Activity-based protein profiling in drug-discovery

Esbroeck, A.C.M. van

1

General introduction: Activity-based protein profiling in drug discovery

Drug discovery & development

The quest for treatments against disease and for symptom relief goes back at least several millennia. Around 1700 BC, the Mesopotamians described the use of drugs for medicinal purposes in a series of 40 tablets collectively known as "Treatise of medical diagnosis and prognosis"[1]. It was not until the beginning of the twentieth century that Paul Ehrlich (1854-1915) hypothesized that chemicals can exert specific biological effects due to the existence of 'chemoreceptors' on cells. With his notion that differential 'chemoreceptors', e.g. in infectious organisms or cancer cells, could be exploited for therapeutic benefit and that the chemical structure of a compound is linked to its specific pharmacological activity, he unknowingly provided the foundation for modern drug discovery[2].

The drug discovery and development process covers all steps from the discovery of a therapeutically relevant biological target (Paul Ehrlich's 'chemoreceptors') to the approval and launch of a drug (Figure 1). The drug discovery process generally starts with target discovery, which aims to identify biological targets in the pathology of interest that can be modified by chemical intervention. Once a target is validated (e.g. using genetically modified animal models), small molecule modulators (hits) are generally identified by

high-throughput screens of chemical libraries. After evaluation and limited optimization, hits can be identified as lead compounds, which show proof-of-principle in an animal model. Analogs with improved potency, reduced off-target activity and optimal physicochemical and metabolic properties are synthesized during lead optimization. The most promising compounds are assessed on pharmacodynamic, pharmacokinetic and toxicological properties. The main goal of the preclinical phase is to successfully demonstrate *in vivo* efficacy in a suitable animal model and to determine the safe dose for first-in-human studies. The investigational new drug then proceeds to the drug development stage, consisting of several clinical testing phases. During clinical phase I the dosage and safety is assessed in a small group of healthy volunteers. Phase II focuses on preliminary efficacy and side effects in a small patient group, whereas during phase III efficacy and adverse reactions are monitored in a large patient group. Roughly 10 percent of the investigational new drugs passes all clinical tests and can be launched as novel drug application after their approval by regulatory bodies[3–5].

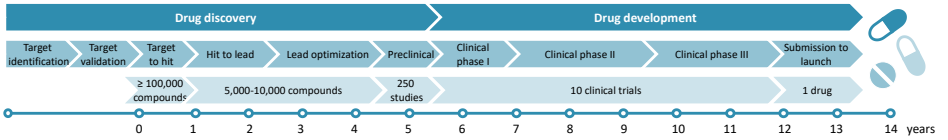

Figure 1 | The drug discovery and drug development process. Schematic representation of the stages in drug discovery and drug development, with their estimated duration and number of compounds/studies required for the development of a single approved drug (modified from literature [3–5]).

Drug discovery and development is a time- and resource-demanding process. On average, it takes more than 13 years to progress from hit to launch and the estimated costs currently exceed $ 2.5 billion[5–7] for each new drug that enters the market. Despite technological advances, the introduction rate of new drugs is at a low, while development costs are rising substantially (mainly due to late-stage clinical failures caused by limited efficacy or unexpected toxicology)[4,8,9]. Although the target-based drug discovery process described above is effective in early stages of drug development, it often fails to predict pharmacological properties and clinical efficacy. The alternative phenotypic drug discovery approach is postulated to have more predictive power on pharmacological success in later stages[10,11]. In phenotypic screens compounds are directly screened in a relevant cellular or animal model for desirable phenotypic effects and responsible target(s) are identified in later stages of the drug discovery process[12]. In either drug discovery approach target identification is a crucial step, because the clinical success of a drug-candidate is highly dependent on understanding the relevance of its biological target(s) in the intended disease. The target identification process is challenging, but advancements are made due to continuous development of novel tools, methods and strategies, e.g. patient-derived induced pluripotent stem cells (iPSCs)[13] and zebrafish[14] as alternative models in phenotypic screening, single-cell analysis techniques[15] like RNA-seq[16], and computational approaches[17]. In the last decades, activity-based protein profiling (ABPP) has emerged as a powerful chemical biology technique in drug discovery as well.

Activity-based protein profiling

ABPP is a chemical proteomic method that uses active-site directed probes to assess the functional state of an entire enzyme class in complex biological samples[18–20]. These probes covalently interact with their target enzyme and report on the abundance of active protein, hence the term activity-based probe (ABP). An ABP typically consists of three main parts (Figure 2A): a reactive group or 'warhead' that covalently interacts with the enzyme's active site, a chemical linker or spacer element, and a reporter tag that enables detection of the probe targets[19]. The reporter tag can be incorporated in the ABP itself (direct probes, Figure 2B) or can be introduced at a later stage by bio-orthogonal chemistry (two-step probe, Figure 2C). Copper(I)-catalyzed azide-alkyne cycloaddition ("click" chemistry) is one of the most common bio-orthogonal reactions to introduce a reporter group[21]. Although the workflow of direct probes is more efficient, a large reporter tag may alter the probe's affinity and selectivity and may affect cell permeability[22].

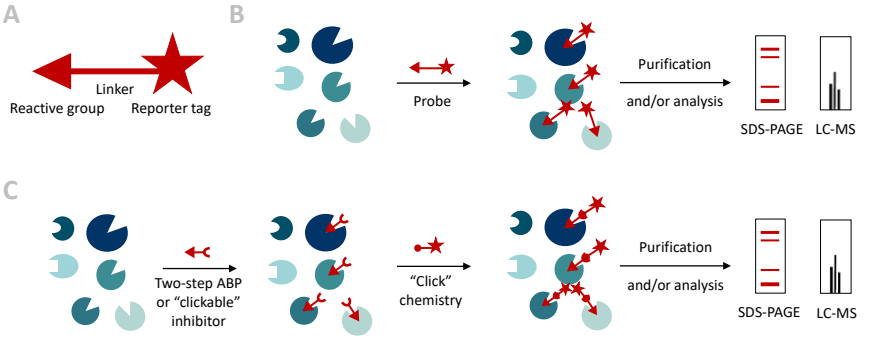

Figure 2 | Activity-based protein profiling. (**A**) An activity-based probe consists of a reactive group coupled to a reporter tag (a fluorophore for gel-based analysis by SDS-PAGE or an affinity tag for purification and analysis by LC-MS) by a chemical linker element. (**B**, **C**) Schematic representation of (**B**) direct ABPP in which a complex proteome is incubated with a probe containing a reporter tag and (**C**) two-step ABPP which requires bio-orthogonal chemistry ("click" chemistry) to introduce a reporter tag to a two-step probe.

Complex protein samples can be labeled with ABPs in different experimental settings, including in cell or tissue lysates (*in vitro*), live cells (*in situ*), and living organisms (*in vivo*)[23]. The readout of the experiment is generally dependent on the type of reporter tag that is used. Fluorescent reporter tags enable rapid analysis in a gel-based assay. The labeled proteome is separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In-gel fluorescence scanning provides a rapid readout, but sensitivity is limited and target identification heavily relies on specific inhibitors. Affinity tags, like biotin, enable target enrichment e.g. by avidin chromatography (pull-down). After tryptic digestion of the enriched targets, the resulting peptides can be identified by liquid chromatography-mass spectrometry (LC-MS) (Figure 2B, C)[19,24]. This MS-based ABPP method (also called chemical proteomics) is much lower throughput, but the high sensitivity and high information content make the technique very attractive nonetheless.

Comparative and competitive ABPP

Two types of experimental setups are commonly used in ABPP: comparative and competitive ABPP (Figure 3). The comparative ABPP approach enables comparison of the activity landscapes of two or more biologically distinct samples[19,25], e.g. healthy versus diseased[26,27] (Figure 3A). Altered enzyme activities can provide insight in (the regulation of) biological pathways and can serve as a starting point in target discovery. An important advantage of ABPP over gene expression studies, is that ABPP provides a direct readout of the enzymatic state of the protein and thus also accounts for the effects post-translational modifications.

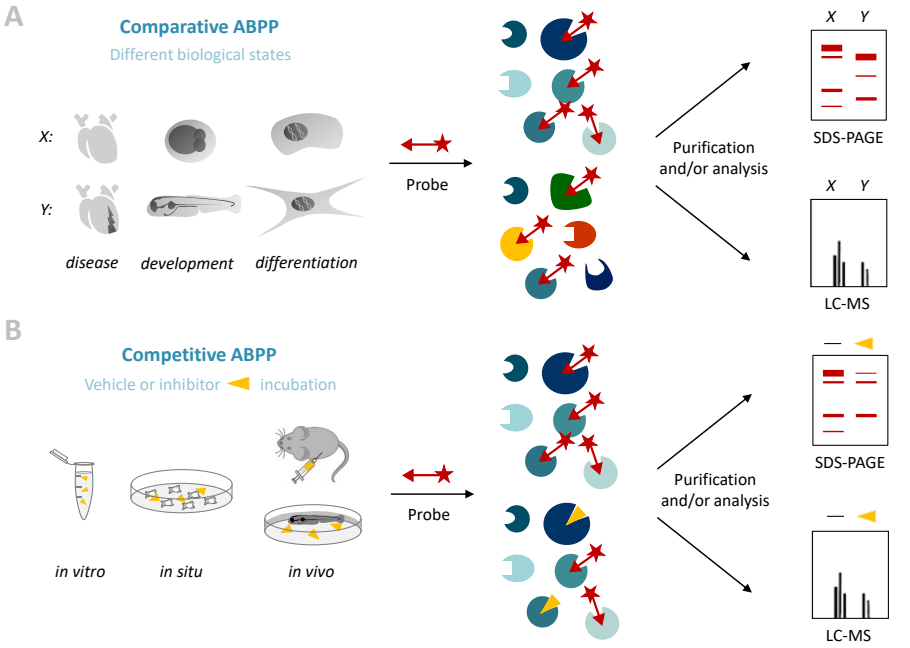

Figure 3 | Competitive and comparative ABPP. (**A**) In comparative ABPP two proteomes from different biological states, e.g. healthy versus diseased or different cell types, are compared by gel- or MS-based analysis of probe treated samples. (**B**) Competitive ABPP enables target-engagement assessment and off-target profiling. Inhibitor treatment can be done *in vitro* (cell or tissue lysate), *in situ* (live cells), or *in vivo* (living organisms), followed by *in vitro* probe incubation and gel- or MS-based analysis.

Competitive ABPP, the second major application, is particularly suitable for inhibitor profiling (Figure 3B)[19,23,24]. It enables simultaneous assessment of inhibitor potency and selectivity and is a powerful tool for target engagement studies. In the drug discovery process, competitive ABPP can guide the lead identification, optimization, and the pre-clinical testing phase. Prior to labeling with an ABP, the proteome is incubated *in vitro, in situ,* or *in vivo* with inhibitor. Targets are identified as those with reduced probe labeling after inhibitor pre-incubation. Target identification is restricted to the enzymes identified by the probe, therefore broad-spectrum ABPs are especially suitable for competitive ABPP. Alternatively, introduction of a ligation handle in the inhibitor enables identification of all covalent interaction partners with two-step ABPP

(Figure 2C). Since the ligation handle can affect potency and selectivity, it is important to cross-check with the original inhibitor[28].

Finally, ABPP can serve as a tool for the characterization of unannoted proteins. Interaction between an ABP and an uncharacterized protein can facilitate the assignment of the protein to a specific enzymatic class or family[19].

Activity-based probes

A variety of enzyme classes can be targeted with ABPs[19,24], including the serine hydrolases[18,29], cysteine and threonine proteases[30,31], kinases[32], phosphatases, cytochrome P450s[33], and glycosidases[34,35]. Photoaffinity probes provide an alternative labeling method for enzymes (e.g. metallohydrolases) or other druggable targets (e.g. receptors) that otherwise cannot interact covalently with a probe. The photoreactive group in these probes can form a covalent bond with the associated protein after UV- irradiation[36].

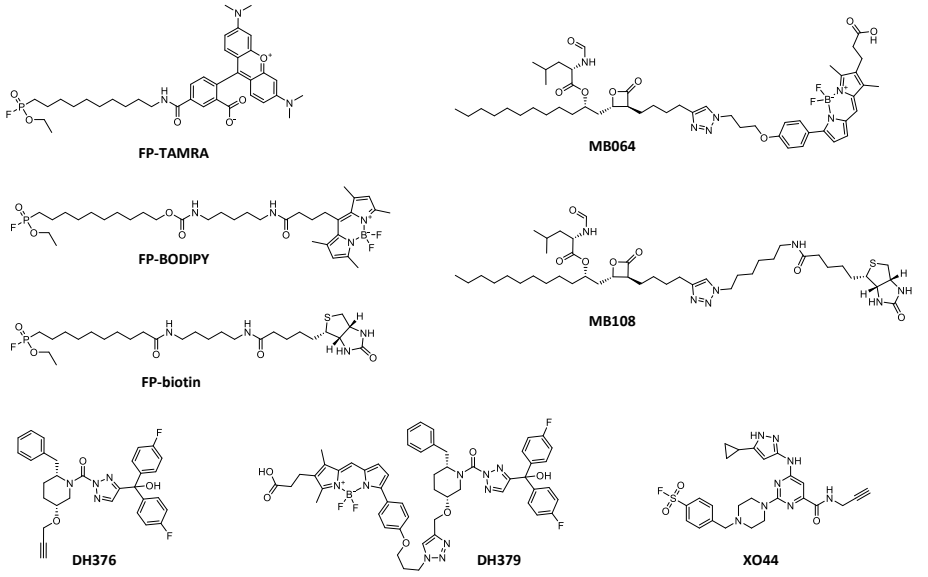

Figure 4 | Activity-based probes. Broad-spectrum serine-hydrolase probes fluorophosphonate (FP)-TAMRA, FP-BODIPY and FP-biotin. Tailored lipase probes MB064 and MB108, targeting e.g. DAGLα/β. Two-step DAGL-probe DH376, containing an alkyne ligation handle, and its derived direct probe DH379. Reactive-lysine targeting kinase probe XO44.

The work in this thesis focuses on activity-profiling of the serine hydrolases, which represent ~ 1 % of all proteins in mammals. The serine hydrolases are characterized by a well conserved GXSXG amino acid motif and are involved in a broad spectrum of physiological and pathological processes[37,38]. The active-site serine can interact covalently with fluorophosphonate (FP)-based probes, such as FP-rhodamine (FP-TAMRA)[18] and FP-BODIPY[22], which are especially suitable for broad-spectrum profiling. Tailored probes like the β-lactone-based MB064[39] and MB108[40] or selective

probes like DH376 and DH379[41] enable assessment of specific hydrolases such as the diacylglycerol lipases (Figure 4) (see *The endocannabinoid system*).

In addition, the kinase-targeting probe XO44 is used (Figure 4)[32]. The kinases, a large protein family comprising over 500 members, are known for their pivotal roles in cell signaling processes, differentiation, proliferation, and disease[42,43]. A conserved lysine in the kinase active-site offers a reactive group for XO44[32]. Of note, in contrast to the serine hydrolase probes, XO44 labels available kinase active-sites dependent on their affinity, but not necessarily on their active state.

The endocannabinoid system

The endocannabinoid system (ECS, Figure 5) plays a central role in the work described in this thesis. The ECS mediates the physiological effects of Δ^9-tetrahydrocannabinol (THC), the psychoactive compound in *Cannabis sativa,* to which the ECS owes its name[44,45]. The ECS is comprised of the cannabinoid receptors type 1 and 2 (CB1R, CB2R), their endogenous ligands, also known as the endocannabinoids, and the enzymes for ligand biosynthesis and degradation. CB1R and CB2R are G protein-coupled receptors and are activated by the endocannabinoids, as well as by plant-derived cannabinoids (phytocannabinoids) like THC[46]. The CB1R is most abundant in the central nervous system[47], while CB2R is mainly expressed in cells of the immune system[48]. The ECS mediates numerous physiological processes, including appetite and metabolism, learning and memory, pain sensation and anxiety, and inflammation[45,49–51].

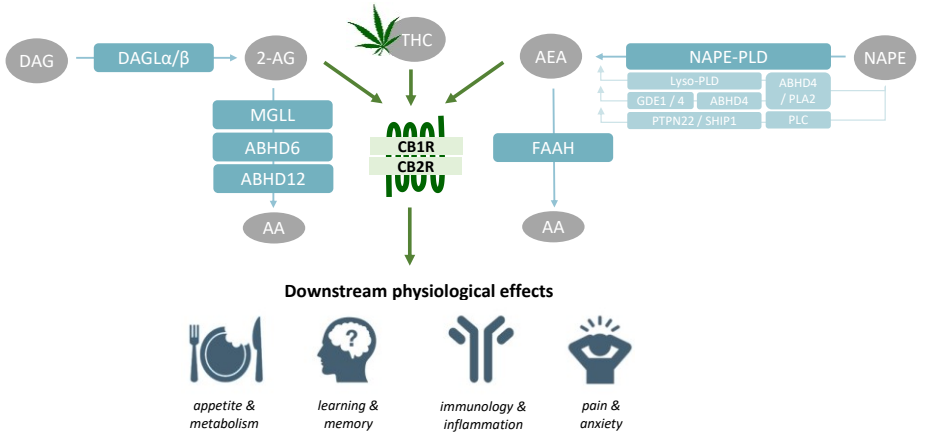

Figure 5 | The endocannabinoid system. The endocannabinoid system is involved in multiple physiological processes and is comprised of the cannabinoid receptors 1 and 2 (CB1R, CB2R), their endogenous ligands (endocannabinoids) and their metabolic enzymes. Ligands & metabolites (grey): DAG: diacylglycerol, 2-AG: 2-arachidonoylglycerol, AA: arachidonic acid, THC: Δ^9-tetrahydrocannabinol, AEA: anandamide, NAPE: *N*-acylphosphatidylethanolamine. Endocannabinoid metabolic enzymes (blue): DAGLα/β: diacylglycerol lipase α and β. MGLL: monoacylglycerol lipase. ABHD4/6/12: α,β-hydrolase domain containing proteins 4, 6 and 12. FAAH: fatty acid amide hydrolase. NAPE-PLD: *N*-acylphosphatidylethanolamine phospholipase D, PLA2: phospholipase A_2, GDE1/4: glycerophosphodiesterase 1 and 4, Lyso-PLD: unidentified lysophospolipase D, PLC: unidentified phospholipase C, PTPN22: tyrosine-protein phosphatase non-receptor type 22, SHIP1: phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1.

In the early 90s *N*-arachidonoylethanolamide (anandamide, AEA) was identified as the first endocannabinoid[52]. In the following years, 2-arachidonoylglycerol (2-AG)[53,54] as well as several other endocannabinoids were identified. However, AEA and 2-AG are considered to be the two main endocannabinoids[45]. In the brain the endocannabinoids are synthesized on-demand at the membrane, in contrast to classical neurotransmitters which are stored in presynaptic vesicles. The endocannabinoids activate the presynaptic CB1R in a retrograde fashion and signaling is terminated by their hydrolysis. This mechanism implies that endocannabinoid levels at the synapse must be tightly controlled by their metabolic enzymes[46].

Several metabolic pathways control the endocannabinoid levels (Figure 5). Diacylglycerol lipase α and β (DAGLα, DAGLβ) are the two main enzymes producing 2-AG, by catalyzing the hydrolysis of diacylglycerol (DAG) to 2-AG [55]. The majority of 2-AG is hydrolyzed to arachidonic acid (AA) by monoacylglycerol lipase (MGLL)[56,57], but α,β-hydrolase domain containing proteins 6 and 12 (ABHD6, ABHD12) can also catalyze this reaction[57–59]. The biosynthesis of AEA is less straightforward, due to the existence of multiple *N*-acylethanolamine (NAE) biosynthetic pathways. Direct hydrolysis of *N*-acylphospatidylethanolamines (NAPEs) to NAEs (including AEA) by NAPE phospholipase D (NAPE-PLD) is considered to be the canonical pathway[60]. The NAEs are hydrolyzed to ethanolamine and free fatty acid by fatty acid amide hydrolase (FAAH)[61,62]. Of note, three alternative multistep pathways exist for the biosynthesis of AEA from NAPEs: (I) phospholipase A_2 (PLA2) and ABHD4 mediated conversion of NAPE to lyso-NAPE, followed by conversion to AEA by an unknown lysophopholipase D (Lyso-PLD); (II) conversion of lyso-NAPE to glycerophospho-AEA by ABHD4 and subsequent hydrolysis to AEA by glycerophosphodiseterase 1 or 4 (GDE1, GDE4); or (III) conversion of NAPE to phospho-AEA by an unidentified phospholipase C and cleavage to AEA by tyrosine-protein phosphatase non-receptor type 22 (PTPN22) or phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1 (SHIP1)[60].

The ECS as a therapeutic target

The role of the ECS in various pathological conditions, including neurological[63] and cardiovascular[64] disorders, makes it a promising target for therapeutic intervention[51,65,66]. At the same time, its involvement in multiple physiological processes makes drug discovery challenging and requires accurate balancing of activation and inhibition to achieve only the desired effects. Several synthetic CB1R and CB2R ligands have been developed, but thus far their clinical success was limited. Rimonabant, a CB1R antagonist, was an efficacious anorectic drug for the treatment of obesity, but two years after its launch in 2006 it was withdrawn from the market due to severe psychiatric side effects[67,68]. Several CB2R agonists were effective in the treatment of (inflammatory) pain in preclinical animal models, but their development has been discontinued due to a lack of analgesic effects in clinical trials[69].

Regulating endogenous ligand levels may provide a more controlled modulation of cannabinoid receptor signaling than direct modulation by synthetic ligands. Therefore, targeting the endocannabinoid metabolic enzymes is a promising avenue for the development of ECS-directed therapeutics. Recently, positive results have been obtained in a phase Ib clinical trial using selective MGLL inhibitor ABX-1431 for the treatment of Tourette syndrome[70–72]. ABPP played a central part in the development of ABX-1431. This technique had previously been used for selectivity profiling of PF04457845, a FAAH inhibitor[73]. Although PF04457845 was effective in increasing plasma levels of NAEs in a first-in-human study[74], the drug exerted no analgesic effects in clinical trials[75]. Several clinical trials for other indications are, however, still on-going[76]. BIA 10-2474, another FAAH inhibitor, produced mild-to-severe neurological symptoms and resulted in the death of a healthy volunteer in a recent phase I clinical trial[77,78]. Given the clinical safety profile of PF04457845, off-target activities of BIA 10-2474 are likely to have contributed to its clinical neurotoxicity. Competitive ABPP may provide insight in the BIA 10-2474 interaction profile.

A better understanding of the ECS in (patho)physiological processes will provide insight in how to regulate cannabinoid receptor signaling and will guide its therapeutic exploitation. Most endocannabinoid metabolic enzymes (including DAGLα, DAGLβ, MGLL, FAAH, ABHD4, ABHD6, ABHD12) belong to the serine hydrolases family and can thus be studied by ABPP using the probes described in Figure 4. ABPP is anticipated to provide new insights in the regulation of the ECS by mapping the endocannabinoid metabolic enzymes in biological processes and facilitate ECS-related drug discovery.

Aim and outline

The aim of the research described in this thesis is to explore activity-based protein profiling (ABPP) as a versatile tool in drug discovery and cell biology.

Chapter 2 describes comparative ABPP as a tool for mapping the serine hydrolase activity profile in clinical samples from patients with terminal-stage heart failure (due to previous ischemic pathology). In **Chapter 3**, the comparative ABPP is method optimized for broad-spectrum profiling of serine hydrolases and kinases in zebrafish larvae during early zebrafish development. In addition, this chapter reports the use of zebrafish larvae as a pre-clinical animal model for *in vivo* target engagement and selectivity screening using FAAH inhibitor PF04457845 in a competitive ABPP setup. In **Chapter 4**, competitive ABPP is employed to investigate the *in vitro* and *in situ* interaction landscape of BIA 10-2474, the FAAH inhibitor that caused severe neurological adverse effects in a recent clinical trial[77,78]. In **Chapter 5**, the enzymes involved in 2-AG biosynthesis during neuronal differentiation are identified using retinoic acid (RA)-induced differentiation of Neuro-2a cells as a model system. A combined strategy of ABPP, CRISPR/Cas9 gene editing, biochemistry and lipidomics identifies ABHD6 as a 2-AG biosynthetic diacylglycerol lipase. **Chapter 6** summarizes the work described in this thesis and discusses the future perspective for ABPP in drug discovery and cellular biology.

References

1. Borchardt, J. K. The Beginnings of Drug Therapy: Ancient Mesopotamian Medicine. *Drug News Perspect.* **15,** 187–192 (2002).
2. Klaeger, S. *et al.* The target landscape of clinical kinase drugs. *Science* **358,** eaan4368 (2017).
3. Scannell, J. W., Blanckley, A., Boldon, H. & Warrington, B. Diagnosing the decline in pharmaceutical R&D efficiency. *Nature Reviews Drug Discovery* **11,** 191–200 (2012).
4. Paul, S. M. *et al.* How to improve RD productivity: The pharmaceutical industry's grand challenge. *Nature Reviews Drug Discovery* **9,** 203–214 (2010).
5. Blass, B. E. *Basic principles of drug discovery and development*.
6. DiMasi, J. A., Hansen, R. W. & Grabowski, H. G. The price of innovation: New estimates of drug development costs. *J. Health Econ.* **22,** 151–185 (2003).
7. DiMasi, J. A., Grabowski, H. G. & Hansen, R. W. Innovation in the pharmaceutical industry: New estimates of R&D costs. *J. Health Econ.* **47,** 20–33 (2016).
8. David, E., Tramontin, T. & Zemmel, R. Pharmaceutical R&D: The road to positive returns. *Nat. Rev. Drug Discov.* **8,** 609–610 (2009).
9. Khanna, I. Drug discovery in pharmaceutical industry: Productivity challenges and trends. *Drug Discovery Today* **17,** 1088–1102 (2012).
10. Nicolaou, K. C. The chemistry-biology-medicine continuum and the drug discovery and development process in academia. *Chemistry and Biology* **21,** 1039–1045 (2014).
11. Eder, J., Sedrani, R. & Wiesmann, C. The discovery of first-in-class drugs: Origins and evolution. *Nat. Rev. Drug Discov.* **13,** 577–587 (2014).
12. Zheng, W., Thorne, N. & McKew, J. C. Phenotypic screens as a renewed approach for drug discovery. *Drug Discovery Today* **18,** 1067–1073 (2013).
13. Avior, Y., Sagi, I. & Benvenisty, N. Pluripotent stem cells in disease modelling and drug discovery. *Nature Reviews Molecular Cell Biology* **17,** 170–182 (2016).
14. MacRae, C. A. & Peterson, R. T. Zebrafish as tools for drug discovery. *Nat. Rev. Drug Discov.* **14,** 721–731 (2015).
15. Heath, J. R., Ribas, A. & Mischel, P. S. Single-cell analysis tools for drug discovery and development. *Nat. Publ. Gr.* **15,** (2015).
16. Tang, F. *et al.* mRNA-Seq whole-transcriptome analysis of a single cell. *Nat. Methods* **6,** 377–382 (2009).
17. Katsila, T., Spyroulias, G. A., Patrinos, G. P. & Matsoukas, M. T. Computational approaches in target identification and drug discovery. *Comput. Struct. Biotechnol. J.* **14,** 177–184 (2016).
18. Liu, Y., Patricelli, M. P. & Cravatt, B. F. Activity-based protein profiling: The serine hydrolases. *Proc. Natl. Acad. Sci.* **96,** 14694–14699 (1999).
19. Cravatt, B. F., Wright, A. T. & Kozarich, J. W. Activity-Based Protein Profiling: From Enzyme Chemistry to Proteomic Chemistry. *Annu. Rev. Biochem.* **77,** 383–414 (2008).
20. Moellering, R. E. & Cravatt, B. F. How chemoproteomics can enable drug discovery and development. *Chemistry and Biology* **19,** 11–22 (2012).
21. Rostovtsev, V. V., Green, L. G., Fokin, V. V. & Sharpless, K. B. A stepwise huisgen cycloaddition process: Copper(I)-catalyzed regioselective 'ligation' of azides and terminal alkynes. *Angew. Chemie - Int. Ed.* **41,** 2596–2599 (2002).
22. Janssen, A. P. A. *et al.* Development of a Multiplexed Activity-Based Protein Profiling Assay to Evaluate Activity of Endocannabinoid Hydrolase Inhibitors. *ACS Chem. Biol.* **13,** 2406–2413 (2018).
23. Niphakis, M. J. & Cravatt, B. F. Enzyme Inhibitor Discovery by Activity-Based Protein Profiling. *Annu. Rev. Biochem.* **83,** 341–377 (2014).
24. van Rooden, E. J., Bakker, A. T., Overkleeft, H. S. & van der Stelt, M. Activity-Based Protein Profiling. *eLS* 1–9 (2018).

25. Baggelaar, M. P. *et al.* Chemical Proteomics Maps Brain Region Specific Activity of Endocannabinoid Hydrolases. *ACS Chem. Biol.* **12,** 852–861 (2017).
26. Nomura, D. K., Dix, M. M. & Cravatt, B. F. Activity-based protein profiling for biochemical pathway discovery in cancer. *Nature Reviews Cancer* **10,** 630–638 (2010).
27. van Rooden, E. J. *et al.* Chemical proteomic analysis of serine hydrolase activity in Niemann-Pick Type C mouse brain. *Front. Neurosci.* **12,** 440 (2018).
28. Van Esbroeck, A. C. M. *et al.* Activity-based protein profiling reveals off-target proteins of the FAAH inhibitor BIA 10-2474. *Science* **356,** 1084–1087 (2017).
29. Simon, G. M. & Cravatt, B. F. Activity-based proteomics of enzyme superfamilies: Serine hydrolases as a case study. *Journal of Biological Chemistry* **285,** 11051–11055 (2010).
30. Kato, D. *et al.* Activity-Based Probes that Target Diverse Cysteine Protease Families. *Nature Chemical Biology* **1,** 33–38 (2005).
31. Li, N. *et al.* Relative quantification of proteasome activity by activity-based protein profiling and LC-MS/MS. *Nat. Protoc.* **8,** 1155–1168 (2013).
32. Zhao, Q. *et al.* Broad-spectrum kinase profiling in live cells with lysine-targeted sulfonyl fluoride probes. *J. Am. Chem. Soc.* **139,** 680–685 (2017).
33. Wright, A. T. & Cravatt, B. F. Chemical Proteomic Probes for Profiling Cytochrome P450 Activities and Drug Interactions In Vivo. *Chem. Biol.* **14,** 1043–1051 (2007).
34. Vocadlo, D. J. & Bertozzi, C. R. A strategy for functional proteomic analysis of glycosidase activity from cell lysates. *Angew. Chemie - Int. Ed.* **43,** 5338–5342 (2004).
35. Kallemeijn, W. W. *et al.* Novel activity-based probes for broad-spectrum profiling of retaining β-exoglucosidases in situ and in vivo. *Angew. Chemie - Int. Ed.* **51,** 12529–12533 (2012).
36. Geurink, P. P., Prely, L. M., Van Der Marel, G. A., Bischoff, R. & Overkleeft, H. S. Photoaffinity labeling in activity-based protein profiling. *Topics in Current Chemistry* **324,** 85–113 (2012).
37. Long, J. Z. & Cravatt, B. F. The metabolic serine hydrolases and their functions in mammalian physiology and disease. *Chem. Rev.* **111,** 6022–6063 (2011).
38. Bachovchin, D. A. & Cravatt, B. F. The pharmacological landscape and therapeutic potential of serine hydrolases. *Nat. Rev. Drug Discov.* **11,** 52–68 (2012).
39. Baggelaar, M. P. *et al.* Development of an activity-based probe and in silico design reveal highly selective inhibitors for diacylglycerol lipase-α in brain. *Angew. Chemie - Int. Ed.* **52,** 12081–12085 (2013).
40. Baggelaar, M. P. *et al.* Highly Selective, Reversible Inhibitor Identified by Comparative Chemoproteomics Modulates Diacylglycerol Lipase Activity in Neurons. *J. Am. Chem. Soc.* **137,** 8851–8857 (2015).
41. Ogasawara, D. *et al.* Rapid and profound rewiring of brain lipid signaling networks by acute diacylglycerol lipase inhibition. *Proc. Natl. Acad. Sci.* **113,** 26–33 (2016).
42. Tarkiainen, T. H. *et al.* Stability over time of short-term heart rate variability. *Clin. Auton. Res.* **15,** 394–399 (2005).
43. Manning, G., Whyte, D. B., Martinez, R., Hunter, T. & Sudarsanam, S. The protein kinase complement of the human genome. *Science* **298,** 1912–34 (2002).
44. Gaoni, Y. & Mechoulam, R. Isolation, Structure, and Partial Synthesis of an Active Constituent of Hashish. *J. Am. Chem. Soc.* **86,** 1646–1647 (1964).
45. Mechoulam, R. & Parker, L. A. The Endocannabinoid System and the Brain. *Annu. Rev. Psychol.* **64,** 21–47 (2012).
46. Di Marzo, V. Endocannabinoid signaling in the brain: biosynthetic mechanisms in the limelight. *Nat. Neurosci.* **14,** 9–15 (2011).
47. Herkenham, M. *et al.* Cannabinoid receptor localization in brain. *Proc. Natl. Acad. Sci.* **87,** 1932–1936 (1990).
48. Cabral, G. A. & Griffin-Thomas, L. Emerging role of the cannabinoid receptor CB 2 in immune regulation: Therapeutic prospects for neuroinflammation. *Expert Reviews in Molecular Medicine* **11,** e3 (2009).

49. Calignano, A., Rana, G. La, Giuffrida, A. & Piomelli, D. Control of pain initiation by endogenous cannabinoids. *Nature* **394,** 277–281 (1998).
50. Di Marzo, V. & Matias, I. Endocannabinoid control of food intake and energy balance. *Nat. Neurosci.* **8,** 585–589 (2005).
51. Pacher, P., Bátkai, S. & Kunos, G. The Endocannabinoid System as an Emerging Target of Pharmacotherapy. *Pharmacol. Rev.* **58,** 389–462 (2006).
52. Devane, W. A. *et al.* Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258,** 1946–1949 (1992).
53. Mechoulam, R. *et al.* Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* **50,** 83–90 (1995).
54. Sugiura, T. *et al.* 2-arachidonoylglycerol: A possible endogenous cannabinoid receptor ligand in brain. *Biochemical and Biophysical Research Communications* **215,** 89–97 (1995).
55. Bisogno, T. *et al.* Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. *J. Cell Biol.* **163,** 463–468 (2003).
56. Dinh, T. P., Freund, T. F. & Piomelli, D. A role for monoglyceride lipase in 2-arachidonoylglycerol inactivation. in *Chemistry and Physics of Lipids* **121,** 149–158 (2002).
57. Savinainen, J. R., Saario, S. M. & Laitinen, J. T. The serine hydrolases MAGL, ABHD6 and ABHD12 as guardians of 2-arachidonoylglycerol signalling through cannabinoid receptors. *Acta Physiologica* **204,** 267–276 (2012).
58. Marrs, W. R. *et al.* The serine hydrolase ABHD6 controls the accumulation and efficacy of 2-AG at cannabinoid receptors. *Nat. Neurosci.* **13,** 951–7 (2010).
59. Blankman, J. L., Simon, G. M. & Cravatt, B. F. A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. *Chem. Biol.* **14,** 1347–1356 (2007).
60. Ueda, N., Tsuboi, K. & Uyama, T. Metabolism of endocannabinoids and related N-acylethanolamines: Canonical and alternative pathways. *FEBS J.* **280,** 1874–1894 (2013).
61. Cravatt, B. F. *et al.* Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* **384,** 83–87 (1996).
62. Mckinney, M. K. & Cravatt, B. F. Structure and function of Fatty Acid Amide Hydrolase. *Annu. Rev. Biochem.* **74,** 411–432 (2005).
63. Di Marzo, V., Stella, N. & Zimmer, A. Endocannabinoid signalling and the deteriorating brain. *Nat. Rev. Neurosci.* **16,** 30–42 (2015).
64. Montecucco, F. & Di Marzo, V. At the heart of the matter: The endocannabinoid system in cardiovascular function and dysfunction. *Trends in Pharmacological Sciences* **33,** 331–340 (2012).
65. Di Marzo, V., Bifulco, M. & De Petrocellis, L. The endocannabinoid system and its therapeutic exploitation. *Nature Reviews Drug Discovery* **3,** 771–784 (2004).
66. Di Marzo, V. Targeting the endocannabinoid system: To enhance or reduce? *Nature Reviews Drug Discovery* **7,** 438–455 (2008).
67. Christensen, R., Kristensen, P. K., Bartels, E. M., Bliddal, H. & Astrup, A. Efficacy and safety of the weight-loss drug rimonabant: a meta-analysis of randomised trials. *Lancet* **370,** 1706–1713 (2007).
68. Moreira, F. A. & Crippa, J. A. S. The psychiatric side-effects of rimonabant. *Rev. Bras. Psiquiatr.* **31,** 145–153 (2009).
69. Dhopeshwarkar, A. & Mackie, K. CB2 Cannabinoid Receptors as a Therapeutic Target--What Does the Future Hold? *Mol. Pharmacol.* **86,** 430–437 (2014).
70. Abide Therapeutics. Abide Therapeutics Presents Positive Data from a Phase 1b Study of ABX-1431 in Tourette Syndrome at the American Academy of Neurology 70th Annual Meeting. Available at: http://abidetx.com/news/abide-therapeutics-presents-positive-data-from-a-phase-1b-study-of-abx-1431-in-tourette-syndrome-at-the-american-academy-of-neurology-70th-annual-meeting/. (Accessed: 28th January 2019)

71. Therapeutics, A. Abide Therapeutics Announces Initiation of Phase 2 Clinical Trial of ABX-1431 in Tourette Syndrome. Available at: http://abidetx.com/news/abide-therapeutics-announces-initiation-of-phase-2-clinical-trial-of-abx-1431-in-tourette-syndrome/. (Accessed: 28th January 2019)
72. Cisar, J. S. *et al.* Identification of ABX-1431, a Selective Inhibitor of Monoacylglycerol Lipase and Clinical Candidate for Treatment of Neurological Disorders. *J. Med. Chem.* **61,** 9062–9084 (2018).
73. Johnson, D. S. *et al.* Discovery of PF-04457845: A highly potent, orally bioavailable, and selective urea FAAH inhibitor. *ACS Med. Chem. Lett.* **2,** 91–96 (2011).
74. Li, G. L. *et al.* Assessment of the pharmacology and tolerability of PF-04457845, an irreversible inhibitor of fatty acid amide hydrolase-1, in healthy subjects. *Br. J. Clin. Pharmacol.* **73,** 706–716 (2012).
75. Huggins, J. P., Smart, T. S., Langman, S., Taylor, L. & Young, T. An efficient randomised, placebo-controlled clinical trial with the irreversible fatty acid amide hydrolase-1 inhibitor PF-04457845, which modulates endocannabinoids but fails to induce effective analgesia in patients with pain due to osteoarthritis of th. *Pain* **153,** 1837–1846 (2012).
76. National Institute of Health. Home - ClinicalTrials.gov. *U.S. National Library of Medicine* (2017). Available at: https://www.clinicaltrials.gov/. (Accessed: 28th January 2019)
77. Bégaud, B. *et al.* BIA 10-2474: Minutes of the Temporary Specialist Scientific Committee (TSSC) meeting on 'FAAH (Fatty Acid Amide Hydrolase) Inhibitors'. *Meet. Minutes* 1–14 (2016).
78. Kerbrat, A. *et al.* Acute Neurologic Disorder from an Inhibitor of Fatty Acid Amide Hydrolase. *N. Engl. J. Med.* **375,** 1717–1725 (2016).