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# Inhibitor discovery of phospholipases and N-acyltransferases

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# 6

## Summary and future prospects

## 6.1 Summary

In **Chapter 1**, the endocannabinoid anandamide (*N*-arachidonoylethanolamine, AEA) is described as an endogenous ligand capable of activating the cannabinoid receptors. A part from AEA, several other structurally related *N*-Acylethanolamines (NAEs), such as Palmitoylethanolamide (PEA), Oleoylethanolamide (OEA), Stearoylethanolamide (SEA) and Docosahexaenoylethanolamide (DHEA), and their biosynthetic pathways were introduced. *N*-acyltransferases (NAT) perform the first rate-limiting step in the biosynthesis of NAEs. There are two classes of NATs: $Ca^{2+}$-dependent NAT (Ca-NAT) and $Ca^{2+}$-independent NATs (PLAAT1-5).[1] Recently, Cravatt and colleagues showed that PLA2G4E (also known as cPLA$_2\varepsilon$) is a Ca-NAT and transfers an acyl chain from the *sn*-1 position of phosphatidylcholine (PC) to the amine of phosphatidylethanolamine (PE), thereby effectively producing NAPEs.[2] PLA2G4E belongs to the family of cytosolic phospholipase A2 group IV (PLA2G4) proteins, of which there are six members (PLA2G4A, PLA2G4B, PLA2G4C, PLA2G4D, PLA2G4E and PLA2G4F). In contrast to the other family members, PLA2G4E has a strong preference for catalyzing the *N*-acyltransferase reaction over phospholipid hydrolysis.

The human phospholipase A/acyl transferase (PLAAT1-5) family consists of five members (namely, PLA/AT 1-5) of which two are absent in rodents (i.e. PLAAT2 and PLAAT4).[3, 4] They are the protein products of the *Hrasls* genes.[5] PLAATs possess $Ca^{2+}$ independent phospholipase activity *in vitro* with both phosphatidylcholine (PC) and phosphatidylethanolamine (PE) acting as substrates. All members also exhibit O-acyl transferase activity with preference for the *sn*-1 position of lysophosphatidylcholine (lyso-PC) as well as *N*-acyltransferase activity with the ability to produce N-acylphosphatidylethanolamines (NAPEs) through catalysis of the acyl chain transfer from the *sn*-1 position of glycerophospholipids to the amine function of PE.[4] All enzymes, except PLAAT3, show a preference for PLA1 activity over PLA2 activity. Depending on the assay conditions the substrate preference of PLAAT3 may shift.

Taken together, PLAAT and PLA2G4E are involved in the main biosynthetic pathways of NAEs, which make them highly interesting drug targets when there is a need to manipulate the level of NAEs in a disease-related situation.[6-8] The aim of the research described in this thesis focused on the development of activity-based protein profiling assays to identify inhibitors for these enzymes.

In **Chapter 2**, activity-based probe (ABP) MB064, previously developed for monitoring diacylglycerol lipase-α/β activity, was used to profile the activity of PLAAT3 enzyme. MB064 was able to label endogenous PLAAT3 in an activity-dependent manner in brown and white adipose tissue. A small focused library of 50 lipase inhibitors was screen at 10 μM in a competitive ABPP format using recombinant overexpressed protein to discover inhibitors for PLAAT3. This resulted in the discovery of an α-ketoamide (compound **1**) that almost completely inhibited the

activity of PLAAT3 at 10 µM. Compound **1** inhibited also other members of PLAAT family, but was selective over other serine hydrolases.

In **Chapter 3** the hit optimization and structure activity relationships of the α-ketoamide inhibitors are described. It was shown that the α-keto and the substituent on the amide group were crucial for the inhibitory activity. The 3-phenylpropanyl moiety (as depicted in compound **37**) was found to be the optimal fragment as the α-keto subsituent. Compounds **49** and **50** were potent and selective inhibitors for PLAAT5, whereas compound **48** and **60** (also named LEI110) were identified as potent and selective inhibitors for PLAAT3 and 5.

In **Chapter 4** the biological profiling of LEI110 is described. LEI110 was found to be a potent, selective and cell permeable inhibitor of PLAAT3. It reduced cellular arachidonic acid levels in PLAAT3 overexpressing U2OS cells and oleic acid-induced steatosis in human HepG2 cells. To gain insight in the molecular interactions of α-ketoamides with PLAAT3, LEI110 and **1** were docked in a PLAAT3 crystal structure. LEI110 and **1** were covalently attached to Cys113 and a molecular dynamics simulation was performed. It was observed that the oxyanion could form a hydrogen bond with His23, as well as π-π stacking with Tyr21. It is anticipated that LEI110 constitutes an excellent starting point for the structure-based drug development of novel molecular therapies for obesity and/or common cold.

**Chapter 5** reports on the development of an ABPP assay to profile the activity of PLA2G4E with TAMRA-FP. A library of more than 200 compounds was screened at 10 µM for human PLA2G4E. Eight inhibitors showed less than 20% residual activity of human PLA2G4E. ESC386 and ESC387 were the most potent inhibitors with $pIC_{50}$ values of 6.2 ± 0.1 and 6.1 ± 0.1, respectively. These compounds represent the first-in-class inhibitors of PLA2G4E and form an excellent starting point for further probe development.

**6.2 Future prospects.**

Although the competitive gel-based APPP assay was successful in the identification of novel inhibitors for PLAAT2-5[9] and PLA2G4E, it cannot be used for high throughput screening. In a classical competitive ABPP assay the workflow is labor intensive and requires the separation of proteins on SDS-PAGE, which is not compatible with an HTS-assay.[10] Therefore, there is an urgent need to develop plate-based assays for high-throughput screening. One option would be to use a fluorescence polarization assay, which has been widely used in the small molecular screening and drug development.[11] Fluorescence polarization activity-based protein profiling (FluoPol-ABPP) was first developed by Cravatt and co-workers to screen for inhibitors of uncharacterized enzymes.[12] The ABP, which contains a small fluorophore, rotates quickly and emit depolarized light when it does not bind to the target protein. Once bound to the target protein, the probe rotates slowly and emits polarized light. This technology was

introduced to address the limitation of traditional competitive ABPP methods. This assay format was also recently applied to identify inhibitors for human non-lysosomal glucosylceramidase (GBA2).[13] In these FluoPol-ABPP studies, tetramethylrhodamine (TMR) was used as a fluorescent dye. MB064 contains a BODIPY-dye, which is very lipophilic and not compatible with screening membrane proteins, such as the PLA2G4E and PLAATs, in solution. Therefore, it would be of interest to synthesize a new probe (Figure 1B) in which the BODIPY is replaced by TMR, to be used in FluoPol-ABPP assays. To determine the selectivity profile of the hits, it is advised to perform competitive ABPP with brain membrane and soluble proteomes with the classical gel-based format.

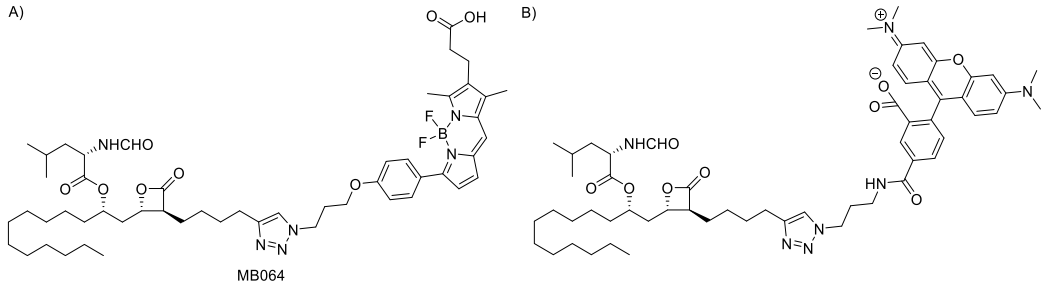


**Figure 1**. (A) Chemical structure of probe MB064; (B) Proposed new probe for FluoPol-ABPP.

Furthermore, there is still a need for more potent and selective inhibitors for each of the individual PLAAT proteins.[9] Since the crystal structure of PLAAT3 has been reported,[14] and the docking study of LEI-110 captured the structure activity relationship of the alpha-ketoamide series, one could use structure-based drug design and homology models of the other PLAATs to develop new and more potent and selective inhibitors for PLAAT3 and PLAAT5.

Alternatively, targeting an allosteric site at a protein represents a novel strategy in drug development to generate more selective compounds.[15-17] Proteins from the same family usually share a highly conserved catalytic domain, which makes it very difficult to generate selective inhibitors when they target the active site.[18] However, allosteric binding sites may be more diverse among proteins within the same family. PLAAT1-5 also share a highly conserved catalytic domain and the current inhibitors target the active site, therefore it would be interesting to investigate with structure-based methods whether potential allosteric sites in PLAATs exist and can be exploited for drug discovery purposes.

With potent inhibitor LEI110 in hand, the biological consequence of inhibiting PLAAT in cells and animal models can be studied in further detail. It has been shown that the dysregulation of the endocannabinoid system (ECS) is associated with the progress of

gynecological disorders and cancer.[7] For example, in ovarian cancers, AEA was found in the follicular fluid after ovarian stimulation by hormones.[19] So it would be interesting to treat ovarian cancer cells with LEI110 and study the maturation of follicles and oocytes. It is also unknown whether cellular NAPEs and NAEs are affected by LEI110 treatment. An activity-based probe can also be designed based on LEI110 (Figure 2). This probe can be applied to visualized the PLAAT activity both in cell lysates and living cells.

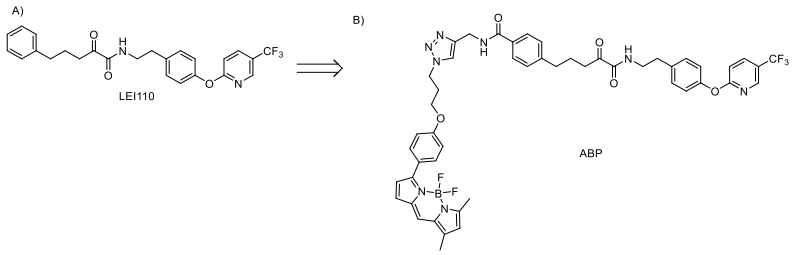


**Figure 2**. (A) Chemical structure of probe LEI110; (B) Proposed activity-based probe.

In summary, in this thesis an activity-based probe was discovered that could visualize the activity of PLAATs. With an optimized gel-based ABPP assay in hand, screening of a compound library led to the discovery of alpha-ketoamides as a hit for PLAAT3. Through extensive structural modifications of the hit, LEI110 was identified as the most potent inhibitor ($K_i$ = 20nM) for PLAAT3. LEI110 reduced cellular arachidonic acid levels in PLAAT3 overexpressing U2OS cells and oleic acid-induced steatosis in human HepG2 cells. Gel-based ABPP and chemical proteomics showed that LEI110 is a selective pan-inhibitor of the *Hrasls*-family of thiol hydrolases (i.e. PLAAT2, PLAAT3 and PLAAT5). LEI110 could be an excellent starting point for the structure-based drug development of novel molecular therapies for obesity and/or common cold. In addition, a competitive, gel-based ABPP method for PLA2G4E using TAMRA-FP was successfully developed and applied to screen a focused library of lipase inhibitors. This resulted in the discovery of two clusters of inhibitors with different scaffolds. Optimization of the potency and selectivity of the inhibitors is required to the study of the biological role of PLA2G4E in an acute and dynamic setting with these novel tools. Together these novel chemical tools and methods will allow for a better understanding of the biosynthesis of the NAPEs and to study their biological role.

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