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# Mechanism-Based Profiling of Enzyme Families

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## 1. Introduction

Genome sequencing projects have ushered biological research into a new age where scientists are confronted with the daunting undertaking of assigning functions to the full repertoire of proteins (the proteome) encoded by eukaryotic and prokaryotic genomes. Toward this goal, the analysis of genomic data itself has advanced some hypotheses regarding protein function. For instance, genomic signatures, such as chromosomal translocation and gene amplification, have identified proteins that contribute to the pathology of cancer.[1,2] Moreover, daughter technologies to genome sequencing, including transcriptional profiling and siRNA-based gene silencing have provided insights into the molecular mechanisms of a range of physiological and pathological processes, including tumor invasion,[3,4] bacterial pathogenesis,[5,6] and insulin signaling.[7] Despite these successes, the general application of such methods for protein function assignment suffers from some inherent limitations, namely, the reliance on profiling and manipulation of gene expression to deduce the roles played by proteins in cellular processes. Most proteins are regulated by a complex array of post-translational events that may or may not be directly reflected in gene expression signatures,[8,9] and therefore, more direct methods are needed to evaluate protein function on a global scale.

Drawing conceptual and methodological inspiration from the success of genomic technologies, the field of proteomics has introduced several strategies for the global analysis of protein expression and function. Examples include liquid chromatography−mass spectrometry (LC−MS) platforms for shotgun analysis of protein expression and modification state,[10−12] yeast two-hybrid assays for the large-scale mapping of protein−protein interactions,[13,14] and protein microarrays for the proteome-wide analysis of the biochemical activities

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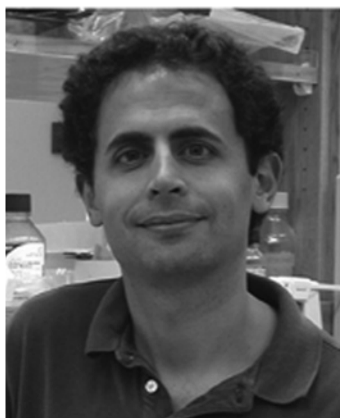


Benjamin Cravatt studied biological sciences (B.S.) and history (B.A.) at Stanford University. He then conducted research with D. Boger and R. Lerner and received his Ph.D. from The Scripps Research Institute (TSRI) in 1996. He joined the faculty at TSRI in 1997 as a member of the Skaggs Institute for Chemical Biology and the Departments of Cell Biology and Chemistry. His research group is developing and applying new technologies to elucidate the roles of enzymes in the physiological and pathophysiological processes of the nervous system and in cancer.

of proteins.[15] These proteomic technologies have greatly enriched our understanding of the expression patterns, interaction maps, and in vitro functional properties of proteins. However, certain key tiers of proteomic information, namely, the functional state of proteins in cells and tissues, still lay beyond the scope of these methods. For example, protein expression profiling by LC−MS does not account for many post-translational events that regulate protein activity in vivo. Conversely, yeast two-hybrid assays and protein microarrays rely on the use of recombinantly expressed proteins for biochemical characterization and, therefore, are not capable of directly reporting on the activity state of proteins in native proteomes. Considering that it is the activity of proteins, rather than mere expression level, that dictates their functional role in cell physiology and pathology, proteomic efforts have emerged to measure this critical parameter for natively expressed proteins in samples of high biological complexity. One such approach is termed activity-based protein profiling (ABPP).

ABPP is a chemical strategy that utilizes active site-directed covalent probes to profile the functional state of enzymes in complex proteomes [Figure 1]. Because many of the post-translational mechanisms that control enzyme activity in vivo involve altering the state of active sites[16−18] (e.g., catalytic residues are tweaked out of alignment in zymogens; endogenous inhibitors sterically occlude active sites), chemical probes that can report on the structure and reactivity of enzyme active sites in cells and tissues have the potential to acquire high-content proteomic information that is beyond the reach of more conventional global profiling technologies. Prototype activity-based probes (ABPs) target a large but manageable fraction of the enzyme proteome, often defined by shared catalytic features. The current suite of ABPs is based on a range of chemical scaffolds, including mechanism-based inhibitors,[19] protein-reactive natural products,[20] and general electrophilic chemotypes.[21] To date, ABPs have been developed for more than a dozen enzyme classes, including proteases, kinases, phosphatases, glycosidases, and oxidoreductases. The application of these reagents to a wide range of cell and animal models, as well as primary human specimens, has produced global portraits of enzyme activity that depict specific physiological and pathological processes. Importantly, these "activity profiles" are often enriched in uncharacterized enzymes for which no previous function had been assigned.

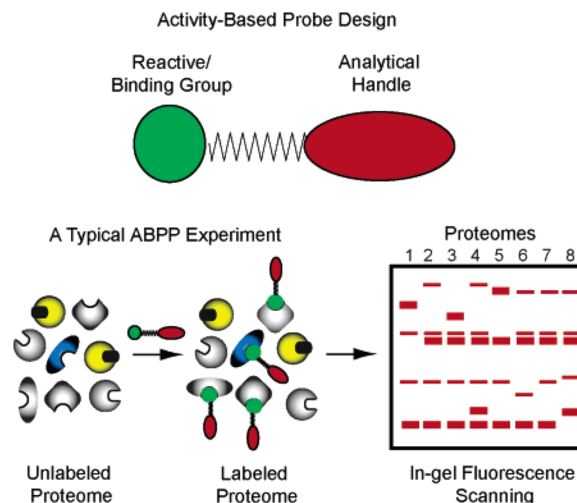



**Figure 1.** Activity-based protein profiling (ABPP): (top) a diagram of the architecture of a typical activity-based probe (ABP), consisting of an electrophilic reactive group (green), which can also be combined with a variable binding group to direct probe reactivity to subsets of the proteome, and an analytical handle (e.g., fluorophore, biotin) for the visualization or characterization of labeling events (red); (bottom) a schematic representation of a typical ABPP experiment, consisting of a labeling step in complex proteomes and an analytical step to visualize and characterize activity-dependent labeling events.

Several excellent reviews describing the principles and early uses of ABPP have appeared in the literature.[22−31] Since then, a number of studies expanding the technological scope and biological applications of ABPP have been reported. The purpose of this review is to describe these advances and situate their findings within the prevailing themes that have served as the foundation for ABPP. Particular attention will be given to the explanation of ABP design to highlight how the structure and reactivity of these reagents influences their target portfolio and the scope of ABPP experiments. This review is organized into the following sections: (1) the design of ABPs for functional proteomics, (2) biological applications of ABPs, (3) methodological advances beyond

the central paradigm, (4) ABPs and their application beyond comparative proteomics, and (5) summary and future directions.

## 2. The Design of Chemical Probes for Activity-Based Protein Profiling (ABPP)

### 2.1. Key Structural Elements and Design Rationale for Activity-Based Probes (ABPs)

It would be naïve to expect interpretable information on the functional state of proteins to emerge from studies that blanket the proteome with highly reactive compounds. Indeed, there are many nucleophilic residues in the proteome with no appreciable catalytic function whose labeling would corrupt ABPP studies.[32−37] Consequently, selective titration of those residues that exist within an active-site microenvironment requires careful consideration of probe structure. ABPs typically possess two main structural components that contribute to their target specificity: (1) a moderately reactive moiety to covalently modify sites in the proteome of heightened nucleophilicity (e.g., catalytic residues) and (2) a binding group to direct ABP reactivity to specific subsets of the proteome (e.g., enzyme active sites) [Figure 1]. In addition to these elements, ABPs should also contain an analytical handle, such as a fluorophore or biotin (or a latent handle, such as an azide or alkyne), for the visualization and characterization of labeling events.

With these parameters in mind, two basic designs have guided the preparation of ABPs. *Directed* strategies for probe development are intended to target enzymes within a mechanistically related family. ABPs that are selective for particular enzyme classes have been generated by at least two approaches. First, employing "mechanism-based" inhibitors as reactive groups can confer an overwhelming chemical preference for certain enzyme classes. Second, incorporation of broad-spectrum, high-affinity binding groups can direct probes to enzymes that share active site structural features. Implicit in the design of directed ABPs is a fundamental knowledge of enzyme mechanism, structure, or both, as well as some residual understanding of the enzyme's small-molecule (inhibitor or substrate) binding preferences. To extend ABPP to less well-characterized enzymes, a *non-directed* strategy has been introduced in which ABP libraries are synthesized that contain mild electrophiles and an array of binding groups to cooperatively drive probes to the active sites of a wide range of mechanistically distinct enzyme classes. In the following sections, prominent examples of ABPs emerging from both directed and nondirected strategies will be described, with special attention paid to their chemical origins, mechanism of action, and proteome-wide target portfolio.

### 2.2. Adapting Mechanism-Based Inhibitors for Class-Selective ABPs

Directed approaches for ABPP have benefited from a rich history of research on the design of mechanism-based inhibitors and affinity labels for enzymes. Indeed, as should become apparent from the examples listed below, many of the most versatile ABPs represent the simple conjugation of well-characterized covalent inhibitors to reporter tags such as fluorophores or biotin. Before proceeding with a discussion of these reagents, it should be noted that many of them do not strictly qualify as "mechanism-based" probes, since they do not require chemical conversion into reactive species by their cognate enzyme targets.[38] However, for the sake of simplicity, we will group all of the probes in this section under the general term "mechanism-based" to signify that they exploit conserved catalytic features of enzymes to gain class-selective reactivity.

#### *2.2.1. Fluorophosphonate (FP) Probes for Serine Hydrolases*

Serine hydrolases are an extremely large and diverse class of enzymes that comprise approximately 1% of the predicted protein products of mammalian genomes.[39] These enzymes play important roles in numerous physiological and pathological processes, including inflammation,[40] angiogenesis,[41] cancer,[42] diabetes,[43] and neural plasticity.[44] This enzyme family catalyzes the hydrolysis of ester and amide bonds in small-molecule and protein substrates. This chemistry is accomplished by engaging substrate esters and amides at the $sp^2$ carbon via a base-activated serine nucleophile [Figure 2, top]. A water molecule cleaves the resulting acyl−enzyme intermediate to complete substrate hydrolysis and restore the hydrolase to its active state.[45]

Fluorophosphonate (FP) reagents, like diisopropyl fluorophosphate (DIFP) [Figure 2, middle], have served as prototype mechanism-based reactive groups for the design of class-wide ABPs for serine hydrolases.[46,47] While many different types of serine hydrolase inhibitors have been described,[48−50] FPs display two desirable features for the global characterization of this enzyme superfamily. First, FPs exploit multiple conserved features of the serine hydrolase active site, including a serine-derived oxygen atom of heightened nucleophilicity (primed for covalent reaction with the electrophilic FP center) and an oxyanion hole that stabilizes the enzyme−substrate tetrahedral intermediate (mimicked by the near-tetrahedral structure of the FP group) to achieve broad target selectivity *within* this enzyme class.[45,51] Second, FPs show minimal *cross-reactivity* with other classes of mechanistically distinct hydrolases, including cysteine-, aspartyl-, and metallohydrolases.[52] Finally, the rate of FP reactivity with active versus inactive (e.g., zymogen) forms of serine hydrolases has been shown to differ by at least 3 orders of magnitude,[52] suggesting that FPs can be used to selectively target functional serine hydrolases in biological samples. Guided by these mechanistic observations, Cravatt and colleagues have prepared a panel of FP inhibitors fitted with various linkers and a fluorophore (e.g., rhodamine, Rh) or biotin for the visualization and characterization of catalytically active serine hydrolases in proteomes [Figure 2, bottom].[46,47,53]

These FP probes have been shown to label active serine hydrolases but not their inactive (e.g., serine nucleophile mutant, zymogen, or inhibitor-bound) forms.[46,47] Extensive application of these reagents to human and mouse proteomes has confirmed their remarkable promiscuity within the serine hydrolase class, as well as selectivity for these enzymes relative to the rest of the proteome. Indeed, a survey of the literature reveals over 100 distinct serine hydrolases, including proteases, peptidases, lipases, esterases, and amidases, that have been identified to date as targets of FP-based probes;[46,47,54−57] in contrast, these reagents have not been found to label other hydrolytic enzymes.

Complementing the broad-spectrum reactivity of FP probes, a small library of arylphosphonate probes has recently been developed by Craik and Mahrus to target the serine

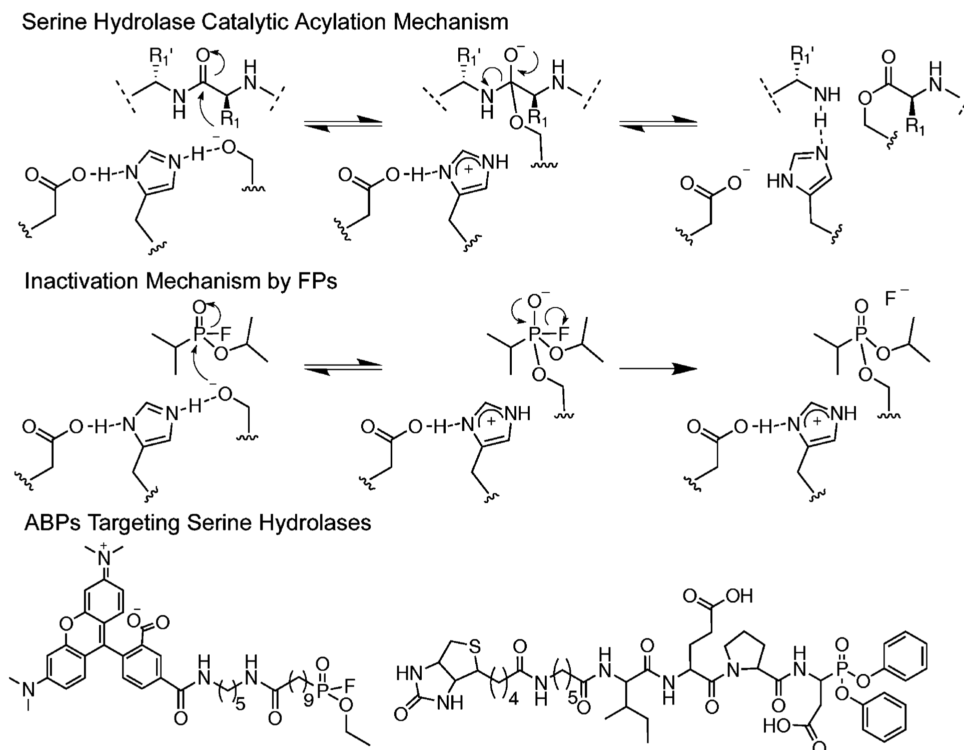



**Figure 2.** ABPs for serine hydrolases: (top) mechanism of acylation for serine hydrolases with amide or ester substrates (shown for a general peptide substrate); (middle) mechanism of inactivation of serine hydrolases by FPs [shown for diisopropyl fluorophosphonate (DIFP)]; (bottom) representative ABPs for serine hydrolases.

proteases granzyme A and B.[58] These probes were fitted with a diphenyl phosphonate electrophile, which displays a more tempered reactivity compared to FPs, and substrate-mimetic recognition groups to promote selective interactions with granzyme A and B [Figure 2, bottom]. Arylphosphonate probes specifically reacted with their respective granzyme targets in purified form and in complex biochemical mixtures. This study demonstrates that the reactivity and binding affinity of promiscuous, class-wide probes can be tuned to create research tools for the functional characterization of individual members of enzyme superfamilies.

### *2.2.2. Acyloxymethyl Ketone (AOMK) Probes for Cysteine Proteases*

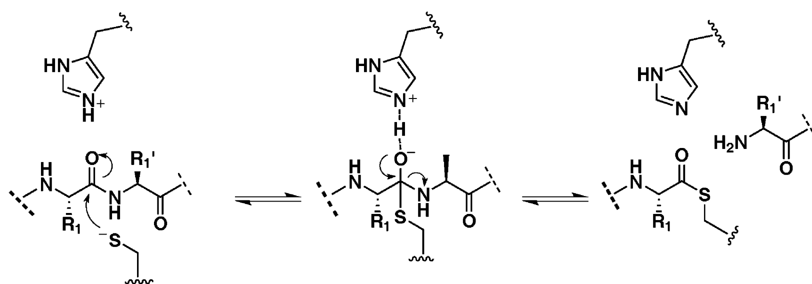
A second large and important class of hydrolases is the cysteine proteases, which have been shown to contribute to arthritis,[59] apoptosis,[60] and tumor metastasis.[61] Like serine proteases, cysteine proteases also form a covalent intermediate with peptide substrates at the $sp^2$ amide carbon, in this case via a histidine-activated sulfhydryl nucleophile [Figure 3, top]. The resulting thioester intermediate, formed between the catalytic cysteine and the carboxy terminus of the substrate, is cleaved by a water molecule to liberate the peptide product and to cycle the enzyme to its catalytically active form.[45]

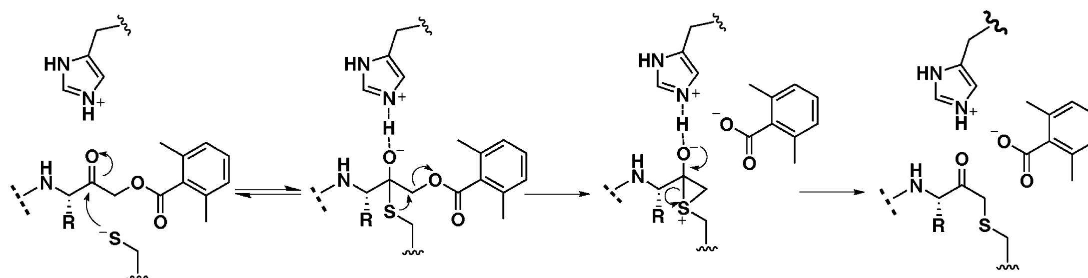
Many electrophilic inhibitors have been developed for cysteine proteases, including diazo- and fluoromethylketones,[62,63] vinyl sulfones[64] (section 2.2.3), epoxides (section 2.3.1), and acyloxymethyl ketones[65−67] (AOMKs). Among these examples, inhibitors bearing an AOMK group have been shown to display exceptional class-wide reactivity with cysteine proteases in complex proteomes. AOMKs likely derive their high selectivity for cysteine proteases from the fact that their reactive character is greatly amplified following engagement of the ketone by a sulfhydryl nucleophile. Specifically, the proximity of the mixed sulfide to the carboxylate moiety from AOMK accelerates the displacement of this otherwise weak leaving group [Figure 3, middle]. Reconstitution of the carbonyl group then opens the intermediate episulfonium species to produce a stable thiomethyl ketone adduct. This complex chemical behavior distinguishes AOMKs from structurally related affinity labels, chloro- and diazoketones, for example, whose heightened electrophilicity promotes direct $S_N2$ displacement by nucleophiles from many hydrolase classes. Accordingly, early biochemical applications incorporated the anchimeric displacement of carboxylate moieties in AOMKs to develop selective affinity probes for cathepsin B[68,69] and interleukin-1$\beta$ converting enzyme.[70]

The full versatility of AOMKs for functional proteomics was recently realized by Bogyo and colleagues, who demonstrated that a structurally diverse library of ABPs incorporating this reactive group [Figure 3, bottom] targeted numerous members of the two major classes of cysteine proteases, the CD (caspase-3, legumain, Arg- and Lys-gingipains) and CA clans (cathepsin B and L) of cysteine proteases.[71,72] These chemical tools were applied to label endogenous legumain, an enzyme whose biochemical properties and cellular functions have, to date, resisted characterization.[73−76]

### *2.2.3. Vinyl Sulfone Probes for the Proteasome and Ubiquitin-Specific Proteases (USPs)*

**2.2.3.1. Vinyl Sulfone Probes for the Proteasome.** The proteasome is the primary protein degradation machinery in the cell and plays a central role in determining the half-lives of proteins in vivo.[77] A barrel-shaped, multimeric macromolecule, the eukaryotic proteasome mediates these processes by catalyzing the hydrolysis of peptide substrates using an activated amino-terminal threonine nucleophile [Figure



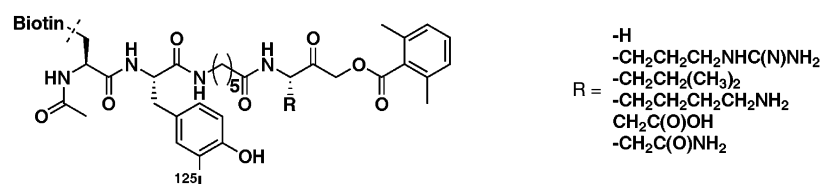
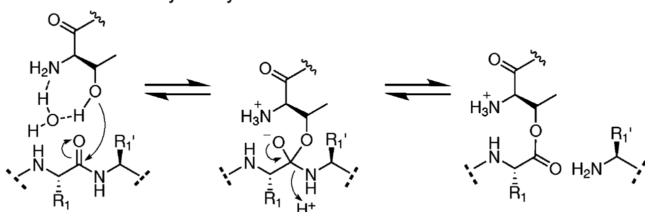




**Figure 3.** ABPs for cysteine proteases: (top) mechanism of acylation for cysteine proteases; (middle) mechanism of inactivation of cysteine proteases by AOMKs; (bottom) representative AOMK-based ABPs for cysteine proteases.



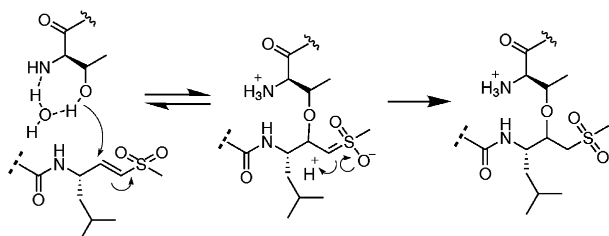




**Figure 4.** ABPs for proteasomal proteases: (top) mechanism of acylation for proteasomal proteases; (bottom) mechanism of inactivation of proteasomal proteases by vinyl sulfones.

4, top].[78-80] Among the catalytic $\beta$-subunits of the proteasome, distinct trypsin-like, chymotrypsin-like, and caspase-like activities are known.[81] Certain cell types, such as plasma cells and cancer cells, are especially dependent on high levels of proteasome activity for survival, which has led to the development of proteasome inhibitors for the treatment of multiple myeloma.[82]

The functions of the proteasome extend beyond simple protein degradation to include events such as antigenic peptide processing.[83] These specialized tasks are accomplished, in part, by altering the subunit composition of the proteasome.[84] The biochemical and cell biological complexities of the proteasome have inspired the creation of inhibitors that target its various catalytic subunits. Several classes of reversible and irreversible inhibitors of the proteasome have been described, including boronic acids,[85] epoxides,[86] lactones,[87] and vinyl sulfones. This last class of covalent inhibitors has been converted into proteasome-directed ABPs.

As activated surrogates of $\alpha,\beta$-unsaturated enones, vinyl sulfones readily form covalent adducts with many nucleophiles ("hard" and "soft") via a Michael-type 1,4-addition [Figure 4, bottom].[88] In early biochemical applications, this reactive group was exploited to develop covalent cysteine protease inhibitors, likely modifying an activated sulfhydryl residue.[64] To selectively engage the catalytic threonine nucleophile within proteasome active sites, Bogyo and coworkers developed a positional scanning library of tetrapeptide vinyl sulfones,[89-91] and showed that substrate recognition by proteasome subunits was mediated by aliphatic residues remarkably distal (P4) from the scissile bond. These studies resulted in the characterization of a highly specific inhibitor targeting the $\beta 2$ subunit, as well as more promiscuous probes for profiling multiple proteasome activities in complex proteomes.

**2.2.3.2. Vinyl Sulfone Probes for USPs.** The attachment of ubiquitin (Ub) and ubiquitin-like (Ubl) peptides to proteins is a broadly applied post-translational regulator of protein stability, subcellular localization, and function.[9,92] The enzymes that conjugate and hydrolyze ubiquitin modifications

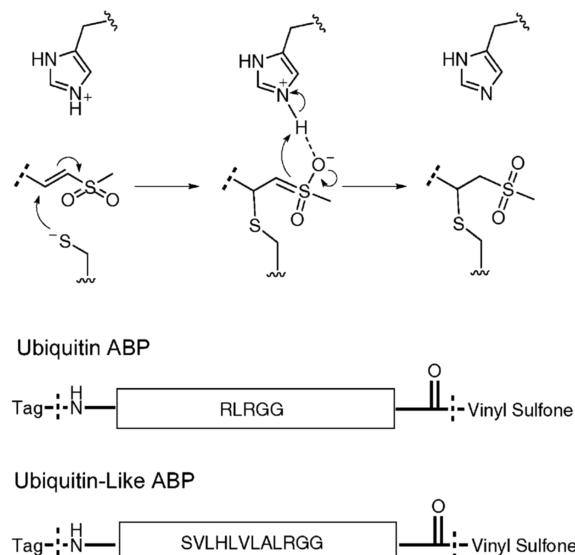
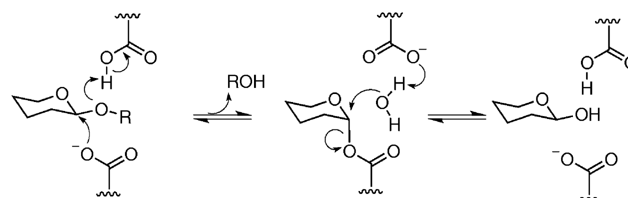
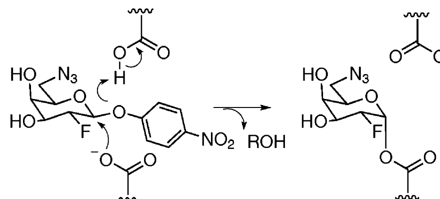



**Figure 5.** ABPs for USPs: (top) USPs are cysteine proteases vulnerable to inactivation by vinyl sulfones; (bottom) representative vinyl sulfone-based ABPs for USPs.









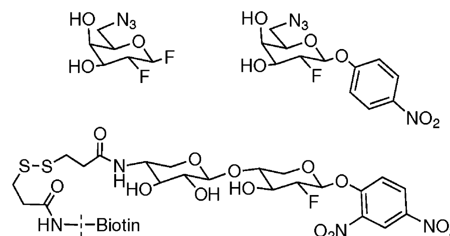




**Figure 6.** ABPs for $\beta$-retaining glycosidases: (top) two-step mechanism of glycoside hydrolysis by $\beta$-retaining glycosidases; (middle) mechanism of inactivation of $\beta$-retaining glycosidases by 2-deoxy-2-fluoro glycosides; (bottom) representative 2-deoxy-2-fluoro glycoside-based ABPs for $\beta$-retaining glycosidases.

constitute large and diverse classes of proteins in humans.[93,94] Indeed, the estimated number of Ub- and Ubl-specific proteases (USPs) far exceeds the handful of functionally annotated enzymes from this class (e.g., more than 50 USPs are encoded by the human genome).[89−101]

To assist in the functional characterization of ubiquitin processing proteases, Ploegh, Kessler, and colleagues have developed several ABPs that target these enzymes.[95−99] Libraries of 5-mer to 18-mer Ub- and Ubl-substrate mimetics were capped with thiol-specific reactive groups, including vinyl sulfones, alkyl halides, and cyanides, to selectively target this enzyme class [Figure 5]. The libraries were biotinylated, radiolabeled, or epitope-tagged to facilitate the characterization of probe-labeled proteins in biological samples. These probes were found to target multiple USP activities in proteomes, including an OTU domain-containing protein, which was shown to represent a prototype member of a novel class of USPs.

### 2.2.4. 2-Deoxy-2-fluoro Glycoside Probes for Retaining exo- and endo-Glycosidases

Glycosidases regulate the structure of carbohydrates and carbohydrate-modified biomolecules (proteins and lipids) in vivo by catalyzing the hydrolysis of anomeric *O*-linked glycosidic bonds.[45] Though formally organized according to three-dimensional structure,[100] glycosidases are further distinguishable according to enzymatic mechanism. The mechanistic class of glycosidases that has received the most attention for functional proteomic studies are the $\beta$-retaining glycosidases. This class employs two conserved carboxylate residues involved in catalysis [Figure 6, top]. One carboxylate functions as a general acid/base catalyst to activate the anomeric leaving group, while the second carboxylate serves as the catalytic nucleophile, displacing the activated anomeric substituent. The resulting glycosyl−enzyme intermediate is hydrolyzed by water, liberating the sugar substrate and priming the enzyme for another cycle of chemistry.[101,102]

Because $\beta$-retaining glycosidases utilize a covalent intermediate, a number of strategies have been developed to irreversibly inhibit this enzyme class.[103] From these examples, two mechanism-based inhibitors have been adapted for functional proteomic studies [Figure 6, middle and bottom]. Bertozzi and colleagues adapted a fluorosugar for the characterization of *exo*-glycosidases.[104] The ABP 6-azido-2,6-dideoxy-2-fluoro-$\beta$-D-galactosyl fluoride (6Az2FgalF) was prepared and shown to inhibit purified and native samples of LacZ, a glycosidase expressed in *Escherichia coli*. Employing the modified Staudinger ligation to attach a biotin moiety (see section 4.1), the authors showed that 6Az2FgalF labeled several structurally unrelated glycosidases, including Abg and Sabg (family 1) and Xbg and Aobg (family 35) in complex proteomes. Considering that substrate modification typically abrogates binding within the shallow active site pockets of *exo*-glycosidases, the success of these initial studies underscore the value of utilizing azide-modified probes in place of probes bearing extended linkers and bulky affinity tags.[105]

Withers and colleagues have reported a complementary strategy for ABPP of $\beta$-retaining *endo*-glycosidases.[106,107] The authors prepared a biotinylated derivative of 2-deoxy-2-fluoroxylobioside primed for glycosidase inhibition with an appropriately positioned 2,4-dinitrophenolic nucleofuge [Figure 6, bottom]. During the course of evaluating probe fidelity for $\beta$-1,4-glycanases in complex proteomes, the authors cleverly circumvented any potential negative impact that the biotin tag might have exerted on probe affinity for *endo*-glycosidases by appending this group to the sugar hydroxyl that is normally linked to the rest of the polysaccharide. The application of this ABP to the secreted proteome of *Cellulomonas fimi* led to the discovery of a novel $\beta$-1,4-glycanase, highlighting the utility of ABPP for the discovery of uncharacterized glycosidase enzymes.

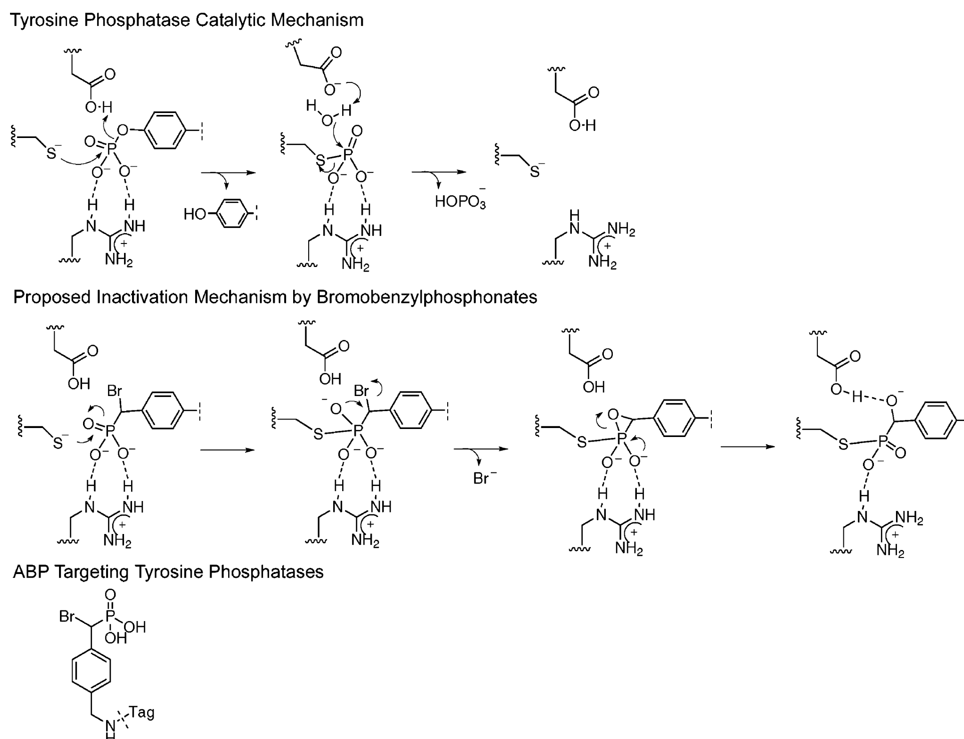



**Figure 7.** An ABP for tyrosine phosphatases: (top) mechanism of phosphate hydrolysis by tyrosine phosphatases; (middle) proposed mechanism of inactivation of tyrosine phosphatases by α-bromobenzylphosphonate; (bottom) an α-bromobenzylphosphonate-based ABP for tyrosine phosphatases.

### 2.2.5. α-Bromobenzylphosphonate Probes for Tyrosine Phosphatases

Estimated to comprise ∼1% of the human genome,[108] phosphatases are responsible for countering the functional impact of kinases by dephosphorylating small-molecule and protein substrates.[45] Phosphatases are classified by substrate specificity as protein tyrosine phosphatases (PTPases or PTPs),[109] serine/threonine phosphatases,[110] and dual specificity (serine/tyrosine) phosphatases.[111] The current dearth of potent and selective small-molecule inhibitors and probes of phosphatases stands as a major challenge for researchers interested in elucidating the functions of individual members of this large enzyme class.

Zhang and co-workers recently attached an affinity handle to α-bromo-benzyl phosphonate, a mechanism-based inhibitor of PTPs [Figure 7], to create an ABP for this enzyme class.[112] Covalent labeling of PTP active sites may derive from a multistep mechanism, where first the conserved cysteine nucleophile attacks the phosphorus center of the probe, and then the bromide is displaced by formation of a transient phosphorane-like intermediate. Subsequent ring opening would give an α-hydroxylbenzylphosphonate stably attached to the active site cysteine. $S_N2$ displacement of the highly electrophilic bromide substituent by the conserved cysteine nucleophile thus exploits the catalytic mechanism utilized by these enzymes to hydrolyze aromatic phosphates [Figure 7].[113] Preliminary investigations demonstrated that the α-bromo-benzyl phosphonate ABP labeled YopH, a PTP from the gram-negative bacterium *Yersinia*, with reasonable affinity and selectivity in complex proteomes. Moreover, the probe displayed broad reactivity with a panel of PTPs, suggesting that it may serve as a useful research tool to globally characterize the activity of this enzyme class in complex proteomes.

### 2.2.6. Latent Electrophilic Probes for Phosphatases, Glycosidases, and Proteases

**2.2.6.1. Tyrosine Phosphatase Quinone Methide Probes.** Two recent reports describe the preparation of fluorescently labeled derivatives of 4-(fluoromethyl)phenyl phosphate and 2-(difluoromethyl)phenyl phosphate to profile PTP activities.[114,115] Upon binding in a PTP active site, dephosphorylation of the probes induces facile rearrangement to an electrophilic quinone methide, which can react with an active site nucleophile [Figure 8, top]. For selected purified PTPs and alkaline phosphatases, these probes showed potent (low

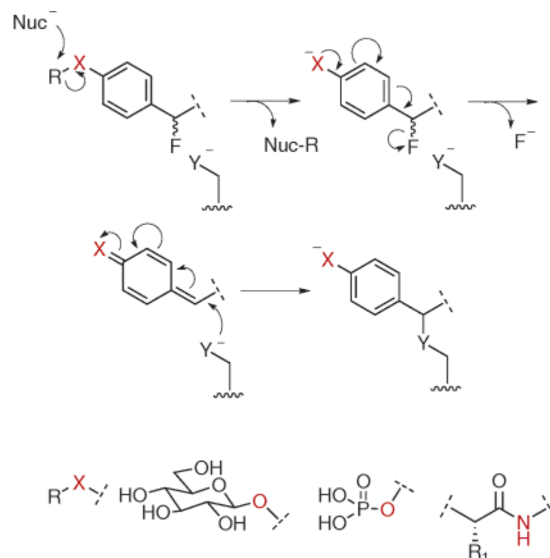



**Figure 8.** Quinone methide-based ABPs for various enzyme classes: (top) general mechanism of enzyme-catalyzed quinone methide production leading to active site labeling; (bottom) Representative quinone methide-based ABPs for glycosidases (left), phosphatases (middle), and proteases (right).

micromolar), heat-sensitive labeling profiles suggestive of specific active site modification. Consistent with this premise, other enzyme classes (e.g., proteases and lipases) were not labeled.

**2.2.6.2 Glycosidase Quinone Methide Probes.** As described above, ABPs for $\beta$-retaining glycosidases have been developed based on 2-deoxy-2-fluoro glycoside inhibitors; however, these probes are not applicable to inverting glycosidases, which do not form a covalent intermediate with substrates. To extend ABPP to inverting glycosidases, several groups have prepared ABPs designed to couple glycosidase activity with the exposure of a quinone methide [Figure 8, bottom].[116−118] In one study,[117] Lo and colleagues demonstrated that an ABP of this type can label purified $\beta$-glucosidase in a manner that appears to constitute active site labeling.

**2.2.6.3. Protease Quinolimine Methide Probes.** Yao and co-workers have further adapted the general quinone methide strategy to prepare ABPs directed toward multiple classes of proteases.[119] In this report, the authors reported a library of ABPs designed to generate a reactive quinolimine methide upon hydrolysis of a peptide motif [Figure 8, bottom]. Applying the library of four probes, the authors demonstrate specific labeling of six purified proteases, with each major protease family represented, in a pattern consistent with the known substrate specificities of these enzymes.

One potential drawback of all probes that depend on the in situ generation of quinone methides is that these reactive groups do not typically maintain strong affinity for their parent enzyme active sites and therefore can diffuse away and nonspecifically label other proteins. The extent to which this issue may confound the use of quinone methide-based probes in ABPP experiments will require a full characterization of their targets in complex proteomes.

## 2.3. Adapting Protein-Reactive Natural Products for Class-Selective ABPs

The development of ABPs has benefited not only from research on mechanism-based inhibitors but also from extensive efforts to characterize the mode of action of bioactive natural products. Many natural products promote their biological effects by covalently labeling enzyme active sites.[20] Notably, some natural products demonstrate broad-spectrum, class-selective reactivity that rivals the most versatile mechanism-based inhibitors. Here, we will review select examples of protein-reactive natural products that have been successfully converted into ABPs.

### *2.3.1. E-64-Based Probes for the Papain Family of Cysteine Proteases*

In 1978, Hanada and co-workers characterized the structure and pharmacological properties of E-64, a metabolite isolated from the mold *Aspergillus japonicus*.[120−122] Evidence from chemical degradation studies, $^1$H NMR, and IR spectroscopy demonstrated that E-64 consisted of the peptide-like structure shown in Figure 9. Further pharmacological investigations revealed that E-64 potently inhibited several papain-like cysteine proteases, including cathepsins B, H, and L.[123] E-64 was shown to be an active-site-directed inhibitor of these proteases, alkylating their conserved catalytic cysteine residue with an electrophilic epoxide.[124] The epoxysuccinyl motif of E-64 has been shown to be remarkably selective for papain proteases—it displays minimal cross-reactivity with other enzyme classes—distinguishing this affinity label among the repertoire of cysteine protease inhibitors.

Recognizing the potential value of E-64 as a probe of papain function in biological systems, Bogyo and colleagues prepared a peptide library of biotinylated ABPs based on this natural product [Figure 9] and showed that probes bearing a leucine residue adjacent to the core epoxysuccinyl reactive group reacted with numerous cathepsins but not other proteases.[125−127] Thus, through clever chemistry, these researchers succeeded in generating an ABP that emulated the proteome reactivity profile of the natural product E-64.

Bogyo and colleagues performed several compelling experiments to showcase the value of E-64-based ABPs for profiling papain activities in biological systems. For example, activity profiles from dendritic cell lysates, an abundant source of lysosomal cathepsins, revealed several E-64 targets that displayed heat- and pH-dependent reactivity consistent with activity-based labeling of these proteases. Extending these findings, the authors labeled and identified multiple cathepsins (cathepsins B, H, and L) from rat kidney proteomes.

Inspired by evidence of the tunable reactivity of the epoxysuccinyl motif, several second-generation libraries have

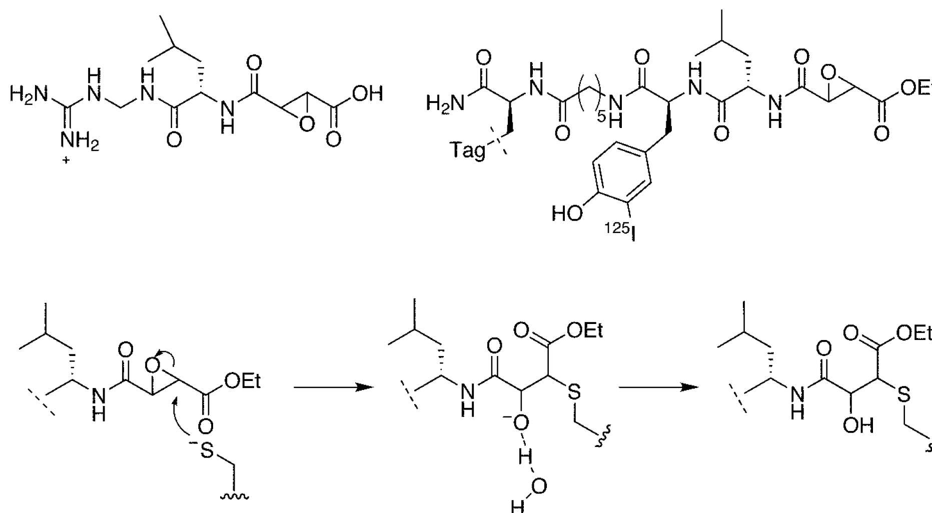



**Figure 9.** E-64-based ABPs for the papain class of cysteine proteases: (top) structures of the natural product E-64 and a representative ABP based on this agent; (bottom) mechanism of inactivation of cysteine proteases by E-64-based ABPs.

been described. These studies have reported the preparation of azapeptide ABPs with improved scaffold diversity and exchangeable electrophiles,[128] "double-headed" epoxysuccinyl probes for increased ABP selectivity among cysteine proteases,[129] a cell-permeable ABP for in situ investigations of enzyme function,[130] and an isotope-coded ABP for the quantitative analysis of cysteine protease activity.[131] An important product of these studies has been the generation of ABPs with strong selectivity for individual cathepsins; as will be further discussed below (section 5.3), these target-specific probes can be used to investigate the function of members of the papain family in living systems.

#### *2.3.2. Wortmannin-Based Probes for Lipid and Protein Kinases*

Virtually all signal transduction cascades are mediated in some part by kinases,[132] which comprise the largest single enzyme class in the human proteome.[133] Kinases transfer phosphate groups from nucleotide cofactors to small-molecule and protein substrates.[45] Though biochemical and cell biology techniques have implicated kinases in inflammation,[134] cell cycle control,[135] and cancer,[136] unambiguously assigning the endogenous functions of individual kinases is complicated by system-wide compensatory effects among related members of this enzyme class.[137] Several innovative chemical biology approaches[138−141] that exert improved temporal and spatial control over kinase activity have contributed key insights into the biology of these enzymes. The success of these approaches has spurred interest in developing new chemical techniques to profile kinase function in biological systems.

Addressing this challenge, two studies have reported the adaptation of the natural product wortmannin for functional proteomic studies of kinases. Wortmannin is a fungal metabolite that has been shown to covalently label kinases of the phosphoinositide-3 kinase (PI3K) family by targeting a conserved lysine residue in the nucleotide binding site [Figure 10].[142] This lysine attacks an electrophilic furan ring in the wortmannin structure, resulting in a stable vinylogous carbamate adduct.[143−145]

Based on this model for inhibition, Cimprich, Wandless, and co-workers prepared a panel of wortmannin analogues fitted with biotin or fluorophores distal to the reactive

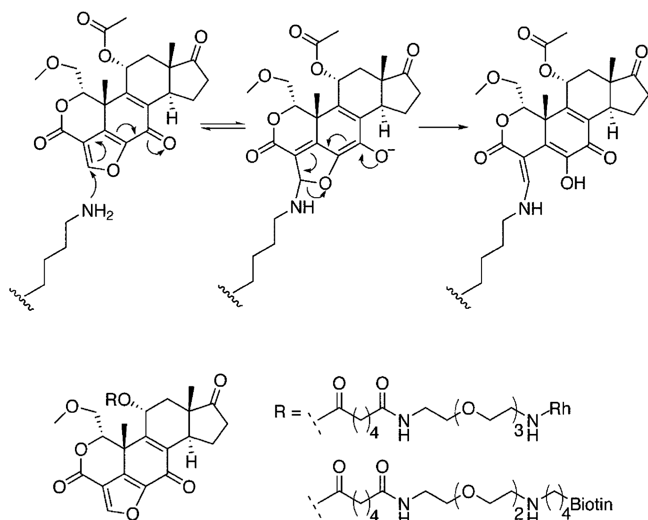



**Figure 10.** Wortmannin-based ABPs for kinases: (top) mechanism of inactivation of kinases by wortmannin; (bottom) structures of wortmannin-based ABPs.

enone.[146] Activity profiles from several cell lysates showed that the wortmannin analogues retained affinity for their known targets, including PI3Ks and PI3K-related kinases (PIKKs).[147] Employing another tagging strategy, Liu and colleagues prepared AX7503, a wortmannin-like ABP derivatized with a Rh fluorophore.[148] Intriguingly, in addition to profiling its expected kinase targets in proteomes, AX7503 was also found to label and inhibit the mammalian polo-like kinase 1 with similar affinity to PI3Ks. These results suggest that some of the pharmacological effects of wortmannin, originally assumed to reflect the inhibition of PI3Ks, may be due to inactivation of polo-like kinases. More generally, these studies promote wortmannin—and perhaps structurally related compounds such as viridin,[149] helenaquinone,[150] and hibiscone C—as a privileged chemical scaffold[151,152] for covalent targeting of kinase active sites.

#### *2.3.3. Microcystin-Based Probes for Serine/Threonine Phosphatases*

Microcystin, a cyclic peptide isolated from cyanobacteria, is a covalent inhibitor of multiple serine/threonine phosphatases.[153,154] This compound inactivates phosphatases of the protein phosphatase 1 (PP-1) and PP-2A type by engaging an active site cysteine residue with an electrophilic enone contained within a dehydroalanine residue [Figure 11].[155,156] Taking advantage of extensive SAR studies, Shreder and co-workers prepared a microcystin derivative with an appended fluorophore positioned to preserve the reactivity of the parent natural product for its known phosphatase targets.[157] On examination of the global reactivity of this ABP in soluble Jurkat lysates, two previously unappreciated phosphatase targets of microcystin, PP-4 and PP-6, were identified. Of perhaps more general significance, these studies provide a prototype example of an ABP that targets a noncatalytic residue in enzyme active sites to achieve class-selective labeling and inhibition in complex proteomes.

### 2.4. Advancing Probe Design for ABPP

The adaptation of mechanism-based inhibitors and protein-reactive natural products has served to establish the methodological parameters and biological utility of ABPP. While continued efforts to transform irreversible inhibitors into functional proteomics probes will surely succeed in addressing additional enzyme classes, an important challenge for ABPP is the development of tools to profile enzymes for which (1) no covalent enzyme−substrate adduct is formed and (2) cognate affinity labels are lacking. The next two sections will describe complementary strategies for probe design to address enzyme classes refractory to the canonical ABPP model. These approaches include the conversion of tight-binding reversible inhibitors into ABPs and the nondirected screening of libraries of candidate ABPs possessing moderately reactive electrophilic groups.

#### *2.4.1. Adapting Tight-Binding Reversible Inhibitors for Class-Selective ABPs*

**2.4.1.1. Photoreactive Hydroxamate Probes for Metalloproteases.** Methods to measure active metalloproteases (MP) in proteomes would be of value for several reasons. First, MPs are a sizable enzyme family (>100 members in the human proteome) that regulate a wide range of physiological and pathophysiological processes, including cancer,[158] tissue remodeling,[159] and hormone signaling.[160] MPs are also

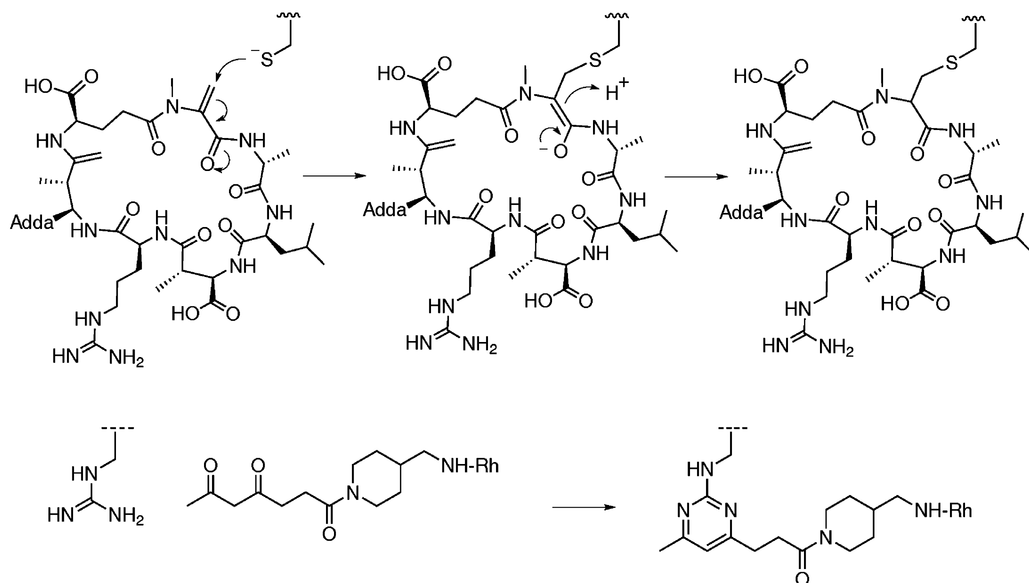



**Figure 11.** Microcystin-based ABPs for serine/threonine phosphatases: (top) mechanism of inactivation of phosphatases by microcystin; (bottom) structure of a microcystin-based ABP (formed via a pyrimidine linker).

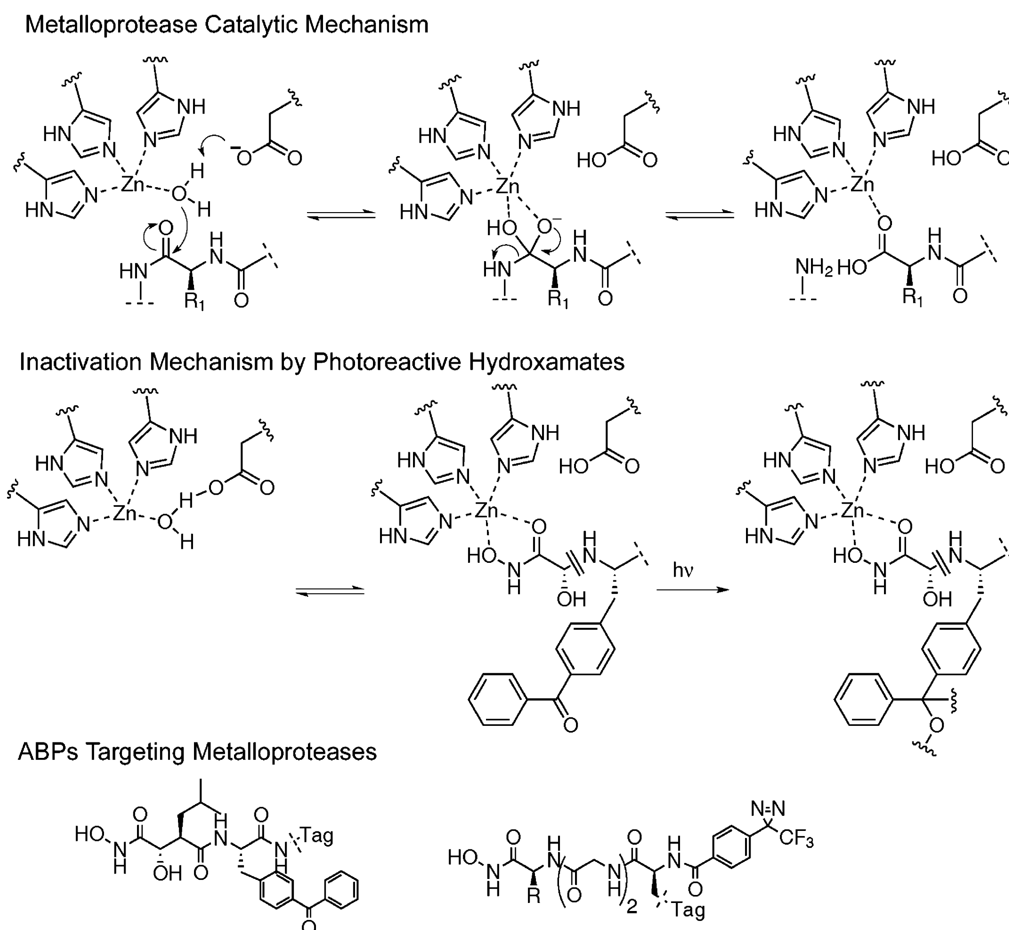



**Figure 12.** Photoreactive hydroxamate-based ABPs for metalloproteases: (top) mechanism of hydrolysis for metalloproteases; (middle) mechanism of inactivation of metalloproteases by photoreactive hydroxamates (shown for a benzophenone reagent); (bottom) representative photoreactive hydroxamate-based ABPs for metalloproteases.

subjected to extensive forms of post-translational regulation, implying that the measurement of MP expression, as judged by conventional genomic or proteomic techniques, may not report accurately on the functional state of these enzymes.[159] Third, multiple broad-spectrum MP inhibitors have entered clinical trials and faltered due, at least in part, to dose-limiting toxicity.[161] Which members of the MP superfamily are responsible for these deleterious side effects remains a critical question that might be effectively addressed by ABPP. Despite these clear motivating factors, MPs do not covalently engage their substrates,[162] which complicates ABP design. Nevertheless, two chemical strategies have been developed to profile the activity of this enzyme class in biological systems.[163,164]

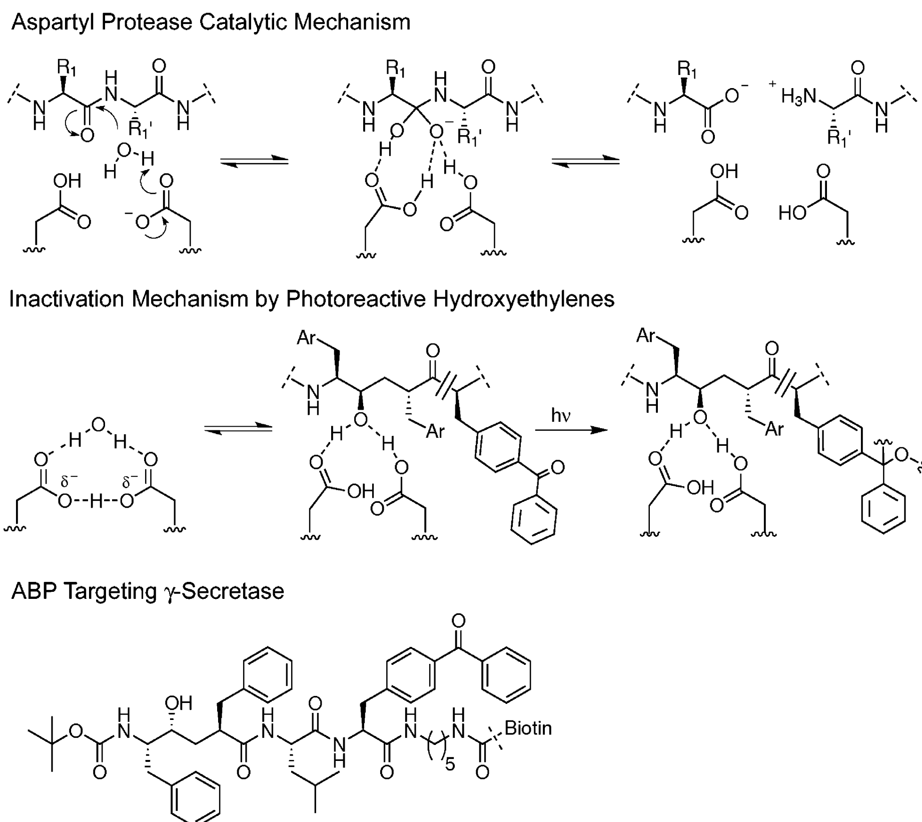



**Figure 13.** Photoreactive hydroxyethylene-based ABPs for aspartyl proteases: (top) mechanism of hydrolysis for aspartyl proteases; (middle) mechanism of inactivation of aspartyl proteases by photoreactive hydroxyethylene inhibitors; (bottom) a photoreactive hydroxyethylene-based ABP for the aspartyl protease $\gamma$-secretase.

Probe design for MPs is challenging because these enzymes use a zinc-activated water molecule, rather than a protein-embedded nucleophile for catalysis [Figure 12, top] and, therefore, are not readily susceptible to inactivation by electrophilic reagents. On the other hand, a number of tight-binding, broad-spectrum reversible inhibitors have been described for matrix MPs (MMPs),[165−167] and these agents have served as the basis for MP-directed ABPs [Figure 12]. Cravatt and co-workers have created ABPs for MPs that possess the following core features:[158] a hydroxamic acid (Hx) moiety to chelate the conserved zinc atom in MP active sites,[168] a peptide-like scaffold that contains a benzophenone (BP) group for photoinduced chemical cross-linking of MP active sites,[169] and a reporter tag consisting of a biotin or Rh for target visualization and enrichment [Figure 12, middle and bottom]. A prototype ABP (HxBP-Rh) was found to inhibit recombinant MMPs and label these enzymes in proteomes following exposure to UV light. Importantly, MMP labeling was exclusively observed with active MMPs but not their zymogen or inhibitor-bound forms. HxBP-Rh was used in competitive ABPP experiments to evaluate the selectivity of the MMP-directed inhibitor GM6001.[170] Interestingly, this inhibitor was found to target several enzymes outside of the MMP family, including MPs from the neprilysin, aminopeptidase, and dipeptidylpeptidase clans. These findings demonstrate that MPs can display overlapping inhibitor sensitivities despite lacking discernible sequence homology, thus underscoring the value of proteome-wide screening methods such as ABPP for evaluating the selectivity of MP-directed inhibitors.

Complementing this work, Yao and colleagues prepared a library of peptidyl hydroxamate ABPs and showed that they target multiple MPs.[164] These probes featured many of the structural elements highlighted above, including a hydroxamic acid, a peptide-based scaffold, a fluorophore, and a diazirine moiety for photoinduced chemical cross-linking [Figure 12, bottom]. The probe design was validated by targeting and labeling thermolysin in an activity-dependent manner from yeast extracts. In particular, one ABP (GGL-$NH_2OH$) labeled as little as 5 ng of purified thermolysin, and cross-reacted with other proteases only at elevated concentrations (>20 $\mu$M). Encouraged by these results, a positional scanning library was prepared to uncover cognate affinity labels for other yeast metalloproteases. The resulting screen uncovered candidate ABPs for 12 of the 17 MPs examined. Many of these proteins have no appreciated biochemical or cellular functions, suggesting that the preparation of cognate ABPs may expedite their characterization.

**2.4.1.2. Photoreactive Hydroxyl-Ethylene Probes for Aspartyl Proteases.** Aspartyl proteases also utilize an activated water molecule for catalysis [Figure 13, top] and therefore present similar technical challenges to MPs as targets for ABPP.[171] Li and colleagues at Merck have succeeded in generating high-affinity, reversible inhibitors for the aspartyl protease $\gamma$-secretase activity and used these agents as templates for the design of an ABP [Figure 13, middle and bottom].[172,173] Key features of the probe included a hydroxyl-ethylene moiety to serve as a transition state mimetic,[174,175] a peptide-like scaffold to maintain high affinity for the $\gamma$-secretase active site, a benzophenone for photoinduced cross-linking, and a biotin group for visualization and identification of labeling events. The fidelity of this probe for aspartyl proteases was demonstrated by selectively labeling solubilized $\gamma$-secretase from HeLa cell lysates. Interestingly, the pro-form of $\gamma$-secretase was not labeled,

confirming that the ABP provided a bona fide readout of the functional state of this protease in proteomes.

Collectively, the ABPP studies of metallo- and aspartyl proteases suggest that the conversion of reversible inhibitors into ABPs can be generally accomplished via the incorporation of a photoreactive group into inhibitor structures. The success of such endeavors for other enzyme classes will likely depend on the availability of high-affinity inhibitors that can be modified to present a photoreactive element without suffering significant losses in potency.

### 2.4.2. Reactive Chemotypes for Nondirected ABPP

**2.4.2.1. Sulfonate Ester Probes That Target Several Mechanistically Distinct Enzyme Classes.** Supplementing the proteome coverage of directed ABPP, nondirected strategies for probe design have emerged to address enzyme classes that lack cognate affinity labels. Cravatt, Sorensen, and co-workers imagined approaching this goal by preparing a structurally diverse library of candidate ABPs bearing a sulfonate ester (SE) reactive group [Figure 14, top].[176−178] It was hoped that the mild reactivity of the SE group would endow this carbon electrophile with an ability to engage a wide range of nucleophilic residues in enzyme active sites. These interactions could be further tuned by introduction of a variable binding group, which was intended to filter SE reactivity to subsets of the proteome.

These hypotheses were tested with a library of 11 sulfonate ester probes fitted with either a biotin or rhodamine tag. Initial experiments in tissue proteomes indicated that SE probes displayed the heat- and pH-dependent profiles characteristic of activity-based labeling.[176] Subsequent identification of the targets of SE probes revealed that they belonged to several mechanistically distinct enzymes classes, including dehydrogenases, glutathione S-transferases, sugar kinases, epoxide hydrolases, and transglutaminases.[177] Substantial indirect evidence was accumulated to support that SE labeling events occurred in enzyme active sites, including heat sensitivity, competition with substrate and cofactor, and dependency on endogenous activators. Among the enzyme classes targeted by SE probes, it is noteworthy to consider that none represented targets of previously described ABPs and several do not engage in covalent catalysis.

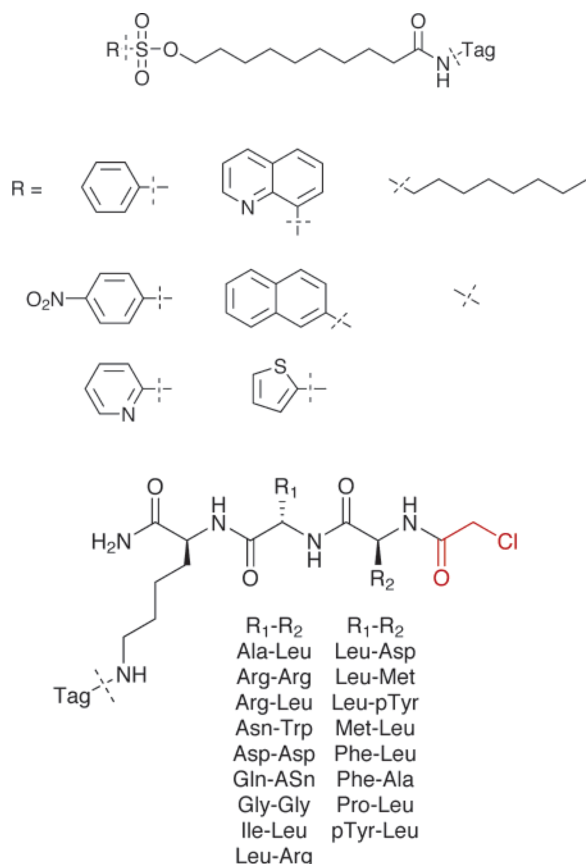



**Figure 14.** General probe classes for nondirected ABPP: (top) a sulfonate ester (SE) probe library, where representative binding groups (R) are shown; (bottom) an α-chloroacetamide probe library, where the variable binding groups ($R_1$ and $R_2$) are shown.

To further understand the mechanism of SE labeling, the probe labeling sites on five enzymes were determined using an advanced liquid chromatography−tandem mass spectrometry (LC−MS/MS) platform for ABPP [see section 4.2.1.1 for more details on the platform].[178] For four of the five enzymes, SEs alkylated known catalytic residues, while in the fifth case (3β-hydroxy steroid dehydrogenase/isomerase-1), labeling occurred on a conserved aspartate residue of unknown function. Subsequent mutagenesis studies provided evidence that this aspartate plays a role in catalysis. More generally, a survey of the active site residues labeled by SE probes revealed little chemical bias, with cysteine, aspartate, glutamate, and tyrosine residues all being targeted. These results indicate that the SE reactive group is a versatile chemotype for the creation of ABPs that target a broad range of enzyme classes.

**2.4.2.2. Additional Probe Libraries for Nondirected ABPP.** Multiple other nondirected ABP libraries have more recently been described in the literature. These include a dipeptide library fitted with an α-chloroacetamide [Figure 14, bottom],[179] and a natural product-like library bearing a reactive spiroepoxide (see section 5.2).[180] Application of these probe libraries has significantly expanded the scope of enzymes addressable by ABPP (see sections 3.1.4 and 5.2 for a more detailed discussion), further underscoring the utility of combinatorial strategies for probe discovery.

## 3. Biological Applications of ABPP

From its inception, proteomics has held as one of its principal goals the elucidation of molecular pathways that promote and support (patho)physiological processes. By monitoring system-wide activity changes in the proteome, it is hoped that ABPP will capture higher-order information on the function of proteins and protein networks that leads to new mechanistic insights to explain and, eventually, diagnose and treat human disease. With this long-term objective in mind, ABPP has been applied to characterize the enzyme activity profiles of several cell and animal models of human disease, as well as of primary human specimens. Here, we highlight representative examples of these efforts.

## 3.1. Profiling Enzyme Activities in Models of Human Disease

### 3.1.1. Cell Models of Human Cancer

Cravatt and co-workers utilized FP probes to profile the activity of the serine hydrolase superfamily across a panel of human cancer cell lines.[57] Groups of hydrolytic enzymes were identified that distinguished cancer cells based on tissue of origin and state of invasiveness. Interestingly, nearly all of the enzymes that contributed to the phenotypic classifica-

tion of cancer cells were found in the secreted and membrane proteome, suggesting that, at least for the serine hydrolase family, these subcellular fractions may be enriched in cancer biomarkers and targets. Among the enzyme activities elevated in invasive cancer cells were uncharacterized proteins such as the integral membrane hydrolase KIAA1363. These findings highlight the value of ABPP for the discovery of novel enzyme activities that depict the origin and pathogenic state of cancer cells.

This trend was corroborated by a recent investigation of USP activities in human cancer lines.[181] Among the soluble proteomes of neuroblastoma, colon, lung, and cervical carcinomas, a broad-spectrum ABP detected both unique and tumor-specific USP activities. Moreover, the activity of at least one USP, UCH-L1, was dramatically elevated in Epstein−Barr virus-immortalized lymphoblastoid cell lines, suggesting a role in advanced cancer phenotypes.

#### *3.1.2. Mouse Models of Cancer*

Cravatt and co-workers have extended their analysis of the invasive human breast cancer line MDA-MB-231 to include a characterization of the enzyme activity profiles of these cells during and after growth as tumors in the mammary fat pad of immune-deficient mice.[56] The mixed species nature of this cancer model enabled discrimination of cancer cell (human) and host (mouse) enzyme activities that accumulated during tumor growth. In vivo-derived lines of MDA-MB-231 cells were found to exhibit dramatic elevations in the levels of several secreted serine protease activities, which correlated with enhanced tumor formation and metastasis in mice. Significantly, the observed changes in protease activity were not reflected at the level of transcription, underscoring the value of functional proteomic methods, like ABPP, that can detect alterations in protein activity that may occur due to post-transcriptional or post-translational mechanisms. These data suggest a potential role for specific proteases in contributing to the tumorigenic properties of cancer cells.

Further evidence supporting a role for proteases in cancer growth in vivo has derived from ABPP studies of the cathepsin family of cysteine proteases. Using E-64-based ABPs, Bogyo, Hanahan, and colleagues profiled cathepsin activities in tumors derived from the RIP1−Tag2 transgenic mouse model of pancreatic cancer.[182,183] Several cathepsins were elevated in activity in tumors compared to normal islets, including cathepsin B, C, L, and Z. In vivo imaging with a fluorescent ABP revealed highest cathepsin activity in the angiogenic vasculature and invasive fronts of tumors, indicating a role for these enzymes in promoting angiogenesis and tumor growth. Consistent with this hypothesis, treatment of transgenic mice with a broad-spectrum cathepsin inhibitor impaired angiogenic switching and tumor growth.

#### *3.1.3. Infectious Diseases*

ABPP has also been applied to functionally characterize enzyme activities in infectious disease models. For example, Bogyo and co-workers have evaluated the cysteine protease activities contributing to host cell invasion by *Plasmodium falciparin*, the human parasite responsible for malaria.[184] Building on previous studies that generally implicated cysteine proteases in *Plasmodium* pathology,[185−187] the authors utilized a broad-spectrum ABP to characterize the functional state of these enzymes across various stages of the parasite life cycle. Striking changes in the activities of falcipain-1, -2, and -3 were observed, including the discovery that falcipain-1 has the most abundant activity in the merozoite (invasive) phase of the life cycle. These activity profiles were generally not observed at the level of protein expression, suggesting post-translational regulation of cysteine protease activities in this system. A more detailed functional analysis of falcipain-1 revealed that this protease localizes to the apical region of the merozoite, and its activity is required for the transition from the merozoite to ring stages of *P. falciparum*. These findings suggest a functional role for falcipain-1 in erythrocyte invasion, promoting it as a target for clinical intervention. More generally, this study shows how the combination of broad-spectrum and selective ABPs can be used to elucidate the function of enzymes in disease models.

#### *3.1.4. Metabolic Disorders*

Barglow and Cravatt recently introduced a novel dipeptide α-chloroacetamide scaffold for ABPs and applied this probe library, along with FP probes, to broadly characterize liver enzyme activities associated with obesity.[179] A comparison of livers from wild-type (lean) mice and mice lacking the leptin gene (*ob*/*ob*; obese) revealed several distinguishing enzyme activities. These included examples of enzyme activities that were elevated (e.g., maleylacetoacetonate isomerase, fatty acid synthase, hydroxypyruvate reductase) and diminished (e.g., glutathione-S-transferase YfYf, liver carboxyesterase) in obese livers. Interestingly, a comparison to previous 2DE-based proteomic studies of lean and obese livers[188] revealed several enzymes that were selectively detected by the ABPP technology. The identification of enzyme activities, like hydroxypyruvate reductase, that were highly elevated in obese livers raises new hypotheses regarding the enzymatic basis for metabolic disorders.

### 3.2. Class Assignment of Uncharacterized Enzymes

Among its more satisfying applications, ABPP has established preliminary functional associations among structurally disparate members of enzyme superfamilies. One notable example is the application of USP-directed ABPs to characterize novel enzymes possessing ubiquitin hydrolase activity.[99] Initial studies in budding yeast indicated that a ubiquitin-like ABP broadly targeted USPs, collectively labeling 6 of the 17 known USPs in this system.[97] Encouraged by these results, the authors profiled EL-4 lysates, a cell line rich with USPs, with a library of ubiquitin-like ABPs. Activity profiles showed that 23 distinct USPs are targeted, of which 10 had no appreciated biochemical or cellular function. An OTU-domain containing protein with no sequence homology to USPs, HSPC263, was labeled by USP-directed ABPs in a ubiquitin-competed manner, suggesting that this protein may represent a new type of deubiquitinating enzyme.

Jessani and colleagues have used FP probes to identify sialyl acetylesterase (SAE) as a member of the serine hydrolase superfamily.[189] Previous efforts to assign SAE to a mechanistic class of hydrolases had been complicated by the lack of sequence homology displayed by this enzyme compared to other known hydrolases.[190] The site of FP labeling on SAE was identified as S127 by LC-MS/MS analysis, a residue completely conserved among SAE-related proteins. Mutation of serine 127 to alanine produced an SAE variant that lacked catalytic activity and FP reactivity, thus supporting a role for this residue as the catalytic nucleophile.

**Table 1. Select Examples of Uncharacterized Enzyme Activities Identified by ABPP**

| name | accession no. | ABP | proteome | suggested class | ref |
|---|---|---|---|---|---|
| KIAA0436 | AB007896 | FP | rat testis | serine hydrolase | 46 |
| KIAA1363 | AB037784 | FP | human cancer cells | serine hydrolase | 57 |
| C9orf77, C19orf27, C20orf22 | AAH38390, AAH94816, CAI23475 | FP | human breast tumors | serine hydrolase | 55 |
| putative lipase | Q9DB29 | FP | mouse liver | serine hydrolase | 54 |
| enoyl-CoA hydratase-like protein | AAH11792 | phenyl SE | human cancer cells | enoyl-CoA hydratase | 200 |
| LOC67914 | NP_080728 | phenyl SE | mouse heart | unknown | 208 |
| Cfx | DQ146941 | 2-deoxy-2-fluoro glycoside | *C. fimi* | glycosidase (family 10) | 106 |
| presenilin | NM008943 | photoreactive hydroxyethylene | HeLa | aspartyl protease | 173 |
| USP13, USP15i, USP19 | Q92995, Q9Y5B5, O94966 | Ub-vinyl sulfone | EL-4 | USP | 99 |

These results provide a compelling example of the remarkable sequence (and structural) diversity that can be found among members of enzyme superfamilies.

Perhaps the most provocative example of the use of ABPs to identify unanticipated members of enzyme superfamilies was described by Li and colleagues, who employed a $\gamma$-secretase-directed probe to discover presenilin-1 (PS-1) as a candidate aspartyl protease.[173] In search of the $\gamma$-secretase activity responsible for the cleavage of amyloid precursor protein,[191] the authors serendipitously discovered that an ABP that inhibited this activity also labeled the C-terminal fragments of PS-1 and PS-2, two polytopic membrane spanning proteins. Though PS-1 and -2 contain no recognizable aspartyl-protease motifs, these findings agree with previous evidence showing (1) a correlation between $\gamma$-secretase activity and the expression of PS-1,[192] (2) that directed mutagenesis of two conserved aspartate residues in PS-1 abolishes $\gamma$-secretase activity,[193] and (3) a correlation between early-onset familial Alzheimer's disease and mutations in PS-1 and -2.[194,195]

Considering the remarkable number of uncharacterized enzymes that have already been identified by ABPP [Table 1], it is likely that this functional proteomic technology will continue to serve as a primary research tool to inventory the complete membership of enzyme superfamilies in proteomes.

## 4. Advances in Analytical Approaches for ABPP

Intense focus has understandably been granted to expanding the proteome coverage of ABPP through the design of new probe classes. This chemical proteomic technology has, however, inspired equally innovative advances in analytical methods for the characterization of probe-labeled proteomes, which we will briefly review in this section.

### 4.1. "Tag-Free" Strategies for ABPP that Exploit Bio-Orthogonal Chemistries

The choice of an appropriate analytical handle impacts the scope of ABPP experiments. Tagging an ABP with biotin, for instance, allows for the visualization of labeling events by avidin blotting and the MS characterization of labeling events by enrichment with avidin-conjugated beads.[196] While biotin moieties remain a central tool for the target identification and certain gel-free profiling platforms (see section 4.2.1), protein blotting is a cumbersome and relatively low-resolution method for the analysis of probe-labeled proteomes. Responding to these limitations, several groups have tagged ABPs with commercially available fluorophores (e.g., fluorescein, Rh, Cy-3, BODIPY) to directly detect probe-labeled proteins. After separating proteomes by 1D SDS−PAGE, fluorescently tagged proteins are visualized using commercial flatbed gel scanners. This technique is sensitive and quantitative, for example, as little as 100 amol of serine hydrolase can be detected with a rhodamine-tagged FP.[53] Fluorophores do not offer a straightforward way to enrich probe-labeled proteins; however, this disadvantage can be circumvented by incorporating both biotin and rhodamine molecules into a given ABP, so-called "trifunctional" ABPs.[197] These various tagging strategies can be interchanged to provide a relatively streamlined process for in vitro ABPP.

Despite their many virtues, reporter tags such as biotin and fluorophores also restrict the range of experiments addressable by ABPP. For instance, these tags are sterically large, which may impede the cellular uptake and distribution of probes, as well as reduce their affinity for certain protein targets. Biotin and fluorophores are also rather costly molecules with complicated solubility properties that limit their suitability for the preparative scale synthesis of ABPs.

Several strategies have been devised to circumvent these challenges by uncoupling the proteome labeling and reporter tagging steps of the ABPP process. The crucial innovation in each of these cases has been the use of a bio-orthogonal chemical reaction to append reporter tags exclusively to the subset of proteins in the proteome that are modified by ABPs. Two bio-orthogonal reactions have emerged as preferred strategies for "tag-free" ABPP: the Cu(I)-catalyzed Huisgen's [3 + 2] azide−alkyne cycloaddition (click chemistry[198]) and the Staudinger ligation [Figure 15].[199]

Cravatt and colleagues have exploited click chemistry to profile enzyme activities in living cells and animal models.[200,201] In this approach, reporter tags on ABPs are replaced by a "sterically inert" azide or alkyne group, and these tag-free probes are then applied to living cells or animal models. After allowing for suitable time for protein labeling, proteomes are harvested and reacted with a complementary "clickable" reporter tag (alkyne- or azide-modified, respectively). The product of this reaction is a 1,4-disubstituted triazole that connects the probe-labeled enzyme to the reporter tag, thus enabling target detection, enrichment, and identification. The chemoselectivity of this "click chemistry" approach was demonstrated in complex proteomes, where

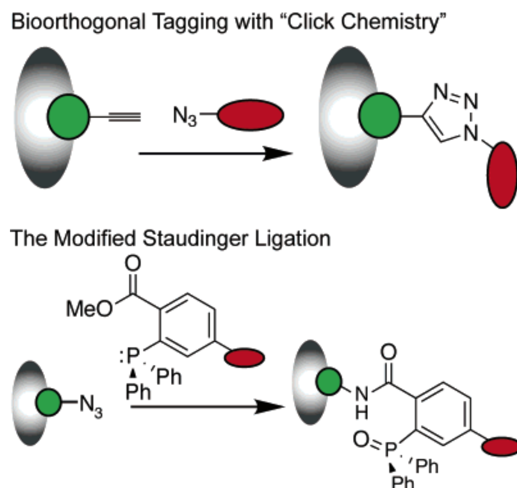



**Figure 15.** Tag-free ABPP using bio-orthogonal reactions. Tag-free ABPP can be accomplished by either click chemistry (top) or the Staudinger ligation (bottom).

several known and novel targets of SE-based ABPs have been identified. Extending this experimental model, several recent reports have described additional applications of click chemistry in proteomics, including a copper-free strain-promoted [3 + 2] cycloaddition[202] and a triazole-activated fluorogenic dye for imaging of labeled proteins.[203]

The bio-orthogonal Staudinger ligation has also been adapted for two-step visualization of activity profiles. Pioneered by Bertozzi and colleagues,[199,204] the modified Staudinger ligation utilizes a highly chemoselective reaction between azide and phosphine functionalities to produce an aza-ylide. In water, rapid hydrolysis of the aza-ylide yields a primary amine and phosphine oxide, a nonproductive reaction for tethering strategies.[205] However, when the aza-ylide forms in the proximity of an electrophilic center, an aryl ester, for example, intramolecular cyclization precedes hydrolysis. Subsequent hydrolysis of the nitrogen−phosphorus bond yields an amide, a robust linkage of the parent pieces. Initially developed to probe protein glycosylation events on cell surfaces,[199] this chemical strategy has more recently been used for the functional analysis of recombinant proteins,[206] elucidating the cellular targets of a class-selective ABP for *exo*-glycosidases (see section 2.2.4 and ref 104), and profiling the activity of the proteasome in situ.[207]

At least two important methodological lessons have been learned using tag-free ABPP. First, comparisons of the in vitro enzyme activity profiles generated with tag-conjugated versus tag-free ABPs have revealed that, in several instances, the reporter tag does indeed influence ABP reactivity. Interestingly, cases have been observed where the tag either hinders or facilitates enzyme labeling (ref 197 and our unpublished findings), thus underscoring that this group is not an inert element of probe structures. Second, a comparison of in situ and in vitro enzyme activity profiles provocatively suggests that tag-free ABPP accesses a dimension of the "functional proteome" whose integrity is dependent on native cellular environments.[200] For instance, several enzyme activities have been identified that show probe labeling selectively in living cells but not in cell homogenates [Table 2]. This phenomenon has also been observed with protease-directed and natural product-like ABPs (see section 5.2). These results highlight the fragility of many biochemical processes in cells, which can be impaired or completely disrupted by routine experimental manipulations (e.g., cell homogenization). Tag-free ABPP should prove of value for characterizing enzymes that require a native cellular environment to maintain activity.

## 4.2. Gel-Free Strategies for ABPP

1D-SDS−PAGE coupled with in-gel fluorescence scanning provides a robust and relatively high-throughput platform for ABPP experiments; however, the inherent resolution and sensitivity limits of 1D-gels precludes a comprehensive analysis of probe-labeled enzyme activities in proteomes using this analytical technique. The recent introduction of multiple gel-free platforms for ABPP has significantly enlarged the information content achievable in chemical proteomics investigations. Specific gel-free approaches and their respective applications will be described in the following sections.

### *4.2.1. Liquid Chromatography−Tandem Mass Spectrometry Platforms*

**4.2.1.1. Active-Site Peptide Profiling.** Initial LC-MS/MS platforms for ABPP were designed with the goal of simultaneously identifying probe-labeled proteins and their specific sites of modification, a strategy referred to as active-site peptide profiling [Figure 16, top].[54,178,189] In this approach, proteomes are digested with trypsin following probe labeling, and the resulting tryptic digest is incubated with anti-rhodamine antibodies (or avidin for biotinylated ABPs) to selectively enrich probe-modified peptides. These peptides are then analyzed by LC−MS/MS and a modified version of the SEQUEST search algorithm to identify targets of ABPs and their sites of modification. Active-site peptide profiling has proven particularly valuable for characterizing the proteome-wide reactivity of novel ABPs, such as those originating from nondirected endeavors [see section 2.4.2.1 and ref 177], and for the functional assignment of sequence-

**Table 2. Enzyme Activities Characterized by Tag-Free ABPP**[a]

| name | ABP | proteome | bio-orthogonal reaction | ref |
|---|---|---|---|---|
| GST-$\omega$ | phenyl SE | human cancer cells | click chemistry | 201 |
| ALDH-1 | phenyl SE | mouse liver | click chemistry | 201 |
| ECH-1 | phenyl SE | mouse heart | click chemistry | 200 |
| *ECH-2* | phenyl SE | human cancer cells | click chemistry | 200 |
| *VLCAD* | phenyl SE | human cancer cells | click chemistry | 200 |
| *PDI* | phenyl SE | human cancer cells | click chemistry | 200 |
| *PGAM1* | MJE3 (spiroepoxide) | human cancer cells | click chemistry | 180 |
| proteasomal subunits | vinyl sulfone | mouse lymphoma cells | Staudinger ligation | 207 |
| Abg, Xbg | 2-deoxy-2-fluoro glycoside | *E. coli* | Staudinger ligation | 104 |

[a] Italicized enzyme names indicate activities that were detected exclusively in situ. Abbreviations: ALDH, aldehyde dehydrogenase; ECH, enoyl-CoA hydratase; GST, glutathione S-transferase; PDI, protein disulfide isomerase; PGAM, phosphoglycerate mutase; SE, sulfonate ester; VLCAD, very long chain acyl-CoA dehydrogenase.

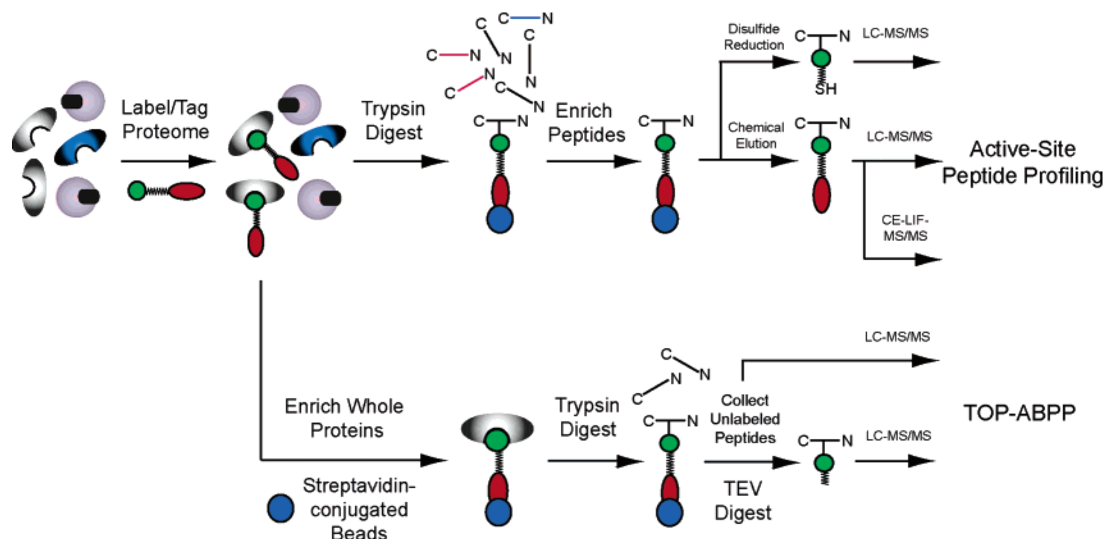



**Figure 16.** Representative gel-free platforms for ABPP: (top) active-site peptide profiling, where proteins and sites of probe modification are identified by LC-MS/MS analysis of affinity-enriched probe-labeled peptides; (bottom) TOP-ABPP, where proteins and sites of probe modification are characterized in sequential LC-MS/MS runs of trypsin and TEV protease digests of affinity-enriched probe-labeled enzymes.

unrelated members of enzyme superfamilies [see section 3.2 and ref 189].

By focusing exclusively on the characterization of probe-labeled peptides, active-site peptide profiling greatly simplifies the complexity of proteomic samples and enhances the detection of low-abundance proteins.[54] However, access to the rest of the sequence of probe-modified proteins would be beneficial for multiple purposes, including to identify post-translational modifications that may influence enzyme activity and to strengthen confidence in the assignment and quantification of proteins by LC−MS/MS through the analysis of multiple peptides per protein. Speers and Cravatt have addressed these limitations by introducing a tandem orthogonal proteolysis (TOP) strategy for ABPP in which probe-labeled proteins and their sites of probe modification are characterized in parallel LC−MS/MS runs [Figure 16, bottom].[208] Click chemistry methods were used to append onto probe-labeled proteins a biotin tag containing a consensus recognition sequence for tobacco etch virus protease (TEV). After enrichment of a labeled proteome with streptavidin beads, bound proteins are digested with trypsin, and probe-unmodified peptides are collected by filtration. Addition of TEV then liberates the probe-modified peptides. Each set of peptides is then analyzed by LC−MS/MS. TOP-ABPP was used to identify SE-labeling sites on more than 30 proteins from mouse heart proteome. These sites represented several types of “functional” residues, including catalytic residues, regulatory residues (e.g., sites of nitrosylation and glutathionylation), and conserved residues of unknown function. Importantly, for the latter two groups of labeling sites, parallel identification of the parent proteins in the trypsin phase increased confidence in the accuracy of their assignment.

Withers and co-workers have also introduced an advanced variant of active-site peptide profiling that incorporates a chemically cleavable reporter tag.[106] This innovation was motivated by the well-appreciated observation that large analytical handles can obscure MS analysis of small peptides. To avoid this complication, reduction of an internal disulfide bond releases enriched peptides from the biotin tag. Utilizing an ABP for glycosidases, the authors isolated active-site peptides for several members of this enzyme class from a panel of complex biochemical mixtures. Analysis of the secreted proteome of *Cellumonas fimi*, a mesophilic aerobic soil bacterium, led to the labeling, cloning, and expression of a novel $\beta$-1,4-glycanase. Notably, although this enzyme displayed high (∼60%) active-site sequence homology to known glycanases from *Streptomyces* sp., it was nevertheless distinguishable by a six-mer active-site peptide. In principle, this approach, like TOP-ABPP, could also accommodate whole protein analysis by switching the order of the trypsin digestion and streptavidin capture steps.

Active-site peptide profiling can also be accomplished using capillary electrophoresis−laser-induced fluorescence (CE−LIF) technologies, as demonstrated by Patricelli and co-workers.[54] CE−LIF exhibits several virtues compared to LC−MS/MS, including greater throughput and sample conservation. The resolution of active-site peptide profiling by CE−LIF is also exceptional, as evidenced by the successful separation of several kallikrein protease activities that comigrated by 1D-SDS−PAGE. Target identification remains a challenge for CE−LIF but can be accomplished by parallel studies using LC−MS/MS methods.

**4.2.1.2. ABPP-MudPIT.** A primary objective of ABPP, and proteomics in general, is the discovery of disease-associated enzymes that may serve as new therapeutic targets or diagnostic markers.[209] The realization of this goal requires analytical platforms for the in-depth, quantitative analysis of proteomes of high biological complexity (e.g., primary human tumors). Jessani and colleagues have addressed this problem by uniting ABPP with the multidimensional protein identification technology (MudPIT[10,210]) originated by the Yates group, to create a streamlined platform for the functional analysis of any biological sample.[55] This approach entails treating proteomes with biotinylated probes, capturing probe-labeled proteins on avidin beads, performing an on-bead trypsin digestion, and analyzing the resulting tryptic peptide mixture by two-dimensional LC−MS/MS. The data sets generated by ABPP-MudPIT are remarkably rich in information content, as evidenced by the identification of more than 50 serine hydrolase activities from individual breast tumor proteomes treated with FP−biotin. Moreover, relative quantification of enzyme activities across different proteomes is possible using spectral counting methods.[211,212] ABPP-MudPIT has been used to discover enzyme activities that are selectively elevated in aggressive breast tumors. The

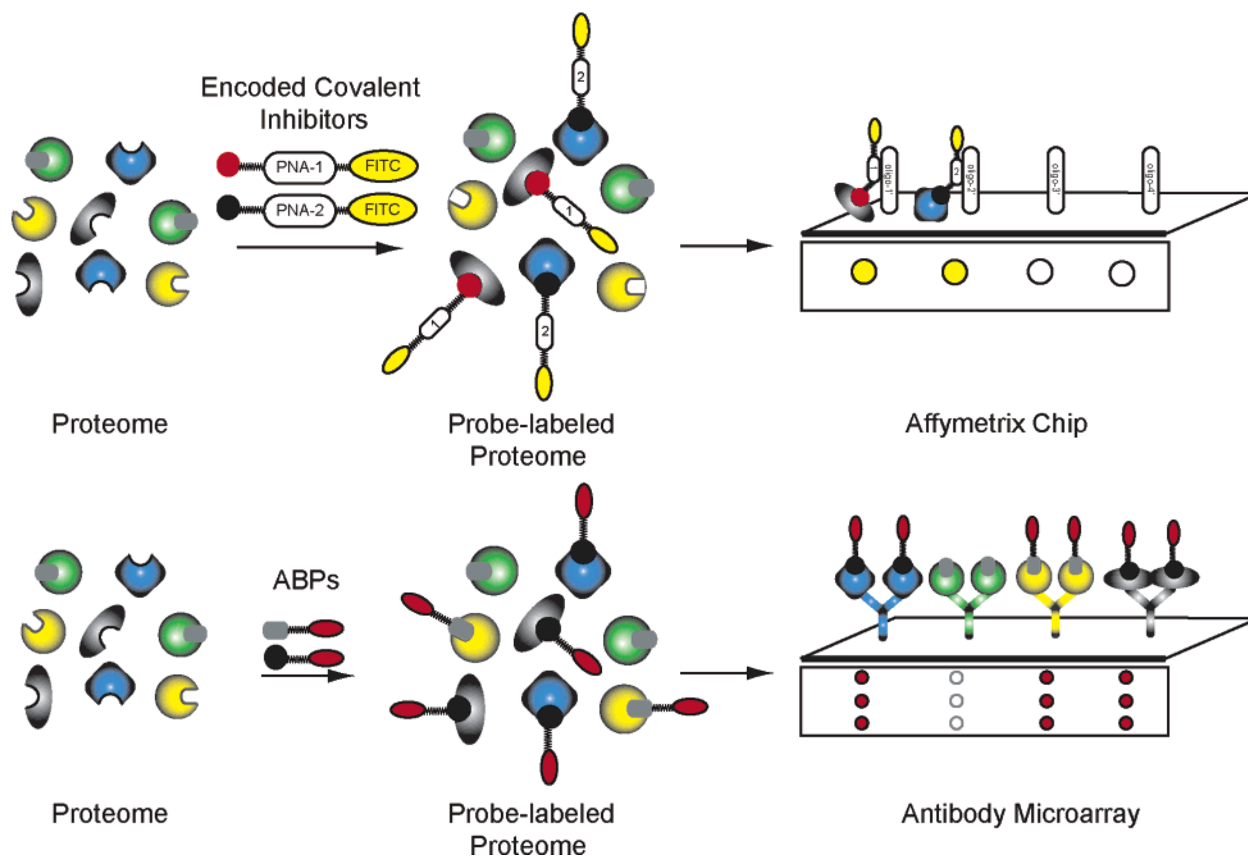



**Figure 17.** Microarray-based platforms for ABPP: (top) small-molecule microarray, where probe-labeled proteins are enriched and visualized via hybridization of a PNA tag to a complementary surface-arrayed oligonucleotide; (bottom) antibody microarray, where probe-labeled enzymes are enriched and visualized via binding to a complementary surface-arrayed anti-enzyme antibody.

merger of ABPP-MudPIT with the TOP method described in section 4.2.1.1 should provide an integrated, high-content platform for the quantitative profiling of enzyme activities and elucidation of sites of probe labeling.

### *4.2.2. Microarray Platforms*

ABPP platforms that rely on LC−MS/MS have addressed many of the limitations of original gel-based approaches. However, LC−MS/MS technologies possess their own drawbacks, including poor throughput and parallelization, as well as high sample demands. In the field of comparative genomics, DNA microarrays have satisfactorily addressed these problems, thus inspiring researchers to consider a similar analytical solution for ABPP. Two general strategies have emerged to date in which either the ABP or its target protein is captured on the microarray by complementary affinity reagents [Figure 17].

**4.2.2.1. Small-Molecule Microarrays for ABPP.** Schultz, Harris, and co-workers have developed small-molecule microarrays for the characterization of cysteine protease activities in complex proteomes.[213,214] A library of micro-array-compatible ABPs was prepared by tethering validated inhibitors [215−217] to a fluorophore via a distinguishing peptide nucleic acid (PNA) linker [Figure 17, top]. Owing to the high affinity of PNAs for complementary oligonucleo-tides,[218,219] the authors sequestered probe-labeled proteins in a spatially discrete fashion with Affymetrix-style gene arrays. The levels of individual cysteine protease activities were determined by measuring the intensity of fluorescent signals within encoded regions of the chip surface. The diagnostic utility of this small-molecule microarray was demonstrated by evaluating changes in caspase-3 activity during apopto-sis.[220] Satisfyingly, the zymogen form of caspase-3 was not enriched from cell lysates, while induction of apoptosis with exogenous granzyme B revealed active caspase-3, detectable to picomole quantities.

**4.2.2.2. Antibody-Microarrays for ABPP.** The afore-mentioned small-molecule microarrays are valuable for the characterization of enzymes that possess cognate high-affinity, high-selectivity ABPs. However, many ABPs are designed to target large swaths of the functional proteome (e.g., FPs, which label numerous serine hydrolases), which limits their utility in platforms that depend on encoded probes for target resolution.

A complementary ABPP microarray platform has been introduced by Cravatt and co-workers that utilizes anti-enzyme antibodies as capture reagents.[221] A virtue of this approach is that, by incorporating orthogonal protein labeling (ABP) and capture (antibody) reagents, it consolidates into a single assay the isolation, detection, and identification of probe-labeled enzyme activities [Figure 17, bottom]. Antibody-based ABPP microarrays were used to profile several protease activities in cell proteomes, exhibiting a sensitivity limit of 2−8 ng of enzyme/mL for the serine protease prostate-specific antigen (PSA). Notably, this detection limit was ∼50× lower than gel-based ABPP and comparable to the normal plasma levels of PSA. Additionally, minute quantities of proteome (low microgram) were required per microarray experiment. These results suggest that ABPP microarrays could be used to profile PSA activity in diseases such as prostate cancer, where this protease is a validated biomarker.[222] The extent to which microarrays will become

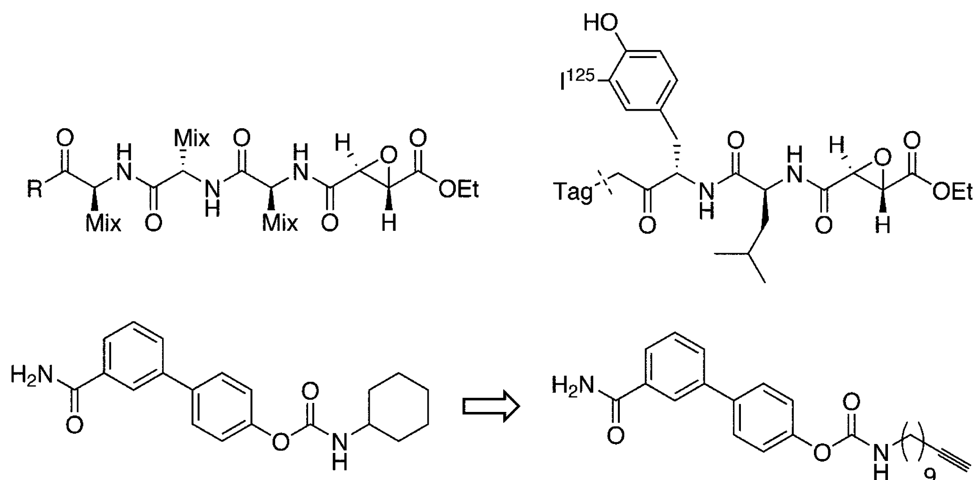



**Figure 18.** Profiling the selectivity of irreversible enzyme inhibitors by ABPP: (top) small-molecule affinity fingerprinting, where libraries of irreversible inhibitors are preincubated with enzymes and screened for their ability to block probe labeling, shown for a set of cysteine protease-directed inhibitors. (bottom) Profiling inhibitor selectivity in vivo. Here, inhibitors are converted into alkyne analogues to enable click chemistry-based conjugation of reporter tags to in vivo-labeled enzymes, as shown for the FAAH-directed covalent inhibitor URB-597.

a preferred mode for performing ABPP experiments will depend on the generation of a proteome-wide set of high-specificity antibodies or equivalent protein capture reagents.[223]

## 5. ABPP—Applications beyond Comparative Proteomics

As chemical tools, and the techniques to define their pharmacology, become integral parts of nearly all systems biology endeavors, protein-reactive compounds[20] have emerged as particularly versatile probes of complex biological systems. While original ABPP endeavors have focused on the comparative analysis of enzyme activities in disease models and specimens, other powerful applications of this chemical proteomic technology are emerging. The following sections describe some of the ongoing efforts to extend the utility of active-site directed covalent probes for the functional characterization of enzymes in physiology and pathology.

### 5.1. Inhibitor Discovery

Genomics-based research has the potential to deliver humankind into the age of molecular medicine, where diseases are treated with targeted therapeutics that possess minimal side effects. In support of this goal, combinatorial synthetic methodologies have provided an unprecedented boon of structurally diverse small-molecule libraries for target-based screening.[224] Nevertheless, appropriately leveraging the volume of available compound diversity requires methods to rapidly assess the potency and selectivity of candidate drugs.[225,226] The principles and tools of ABPP have been brought to bear on this problem to expedite discovery and characterization of enzyme inhibitors.

#### *5.1.1. Discovery of Irreversible Inhibitors*

Many natural products and drugs produce their biological effects through the covalent inactivation of proteins.[227] The inherent reactivity of these bioactive small molecules raises concerns about their specificity in proteomes, an important question that has been the ongoing focus of research in the ABPP field. Initial studies by Bogyo and colleagues showcased the utility of ABPP for characterizing the potency and selectivity of irreversible cysteine protease inhibitors.[127] The researchers screened a library of ABPs against a panel of papain cysteine proteases, resulting in the identification of reagents that selectively targeted cathepsin B [Figure 18, top]. This method, referred to as small-molecule affinity fingerprinting, has been extended to classify many papain proteases based on their inhibitor sensitivity profiles, revealing functional relationships among these enzymes that are not reflected in their linear amino acid sequences.

The target selectivity of irreversible inhibitors can also be evaluated in vivo using tag-free ABPP, as recently demonstrated for the endocannabinoid-degrading enzyme fatty acid amide hydrolase (FAAH). Alexander and Cravatt prepared a series of carbamate-directed FAAH inhibitors and, after confirming that these reagents covalently modified the serine nucleophile of this enzyme, synthesized an alkynyl carbamate for in vivo profiling [Figure 18, bottom].[228] Administration of this probe to mice, followed by tissue homogenization and conjugation of a rhodamine reporter tag to probe-labeled proteins by click chemistry, revealed the proteome-wide in vivo target selectivity of FAAH-directed carbamates. These reagents were selective for FAAH in the nervous system, but labeled several additional enzymes in peripheral tissues, including multiple carboxylesterases. Dose-dependence studies identified a restricted concentration window in which selective inactivation of FAAH could be achieved without substantial labeling of other targets in vivo. This general approach could be used to establish the in vivo selectivity profile of any covalent inhibitor and thereby assist in the characterization and refinement of these reagents for pharmacological and medicinal studies.

#### *5.1.2. Discovery of Reversible Inhibitors*

ABPP, when performed in a competitive mode, can also be used to identify and characterize reversible enzyme inhibitors [Figure 19], as described by Cravatt and coworkers.[229,230] In this approach, the kinetics of proteome labeling are determined for a given ABP, allowing for the selection of a time point at which the labeling of most of the enzymes in the sample has not yet reached completion. Inhibitor libraries and the ABP are then co-incubated in proteomes for this defined period of time, over which the

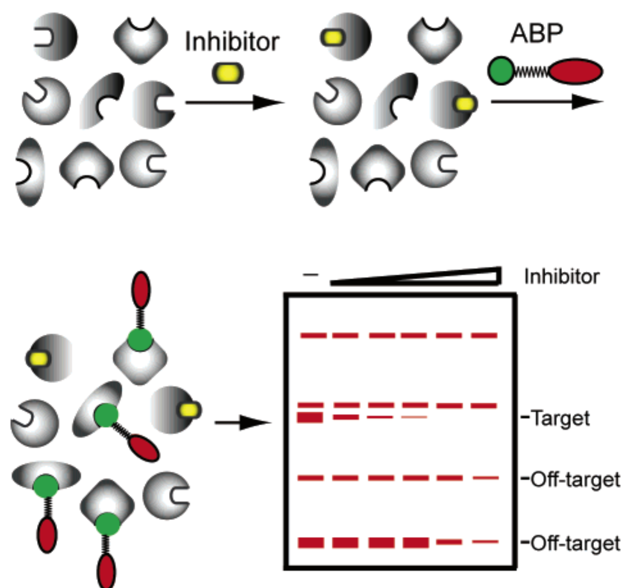



**Figure 19.** Profiling the selectivity of reversible enzyme inhibitors by competitive ABPP. The ability of an inhibitor to slow the rate of ABP labeling of one or more enzymes in the proteome is recorded as a reduction in fluorescent labeling intensity. Concentration-dependence curves can provide quantitative information on the potency of inhibitors for both the intended enzyme target and potentially unanticipated off-target enzymes.

binding of an inhibitor to one or more enzymes is detected as a quantitative reduction in probe labeling. The researchers applied competitive ABPP to characterize the proteome-wide selectivity of a library of reversible inhibitors directed toward FAAH. Concentration curves were generated for each inhibitor to provide $IC_{50}$ values that approximated the $K_i$ values determined using standard substrate assays. From these data sets, selective FAAH inhibitors could be readily distinguished from promiscuous agents that targeted multiple enzymes. Notably, these additional "off-target" enzymes shared no sequence homology with FAAH (or with one another), indicating that substantial active site homology can be found among enzymes that lack sequence-relatedness. The advantages of inhibitor discovery by competitive ABPP include the following: (1) enzymes are screened in native proteomes, thus eliminating the need for recombinant expression or purification of proteins, (2) enzymes are screened in "substrate-free" mode, thus facilitating the identification of inhibitors for uncharacterized enzymes, and (3) many enzymes are screened in parallel, thus assigning both potency and selectivity factors to each inhibitor.

Complementing de novo inhibitor screens, ABPP tools have been applied to characterize the inhibition profiles of existing drugs and natural products. Ploegh, Ovaa, and colleagues evaluated the specificity of bortezomib, a clinically approved proteasome inhibitor for the treatment of multiple myeloma,[231] using a two-step strategy.[232] EL-4 cells were first treated with this inhibitor followed by addition of a cell-permeable, proteasome-directed ABP. Comparison to cells treated only with the ABP revealed which catalytic subunits of the proteasome are affected by bortezomib in living cells. Bortezomib was shown to inhibit the $\beta1/\beta1i$ and $\beta5$ subunits, corresponding to caspase-like and chymotrypsin-like protease activities, respectively. Inhibitor profiling by ABPP has also been extended to characterize the selectivity of other protease-directed agents,[233,234] as well as map the endogenous targets of natural products, such as FR182877.[235]

## 5.2. Cell-Based Screening

Cell-based screens offer a powerful strategy to identify new bioactive small molecules that perturb protein function in living systems. This process, when carried out in systematic form, is called chemical genomics.[236,237] Numerous sophisticated platforms have been developed to generate[238,239] and assay[240,241] structurally diverse compound libraries in cell biological settings. However, unambiguously assigning the protein target(s) of bioactive small molecules emerging from these screens remains a pressing technical challenge that complicates downstream mechanism-of-action studies and the pharmacological refinement of lead compounds.[242]

Cravatt and co-workers have invoked the tools and principles of ABPP to develop a potentially general method for accelerated target discovery in small-molecule cell-based screens.[180] Specifically, a natural products-inspired library was designed with two features to assist target identification: (1) an electrophilic spiroepoxide to covalently modify cellular target(s) and (2) an alkyne for the visualization/identification of target proteins via click chemistry [Figure 20]. The inclusion of a variable binding group was intended to direct members of the probe library to distinct fractions of the proteome. The identification of proteins selectively targeted by bioactive probes could then be accomplished by comparing their in situ proteome reactivity profiles to those of inactive probes.

The spiroepoxide library was screened for anti-proliferation activity against the invasive human breast cancer cell line MDA-MB-231. One compound, MJE3 [Figure 20], exhibited

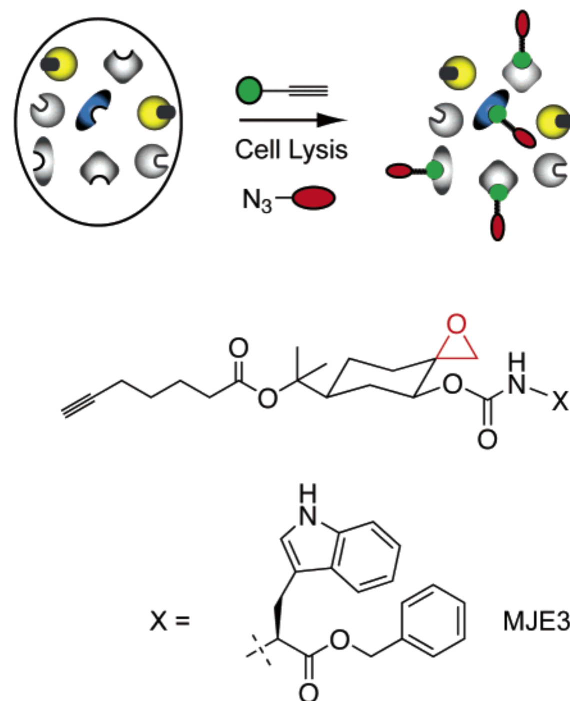



**Figure 20.** In situ proteome reactivity profiling to identify targets of ABPs in cell-based screens. Cells are treated with alkyne-modified probes, after which specific protein targets are identified by click chemistry addition of azide-modified reporter tags. At the bottom is shown the general structure of a natural products-inspired ABP library for cell-based screening, including the specific structure of one member, MJE3, that was found to display anti-proliferative effects and inactivate the glycolytic enzyme phosphoglycerate mutase 1 (PGAM1) in human breast cancer cells.

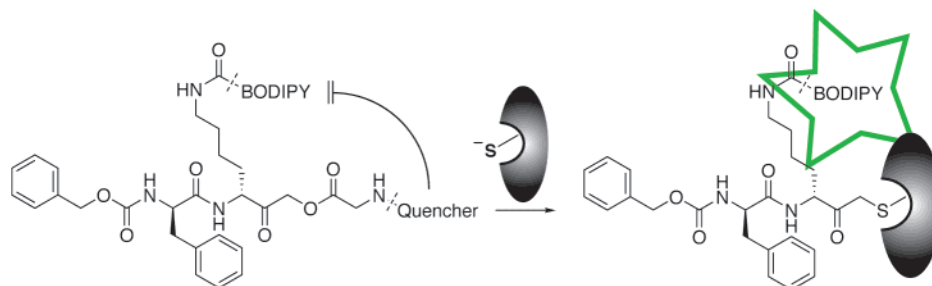



**Figure 21.** A fluorescently quenched ABP for in situ imaging of cysteine protease activities.

substantially greater inhibitory effects on proliferation compared to other members of the probe library. On analysis of the in situ reactivity profile, a single protein was found to be uniquely labeled by MJE3. This protein was identified as the glycolytic enzyme phosphoglycerate mutase B (PGAM1). Additional studies determined that MJE3 labeled PGAM1 on an active site peptide containing key substrate binding residues, which in turn resulted in enzyme inhibition. These data, along with complementary studies from other groups using peptide inhibitors[243] and siRNA probes,[244] suggest that PGAM1 activity is important for cancer cell growth and proliferation.[245]

Provocatively, the labeling and inactivation of PGAM1 by MJE3 were exclusively observed in intact cells, suggesting that key (as of yet unidentified) in situ factors influence this protein−small-molecule interaction. This result is instructive for future chemical genomics experiments, which may benefit from the inclusion of protein-reactive compounds that facilitate the covalent trapping of low-affinity and context-dependent protein targets.

## 5.3. Imaging Enzyme Activities

The adaptation of ABPP to increasingly sophisticated biological systems would benefit from tools that can sensitively and continuously monitor enzyme activities in vivo. Addressing this challenge, Bogyo and colleagues have developed a fluorescently quenched ABP (GB117) for the visualization of cathepsin activities in living cells [Figure 21].[246] An acyloxymethyl ketone probe was capped with a BODIPY fluorophore and a quencher moiety (QSY7), such that displacement of the leaving group, which occurs upon protease labeling, will liberate QSY7. Consequently, GB117 labeling is coupled with the appearance of a fluorescent signal. After demonstrating suitable probe affinity for cathepsins B and L in vitro, the authors explored the localization of GB117 in cultured monolayers of the murine fibroblast cell line NIH-3T3. Probe incubation illuminated distinct clusters of fluorescent signals in the cells. Significantly, the probe-induced distribution of fluorescent signal closely overlapped with the immunofluorescent staining pattern observed with anti-cathepsin B antibodies or with a fluorescent lysosomal marker. Fluorescently quenched ABPs should offer a useful design strategy for imaging enzyme activities in living systems.

# *6. Summary and Future Directions*

Over the past several years, the field of ABPP has enjoyed tremendous growth in terms of both technology development and biological applications. These advances can be traced not only to a rich history of research on mechanism-based enzyme inhibitors but also to the success of genome sequencing projects and innovations in mass spectrometry. Within the realm of systems biology, ABPP distinguishes itself as a highly interdisciplinary technology, integrating the more classical fields of organic synthesis and mechanistic enzymology with contemporary analytical methods. By development of chemical probes that capture fractions of the proteome based on shared functional properties, rather than mere abundance, ABPP interrogates portions of biomolecular space that are inaccessible to other large-scale profiling methods. More than a dozen enzyme classes are now addressable by ABPP, including all major classes of proteases, kinases, phosphatases, glycosidases, and oxidoreductases. The application of ABPP to a number of cell and animal models has succeeded in identifying enzyme activities associated with a range of diseases, including cancer, malaria, and metabolic disorders. The ABPP method also facilitates the generation of selective inhibitors for disease-linked enzymes, including enzymes of uncharacterized function. In summary, ABPP constitutes a powerful *hypothesis-generating technology engine*, illuminating which members of enzyme superfamilies are associated with specific physiological or pathological processes and, at the same time, facilitating the creation of selective chemical reagents to test the functions of these proteins.

Looking forward, several challenges remain for researchers interested in using ABPP to investigate biological processes. Many of these problems are technical and include expanding the proteome coverage of ABPP and increasing the sensitivity, resolution, and throughput of the analytical platforms used for data analysis. However, perhaps the most provocative frontier facing ABPP is the integration of this functional proteomic method with other large-scale profiling technologies and more targeted experimental approaches to achieve a deeper understanding of the biochemical mechanisms for health and disease. Indeed, it is important to recognize that the data sets generated by ABPP and, for that matter, other global molecular profiling methods are largely associative in nature. Elucidation of the functional significance of these relationships still falls mostly in the domain of more classical "one protein at a time" style research. Although ABPP does provide a conduit for inhibitor discovery that can accelerate the pharmacological characterization of individual enzymes, an understanding of the molecular mechanisms by which these proteins regulate disease processes requires that their *endogenous biochemical activities* also be elucidated. Characterization of these activities, which includes the identification of physiological substrates and products, is difficult to accomplish using genomic and proteomic methods alone.

It is possible that, in the future, ABPP could be united with complementary "systems biology" methods for profiling the metabolome,[247,248] a portion of biomolecular space that constitutes the major biochemical output of enzyme activity in vivo. By perturbing enzyme activity in living systems with

specific pharmacological or molecular biology (e.g., RNA interference) tools and then profiling the metabolic consequences, researchers may succeed in integrating both known and uncharacterized enzymes into the higher-order signaling and metabolic networks of cells and tissues. In this way, a relatively streamlined experimental platform could be established for rapidly moving from the discovery of enzyme activities associated with biological processes to elucidation of mechanistic basis and functional significance of these relationships. Those enzymes that emerge as key contributors to disease processes should constitute outstanding candidates for next-generation therapeutics.

## 7. Acknowledgments

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