Activity-based protein profiling of diacylglycerol lipases

Baggelaar, M.P.

CHAPTER 1

Activity-based Protein Profiling Drives Inhibitor Discovery

1. Activity-based protein profiling

Over the years extensive enzyme expression databases have been generated by *in-situ* hybridization and global proteomics.[1,2] However, the activity of enzymes in complex proteomes does not always correlate directly with their expression, due to multiple factors, including post-translational modifications, allosteric control, and the presence of endogenous small molecule inhibitors that can regulate enzyme activity.[3,4] Activity-based protein profiling (ABPP) has emerged as a powerful technique to analyze the functional states of enzymes in complex biological systems.

ABPP relies on active site directed small molecules, termed "activity-based probes" (ABPs) that inhibit a specific enzyme or an entire enzyme family.[5] They react in a mechanism-based fashion, thereby establishing a covalent interaction with the catalytic residue of only the active form of its target enzyme(s). ABPs generally consist of three fundamental features with distinct functions (Figure 1). *1)* A reactive group, the so-called "warhead" that establishes a covalent and irreversible interaction with the catalytic residue of the target enzyme; *2)* a recognition element that directs the ABP towards its specific target *3)* and a reporter tag that enables visualization, identification and/or quantification of labeled proteins. A biotin reporter group is commonly used for enrichment and subsequent mass spectrometric analysis. Fluorescent reporter groups are often used for visualization by microscopy or in-gel fluorescence detection after the proteome is resolved on SDS-PAGE. These reporter groups can be directly incorporated in the ABP (direct ABP), or a detection

element can be introduced in a later stage via ligation chemistry (two-step ABP).[6] Two-step ABPs contain a latent affinity handle, which can be coupled to a reporter group via biorthogonal ligation chemistry. Multiple ligation strategies have been employed for ABPP, including Staudinger ligation, copper(I) catalyzed alkyne-azide [3+2] cycloaddition (click) reaction and reverse electron demand Diels Alder ligation.[7] The advantage of using two-step probes is that the bioorthogonal ligation handles are generally very small and, therefore, they may not interfere with protein binding and cellular penetration, which contrasts bulky fluorophores and biotin reporter groups.

One of the advantages of ABPP is that it can assess enzyme activity in diverse biological systems, including cell and tissue lysates, cell cultures and even living organisms. ABPP can be employed for a wide variety of purposes, such as the discovery of new enzyme inhibitors, identification of down-regulated enzyme activities in disease states, enzyme activity assays, target elucidation, and localization of enzyme activity in cells and *in vivo*.[5, 8-10]

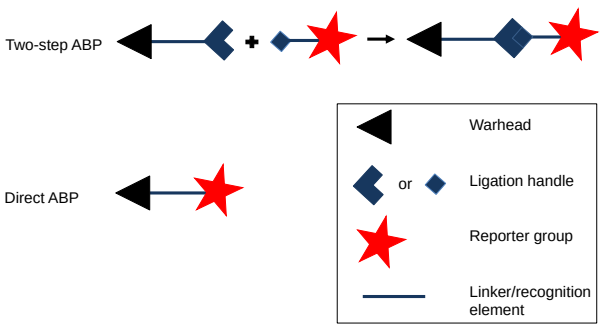

Figure 1. Schematic representation of a direct and a 2-step ABP.

2. Competitive ABPP

Competitive ABPP involves treatment of a proteome with a small molecule inhibitor before labeling with an ABP. Reduction of probe labeling provides information on the affinity of the small molecule inhibitor for the targeted enzyme(s). A schematic representation of a competitive ABPP workflow is given in Figure 2.[11] This methodology to monitor enzyme inhibition has several advantages compared to conventional substrate assays. First, the inhibitor activity measurement can be conducted directly in native proteomes. Therefore, recombinant expression and purification are not required. Second, when broad spectrum ABPs are used, evaluation of selectivity and potency against many enzymes can be performed in parallel. Finally, probe labeling acts as a surrogate for substrates, thus inhibitors can also be developed for enzymes with unknown substrates.

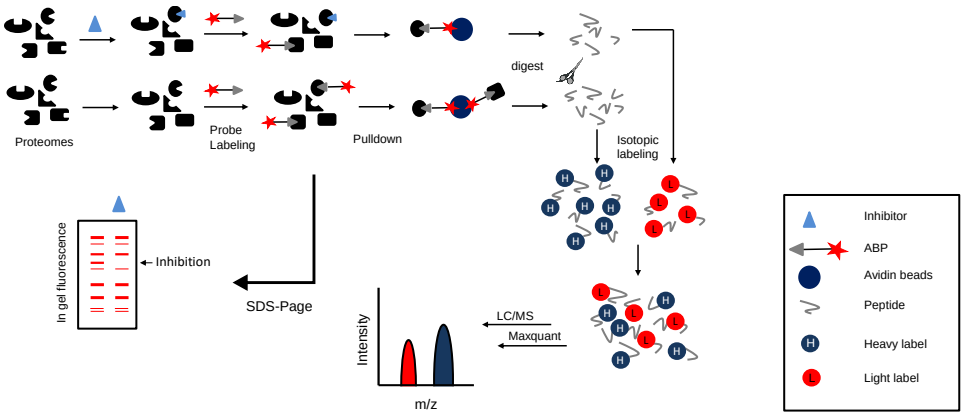

Figure 2. Schematic representation of competitive ABPP. Proteomes are treated with inhibitor or vehicle and subsequently labeled with ABP. At this stage, the proteome can be resolved on SDS-PAGE and the in gel fluoresce can be analyzed when the ABP is equipped with a fluorescent reporter group. Reduction of the intensity of fluorescence of specific bands in inhibitor treated samples compared to control samples indicates inhibition of that specific protein. Alternatively, for biotinylated ABPs, the labeled proteome can be analyzed by mass-spectrometry. Therefore, labeled proteomes are enriched using avidin beads and subsequently digested by trypsin. Peptides are labeled with different isotopic labels for comparison between inhibitor treated and untreated proteomes. The differentially labeled peptides are mixed and analyzed by mass spectrometry. The identity of the proteins can be determined by correlating measured tryptic peptides with predicted typtic peptides. Information on inhibition can be retrieved from the isotopic ratio between the inhibitor treated and untreated proteome.

3. Small molecule inhibitors to study enzyme function

In the postgenomic era, the identification and characterization of the biological functions of proteins that coordinate and control biological processes in health and disease has become one of the most important challenges in the field of chemical biology. Although predicted gene products can be divided in sequence related protein families such as receptors, enzymes or transport channels, their exact biological function remains often obscure. Understanding the role and importance of a given gene product in (patho)physiological processes, requires knowledge of its substrates, ligands and other interacting biomolecules.[12] Congenital deletion and targeted gene disruption by siRNA and CRISPR-Cas9 are regularly used tools to investigate the biochemical function of enzymes in cells and animals. However, they may produce outcomes that are difficult to interpret. Congenital deletion of genes may lead to developmental defects and cellular compensation by other proteins. Additionally, genetic techniques are not suitable for separating a scaffolding function from the catalytic activity of enzymes.

Small molecule inhibitors that inhibit enzyme activity in an acute fashion are a complementary approach and can address some of the above mentioned shortcomings of the genetic techniques. Perturbation of enzyme activity using selective small molecule inhibitors has several distinct advantages over genetic approaches. They allow control of

enzyme activity rapidly, reversibly and without compensation of enzyme expression. In addition, small molecule probes provide the possibility to tune the extent and timing of inhibition and can serve as starting points for the development of drugs for human diseases.

A complete understanding of the target profile of small molecule inhibitors in native biological systems is highly important to retrieve reliable information on the function of specific enzymes. ABPP is a powerfull technique to evaluate the activity and selectivity of small molecule inhibitors over specific enzyme families. In contrast to genetic techniques, not all protein classes can be addressed by activity-based protein profiling. Enzymes require a nucleophilic amino acid in the active site that can covalently bind with the warhead of an ABP, which is in general an electrophilic trap. ABPs have been developed to covalently bind to aspartate, glutamate, cysteine, lysine, tyrosine, and serine residues across a mechanistically diverse set of enzymes, e.g. cysteine proteases,[13] serine hydrolases,[14] phosphatases,[15] glycosidases[16] and palmitoyl transferases.[17]

The main focus of the work described in this thesis is the development of ABPs and inhibitors for endocannabinoid hydrolases. Many of these enzymes belong to the serine hydrolase family and regulate the biosynthesis and degradation of endocannabinoids. The activated nature of the catalytic serine makes these enzymes susceptible for covalent modification by a wide array of electrophiles, such as lactones,[14] triazole ureas,[18] flurophosphonates[19] and carbamates.[20] Therefore, they are highly suitable to study by ABPP. Fluorophosphonates have proven to be exceptionally useful for the generation of broad-spectrum ABPs for this enzyme family.[21] It has been shown that the large majority (>80%) of the mammalian serine hydrolases are targeted by a single fluorophosphonate based ABP.[19] Tailor-made ABPs with different warheads, such as carbamates and triazole ureas, are employed to study the remaining enzymes.[18,22]

In particular the diacylglycerol lipases were studied in depth. There are two diacylglycerol lipase isoforms, diacylglycerol lipase-α and -β (DAGL-α and DAGL-β). These enzymes play a key role in the biosynthesis of the major endocannabinoid 2-arachidonoylglycerol. This signalling lipid is involved in multiple (patho)physiological processes, including neuroinflammation, anxiety, metabolism and addiction. Tools to study and modulate the activity of these enzymes can be used to provide insight in the physiological role of 2-arachidonoylglycerol. In addition, these tools may lead to novel therapeutics for the treatment human disorders such as the metabolic syndrome, Parkinson's disease, Alzheimer's disease and Multiple Sclerosis.

5. Aim and outline of the Thesis:

The aim of the research described in this thesis is to identify, develop and apply novel chemical tools and methodologies to study the function of endocannabinoid hydrolases in complex proteomes.

2-Arachidonoylglycerol (2-AG) is one of the most extensively studied monoacylglycerols. It is an important signaling lipid as well as an intermediate in lipid metabolism in the brain. **Chapter 2** describes the physiological role of 2-AG, which is tightly regulated by enzymes responsible for its biosynthesis and catabolism. The function of each of these enzymes is described in detail.

Diacylglycerol lipase α (DAGL-α) is a key enzyme involved in the biosynthesis of 2-AG in the brain. To gain insight in the physiological role of 2-AG, selective inhibitors for DAGL-α are required. An ABP that detects diacylglycerol lipases in native biological systems is a highly valuable tool for the identification and characterization of inhibitors for DAGL-α. **Chapter 3** describes the synthesis and characterization of a tetrahydrolipstatin (THL)-derived ABP (MB064) for DAGL-α. This ABP is required, because the commonly used fluorophosphonate-based broad-spectrum serine hydrolase ABPs do not react well with DAGL-α.
MB064 was used to screen a targeted compound library against DAGL-α activity in the mouse brain membrane proteome. This screen revealed a new inhibitor for DAGL-α and DAGL-β. The inhibitor was termed "LEI104". A gel-based assay in the mouse brain membrane proteome revealed that the inhibitor had an important off-target that could complicate analysis when using this inhibitor to study the physiological function of 2-AG.

The aim of the research described in **Chapter 4** was to optimize LEI104 towards a more selective DAGL-α/β inhibitor. This optimization requires an assay that can determine the target interaction profile of reversible serine hydrolase inhibitors in complex proteomes. To this end, a chemoproteomic assay using MB108, a biotinylated version of MB064, was developed and applied. MB108 was instrumental in the discovery of LEI105, the most selective inhibitor for DAGL-α and DAGL-β to date.

The aim of the research described in **Chapter 5** was to investigate the structure activity relationship of LEI105 on DAGL-α. This may provide insight in important enzyme-inhibitor interactions and lead to the discovery of potent inhibitors with improved physicochemical properties. Competitive and comparative ABPP assays enabled the determination of their selectivity profile across a large panel of serine hydrolases.

The aim of the research described in **Chapter 6** was to use the chemoproteomics assay to study the relative activity of different hydrolases involved in endocannabinoid biosynthesis and metabolism in various mouse brain regions, such as the hippocampus, cerebellum, striatum and frontal cortex. Comparison of specific serine hydrolase activities between the brain regions provided insight in which endocannabinoid metabolic pathway is important in specific brain regions. Subsequently the regulatory function of the CB_1 receptor on

endocannabinoid hydrolase activities was investigated by comparing serine hydrolase activity in brain regions from CB_1 knockout and wild type mice.
The aim of the research described in **Chapter 7** was to investigate the interaction landscape of MB064 and MB108. In this Chapter a chemoproteomic mouse tissue screen was performed to identify all targets of MB064 and MB108. This revealed that these ABPs react with multiple members of the α,β-hydrolase family. Several members of this class were recombinantly expressed and screened against a focused library of lipase inhibitors. This led to the identification of new inhibitors for ABHD2 and ABHD3.

Chapter 8 summarizes the work described in this thesis and presents some future challenges.

References

1. Sharma, K.; Schmitt, S.; Bergner, C. G.; Tyanova, S.; Kannaiyan, N.; Manrique-Hoyos, N.; Kongi, K.; Cantuti, L.; Hanisch, U. K.; Philips, M. A.; Rossner, M. J.; Mann, M.; Simons, M. *Nat. Neurosci.* **2015**, *18*, 1819.
2. Uhlen, M.; Fagerberg, L.; Hallstrom, B. M.; Lindskog, C.; Oksvold, P.; Mardinoglu, A.; Sivertsson, A.; Kampf, C.; Sjostedt, E.; Asplund, A.; Olsson, I.; Edlund, K.; Lundberg, E.; Navani, S.; Szigyarto, C. A.; Odeberg, J.; Djureinovic, D.; Takanen, J. O.; Hober, S.; Alm, T.; Edqvist, P. H.; Berling, H.; Tegel, H.; Mulder, J.; Rockberg, J.; Nilsson, P.; Schwenk, J. M.; Hamsten, M.; von Feilitzen, K.; Forsberg, M.; Persson, L.; Johansson, F.; Zwahlen, M.; von Heijne, G.; Nielsen, J.; Ponten, F. *Science* **2015**, *347*, 1260419.
3. Otlewski, J.; Jelen, F.; Zakrzewska, M.; Oleksy, A. **2005**, *24*, 1303.
4. Lindsley, J. E.; Rutter, J. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 10533.
5. Moellering, R. E.; Cravatt, B. F. *Chem. Biol.* **2012**, *19*, 11.
6. Willems, L. I.; Overkleeft, H. S.; van Kasteren, S. I. *Bioconjugate Chem.* **2014**, *25*, 1181.
7. Debets, M. F.; van Hest, J. C.; Rutjes, F. P. *Org. Biomol. Chem.* **2013**, *11*, 6439.
8. Niphakis, M. J.; Cravatt, B. F. *Annu. Rev. Biochem.* **2014**, *83*, 341.
9. Hoover, H. S.; Blankman, J. L.; Niessen, S.; Cravatt, B. F. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5838.
10. de Bruin, G.; Xin, B. T.; Kraus, M.; van der Stelt, M.; van der Marel, G. A.; Kisselev, A. F.; Driessen, C.; Florea, B. I.; Overkleeft, H. S. *Angew. Chem. Int. Ed.* **2016**, *55*, 4199.
11. Niphakis, M. J.; Cravatt, B. F. *Annu. Rev. Biochem.* **2014**, *83*, 341.
12. Saghatelian, A.; Cravatt, B. F. *Nat. Chem. Biol.* **2005**, *1*, 130.
13. Fonovic, M.; Bogyo, M. *Expert Rev. Proteomics* **2008**, *5*, 721.
14. Baggelaar, M. P.; Janssen, F. J.; van Esbroeck, A. C.; den Dulk, H.; Allara, M.; Hoogendoorn, S.; McGuire, R.; Florea, B. I.; Meeuwenoord, N.; van den Elst, H.; van der Marel, G. A.; Brouwer, J.; Di Marzo, V.; Overkleeft, H. S.; van der Stelt, M. *Angew. Chem. Int. Ed.* **2013**, *52*, 12081.
15. Kumar, S.; Zhou, B.; Liang, F.; Wang, W. Q.; Huang, Z.; Zhang, Z. Y. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 7943.
16. Willems, L. I.; Jiang, J.; Li, K. Y.; Witte, M. D.; Kallemeijn, W. W.; Beenakker, T. J.; Schroder, S. P.; Aerts, J. M.; van der Marel, G. A.; Codee, J. D.; Overkleeft, H. S. *Chem. Eur. J.* **2014**, *20*, 10864.
17. Zheng, B.; DeRan, M.; Li, X.; Liao, X.; Fukata, M.; Wu, X. *J. Am. Chem. Soc.* **2013**, *135*, 7082.

18. Ogasawara, D.; Deng, H.; Viader, A.; Baggelaar, M. P.; Breman, A.; den Dulk, H.; van den Nieuwendijk, A. M.; Soethoudt, M.; van der Wel, T.; Zhou, J.; Overkleeft, H. S.; Sanchez-Alavez, M.; Mori, S.; Nguyen, W.; Conti, B.; Liu, X.; Chen, Y.; Liu, Q. S.; Cravatt, B. F.; van der Stelt, M. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113*, 26.
19. Bachovchin, D. A.; Ji, T.; Li, W.; Simon, G. M.; Blankman, J. L.; Adibekian, A.; Hoover, H.; Niessen, S.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 20941.
20. Hsu, K. L.; Tsuboi, K.; Adibekian, A.; Pugh, H.; Masuda, K.; Cravatt, B. F. *Nat. Chem. Biol.* **2012**, *8*, 999.
21. Liu, Y.; Patricelli, M. P.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 14694.
22. Chang, J. W.; Cognetta, A. B., 3rd; Niphakis, M. J.; Cravatt, B. F. *ACS Chem. Biol.* **2013**, *8*, 1590.