# Discovery of novel inhibitors to investigate diacylglycerol lipases and α/β hydrolase domain 16A

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# CHAPTER 9

## Summary and future prospects

This Thesis reports on the discovery and optimization of potent inhibitors for the serine hydrolases *sn*-1 diacylglycerol lipase α (DAGLα) and α/β hydrolase domain 16A (ABHD16A). Several structure- and ligand-based drug discovery methodologies were employed in combination with activity-based protein profiling (ABPP).

DAGLs are multidomain membrane proteins belonging to the large family of serine hydrolases. They contain a typical α/β hydrolase fold and employ a Ser-His-Asp catalytic triad for specific hydrolysis of *sn*-1 fatty acid chains of arachidonate containing 1,2-diacylglycerols. As such, DAGLs are key proteins involved in the formation of 2-monoacylglycerols, including 2-arachidonoylglycerol (2-AG, see Figure 1). The two DAGL isoforms (α and β) share extensive homology and differ mostly in a large C-terminal tail, which is present in DAGLα, but not in DAGLβ.[1] Genetic disruption of DAGLα in mice results in a strong reduction of 2-AG levels in the brain (80-90%), whereas in DAGLβ$^{-/-}$ mice the 2-AG level is approximately 50% reduced in the brain.[2,3] As one of the two major endocannabinoids, 2-AG contributes to cannabinoid type 1 receptor (CB1R) mediated synaptic plasticity and acts as a retrograde messenger inhibiting GABAergic and glutamatergic neurotransmission.[2,3]

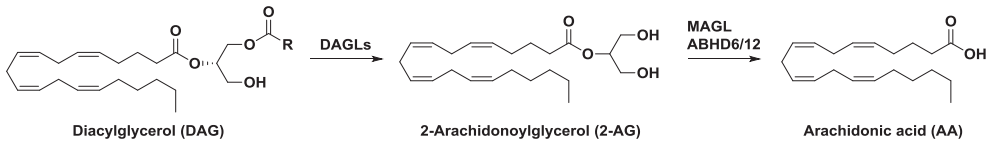



**Figure 1.** Diacylglycerol (DAG) is a substrate for *sn*-1 specific diacylglycerol lipases α and β (DAGLs) which produce the endocannabinoid 2-arachidonoylglycerol (2-AG), a ligand for the cannabinoid receptors type 1 and 2 (CB1R and CB2R).[1] 2-AG is degraded by several enzymes including monoacylglycerol lipase (MAGL), α/β hydrolase domain 6 and 12 (ABHD6 and ABHD12) to arachidonic acid (AA), which serves as a precursor for the formation of several distinct eicosanoids, such as proinflammatory prostaglandins.

The CBRs are involved in many physiological functions, including food intake,[4–6] inflammation,[7,8] memory formation,[9–11] mood,[12,13] locomotor acivity,[14,15] pain sensation,[16] addiction and reward.[17] In fact, the endocannabinoid system is a clinically proven signaling

pathway controlling the energy balance in humans. The first generation CB1R antagonist/inverse agonist Rimonabant was considered one of the most promising therapeutic drugs to treat human obesity, until the appearance of central psychiatric side effects resulted in its removal from the market in 2008.[18–20] Rimonabant reduces food intake, body weight and waist circumference in obese patients and improves cardiovascular risk factors.[18,19,21] Currently, several lines of evidence suggest that 2-AG, and not anandamide (nor constitutively active CB1Rs), regulates CB1R-dependent food intake. 2-AG levels are increased in the hypothalamus of fasting mice[5] and pharmacological intervention leads to reduced food intake in mice.[22] Third, DAGLα$^{-/-}$ mice showed hypophagia and leanness similar to that of CB1R$^{-/-}$ mice, while knockout mice of DAGLβ and *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD, the main enzyme responsible for anandamide synthesis) did not share this phenotype.[23,24] Interestingly, DAGLα knockout mice also had low fasting insulin, triglyceride, and total cholesterol levels, and after glucose challenge had normal glucose but very low insulin levels.[24] Taken together, this data suggests that selective interference with DAGLα signaling represents a novel therapeutic avenue to treat obesity and the metabolic syndrome.

Inflammatory processes are associated with obesity and with many neurodegenerative diseases, including stroke, Parkinson's and Alzheimer's disease.[25] Prostaglandins produced by cyclooxygenases from arachidonic acid (AA) are important pro-inflammatory stimuli. Cyclooxygenase inhibitors show neuroprotection in animal models of Parkinson's and Alzheimer's disease, but their gastrointestinal and cardiovascular actions have limited their use in humans.[26] Nomura *et al.* discovered that monoacylglycerol lipase (MAGL) regulates AA levels in specific tissues, which is required for prostaglandin synthesis by cyclooxygenases type 1 and 2 (COX1 and COX2).[27] For instance, MAGL is the predominant enzyme producing AA in the brain, liver and lung, whereas phospholipase A2 (PLA2) regulates AA levels in the gut and spleen. Inhibition of MAGL activity in LPS-treated mice resulted in an attenuated neuroinflammatory response as witnessed by a marked decrease in pro-inflammatory prostaglandins and cytokine formation in the brain. MAGL inhibitors improved neurological outcome in animal models of Multiple Sclerosis,[28] Parkinson's[27,29] and Alzheimer disease.[30] Of note, CB1R activation by elevated 2-AG levels did not seem to be involved in the protective response. Concomitant chronic activation of the CB1R by elevated 2-AG levels has previously been shown to lead to adaptations of the endocannabinoid system (*e.g.,* downregulation of CB1R and physical dependence).[31] It is currently unknown how elevated 2-AG levels will impact CB1R-mediated signaling under chronic neurodegenerative conditions. Therefore, DAGL inhibition may provide an alternative approach to reduce AA formation in the brain without accumulation of 2-AG and (chronic) CB1R activation. However, pharmacological characterization of DAGLs has long been hampered due to the lack of multi-well activity assays and potent and selective DAGL inhibitors. Several small molecule DAGL inhibitors have been reported, yet most lack selectivity, *in vivo* activity or pharmacokinetic properties to act as drug candidates, or to

study the role of 2-AG. Thus, there is an unmet need to identify novel chemotypes to modulate DAGL activity. Throughout this Thesis, five DAGL chemotypes are reported, the glycine sulfonamides and newly discovered α-ketoheterocycles, α-keto amides, β-keto difluoroamides and sulfonyl 1,2,4-triazole ureas.

**α-Keto heterocycles** were discovered as potent DAGLα inhibitors in **Chapter 2** by ligand-based pharmacophore screening. Two pharmacophore models were constructed based on a known previously published bioactive conformation of a non-selective DAGLα inhibitor, tetrahydrolipstatin (THL). *In silico* screening of a focused library of 16 lipase and endocannabinoid system associated inhibitors identified LEI103, an oxadiazolone and LEI104, an α-keto heterocycle (**1**, Table 1), as two high ranking binding poses in both models. The potency of LEI103 and LEI104 was confirmed in a DAGLα surrogate substrate activity assay using PNP-butyrate. This assay showed that, of both DAGLα inhibitors, LEI104 is most potent. To determine a potential binding mode, a homology model was constructed based on a previously reported co-crystal structure which was used as template. The DAGLα model contains an α/β hydrolase fold and well-aligned catalytic triad consisting of the previously reported residues. LEI104 was covalently docked, forming a hemiketal adduct and subsequently optimized by molecular dynamics (MD) in the presence of the model. MD simulations indicated that the formed oxyanion is stabilized by hydrogen bonding interactions with Thr400 OH, backbone NH and Leu473 backbone NH (the 'oxyanion hole'). The binding mode via the ketone is supported by the alcohol precursor of LEI104 being inactive. The pyridine and oxazole nitrogens form crucial interactions in the DAGLα homology model with His471 and His650 respectively. The importance of the pyridine nitrogen was confirmed, as its removal, leading to the corresponding benzoxazole, resulted in significantly decreased inhibitor potency. The structure-activity relationships of LEI104 (Figure 2A) were thoroughly investigated in **Chapter 3** by synthesis of several analogs and screening of a 1040 focused library of α-keto heterocycle derivatives. This Chapter shows that the heterocyclic scaffold is crucial for inhibitor potency, as many tested hetrocycles (*e.g.* benzoxazole, -imidazole, -thiazoles, imidazolopyridine, thiazolopyridine and oxadiazoles) are less potent on DAGLα compared to oxazolopyridines. By synthesis of all possible oxazolopyridine regioisomers, it was shown that the original *4N*-oxazolopyridine regioisomer is optimal. This is in line with the reported homology model, as only this specific position can form hydrogen bonding interactions with His471 and His650. Potency of oxazole inhibitors can be increased by introduction of electron withdrawing substituents at the *meta*- but not at the *para*-position. The 2-acyl substituent on the heterocyclic scaffold was also investigated. In the case of a 2-acyl phenylmethylene spacer, a C8 spacer is most potent ($pIC_{50}$ = 8.44, lipophilic efficiency, LipE = 2.78) and a C5 spacer is most efficient ($pIC_{50}$ = 7.43, LipE = 3.35). 2-Acyl fatty acid spacers are also allowed. Interestingly, in terms of potency oleoyl >> arachidonoyl, indicating that the 2-acyl spacer is possibly situated in the pocket normally accommodating the *sn*-1 acyl chain that is specifically cleaved by DAGLα. Most α-keto heterocycles that have previously been published are dual FAAH and DAGLα inhibitors. The structure-activity relationships in this study are fully in line with the homology model,

which was instrumental in the optimization towards LEI105, a potent dual DAGLα/DAGLβ inhibitor that is selective over FAAH and modulates DSI in hippocampal CA1 pyramidal neurons.[32] The lead optimization of LEI105 to optimize its physicochemical properties is described in **Chapter 4**, starting from optimization of the 2-acyl substituent (Figure 2B). Synthesis of shorter spacer analogs of LEI105 showed that the C5 methylenephenyl spacer in LEI105 is most potent, but a C3 spacer has optimal LipE. Introduction of aliphatic spacers containing terminal trifluoromethyl ($CF_3$) groups increased LipE 100-fold. In particular, a 4,4,4-trifluoromethylbutanoyl spacer was further optimized, focusing primarily on the heterocyclic scaffold. Methoxy- and cyano-groups were incorporated to assess the electronic effects on the phenyl ring, moreover *p*-chloro and *p*-fluoro groups were introduced to increase metabolic stability. All inhibitors were also tested in a DAGLα natural substrate assay. The selectivity of the highest LipE compounds were assessed over endogenous serine hydrolases in mouse brain proteome using ABPP on two broad-spectrum probes MB064 and TAMRA-FP. Ultimately, *p*-fluorophenyl derivative LEI107, an exquisitely selective DAGLα inhibitor with drug-like chemical properties was discovered. LEI107 is more potent than LEI105 and is 1000+ fold improved in lipophilic efficiency (Figure 2B and **2** in Table 1).

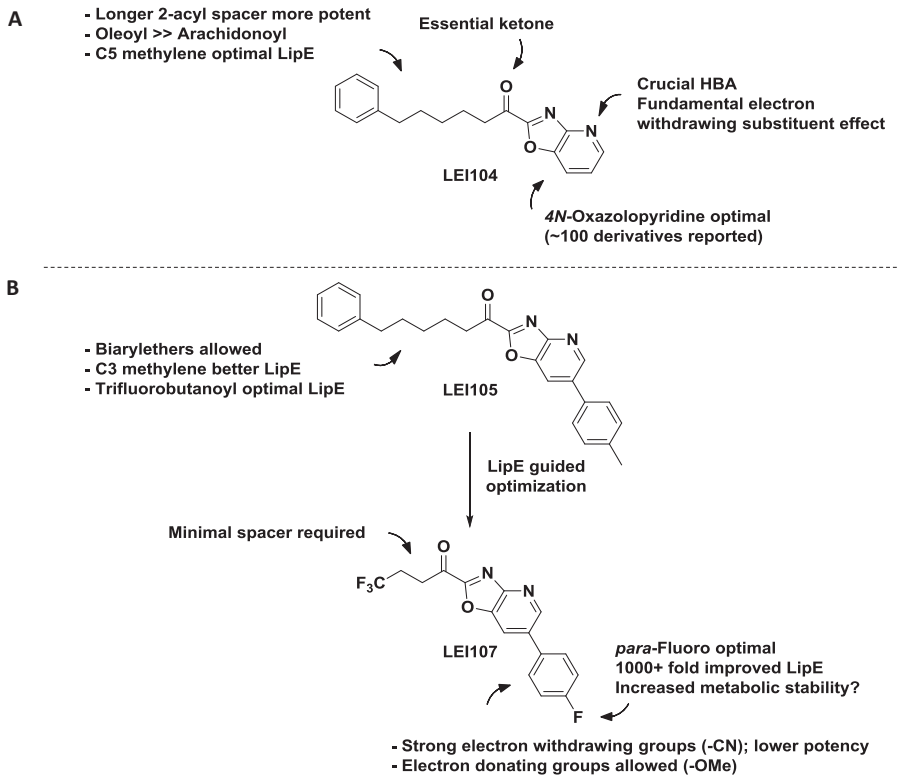



**Figure 2. A)** Structure-activity relationships of LEI104. **B)** Structure-activity relationships of the optimization of LEI105 to LEI107.

**Glycine sulfonamides** were investigated in **Chapter 5** as DAGLα inhibitors via a systematic structure-activity relationship analysis. Glycine sulfonamides were identified as DAGLα inhibitors by a high throughput screening (HTS) performed by researchers of Bristol Meyers-Squibb (BMS). Starting from these reported compounds,[33,34] the biaryl-ether scaffolds, side groups and carboxylic acid were investigated. All compounds were tested in a 96-well DAGLα surrogate substrate assay. The glycine carboxylic acid was an important focus of the investigation, as this chemotype was the only reported inhibitor without a clear warhead or mechanism of action. Hence, the carboxylic acid was investigated by synthesis of the corresponding methyl ester, alcohol, secondary and primary amide, nitrile, tetrazole and a piperidine carbamate**,** all of which were uniformly inactive (Figure 3). Activity is lost upon derivatization of the carboxylic acid moiety, which indicates that it is an essential feature for inhibiting DAGLα.[35,36] By synthesis of 13 different scaffold analogs it was discovered that DAGLα tolerates all substitution patterns on the scaffold, however *para* > *meta* > *ortho* indicating a large linear-like binding groove. Several sulfonamide side groups are tolerated, although the originally reported 2,2-dimethylchroman sulfonamide remains the most potent. Ultimately, systematic analysis of the structural requirements in this study led to the discovery of LEI106, a highly potent DAGLα inhibitor (Figure 3 and compound **3** in Table 1). Due to the presence of the essential carboxylic acid, the binding mode of LEI106 was investigated using docking in the newly developed DAGLα homology model (see Chapter 2 for model development). The modeling indicated that the carboxylic acid might interfere with the hydrogen bonding network of the catalytic triad. The activity and selectivity of LEI106 was assessed over endogenous serine hydrolases in mouse brain proteome using ABPP. The ABPP approach showed that glycine sulfonamide LEI106 is selective over several endocannabinoid system related enzymes, including monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH). However, LEI106 did inhibit α/β hydrolase domain 6 (ABHD6) in the ABPP setting used. Testing of LEI106 in a natural substrate assay based on the conversion of 2-AG by ABHD6, confirmed that LEI106 potently inhibited ABHD6 ($K_i$ = 0.8 ± 0.1 μM). LEI106 inhibited DAGLα in a natural substrate assay based assay ($K_i$ = 0.7 ± 0.08 μM) making LEI106 the first reported reversible dual DAGLα/ ABHD6 inhibitor.

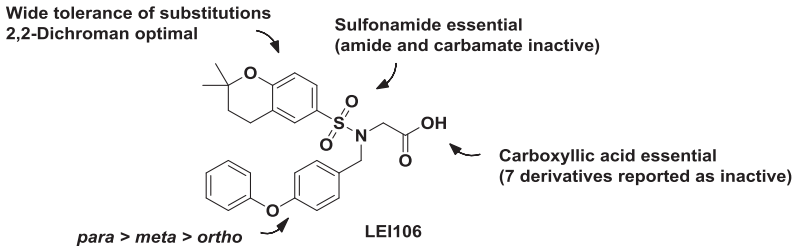



**Figure 3.** Structure-activity relationships of LEI106.

**Chapter 6** encompassed the miniaturization of the PNP-butyrate DAGLα activity assay from 96-well plate to 1536-well plate and subsequent HTS-ABPP. The 96-well plate assay was first

optimized to 354-well plate by optimizing total volume (30 µL), enzyme concentration (0.05 µg/µL) and changing readout from kinetic readout to endpoint determination. The assay was used in a proof-of-principle screen on the commercially available 'List of pharmaceutically active compounds' (LOPAC®, Sigma-Aldrich) library. This screen showed that the assay is of high quality and robust over multiple screening days. The 384-well plate assay was subsequently optimized to 1536-well plate within the European Lead Factory (ELF). Thorough optimization of assay plates, substrate storage conditions, assay volume, enzyme and substrate concentration resulted in an optimized 1536-well plate protocol that was robust and of high quality. The protocol was used in automated HTS as primary screen (single point 10 µM inhibitor) of the Joint European Compound Library (JECL), consisting of 300.000+ compounds. The primary assay was followed up by active conformation at two inhibitor concentrations, resulting in a total of 263 confirmed actives. Orthogonal ABPP was employed to assess activity and selectivity of these actives over multiple serine hydrolases in mouse brain proteome. For high throughput purposes, an ABPP assay protocol was developed that uses incubation in 384-well plate. DAGLα activity was investigated by labeling with ABP MB064 and subsequent in-gel analysis using SDS-PAGE and fluorescence scanning. In addition to DAGLα, MB064 reports on DDHD2, ABHD6, ABHD12, ABHD16A off-target activity. After triaging based on the orthogonal assay activity and selectivity, chemical eye, purity analysis and legal clearance, 46 actives were obtained forming the Qualified Hit List (QHL). The list contains 10 clusters and 10 singletons, including previously reported glycine sulfonamides and three important novel chemotypes: α-keto amides, β-keto difluoro amides and sulfonyl 1,2,4-triazole ureas.

**Glycine sulfonamides** IMI4906626 and IMI4749305 were identified as the most potent DAGLα inhibitors across the entire screening campaign (**4** and **5**, Table 1). Compound **5** was resynthesized and retested by the European Screening Centre. Similar ABPP assessment (as with LEI106) on **5** indicated that it is selective over ABHD6, making this the first reported glycine sulfonamide DAGL inhibitor that seems selective over this specific off-target on ABPP. Compound **4** has not been resynthesized and retested, nevertheless **4** did significantly inhibit ABHD6 in the orthogonal ABPP assay. **α-Keto amides** and **β-keto difluoro amides** are reversible inhibitors that use an activated ketone as electrophilic trap, presumably reacting with Ser472 of DAGLα. Both chemotypes are interesting leads for inhibitor development as IMI0226509, IMI8042748, IMI7294928 and IMI6975607 (**6**-**9**, Table 1) have great physicochemical properties and good LipE (~2) and ligand efficiency, LE (~0.35). These compounds were all resynthesized and retested by the European Screening Centre. ABPP analysis revealed that α-keto amides **6** and **7** did not appear to inhibit ABHD6 to a large extent in the ABPP setting used, whereas β-keto difluoro amides **8** and **9** did inhibit ABHD6 labeling. Both chemotypes can be important for inhibitor development on future uncharacterized serine hydrolases or cysteine proteases in which the QHL can serve as important screening tool. **Sulfonyl 1,2,4-triazole ureas** IMI18721890, IMI18721890 and IMI1788117 (**10**-**12**, Table 1) were highly potent inhibitors in both the primary and orthogonal DAGLα assays. This chemotype uses an activated carbonyl as covalent

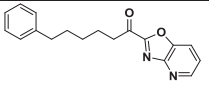
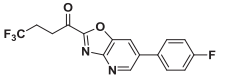
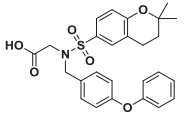
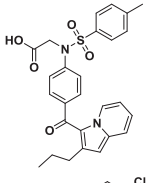
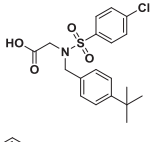
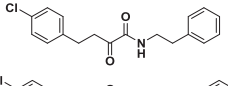
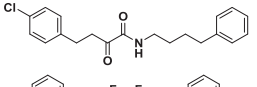
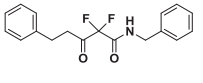
irreversible electrophilic trap. Compounds **10** and **11** were resynthesized and retested by the European Screening Centre. Selectivity assessment of **10** and **11** on ABPP reveals that ABHD6 as well as FAAH and DDHD2 are targeted. Due to their potency, sulfonyl 1,2,4-triazole ureas were selected for subsequent lead optimization and a focused library of ~100 derivatives was synthesized within the ELF consortium by the European Screening Centre. The focused library can serve as an important tool for inhibitor optimization on DAGLs and other targets, such as future uncharacterized serine hydrolases or cysteine proteases.

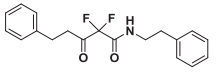
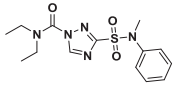
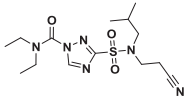
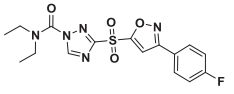
**Chapter 8** encompassed an extensive review on the effect of several DAGL inhibitors in pre-clinical models of metabolic disorders and neurodegenerative diseases. Several parts of this Chapter have been mentioned throughout this summary.

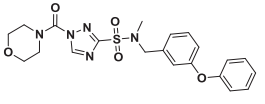
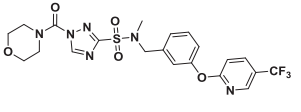
The development of DAGL knockout mice in combination with *in vivo* active DAGL inhibitors has greatly contributed to the understanding of the physiological role of DAGLs. Studies using 1,2,3-triazole ureas DO34 and DH376 have demonstrated that DAGLs regulate the formation of proinflammatory prostaglandins and cytokines under neuroinflammatory conditions.[37] The efficacy of DO34 and DH376 in mouse models of disease has not been reported to date and could provide important insights in the contribution of 2-AG signaling and metabolites in neurodegenerative diseases. One study does show that O-3841, a fluorophosphonate DAGL inhibitor, was neuroprotective in a malonate model of Huntington's disease. Interestingly, MAGL inhibitors exacerbated neuronal damage and oxidative metabolism of 2-AG by COX2 was suggested to result in the formation of the toxic metabolites.[38,39] Considering that several studies indicate that 2-AG signaling, via DAGLα, is involved in the regulation of neuropsychiatric behavior, application of centrally active inhibitors for DAGLα necessitates caution *in vivo*.[23,24,40] Therefore, it will be highly important to determine the efficacy and therapeutic window of both MAGL and DAGL inhibitors in parallel with respect to CB1R mediated adverse effects and activation of alternative metabolic pathways during neuroinflammation.

The 1,2,3-triazole ureas provide a structural template bearing a reactive urea with tunable reactivity, which seems ideal for optimization of serine hydrolase inhibition. It is expected that the newly discovered sulfonyl 1,2,4-triazole ureas are very similar in that aspect. Moreover, their reactivity can potentially be tuned by the sulfur oxidation state, which could influence the triazole pKa, and thereby leaving group capacity. The sulfonyl 1,2,4-triazole ureas have very good physicochemical properties (*e.g.* low MW and low cLogD) although their tPSA is relatively high (>96 $A^2$). Potential drawbacks of the triazole ureas are their off-target activity and covalent irreversible mode of action. This makes the 1,2,3-triazole ureas and sulfonyl 1,2,4-triazole ureas less suitable as potential drug candidates. As such, triazole ureas can best be viewed as tool compounds to report on DAGL function in health and disease, and possibilities regarding DAGLα or DAGLβ subtype selectivity might be explored. To this end, development of paired control compounds is necessary. Reversible inhibitors, like the ketones reported in this Thesis, are perhaps more suitable as drug candidates.

**Table 1.** Overview of the all inhibitors discovered in this Thesis.

| Entry | Code | Structure | DAGLα PNP ($pIC_{50}$) | cLogD | LipE | HBA | HBD | LE | MW | tPSA | Off-targets |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | LEI104 (OL-100) |  | 7.4 | 4.1 | 3.4 | 4 | 0 | - | 294 | 51 | FAAH |
| **2** | LEI107 |  | 8.6 | 3.3 | 5.3 | 4 | 0 | - | 338 | 51 | No off-targets identified |
| **3** | LEI106 |  | 7.7 | 3.7 | 4.0 | 7 | 1 | - | 482 | 93 | ABHD6 2 unknown |
| **4** | IMI4906626 ESC1000043-01 |  | 7.5 | 3.9 | 3.6 | 7 | 1 | 0.29 | 491 | 105 | No off-targets identified |
| **5** | IMI4749305 |  | 6.9 | 2.6 | 4.3 | 5 | 1 | 0.36 | 396 | 83 | ABHD6 |
| **6** | IMI0226509 ESC1000025-01 |  | 6.0 | 4.0 | 2.0 | 2 | 1 | 0.37 | 316 | 46 | - |
| **7** | IMI8052748 ESC1000026-01 |  | 6.0 | 4.9 | 1.9 | 2 | 1 | 0.36 | 344 | 46 | - |
| **8** | IMI7294928 ESC1000042-01 |  | 6.1 | 3.5 | 2.6 | 2 | 1 | 0.36 | 317 | 46 | ABHD6 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **9** | IMI6975607<br>ESC1000044-01 |  | 6.2 | 3.8 | 2.4 | 2 | 1 | 0.35 | 331 | 46 | ABHD6 |
| **10** | IMI18721890<br>ESC1000032-01 |  | 5.9 | 1.7 | 4.2 | 8 | 0 | 0.35 | 337 | 97 | ABHD6<br>FAAH<br>DDHD2 |
| **11** | IMI18721890<br>ESC1000048-01 |  | 5.5 | 1.4 | 4.1 | 9 | 0 | 0.31 | 356 | 121 | ABHD6<br>FAAH<br>DDHD2 |
| **12** | IMI1788117 |  | 5.6 | 2.4 | 3.2 | 9 | 0 | 0.28 | 393 | 115 | ABHD6<br>FAAH<br>DDHD2 |

| Entry | Code | Structure | ABHD16A ABPP ($pIC_{50}$) | cLogP | LipE | HBA | HBD | LE | MW | PSA | Selectivity |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **13** | XGN67 |  | 8.1 | 3.0 | 5.1 | 10 | 0 | - | 458 | 104 | ABHD6<br>FAAH<br>CES3<br>And others |
| **14** | XGN75 |  | 7.4 | 2.5 | 4.9 | 11 | 0 | - | 526 | 117 | ABHD6<br>FAAH<br>CES3<br>And others |

In particular, the α-keto heterocycles such as LEI105 and LEI107 are highly potent and selective. LEI107 shows an ideal combination of high potency, high selectivity and drug-like physicochemical properties, making it the most suitable candidate in this Thesis for *in vivo* studies. LEI107 can be used to assess the effect of pharmacological intervention of DAGL in mouse models of obesity, Multiple Sclerosis, Alzheimer's and Parkinson's disease. Moreover, it can be used to investigate whether a therapeutic window can be established over possible CB1R-related adverse side effects. Proof of target engagement in mice is still required for the α-keto heterocycles, as well as investigation into its *in vivo* stability. Important to keep in mind is that hemiacetal formation can occur at the ketone and its consequences *in vivo* are not known to date.

Interestingly, very recently it has been demonstrated that disruption of DAGLβ alone contributes to lowering the neuroinflammatory response *in vivo*.[41] DAGLβ is key in regulating 2-AG levels in microglia and LPS treated DAGLβ$^{-/-}$ mice show attenuated microglial activation without changes in overall 2-AG and prostaglandin levels in brain. Therefore, DAGLβ can be an important target to attenuate the neuroinflammatory response *in vivo* without affecting synaptic transmission,[41] and therefore could be potentially be devoid of CNS mediated side-effects. Consequently, orally bioavailable, centrally active and selective DAGLβ inhibitors are highly desired and could play a pivotal role in the potential treatment of neurodegenerative diseases such as Multiple Sclerosis, Parkinson's, Alzheimer's and Huntington's disease (Table 2).

**Table 2.** Potential therapeutic areas for subtype selective DAGL inhibitors with central and peripheral activity.

| | **DAGLα selective** | **DAGLβ selective** | **Dual DAGLα/β** |
|---|---|---|---|
| **Central nervous system (CNS)** | Addiction<br>Obesity<br>Neuroinflammation<br>Therapeutic window?<br>Adverse side effects? | **Neuroinflammation** | Therapeutic window?<br>Adverse side effects? |
| **Peripheral system** | **Metabolic syndrome**<br>**Diabetes**<br>**Pheripheral obesity** | **Pathological pain** | **Metabolic syndrome**<br>**Diabetes**<br>**Pheripheral obesity**<br>**Pathological pain** |

Another important line of research is the development of peripherally restricted DAGL inhibitors (Table 2). Inhibitors that do not enter the central nervous system (CNS) are most likely devoid of adverse central side effects and may, therefore, not require full DAGLα/β subtype selectivity. The most important chemotype for this application are the glycine sulfonamides, as they are reversible and have been reported as non-brain penetrable. As a matter of fact, after the publication of LEI106, BMS published a full account of their SAR studies on glycine sulfonamides from the lead optimization program that followed after the

HTS.[33,36] As such, Compounds **3** and **24** (Chapter 8) were identified as highly potent DAGLα/β inhibitors. Reportedly, **3** is cellular active and orally bio-available and **24** is peripherally restricted, picomolar potent and shows a good pharmacokinetic profile. Unfortunately no additional functional or *in vivo* efficacy data have been reported with this series to date. Due to an improved selectivity profile, glycine sulfonamide IMI4906626 (**4**) could provide an interesting starting point for optimization. The key features of **4** that provide selectivity over ABHD6 are not known to date ans is important to address. Investigation into the effect of peripheral inhibition of DAGLα/β *in vivo* for the potential treatment of obesity and metabolic disorders is also important. The main questions are whether glycine sulfonamides can induce weight loss, decrease insulin resistance and/or improve cardiovascular risk factors via perturbation of DAGL α/β activity. The effect of ABHD6 as a peripheral off-target needs to be addressed as well and could potentially be beneficial through polypharmacology.[35] Moreover, local application of peripheral DAGLβ inhibitors, like the glycine sulfonamides, for treatment of pathological pain is another avenue to explore.[42]

**Chapter 7** describes the application of ABPP for the development of *in vivo* active inhibitors for ABHD16A, a principal phosphatidyl serine (PS) lipase. ABHD16A cleaves the fatty acyl chain specifically at the *sn*-1 position (Figure 4), forming *lyso*-PS as the main product.[43] *Lyso*-PS is an important signaling phospholipid involved in T-cell growth,[44] mast cell activation[45,46] and neurite outgrowth.[47] Moreover, it is a toll-like receptor 2 (TLR2) agonist.[48] α/β Hydrolase domain type 12 (ABHD12) hydrolyses *lyso*-PS *in vitro* and indeed, ABHD12$^{-/-}$ mice have been shown to accumulate several distinct long chain *lyso*-PS lipids in the brain.[49] Human genetic studies identified null-mutations of ABHD12 as the cause for the onset of a rare neurological disease: polyneuropathy, hearing loss, ataxia, retinitis pigmentosa and cataract (PHARC).[50,51] ABHD12 knockout studies confirmed that mice devoid of ABHD12 activity suffer from multiple symptoms of PHARC.[49] Therefore, ABHD12$^{-/-}$ mice may serve as an excellent mouse model to investigate this neurological disease.[49] The exact molecular mechanism of the development of PHARC is currently unknown, but the accumulation of *lyso*-PS and subsequent excessive signaling via TLR2 are hypothesized to be involved in the neuroinflammatory response. To test this hypothesis, *in vivo* active inhibitors for ABHD16A are required. In this chapter, ABPP on a focused library of several sulfonyl 1,2,4-triazole ureas revealed that this chemotype can target ABHD16A.

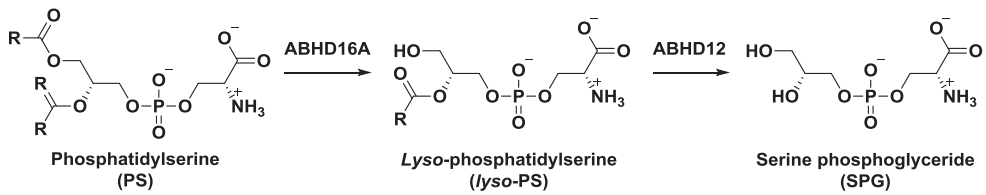



**Figure 4.** Phosphatidylserine (PS) and lyso-phosphatidylserine (lyso-PS) levels are regulated by α/β hydrolase domain type 12 and 16A (ABHD12 and ABHD16A). ABHD16A cleaves specifically at the PS *sn*-1 fatty ester, whereas ABHD12 cleaves the remaining *sn*-2 fatty ester of *lyso*-PS.

A particular sulfonyl 1,2,4-triazole urea was used as a starting point for further optimization. A ligand-based rational design approach led to XGN67 and XGN75 (**13** and **14**, Table 1). Cellular experiments strongly indicate that **13** and **14** do not efficiently penetrate cells, but do potently inhibit extracellular ABHD16A *in situ*. 1,2,4-Triazole urea sulfonamides **13** and **14** partially, but significantly, inhibit ABHD16A *in vivo* after *i.p.* administration. This makes compounds **13** and **14**, the first *in vivo* active ABHD16A inhibitors in the literature. As brain ABHD16A was not completely inhibited at high doses of both compounds, future work should focus on optimization of brain penetration by decreasing the polar surface area (< 100 $A^2$) and increasing metabolic stability. This, however, is likely to increase off-target activity *in vivo*. Hence the development of a paired inactive control compound is required, to exclude potential off-target effects in animal models. The structure-activity relationships from this chapter provide an excellent starting point for further optimization of the next generation *in vivo* active ABHD16A inhibitors. These inhibitors could provide proof-of-principle for substrate reduction therapy by lowering *lyso*-PS levels through ABHD16A inhibition as a potential treatment for the debilitating disease PHARC.

In general, drug discovery is best practiced by applying multiple methods for inhibitor discovery in parallel. In target-based drug discovery, HTS can be considered the best method to obtain novel hits if multi-well assays are available (or can be developed) for the target of interest. Provided that the screening library is of high quality, the resulting hits should be drug-like and suitable for medicinal chemistry optimization. Academic access to high quality drug-like libraries, such as the JECL, is therefore of paramount importance to boost academic research and hit discovery (in Europe). However, HTS is time-consuming, costly and good hits are not guaranteed. Therefore other (higher risk) methods should be performed as well. For serine hydrolases in particular, library screening in combination with ABPP (for 'target hopping') has proven a rewarding strategy. Its success is twofold; off-target activity is highly common among serine hydrolase inhibitors as this large protein family shares many common substrates and therefore often has similar binding pockets. In addition, all members have a highly conserved catalytic mechanism. These features, combined with readily available broad-spectrum probes, such as TAMRA-fluorophosphonate, allow rapid assessment of off-target selectivity. Recent developments of activity assays for serine hydrolases, such as Enplex, enable high-throughput superfamily-wide profiling of activity and selectivity of inhibitors over many targets.[52] This method is expected to have high impact in serine hydrolase drug discovery and should be applied as early in the pipeline as possible for counter screening and inhibitor optimization purposes. Lastly, ligand- and structure-based *in silico* strategies, such as high throughput docking and pharmacophore modeling, are powerful low-cost alternatives that can be performed in parallel as well. *In silico* strategies require thorough knowledge of the field and of the methods, algorithms and scoring functions that can be applied. Phenotypic approaches are considered very promising as well, although these are not discussed or applied in this Thesis. Phenotypic screening in combination with novel chemical biology methods (such as incorporation of bio-orthogonal handles and subsequent chemical proteomics) can provide powerful strategies to

interrogate biological systems.

Projecting forward, glycine sulfonamides, such as LEI106, are important peripherally restricted inhibitors that can be used to evaluate the contribution of perturbing DAGL activity in the potential treatment of metabolic syndrome, diabetes and pheriphiral obesity. α-Keto heterocycles, such as LEI107, could be important inhibitors to evaluate if a therapeutic window can be established for (central) DAGL inhibitors in the potential treatment of addiction, obesity and neuroinflammation. Lastly, 1,2,4-triazole urea sulfonamides can be used as novel tool compounds to evaluate DAGL and ABHD16A function in both health and disease.

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