; Peng et al., 2006). These studies revealed that the acetylcholine binding site was located within the transmembrane domains with residues contributed by TM3, TM4, TM6 and TM7. With the discovery that orthosteric agonist activation of the M1 muscarinic receptor could be modulated by allosteric potentiators such as brucine and allosteric inhibitors, such as gallamine, mutagenesis studies focused on residues within the extracellular loops, as it was hypothesized that allosteric sites resided outside of the transmembrane domains (Matsui et al., 1995; Birdsall et al., 2001). Recently, BQCA, a highly selective M1 allosteric potentiator has been described (Ma et al., 2009) and proposed to bind to an extracellular site between transmembrane IV and V based on site directed mutagenesis

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

and molecular modeling. With the recent discovery of M1 allosteric agonists, AC-42 and its analogs, mutagenesis studies have begun to explore residues for their role in binding and define the proximity of allosteric sites to orthosteric sites on the receptor (Spalding et al., 2006; Lebon et al., 2008). In this report, we have provided further evidence supporting a model where AC-42 binds at an allosteric site distinct from the orthosteric site and demonstrated that the structural diverse allosteric agonist, TBPB binds to a similar site on the M1 receptor.

In this study, we compared the activity the M1 orthosteric agonists, acetylcholine and xanomeline, the allosteric agonist, AC-42 and TBPB, and an active metabolite of clozapine, N-desmethylclozapine on chimeric M1M3 receptors and point mutants of M1 receptors to elucidate their binding sites. An M1 receptor homology model was constructed based on the rhodopsin crystallography structure and the orientation of the M1 ligands were supported by the changes in functional potency observed on the mutant constructs. As a result, we have determined that AC-42 and TBPB share a similar binding pocket adjacent to the orthosteric binding site. In contrast, results supported N-desmethylclozapine binding to a site distinct from both AC-42 and TBPB, which overlaps with the orthosteric site.

In the present study, the allosteric mode of action of TBPB was evaluated in dissociation kinetic binding assays. Similar to AC-42 (Langmead et al., 2006; Spalding et al., 2006), TBPB was determined to reduce the [^{3}H]NMS dissociation rate from the M1 receptor. In contrast, Spalding et al. (2006) also showed that N-desmethylclozapine did not exhibit allosteric properties on M1 in dissociation binding studies. These findings further established the functional similarity of AC-42 and TBPB and difference with N-desmethylclozapine.

Both AC-42 and TBPB have been shown to retain functional activity on a point mutant, Y381A in TMVI of the M1 receptor, which in contrast results in diminishing the receptor's potency for the orthosteric agonist, acetylcholine. It was therefore postulated that AC-42 and TBPB might bind to a different site than acetylcholine. In comparison,

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

we previously reported that N-desmethylclozapine retained functional activity on Y381A mutant suggesting that it also acted at a different site from acetylcholine, however it was unclear without further investigation, whether N-desmethylclozapine bound to a similar or different site on the M1 receptor as AC-42 and TBPB. In the homology model, Y381 contributes to the stabilization of the hydrophobic cage proposed by Hulme et al., (2003), surrounding the critical D105 reside in the orthosteric acetylcholine binding site. Our model also predicts that the allosteric binding pocket for AC-42 and TBPB resides at the opposite interface of W101 and Y408 and therefore, Y381 being far removed from this site, would not be expected to participate in the binding of AC-42 and TBPB. In contrast to AC-42 and TBPB, the binding site for N-desmethylclozapine is proposed to overlap with the orthosteric agonist binding site and in part, hydrophobic contacts are contributed by Y381. The substitution of alanine for phenylalanine at position 381 would be expected to allow N-desmethlyclozapine to more effectively occupy the hydrophobic cage. The homology model supports the functional activity data measured with AC-42, TBPB and N-desmethylclozapine on the Y381A mutant whereby, in contrast to orthosteric agonists, an increase in potency and efficacy was observed with all three ligands.

The minimal epitope for AC-42 binding to the M1 receptor was first described by Spalding et al. (2002) to be comprised of the first 45 amino acids of M1 corresponding to the amino terminus and TMI, the third extracellular loop and TVII. Our data utilizing M1M3 chimeric receptors now extends this binding domain to include residues within TMII, specifically Phe 77 for both AC-42 and TBPB. Phe 77 was not previously implicated in the first report describing AC-42 as M1-M5 chimeric receptors were utilized in these studies. The phenylalanine residue at this position is conserved between M1 and M5 muscarinic receptor subtypes and was obscured in the initial AC-42 mapping utilizing the M1M5 chimeras. In the present study, the importance of the phenylalanine residue was realized as the corresponding position contributed by the M3 sequence was an isoleucine substitution. Interestingly, the Phe 77 residue resides at the base of a hydrophobic network of amino acids contributed between TMII, VI and VII which constitutes the binding site for AC-42 and TBPB and may function by providing stabilization to the receptor-ligand complex.

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

In this report, we evaluated the impact of substitution of W101 or Y404 residues with phenylalanine or alanine to examine the contribution of the hydroxyl group compared to the aromatic side chain on the ability of the allosteric agonists, TBPB an AC-42 to produce receptor activation. The greater effect observed on functional potency when W101 and Y404 were mutated to alanine compared to that seen with phenylalanine substitution, suggests that there is an enhancement in accessing the allosteric agonist binding pocket for TBPB and AC-42 when the steric constraint of the aromatic ring is removed. Hulme et al., (2003) have suggested that a hydrophobic cage exists around Asp 105, a critical residue required to form a salt bridge with the choline head group of acetylcholine comprised of residues contributed by TM3 (W101), TM6 (Y381) and TM7 (Y404, Y408). It is postulated that an activated state of the receptor is favored by rearrangement of the hydrophobic cage allowing for AC-42 or TBPB to participate in the hydrophobic cage to favor an activated conformation of the receptor.

Lebon et al., (2009) have proposed a similar location for AC-42 binding to the M1 receptor. In comparison to our study, a homology model based on the β2-adrenergic receptor was employed to interpret the mutagenesis studies and the mapping of TBPB was not evaluated. Lebon et al., also found a similar large increase in the functional potency for AC-42 when W101 was mutated to alanine. In contrast, on the Y404A mutant, we observed significant enhancement of both AC-42 and TBPB whereas Lebon et al reported only a slight enhancement of activity.

In summary, the results of this study further defined the binding site for the allosteric agonists, AC-42 and TBPB to be at a similar site distinct from the orthosteric agonist site. Comparing the functional activity of AC-42, TBPB and N-desmethylclozapine and the orthosteric agonists acetylcholine and xanomeline on mutant constructs, the ligands were found to generally cluster into three groups supporting the hypothesis that the M1 receptor activation can occur though at least three different binding domains (Spalding et al., 2006). Additional evidence is now provided from the homology model which illustrates the location of the three binding domains: the

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

orthosteric agonist site for acetylcholine and xanomeline stabilized by charge interactions, a defined allosteric site proximal to the orthosteric site for AC-42 and TBPB stabilized by hydrophobic interactions, and a third site positioned above the orthosteric binding site for binding N-desmethlyclozapine. Modeling allosteric binding sites on M1 muscarinic receptors could facilitate the optimization and development of novel treatments for psychiatric disorders and cognition impairment in Alzheimer's disease.

References

Ballesteros JA and Weinstein H (1995) Integrated Methods for the Construction of Three-Dimensional Models and Computational Probing of Structure-Function Relations in G Protein-Coupled Receptors. *Methods Neurosci* **25**:366-428.

Birdsall NJM, Lazareno S, Popham A and Saldanha J (2001) Mutiple allosteric sites on muscarinic receptors. *Life Sci* **68**:2517-2524.

Birdsall NJM. and Lazareno S (2005) Allosterism at muscarinic receptors: ligands and mechanisms. *Mini Rev Med Chem* **5**:523-543.

Bodick NC, Offen WW, Levey AI, Cutler NR, Gauthier SG, Satlin A, Shannon HE, Tollefson GD, Rasmussen K, Bymaster FP, Hurley DJ, Potter,WZ and Paul SM (1997) Effects of xanomeline, a selective muscarinic receptor agonist, on cognitive function and behavioral symptoms in Alzheimer disease. *Arch Neurol* **54**:465-473.

Bymaster FP, McKinzie DL, Felder CC and Wess J (2003) Use of M1-M5 muscarinic receptor knockout mice as novel tools to delineate the physiological roles of the muscarinic cholinergic system. *Neurochem Res* **28**:437-442.

Caccamo A, Fisher A and LeFerla FM (2009) M1 agonists as a potential disease-modifying therapy for Alzheimer's disease. *Curr Alzheimer Res* **6**: 112-117.

CCG (2005) Molecular Operating Environment, Chemical Computing Group, Montreal, Canada.

De Amici M, Dallanoce C, Holzgrabe U, Trankle C and Mohr K (2010) Allosteric ligands for G protein-coupled receptors: a novel strategy with attractive therapeutic opportunities. *Med Res Rev* **30**:463-549.

DeLano WL (2002) The PyMOL Molecular Graphics System, DeLano Scientific, Palo Alto, CA

Fredriksson R, Lagerstrom MC, Lundin L and Schioth HB (2003) The G-Protein-Coupled Receptors in the Human Genome Form Five Main Families. Phylogenetic Analysis, Paralogon Groups, and Fingerprints. *Mol Pharmacol* **63**:1256-1272.

Halgren TA (1999) MMFF VI. MMFF94s option for energy minimization studies. *J Comput Chem* **20**:720-729.

Hulme EC, Lu ZL and Bee MS (2003) Scanning mutagenesis studies of the M1 muscarinic acetylcholine receptor. *Receptors & Channels* **9**:215-228.

Jacobson MP, Pincus DL, Rapp CS, Day TJ, Honig B, Shaw DE and Friesner RA (2004) A hierarchical approach to all-atom protein loop prediction. *Proteins* **55**:351-67.

Jones CJ, Brady AE, Davis AA, Xiag Z, Bubser M, Tantawy MN, Kane AS, Bridges TM, Kennedy JP, Bradley SR, Peterson TE, Ansari MS, Baldwin RM, Kessler RM, Deutch AY, Lah JJ, Levey AI, Lindsley CW and Conn PJ (2008) Novel selective allosteric activator of the M1 muscarinic acetylcholine receptor regulates amyloid processing and produces antipsychotic-like activity in rats. *Journal of Neuroscience* **28** : 10422-10433.

Langmead CJ and Christopoulos A (2006) Allosteric agonists of TM7 receptors: expanding the pharmacological toolbox. *TIPS* **27**:475-481.

Langmead, CJ, Fry VAH, Forbes, IT, Branch, CL, Christopoulos A, Wood, MD and Herdon HJ (2006) Probing the molecular mechanism of interaction between 4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1butyl]-piperidine (AC-42) and the muscarinic M1 receptor : direct pharmacological evidence that AC-42 is an allosteric agonist. *Mol Pharmacol* **69**:236-246.

Langmead CJ, Watson J and Reavill C (2008) Muscarinic acetylcholine receptors as CNS drug targets. *Pharm. Therap.* **117**: 232-243.

Lazareno S, Gharagozloo P, Kuonen D, Popham A and Birsdall NJM(1998) Subtype-selective positive cooperative interactions between brucine analogues and acetylcholine at muscarinic receptors : radioligand binding studies. *Mol Pharmacol* **53**:573-589.

Lebon G, Langmead CJ, Tehan BG and Hulme EC (2008) Mutagenic mapping suggests a novel binding mode for selective agonists of M1 muscarinic acetylcholine receptors. (2009) Mol Pharmacol **75:**331-341**.**

Lu ZL and Hulme EC (1999) The functional topography of transmembrane 3 of the M_1 muscarinic acetylcholine receptor, revealed by scanning mutagenesis. *J Biol Chem* **274**:7309-7315.

Lu ZL, Saldanha JW and Hulme EC (2001) Transmembrane domains 4 and 7 of the M1 muscarinic acetylcholine receptor are critical for ligand binding and the receptor activation switch. *J Biol Chem* **276**:34098-34104.

Ma L, Seager M, Wittmann M, Jacobson M, Bickel D, Burno M, Jones K, Kuzmick-Graufelds V, Xu G, Pearson M, McCampbell A, Gaspar R, Shughrue P, Danziger A, Regan C , Flick R , Pascarella D, Garson S, Doran S, Kreatsoulas C, Veng L, Lindsley, C, Shipe W, Kuduk S, Sur C, Kinney G, Seabrook GR, Ray WJ (2009) Selective activation of the M1 muscarinic acetylcholine receptor achieved by allosteric potentiation. *Proc Natl Acad Sci USA* **106**:15950-15955.

Marlo JE, Niswender CM, Days EL, Bridges TM, Xiang Y, Rodriguez AL, Shirey JK, Brady AE, Nalywajko T, Luo Q, Austin CA, Williams MB, Kim K, Williams R, Orton D, Brown HA, Lindsley CW, Weaver CD, Conn PJ (2009) Discovery and Characterization of Novel Potentiators of M_1 Muscarinic Receptors Reveals Multiple Modes of Activity. *Mol Pharmacol* **75**:577-588.

Murakami M and Kouyama T (2008) Crystal structure of squid rhodopsin. *Nature* **453**:363-367.

May LT, Leach K, Sexton PM and Christopoulos A (2007) Allosteric modulation of G protein-coupled receptors. *Annu Rev Pharmacol Toxicol* **47**:1-51.

Matsui H, Lazareno S and Birdsall NJM (1995) Probing the location of the allosteric site on the m1 muscarinic receptors by site-directed mutagenesis. *Mol Pharmacol* **47**:88-98.

Page KM, Curtis CA, Jones PG and Hulme EC (1995) The functional role of the binding site aspartate in muscarinic acetylcholine receptors, probed by site-directed mutagenesis. *Eur J Pharmacol.* **289**:429-437.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M and Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289**:739-45.

Peng JY-C, Vaidehi N, Hall SE and Goddard WA (2006) The predicted 3D structures of the human M1 muscarinic acetylcholine receptor with agonist or antagonist bound. *Chem Med Chem* **1**:878-890.

Schrodinger LLC (2006) Maestro, Schrodinger, LLC, Portland, OR.

Shekhar, A, Potter WZ, Lightfoot J, Lienemann J, Dube S, Mallinckrodt C, Bymaster FP, McKinzie DL and Felder CC (2008) Selective Muscarinic Receptor Agonist Xanomeline as a Novel Treatment Approach for Schizophrenia. *Am J Psychiatry* **165**:1033-1039.

Sherman W, Beard HS and Farid R (2006) Use of an induced fit receptor structure in virtual screening. *Chem Biol Drug Des* **67**:83-4.

Spalding TA, Trotter C, Skjaerbaek N, Meisser TL, Currier EA, Burstein ES, Li D, Hacksell U and Brann MR (2002) Discovery of an ectopic activation site on the M_1 muscarinic receptor. *Mol Pharmacol* **61**: 1297-1302.

Spalding TA, Ma JN, Ott TR, Friberg M, Bajpai A, Bradley SR, Davis RE, Brann MR and Burstein ES (2006) Structural requirements of transmembrane domain 3 for activation by the M_1 muscarinic receptor agonists AC-42, AC-260584, clozapine and N-desmethylclozapine: Evidence for three distinct modes of receptor activation. *Mol Pharmacol* **70**:1974-1983.

Sur C, Mallorga PJ, Wittmann M., Jacobson MA, Pascarella D, Williams JB, Brandish PE, Pettibone DJ, Scolnick EM and Conn, PJ (2003) N-desmethlyclozapine, an allosteric agonist at muscarinic 1 receptors, potentiates N-methyl-D-aspartate receptor activity. *Proc Natl Acad Sci.* **100**:13674-13679.

Voigtlander U, Johren K, Mohr M, Raasch A, Trankle C, Buller S, Ellis J, Holtje HD and Mohr K (2003) Allosteric site on muscarinic acetylcholine receptors: identification of two amino acids in the muscarinic M2 receptor that account entirely for the M2/M5 subtype selectivities of some structurally diverse allosteric ligands in N-methylscopolamine-occupied receptors. *Mol Pharmacol* **64:21-31.**

Ward SDC, Curtis CAM and Hulme EC (1999) Alanine-scanning mutagenesis of transmembrane domain 6 of the M_1 muscarinic acetylcholine receptor suggests that Tyr381 plays a key role in receptor function. *Mol Pharmacol* **56**:1031-1041.

Wess J, Duttaroy A, Zhang W, Gomeza J, Cui Y, Miyakawa T, Bymaster FP, McKinzie L, Felder CC, Lamping KG, Faraci FM, Deng C and Yamada M (2003) M1-M5 muscarinic receptor knockout mice as novel tools to study the physiological roles of the muscarinic cholinergic system. *Receptors Channels* **9**:279-290.

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures

Figure 1. Structures of M1 muscarinic receptor orthosteric and allosteric agonists

Figure 2. Effect of TBPB (100 μM) (filled squares), gallamine (1 mM) (open circles), and clozapine (10 μM) (filled triangles) on the atropine induced dissociation of [^{3}H]NMS binding to human M1 receptor expressed on CHO-K1 membranes. Atropine alone (1 μM) (open squares) was measured as a control. The k_{off} values measured in the presence of TBPB (k_{off} = 0.034 ± 0.002 min^{-1}), gallamine (k_{off} = 0.0071 ± 0.0016 min^{-1}) and clozapine (k_{off} = 0.057 ±0.004 min^{-1}) compared with atropine alone (k_{off} = 0.060 ± 0.004 min^{-1}).

Figure 3. Sequence alignment of the first intracellular loop (i1), transmembrane II (TMII), extracellular loop 1 (o1) and transmembrane III (TMIII) of human M1, M3 and M5 muscarinic receptors. Gray shading indicates 100% identity. Bolded residues indicate residues modified in NC4M1M3 chimeric receptor by site directed mutagenesis

Figure 4. Functional activity of acetylcholine (filled squares), AC-42 (filled triangles) and TBPB (filled diamonds) on point mutations selectively introduced into chimeric receptor NC4M1M3 measured by calcium mobilization assay in FLIPR. Results are expressed as percentage of maximum acetylcholine activity and are the means ± SEM from three individual experiments performed in triplicate.

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5. Models (gold sticks) of A) acetylcholine and B) xanomeline bound to M1 receptor. Residues labeled have favorable interactions with either the acetylcholine or xanomeline.

Figure 6. Models (gold sticks) of A) AC-42 and B) TBPB bound to M1 receptor. Residues labeled have favorable interactions with either the ligand or acetylcholine. Mesh representation of acetylcholine binding is shown for reference

Figure 7. Model of N-desmethylclozapine (gold sticks) bound to M1 receptor. Residues labeled have favorable interactions with either the ligand or acetylcholine. Mesh representation of acetylcholine binding is shown for reference.

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Functional activity of muscarinic agonists at wild type and Y381A mutated M1 receptors.

	hM1-WT		Y381A		
	EC50	% Max	EC50	% Max	shift
Acetylcholine	21± 6	100	3837± 677	100	183
Xanomeline	19± 7	81±3	42± 7	69± 3	2.2
AC-42	805± 251	71± 2	220± 16	83± 3	0.27
TBPB	22± 2	77± 2	2±1	90±1	0.09
N-desmethylclozapine	115±28	50±5	14±2	96±2	0.12

EC50 values (nM) and % Max of acetylcholine response are the mean ± SEM of 3-6 determinations. Shift is EC50 value on Y381A mutant/ EC 50 value on wild type M1.

Molecular Pharmacology Fast Forward. Published on July 21, 2010 as DOI: 10.1124/mol.110.065771
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2. Functional activity of muscarinic agonists at wild type M1, NC4M1M3 and NC3M1M3 chimeric receptors measured in calcium mobilization assays by FLIPR

	hM1-WT		NC4M1M3		NC3M1M3	
	EC50, nM	% Max	EC50, nM	% Max	EC50, nM	% Max
Acetylcholine	5 ± 0.2	100	3.4± 1.5	100	40 ± 14	100
Xanomeline	3.2 ± 0.2	81±3	18 ± 4	82± 2	44 ± 9	69±5
AC-42	128± 7	74± 1	>100,000		922 ± 11	48±3
TBPB	5.3 ± 0.2	70± 0.4	>100,000		117±42	36±4
N-desmethylclozapine	20 ±1	65±0.4	59 ±18	43±2	83 ±5	47±3

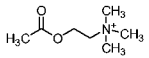
EC 50 values (nM) and % maximum acetylcholine activity are the mean ± SEM from three individual experiments performed in triplicate.

Table 3. Functional activity of muscarinic agonists on M1 point mutations measured in calcium mobilization assays with FLIPR

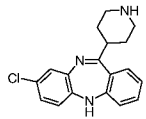
Receptor	Acetylcholine			Xanomeline			AC-42			TBPB			NDMC			Bmax pmol/mg protein
	EC50	% Max	shift	EC50	% Max	shift	EC50	% Max	shift	EC50	% Max	shift	EC50	% Max	shift	
M1 WT	5.0±0.2	100	---	3.2±0.2	81 ± 3	---	128 ± 7	74 ± 1	---	5.3±0.2	70 ±0.4	---	20 ± 1	65 ±0.4	---	2.7± 1
W101A	15175 ± 823	100	3035	82 ± 26	77 ± 2	26	1.7±0.2	73 ± 2	0.013	1.1±0.1	57.5± 4	0.2	43 ± 6	68 ±3	2	1.9±0.1
W101F	12.8±0.9	100	2.6	4.0±0.5	89 ± 2	1.2	73 ± 7	72 ±1.5	0.6	2.1 ±0.3	77±1.5	0.4	16 ± 2	78 ±3	0.8	3.8±0.4
Y179A	12.4±0.9	100	2.5	3.6±0.3	83 ± 1	1.1	101 ± 14	72 ± 2	0.8	1.8±0.2	75 ± 2	0.34	12± 1	72 ± 2	0.6	2.9±0.2
Y179F	10.5±0.6	100	2	4.5±0.5	84 ±0.3	1.4	359 ± 67	74 ± 3	2.8	5.4±0.1	65 ±1	1	37 ± 6	66 ± 1	1.8	3.0±0.0
F374A	90 ± 4	100	18	19.4±1.5	79 ± 2	6	470 ± 37	69 ±1.6	3.7	0.4±0.0	77 ± 2	0.08	106 ±14	70 ± 3	5.2	0.12± 0.01
Y404A	18660 ± 498	100	3732	85 ± 13	80 ± 1	26	2.2±0.1	76 ±0.7	0.017	1.7±0.1	59± 1	0.2	63 ±6.1	71± 3	3.1	0.93± 0.06
Y404F	809 ± 12	100	162	20 ± 3	55 ±1.0	6	75 ± 8	62 ±0.5	0.6	2.1±0.3	63 ±0.5	0.4	25.4±2.7	49 ±1	1.25	1.8± 0.8
Y408A	8500 ± 358	100	1700	252 ± 34	74 ± 3	79	ND	ND		1304±52	34 ± 1	246	63± 8.5	34 ±1	3.1	0.13
Y408F	75 ± 4	100	35	1.8±0.4	84 ± 3	0.6	1339 ± 16	55 ± 5	10.5	47 ± 2	59 ± 4	9	9.7±0.7	67 ± 4	0.48	1.2±0.4

Values for EC_{50} (nM) and % maximum of acetylcholine response, represent the mean of five to seven determinations +/- SEM measured on transiently expressed receptors. The shift represents the EC_{50} measured for the mutated receptor divided by the EC_{50} measured for the wild type receptor. If the response was too low to quantify, EC_{50} and % max is designated ND (not determined). Receptor expression levels for wild type and point mutant receptors were measured with [^{3}H]-QNB binding on membranes prepared from transfected CHONFAT cells.

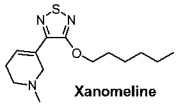
Figure 1



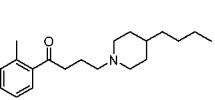
Acetylcholine



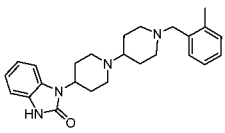
N-desmethylclozapine



Xanomeline



AC-42



TBPB

Figure 2

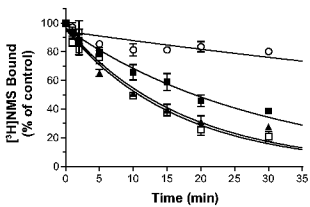

[3H]NMS Bound
(% of control)
100
80
60
40
20
0
0
5
10
15
20
25
30
35
Time (min)

Figure 3


i1
TMII
o1
TMIII
HUMAN M5 (50) LVMISFKVNSQLKTVNNYYLLSLACADLIIGIF YTTYIILMGRWALGSLACDLWLAL
HUMAN M3 (88) LVIVSFKVNKQLKTVNNYFLLSLACADLIIGVISMNLFTTYIIMNRWALGNLACDLWLAI
HUMAN M1 (45) LVLISFKVNTELKTVNNYFLLSLACADLIIGTFSMNLYTTYLLMGHWALGTLACDLWLAL

Figure 4

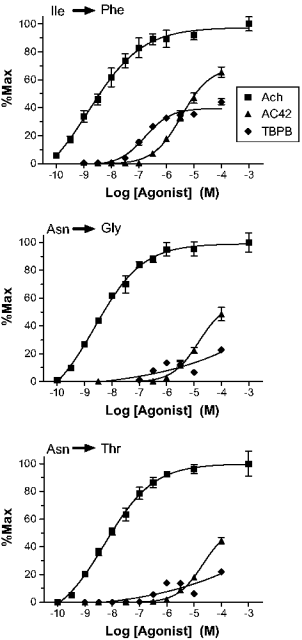
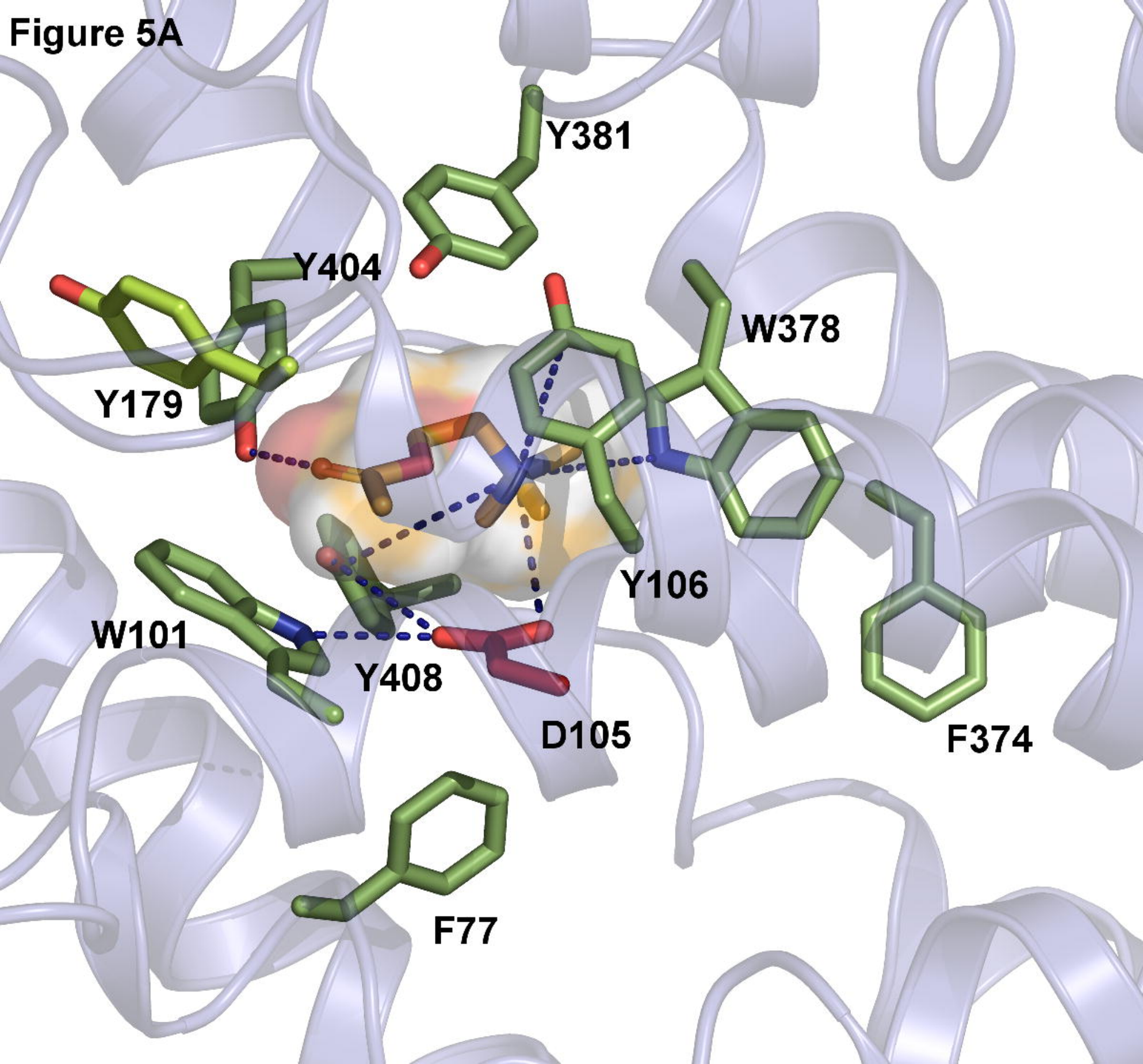

Ile → Phe
%Max
Log [Agonist] (M)
Ach
AC42
TBPB
Asn → Gly
%Max
Log [Agonist] (M)
Asn → Thr
%Max
Log [Agonist] (M)

Figure 5A



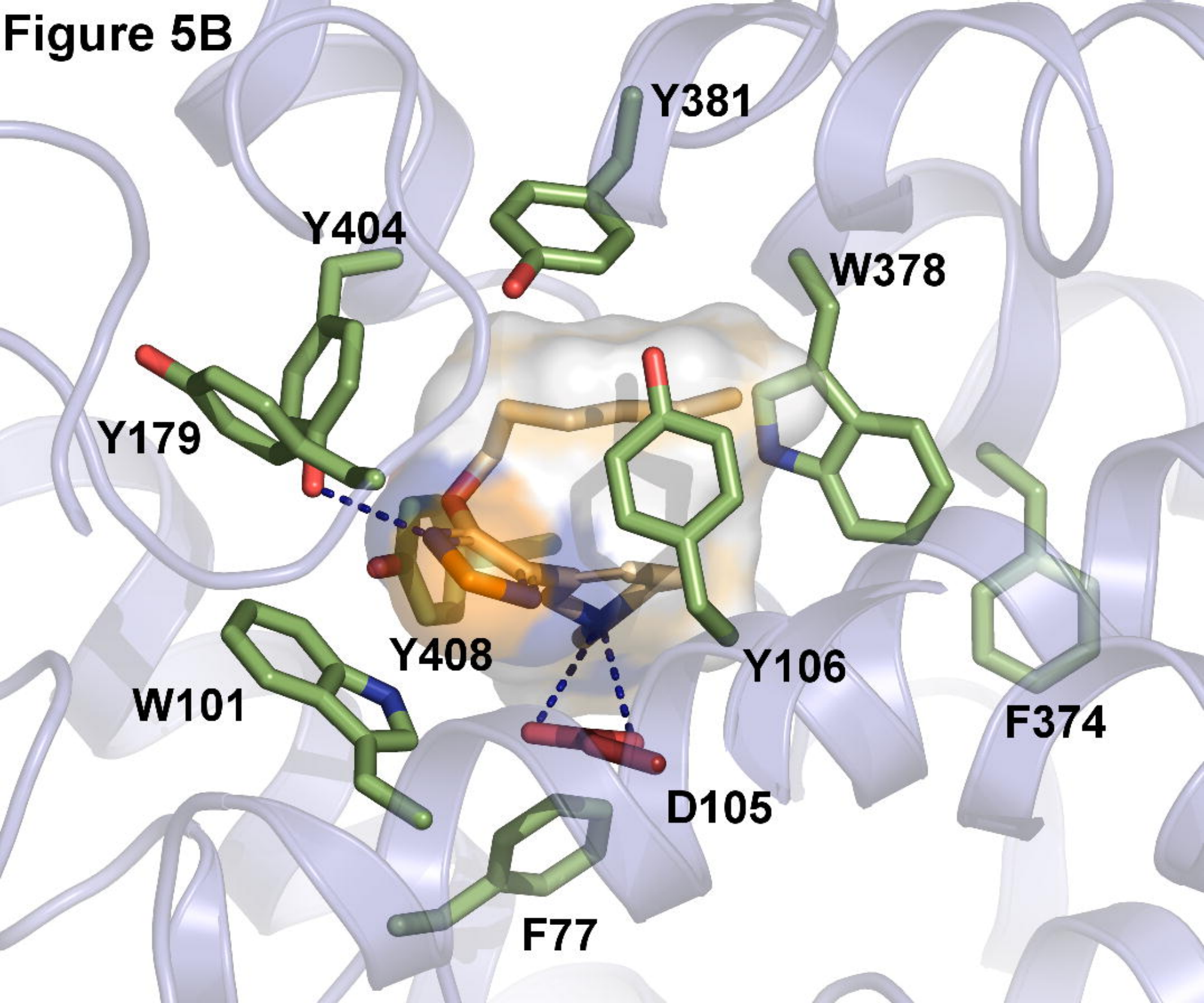


Figure 5B
Y381
Y404
W378
Y179
Y408
Y106
W101
F374
D105
F77

Figure 6A

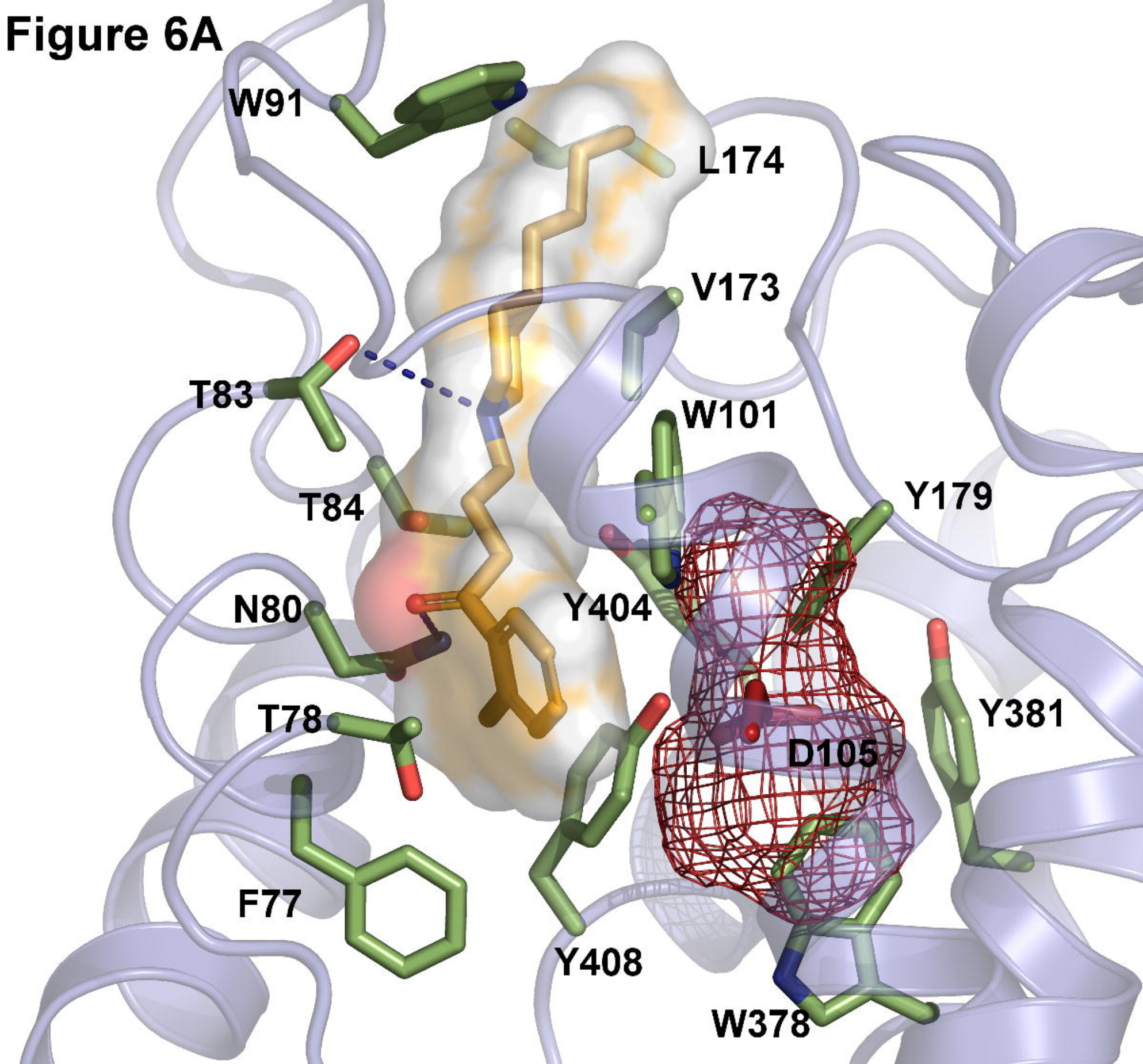

W91
L174
V173
T83
W101
T84
Y179
N80
Y404
T78
Y381
D105
F77
Y408
W378

Figure 6B

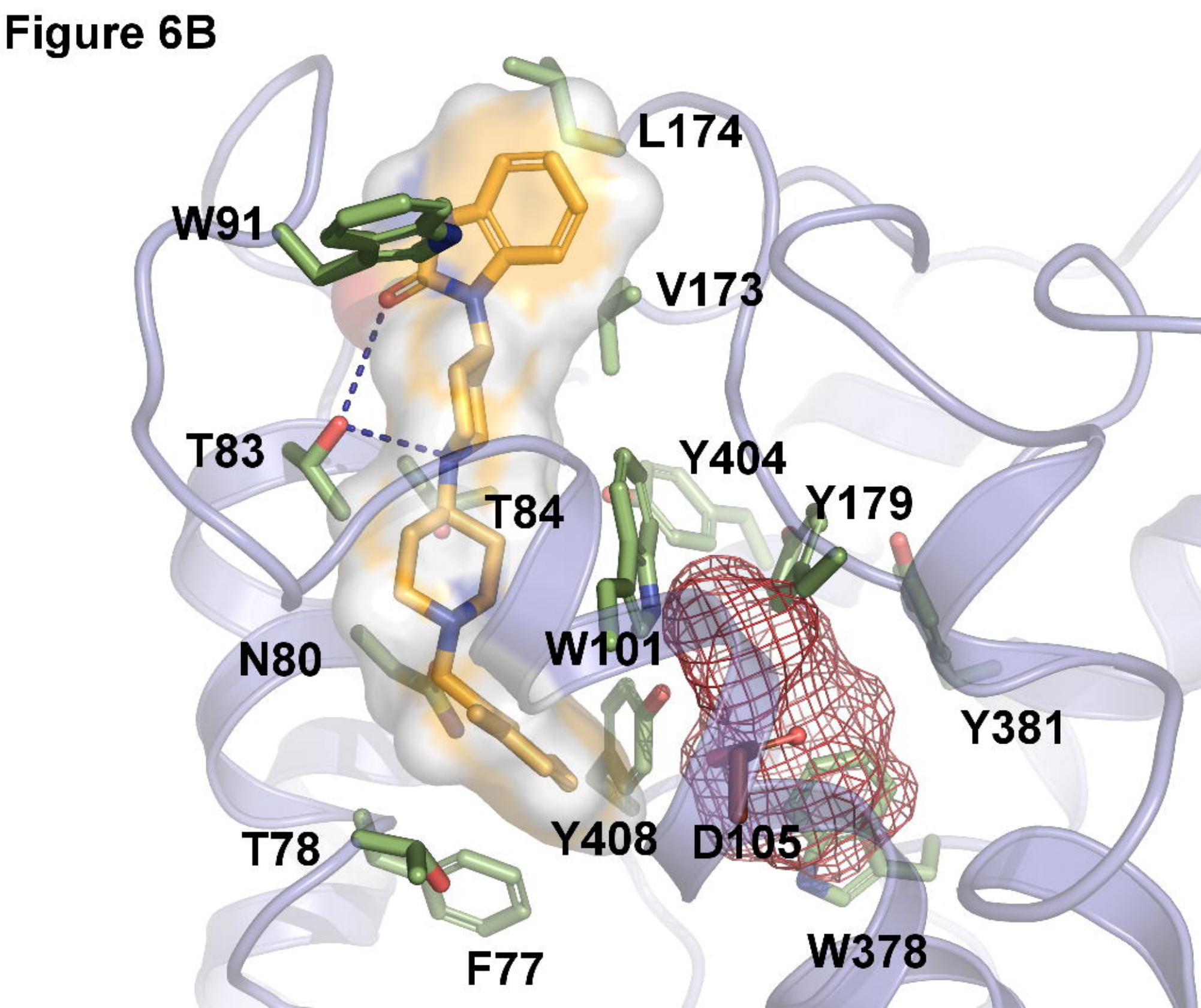
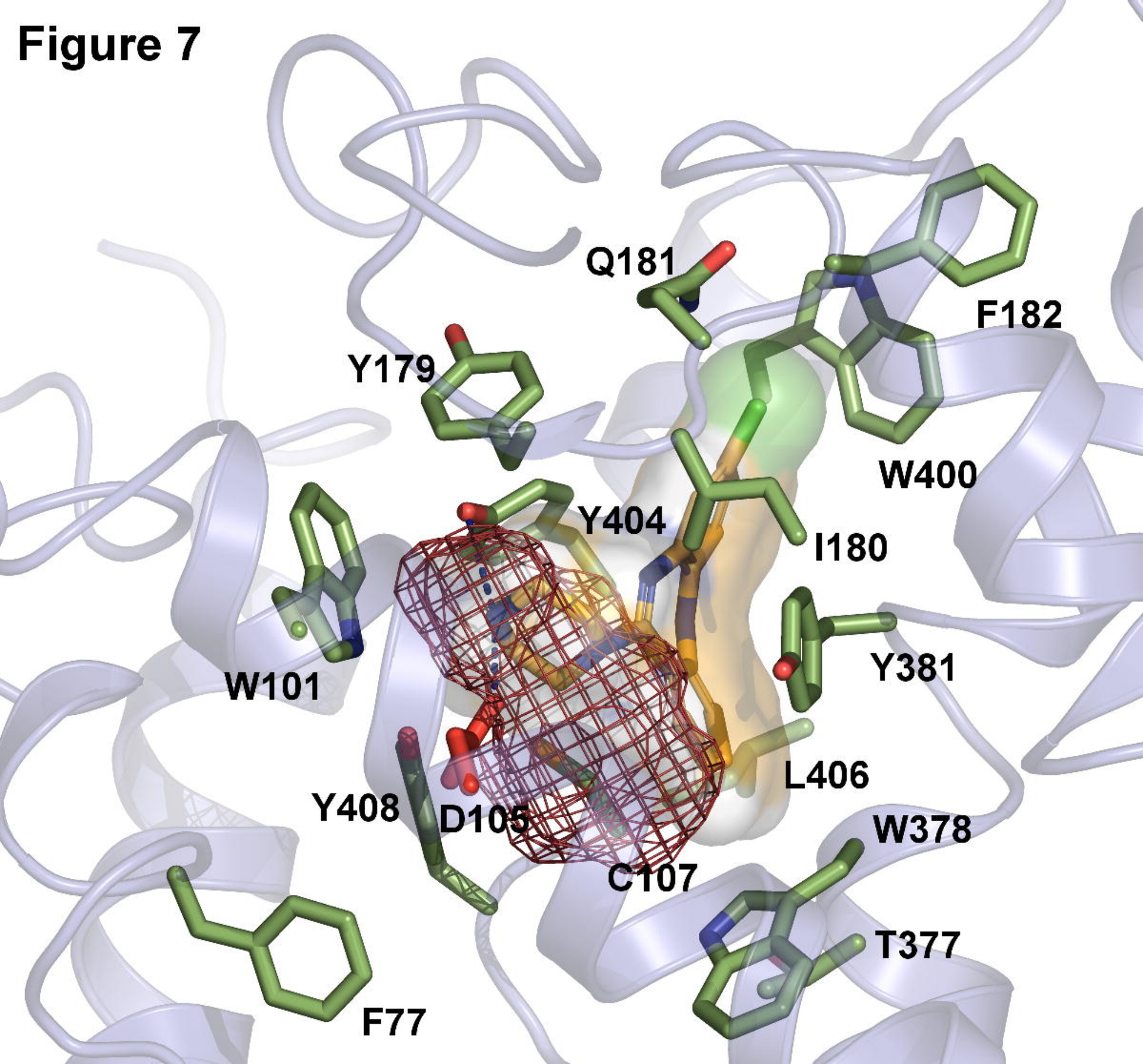

L174
W91
V173
T83
Y404
T84
Y179
N80
W101
Y381
T78
Y408
D105
F77
W378

Figure 7


Q181
F182
Y179
W400
Y404
I180
W101
Y381
L406
Y408
D105
W378
C107
T377
F77