The binding orientation of epibatidine at α7 nACh receptors

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ABSTRACT

Epibatidine is an alkaloid toxin that binds with high affinity to nicotinic and muscarinic acetylcholine receptors, and has been extensively used as a research tool. To examine binding interactions at the nicotinic receptor, it has been co-crystallised with the structural homologue acetylcholine binding protein (AChBP; PDB ID 2BYQ), and with an AChBP chimaera (3SQ6) that shares 64% sequence identity with the α7 nACh receptor. However, the binding orientations revealed by AChBP co-crystal structures may not precisely represent their receptor homologues and experimental evidence is needed to verify the ligand poses. Here we identify potential binding site interactions between epibatidine and AChBP residues, and substitute equivalent positions in the α7 nACh receptor. The effects of these are probed by [3H]epibatidine binding following the expression of α7 nACh receptor cysteine mutants in HEK 293 cells. Of the sixteen mutants created, the affinity of epibatidine was unaffected by the substitutions Q55C, L106C, L116C, T146C, D160C and S162C, reduced by C186A and C187A, increased by Q114C and S144C, and abolished by W53C, Y91C, N104C, W145C, Y184C and Y191C. These results are consistent with the predicted orientations in AChBP and suggest that epibatidine is likely to occupy a similar location at α7 nACh receptors. We speculate that steric constraints placed upon the C-5 position of the pyridine ring in 3SQ6 may account for the relatively poor affinities of epibatidine derivatives that are substituted at this position.

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1. Introduction

α7 nACh receptors are considered to be valuable drug targets as they modulate many physiological responses and are associated with neurological disorders and pathological conditions such as Alzheimer’s, Parkinson’s and schizophrenia (Hernandez and Dineley, 2012; Mazurov et al., 2011; Thomsen et al., 2010). Consequently there have been continued efforts to develop new ligands that target these receptors, both as drug candidates and as pharmacological research tools. Knowledge of how the ligands orientate in the receptor binding site can provide insights into where modifications might be tolerated. Such changes can be used to improve their pharmacological properties and aid the coupling of functional groups such as fluorescent labels, thiol-reactive moieties and photo-affinity probes (Lochner and Thompson, 2015).

α7 nACh receptors belong to the Cys-loop family of transmembrane ligand-gated ion-channels that are responsible for fast synaptic neurotransmission in the central and peripheral nervous systems. All members of this family are composed of five subunits, each of which contains an extracellular, a transmembrane and an intracellular domain (Thompson et al., 2010; Unwin, 2005). Competitive ligands such as acetylcholine or epibatidine bind at an extracellular, orthosteric binding site that is located at the interface of two adjacent subunits (Fig. 1). This site is a hydrophobic cavity created by the convergence of loops A − C from the principal subunit and loops D − F from the complementary subunit (Fig. 1). However, owing to the difficulty of crystallising membrane proteins, a soluble structural homologue (acetylcholine binding protein; AChBP), has been commonly used to study the binding orientations of α7 nACh receptor ligands. Examples include the

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Fig. 1. Locations of residues mutated in this study. A, A cartoon showing the binding site of AChBP with binding loops A – F highlighted in colour. The binding site is found at the interface of two adjacent subunits, and for clarity only two of the five subunits of the native receptor are shown. The structure is 3SQ6, but the location of the membrane has been indicated to show where it would be located in the α7 nACh receptor. B, Amino acid sequence alignment of the α7 nACh receptor and sequences from the two crystal structures (2BYQ & 3SQ6) in which epibatidine has been co-crystallised. The binding loops are shown in the same colours as in panel A, and amino acids that are within 5 Å of epibatidine are highlighted as white text in grey boxes on the AChBP structures. The equivalent α7 nACh residues substituted in this study are shown as white text in black boxes. α7m5HT3 = chick α7nACh chimera; 3SQ6 = Ls-AChBP-α7 nACh chimera; 2BYQ = Ac-AChBP. The residue numbering used in this manuscript corresponds to residues in the crystal structure 3SQ6. 2BYQ and 3SQ6 share 31.8% identity and 49.8% similarity in their sequences (EMBOSS Needle; Rice et al., 2000). C, The structure and atom numbering of protonated epibatidine is shown below the alignment.
agonists acetylcholine (3WIP), anabasine (2WNL), carbamylcholine (1UV6), cytisine (4BQT), epibatidine (2BYQ, 3SQ6), nicotine (1UW6), tropisetron (2WNC) and varenicline (4AFT, 4AFG), and antagonists such as lobeline (2BYS, 4AFH, 5AFH) and methyllycaconitine (2BYR). Owing to the relatively high sequence similarity of AChBP and the α7 nACh receptor (<64%) it is generally thought that the orientations of ligands in the crystals will be similar to those in the α7 nACh receptor. Consequently these structures have been used to direct efforts in the chemical synthesis of novel ligands and for computational methods such as molecular dynamic simulations (Akdemir et al., 2012; Camacho-Bustamante et al., 2016; Yamauchi et al., 2012). While several studies report the successful creation of novel α7 nACh receptor ligands, AChBP co-crystal structures should still be viewed with caution as some of the ligands they contain also bind to other Cys-loop family members where they have different affinities and functional effects. For example, varenicline inhibits nACh receptors, but is a partial agonist of 5-HT3 receptors (Lummis et al., 2011). Similarly, tropisetron is a partial agonist of 5-HT3 receptors (Eiselé et al., 1993) and was cloned into pcDNA3.1 for expression in HEK293 cells. Human 5-HT3A cDNA (accession number: P46098) and mouse 5-HT3A cDNA (Q6J1J7) were cloned into the same vector. Cells were transiently transfected with this cDNA using either polyethylenimine (PEI: 25 kDa, linear, powder, Polysciences Inc., Eppelheim, Germany) or Lipofectamine 2000 (Life Technologies, CA, USA) according to the manufacturer’s instructions. 30 μl of PEI (1 mg ml⁻¹), 5 μg cDNA and 1 ml DMEM/F12 were incubated for 10 min at room temperature, added drop wise to a 90 mm plate of 70–80% confluent HEK293 cells, and incubated for 2–3 days before use.

2. Materials and methods

2.1. Computational analysis

To gain insights into potential binding-site interactions, residues located within 5 Å of bound epibatidine in the AChBP crystal structures 2BYQ and 3SQ6, and residues identified by in silico docking into AChBP homology models. By substituting the equivalent residues at α7 nACh receptors we determine whether their effects on [³H]epibatidine binding are consistent with the ligand orientations predicted from the structures.

2.2. Receptors

Mutagenesis was performed using the QuikChange method (Agilent Technologies Inc., CA, USA) to substitute amino acids throughout each of the binding loops A–F (Fig. 1). The α7 nACh-5-HT3 subunit cDNA was as described by Bertrand (Eiselé et al., 1993) and was cloned into pcDNA3.1 for expression in HEK293 cells. Human 5-HT3A cDNA (accession number: P46098) and mouse 5-HT3A cDNA (Q6J1J7) were cloned into the same vector. Cells were transiently transfected with this cDNA using either polyethylenimine (PEI: 25 kDa, linear, powder, Polysciences Inc., Eppelheim, Germany) or Lipofectamine 2000 (Life Technologies, CA, USA) according to the manufacturer’s instructions. 30 μl of PEI (1 mg ml⁻¹), 5 μg cDNA and 1 ml DMEM/F12 were incubated for 10 min at room temperature, added drop wise to a 90 mm plate of 70–80% confluent HEK293 cells, and incubated for 2–3 days before use.

2.3. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were grown on 90 mm round tissue culture plates as monolayers in DMEM/F12 (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich) at 37°C in a moist atmosphere containing 5% CO₂.

2.4. Radioligand binding

Transfected HEK 293 cells were scraped into 0.6 ml of ice-cold 10 mM HEPES buffer (pH 7.4) and stored frozen. After thawing, they were washed with HEPES buffer, homogenised by passage through a 21 G needle, and 50 μl of cell suspension incubated in 0.5 ml HEPES buffer containing [³H]epibatidine (55.8 Ci/mmol, Waltham, MA). Non-specific binding was determined using 300 μM (–)nicotine. Equilibrium reactions were incubated on ice for at least 1 h. Incubations were terminated by vacuum filtration onto Whatman GF/B filters wetted with 0.3% polyethyleneimine, followed by two rapid washes with 2.5 ml ice cold buffer. Radioactivity was determined by scintillation in Ultima Gold XR (Perkin Elmer) using a Tri-Carb 2100 TR (PerkinElmer) scintillation counter. Final counts were monitored to ensure that binding never exceeded 10% of the added concentrations of radioligands.

2.5. Data analysis

All data were analysed using GraphPad Prism v5. Individual saturation binding experiments were fitted to Eq. (1):

\[ y = B_{\text{max}} \times \frac{[L]}{K_d + [L]} \]  

(1)

where \( B_{\text{max}} \) is maximum binding at equilibrium, \( K_d \) is the equilibrium dissociation constant and \([L]\) is the free concentration of radioligand. Values from individual experiments were averaged to yield the mean ± sem. Affinities were compared using 1-way ANOVA and a Dunnett’s Post-Test.

2.6. Detection

48 h post transfection, HEK293 cells were harvested in 10 ml phosphate buffered saline (137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 2 mM KH₂PO₄, pH 7.4), centrifuged 5 min at 220 g and the pellets stored at −80°C. Total extracts were prepared by resuspension of 1 × 10⁷ cells in 1 ml lysis buffer (10 mM Tris–HCl pH 7.5, 10 mM NaCl, 2 mM EDTA, 5 mM MnSO₄, 4× Halt Protease Inhibitor
nACh receptors with a binding at (Hansen et al., 2005; Li et al., 2011). Here we examined whether homologous AChBP have been used as substitutes for this receptor that govern this binding interaction, elsewhere crystal structures of (Li et al., 2011; Xiao et al., 2012). To better understand the residues that its orientation is tightly con
orientations of epibatidine (RMSD
poses were compared there was very little difference between conformation. For each of the main residues identi
the crystal structures are considered (Table 1). However, when all
a total of 72 independent ligand orientations when both these and
(4AFT). For each template 10 docked poses were generated, yielding
(4BQT), nicotine (1UW6), tropisetron (2WNC) and varenicline (3WIP), anabasine (2WNL), carbamylcholine (1UV6), cytisine (1CC)
epibatidine bound to AChBP. Epibatidine has been docked into the remaining structures shown. A – F – binding loops (see Fig. 1).

3. Results & discussion

[^3H]epibatidine displayed high-affinity saturable binding at α7 nACh receptors with a Kd (8 nM) similar to that reported elsewhere (Li et al., 2011; Xiao et al., 2012). To better understand the residues that govern this binding interaction, elsewhere crystal structures of homologous AChBP have been used as substitutes for this receptor (Hansen et al., 2005; Li et al., 2011). Here we examined whether these co-crystal structures are good representations of epibatidine binding at α7 nACh receptors and used them to speculate why chemical modification of this ligand may have previously generated much lower affinity fluorescent derivatives (Grandl et al., 2007). To this end we identified potential ligand-receptor interactions by examining the orientations of epibatidine in AChBP crystal structures and following in silico docking into other agonist-bound AChBP structures. Using radioligand binding we determined whether these interactions are consistent with the effects of substituting equivalent residues in the α7 nACh receptor.

Residues within 5 Å of epibatidine were identified in the co-crystal structures 2BYQ and 3SQ6, and following in silico docking into seven other agonist-bound AChBP templates; acetylcholine (3WIP), anabasine (2WNL), carbamylcholine (1UV6), cytisine (4BQT), nicotine (1UW6), tropisetron (2WNC) and varenicline (4AFT). For each template 10 docked poses were generated, yielding a total of 72 independent ligand orientations when both these and the crystal structures are considered (Table 1). However, when all poses were compared there was very little difference between orientations of epibatidine (RMSD = 0.78 Å average), suggesting that its orientation is tightly confined by the agonist-bound conformation. For each of the main residues identified in these poses, Cys substitution at the equivalent residue positions in the α7 nACh receptor was undertaken and the binding affinity of[^3H] epibatidine at these mutants was measured (Fig. 2, Table 2). Changing 6 of the 16 residues resulted in no significant change in

| PDB id | Principal Face | Complementary Face |
|--------|----------------|-------------------|
| 3SQ6   | Y91            |       | Y         |       |
| 2BYQ   | Y          | Y       | Y         | Y       |
| 1UV6   | S          | S       | S         | S       |
| 1W6M   | Y           | V       | V         | Y       |
| 3WIP   | Y           | Y       | Y         | Y       |
| 4AFT   | Y           | Y       | Y         | Y       |
| 2WNC   | Y           | Y       | Y         | Y       |
| 2WNL   | Y           | Y       | Y         | Y       |
| 4BQT   | Y           | Y       | Y         | Y       |

Locations of the most predominant residues can be found in Figs. 1 and 4. Only the numbering for 3SQ6 has been included to avoid confusion. 3SQ6 and 2BYQ are co-crystal structures of epibatidine bound to AChBP. Epibatidine has been docked into the remaining structures shown. A – F – binding loops (see Fig. 1).
affinity when compared to wild type \( \alpha 7 \) nACh receptors (Q55, L106, L116, T146, D160, S162), suggesting these residues do not play a significant role in ligand binding or the conformation of the binding site. For the remaining 10 mutants there were differences in the binding affinities. Of these, 2 had increased affinities (Q114C, S144C), 2 had decreased affinities (C186A, C187A) and 6 showed no saturable binding (\( K_d > 20 \) nM; W53C, Y91C, N104C, W145C, Y184C, Y191C). For mutants that did not show saturable binding, Western blots were performed to confirm receptor expression (Fig. 3). The observed signals showed that all mutants were expressed and the absence of measurable binding could be attributed to changes in binding site interactions, or to altered gating which can also impact the affinity (Colquhoun, 1998).

In the principal binding interface of the \( \alpha 7 \) nACh receptor, Cys substitution of Y91 in loop A abolished binding, indicating a key interaction. In the AChBP co-crystal structure 3SQ6, Y91 forms a cation-\( \pi \) interaction with K141 and a hydrogen bond with epibatidine (Fig. 5A), both of which have also been observed in functional assays on \( \alpha 7 \) nACh receptors. For example, removing the hydrogen bonding capability of Y91 by substitution with Phe caused a dramatic reduction in the affinity of \( \alpha 7 \) nACh for epibatidine, while unnatural amino acid mutagenesis identified a cation-\( \pi \) interaction at the same location (Li et al., 2011; Puskar et al., 2011). In loop B, Cys substitution of the aromatic residue W145 also abolished epibatidine binding, indicating another important effect. This centrally located aromatic residue is highly

| Loop | Mutant | \( pK_a \) Mean ± SEM | \( n \) | \( K_d \) (nM) | \( pK_{(WT)} - pK_{(mut)} \) |
|------|--------|----------------------|-----|-------------|------------------|
| D    | Wild type | 8.09 ± 0.06 | 6 | 8.0 |  |
|      | W53C | NB | 4 | - |  |
|      | Q55C | 8.16 ± 0.09 | 3 | 6.9 |  |
|      | Y91C | NB | 6 | - |  |
|      | N104C | NB | 6 | - |  |
| E    | L106C | 8.08 ± 0.12 | 5 | 8.3 |  |
|      | Q114C | 8.82 ± 0.09* | 6 | 1.5 |  |
|      | L116C | 8.29 ± 0.05 | 4 | 5.1 |  |
|      | S144C | 8.64 ± 0.12* | 4 | 2.3 |  |
| B    | W145 | NB | 3 | - |  |
|      | T146C | 8.01 ± 0.07 | 4 | 9.8 |  |
|      | D160C | 8.06 ± 0.10 | 5 | 8.7 |  |
|      | S162C | 8.14 ± 0.09 | 5 | 7.2 |  |
| C    | Y184C | NB | 5 | - |  |
|      | C186A | 7.48 ± 0.10* | 3 | 33 |  |
|      | C187A | 7.22 ± 0.07* | 4 | 60 |  |
|      | Y191C | NB | 3 | - |  |

* = significantly different to wild type receptors (1-way ANOVA and a Dunnett’s Post-Test). \( pK_{(WT)} \): - \( pK_{(mut)} \) is the difference between wild type and mutant receptors ± the standard error of the difference.

Fig. 3. Expression of mutant \( \alpha 7 \)nACh receptors. Several mutant receptors did not bind [\( ^3H \)]epibatidine. To confirm that this was a change in the binding affinity rather than altered expression, these mutants were probed by Western blot using a receptor-specific antibody directed against the 5-HT3 region of the chimaeric receptor. Expression levels of the mutants and wild type receptors were comparable (black arrow), and there was no detection in untransfected cells. In each lane 10⁵ cell equivalents were loaded. The membrane was cut at 70 kDa and the upper section was incubated with an antibody of cleavage and polyadenylation specificity factor (CPSF) to provide an internal control. For both Western blots and radioligand binding the same whole-cell homogenates were used, ensuring that the same population of receptors was probed.
epibatidine via its azabicyclo moiety (Hansen et al., 2005; Li et al., 2011). In our study of \(7\) nACh receptors, epibatidine binding was not detectable following Y184C substitution, a finding that is comparable to the >250-fold change that was previously reported following the modest substitution Y184F (Li et al., 2011). We also found that Ala mutation of either of the closely located vicinal disulphides (C186, C187) decreased the affinity for epibatidine by >30-fold. This is consistent with the increased \(E_{C50}\) of ACh that is seen when the equivalent residue is mutated in other nACh receptor subtypes such as \(\alpha1\) subunit-containing nACh receptors (Blum et al., 2011; Karlin and Bartels, 1966). In \(\alpha1\) nicotinic subunits the disulphide bond formed by these adjacent Cys is proposed to distort loop C and enable key residues to interact with loop F on the opposite side of the binding interface, and at \(7\) nACh receptors they may have a similar role (Blum et al., 2011).

On the complementary face of the binding site, W53 in loop D also constitutes part of the aromatic box. We show that binding is undetectable following Cys mutation of this residue at \(7\) nACh receptors. In the AChBP crystal structure 3SQ6, W53 stabilises W145 via edge-to-face interactions rather than making a direct contact with epibatidine. Such an interaction is consistent with the relatively small affinity change (4-fold) for epibatidine when a common aromatic W53F substitution is made. However, the larger W145F mutation at \(\alpha7\) receptors has little effect on the binding of epibatidine, but the affinity of epibatidine at the mutant receptor was unaltered when compared to wild type \(7\) nACh receptors. A similar absence of major effects following Cys substitution of this residue have been reported in functional studies of other agonists at \(7\) nACh receptors (Papke et al., 2011). Based on these findings and the orientation of epibatidine in both of the co-crystal structures it is unlikely that this residue makes substantial contact with the ligand or affects gating. Also present in the complementary binding interface, the AChBP crystal structures indicate that the equivalent of loop E residues N104, L106, Q114 and L116 stabilise epibatidine via Van der Waals interactions with its chloropyridine ring (Hansen et al., 2005; Li et al., 2011). We found that the binding affinity was unaltered by Cys substitution of L106 and L116, possibly owing to the similar size and hydrophobicity of leucine and Cys (Nagano et al., 1999). For L106 our results are the same as those for L106A which does not affect the \(E_{C50}\) of epibatidine, ACh or varenicline (Van Arnam et al., 2013). In contrast, our results for L116 appear to conflict with reports that showed Ala mutation causes a 9-fold change in the \(E_{C50}\) of epibatidine, and that the equivalent residue makes an important hydrogen-bond with agonists in \(\alpha4\beta2\) nACh receptors (Blum et al., 2013; Van Arnam et al., 2013). However, when the backbone of L116 was probed by unnatural amino acid mutagenesis to determine if hydrogen-bonding was important at \(7\) nACh receptors, there were only minor effects on the binding of epibatidine, ACh and varenicline, suggesting that this residue does not have the same role as that found in \(\alpha4\beta2\) nACh receptors (Van Arnam et al., 2013). Furthermore, a lack of effects on activation by other agonists has been previously seen following Cys substitution at L116, and changes may be dependent upon the ligand and the residue substitution (Amiri et al., 2008; Papke et al., 2011). For Q114 a halogen-bonding interaction between the backbone carbonyl and the pyridine chloride of epibatidine is seen in AChBP (Li et al., 2011). Consistent with this, removal of the side chain of Q114 by substitution to Ala has little effect on the \(E_{C50}\) of epibatidine and other \(\alpha7\) nACh
receptor agonists (Van Arnam et al., 2013). We did not probe main chain interactions, but our findings suggest that Cys mutation may have additional effects. For example, as Q114C increased the affinity for epibatidine by 5-fold, we speculate that this could reflect the formation of an attractive non-covalent interaction between the sulfhydryl group and the chlorine atom. Elsewhere in AChBP mutation of residues in loop C is similarly reported to increase the affinity of ACh and it was also speculated that this might reflect changes in electrostatic interactions (Li et al., 2011). In loop F, neither D160C nor S162C mutations had any effect on the affinity of epibatidine, nor indeed are there any previous reports of these residues mediating ligand interactions or functional effects. In other studies, mutation of the closely located residue G167 also had no effect on the function of epibatidine when mutated to a range of other amino acids, and I161C and G167C had no effect on several other α7 nACh receptor agonists, further indicating that this region may not have a major role in binding (Matsuda et al., 2000; Papke et al., 2011). High-affinity epibatidine binding has also been reported at 5-HT3 receptors, similar to the binding of ACh, nicotine, tropisetron and o-tubocurarine at the two receptor types (ChavezNoriega et al., 1997; Drisdel et al., 2008; Thompson et al., 2014). In light of this we also measured binding at the 5-HT3 receptor to establish whether epibatidine could have utility for studying this other member of the Cys-loop receptor family. However, saturable binding with [3H] epibatidine was not detected at either human or mouse 5-HT3 receptors at a concentration of up to 25 nM. This result was confirmed when competition of unlabelled epibatidine with the 5-HT3 receptor antagonist [3H]tropisetron yielded a pK_i of 5.60 ± 0.02 (K_i = 2.51 μM, n = 3) for human and a pK_i of 4.91 ± 0.12 (K_i = 12.3 μM, n = 4) for mouse, but contrasts with a report from Drisdel et al. (2008) who found a nanomolar affinity for epibatidine at 5-HT3 receptors. It is unclear why there is a 100-fold difference between our results, but the larger value we measured precludes the possibility of using this ligand as precursor for developing novel molecular probes for studying 5-HT3 receptors. In contrast, epibatidine may provide an opportunity for developing α7 nACh receptor probes, but previous efforts to couple fluorophores at the C-5' position (see Fig. 1 for atom numbering) caused a dramatic reduction in affinity (at worst, from nM to mM) and made the resultant fluorescent derivatives unsuitable for fluorescence-based experiments (Grandl et al., 2007). However, further analysis of 3SQ6 suggests that the C-4' position is more accessible than the C-5' position. Indeed, all ten of the binding orientations in 3SQ6 (Fig. 5B) expose the C-4' position, while orientations in 2BYQ show that either the C-5' is more accessible or that the pyridine ring is flipped by 180° which makes the C-2' most accessible (Fig. 5C and D). The finding that C-5' substituted epibatidine derivatives still bind within the tightly confined space of the ligand binding site shows that conjugation at this location does not prevent binding,
but the modification is not optimal. Our finding that the orientations of epibatidine in the AChBP co-crystal structures are broadly correct, and that 3SQ6 shows improved accessibility at the C′-4′ position, suggests that there may still be opportunities to develop epibatidine-based probes with higher affinities for the α7 nACh receptor. In our previous work the development of similar probes for targeting 5-HT3 receptors has enabled quantitative fluorescent measurements, receptor localisation and photo cross-linking to be performed, and shows the value of pursuing their synthesis (Lochner and Thompson, 2015; Jack et al., 2014).

To visualise ligand-receptor interactions homologous proteins are often used as substitutes for the native receptor, and AChBP is a popular substitute for all Cys-loop receptors despite often quite distinct pharmacologies for the same ligands at different receptors. Here we specifically examined another of the structures, to determine whether differences in the affinity of epibatidine caused by point mutations in the α7 nACh receptor are consistent with residues identified in epibatidine-bound AChBP crystals. The results presented here suggest that the orientations of epibatidine in the AChBP crystal structures are a reasonable approximation of its orientation at α7 nACh receptors. It is also striking how similarly epibatidine binds in the two crystal structures despite the only 31.8% sequence identity between 2BQX and 3SQ6. This similarity in ligand orientation extends to the in silico docking presented here and perhaps reflects the high conservation of major aromatic residues that constitute the binding site (in 3SQ6 and the α7 nACh receptor these residues are identical) and the tight pocket imposed by the agonist-bound conformation. Furthermore, while the accessibility of locations on the pyridine ring of epibatidine still needs to be clarified in more detail, we can now be more confident of the crystal structures and use them as models for our ongoing efforts to attach fluorescent moieties to this ligand in order to develop specific high-affinity molecular probes for studying the α7 nACh receptor subtype. The use of cysteine mutants could also provide a stimulus for probing ligand interactions by chemical modification (e.g. by methanethiosulfonate (MTS) reagents) of the thiol side chains.

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