The acetylcholine binding protein as a template for the ligand binding domains of the homologous nicotinic receptors

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Summary, Conclusions and Future Perspectives

Summary

At the start of this project in November 2004, acetylcholine binding proteins (AChBPs) had just been established as an interesting extracellular domain (ECD) mimic for ligand-gated ion channels (LGICs). [1, 2] The extracellular AChBPs are secreted by glial cells into the synapses to scavenge their endogenous ligand acetylcholine. AChBP was identified in different species.[1-5] Sequence analysis reveals that these proteins have low similarity with the extracellular ligand binding domains of the nicotinic acetylcholine receptors (nAChRs) and other Cys-loop receptors, but pharmacological and available structural data indicate that AChBP has striking similarities with the ECD of LGICs (**Chapter 1**). AChBPs lack the transmembrane and intracellular domains of the Cys-loop receptors, which is advantageous for their water solubility and crystallizability. Interestingly, the sequence similarity in the binding pocket is much higher compared to the overall sequence similarity (**Chapter 1**). Probably as a result of this, several AChBPs show some similarity in pharmacology to the nAChRs and bind several ligands that show high affinity for several nAChR subtypes.[1, 2, 4-9] For example, Ls-AChBP shows affinity for the α7 selective antagonists α-Bgt, while Ac-AChBP shows affinity for the α7 selective antagonists MLA (**Chapter 1**).

All together, we and others considered AChBPs as promising templates to investigate the ligand-receptor interactions of the homologous nAChRs.[1, 2, 10-12] At the start of my research project, only a few cocrystal structures of AChBPs were available, e.g., Ls-AChBP in complex with nicotine has just become available.[6] At that time, few homology models of the α4β2 and α7 receptors had been constructed,[10, 11] but to the best of our knowledge, no systematic in silico screening procedures, structure-based hit optimization procedures and mutagenesis studies to mimic the binding pocket of the nAChRs had been performed. Therefore, the main goal of this PhD thesis was to evaluate the use of AChBP as a template to obtain selective ligands for the nAChRs. To this end, we defined the following three research questions for our thesis:

- Can we use AChBP as a tool to identify novel chemotypes for the nAChRs?
- Can we use AChBP in structure-based hit optimization procedures to optimize the ligand-protein interactions of both AChBPs and the nAChRs?
- Can we construct AChBP mutants that are good models for the orthosteric binding pockets of the nAChRs?

In **Chapter 1**, the nAChRs are introduced and their physiological importance and pharmacological relevancy is described.[13-17] Attempts that have been made by various research groups to obtain structural information of the nAChRs and its eukaryotic subtypes (i.e., mouse α1 subunit and Torpedo receptor) and prokaryotic analogues (i.e., GLIC and ELIC) are being described.[18-25] Even though these receptors show similarity in sequence and structure, they did not reveal detailed information of the binding pocket architecture on an atomic level.

A major breakthrough in the understanding of the nAChR binding pockets was made possible with the identification of the AChBPs.[1, 2] The three AChBPs from different species are introduced, their sequence and structural features are described and an overview is presented of the obtained cocrystal structures of AChBPs with different classes of nAChR ligands. Finally, the research questions of this Thesis are defined.

Chapter 2 reports the use of AChBP as a template for an in silico screening procedure to identify structurally novel ligands for the nAChRs.[12] An in silico screening protocol was set up using three different cocrystal structures of AChBP, i.e., in complex with HEPES, carbamylcholine and nicotine.[6, 12] The screening procedure was applied to a proprietary compound collection of druglike molecules and consists of the actual docking, visual inspection of the binding pose, pharmacological assays and an analogue search. Using this approach, we discovered novel classes of small molecule AChBP ligands. Several hit compounds were shown to also bind to the α7 nAChR.[12] We selected two high affinity ligands that contained a dibenzosuberyl-moiety for cocrystallion with Ac-AChBP. These compounds were selected because they show resemblance to several antidepressants such as carbamazepine.[12] These tricyclic compounds are known in channel blockers but recent studies suggest a possible interaction with the ligand binding domain of LGICs as well. The obtained cocrystal structures of both compounds in complex with Ac-AChBP and additional binding studies suggest that the orthosteric site of the nAChRs could be targeted.[12] Functional studies reveal that these two cocrystallized ligands cause, amongst others, inhibition of the α7, α4β2 and $5HT_3$ receptors.[12] The non-competitive blockade of these receptors suggests that these compounds act by steric hindrance of the channel. These data suggest a dual binding mode for these dibenzosuberyl-containing compounds, e.g., blocking of the ion channel and binding to the orthosteric site.

The cocrystal structures obtained from this in silico screening study were used in structure-based hit optimization procedures (**Chapter 3**). We designed two novel classes of ligands, i.e., dibenzosuberyl- and benzoate-substituted tropines, which aimed at addressing a region within the binding pocket that we have called the lobeline pocket. To the best of our knowledge, no crystal structures exist to date besides of the structure of Ac-AChBP in complex with lobeline in which the lobeline pocket is being addressed by a ligand (**Chapter 1**). Cocrystallization with Ac-AChBP of one of our benzoate-substituted tropines confirmed that we addressed the lobeline pocket successfully for the first time by rationally designed ligands (Figure 4 of **Chapter 3**). Interestingly, the compounds show selectivity for the α7 receptor and lack affinity for the α4β2 receptor. In addition, Ls-AChBP and Ac-AChBP respond differently to structural modifications of the ligands. The newly obtained cocrystal structures can be used to focus on the subtle differences between the AChBPs and nAChRs. Thus, we have identified a region within the pocket that can be explored for gaining activity and selectivity amongst nAChR subtypes.

Chapter 4 describes a more extensive hierarchical in silico screening protocol against the agonist (nicotine) bound AChBP cocrystal structure.[6] This led to the efficient identification of novel chemotypes for AChBP and the human α7 receptor. Two novel ligands were identified that act as competitive α7 receptor antagonists and as non-competitive α4β2 receptor inhibitors. These ligands were cocrystallized with Ac-AChBP revealing previously unobserved binding modes in AChBP crystal structures. Interestingly, the structures reveal that no hydrogen bonding interactions occur but intermolecular cation-π interactions with loop C are identified. These studies highlight the use of AChBP as a structural template in structure-based in silico screening studies to find new chemical scaffolds for the α7 receptor. These studies also reveal challenges in finding competitive α4β2 receptor ligands and α7 receptor agonists and the computational prediction of ligand binding modes.

In **Chapter 5**, several mutants of Ac-AChBP have been designed to mimic the orthosteric binding pocket of the human nicotinic α4β2 and α7 receptors. The expressed proteins of these mutants were pharmacologically evaluated by measuring the affinity for a set of reference ligands that contain subtype selective nAChR ligands. The α4β2 receptor mimics did not change the ligand selectivity profile of Ac-AChBP. The α7 mimics of

AChBP did have some increased affinity for some α7-selective ligands. However, mutagenesis of residues located in or close to the Ac-AChBP ligand binding pocket did not yield proteins which fully mimic the affinity profile of closely related nAChRs. These studies indicate that the molecular features that are responsible for subtype selectivity are complex and might be located further away from the ligand binding site.

Conclusions and Future Perspectives

At the beginning of this PhD project, three research questions were defined to evaluate the use of AChBP as a molecular tool to investigate the homologous binding pockets of the related and pharmaceutically relevant human nAChRs. In the following paragraphs, the outcome of this PhD project will be discussed in the light of these research questions.

Can we use AChBP as a tool to identify novel chemotypes for the nAChRs?

In silico screening procedures are used to predict whether ligands will bind to the binding pocket of the target proteins under investigation.[12, 26-29] We have applied two different in silico screening protocols to a proprietary compound library of druglike ligands using agonist bound Ls-AChBP cocrystal structures.[6, 12] The first in silico screening consisted of a docking step, visual inspection of the obtained binding poses, pharmacological procedures, and an analog search (**Chapter 2**).[12] The second in silico screening procedure was more complex and consisted of a pharmacophore screening, molecular docking, automated post-processing of the obtained binding poses, visual inspection and pharmacological assays (**Chapter 4**). The second in silico screening showed a better enrichment compared to the first in silico screening procedure. Validation experiments confirmed that the high hit rate was not the result of artificial enrichment[30] of the databases with compounds similar to nAChR ligands. We assume that the strength of the second in silico screening procedure lies in the combination of hierarchical filters with increasing complexity, adapted to the specific characteristics of the Ls-AChBP binding pocket.

We have identified new chemotypes with affinity for AChBPs and the human α7 receptor with both in silico screening procedures. Several of these ligands have been cocrystallized with Ac-AChBP. Remarkably, the obtained cocrystal structures clearly show that the binding pocket conformation was changed upon ligand binding, i.e., the loop C conformation adopts a different conformation. The experimentally determined ligand binding mode did therefore differ from the predicted in silico docking binding mode. The inclusion of protein flexibility in docking studies is computationally demanding and therefore was not taken into account in our docking studies.[26-29] However, the limitations identified in this thesis has prompted our research group to perform docking studies that allow protein flexibility.[31] These studies indeed indicate flexibility of the binding pocket upon ligand binding. The use of flexibility in virtual screening campaigns still remains to be

explored. In an alternative approach, Taylor and coworkers performed molecular dynamics combined with molecular docking to set up a virtual screening protocol that allows protein flexibility. Unfortunately, no affinity data and structural validation of the binding mode in AChBP were reported. Also no determination of affinities and functional activities on nAChRs were reported. Nevertheless, these recent studies underline both the importance as well as difficulty to determine induced-fit effects in virtual screening campaigns.

Remarkably, all hits obtained from our in silico screening procedures showed affinity for AChBPs and the α7 receptor, but lacked affinity for the α4β2 receptor. In **Chapter 2**, we suggested that a phenylalanine in the human α4β2 receptor, which is located at the M116 position of Ac-AChBP, might be causing this effect by steric clashes with the dibenzosuberol moiety of several obtained hit ligands. In **Chapter 3** and **Chapter 4**, we obtained ligands that do not have the dibenzosuberol moiety, but still the ligands did not show any affinity for the human α4β2 receptor. This result hints at more complex mechanisms for selectivity between the nAChR subtypes and the AChBPs. Residues not directly contacting the ligands can influence the binding properties through various mechanisms including electrostatic interactions.[3, 6, 32]

Finally, it should be noted that in both in silico screening procedures agonist-bound Ls-AChBP cocrystal structures were used.[6] Nevertheless, none of the obtained ligands behaved as agonists in our pharmacological assays.[12] In **Chapter 2**, the hit compounds showed non-competitive inhibition of α7 and α4β2 receptors by ion channel blockade. In **Chapter 4**, the hit compounds were competitive antagonists of the α7 receptor and non-competitive inhibitors of the α4β2 receptor.

It is apparent that not all AChBP hits show affinity for nAChRs and functional activity of the nAChR hits is difficult to predict. This clearly illustrates limitations of AChBP as a model for the extracellular LBDs of nAChRs. Despite these shortcomings, we have found that the use of AChBP enables us to find novel AChBP and nAChR binding compounds that fuel our chemistry programs in hit exploration and optimization campaigns.

Can we use AChBP in structure-based hit optimization procedures?

Structure-based hit optimization procedures were performed using cocrystal structures of the proprietary hit compound **31** and lobeline (**Chapter 3**). Using a fragment-merging strategy, we rationally designed ligands that interact with the lobeline pocket. Cocrystallization studies of these ligands show that we were indeed successful in addressing the lobeline pocket. In fact, to the best of our knowledge, our rationally designed ligands are the first class of ligands besides of lobeline that address the lobeline pocket.

Interestingly, the obtained ligands show affinity for AChBPs and the human α7 receptor, while they lack affinity for the α4β2 receptor (**Chapter 3**). This might be related to the differences in accessibility and behavior of the lobeline pocket amongst the AChBPs and the nAChR subtypes. The subtle differences in ligand binding that the AChBPs and the nAChRs display cannot be explained completely. This is also illustrated by the limited success in the studies that were aimed to construct mutant AChBPs to better mimic the α7 and α4β2 receptor binding sites. Clearly more studies are needed to unravel the differences, including cocrystallization studies with selective ligands and additional biophysical characterization. It would be an important step forward if more detailed information of the human nAChRs or other LGICs could be obtained.

Is AChBP a good model for the nAChR orthosteric binding pockets?

It is not straightforward to mimic the binding pocket of the nAChRs by simple mutagenesis of the binding pocket residues of the homologous AChBPs.[3, 32] Key residues lining the Ac-AChBP binding pocket and that are within interaction distance to the ligands, were mutated to their counterparts in the human α7 and α4β2 receptors. In addition, other amino acids that were not lining the binding pocket, but that were in interaction distance to the previously mutated key residues, were also mutated to their counterparts in the nAChRs. These mutations were not sufficient to obtain good mimics of the nAChR binding pockets. This might be related to the influence of loop F, which we did not include in our mutagenesis studies, in subtype-selectivity.[9] Other possible reasons might be electrostatic interactions, architecture and folding of the binding pocket and differences in ligand entrance sites.[3, 32] These should be investigated by further mutagenesis studies of AChBPs and cocrystallization studies in the presence of selective nAChR ligands.

Having identified several shortcomings and complications of AChBP as a mimic of the ECD of LGICs, the present studies also illustrate some unique opportunities for using AChBP. This protein was successfully used in virtual screening campaigns and structure-based hit optimization procedures to yield new ligand chemotypes for the α7 receptor and AChBPs. However, the functional activity on nAChRs and the binding modes for AChBPs were difficult to predict. Mutagenesis studies showed that ligand selectivity was more complex than assumed and probably involved more residues than those lining the binding pocket.

Still AChBP provides some unique opportunities since this protein is easy to crystallize in the presence of selective nAChR ligands and the complexes do give insights in the ligand-protein interactions that occur. Multidisciplinary research combining more sophisticated mutagenesis studies of AChBP, cocrystallization studies with (mutated) AChBPs in the presence of selective ligands, biophysical screening techniques (e.g., SPR), and more advanced computational chemistry calculations (flexible docking, molecular dynamics simulations) could provide more knowledge on the selectivity and functional activity issues of the related nAChRs.

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