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The existence of a second allosteric site on the M_1 muscarinic acetylcholine receptor and its implications for drug design

L. Michel Espinoza-Fonseca[a,*] and José G. Trujillo-Ferrara[b,c]

[a]*Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN 55455, USA*
[b]*Sección de Estudios de Posgrado e Investigación, Escuela Superior de Medicina del Instituto Politécnico Nacional, Apartado Postal 42-161, C.P. 11340, Mexico City, Mexico*
[c]*Departamento de Bioquímica, Escuela Superior de Medicina del Instituto Politécnico Nacional, Apartado Postal 42-161, C.P. 11340, Mexico City, Mexico*

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Abstract—Fully flexible docking of KT5720, an allosteric modulator of the muscarinic receptors, was performed on a dynamic model of the M_1 muscarinic acetylcholine receptor. The results confirmed the existence of a second allosteric site, located on the intracellular face of the receptor. These results would be beneficial for the design of modulators of this receptor to be used as an effective alternative against the Alzheimer's disease.

Over the past few years, multiple proteins involved in the pathogenesis of the Alzheimer's disease have been identified as potential drug-design targets against this disease.[1] Among these targets, the M_1 muscarinic acetylcholine receptor recently received attention as a promising target for the design of anti-Alzheimer's disease drugs.[2] Its activation by agonists has been found to reduce the amyloid precursor protein levels and to decrease the *tau* protein hyperphosphorylation/phosphorylation and therefore to contribute to achieve improvements of cognitive abilities.[3] Unfortunately, this receptor, which is a member of the superfamily of G-protein coupling receptor, shares similar structural features with other types of muscarinic receptors (M_2–M_5). Particularly, their orthosteric site where acetylcholine binds has been shown to lack selectivity when binding agonists.[4] This fact has hindered the design of specific agonists of the M_1 muscarinic receptor.

On the other hand, it has been found that ligands that allosterically regulate the binding and actions of acetylcholine at muscarinic receptors have the potential capability to selectively regulate the function of a single receptor subtype in novel ways.[5–8] These allosteric agents would be capable of inducing favorable conformational changes in the receptor, increasing the affinity of acetylcholine and therefore improving the G-protein coupling to the receptor. Thus, the possibility to develop novel allosteric agents could be a promising alternative to attack one of the multiple pathways involved in the etiology of the disease.

Recently, it was experimentally shown that the muscarinic receptors possessed multiple allosteric sites, but there has been no strong evidence on their exact location.[9] Flexible docking simulations of staurosporine and four staurosporine derivatives to a homology model of the M_1 muscarinic receptor showed that there were at least two different allosteric sites, one of them located in the extracellular face of the receptor, and the other one in the intracellular face, very close to the intracellular loop 3 (i3).[10] However, in these simulations only the ligands were flexible, while the receptor was kept rigid. Therefore, given the existence of a second allosteric site on this receptor, new possibilities for the design of drugs to treat Alzheimer's disease can be explored.

In this contribution, we combined two successful computational methodologies, the 'blind' docking

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* Corresponding author. E-mail: mef@ddt.biochem.umn.edu

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approach[11] and the relaxed complex scheme[12], as a reliable tool to establish the existence of a second allosteric site on the M_1 muscarinic receptor.

We chose the compound KT5720 previously reported by Lazareno et al. (Chart 1) because it was observed that it did not compete with gallamine, a well-known allosteric modulator, and because it displayed neutral cooperativity with the latter, suggesting that it binds to a different site.[9] This allowed us to correctly identify not only the second allosteric site, but also the binding mode of this compound on the allosteric site.

KT5720 was modeled using the program MOE [13] and was optimized using the semi-empirical AM1 level. Partial charges, necessary for the docking protocol, were assigned according to the Gasteiger–Marsili scheme.[14]

In order to allow the protein and ligand to flex, we combined both 'blind' docking approach and the relaxed complex scheme, which allows implicit flexibility to the protein by capturing rare conformations that are not observed when using a rigid model. For this purposes, a 3 ns molecular dynamics (MD) simulation of the homology model of the M_1 muscarinic acetylcholine receptor reported by Hulme and co-workers[15] was performed using the program **NAMD** 2.6[16] and the CHARMM 27 force field.[17] The protein was inserted in a pre-equilibrated 80×80 Å POPC bilayer slab, and the protein-membrane system was embedded in a TIP3P water box. Finally, counterions were added to the system in order to produce a neutral charge on it. For the MD simulations, periodic boundary conditions were applied to the system and the particle mesh Ewald method was employed. Nonbonded cutoff, switching distance, and nonbonded pair-list distance were set to 9, 8, and 11 Å, respectively. The use of the SHAKE algorithm allowed a 2-fs time step. NPT ensemble was maintained throughout the simulation. The system was first minimized, warmed up, and equilibrated for a total of 400 ps; finally, the simulation was continued with no restrains for 3 ns.[18]

Docking simulations were performed with the program AutoDock 3.0.5.[19] This program uses the Lamarckian Genetic algorithm and its scoring function comprised by van der Waals, Coulomb potential electrostatics, hydrogen bonding, a volume-based solvation term, and an estimation of the entropic cost of binding through a weighted sum or torsional degrees of freedom terms. These scoring parameters are also used for the estimation of the free energy of binding. Additionally, one does not need to specify the possible binding site, since the algorithm allows an efficient searching of the entire surface of the target.

Fifty snapshots from the 3-ns MD simulation of the receptor were taken (e.g., each 60 ps), and water, ions, and lipid molecules were removed. Grid maps of 126 Å and spacing of 0.6 Å in each direction were calculated with AutoGrid. The Lamarckian Genetic Algorithm was used for the actual docking simulations, using an initial population of 500 individuals, a maximum number of 50,000,000 energy evaluations, and a maximum number of 50,000 generations. Docking simulations consisted of 100 independent runs (for a total of 5000 runs), and resulting orientations lying within 2 Å were clustered together and represented by the orientation with most favorable free energy of binding.

Our results show that, when KT5720 was docked to the rigid homology model of the receptor, three binding sites were found, as previously reported. Two of those binding sites were located in the extracellular face of the receptor, while the third one was found in the intracellular face of the receptor. In order to better determine the preferred binding site by KT5720, the protein was allowed to flex by means of an MD simulation. It was observed that fully flexible docking preferably placed KT5720 in the allosteric site located at the intracellular face of the receptor (Fig. 1). KT5720 had extensive interaction with the receptor. The aliphatic chain of the compound makes important contacts with residues T58, N60, N61, L64, Y418, and N422 (Fig. 2), principally via van der Waals and hydrophobic interactions. As shown in Figure 1, this aliphatic chain is docked toward the transmembrane domain of the receptor and

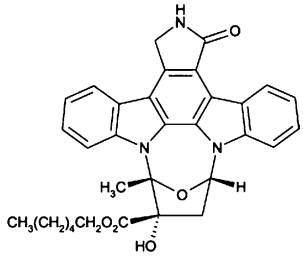

Chart 1. Chemical structure of the allosteric modulator KT5720.

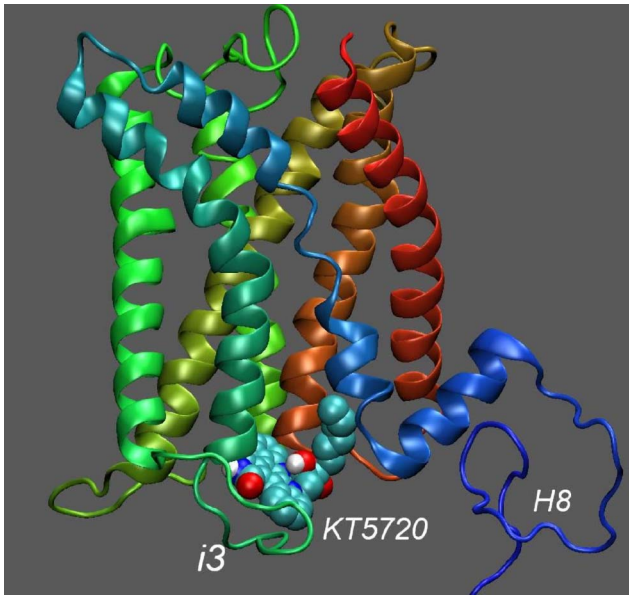

Figure 1. Location of KT5720 in the M_1 muscarinic receptor (van der Waals spheres). The helix 8 (H8) and the intracellular loop 3 (i3) are shown. The seven transmembrane (TM) helices are colored as follows: TM1, red; TM2, brown; TM3, olive; TM4, dark green; TM5, light green; TM6, cyan; TM7, light blue.

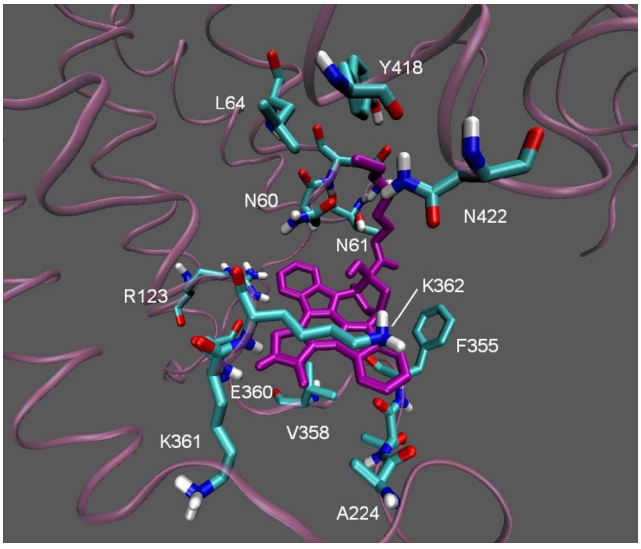

Figure 2. Binding mode of KT5720 (purple) in the second allosteric site described here. The residues that play an important role in binding and stabilizing the complex are displayed as sticks. The rest of the protein is shown as ribbons (mauve).

surrounded by transmembrane (TM) helices 2 and 8. Thus, this fragment of KT5720 could partially serve as an anchor to favor (or 'allow') the binding to this allosteric site of the receptor.

The aromatic moiety of the compound (indolocarbazol fragment) interacts with most of the residues reported in our previous study.[10] The indolocarbazol moiety interacts with R123, A224, T354, F355, V358, E360, K361, and K362 (via π-cation interaction). Most of the interactions between the allosteric modulator and the receptor were van der Waals and hydrophobic ones. Interestingly, it was observed that a positively charged residue (K362) interacted with the aromatic indolocarbazol moiety of the compound. This suggests that the molecular recognition and binding mechanisms of this compound might be driven by π-cation interactions, while extensive van der Waals and hydrophobic interactions play an important role in stabilizing the modulator–receptor complex at the allosteric site.

Another aspect of or in this study is the calculation of the binding affinity of KT5720. As observed in Table 1, a single docking simulation on the original (rigid) homology model of the M_1 muscarinic receptor shows that the binding energy is more favorable than the energy obtained using the fully flexible protein. When the free energies of binding calculated for the rigid and for the flexible proteins are compared, a difference of 3.07 kcal/mol is obtained. Interestingly, the pK_i computed for the binding of KT5720 to the rigid muscarinic receptor is significantly higher than the pK_i experimentally obtained by Lazareno et al.[9] In contrast, the pK_i calculated for the allosteric agent binding to the fully flexible receptor is similar to the value obtained experimentally (see Table 1). As previously observed by McCammon and co-workers, the use of a fully flexible model allows us to evaluate several conformations of the protein that will permit the ligands to better accommodate on the binding sites. Thus, the difference of 3.07 kcal/mol between the rigid and the flexible models of the receptor reflects the importance of the dynamics of the receptor not only in drug-design efforts, but also in finding other allosteric/orthosteric putative sites in silico.[20]

Table 1. Free energies of binding and inhibition constants calculated for KT5720 binding to the intracellular site of both rigid model and the flexible docking approach used here

Type of docking	$\Delta G_{binding}$ (kcal/mol)	K_i[b] (nM)	pK_i[b]
Rigid[a]	−11.71	2.96	8.53
Flexible[a]	−8.64	447 (±14)	6.35 (±0.84)

[a] Value is the means of the docking simulations, standard deviation is given in parentheses.
[b] Experimental pK_i value is 6.42 ± 0.09.

These observations support the fact that KT5720 does not bind to the previously studied allosteric site located at the extracellular face of the receptor. Instead, it binds to the intracellular face of the receptor, which is very close to the intracellular loop 3 and the helix 8. These findings are of great interest in medicinal chemistry due to the following: (a) it is possible to exploit this allosteric site by selectively targeting it with improved compounds. For example, structural modifications of KT5720, such as the modification of the length of the aliphatic chain or the addition of substituents in order to improve KT5720 affinity; (b) the present model lays the ground for future experimental and theoretical studies of the second allosteric site. Mutagenesis studies in particular would be helpful to better understand the complex allosteric behavior experimentally observed by KT5720; (c) the localization of this allosteric site is not random: it is placed very close to the regions that are important for the G-protein coupling. Therefore, we can hypothesize that the formation of the complex between the receptor and the G-proteins could be modulated in situ.[10] In this case, binding of acetylcholine to the receptor activates it and induces conformational changes along the transmembrane domain of the receptor. Consequently, the energy transduction will be modulated not only at the extracellular domain of the receptor, but also at the intracellular domain. Thus, this second allosteric site would serve as a 'switch' in order to continue or stop the energy flow through the receptor.

Considering the results obtained in the present study, several experiments can be suggested. For example, extensive mutagenesis analysis of an allosteric site that is located close to the entrance to the orthosteric site has been performed.[8] Point mutagenesis of the key residues found in this study such as A224, F355, V358 or K362 can be performed. Additionally, more complex simulations are in course, including the docking and molecular dynamics simulations of ternary and quaternary complexes (receptor/gallamine/KT5720, receptor/KT5720/ACh, receptor/gallamine/KT5720/ACh), as well as more accurate calculations of the free energy of binding by using methodologies such as the MM-PBSA. By combining the latter and the former calculations, it will be possible to measure the effect of the allosteric site over the orthosteric one.

In summary, in the present study we confirm the existence of a second allosteric site on the M_1 muscarinic acetylcholine receptor by using the blind docking approach and the relaxed complex scheme method. The existence of this second allosteric site would be of great importance in the design and development of novel allosteric modulators of this receptor. While more experimental and theoretical work needs to be performed, the main goal of this study is to set up a basis for the design of efficient agents to be used in the treatment of the Alzheimer's disease.

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