Structural and pharmacological validation of allosteric sites at the M_5 Muscarinic acetylcholine receptor – a target for CNS disorders

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G protein-coupled receptors (GPCRs) are preeminent drug targets accounting for a third of approved medicines. Despite this success, the discovery of new drugs that selectively target GPCRs has been a challenge due to many GPCRs being activated by similar types of ligands. It is now well-appreciated that GPCRs contain allosteric sites, which are binding sites that are distinct, but conformationally linked to the endogenous/orthosteric binding site. A key feature of allosteric modulators is their capacity to specifically bind to one GPCR subtype due to allosteric sites being less conserved. This ability allows them to circumvent the challenge associated with targeting the conserved orthosteric-binding site found on closely related receptors. The muscarinic acetylcholine receptors (mAChRs) are a five-membered (M_1-M_5) subfamily of Class A GPCRs that are an exemplary example of therapeutically relevant GPCRs that can be selectively targeted by allosteric ligands. The mAChR subtypes play a critical role in neurological functioning, and M_5R knockout mice suggest a physiological role in CNS disorders. Although there is strong data supporting the M_5 mAChR as a potential therapeutic target, further clinical research has been hindered due to a lack of selective drug-like molecules for the receptor. Thus, researchers have focused on finding allosteric ligands that selectivity modulate the M_5 mAChR. Here, we report a 2.6 Å cryo-EM structure of the human M_5 mAChR bound to acetylcholine (ACh) and a distinct positive allosteric modulator ML129 (Isatin PAM). ML129 covalently interacts with cysteine residue (Cys 214 $^{5.59}$) at a previously unidentified allosteric site in TM5. Further analysis revealed that this compound also interacts with another cysteine residue (Cys $484^{7.42}$) in TM7, though the electron density for this interaction is less well-defined. Moreover, we believe that ML129 forms a transient interaction with two cysteine in TM7: one is the previously mentioned $C484^{7.42}$ and another one is $C494^{7.52}$. Further to validate this site, we designed alanine point mutations of key interacting cysteine's. Our previous study with a different PAM, VU6007678, revealed an intracellular allosteric site located at the receptor–lipid interface, interacting with key activation motifs. In contrast, the structurally distinct ML129 binds to a separate region of the receptor, highlighting the structural and topological diversity of allosteric modulation at the M_5 mAChR. Overall, our study has identified a new mAChR allosteric site that may be useful for the design of selective allosteric modulators.