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Activity-based protein profiling in drug-discovery

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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 discovery².

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 bodies³⁻⁵.

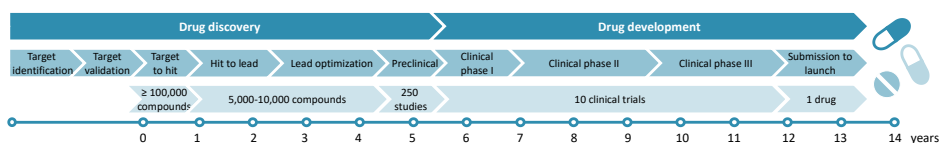


Figure 1 | The drug discovery and drug development process. Schematic representation of the stages in drug discovery and drug development, with their estimated duration and number of compounds/studies required for the development of a single approved drug (modified from literature³⁻⁵).

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 billion⁵⁻⁷ 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 process¹². 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 zebrafish¹⁴ as alternative models in phenotypic screening, single-cell analysis techniques¹⁵ like RNA-seq¹⁶, and computational approaches¹⁷. 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 targets¹⁹. 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 group²¹. 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 permeability²².

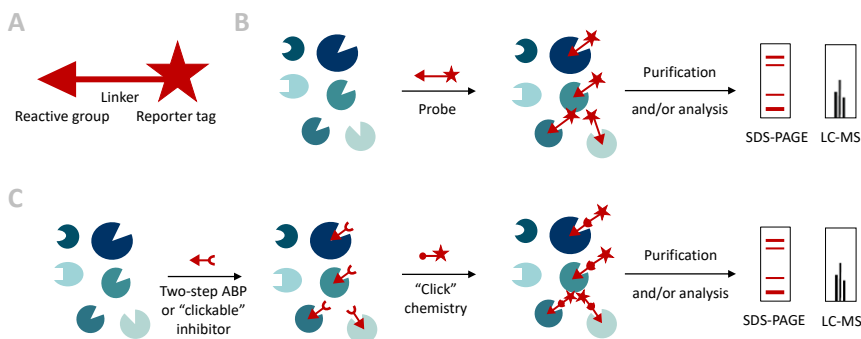


Figure 2 | Activity-based protein profiling. (A) An activity-based probe consists of a reactive group coupled to a reporter tag (a fluorophore for gel-based analysis by SDS-PAGE or an affinity tag for purification and analysis by LC-MS) by a chemical linker element. (B, C) Schematic representation of (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.

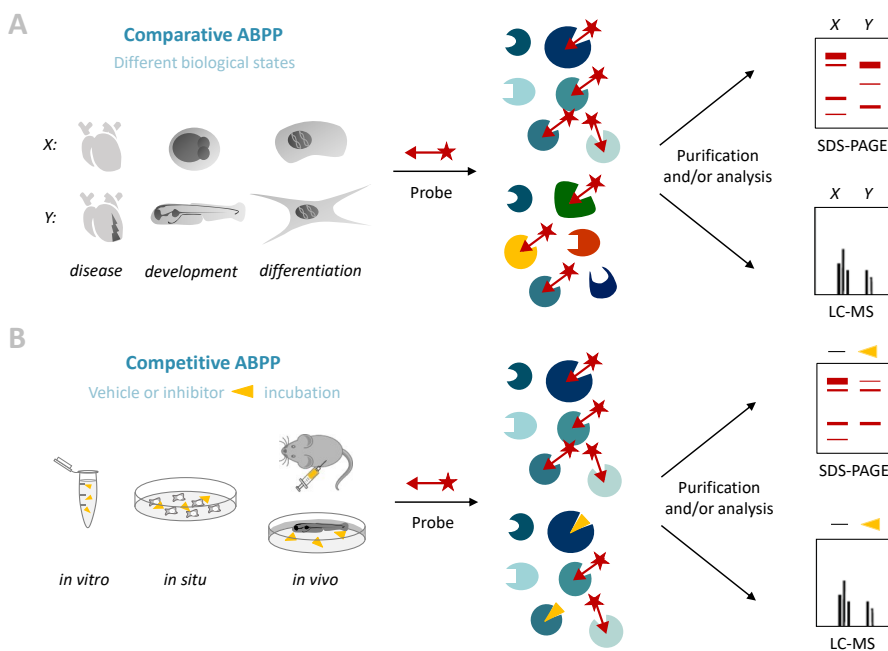


Figure 3 | Competitive and comparative ABPP. (A) In comparative ABPP two proteomes from different biological states, e.g. healthy versus diseased or different cell types, are compared by gel- or MS-based analysis of probe treated samples. (B) Competitive ABPP enables target-engagement assessment and 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 inhibitor²⁸.

Finally, ABPP can serve as a tool for the characterization of unannotated proteins. Interaction between an ABP and an uncharacterized protein can facilitate the assignment of the protein to a specific enzymatic class or family¹⁹.

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}, kinases³², phosphatases, cytochrome P450s³³, 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- irradiation³⁶.

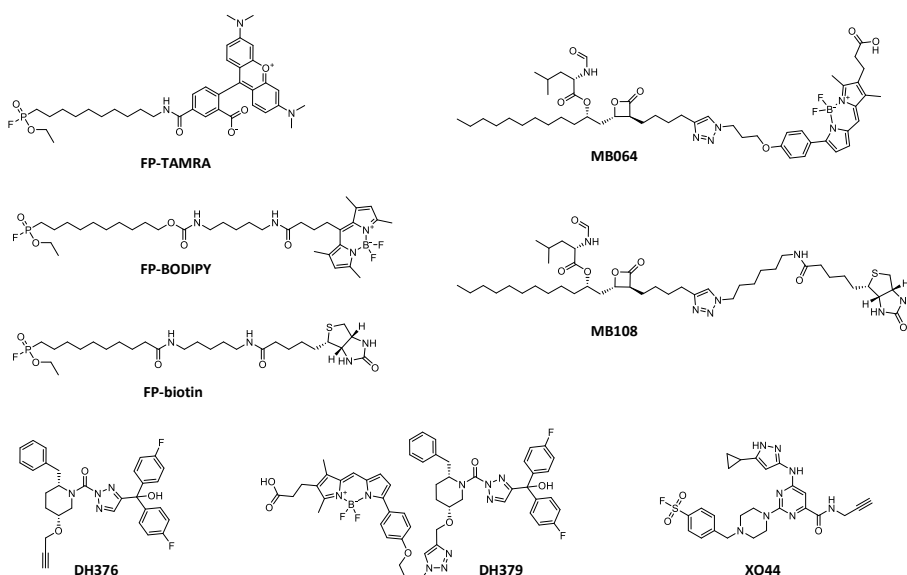


Figure 4 | Activity-based probes. Broad-spectrum serine-hydrolase probes fluorophosphonate (FP)-TAMRA, FP-BODIPY and FP-biotin. Tailored lipase probes MB064 and MB108, targeting e.g. DAGL α/β . Two-step DAGL-probe DH376, containing an alkyne ligation handle, and its derived direct probe DH379. Reactive-lysine 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 GX SXG 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-BODIPY²², which are especially suitable for broad-spectrum profiling. Tailored probes like the β -lactone-based MB064³⁹ and MB108⁴⁰ or selective

probes like DH376 and DH379⁴¹ 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 XO44³². 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 THC⁴⁶. The CB1R is most abundant in the central nervous system⁴⁷, while CB2R is mainly expressed in cells of the immune system⁴⁸. The ECS mediates numerous physiological processes, including appetite and metabolism, learning and memory, pain sensation and anxiety, and inflammation^{45,49–51}.

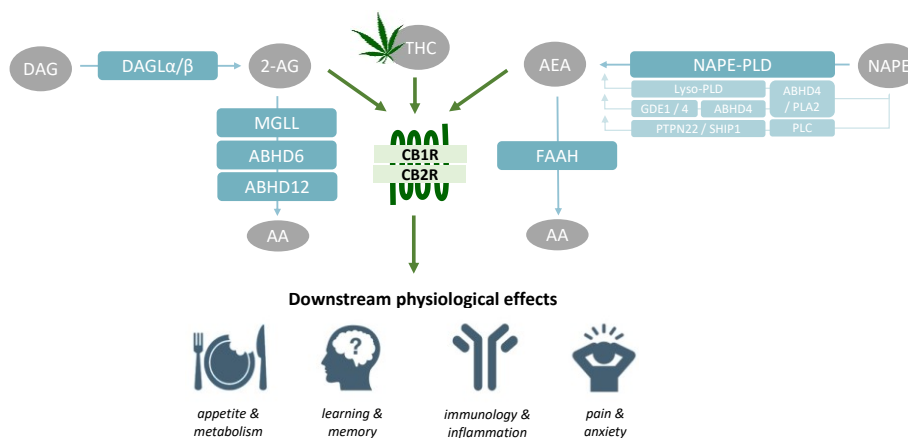


Figure 5 | The endocannabinoid system. The endocannabinoid system is involved in multiple physiological processes and is comprised of the cannabinoid receptors 1 and 2 (CB1R, CB2R), their endogenous ligands (endocannabinoids) and their metabolic enzymes. Ligands & metabolites (grey): DAG: diacylglycerol, 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 A₂, GDE1/4: glycerophosphodiesterase 1 and 4, Lyso-PLD: unidentified lysophospholipase 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*-arachidonylethanolamide (anandamide, AEA) was identified as the first endocannabinoid⁵². 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 endocannabinoids⁴⁵. 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 enzymes⁴⁶.

Several metabolic pathways control the endocannabinoid levels (Figure 5). Diacylglycerol lipase α and β (DAGL α , DAGL β) are the two main enzymes producing 2-AG, by catalyzing the hydrolysis of diacylglycerol (DAG) to 2-AG⁵⁵. 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*-acylphosphatidylethanolamines (NAPEs) to NAEs (including AEA) by NAPE phospholipase D (NAPE-PLD) is considered to be the canonical pathway⁶⁰. 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 A₂ (PLA2) and ABHD4 mediated conversion of NAPE to lyso-NAPE, followed by conversion to AEA by an unknown lysophospholipase D (Lyso-PLD); (II) conversion of lyso-NAPE to glycerophospho-AEA by ABHD4 and subsequent hydrolysis to AEA by glycerophosphodisesterase 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 neurological⁶³ and cardiovascular⁶⁴ 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 trials⁶⁹.

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 inhibitor⁷³. Although PF04457845 was effective in increasing plasma levels of NAEs in a first-in-human study⁷⁴, the drug exerted no analgesic effects in clinical trials⁷⁵. Several clinical trials for other indications are, however, still on-going⁷⁶. 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 DAGL α , DAGL β , 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.

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