1. Research aims

The work described in this thesis focuses on structure- and fragment-based approaches on acetylcholine-binding protein (AChBP). This water-soluble pentameric protein is widely recognized and used as a structural homolog of the ligand-binding domain (LBD) of the nicotinic acetylcholine receptor (nAChR).\(^1,2\) Due to its water-soluble nature, AChBP provides relative easy access to X-ray co-crystal structures enabling structure-based optimization. Moreover, compared to its structural analog, the membrane bound nAChR, AChBP is easier to incorporate in biological assays and better suited for studying ligand-protein interactions with biophysical techniques such as surface plasmon resonance (SPR) biosensor analysis and isothermal titration calorimetry (ITC). For these reasons, we have embarked on a fundamental study in which AChBP is used to increase our knowledge on how to efficiently optimize fragment- and virtual screening hits and in the process gain more understanding on ligand-protein interactions. In addition, we have investigated which techniques are most suitable for monitoring the hit optimization process. While generating new compounds to increase our molecular insights in ligand-AChBP molecular interactions, we also considered translational aspects by comparing our AChBP findings with the therapeutically relevant nicotinic acetylcholine receptors. At the beginning of this project, we set ourselves with the following research aims:

- Increase our knowledge on how to efficiently optimize fragments towards high affinity binders using AChBP as a model protein.
- Investigate if thermodynamic analysis can contribute to a more efficient fragment-optimization process using AChBP as a model protein.
- Investigate if our fragment-optimization studies on AChBP can contribute to our understanding on how to design subtype-selective ligands for the therapeutically relevant human nAChRs.

In this final chapter, the most important findings in this thesis will be summarized and the outcome of the research aims will be evaluated.

2 Efficient fragment hit optimization

The water-soluble nature of AChBP facilitates studying ligand-protein interactions and having x-ray co-crystal structures at hand, we decided to embark on a structure-based optimization project. The aim of this project was to increase our understanding on how to efficiently optimize fragment- and virtual screening hits. Several aspects are known to complicate fragment hit optimization. For example, maintaining the original binding mode of the fragment hit throughout the optimization process is often difficult as the ligand adjusts the orientation of the different substructures to find the pose that is the energetically most favorable pose for the complete molecule.\(^3,4\) At the same time, the protein target can adjust its conformation, leading to induced-fit phenomena.\(^5,6\) The conformational changes of the protein are difficult to predict and most computational tools consider the protein as rigid entities.\(^7\) Accurate first principle (QM) calculations are possible for bigger systems, such as AChBP-fragment complexes but these studies remain computationally too expensive to guide chemistry programs.
Another factor that complicates fragment optimization is that establishing additional interactions with the protein binding site not always translates into higher binding affinities. This is because binding affinity does not correlate directly to the physical forces that drive complex formation (enthalpy) but rather quantifies an average value that also originates from conformational changes of the ligand and its binding partner and changes in their solvation shells (entropy) (Chapter 3). Interestingly, biophysical approaches, such as the well established isothermal titration calorimetry (ITC) and the newer surface plasmon resonance (SPR) biosensor analysis, are able to determine the thermodynamical aspects of interactions, and allows dissection of binding affinity into the separate enthalpic and entropic contributions. Biophysical analysis of molecular recognition events will therefore increase our understanding of ligand-protein interactions and may ultimately lead to a more efficient fragment optimization process. Clearly, structure- and fragment-based approaches can reach a next level of efficiency if the dynamic and thermodynamic aspects of ligand-receptor binding can be addressed.

2.1 Conformational changes induced by fragment growing

By comparison of previously obtained AChBP co-crystal structures with an X-ray structure of lobeline bound to Ac-AChBP, an interesting conformational change of the binding site was observed that leads to the opening of a subpocket, enabling the binding of the α-hydroxyphenethyl moiety of the ligand. This subpocket, referred to as the lobeline-pocket, becomes accessible after a change in rotameric state of Tyr91 (g- to t conformation8, hereafter referred to as the tyrosine-flip). At the time the various crystal structures became available, lobeline was the only ligand known to be able to induce this conformational change in the protein and address this subpocket. Considering the partially overlapping binding modes of fragment 1 and lobeline (2), we designed a fragment growing optimization study to induce the tyrosine-flip and grow the fragment into the lobeline-pocket (Figure 1). This structure-based fragment optimization study that is described in Chapter 4 was found to be an efficient method to increase binding affinity for Ac-AChBP. The compound designed in the first iteration displayed a 50-fold improvement in binding affinity ($pK_i = 7.0$, Table 1) compared to the starting fragment. The successful insertion into the ligand-inducible lobeline-pocket was confirmed by an X-ray co-crystal structure and the experimentally determined binding mode corresponded reasonably well to the predicted binding mode that was obtained by molecular docking (rmsd of 1.1 Å). The most pronounced deviation was that in the experimentally determined binding mode the hydroxyl group of compound (3) was not engaged in hydrogen bonding to the backbone carbonyl oxygen atom of Ser144 or Trp145, but instead was facing the hydrophobic ring of Tyr193 (Figure 2).

Since the hydroxyl group of the optimized fragment 3 is not involved in formation of hydrogen bonds with the binding site, it was expected that its removal would lower the desolvation penalty and thereby result in an increase in binding affinity. Indeed, nor-hydroxyl derivative 4 exhibited higher Ac-AChBP affinity ($pK_i = 7.5$, Table 1) than 3 and when compared with the starting fragment 1, a 150-fold increase in binding affinity was obtained (Figure 4). To determine if optimized fragment 4 induces the tyrosine-flip and interacts with the lobeline-pocket, an additional co-crystal structure was generated that shows an almost identical binding mode to 3.
Figure 1. Fragment optimization strategy. Surface representations of the crystal structures of fragment 1-bound Ac-AChBP (a) and of lobeline-bound Ac-AChBP (PDB 2BYS)\textsuperscript{16} (b). (a) In the fragment 1-Ac-AChBP complex, Tyr91 is stabilized in the g- conformation through a hydrogen bond with Ser144, rendering the lobeline-pocket inaccessible. (b) However, Tyr91 adopts a t conformation in the lobeline-Ac-AChBP complex interacting with Tyr53 and Ser165 through hydrogen bonds, thus leading to the opening of the lobeline-pocket. (c) The superposition of the fragment 1 and lobeline (2) molecules indicates that the fragment may be grown into the lobeline-pocket by extending its tropine nitrogen atom with the α-hydroxyphenethyl moiety of lobeline generating compound (3). A version of this figure in full color is shown on page 63.
Figure 2. Predicted binding mode (light grey-colored sticks) versus the experimentally determined binding mode (dark grey-colored sticks) of optimized fragment 3 (rmsd = 1.1 Å). The orientation of the α-hydroxyl moiety is different than was predicted by molecular docking. Instead of hydrogen bond formation with the carbonyl backbone of Ser144 (predicted hydrogen bonds are shown in black dashed lines), it facing a lipophilic part of the binding site; Tyr193.

(rmsd of 0.6 Å), exemplifying that a hydroxyl functionality is not required for opening of and insertion into the lobeline-pocket.

In the performed hit optimization procedures, we found that that binding affinity for AChBP could be readily increased by the introduction of lipophilic phenethyl moieties. It was also found that optimization by the formation of additional hydrogen bonds with the binding site residues was substantially more difficult. Molecular docking studies had suggested that incorporation of hydroxyl substituents at the α- or meta positions of the N-phenethyl substituents of the dibenzosuberyl as well as the benzoate series could enable the formation of additional hydrogen bonds with the lobeline-pocket (Chapter 5). However, for both series of compounds, introduction of hydroxyl moieties at the α- or meta-positions of the N-phenethyl moiety diminished binding affinity for Ls-AChBP as well as Ac-AChBP (Table 1 and 2). For Ls-AChBP and the dibenzosuberyl substituted tropines, this can be explained by a different binding mode than predicted, i.e., no interaction of the N-phenethyl moiety with the lobeline-pocket (see section 2.2 in this chapter). However, for substituted tropine benzoates 3 and 4, it has been shown by X-ray co-crystal structures that their N-phenethyl moieties were interacting with
Table 1. The binding affinity (pKᵢ) of benzoate substituted tropines

<table>
<thead>
<tr>
<th>Cpd</th>
<th>R₁</th>
<th>R₂</th>
<th>Ls-AChBP pKᵢ ± SEMᵃ</th>
<th>Ac-AChBP pKᵢ ± SEMᵃ</th>
<th>α7 pKᵢ ± SEMᵇ</th>
<th>α4β2 pKᵢ ± SEMᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td></td>
<td></td>
<td>6.5 ± 0.1</td>
<td>5.6 ± 0.1</td>
<td>6.0 ± 0.1</td>
<td>7.9 ± 0.1</td>
</tr>
<tr>
<td>α-lolobeline</td>
<td></td>
<td></td>
<td>6.2 ± 0.1</td>
<td>8.6 ± 0.1</td>
<td>5.1 ± 0.1</td>
<td>8.3 ± 0.1</td>
</tr>
<tr>
<td>1</td>
<td>H</td>
<td>H</td>
<td>6.1 ± 0.1</td>
<td>5.3 ± 0.1</td>
<td>5.5 ± 0.1</td>
<td>&lt; 4.5</td>
</tr>
<tr>
<td>3</td>
<td>OH</td>
<td>H</td>
<td>6.1 ± 0.1</td>
<td>7.0 ± 0.1</td>
<td>4.9 ± 0.1ᵇ</td>
<td>&lt; 4.5ᵇ</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>H</td>
<td>7.1 ± 0.1</td>
<td>7.5 ± 0.1</td>
<td>5.1 ± 0.1</td>
<td>&lt; 4.5</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Me</td>
<td>6.9 ± 0.1</td>
<td>6.1 ± 0.1</td>
<td>5.0 ± 0.1ᵇ</td>
<td>&lt; 4.5ᵇ</td>
</tr>
</tbody>
</table>

a) [3H]epibatidine displacement studies, pH 7.4; b) [3H]MLA displacement studies, pH 7.4; c) tested as a racemic mixture n.d. = not determined; Cpd = compound

the lobeline-pocket in Ac-AChBP (Chapter 4). As discussed in Chapter 3, it can be anticipated that due to desolvation penalties, the positioning of hydrogen-bonding groups needs to be near-optimal in order to be beneficial in terms of binding affinity.⁹,¹⁰ Apparently, upon introduction of one or two meta-hydroxyl groups to benzoate ester 3, the desolvation penalty dominates. This indicates that the positioning of the α-hydroxyphenethyl moiety in the lobeline-pocket does not allow for strong hydrogen bond formation with the carbonyl backbone of Thr89 and/or the side chain of Asp195. Interestingly, ionic and hydrogen bond interactions with Asp195 have been observed for α-conotoxins in complex with Ac-AChBP with Tyr91 in a g- conformation (closed lobeline-pocket, PDB: 2UZ6 and 2BYP), showing that it is possible for ligands to interact with this residue.¹¹,¹²

In these studies that explore structure-activity relationships between different series of compounds, we found the use of Group Efficiency (GE) measurements very useful. For Ls-AChBP, the GE’s of the tropine substituents are almost identical for the dibenzosuberyl and benzoate series (Figure 3). These similar GE’s that were observed for modifications on the tropine nitrogen atom of the dibenzosuberyl- and benzoate derivatives, are indicative of similar binding modes between the two compound series in Ls-AChBP. In contrast, for Ac-AChBP clear GE differences are
Table 2. The binding affinity (pK$_i$) of dibenzosuberyl substituted tropines

<table>
<thead>
<tr>
<th>Cpd</th>
<th>R$_1$</th>
<th>R$_2$</th>
<th>Ls-AChBP pK$_i$ ± SEM$^a$</th>
<th>Ac-AChBP pK$_i$ ± SEM$^a$</th>
<th>α7 pK$_i$ ± SEM$^b$</th>
<th>α4β2 pK$_i$ ± SEM$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td></td>
<td>H</td>
<td>7.0 ± 0.1</td>
<td>5.0 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>&lt; 4.5</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>H</td>
<td>5.7 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>4.7 ± 0.1</td>
<td>&lt; 4.5</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>H</td>
<td>5.7 ± 0.1</td>
<td>4.5 ± 0.1</td>
<td>4.4 ± 0.1</td>
<td>&lt; 4.5</td>
</tr>
</tbody>
</table>

a) [3H]epibatidine displacement studies, pH 7.4; b) [3H]MLA displacement studies, pH 7.4; n.d. = not determined; Cpd = compound

observed when focusing on the tropine substituent, indicative of different binding modes between the two compound series. Since we have provided structural evidence that benzoate ester 4 is interacting with the lobeline-pocket in Ac-AChBP (2Y57.pdb, Chapter 4), a likely explanation for the observed SAR differences between the compound series is that dibenzosuberyl ether 6 is not interacting with the lobeline-pocket. In Ls-AChBP, the N-phenethyl substituents of the dibenzosuberyl- and benzoate- substituted ligands are likely to be accommodated by a (hydrophobic) part of the binding site different than the lobeline pocket.
Figure 3. Calculation of ligand efficiency (LE) and group efficiency (GE) allows easy comparison of and the average affinity contributions per heavy atom of compounds and functional groups of different sizes. Comparison of GE's underlines that there are clear SAR differences between both proteins and the two compound series, indicative of binding mode differences. For each compound, the LE and EE have been calculated in kcal·mol$^{-1}$ per heavy atom. $\Delta G^0$ = Gibbs energy change, and HA = number of heavy atoms.

2.2 Thermodynamic analysis in Fragment-based drug discovery

In Chapter 3, we analyzed a significant number of datasets and studied the information that can be gained from thermodynamic analysis in a fragment-based drug discovery (FBDD) context. The small size of fragments simplifies the process of relating the thermodynamic profile of fragments to the interactions made during binding. When combined with structural data, the stepwise fragment growing process provides an ideal dataset with which to improve our understanding of the thermodynamics of binding. In this manner both FBDD, and our understanding of the thermodynamics of binding, have much to gain from the application of thermodynamic analysis in FBDD.

As exemplified by our studies on AChBP (Chapter 4 and 5), enthalpic optimization by the formation of additional polar interactions with the binding site is a difficult route to improve binding affinity. A poorly optimized polar interaction may not simply provide a lower favorable contribution to the enthalpy change on binding, it may actually decrease binding affinity. Desolvation of polar functionalities comes at a cost, which can only be overcome when the positioning of interacting groups obeys strict angle and distance requirements.$^{10,14}$ Nevertheless, due to the
specificity of polar interactions, enthalpy driven ligand binding may provide a major selectivity advantage. The examples of HIV-protease inhibitors and cholesterol lowering statins illustrate that the best in class compounds had been better optimized with respect to enthalpy compared to the first in class compounds (Chapter 3). While enthalpic optimization can provide highly selective high affinity drugs, medicinal chemists tend to optimize entropy, as shown in a recent study comparing synthetic and natural drugs. Unfortunately, too much focus on entropic optimization by constraining ligands in their bioactive conformation, and by the addition of hydrophobic groups, may in the end result in poorly soluble compounds, with reduced selectivity and higher chances of attrition. Very appropriately, Michael Hann has recently introduced the term “Molecular Obesity” to describe our tendency to build potency into molecules by the inappropriate use of lipophilicity, leading to the premature demise of drug candidates.

Since enthalpic optimization is difficult to achieve, choosing a fragment in which binding is enthalpically driven as a starting point and adding hydrophobic groups during optimization, may provide the easiest route to a high affinity compound with favorable changes in both enthalpy and entropy and a reduced risk of attrition. In this respect, nicotine can be considered a fragment that is better suited for further optimization than the fragment that we optimized in Chapter 4, both in terms of ligand efficiency (LE) and enthalpic efficiency (EE). Since we had not determined the thermodynamic profiles before initiating our fragment optimization study, the thermodynamic profiles of binding were not taken into account upon selecting which fragment to pursue. At that time, fragment 1 was selected as it represented a novel chemotype with affinity for AChBP. Nevertheless, the future synthesis of a hybrid molecule of nicotine and lobeline would be a nice opportunity to challenge the above consideration.

In order to increase our understanding of the thermodynamic aspects of fragment-protein binding, we have constructed a database containing 162 unique ligand-protein complexes in which the molecular weight of the ligand was smaller than 300 Da (Chapter 3). The thermodynamic data that was collected using ITC is summarized in Figure 4a and data that was obtained using van ‘t Hoff analysis is summarized in Figure 4b.

Several trends were observed from the enthalpy-entropy plots depicted in Figure 4. First of all, data obtained using van ‘t Hoff analysis is characterized by a greater amount of entropy driven binding. This is not a trend resulting from the techniques, but rather a trend resulting from the different targets being tested. The van ‘t Hoff experiments were almost exclusively performed on membrane-bound receptors, while the ITC experiments tend to be performed on water-soluble enzymes. The trend of wider distribution of thermodynamic binding signatures in the case of membrane-bound receptors is likely to originate from the conformational changes that are required for receptors to switch between active and inactive states. This explanation is in line with an additional trend that is visible in Figure 4b; the thermodynamic discrimination between agonists and antagonists in many of the tested receptors. This phenomenon, in which agonist binding to a receptor is entropy-driven and the binding of its antagonist is enthalpy-driven, or vice versa, has been observed for different receptor families such as G-protein-coupled
receptors (GPCRs), and ligand-gated ion channels (LGICs).20-27 The phenomenon of thermodynamic discrimination illustrates that fragments that exhibit enthalpy driven complex formation are not always the preferred candidates for further optimization. The preferred thermodynamic profile of the fragment may depend on the target protein being an enzyme, or a receptor, and on what kind of functional profile (antagonist or (partial) agonist) is required to obtain the anticipated therapeutic effect. A third trend that can be observed is that the distribution in Gibbs energy of binding (ΔΔG° = 11 kcal·mol⁻¹) is significantly smaller than the distribution in enthalpy and entropy (ΔΔH° = TΔΔS° = 63 kcal·mol⁻¹). These differences reflect enthalpy-entropy compensation; large favorable changes in enthalpy are compensated by unfavorable changes in entropy, and vice versa.

The analysis described in Chapter 3 also revealed that sometimes fragment growing can result in simultaneous enthalpic and entropic optimization. An example on the adenosine A₁ receptor illustrates that enormous increases in binding affinity (900-fold) can be obtained by the introduction of relative small substituents (cyclopentyl) (Figure 5).21 The unusual simultaneous optimization of enthalpy and entropy by extending the fragment with a cyclopentyl moiety may result from additional flexibility in the ligand-protein complex (favorable ΔS°) from which the loss in enthalpy by enthalpy-entropy compensation is overcompensated by the formation of strong enthalpic van der Waals interactions. An alternative explanation may be that the introduction of the cyclopentyl substituent, displaces water molecules from a hydrophobic subpocket that are not engaged in hydrogen bonds prior to complex formation. As optimization of both thermodynamic binding parameters at the same time is the most efficient way of increasing binding affinity, this specific example warrants further investigation. For example, the generation of X-ray co-crystal structures of the starting fragment and its 8-cyclopentyl substituted derivative may provide insights in how to successfully escape from enthalpy-entropy compensation and thereby optimize compounds in the most efficient manner.
Figure 4: Enthalpy vs. entropy plot of ligand-protein complexes that were obtained using ITC (a) and van 't Hoff analysis (b). The two diagonal lines show Gibbs energy changes of $-4.1 \text{ kcal mol}^{-1}$ and $-12.4 \text{ kcal mol}^{-1}$ equivalent to affinities of 1 mM and 1 nM, respectively. A version of this figure in full color is shown on page 47.
**Figure 5:** Changes in Gibbs energy ($\Delta\Delta G^0$), enthalpy ($\Delta\Delta H^0$) and entropy ($-T\Delta\Delta S^0$) in kcal·mol$^{-1}$ upon growing the fragment theophylline towards the high affinity adenosine A$1$ antagonist CPT. For each compound, the ligand efficiency (LE), enthalpic efficiency (EE) and group efficiency (GE) have been calculated in kcal·mol$^{-1}$ per heavy atom.

Polar contacts are generally associated with enthalpic rather than entropic interactions, and as a consequence, enthalpic binders are likely to be more hydrophilic than entropic binders. One may therefore argue that keeping control of lipophilicity, for example by taking LE as well as logP into account, is likely to be as useful and less complicated than the determination of thermodynamic binding parameters. However, as exemplified in Chapter 3 and 4 this is not always true. In our work that is described in Chapter 4 we have shown that enthalpic optimization can also be established by the incorporation of lipophilic substituents. Moreover, in Chapter 3, a thermodynamic study on carbonic anhydrase is analyzed, that shows that selecting the more enthalpy efficient (12) instead of the most ligand efficient fragment (14) for further optimization from two fragments with similar logP values, may ultimately result in a more potent compound with a better thermodynamic profile (compare 15 and 17, Figure 6). These examples show that, compared to binding affinity data alone, thermodynamic data can provide additional information and can be utilized in a more efficient hit optimization process.
Figure 6: Changes in Gibbs energy ($\Delta G^\circ$), enthalpy ($\Delta H^\circ$) and entropy ($-T\Delta S^\circ$) in kcal·mol$^{-1}$ upon growing benzene sulfonamide (6) towards more potent carbonic anhydrase inhibitors. For each compound, the LE and EE have been calculated in kcal·mol$^{-1}$ per heavy atom.
2.3 Thermodynamic analysis provides novel insights in ligand recognition

Our site-directed mutagenesis work on AChBP suggest differences in accessibility of the lobeline-pocket and therefore indicates that the benzoate esters 3 and 4 are likely to exhibit different binding modes between Ac- and Ls-AChBP. Nevertheless, ligand 4 binds with similar affinity to Ac-AChBP and Ls-AChBP. The affinity data therefore do not provide any indication for a difference in binding modes. However, the thermodynamic analysis that is described in Chapter 4, reveals significant differences that are indicative of different binding modes between both protein species (Figure 7). We therefore conclude that more than affinity data alone, dissection of binding affinity into the separate enthalpic and entropic contributions provides valuable information with regards to the binding mode of a ligand and has therefore a high added value in structure- and fragment-based hit optimization.

Another interesting observation from the thermodynamic analysis experiments in Chapter 4 is that the favorable change in enthalpy upon growing fragment 1 into the lobeline-pocket is partially compensated by an entropic penalty, illustrative of the phenomenon known as enthalpy-entropy compensation. In our case, it is likely that the favorable enthalpic interactions that are made upon growing into the lobeline-pocket, restrict the ligand as well as the protein binding site in their conformational flexibility, resulting in the observed entropic penalty. Furthermore, comparing the thermodynamic profiles of binding to Ac-AChBP of optimized fragments 3 and 4 shows that binding of 4 is driven by more enthalpic contributions than its hydroxyl substituted analog 3 (Chapter 4). This difference in thermodynamic binding profiles is likely to originate from a desolvation penalty for 3 from which its hydroxyl group is not engaged in hydrogen bonds with the AChBP binding site (Figure 2).

Inspection of the co-crystal structures of lobeline and optimized fragment 4 with Ac-AChBP reveals that lobeline is involved in four hydrogen bonds with the Ac-AChBP binding site whereas 4 only forms one hydrogen bond. Therefore, one would expect that the binding of lobeline that is driven by extensive polar interactions would be characterized by more favorable changes in enthalpy than 4. Surprisingly, this is not the case (Chapter 4). This moderate enthalpy driven binding of lobeline to Ac-AChBP may be explained by a more flexible lobeline-Ac-AChBP complex compared to the compound 3-Ac-AChBP complex, reducing enthalpic contributions but also preventing a significant entropic penalty. Accordingly, lobeline can be considered a more flexible molecule than 4 as it contains more rotatable bonds and the center of the molecule consists of a flexible piperidine ring instead of a rigid bicyclic tropane moiety. As a consequence, lobeline may be better able to adopt to the dynamic movements of the Ac-AChBP binding site (low entropic penalty) whereas the more rigid compound 4 locks the protein in a minimal ensemble of conformations (high entropic penalty). As lobeline binds to Ac-AChBP with higher affinity than 4, these results are in line with the hypothesis postulated by Williams and Whitesides that a so-called 'sloppy' fit, one that is sufficiently complementary in shape to allow some favorable enthalpy, but loose enough not to be entropically too unfavorable may result in the strongest associations.29,30
Figure 7. Thermodynamic analysis provides indication of successful insertion into the lobeline-pocket. Chemical structures and binding modes as determined by X-ray analysis of Ac-AChBP co-crystal structures and the thermodynamic binding signatures for Ac-AChBP and Ls-AChBP of optimized fragment 4 (a) fragment 1 (b) and quaternary ammonium derivative 5 (c) as determined by SPR biosensor analysis (dark bars ± SEM) and ITC (light bars ± fitting errors). Shown are the changes that occur upon ligand binding in Gibbs energy (ΔG°) (SPR: dark blue; ITC: light blue), enthalpy (ΔH°) (SPR: dark green; ITC: light green) and entropic contributions (-TΔS°) (SPR: dark red; ITC: light red). All thermodynamic parameters shown are in kcal·mol⁻¹. A version of this figure in full color is shown on page 72.
The obtained thermodynamic results also show that the fundamentally different biophysical techniques van ‘t Hoff analysis (by SPR) and ITC result in very comparable thermodynamic binding profiles (Figure 8). Previously, discrepancies between calorimetry and van ‘t Hoff analysis have been reported, but our current work and other recent comparison studies show that application of both techniques result in similar values for the thermodynamic binding parameters (Chapter 3 and 4), at least when using these protein targets and sets of compounds. When comparing both techniques, the main advantages of ITC experiments are that set-up is straightforward, as calorimetric measurements do not require immobilization or labeling. Furthermore, all binding parameters ($n$, $K_B$, $\Delta H^0$ and $\Delta S^0$) are determined in a single experiment. A major disadvantage is the large amounts of protein needed for a single experiment. If the target protein is more difficult to obtain, SPR biosensor analysis is a more sensible technique to use, as the protein consumption is very low. SPR biosensor analysis has an additional advantage of being able to provide information about the kinetic parameters of binding ($k_{on}$ and $k_{off}$). This enables combining kinetic and thermodynamic data providing insights into transition state thermodynamics, e.g., enthalpy of association. Unfortunately, both ITC- and SPR biosensor-based thermodynamic analysis are hampered by low throughput and are therefore not suitable for primary screening purposes. Instead, these techniques are more appropriate for secondary screening and are excellent ways for hit validation and to obtain more information about the process of binding by characterization of the thermodynamic binding profile.

3 AChBP: A close mimic or far homolog to the nAChR LBD?

The first chapter provides an introduction to the nAChRs and their water-soluble LBD structural homolog, AChBP. In Chapter 1, various studies are summarized that exemplify how AChBP X-ray structures have significantly improved our understanding on the overall molecular structure of the nAChR LBD and its molecular interactions with nicotinic ligands. Particularly, the validation of AChBP-derived hypotheses by site-directed mutagenesis with unnatural amino acids in nAChRs is a powerful method to increase our understanding on the nAChR LBD. Examples of insights in the interactions between ligands and the nicotinic receptors that have been obtained using this strategy include; the formation of cation-π interactions of cationic ligands with specific aromatic residues within the binding pocket, the formation of charged hydrogen bonds with the carbonyl backbone oxygen atom of Trp145 (Ls-AChBP numbering), and the formation of a hydrogen bond between the pyridine nitrogen atom of nicotine and a conserved water molecule (Figure 8).

An important issue that has been raised in this thesis, is the role of amino acid residues located outside the first shell of binding site residues in molecular recognition processes. It is tempting to focus solely on residues directly aligning the protein binding site but site-directed mutagenesis work on AChBP (see also Chapter 4) as well as nAChRs illustrate that in certain cases amino acid residues in the second or third shell should be considered as well. Structural comparisons between AChBPs originating from different species revealed differences in stabilization of the ligand-induced tyrosine-flip. Site-directed mutagenesis
experiments were performed in which the tyrosine-flip stabilizing residues in Ac-AChBP were substituted for their Ls-AChBP counterparts (Figure 9). The results strongly indicate that the side chain of a serine residue (Ser165) is essential for stabilizing the tyrosine-flip and that the lobeline-pocket is less accessible in Ls-AChBP compared to Ac-AChBP. It is worth mentioning that the residues that putatively stabilize the tyrosine-flip are not directly aligning the binding site (i.e., are not in contact with ligands) and the obtained results therefore underline the important role that residues outside the first shell of binding site residues may play in ligand recognition.

In line with the site-directed mutagenesis results is the affinity data that is depicted in Table 1. Ligands that are known to interact with the lobeline-pocket in Ac-AChBP (2, 3 and 4) have higher affinity for Ac-AChBP whereas ligands that do not interact with the lobeline-pocket (nicotine, 1 and 5) have higher affinity for Ls-AChBP. Thus, our investigations reveal that growing the fragment 1 into the lobeline-pocket shifts the species preference towards Ac-AChBP whereas introduction of an additional methyl substituent preventing interactions with the lobeline-pocket reverses the preference back to Ls-AChBP. These subtle differences in protein conformational changes that induce the lobeline-pocket may be of interest in the design of subtype-selective ligands for human nicotinic receptors as well. The gatekeeper tyrosine residue is conserved amongst the human nAChR subtypes whereas the residue (Ser165 in Ac-AChBP) that stabilizes the open lobeline-pocket conformation is located in a highly variable region (Chapter 1). As can be seen in Table 1, lobeline exerts a 250-fold selectivity for Ac-AChBP (accessible lobeline-pocket) over Ls-AChBP (inaccessible lobeline-pocket) and ~1000-fold selectivity for α4β2 over α7 nAChRs. Differential stabilization of the rotameric states of the gatekeeper tyrosine residue may provide an explanation for the observed nAChR subtype selectivity of lobeline and the lobeline-pocket may therefore be targeted as a nAChR subtype-selectivity pocket, as well. Alas, our initial site-directed mutagenesis studies that were aimed to validate this hypothesis give ambiguous results (unpublished results), indicating that other differences in the ligand binding pockets may play a complementary role. Additional experiments using site-directed mutagenesis and/or molecular probes are therefore required to determine if the lobeline-pocket may be of use in addressing nAChR subtype selectivity.

Another example of the important role that amino acid outside the first shell of amino acid residues aligning the binding site may play in nAChR subtype selectivity was already discussed in Chapter 1. A backbone mediated hydrogen bond between two residues in the second shell is likely to be responsible for shaping the α4β2 nAChR binding site in such a way that nicotine establishes a very strong cation-π interaction with a tryptophan residue (Figure 8). These findings are in line with the selectivity of nicotine for the α4β2 over the α7 and muscle nAChR subtypes.
Figure 8: X-ray structure of Ls-AChBP in complex with nicotine (PDB: 1UW6) shows that the formation of a cation-π interaction between the charged pyrolidine nitrogen atom and the aromatic side chain of Trp143 is a major contributor to complex formation. In addition, two hydrogen bonds (black dashed lines) with the AChBP binding site are made upon binding. A charged hydrogen bond is established between the positively charged pyrolidine nitrogen atom of nicotine and the backbone carbonyl oxygen atom of Trp143 and the pyridine nitrogen atom is involved in hydrogen bond formation with a conserved water molecule. The residues Ser147 and Ala191 are engaged in a backbone mediated hydrogen bond (black dashed line indicated by the white arrow) between Ser147 and Ala191. Combining the results of molecular dynamics and site-directed mutagenesis experiments indicates that compared to the muscle-type (Ser147 = Gly175) and α7 nAChR (Ser147 = Gly148), this hydrogen bond is much stronger in the α4β2 nAChR (Ser147 = Lys150), shaping the binding pocket in such a way that nicotine establishes a very strong cation-π interaction with Trp143.
**Figure 9.** AChBP species differences in stabilization of the tyrosine-flip. The superposition of crystal structures of Ac-AChBP (in light grey) and Ls-AChBP (in dark grey) suggests that Trp53 and Tyr164 in Ls-AChBP (represented as blue sticks) cannot stabilize the tyrosine-flip contrary to Tyr53 and Ser165 in Ac-AChBP. A colored version of this figure is shown on page 71.

It should be noted that due to its moderate overall sequence identity (24% with the human α7 nAChR), AChBP cannot be considered an exact water-soluble mimic of the nicotinic receptor LBD. Some of the work that is discussed in this thesis, exemplifies the limitations of AChBP in providing a structural template for nAChRs. Previously, using a virtual screening exercise based on AChBP X-ray structures, we have found that only a limited amount of AChBP derived in silico hits displays affinity for the α7 nAChR and none of the hits bind to the less homologous α4β2 nAChR LBD. In our current work, we have found that ligands that have been optimized for AChBP do not display affinity for the α4β2 nAChR subtype and that the SAR that was identified for AChBP often does not correlate with SAR for the α7 nAChRs (Chapter 5 and Table 1 in this chapter). Nevertheless, until structures of nAChR LBDs become available, AChBP can be considered a valuable tool to elucidate general features of the molecular structure of nicotinic receptors. However, when using AChBP as a structural template for nAChRs, one should be aware of its limitations and validate AChBP-derived hypotheses in the membrane bound nicotinic receptors.

Obviously, an alternative to using AChBP directly for nAChRs research is to use the structural AChBP data for homology modeling. However, homology models often have small inaccuracies that compromise the success of the computational approaches. More importantly for the studies described in this thesis is that we would have lost the ability to validate and guide the fragment- and structure-based approaches by using the water soluble AChBP that allows structural and
biophysical evaluation next to determining binding affinities of the reference and the novel ligands. In an effort to combine the best of both worlds (i.e., nAChR ligand binding characteristics and water soluble AChBP properties), mutant AChBP proteins are being constructed and tested in our labs that are designed to better mimic the human nAChRs, but until now the results have been ambiguous when evaluating panels of reference nAChRs ligands (unpublished results). Until better nAChRs mimics are available, the direct use of AChBP as bait for nAChRs remains problematic. Ultimately, it can be anticipated that LBD or full length receptors of the nAChR subtypes themselves can be crystallized. A similar revolution is now taking place for the other membrane-bound receptors that are often targeted in drug development programs as in recent months several structures for G-protein coupled receptors have been elucidated. At that time when more detailed structural information for human nAChRs becomes available, the computational protocols and approaches described in this thesis can be revisited by replacing the AChBP structural information with the nAChRs data.

4 Fragment optimization towards dual-action NSAID ester prodrugs

In the final chapter of this thesis, we have used crystal structure data of acetylcholine-binding protein (AChBP), to design NSAID ester prodrugs that are capable of activating α7 nicotinic acetylcholine receptors (nAChRs) (Chapter 6). The α7 nAChR is expressed on macrophages and other immune cells and has recently been identified as a target for treating inflammation-related disorders. Thus, by incorporating α7 nAChR activity, we hope to obtain dual-action anti-inflammatory NSAID prodrugs. After suppressing inflammatory disorders via the α7 nicotinic receptor, hydrolysis of the prodrug will release the parent NSAID that will exert an additional anti-inflammatory effect by acting on COX-2. In chapter 4 and 5, we have described a fragment (1, Chapter 4) that exhibits good ligand efficiency (LE = 0.43) for AChBP, as well as for the α7 nicotinic receptor (LE = 0.44, Chapter 5). This fragment is an ester of benzoic acid and nortropinol. Since the fragment displays affinity for the α7 receptor and the benzoic acid part exhibits high structural resemblance to certain NSAIDs or structural fragments of NSAIDs, we investigated if esterification of NSAIDs with tropine-like moieties would afford NSAID prodrugs with intrinsic α7 nAChR activity, see Figure 10.

To select which NSAIDs are most suitable for modification towards α7 nAChR activation we have used molecular docking and X-ray structures of acetylcholine-binding protein (AChBP) in complex with (partial) agonists for the α7 nAChRs. Although our later work has given indications that AChBP is compromised for use in structure-based optimization of ligands for the human nAChRs, at that time it was anticipated that AChBP can still be used to discriminate binders from non-binders of the α7 nicotinic receptors and therefore useful in designing the NSAID ester prodrugs. Based on the in silico results, a selection of NSAIDs was chemically modified with tropine moieties to obtain ester prodrugs with potential α7 nAChR activity.
Figure 10: Fragment VUF10663 and the 5 NSAID derivatives that were selected in order to obtain NSAID prodrugs with an additional α7 nAChR mediated anti-inflammatory effect.

Radioligand displacement studies showed that all designed NSAID tropinyl esters exhibit affinity for the α7 nAChR (pKᵢ > 4.8). Using electrophysiological recordings from Xenopus leavis oocytes expressing the human α7 nAChR, two salicylate tropine esters were identified that acted as α7 partial agonists and induce maximal currents of 7-10% compared to the maximal current that is induced by the endogenous ligand acetylcholine. It is not known how much receptor activation is required for an α7 nicotinic partial agonist to reduce the release of proinflammatory mediators from macrophages in vivo. This minimal efficacy is likely to be dependent on the frequency of acetylcholine release near macrophages by the “cholinergic anti-inflammatory pathway”. When this frequency is relatively high, a low efficacious agonist that is bound to the receptor, prevents acetylcholine from activating the α7 receptor and may not lower but instead increase the release of proinflammatory mediators from immune cells. On the other hand, if the frequency of “cholinergic anti-inflammatory pathway” stimulation is low, which is likely to be the case in chronic inflammatory diseases, a low efficacious agonist will still be able to lower the release of proinflammatory cytokines from macrophages and other immune cells.

Having identified esters of salicylic acid that are capable of activating the α7 nAChR, we subsequently investigated if the ester linkage is likely to be hydrolyzed in vivo. We found that the salicylate ester prodrug is likely to be present long enough to exert its effect on the α7 nicotinic receptor but eventually will be hydrolyzed to release salicylic acid producing an additional anti-inflammatory effect by affecting COX-2. Thus, the salicylate ester prodrugs are likely to exhibit a dual anti-inflammatory action combined with an improved GI toxicity profile. At the moment of finalizing this thesis, we are performing in vitro experiments to determine if the dual targeting of α7 nAChRS and COX-2 translates into an improved anti-inflammatory action compared to the parent NSAID. Ultimately, the pharmacokinetic properties of the compounds including hydrolysis of the ester bond, key for the COX-2 activity, needs to be evaluated in vivo.
5 Conclusion
The work described in this thesis exemplifies that AChBP is a very useful tool to study fragment- and structure-based optimization procedures. One of our research aims was to increase our knowledge on how to efficiently optimize fragments. Using an X-ray co-crystal structure of a fragment in complex with AChBP, an efficient fragment hit optimization was established. The obtained results show that fragment merging can be a very efficient method for increasing binding affinity. Furthermore, the work described in this thesis demonstrates that anticipating on ligand-induced conformational changes of the protein binding site can be of great value when optimizing fragment hits. The second research aim was to determine if thermodynamic analysis can contribute to a more efficient fragment-optimization process. Our findings exhibit that the monitoring of the optimization process by thermodynamic analysis provided novel insights into molecular recognition principles and in combination with detailed structural information (X-ray of co-crystals), thermodynamic data provides crucial insights that can enable efficient fragment optimization.

In addition, our work underlines the importance in ligand recognition of amino acid residues that are outside of the first shell aligning the binding site. Our work also suggests that targeting ligand-inducible subpockets may be a fruitful strategy to obtain selectivity between highly homologous proteins. These findings may be of relevance to the human nicotinic receptors, as well, as sequence alignments indicate that the lobeline-pocket can be addressed to obtain selectivity between the human nAChR subtypes. As such, the third research aim that we set ourselves with at the beginning of this research has also been met. Although, not finalized yet, our AChBP-related findings concerning the lobeline pocket may be translated to the therapeutically relevant human nAChRs. Furthermore, the work that is described in this thesis has led to the development of novel potential dual-action anti-inflammatory compounds that are capable of activating \( \alpha_7 \) nicotinic receptors. These dual action NSAID ester prodrugs may have clinical benefit over traditional NSAIDs in treating chronic inflammatory disorders such as rheumatoid arthritis.

6 References


