Bioconjugate Chemistry

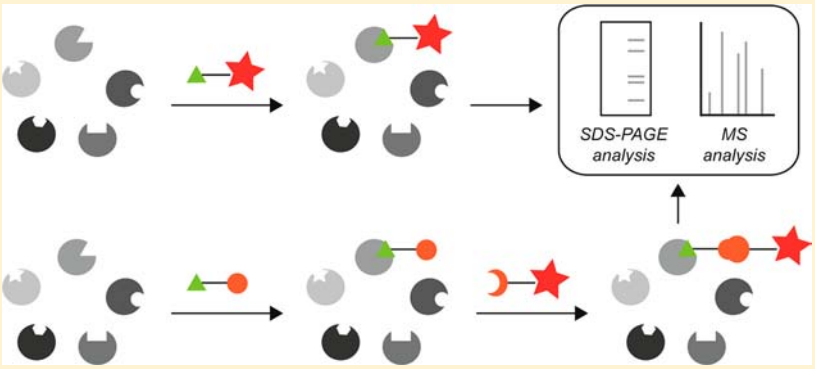
Review

pubs.acs.org/bc

Current Developments in Activity-Based Protein Profiling

Lianne I. Willems,[†] Herman S. Overkleeft,*[,†] and Sander I. van Kasteren*[,†]

[†]Leiden University, Leiden Institute of Chemistry, Gorlaeus Laboratories, Einsteinweg 55, 2333 CC Leiden, The Netherlands



ABSTRACT: Activity-based protein profiling (ABPP) has emerged as a powerful strategy to study the activity of enzymes in complex proteomes. The aim of ABPP is to selectively visualize only the active forms of particular enzymes using chemical probes termed activity-based probes (ABPs). These probes are directed to the active site of a particular target protein (or protein family) where they react in a mechanism-based manner with an active site residue. This results in the selective labeling of only the catalytically active form of the enzyme, usually in a covalent manner. Besides the monitoring of a specific enzymatic activity, ABPP strategies have also been used to identify and characterize (unknown) protein functions, to study up- and down-regulation of enzymatic activity in various disease states, to discover and evaluate putative new enzyme inhibitors, and to identify the protein targets of covalently binding natural products. In this Topical Review we will provide a brief overview of some of the recent developments in the field of ABPP.

Activity-based protein profiling (ABPP) has emerged as a powerful strategy to study the activity of enzymes in complex proteomes.[1] Enzyme expression levels often do not correlate directly to their activity, as the activities of many enzymes are regulated by post-translational modifications, protein−protein interactions, and/or the action of endogenous small molecules and protein attenuators.[2] This importance of monitoring enzymatic activity in relation to their expression levels can be illustrated using the endolysosomal proteases. These enzymes are highly proteolytic with limited specificity[3] and therefore inherently destructive even to self-proteins. The presence of proteolytically active endolysosomal cysteine cathepsins in the cytosol, for example, is a strong enough danger to instigate inflammatory apoptosis.[4] In order to carry out their function in their designated organelle, endolysosomal proteases are synthesized as zymogens with an inhibitory peptide blocking the active site. Only in the acidic environment of the endolysosomal system are these pro-peptides removed, liberating the active enzyme.[5] Methods to quantify mRNA or protein levels could therefore result in misinterpretation of the role of these proteases, as the active populations of the enzymes are most important for their biological function.

The aim of ABPP is to selectively visualize only the *active* forms of particular enzymes using chemical probes termed activity-based probes (ABPs). These probes are directed to the active site of a particular target protein (or protein family) where they react in a mechanism-based manner with an active site residue. This results in the selective labeling of only the catalytically active form of the enzyme, usually in a covalent manner. Since the first description of a radioactive proto-ABP against esterases by Ostrowski and Barnard in 1961,[6] a large number of ABPs that target many different enzyme classes has been developed.[7] These probes have enabled the selective labeling of known enzymatic activity in complex biological samples such as cell lysates, cell cultures, and living organisms.[1a−c] Besides monitoring a specific enzymatic activity, ABPP strategies have also been used to identify and characterize (unknown) protein functions, to study up- and down-regulation of enzymatic activity in various disease states, to discover and evaluate putative new enzyme inhibitors, and to identify the protein targets of covalently binding natural products.[1] In this Topical Review we will provide a brief overview of some of the recent developments in the field of ABPP.

GENERAL STRATEGY FOR ABP DEVELOPMENT

In general, ABPs contain three essential structural elements (Figure 1A). These elements are a *reactive group*, which reacts with the active site of the target enzyme (also called the

Received: May 13, 2014
Revised: June 16, 2014
Published: June 19, 2014

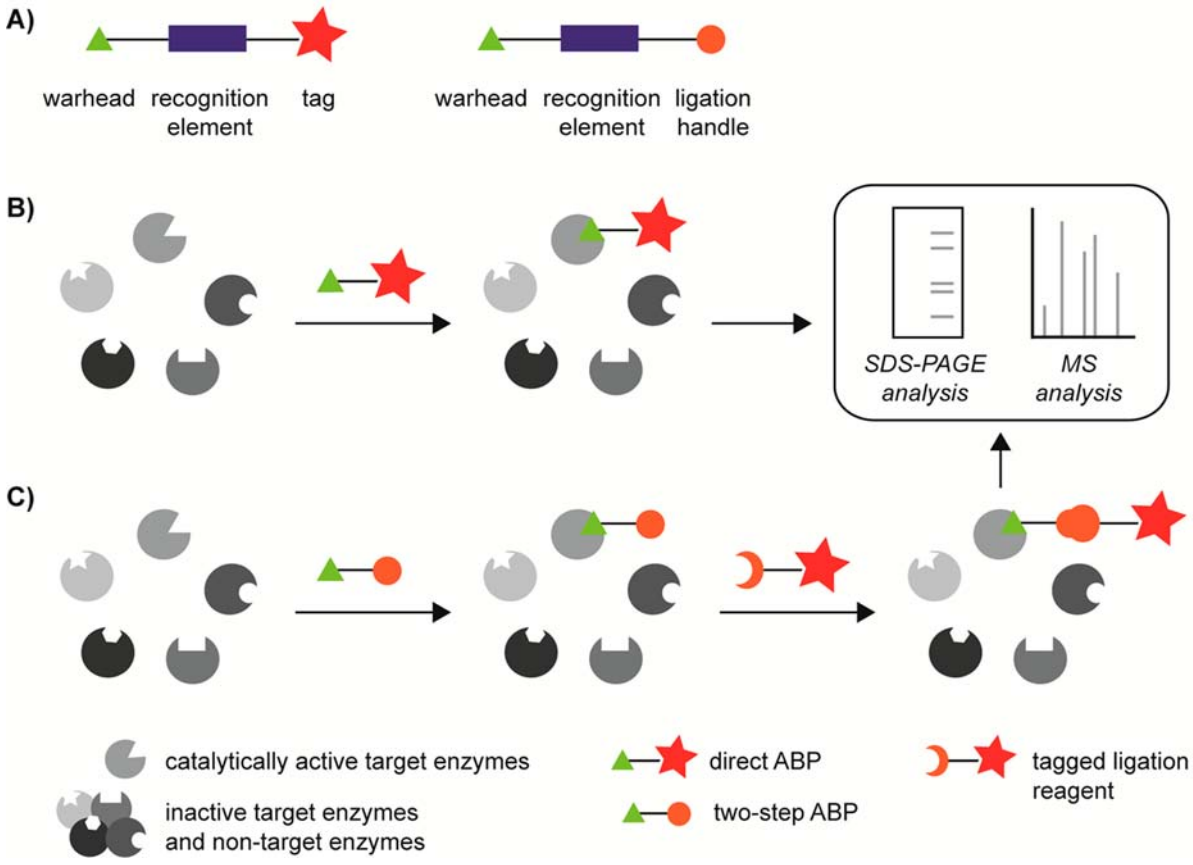


Figure 1. Schematic representations of one- and two-step ABPP strategies. (A) General composition of a direct ABP (left) and a two-step ABP (right). (B,C) Labeling of enzymatic activity by a direct ABP (B) or a two-step ABP (C). SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis. MS: mass spectrometry.

"warhead"); a *recognition element*, which imposes selectivity upon the warhead for a specific enzyme or enzyme class; and finally, a *detectable agent* that allows the retrieval or imaging of the enzymes in question.

The warhead mediates irreversible binding of the probe to the enzyme of interest by reacting in a mechanism-based manner with the catalytically active amino acid residue of the enzyme. It is often an electrophilic moiety that is susceptible to attack by the active site nucleophile. The warhead needs to be sufficiently reactive to allow reaction with the intended active site residues. However, it should be unreactive enough not to react with off-target amino acids or any other functionality present in the sample.

The recognition element confers selectivity toward a specific protein of interest by binding the target protein and bringing the warhead of the probe and the active site nucleophile in close proximity, thereby stimulating their reaction. Together, the structure of the recognition element and the reactivity of the warhead can be tuned to provide selective targeting of a specific enzyme or multiple members of an enzyme class.

ABPs attached to active enzymes can be detected using a detectable group (Figure 1B). Commonly used reporter entities for ABPP studies include fluorescent tags that enable visualization of the labeled proteins in an SDS-PAGE experiment and by fluorescence microscopy, affinity tags such as biotin for detection in blotting experiments or even the retrieval of labeled proteins using a solid-support resin for subsequent mass spectrometry analysis, or a combination of these.[8] Quenched fluorophores that only become fluorescent after binding of the ABP to a target enzyme can strongly reduce background labeling and thereby provide significant advantages for (in vivo) imaging applications.[9] An alternative approach is two-step labeling, in which the ABP is first modified with a small bioorthogonal chemical group[10] and subsequently modified with a reporter entity (Figure 1C). This approach provides the advantages that multiple visualization and retrieval handles can be introduced in one experiment and that cell-permeability of such two-step ABPs carrying small bioorthogonal handles is generally improved. The use of two-step labeling strategies will be discussed in detail further in this Topical Review.

The three main elements of ABPs may be separated by additional linkers that can be used to minimize steric hindrance or modulate solubility and membrane-permeability of the probe. Moreover, chemically cleavable linkers may be included to facilitate the selective release of target proteins after affinity purification[10,11] and targeting elements can be added for targeted delivery of the probe to a specific cell type or cellular compartment.[12]

The enzymes that are most readily targeted by ABPs are those that form a covalent intermediate with their substrate at some point in the catalytic mechanism. The formation of such an intermediate can be exploited for the design of a probe that reacts in a manner similar to the substrate and thereby forms a covalent and irreversible bond with the active site of the enzyme. An example is the labeling of cysteine proteases using the biotinylated peptide epoxysuccinate DCG-04 (**1**; Figure 2A), which was shown to react with a number of cysteine

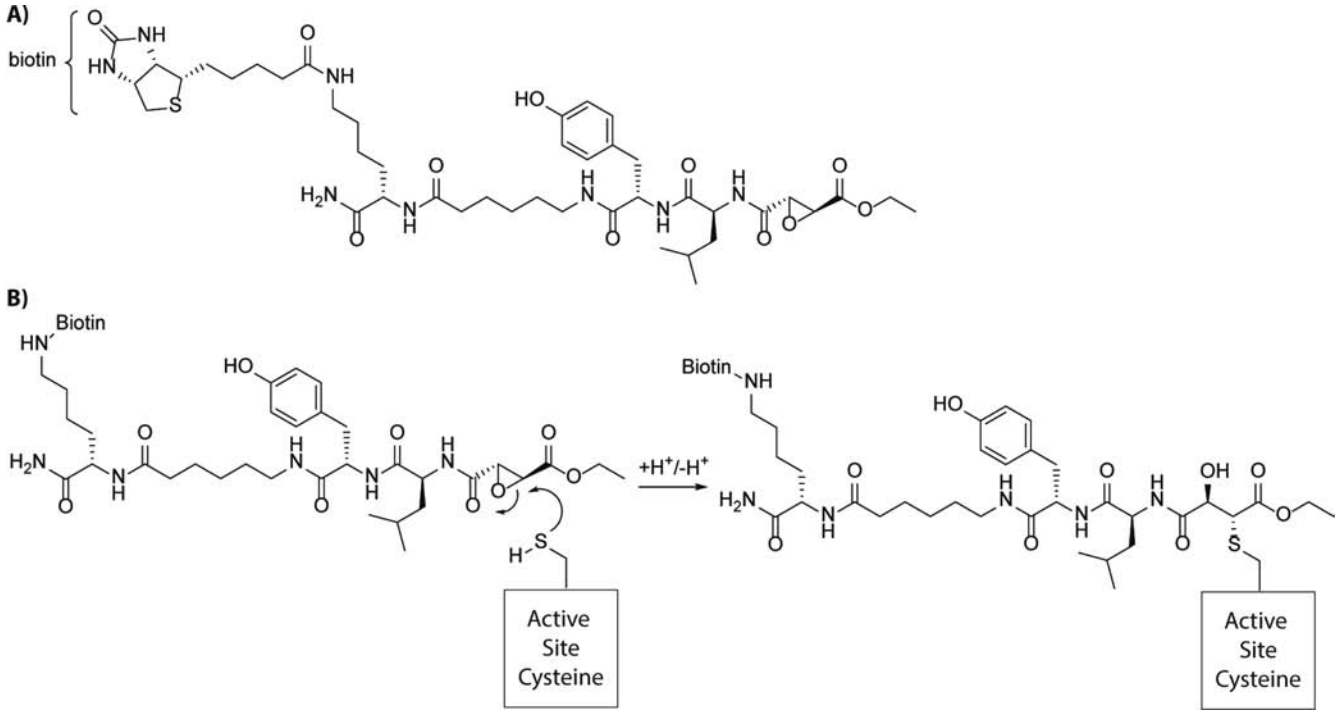


Figure 2. (A) DCG-04, a biotin-tagged cysteine protease ABP (**1**). (B) Mechanism of action of **1** reacting with the active-site cysteine nucleophile in papain-like cysteine proteases.

proteases of the cathepsin family in cell extracts.[13] The epoxide moiety of the ABP, which functions as the warhead, is attacked by the catalytically active cysteine residue in the active site, resulting in opening of the epoxide and the formation of a stable covalent bond between the enzyme and the probe (Figure 2B).[14]

In cases where no covalent enzyme−substrate intermediate is formed, activity-based profiling can be achieved by applying suicide substrates that act via an indirect binding mechanism. For example, fluorogenic ABP **2** (Figure 3A) was developed to report on the activity of β-galactosidases by reacting as shown in Figure 3B.[15] This probe contains a latent quinone methide precursor which is released upon hydrolysis by a target enzyme. The highly reactive species that is formed can subsequently react with a nearby nucleophilic amino acid residue, yielding an immobilized fluorescent label. Although suicide substrates bind in a mechanism-based manner, the reactive species that is released may react with a nucleophile distant from the active site or even another component in the sample, so that this type of ABP does not specifically label the protein of interest. In this Topical Review the focus is on the first class of ABPs which directly modify a catalytically active amino acid residue. It should be kept in mind that the use of these probes results in the subsequent inhibition of catalytic activity and may thereby potentially disturb the system at hand, especially when applied to living organisms.

SELECTIVITY VERSUS REACTIVITY

One of the main challenges when designing an ABP is to find the appropriate balance between the selectivity of the probe (that is its target-to-background reactivity) and the reactivity toward the target protein. A fine balance between reactivity and selectivity of an ABP is essential to achieve specific labeling of a desired protein or set of proteins. The rational design of a warhead, in particular, its reactivity, can be an important factor to modify the range of enzymes that is targeted by an ABP. This aspect of ABP design is clearly illustrated by the development of probes **3**−**5** (Figure 4), which contain either an α-halogenated acetamide or a fluoromethylketone as the warhead.[16] Iodide is a good leaving group and the iodoacetamides are excellent electrophiles for S_N2 reactions with soft nucleophiles such as thiolates, while being stable toward nucleophilic attack by hard nucleophiles such as water. This results in a high intrinsic reactivity toward cysteine residues, so that tagged derivatives such as biotinylated probe **3**[16a] and two-step probe **4**[16b] are capable of labeling virtually all cysteine residues within a biological sample. Consequently, these reagents are not applicable as ABPs but they are generally used for the global profiling of protein expression by mass spectrometry analysis. The incorporation of a "light" (hydrogen) or "heavy" (deuterium) isotope-labeled linker into the ABP (**3**) or, in case of probe **4**, into the ligation reagent enables the relative quantification and parallel identification of individual proteins from two complex biological samples. In addition to these global labeling experiments, probe **4** was used to demonstrate how selectivity can be realized without lowering the reactivity of the probe.[16b] Highly reactive cysteine residues, which likely include catalytic residues and, for example, sites of post-translational modification, were discriminated from cysteine residues with low reactivity by using varying concentrations of the iodoacetamide reagent. While the cysteine residues with low reactivity are labeled in a concentration-dependent manner, labeling of the most reactive cysteine residues is equally efficient when a sub-stoichiometric amount or an excess of the reagent is used. This strategy enables the annotation of highly reactive cysteines regardless of their natural abundance and can be used to identify new residues with putative catalytic activity.

In contrast to the iodoacetamide reagents, fluoromethylketone inhibitors and their derivatives, such as the fluorescein-

dx.doi.org/10.1021/bc500208y | Bioconjugate Chem. 2014, 25, 1181−1191

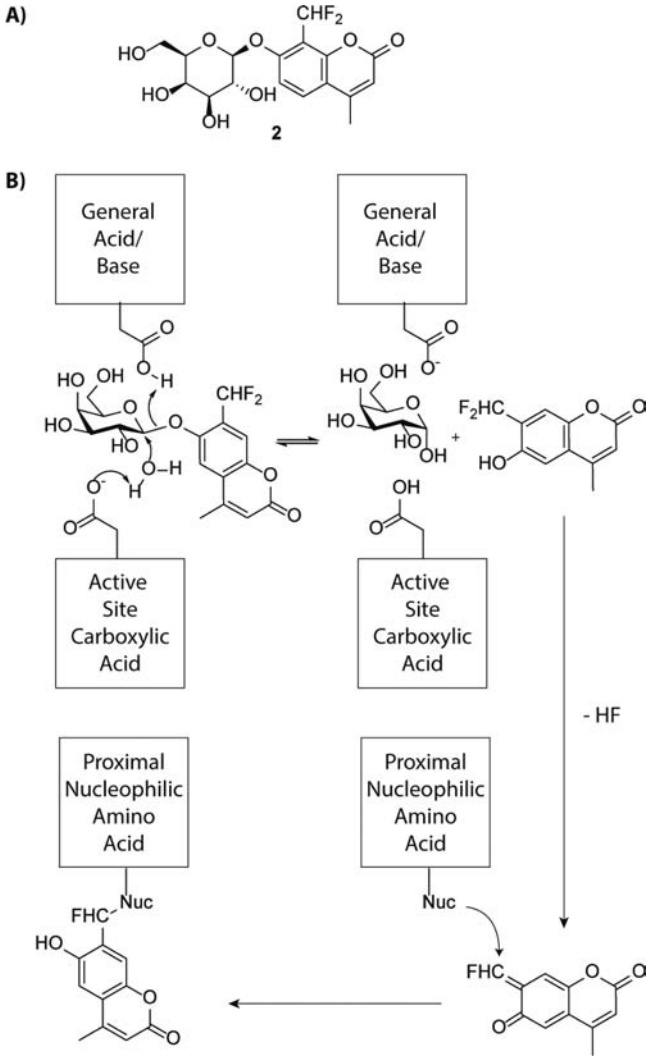


Figure 3. (A) Indirect alkylation-based ABP for β-galactosidases (**2**). (B) Mechanism of action of ABP **2** labeling a nearby nucleophilic amino acid residue after cleavage in the active site of a target enzyme.

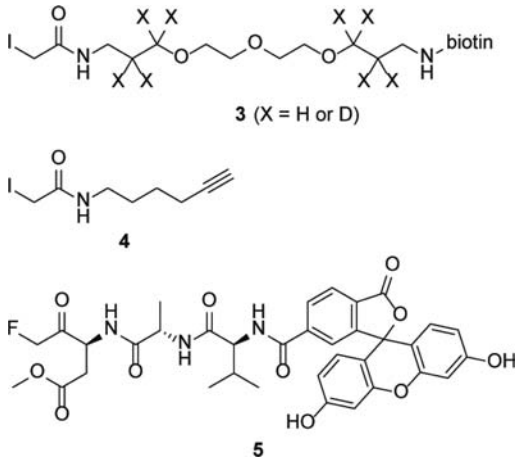


Figure 4. Structures of reactivity-based probes: biotinylated (**3**) and alkyne-functionalized (**4**) iodoacetamide reagents react with cysteine nucleophiles without a recognition element imbuing specificity. ABP **5** reacts selectively with caspases due to the relatively low nucleophilicity of the fluoromethylketone warhead and a small caspase recognition element.

functionalized ABP **5** (Figure 4),[16c] have a very low intrinsic reactivity toward cysteine residues because the fluoride is a very poor leaving group and the carbon−fluorine bond is much more stable than the carbon−iodine bond. In this case, selective activity-based labeling of a specific class of cysteine proteases, the caspases, was achieved by incorporation of a peptide into the ABP that specifically binds these enzymes and thereby brings the warhead in close proximity to the active site cysteine residue so that a reaction takes place.

ABPs based on the small (76 amino acids) protein ubiquitin offer another example of reactivity vs selectivity tuning (**6a−f**, Figure 5). These ABPs can be used to target deubiquitinating

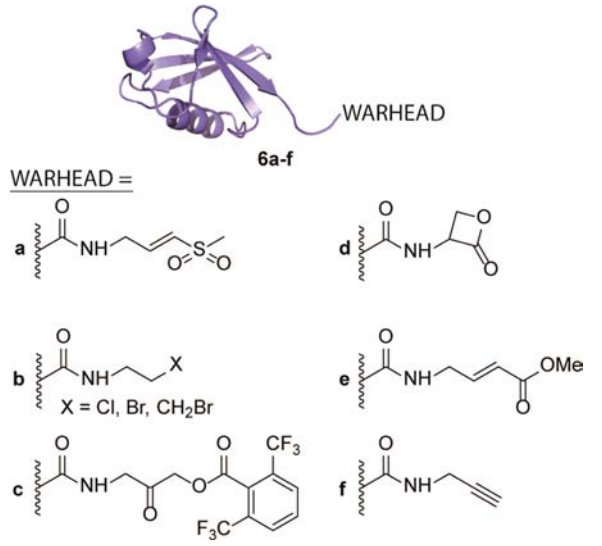


Figure 5. Structures of ABPs for deubiquitinating enzymes and ubiquitin ligases: the *C*-terminal glycine residue of ubiquitin can be replaced by a series of warheads, each with different DUB selectivity and specificity.

enzymes (DUBs), a class of proteases that remove ubiquitin-proteins from substrate proteins which are linked via their *C*-terminus to the ε-amine group of a lysine residue on the substrate or on another ubiquitin. The first example of an ABP for the cysteine-DUBs was reported by the Ploegh lab.[17] They used a native chemical ligation strategy to substitute the *C*-terminal glycine residue of recombinant ubiquitin with a vinyl sulfone warhead (**6a**). Subsequent radioiodination led to an ABP that labels a number of deubiquitinating enzymes in cell extracts. In later studies, other thiol-reactive electrophiles were attached to epitope-tagged ubiquitin to modify the reactivity of the probes toward specific deubiquitinating and ubiquitin-conjugating enzymes.[18] It was found that different classes of deubiquitinating enzymes were isolated depending on the nature of the warhead: those based on Michael-addition reactions resulted in the retrieval of different classes of DUBs than those based on substitution reactions.[18a] The vinyl sulfone warhead, for example, did not react with cysteine DUBs from the OTU-family, whereas the bromoalkyl warhead (**6b**, X = Br) did react with these. The recently reported 2,6-trifluoromethylbenzyloxymethylketone (TF_3BOK, **6c**) and β-lactone (**6d**) warheads were shown to be reactive toward cysteine residues with pK_a values 2 units higher than those of DUBs reactive with vinyl methylester (**6e**). Vinyl sulfone warheads were even used to label the cysteine residues of ligases from the ubiquitin-conjugating machinery.[18b] Ekkebus et al.[19] and Sommer et al.[20]

dx.doi.org/10.1021/bc500208y | *Bioconjugate Chem.* 2014, 25, 1181−1191

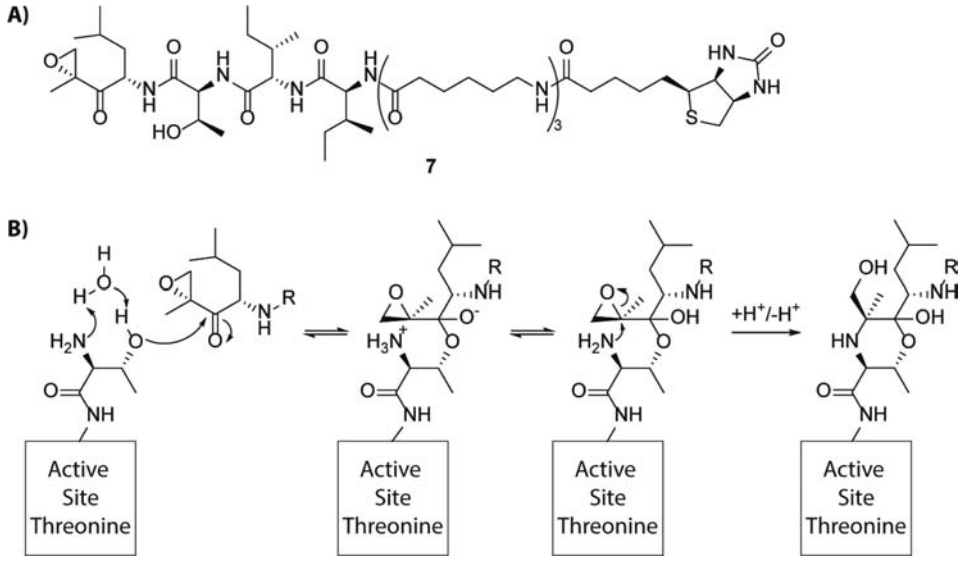


Figure 6. (A) Biotinylated epoxomicin-derived proteasome probe **7**. (B) Binding mechanism of ABP **7** to active proteasome β-subunits.

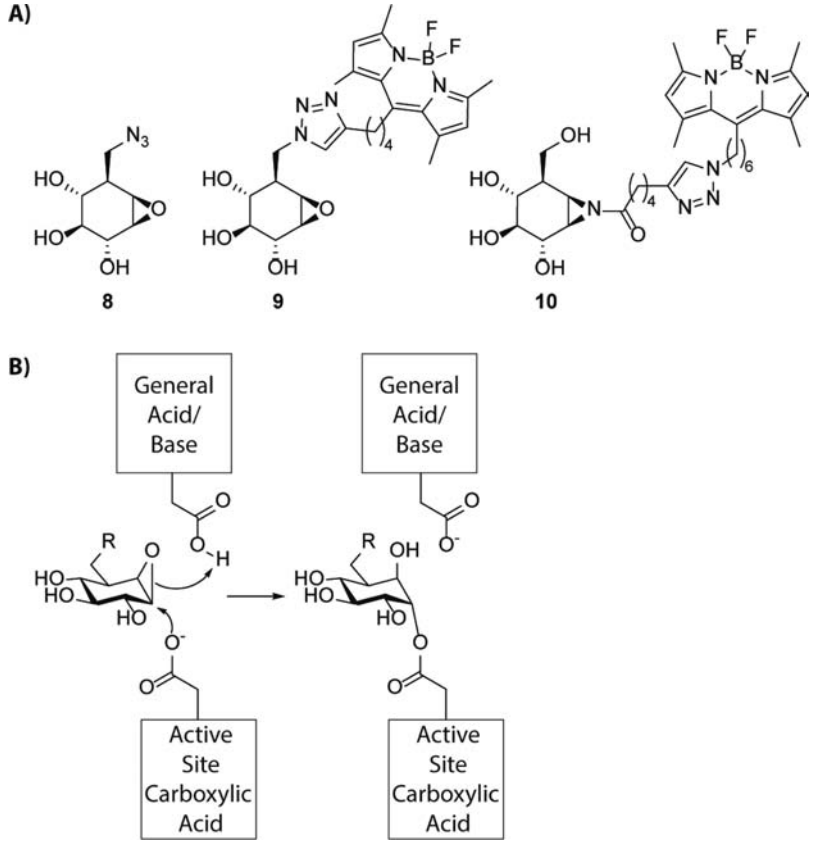


Figure 7. (A) Azide-tagged (**8**) and fluorescently labeled (**9**) cyclophellitol derivatives and fluorescently labeled aziridine-based probe **10** to target retaining β-glucosidases. (B) Mechanism of retaining β-glucosidase labeling by ABPs **8** and **9**.

recently reported DUB- and SUMO-ABPs based on an alkyne as a warhead (**6f**). This was surprising as the alkyne is essentially a very poor electrophile and is therefore frequently used for bioorthogonal ligation reactions. When attached to the *C*-terminus of ubiquitin or SUMO, however, it can be used to label deubiquitinating enzymes in an activity-based manner; even those that are not labeled by existing vinyl methylester and vinyl sulfone probes. These results nicely reveal that attachment of a warhead, that is generally considered to be unreactive toward nucleophiles, to a specific and tightly binding substrate can induce reaction with the catalytically active cysteine residue. The reactivity vs selectivity balance was further highlighted by comparing caspase probes bearing either an aldehyde or an alkyne warhead. The peptide-aldehyde inhibitor of caspase-1, Ac-YVAD-aldehyde, is a potent caspase inhibitor.[21] Conversion of the *C*-terminal aldehyde to an alkyne using the Bestmann-Ohira reagent[22] completely ablated the inhibitory potential of this compound, presumably due to the low affinity of this

dx.doi.org/10.1021/bc500208y | *Bioconjugate Chem.* 2014, 25, 1181−1191

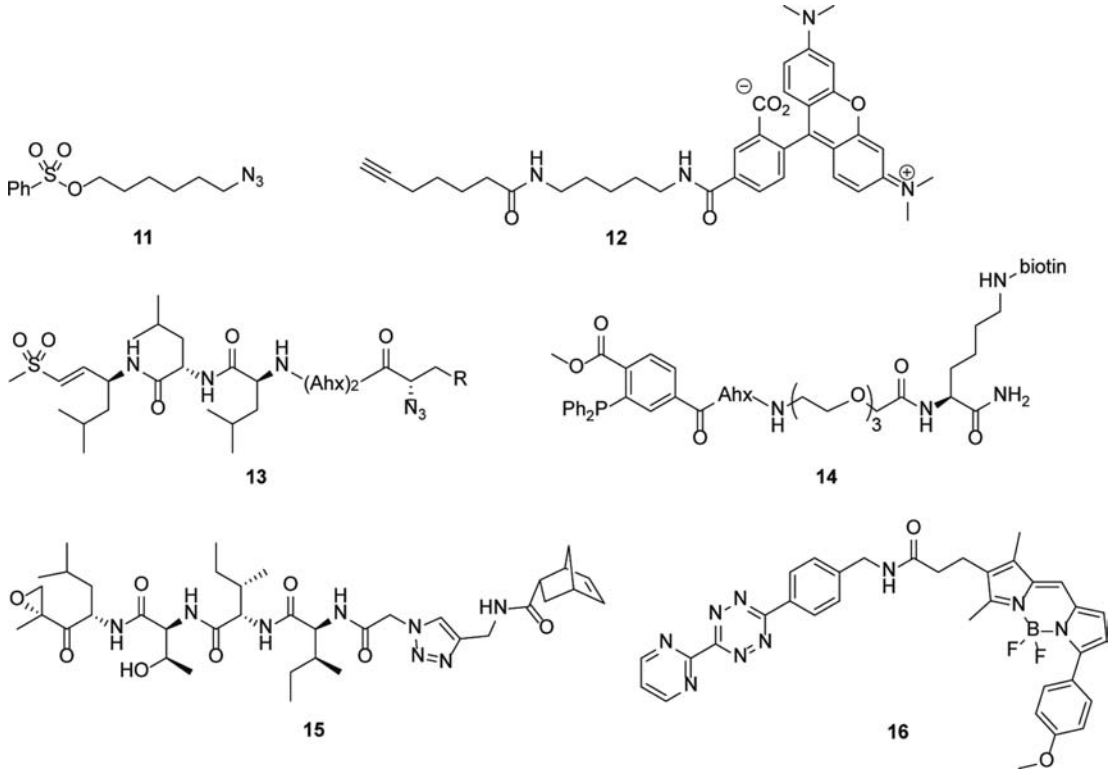


Figure 8. Examples of two-step ABPP reagents: azide-functionalized ABPs **11** and **13**, norbornene-modified ABP **15**, rhodamine-alkyne **12**, biotin-phosphine **14**, and BodipyTMR-tetrazine **16**.

acylated tetrapeptide for these enzymes.[19] In contrast, the functionalization of larger peptide fragments of a natural caspase-1 substrate with an alkyne warhead yielded selective caspase-1 ABPs.[19]

An exciting recent development in the field of DUB-labeling has been the recent report of interchain warheads to probe ubiquitin−ubiquitin linkage specificity of certain deubiquitinating enzymes. McGouran et al.,[23] followed by Li et al. and Mulder et al.,[24] reported the use of diubiquitin-based ABPs where the warhead was positioned between two regiospecifically linked ubiquitin chains. With this approach, they showed that most DUBs have no linkage preference while certain DUBs show a very high selectivity for cleavage of specific poly ubiquitin chains.

■ INHIBITOR-BASED PROBE DESIGN

In the above approach, the natural substrate of deubiquitinating enzymes was used as the starting point for ABP design by modifying it with a detectable group and a reactive warhead in such a way that reaction with the enzyme results in covalent and irreversible binding. Knowledge of the catalytic mechanism and/or reactivity of an enzyme toward particular electrophiles is essential for the design of substrate-based probes. Alternatively to substrate-based design strategies, irreversible inhibitors (often natural products) can provide excellent starting points for the design of an ABP. For example, the structure of the potent biotin-tagged proteasome ABP **7** (Figure 6A) was derived from the naturally occurring irreversible proteasome inhibitor epoxomicin by extension of the *N*-terminus of the peptide epoxyketone with a biotinylated linker.[25] The proteasome contains three catalytically active β-subunits that have different substrate preferences but each employ an *N*-terminal threonine residue as the active site nucleophile, a distinctive feature that is only found in a few other proteases. This particular aspect of the proteasome's catalytic mechanism is exploited by the inhibitor to achieve selective targeting of this enzyme. The binding mechanism involves reaction of both the hydroxyl group and the primary amine of a catalytic threonine residue with the ketone and the epoxide of the inhibitor warhead, respectively, to form a stable morpholine ring (Figure 6B).[25c]

Another example of ABP development guided by a mechanism-based inhibitor is based on the natural product cyclophellitol.[26] This irreversible retaining β-glucosidase inhibitor has served as a basis for the synthesis of direct and two-step ABPs (**8** and **9**, Figure 7A) that selectively label the human enzyme glucocerebrosidase in cell extracts and cultured cells.[27] Interestingly, the introduction of the hydrophobic fluorescent tag in probe **9** proved to strongly enhance its affinity as compared to the parent compound cyclophellitol. Irreversible binding of these probes is thought to occur by attack of the nucleophilic glutamic acid residue in the active site of retaining β-glucosidases on the epoxide moiety of the ABPs (Figure 7B).[28] A second glutamic acid residue, which is essential for substrate hydrolysis by acting as a general acid/base, was shown to be also requisite for binding of the epoxide probes.[27] In a later study, an aziridine-based ABP (**10**, Figure 7A) was revealed to inhibit retaining β-glucosidases with enhanced potency and enabled in vivo profiling of enzymatic activity.[29]

The biotinylated peptide epoxysuccinate DCG-04 (**1**, Figure 2) represents a further example of an ABP that is derived from a natural product, the irreversible cysteine protease inhibitor E-64.[30] The structure of this inhibitor, which contains the thiol-reactive epoxysuccinate warhead, was modified by elaboration

dx.doi.org/10.1021/bc500208y | *Bioconjugate Chem.* 2014, 25, 1181−1191

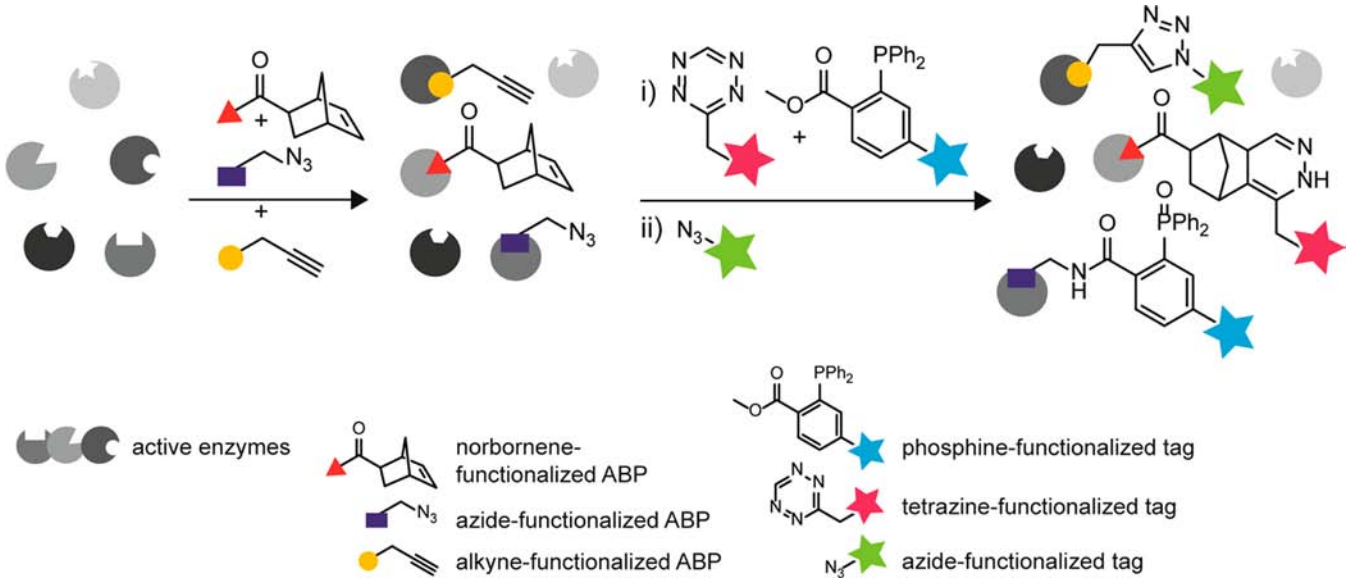


Figure 9. Triple bioorthogonal ligation strategy for simultaneous labeling of three different enzymatic activities. The three active proteasome β-subunits were targeted by norbornene-, azide-, and alkyne-modified ABPs and subsequently labeled via tetrazine ligation, Staudinger-Bertozzi ligation, and copper(I)-catalyzed click reaction, respectively.

of the peptide portion to allow incorporation of an affinity tag.[13]

1-STEP AND 2-STEP DETECTION STRATEGIES

The third common feature of all ABPs is a means to visualize, isolate, identify, and/or quantify the labeled proteins. This can be done, for example, using SDS-PAGE, microscopy, or mass spectrometry analysis. The required functionality for this detection can either be a reporter group that enables the direct visualization and/or isolation of target enzymes (see Figure 1B) or a small bioorthogonal "ligation handle", that can be used to introduce the tag after binding of the probe to a target enzyme (see Figure 1C). Two-step labeling experiments are particularly useful when the covalent attachment of a fluorescent group or an affinity tag to an ABP interferes with the interaction between the ABP and the enzyme. An inherent consequence of the introduction of a tag can be that it reduces the resemblance a probe may possess to an enzyme substrate. Furthermore, the presence of a relatively bulky reporter group is not always tolerated in the active site of an enzyme and the tag might also render the ABP less cell-permeable or reduce its bioavailability. Two-step ABPP strategies can solve these issues by allowing the temporal separation of an ABP and a reporter group. A further advantage as compared to direct ABPP is the option to use different reporter groups depending on the experimental settings and the desired method of analysis while using a single ABP.

The introduction of a tag in two-step ABPP experiments is achieved by reacting the ligation handle of the probe selectively and efficiently with a second reactive group to which the tag is attached. At the same time interference with any other (biological) functionalities should be avoided. Therefore, reactions are used which are commonly referred to as bioorthogonal ligation reactions.[31] A frequently used example of a bioorthogonal ligation reaction is the copper(I)-catalyzed [2 + 3] cycloaddition of an azide with an alkyne,[32] a reaction generally referred to as "click" reaction.[33] Both of the reactive moieties are small and can be incorporated into an ABP without significantly affecting the properties of the probe. The power of this strategy was first illustrated by the labeling of enzymatic activity in cell extracts with azide-functionalized sulfonate **11** followed by reaction with rhodamine-alkyne **12** and copper(I) as the catalyst (Figure 8).[10] A second example of a bioorthogonal ligation reaction is the Staudinger-Bertozzi ligation, in which an azide is again used as a ligation handle. In this case two-step labeling is achieved via selective reaction with a phosphine reagent.[34] This strategy has been applied to profile the activity of the proteasome β-subunits by treating cultured cells with an azide-modified proteasome inhibitor (**13**) and labeling the target proteins after cell lysis with biotinylated phosphine **14**.[35] More recently, selective two-step profiling of enzymatic activity was achieved in living cells by means of the inverse-electron-demand Diels–Alder reaction between 1,2,4,5-tetrazine and norbornene, a bioorthogonal ligation reaction originally developed by Devaraj et al.[36] for the live-cell imaging of cell surface receptors. This method was adapted for the labeling of endogenous proteasome activity by making use of a norbornene-modified epoxomicin derivative (**15**).[37] Two-step labeling of the catalytically active proteasome β-subunits was accomplished by reaction with a number of fluorescently labeled and biotin-tagged tetrazine reagents, for instance, Bodipy-tetrazine **16**.

In addition to the fact that the tetrazine ligation provides excellent selectivity and fast reaction kinetics, the orthogonality of the reagents with respect to those of other commonly used ligation reactions opens up the possibility to perform multiple ligation reactions concurrently in a single experiment. Such tandem ligation strategies can be used to study various enzymatic activities simultaneously. We have demonstrated this aspect of two-step ABPP by labeling three enzymatic activities with different tags in a single experiment via a triple ligation procedure that involves the tetrazine ligation, the copper(I)-catalyzed click reaction and the Staudinger-Bertozzi ligation.[37] In this strategy, each of the proteolytically active proteasome β-subunits was targeted with an azide-, alkyne-, or norbornene-functionalized ABP (Figure 9). Subsequently, simultaneous labeling of the three subunits in the same sample was accomplished by reaction with a fluorescently labeled

dx.doi.org/10.1021/bc500208y | Bioconjugate Chem. 2014, 25, 1181–1191

tetrazine and a biotinylated phosphine reagent, followed by copper(I)-catalyzed click reaction with an azide-modified Bodipy tag.

■ ABPP OF OTHER ENZYME CLASSES

Originally, the development of ABPs was mainly directed toward a few classes of hydrolytic enzymes with well-defined catalytic mechanisms, mainly serine hydrolases and cysteine/threonine proteases.[1a−c] Novel ABPP strategies are increasingly directed toward the development of ABPs that target other enzyme families, often with different catalytic mechanisms or low expression levels. Recent advances in the field of ABPP include the development of specific ABPs for carbohydrate processing enzymes,[38] protein tyrosine phosphatases,[39] monooxygenases,[40] and monoamine oxidases.[41]

One enzyme class that has remained somewhat resistant to ABPP is that of the glycosidases despite at least a number of glycosidases employing a mechanism involving a covalent enzyme−substrate intermediate. The first ABPs for this class of enzymes were developed by Vocadlo et al.[42] and were based on 2-deoxy-2-fluoroglycosides, substrate mimics in which the hydroxyl group next to the anomeric center has been substituted with a fluorine. A drawback of these inhibitors, though well suited for structural studies on transition states, is that they bind in a reversible manner. Nonetheless it was shown that the covalent intermediate formed between a number of these ABPs and their respective target enzymes is sufficiently stable to enable visualization of the labeled proteins on gel.[43] With the aim to generate more potent glycosidase ABPs, we developed another type of probe that is based on the irreversible retaining β-glucosidase inhibitor cyclophellitol, as described above (see Figure 7).[27,29] In a comparative study the cyclophellitol derivatives proved to be superior to 2-deoxy-2-fluoroglucosides in terms of potency and labeling efficiency on two retaining β-glucosidases.[43] In a later study, we expanded the cyclophellitol-toolkit by synthesizing differently configured isomers of these cyclophellitol-based ABPs. This, for the first time, has allowed the labeling of a mechanistically related class of glycosidases, the retaining α-galactosidases.[44] By changing the configuration of the probes to that of an α-galactopyranoside, selective profiling of these enzymes was achieved, both the recombinant enzyme as well as endogenous levels of α-galactosidase activity in cell extracts.

Tyrosine phosphatases utilize an active site cysteine residue for their catalytic activity, which in principle makes them suitable candidates for ABP development. However, the cysteine nucleophile appears to be less reactive toward electrophiles than the active cysteine residues in proteases, so that protonation of the leaving group oxygen of a substrate is necessary to enable nucleophilic attack.[45] Nonetheless, a number of ABPs for this class of enzymes has been synthesized that either directly form a covalent bond with an active site residue[39b] or act as a suicide substrate.[39a] The cytochrome P450 family of monooxygenases does not contain a catalytically active nucleophilic amino acid residue for direct targeting by an ABP. The development of ABPs for these enzymes was therefore guided by existing suicide inhibitors, which act via a conceptually different mechanism than the quinone methides described earlier.[40] In these probes, a highly reactive ketene group is generated upon oxidation of an aryl acetylene by a target enzyme. The ketene intermediate then reacts with a nearby nucleophilic amino acid residue to label the enzyme. A similar design was used to generate specific ABPs for monoamine oxidases.[41] These probes contain an *N*-propargylamine group that is oxidized by the enzyme to an iminium cation. The resulting Michael acceptor then reacts with the flavin cofactor to form a covalent adduct.

■ RECENT DEVELOPMENTS IN ABPP

Recent developments in ABPP have opened opportunities for in vivo imaging and drug discovery. Comparative ABPP, for example, can be used to identify new drug targets within a specific enzyme class, and competitive ABPP can be used to readily study the binding profile of new drug candidates in complex lysates. Comparative ABPP is the use of ABPP to compare the activity levels of an enzyme class (usually measured with a broad spectrum inhibitor) between different samples. This approach can be used to, for example, determine whether specific enzyme activities are up- or down-regulated in a specific disease state, such as a malignant transformation.[1d] Combination of this approach with mass spectrometry allows identification and quantification of newly up-regulated enzyme activities, providing new targets for drug discovery.

Competitive ABPP[46] is another application of ABPP that is very powerful in the drug discovery process. Here, a broad spectrum ABP is used to visualize a class of enzymes in, for example, a cell lysate (e.g., all reactive cysteine thiol containing enzymes[16]). Addition of a drug prior to ABP-addition will result in reduced intensity of the signal coming from the target of the drug while leaving the signal from those enzymes not interacting with the drug intact. This allows the rapid identification of both targets and off-targets in complex mixtures in a single experiment (at least those off-targets labeled by the ABP) without the need for isolating and/or cloning the enzymes involved. This approach could be of great benefit for streamlining drug discovery.

Until recently, the use of ABPP to profile enzymes in vivo was difficult. All available probes were fluorescent even when they were not bound to their target enzyme, and therefore the signal of probes bound to an enzyme could not be visually distinguished from unbound probe. Recently, two new approaches have been reported that circumvent this problem. In 2005, Blum et al. published a new approach in which a fluorescent quencher group was introduced into the warhead of a fluorescent cysteine protease probe, which resulted in a "dark" protease ABP.[9] Upon reaction with a cathepsin the quenching group is hydrolyzed, resulting in a fluorescent ABP "lighting up" upon covalent attachment to the enzyme. This strategy allowed for in vivo imaging of cysteine cathepsin activity associated with tumors—an important parameter relating to tumor progression and metastasis. These first generation probes were recently superseded by more stable probes for cathepsins with broader enzyme specificity.[47] The same group also published a similar probe targeting the regulator-protease legumain/AEP and used it to image its activity selectively in vivo.[48] This approach offers an exciting prospect for the imaging of these, and other, enzymes in vivo.

A second new approach that allows the distinction of unbound probe from bound probe is fluorescence polarization-based profiling: FluoPol-ABPP.[49] In this approach, the read-out is not fluorescence (which remains identical throughout the experiment), but the polarization of the fluorescent signal. When a fluorophore is excited by a polarized light source, it will emit partially polarized light. The amount of depolarization is dependent on the rotation speed of the fluorophore. As most fluorescent ABPs are small, their depolarization rate is high

when they are not bound to a target enzyme. Upon binding a (relatively large) target enzyme, the fluorescent ABP−enzyme complex will depolarize the light much less than the unbound ABP, resulting in a lower amount of depolarization. This difference in (de)polarization properties can be measured on a modified fluorescence plate reader in real time, opening up the possibility for high throughput screening of ABP−enzyme binding kinetics. It was first employed by Bachovchin et al. to identify new inhibitors for the hydrolase RBBP9 from publically available libraries: first, the enzyme was incubated with a series of inhibitors, followed by the addition of a FluoPol-ABPP. If the inhibitor bound to the substrate strongly enough to prevent competition with the ABPP, no change in depolarization of the probe was observed, whereas if a compound was not a hit a large decrease in depolarization could be seen. This provides a rapid route to measuring enzyme activity. It was used, for example, to discover new inhibitors of protein arginine methyltransferases by targeting the Fluo-Pol-ABP to the active site cysteine of this enzyme followed by competitive ABPP.[50] The approach is also compatible for use with membrane proteins, which are notoriously difficult to work with. Wolf et al. showed this potential by identifying β-lactones as a new class of inhibitors of the *E. coli* rhomboid serine protease GlpG.[51] Finally, Geurink et al. showed that this approach can also be used for large protein-based ABPs. They quantified the binding of active-site directed ubiquitin probes to various deubiquitinating enzymes and determined the kinetics of inhibition of these ABPs.[52] The sensitivity, ease of assay, and high throughput nature of this approach will likely make it an important tool for drug discovery in the future.

CONCLUSION AND FUTURE OUTLOOK

Over the past decades, the field of ABPP has developed into an important technique for the profiling of a wide variety of enzymatic activities. In this Topical Review we have given an overview of the general design principles underlying ABP development, often inspired by mechanism-based inhibitors. For those enzymes that do not make use of an active site nucleophile in their catalytic mechanism or that are not susceptible to targeting by ABPs for another reason, the use of affinity-based probes (A_fBPs) can provide a valuable alternative. Although these probes do not label their target proteins in an activity-based manner, the careful design of a probe such that binding is dependent on the integrity of the active site may provide a means to monitor the functional state of an enzyme.

As the field of ABPP is gradually moving toward the targeting of enzyme classes with different and sometimes poorly understood catalytic mechanisms, tight substrate specificity, and/or low expression levels, efforts are increasingly directed more toward the development of novel strategies for the activity-based profiling of such difficult to target enzymes. Creative thinking and clever design of novel ABPs results in the rapid expansion of the ABPP toolkit, both for the profiling of specific enzymes as well as for the broad-spectrum profiling an enzyme family. At the same time, the application of ABPP is shifting from the mere labeling of a specific enzymatic activity to the identification and characterization of new enzymatic activities, the discovery of novel enzyme inhibitors, the in situ and in vivo imaging of enzymatic activity, and the study of enzymatic activity in relation to disease.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: S.I.van.Kasteren@chem.leidenuniv.nl.
*E-mail: H.S.Overkleeft@lic.leidenuniv.nl.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) (a) Cravatt, B. F., Wright, A. T., and Kozarich, J. W. (2008) Activity-based protein profiling: from enzyme chemistry to proteomic chemistry. *Annu. Rev. Biochem. 77*, 383−414. (b) Fonovic, M., and Bogyo, M. (2008) Activity-based probes as a tool for functional proteomic analysis of proteases. *Expert Rev. Proteomics 5* (5), 721−30. (c) Schmidinger, H., Hermetter, A., and Birner-Gruenberger, R. (2006) Activity-based proteomics: enzymatic activity profiling in complex proteomes. *Amino Acids 30* (4), 333−50. (d) Nomura, D. K., Dix, M. M., and Cravatt, B. F. (2010) Activity-based protein profiling for biochemical pathway discovery in cancer. *Nat. Rev. Cancer 10* (9), 630−8. (e) Heal, W. P., Dang, T. H., and Tate, E. W. (2011) Activity-based probes: discovering new biology and new drug targets. *Chem. Soc. Rev. 40* (1), 246−57. (f) Krysiak, J., and Breinbauer, R. (2012) Activity-based protein profiling for natural product target discovery. *Topics Curr. Chem. 324*, 43−84.

(2) Ryšlavá, H., Doubnerová, V., Kavan, D., and Vaněk, O. (2013) Effect of posttranslational modifications on enzyme function and assembly. *J. Proteomics 92*, 80−109.

(3) Sanman, L. E., and Bogyo, M. (2014) Activity-based profiling of proteases. *Annu. Rev. Biochem. 83*, 249−273.

(4) Martinon, F., Mayor, A., and Tschopp, J. (2009) The inflammasomes: guardians of the body. *Annu. Rev. Immunol. 27*, 229−265.

(5) (a) Cygler, M., Sivaraman, J., Grochulski, P., Coulombe, R., Storer, A. C., and Mort, J. S. (1996) Structure of rat procathepsin B: model for inhibition of cysteine protease activity by the proregion. *Structure 4* (4), 405−16. (b) Coulombe, R., Grochulski, P., Sivaraman, J., Menard, R., Mort, J. S., and Cygler, M. (1996) Structure of human procathepsin L reveals the molecular basis of inhibition by the prosegment. *EMBO J. 15* (20), 5492−503.

(6) Ostrowski, K., and Barnard, E. A. (1961) Application of isotopically labelled specific inhibitors as a method in enzyme cytochemistry. *Exp. Cell. Res. 25*, 465−8.

(7) Serim, S., Haedke, U., and Verhelst, S. H. L. (2012) Activity-based probes for the study of proteases: recent advances and developments. *ChemMedChem 7* (7), 1146−1159.

(8) Jessani, N., Niessen, S., Wei, B. Q., Nicolau, M., Humphrey, M., Ji, Y., Han, W., Noh, D. Y., Yates, J. R., 3rd, Jeffrey, S. S., and Cravatt, B. F. (2005) A streamlined platform for high-content functional proteomics of primary human specimens. *Nat. Methods 2* (9), 691−7.

(9) Blum, G., Mullins, S. R., Keren, K., Fonovic, M., Jedeszko, C., Rice, M. J., Sloane, B. F., and Bogyo, M. (2005) Dynamic imaging of protease activity with fluorescently quenched activity-based probes. *Nat. Chem. Biol. 1* (4), 203−209.

(10) Speers, A. E., Adam, G. C., and Cravatt, B. F. (2003) Activity-based protein profiling in vivo siung a copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. *J. Am. Chem. Soc. 125* (16), 4686−4687.

(11) (a) Geurink, P. P., Florea, B. I., Li, N., Witte, M. D., Verasdonck, J., Kuo, C. L., van der Marel, G. A., and Overkleeft, H. S. (2010) A cleavable linker based on the levulinoyl ester for activity-based protein profiling. *Angew. Chem., Int. Ed. 49* (38), 6802−5. (b) Verhelst, S. H., Fonovic, M., and Bogyo, M. (2007) A mild chemically cleavable linker system for functional proteomic applications. *Angew. Chem., Int. Ed. 46* (8), 1284−6. (c) Dirksen, A., Yegneswaran, S., and Dawson, P. E. (2010) Bisaryl hydrazones as exchangeable biocompatible linkers. *Angew. Chem., Int. Ed. 49* (11), 2023−7. (d) Yang, Y., Hahne, H., Kuster, B., and Verhelst, S. H. (2013) A simple and effective cleavable linker for chemical proteomics applications. *Mol. Cel. Proteomics 12* (1), 237−44. (e) Gartner, C. A., Elias, J. E., Bakalarski, C. E., and Gygi,

S. P. (2007) Catch-and-release reagents for broadscale quantitative proteomics analyses. *J. Proteome Res. 6* (4), 1482−91.
(12) (a) Hillaert, U., Verdoes, M., Florea, B. I., Saragliadis, A., Habets, K. L., Kuiper, J., Van Calenbergh, S., Ossendorp, F., van der Marel, G. A., Driessen, C., and Overkleeft, H. S. (2009) Receptor-mediated targeting of cathepsins in professional antigen presenting cells. *Angew. Chem., Int. Ed. 48* (9), 1629−32. (b) Edgington, L. E., Berger, A. B., Blum, G., Albrow, V. E., Paulick, M. G., Lineberry, N., and Bogyo, M. (2009) Noninvasive optical imaging of apoptosis by caspase-targeted activity-based probes. *Nat. Med. 15* (8), 967−973.
(13) Greenbaum, D., Medzihradszky, K. F., Burlingame, A., and Bogyo, M. (2000) Epoxide electrophiles as activity-dependent cysteine protease profiling and discovery tools. *Chem. Biol. 7* (8), 569−81.
(14) Matsumoto, K., Mizoue, K., Kitamura, K., Tse, W. C., Huber, C. P., and Ishida, T. (1999) Structural basis of inhibition of cysteine proteases by E-64 and its derivatives. *Biopolymers 51* (1), 99−107.
(15) Kwan, D. H., Chen, H. M., Ratananikom, K., Hancock, S. M., Watanabe, Y., Kongsaeree, P. T., Samuels, A. L., and Withers, S. G. (2011) Self-immobilizing fluorogenic imaging agents of enzyme activity. *Angew. Chem., Int. Ed. 50* (1), 300−3.
(16) (a) Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol. 17* (10), 994−9. (b) Weerapana, E., Wang, C., Simon, G. M., Richter, F., Khare, S., Dillon, M. B., Bachovchin, D. A., Mowen, K., Baker, D., and Cravatt, B. F. (2010) Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature 468* (7325), 790−5. (c) Bedner, E., Smolewski, P., Amstad, P., and Darzynkiewicz, Z. (2000) Activation of caspases measured in situ by binding of fluorochrome-labeled inhibitors of caspases (FLICA): correlation with DNA fragmentation. *Exp. Cell Res. 259* (1), 308−13.
(17) Borodovsky, A., Kessler, B. M., Casagrande, R., Overkleeft, H. S., Wilkinson, K. D., and Ploegh, H. L. (2001) A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14. *EMBO J. 20* (18), 5187−5196.
(18) (a) Borodovsky, A., Ovaa, H., Kolli, N., Gan-Erdene, T., Wilkinson, K. D., Ploegh, H. L., and Kessler, B. M. (2002) Chemistry-based functional proteomics reveals novel members of the deubiquitinating enzyme family. *Chem. Biol. 9* (10), 1149−1159. (b) Love, K. R., Pandya, R. K., Spooner, E., and Ploegh, H. L. (2009) Ubiquitin C-terminal electrophiles are activity-based probes for identification and mechanistic study of ubiquitin conjugating machinery. *ACS Chem. Biol. 4* (4), 275−287.
(19) Ekkebus, R., van Kasteren, S. I., Kulathu, Y., Scholten, A., Berlin, I., Geurink, P. P., de Jong, A., Goerdayal, S., Neefjes, J., Heck, A. J., and Ovaa, H. (2013) On terminal alkynes that can react with active-site cysteine nucleophiles in proteases. *J. Am. Chem. Soc. 135* (8), 2867−2870.
(20) Sommer, S., Weikart, N. D., Linne, U., and Mootz, H. D. (2013) Covalent inhibition of SUMO and ubiquitin-specific cysteine proteases by an in situ thiol-alkyne addition. *Bioorg. Med. Chem. 21* (9), 2511−2517.
(21) Chapman, K. T. (1992) Synthesis of a potent, reversible inhibitor of interleukin-1β converting enzyme. *Bioorg. Med. Chem. Lett. 2* (6), 613−618.
(22) Roth, G. J., Liepold, B., Muller, S. G., and Bestmann, H. J. (2004) Further improvements of the synthesis of alkynes from aldehydes. *Synthesis* No. 1, 59−62.
(23) McGouran, J. F., Gaertner, S. R., Altun, M., Kramer, H. B., and Kessler, B. M. (2013) Deubiquitinating enzyme specificity for ubiquitin chain topology profiled by di-ubiquitin activity probes. *Chem. Biol. 20* (12), 1447−55.
(24) (a) Li, G., Liang, Q., Gong, P., Tencer, A. H., and Zhuang, Z. (2014) Activity-based diubiquitin probes for elucidating the linkage specificity of deubiquitinating enzymes. *Chem. Commun. 50* (2), 216−18. (b) Mulder, M. P., El Oualid, F., Ter Beek, J., and Ovaa, H. (2014) A native chemical ligation handle that enables the synthes of advanced activity-based probes: diubiquitin as a case study. *ChemBioChem 15* (7), 946−9.
(25) (a) Hanada, M., Sugawara, K., Kaneta, K., Toda, S., Nishiyama, Y., Tomita, K., Yamamoto, H., Konishi, M., and Oki, T. (1992) Epoxomicin, a new antitumor agent of microbial origin. *J. Antibiot. 45* (11), 1746−52. (b) Sin, N., Kim, K. B., Elofsson, M., Meng, L., Auth, H., Kwok, B. H., and Crews, C. M. (1999) Total synthesis of the potent proteasome inhibitor epoxomicin: a useful tool for understanding proteasome biology. *Bioorg. Med. Chem. Lett. 9* (15), 2283−8. (c) Groll, M., Kim, K. B., Kairies, N., Huber, R., and Crews, C. M. (2000) Crystal structure of epoxomicin:20S proteasome reveals a molecular basis for selectivity of α',β'-epoxyketone proteasome inhibitors. *J. Am. Chem. Soc. s122* (6), 1237−1238.
(26) (a) Caron, G., and Withers, S. G. (1989) Conduritol aziridine: a new mechanism-based glucosidase inactivator. *Biochem. Biophys. Res. Commun. 163* (1), 495−9. (b) Tatsuta, K., Niwata, Y., Umezawa, K., Toshima, K., and Nakata, M. (1990) Enantiospecific total synthesis of a β-glucosidase inhibitor, cyclophellitol. *Tetrahedron Lett. 31* (8), 1171−1172. (c) Atsumi, S., Umezawa, K., Iinuma, H., Naganawa, H., Nakamura, H., Iitaka, Y., and Takeuchi, T. (1990) Production, isolation and structure determination of a novel beta-glucosidase inhibitor, cyclophellitol, from Phellinus sp. *J. Antibiot. 43* (1), 49−53.
(27) Witte, M. D., Kallemeijn, W. W., Aten, J., Li, K. Y., Strijland, A., Donker-Koopman, W. E., van den Nieuwendijk, A. M., Bleijlevens, B., Kramer, G., Florea, B. I., Hooibrink, B., Hollak, C. E., Ottenhoff, R., Boot, R. G., van der Marel, G. A., Overkleeft, H. S., and Aerts, J. M. (2010) Ultrasensitive in situ visualization of active glucocerebrosidase molecules. *Nat. Chem. Biol. 6* (12), 907−13.
(28) Gloster, T. M., Madsen, R., and Davies, G. J. (2007) Structural basis for cyclophellitol inhibition of a beta-glucosidase. *Org. Biomol. Chem. 5* (3), 444−6.
(29) Kallemeijn, W. W., Li, K. Y., Witte, M. D., Marques, A. R., Aten, J., Scheij, S., Jiang, J., Willems, L. I., Voorn-Brouwer, T. M., van Roomen, C. P., Ottenhoff, R., Boot, R. G., van den Elst, H., Walvoort, M. T., Florea, B. I., Codee, J. D., van der Marel, G. A., Aerts, J. M., and Overkleeft, H. S. (2012) Novel activity-based probes for broad-spectrum profiling of retaining beta-exoglucosidases in situ and in vivo. *Angew. Chem., Int. Ed. 51* (50), 12529−33.
(30) Barrett, A. J., Kembhavi, A. A., Brown, M. A., Kirschke, H., Knight, C. G., Tamai, M., and Hanada, K. (1982) L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L. *Biochem. J. 201* (1), 189−98.
(31) (a) Sletten, E. M., and Bertozzi, C. R. (2011) From mechanism to mouse: a tale of two bioorthogonal reactions. *Acc. Chem. Res. 44* (9), 666−676. (b) Lang, K., and Chin, J. W. (2014) Bioorthogonal reactions for labeling proteins. *ACS Chem. Biol. 9* (1), 16−20. (c) Ramil, C. P., and Lin, Q. (2013) Bioorthogonal chemistry: strategies and recent developments. *Chem. Commun. 49* (94), 11007−22.
(32) (a) Tornøe, C. W., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on solid phase: [1,2,3]-Triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem. 67* (9), 3057−3064. (b) Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem., Int. Ed. 41* (14), 2596−9.
(33) Kolb, H. C., Finn, M. G., and Sharpless, K. B. (2001) Click chemistry: diverse chemical function from a few good reactions. *Angew. Chem., Int. Ed. 40* (11), 2005−2021.
(34) Saxon, E., and Bertozzi, C. R. (2000) Cell surface engineering by a modified Staudinger reaction. *Science 287* (5460), 2007−2010.
(35) Ovaa, H., Van Swieten, P. F., Kessler, B. M., Leeuwenburgh, M. A., Fiebiger, E., Van den Nieuwendijk, A. M. C. H., Galardy, P. J., Van der Marel, G. A., Ploegh, H. L., and Overkleeft, H. S. (2003) Chemistry in living cells: Detection of active proteasomes by a two-step labeling strategy. *Angew. Chem., Int. Ed. 42* (31), 3626−3629.
(36) Devaraj, N. K., Weissleder, R., and Hilderbrand, S. A. (2008) Tetrazine-based cycloadditions: application to pretargeted live cell imaging. *Bioconjugate Chem. 19* (12), 2297−9.

(37) Willems, L. I., Li, N., Florea, B. I., Ruben, M., van der Marel, G. A., and Overkleeft, H. S. (2012) Triple bioorthogonal ligation strategy for simultaneous labeling of multiple enzymatic activities. *Angew. Chem., Int. Ed. 51* (18), 4431−4.
(38) (a) Stubbs, K. A. (2014) Activity-based proteomics probes for carbohydrate-processing enzymes: current trends and future outlook. *Carbohydr. Res. 390C*, 9−19. (b) Witte, M. D., van der Marel, G. A., Aerts, J. M., and Overkleeft, H. S. (2011) Irreversible inhibitors and activity-based probes as research tools in chemical glycobiology. *Org. Biomol. Chem. 9* (17), 5908−26.
(39) (a) Lo, L. C., Pang, T. L., Kuo, C. H., Chiang, Y. L., Wang, H. Y., and Lin, J. J. (2002) Design and synthesis of class-selective activity probes for protein tyrosine phosphatases. *J. Proteome Res. 1* (1), 35−40. (b) Kumar, S., Zhou, B., Liang, F., Wang, W. Q., Huang, Z., and Zhang, Z. Y. (2004) Activity-based probes for protein tyrosine phosphatases. *Proc. Natl. Acad. Sci. U.S.A. 101* (21), 7943−8. (c) Lee, K., Kang, H. J., Xia, Y., and Chung, S. J. (2011) Recent advances in protein tyrosine phosphatase detection ung chemical probes. *Anti-cancer agents in medicinal chemistry 11* (1), 54−63.
(40) Wright, A. T., Song, J. D., and Cravatt, B. F. (2009) A suite of activity-based probes for human cytochrome P450 enzymes. *J. Am. Chem. Soc. 131* (30), 10692−700.
(41) Krysiak, J. M., Kreuzer, J., Macheroux, P., Hermetter, A., Sieber, S. A., and Breinbauer, R. (2012) Activity-based probes for studying the activity of flavin-dependent oxidases and for the protein target profiling of monoamine oxidase inhibitors. *Angew. Chem., Int. Ed. 51* (28), 7035−40.
(42) Vocadlo, D. J., and Bertozzi, C. R. (2004) A strategy for functional proteomic analysis of glycosidase activity from cell lysates. *Angew. Chem., Int. Ed. 43* (40), 5338−42.
(43) Witte, M. D., Walvoort, M. T., Li, K. Y., Kallemeijn, W. W., Donker-Koopman, W. E., Boot, R. G., Aerts, J. M., Codee, J. D., van der Marel, G. A., and Overkleeft, H. S. (2011) Activity-based profiling of retaining beta-glucosidases: a comparative study. *ChemBioChem 12* (8), 1263−9.
(44) Willems, L. I., Beenakker, T. J. M., Murray, B., Scheij, S., Kallemeijn, W. W., Boot, R. G., Verhoek, M., Donker-Koopman, W. E., Ferraz, M. J., van Rijssel, E. R., Florea, B. I., Codée, J. D. C., van der Marel, G. A., Aerts, J. M. F. G., and Overkleeft, H. S. (2014) Potent and selective activity-based probes for human retaining α-galactosidases, submitted for publication.
(45) Asthagiri, D., Dillet, V., Liu, T., Noodleman, L., Van Etten, R. L., and Bashford, D. (2002) Density functional study of the mechanism of a tyrosine phosphatase: I. Intermediate formation. *J. Am. Chem. Soc. 124* (34), 10225−35.
(46) Leung, D., Hardouin, C., Boger, D. L., and Cravatt, B. F. (2003) Discovering potent and selective reversible inhibitors of enzymes in complex proteomes. *Nat. Biotechnol. 21* (6), 687−91.
(47) (a) Verdoes, M., Oresic Bender, K., Segal, E., van der Linden, W. A., Syed, S., Withana, N. P., Sanman, L. E., and Bogyo, M. (2013) Improved quenched fluorescent probe for imaging of cysteine cathepsin activity. *J. Am. Chem. Soc. 135* (39), 14726−14730. (b) Verdoes, M., Edgington, L. E., Scheeren, F. A., Leyva, M., Blum, G., Weiskopf, K., Bachmann, M. H., Ellman, J. A., and Bogyo, M. (2012) A nonpeptidic Cathepsin S activity-based probe for non-invasive optical imaging of tumor-associated macrophages. *Chem. Biol. 19* (5), 619−628.
(48) Edgington, L. E., Verdoes, M., Ortega, A., Withana, N. P., Lee, J., Syed, S., Bachmann, M. H., Blum, G., and Bogyo, M. (2013) Functional imaging of legumain in cancer using a new quenched activity-based probe. *J. Am. Chem. Soc. 135* (1), 174−182.
(49) Bachovchin, D. A., Brown, S. J., Rosen, H., and Cravatt, B. F. (2009) Identification of selective inhibitors of uncharacterized enzymes by high-throughput screening with fluorescent activity-based probes. *Nat. Biotechnol. 27* (4), 387−94.
(50) Dillon, M. B. C., Bachovchin, D. A., Brown, S. J., Finn, M. G., Rosen, H., Cravatt, B. F., and Mowen, K. A. (2012) Novel inhibitors for PRMT1 discovered by high-throughput screening using activity-based fluorescence polarization. *ACS Chem. Biol. 7* (7), 1198−1204.
(51) Wolf, E. V., Zeissler, A., Vosyka, O., Zeiler, E., Sieber, S., and Verhelst, S. H. L. (2013) A new class of rhomboid protease inhibitors discovered by activity-based fluorescence polarization. *PLoS One 8* (8), e72307.
(52) Geurink, P. P., El Oualid, F., Jonker, A., Hameed, D. S., and Ovaa, H. (2012) A general chemical ligation approach towards isopeptide-linked ubiquitin and ubiquitin-like assay reagents. *ChemBioChem 13* (2), 293−97.