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Activity-Based Protein Profiling: From Enzyme Chemistry to Proteomic Chemistry

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Abstract

Genome sequencing projects have provided researchers with a complete inventory of the predicted proteins produced by eukaryotic and prokaryotic organisms. Assignment of functions to these proteins represents one of the principal challenges for the field of proteomics. Activity-based protein profiling (ABPP) has emerged as a powerful chemical proteomic strategy to characterize enzyme function directly in native biological systems on a global scale. Here, we review the basic technology of ABPP, the enzyme classes addressable by this method, and the biological discoveries attributable to its application.

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INTRODUCTION

Explosive advances in genomics have spawned multidisciplinary efforts to develop new technologies for proteomics. To date, genome sequences of 639 organisms have been completed, including three primates—rhesus, chimpanzee, and man. Over 2000 other genome projects are underway. This embarrassment of riches for proteomics has put in sharp relief the daunting challenge of assigning functional, evolutionary, and interactive relationships to the full complement of proteins (the proteome) encoded by genomes. The context and temporal variability of the proteome distinguishes it from the background of a relatively stable genome and layers a manifold of complexity on the problem of developing large-scale methods for protein characterization.

Genomic signatures, such as chromosomal translocation and gene amplification, have provided some insights into protein expression (1, 2). Other genomic technologies, including transcriptional profiling and RNA-interference-based gene silencing, have provided insights into physiological and pathological processes, e.g., tumorigenesis (3, 4), bacterial pathogenesis (5, 6), and insulin signaling (7). However, these methods rely on profiling and manipulation of gene expression to deduce protein function and do not generally capture the myriad of posttranslational events that regulate protein activity (8). Sparked by this necessity, proteomics has introduced several strategies for the global analysis of protein expression and function. Liquid chromatography-mass spectrometry (LC-MS) platforms for shotgun analysis (9, 10), yeast two-hybrid methods (11), and protein microarrays (12) have greatly enriched our understanding of the expression patterns, interaction maps, and *in vitro* functional properties of proteins. However, an accurate assessment of the functional state of proteins in cells and tissues requires more direct methods to assess protein activity. Activity-based protein profiling (ABPP) has emerged as a key technology in the evolution of functional proteomics.

ABPP relies on the design of active-site-directed covalent probes to interrogate specific subsets (families) of enzymes in complex proteomes and to provide the basis for a quantitative readout of the functional state of individual enzymes in the family. Prototypic ABPP probes target a large, but manageable, fraction of the enzyme proteome, often defined by shared catalytic features. The size of the enzyme subset, however, can range from a few proteins to several hundred. ABPP probes utilize a range of chemical scaffolds, including mechanism-based inhibitors, protein-reactive natural products, and general electrophiles. Because the activity state of an enzyme is regulated by a variety of posttranslational mechanisms (8, 13), chemical probes

that report on the structure and reactivity of enzyme active sites in cells and tissues can provide high-content proteomic information that is beyond the reach of standard expression profiling technologies.

Although ABPP derives its enormous potential from recent genomic advances, its roots extend back for nearly a century. ABPP evolved out of the preconvergence of organic chemistry and enzymology (14). Motivated by a desire to glean mechanistic and structural information on the nature of enzymatic catalysis, enzyme chemists studied the modification of purified proteins by reagents ranging from simple organic compounds to affinity labels to elaborate mechanism-based inhibitors. With the goal of achieving a physical chemist's simplicity of experimental design, major effort was focused on the up-front purification of specific proteins by often heroic protocols to isolate a few units of active enzyme for downstream modification and analysis. The results were impressive; the work of Balls & Jensen (15–16) and their coworkers (17–19) in the early 1950s on the stoichiometric inhibition of the serine hydrolases, chymotrypsin, trypsin, and cholinesterase by diisopropyl fluorophosphate was relevant to the future design of ABPP probes. Subsequent work on the irreversible inactivation of serine, cysteine, and threonine proteases by a wide variety of molecules has been extensively reviewed (20).

There is no doubt that early experiments in the style of ABPP were performed before the word “proteomics” was invented. A case can arguably be made that the first such experiments were reported in the early 1970s using the classic serine-modifying antibiotic penicillin (21). Tipper & Strominger (22) had proposed that the β -lactam nucleus of penicillin was a structural analog of the D-ala-D-ala terminus of bacterial peptidoglycan and that the chemically reactive β -lactam was positioned to react covalently with the catalytic serine of the transpeptidase(s) responsible for cell

wall biosynthesis. Using radiolabeled penicillin G, whole cells or membrane fractions, and primitive gel electrophoretic methods by today's standards, Strominger and colleagues (23, 24) established that multiple penicillin-binding proteins (termed PBPs) are present in a bacterium. The number and function of these serine hydrolases vary in different bacteria. Using radiolabeled penicillin G as a probe, they were subsequently able to profile the PBP selectivity of other penicillin and cephalosporin analogs and developed covalent affinity chromatography methods for capturing and releasing PBPs for rapid purification (25, 26). Ultimately, this early approach linked enzyme class function to biology by demonstrating that, by selectively inhibiting or mutating single PBPs, one could infer specific roles for PBPs in cell wall biosynthesis and cell shape determination (27). The limiting factor in these studies was the difficulty in obtaining protein sequence information and in linking it to genomics information, which was nonexistent at that time.

The advances in protein separation technology and mass spectrometry over the past 30 years, coupled with genomics, now allow routine downstream deconvolution of complex proteomic samples. ABPP probes play a crucial role in fractionating these samples on the basis of a key aspect of enzyme function—catalytic activity. In addition, the interplay between new ABPP methods and the downstream analytical technologies has created a synergistic cycle of advancement of both disciplines. To date, probes have been developed for more than a dozen enzyme classes, including proteases, kinases, phosphatases, glycosidases, and oxidoreductases. They have contributed to our understanding of enzyme activity in specific physiological and pathological processes on a proteome-wide scale. Uncharacterized enzymes for which no previous function had been assigned abound in these profiles, providing new insight into their biology.

Several excellent reviews on ABPP have appeared (28–33). The purpose of this review is to describe the most recent advances and place them thematically within the field.

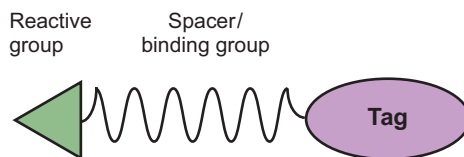
THE BASIC TECHNOLOGY OF ABPP

Before describing specific examples of ABPP probes and their biological applications, we briefly review the general design concepts and analytical platforms that support the ABPP technology.

General Design Features for ABPP Probes

The fundamental building blocks of ABPP are small-molecule probes that covalently label the active site of a given enzyme or enzymes. Ideal ABPP probes would target a large, but manageable, number of enzymes (tens to hundreds) to provide researchers with a global view of the functional state of the proteome. This level of target promiscuity must, however, be counterbalanced by limited cross-reactivity with other proteins in the proteome. Most ABPP probes achieve a desired level of intraclass coverage and minimal extra-class cross-reactivity by displaying a combination of reactive and binding groups that target conserved mechanistic or structural features in enzyme active sites (**Figure 1**). Reactive groups can be further divided into two general classes: (a) electrophilic groups that modify conserved active-site nucleophiles, and (b) photoreactive groups that label proximal residues in enzyme active sites following UV irradiation. ABPP probes must also possess a third element, a reporter tag, to facilitate target characterization (**Figure 1**). Examples of reporter tags include fluorophores, biotin, and latent analytical handles such as alkynes or azides, which can be modified by click chemistry [copper-catalyzed Huisgen's azide-alkyne cycloaddition (34)] methods to visualize protein targets postlabeling (35, 36). As is elaborated in the following section, the iden-

a ABPP probe:



b Tags:

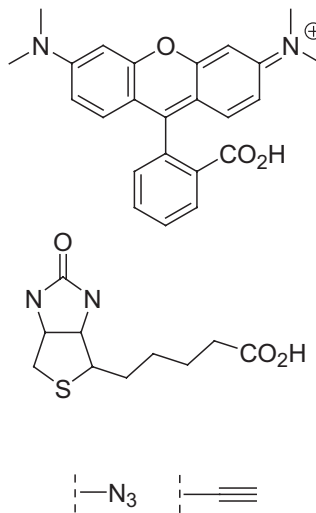


Figure 1

(a) Representative structure of an ABPP probe, which contains a reactive group (green), a spacer or binding group (black), and a reporter tag (purple). (b) A variety of reporter tags can be used for enzyme visualization and enrichment, including fluorophores (e.g., rhodamine) and biotin, as well as “clickable” handles, such as azides and acetylenes.

tity of the reporter tag dictates experimental options for downstream analysis in ABPP experiments.

Analytical Platforms for ABPP

With a proficient ABPP probe in hand, researchers can investigate proteomes using a number of analytical platforms. These platforms exhibit distinct advantages and limitations that must be matched with the

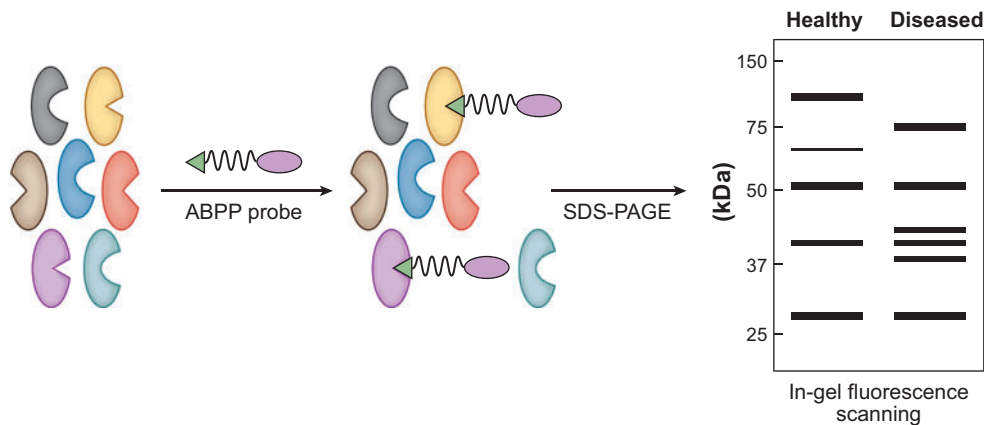


Figure 2

Gel-based activity-based protein profiling (ABPP), where probe-labeled enzymes are visualized and quantified across proteomes by in-gel fluorescence scanning.

specific experimental problem to maximize the information content acquired in ABPP investigations.

Gel electrophoresis platforms for ABPP.

Original versions of ABPP utilized gel-based methods for the detection of enzyme activities (37–40). In this approach, probe-treated proteomes are first resolved by one- (1D) or two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE), and the labeled enzymes then are visualized by either in-gel fluorescence scanning (for fluorescent probes) or avidin blotting (for biotinylated probes) (Figure 2). A typical functional proteomic investigation would likely employ both modes of detection, first using fluorescent probes for rapid screening of proteomes by 1D-sodium dodecyl sulfate (SDS)-PAGE, and then applying biotinylated probes in combination with (strept)avidin chromatography, gel separation, and mass spectrometry analysis to identify probe-labeled enzymes. Although higher-resolution, “gel-free” analytical platforms have since emerged for ABPP (see below), gel-based methods are still widely employed today owing to their robustness and throughput. Indeed, hundreds of proteomes can be analyzed per day by an individual research group using fluorescent probes and

1D-SDS-PAGE, a level of throughput that vastly exceeds most other methods. Gel-based ABPP is thus still preferred for research problems that require the rapid comparative analysis of many proteomes in parallel.

Liquid chromatography-mass spectrometry (LC-MS) platforms for ABPP.

Recognizing that the principal limitation of gel-based methods was resolution, researchers have introduced multiple LC-MS strategies for analyzing probe-treated proteomes that vastly improve the information content acquired in ABPP experiments. LC-MS approaches for ABPP can be generally divided into two categories—those that analyze the protein targets of probes and those that specifically analyze probe-labeled peptides derived from these targets. The former strategy involves using biotinylated probes to label proteomes, which are then directly incubated with (strept)avidin beads to enrich probe-labeled proteins (41) (Figure 3a). Enriched proteins are then digested on-bead with trypsin and analyzed by multidimensional LC-MS/MS. This approach, which essentially represents a fusion of ABPP and the multidimensional protein identification technology (ABPP-MudPIT), has proven capable of identifying 50–100+ enzyme

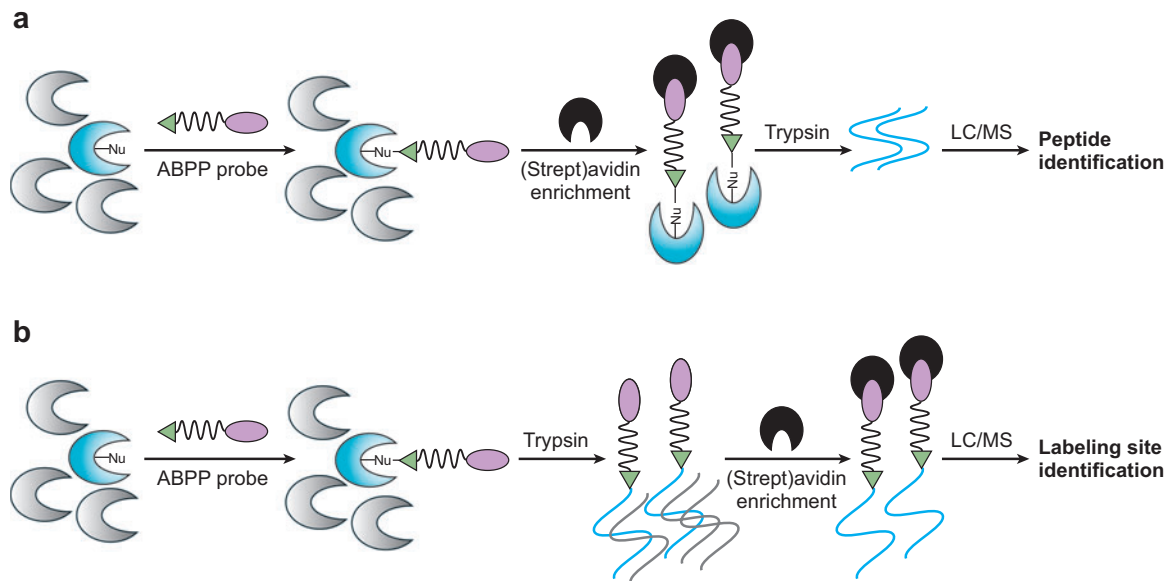


Figure 3

Liquid chromatography-mass spectrometry (LC/MS)-based platforms for activity-based protein profiling (ABPP). (a) ABPP and multidimensional protein identification technology (41), whereby probe-labeled enzymes are first captured on (strept)avidin beads and then digested with trypsin and the resulting peptide mixture analyzed by LC-MS/MS. (b) Active-site peptide profiling (44, 45), whereby probe-treated proteomes are digested with trypsin prior to affinity enrichment. Enriched peptides are then eluted with organic solvents and analyzed by LC-MS/MS.

activities in individual proteomes. Moreover, the relative levels of enzyme activities in two or more proteomes can be determined by ABPP-MudPIT using simple, semiquantitative parameters such as spectral counting (41–43).

One shortcoming of ABPP-MudPIT is that this method does not offer a straightforward way to identify the probe-labeled peptides of enzyme targets. This goal has been achieved using a distinct LC-MS platform, termed active-site peptide profiling (ASPP), that involves digesting probe-treated proteomes with trypsin prior to incubation with affinity enrichment resins (**Figure 3b**) (44, 45). Probe-labeled peptides are thus selectively enriched using either (strept)avidin or antibody resins (for biotinylated and fluorescent probes, respectively) and then eluted with organic solvents and analyzed by LC-

MS/MS. Modified versions of the SEQUEST search algorithm are employed to simultaneously identify enzyme targets and their specific sites of probe labeling. If ABPP probes that contain a disulfide linker are utilized, labeled peptides can be eluted with excess free thiols and analyzed by LC-MS/MS (46).

ABPP-MudPIT and ASPP possess complementary advantages and limitations. Because the former method analyzes many tryptic peptides for each probe target, the likelihood of accurately identifying and quantifying proteins improves. In contrast, the site of labeling data provided by ASPP greatly strengthens confidence that probes are targeting active sites to provide a legitimate readout of the functional state of enzymes. Could both sets of information be acquired in a single ABPP experiment? Speers & Cravatt (47) broached this subject by introducing

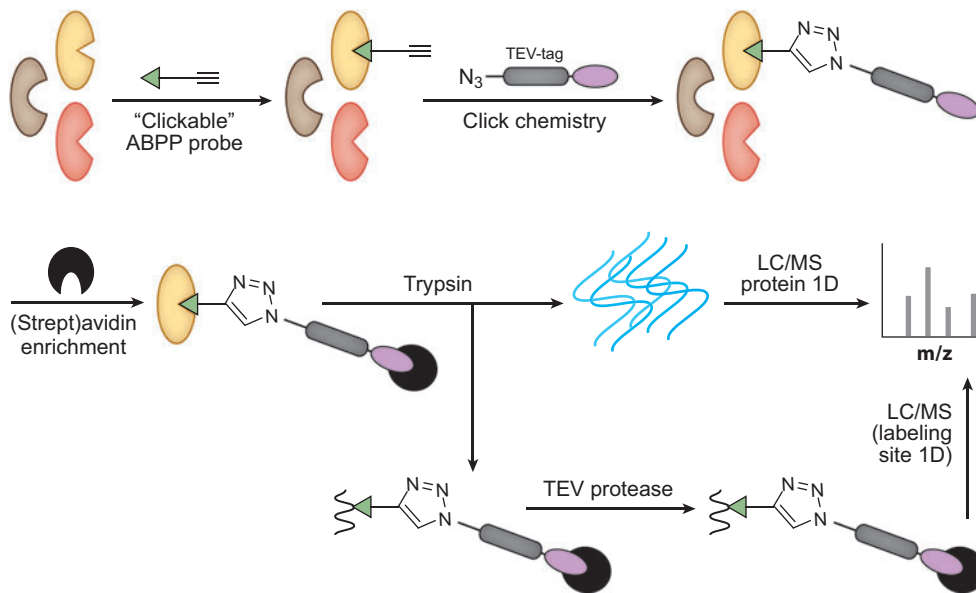


Figure 4

Tandem-orthogonal proteolysis (TOP)-activity-based protein profiling (ABPP) for simultaneous characterization of protein targets of probes and sites of probe labeling (47). Abbreviations: 1D, one dimensional; LC-MS, liquid chromatography-mass spectrometry; m/z , mass/charge; TEV, *Tobacco etch virus*.

a *Tobacco etch virus* (TEV) protease cleavage site between the reactive group and biotin tag of ABPP probes. Specifically, the researchers made use of click chemistry to conjugate an azide-TEV cleavage peptide-biotin tag onto enzymes labeled by an alkyne-sulfonate ester ABPP probe (**Figure 4**). The implementation of click chemistry methods (35, 36) circumvented potential problems that the large reporter tag might have caused for probe activity. Probe-labeled proteins were then captured on (strept)avidin beads and subject to tandem digestions with trypsin and TEV protease to release bulk (unlabeled) and probe-labeled peptides. These protease digestions were then analyzed in sequential LC-MS runs. Cross-correlating the data from each protease digestion greatly improved confidence in protein assignments, allowing for the removal of false-positive signals and the identification of unanticipated targets of ABPP probes. This platform, dubbed tandem-orthogonal proteol-

ysis (TOP)-ABPP, thus allows researchers to simultaneously profile enzyme targets of ABPP probes and their specific sites of probe modification. Recently, chemical strategies have been introduced for the cleavage of probe-modified proteins/peptides from affinity resins (48, 49), which should serve as useful complements to the TOP-ABPP method.

It is important to recognize that the superior resolution and information content afforded by LC-MS approaches for ABPP come with a cost to investigators. First, LC-MS requires much larger quantities of proteome compared to gel-based methods (0.5–1.0 mg versus 0.01–0.02 mg, respectively). Although for many studies, including those that analyze rodent tissues and cell lines, proteome may be in ample supply, other samples, such as primary human biopsies, are of a finite amount, which may hinder their analysis by LC-MS. LC-MS methods for ABPP are also much slower than 1D-SDS-PAGE, especially when performed to maximize resolution

(i.e., multidimensional separations, which require several hours per sample). Thus, even though LC-MS would certainly be a preferred method for the in-depth comparison of a handful of proteomes, the analysis of dozens or hundreds of samples will likely require other approaches (or access to several LC-MS instruments).

Emerging platforms for high-throughput, high-resolution ABPP.

An ideal ABPP platform would combine the throughput and minimal sample requirements of gel-based analysis with the resolution afforded by LC-MS. Although no such platform yet exists, substantial progress has been made toward this goal by combining ABPP with analytical methods that require neither gel nor MS readouts. One such strategy exploits capillary electrophoresis (CE) coupled with laser-induced fluorescence (LIF) scanning as separation and detection techniques, respectively (45). In this method, proteomes are treated with fluorescent probes, digested with trypsin, and the resulting probe-labeled peptides enriched with antiferrofluorophore antibody resins. Enriched peptides are then eluted and analyzed by CE-LIF, which was shown to provide vastly superior resolution compared to 1D-SDS-PAGE, especially for enzyme targets that share similar molecular mass. CE-LIF ABPP also has the advantages of consuming minimal amounts of sample and of potentially achieving high throughput. Indeed, CE run times are very short (15–20 min), and many samples can be analyzed in parallel on 96-channel instruments. One potential drawback of CE-LIF ABPP is that the identity of enzyme targets initially remains unclear. Although this information can be obtained in complementary LC-MS experiments, eventually leading to unequivocal assignment of individual CE peaks to specific enzyme targets, this process is slow. CE-LIF ABPP may therefore be most applicable for the repetitive analysis of well-characterized proteomes, as is required when screening large chemical libraries for target selectivity.

A second emerging platform that holds potential for satisfying the requirements of low-sample demand and high-throughput/high-resolution analysis is the ABPP microarray (50). In this method, antibodies that specifically recognize enzyme targets of ABPP probes are arrayed on glass slides and used as capture tools. Once bound to their cognate antibodies on the microarray, probe-labeled enzymes can be directly detected by fluorescence scanning. ABPP microarrays were shown to exhibit significantly improved sensitivity compared to gel-based methods for the detection of protease activities in proteomes. Additionally, minimal amounts of proteome (<0.01 mg) were required for ABPP microarray experiments. The throughput of ABPP microarrays is also potentially excellent, as many antibodies can be arrayed in parallel on a single slide. Finally, ABPP microarrays, by removing the requirement for a second antibody detection agent, address one of the main technical challenges facing the implementation of standard protein microarrays. The principal limitation that currently hinders the routine application of ABPP microarrays is a dearth of commercially available antibodies that can selectively recognize enzyme targets in this assay format. Alternative microarray formats for ABPP have also been introduced that couple probes to peptide-nucleic acid tags (51). Probe-labeled enzymes are then captured by hybridization to arrays bearing complementary oligonucleotides. Using this technology, Harris and colleagues (52) identified a selective inhibitor of the dust mite protease Der p 1 and provided evidence that this protease plays a role in allergy progression.

Types of Biological Experiments that Can Be Performed with ABPP

As the methods for performing ABPP have diversified and matured, multiple types of biological applications for this technology have emerged. In this section, we briefly summarize the most common uses of ABPP. Specific

examples of the biological impact of ABPP applied in these various formats are covered in the following section on enzyme classes addressable by ABPP.

Comparative ABPP for target discovery.

The original, and still most common application of ABPP is for “target discovery” in biological systems. A typical target discovery experiment would comparatively analyze two or more proteomes by ABPP to identify enzymes with differing levels of activity (Figure 2). If the proteomes under comparison in turn display distinct biological properties (e.g., healthy versus normal), then the altered enzyme activities identified by ABPP can be hypothesized to regulate these phenotypes. The testing of such hypotheses, of course, requires further experimentation; examples are detailed below. In comparison to more conventional expression-based genomics and proteomics, ABPP has multiple advantages for target discovery experiments. First, ABPP accounts for myriad posttranslational mechanisms that regulate enzyme activity (but not necessarily expression) in living systems. Second, because ABPP probes label enzymes using conserved active-site features rather than mere expression level, these reagents provide exceptional access to

low-abundance proteins in samples of high complexity.

Competitive ABPP for inhibitor discovery.

ABPP has been adapted for a second major application—the discovery of enzyme inhibitors. This pursuit involves performing ABPP in a competitive mode, during which inhibitors are identified by their ability to block probe labeling of enzymes (38, 53, 54) (Figure 5). Competitive ABPP offers several advantages over conventional inhibitor screening methods. First, enzymes are tested in native proteomes, alleviating the need for recombinant expression and purification. Second, probe labeling acts as a surrogate for substrate assays, meaning that novel enzymes that lack known substrates are amenable to analysis. Finally, because ABPP tests inhibitors against many enzymes in parallel, potency and selectivity factors can be simultaneously assigned to these compounds. Both reversible (54) and irreversible (53) inhibitors of enzymes can be characterized by competitive ABPP, although the details of the assay format differ slightly. Reversible inhibitors must be screened under kinetically controlled conditions where probe labeling of enzyme targets has not yet reached completion. Analysis of irreversible inhibitors, which is more straightforward and can even be performed in

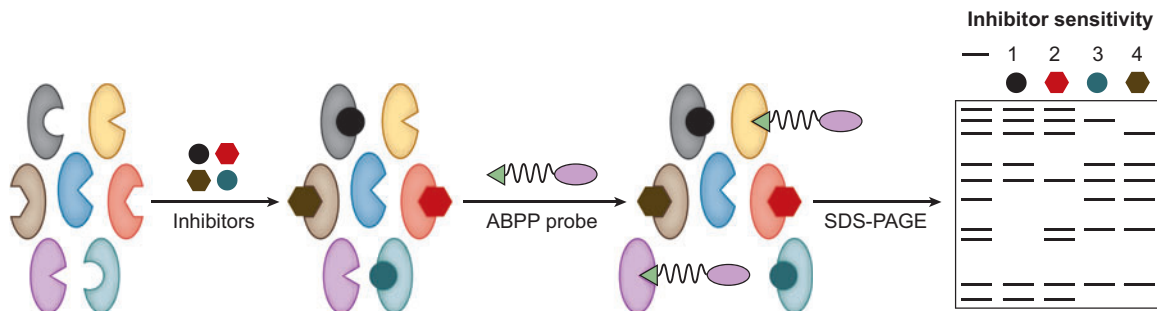


Figure 5

Inhibitor discovery by competitive activity-based protein profiling (ABPP). The selectivity and potency of enzyme inhibitors can be determined by initial incubation of a proteome with inhibitors followed by probe treatment. Inhibitor-bound enzymes are detected by a reduction in probe labeling intensity.

Abbreviation: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

vivo, involves preincubation with proteomes for various amounts of time, after which residual enzyme activity is monitored by addition of ABPP probes (43, 55, 56).

Characterization of enzyme active sites by ABPP. Genome sequencing projects have revealed that eukaryotic and prokaryotic organisms possess a remarkable number of unannotated proteins. ABPP offers a potentially powerful means to gain functional insights into uncharacterized enzymes. For example, discovery that an uncharacterized enzyme reacts with an ABPP probe may facilitate assignment of the enzyme to a specific mechanistic class (57, 58). Similarly, characterization of unanticipated sites of labeling in enzyme active sites can designate potential catalytic roles for residues of previously unknown function (44). Finally, the reactivity profile of uncharacterized enzymes with libraries of ABPP probes offers insights into active-site recognition, which may in turn lead to hypotheses about the structures of endogenous substrates and products (59).

ENZYME CLASSES ADDRESSABLE BY ABPP

Research efforts over the past decade have engendered ABPP probes for numerous enzyme classes. These probes have been used to garner striking insights into enzyme function in a wide range of biological systems. Here, we summarize the progress made to date in the development and biological application of ABPP probes for individual enzyme classes.

Serine Hydrolases

Serine hydrolases represent a large and diverse enzyme class that includes proteases, peptidases, lipases, esterases, and amidases. These enzymes collectively constitute approximately 1% of the predicted protein products encoded by most eukaryotic genomes. Serine hydrolases are united by a common catalytic mechanism that involves

activation of a conserved serine nucleophile for attack on a substrate ester/thioester/amide bond to form an acyl-enzyme intermediate, followed by water-catalyzed hydrolysis of this intermediate to liberate the product. The greatly enhanced nucleophilicity of the catalytic serine renders it susceptible to covalent modification by many types of electrophiles, including fluorophosphonates (FPs) and aryl phosphonates, sulfonyl fluorides, and carbamates. Phosphonate electrophiles have emerged as a particularly powerful class of affinity labels for the design of ABPP probes that target serine hydrolases (**Figure 6**).

Phosphonate probes for serine hydrolases.

Reporter-tagged FPs (**Figure 6a**) exhibit remarkably broad reactivity with enzymes from the serine hydrolase class (37–39). To date, more than 80 distinct serine hydrolases have been identified in the literature as targets of FP probes in human and mouse proteomes (41, 43, 45, 60, 61). A provocative number of these enzymes are uncharacterized in terms of their endogenous substrates and products, reflecting that less than half of the members of the mammalian serine hydrolase family have a defined metabolic function. Importantly, FPs have been shown to serve as bona fide activity-based probes for serine hydrolases, reacting with active enzymes but not with their inactive precursor (i.e., zymogen) or inhibitor-bound forms (38, 60). This factor has enabled researchers to identify serine hydrolases that show altered activity, but not expression, in biological systems (60, 61). The activity-based nature of FP labeling has also bolstered confidence in the functionality of serine hydrolases that lack known substrates, such as the predicted peptidase PREPL, which is deleted in hypotonia-cystinuria syndrome (62). Finally, high-resolution MS platforms have been developed to map the precise sites of FP probe labeling (45), facilitating the identification of novel, sequence-unrelated members of the serine hydrolase class (58).

Although the selectivity of FP probes can be partially tuned by varying the linker

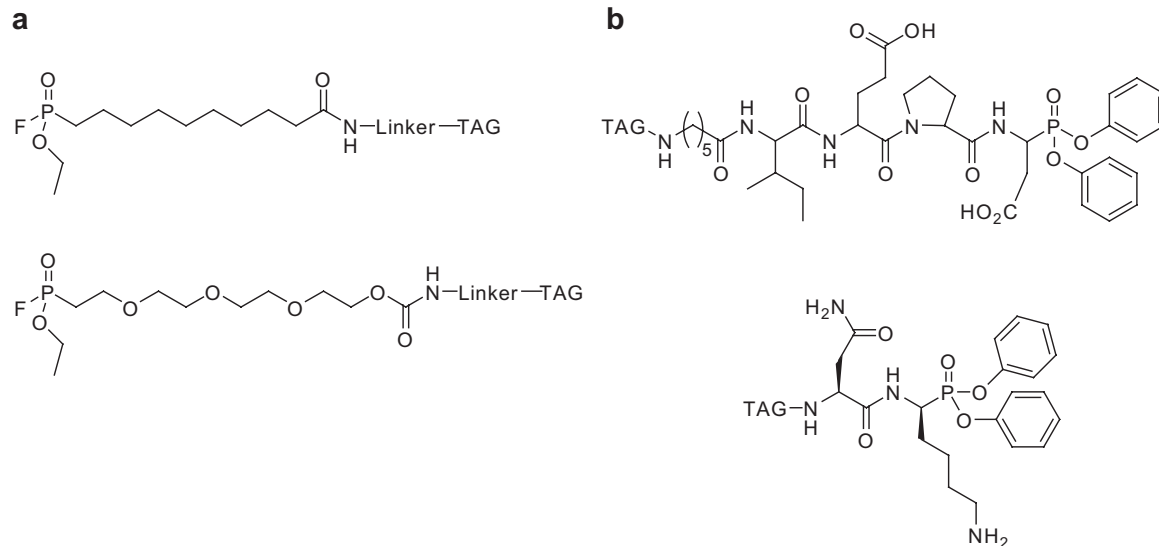


Figure 6

Electrophilic phosphonates as prototypic activity-based protein profiling probes for serine hydrolases. (a) Representative fluorophosphonate (FP) probes (37, 38). (b) Representative aryl phosphonate probes (63, 64).

unit that connects this reactive group to the reporter tag [i.e., switching from an alkyl to a pegylated chain (38) (**Figure 6a**)], these probes, in general, show little dependence on additional binding groups for reactions with most serine hydrolases. There are exceptions, however, including certain serine proteases that display restricted substrate selectivities that reduce their rates of labeling with generic FP probes. To address this problem, peptidic arylphosphonate ABPP probes have been introduced (**Figure 6b**) that show enhanced rates of labeling (63) and, in certain cases, high specificity for individual serine proteases (64). Collectively, these studies suggest that electrophilic phosphonates should offer a universal strategy to profile the activity of essentially all members of the serine hydrolase superfamily.

Biological applications of ABPP probes for serine hydrolases. ABPP has been used to profile the activity of serine hydrolases in numerous biological settings, including cancer

(41, 60, 61, 65), atherosclerosis (66), immune cell activation (64), and nervous system signaling (56, 67). In certain instances, these studies have facilitated the assignment of metabolic and cellular functions to previously uncharacterized members of the SH family (68).

Profiling serine hydrolase activities in cancer. Many serine hydrolases, including proteases, lipases, and esterases, have been postulated to play important roles in cancer. These enzymes, by controlling extracellular matrix structure, growth factor activation, and the metabolism of small-molecule signals, may contribute to the malignant behavior of aggressive cancer cells. To explore this premise further, Jessani and colleagues (60) globally profiled serine hydrolase activities across a panel of human breast carcinoma and melanoma cell lines that differed in pathogenic properties. The researchers first used fluorescent FP probes to generate gel-based profiles for the quantitative comparison of enzyme activities and then

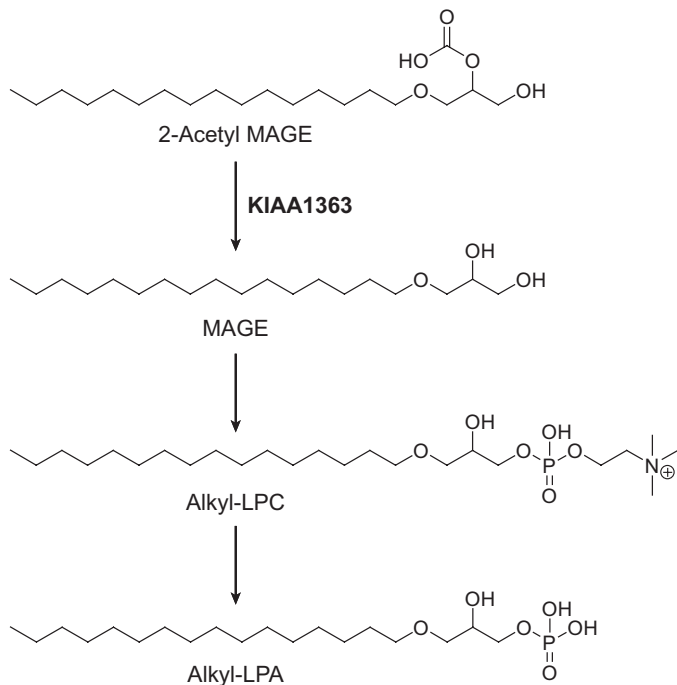


Figure 7

The uncharacterized serine hydrolase KIAA1363, which was found by activity-based protein profiling to be elevated in aggressive cancer cells, regulates an ether lipid signaling network that includes monoalkylglycerol ethers (MAGEs), alkyl-lysophosphatidylcholine (LPC), and alkyl-lysophosphatidic acid (LPA) (68).

biotinylated FP probes coupled with avidin chromatography and LC-MS analysis to enrich and identify these enzymes. Hierarchical clustering analysis identified enzyme activity signatures that segregated cancer cells into subgroups on the basis of tumor of origin or state of invasiveness. These enzyme activities included known markers of cancer malignancy, such as the serine protease urokinase plasminogen activator (uPA) and uncharacterized proteins, such as the integral membrane protein KIAA1363, for which no prior association with cancer had been made. Subsequent studies using ABPP-MudPIT confirmed that KIAA1363 activity is highly elevated in aggressive estrogen receptor-negative [ER(-)] primary human breast tumors relative to less aggressive ER(+) tumors or normal breast tissue (41).

Intrigued by the strong correlation between KIAA1363 activity and the pathogenic state of cancer cells, Chiang and colleagues (68) pursued the functional characterization of this enzyme. A potent and selective carbamate inhibitor of KIAA1363 was developed using competitive ABPP methods and applied to aggressive cancer cell lines that contain high levels of the enzyme. Analysis of the inhibitor-treated cells using global metabolite profiling methods (69) revealed a selective decrease in an unusual class of lipids—the monoalkylglycerol ethers (MAGEs). Biochemical studies confirmed that KIAA1363 regulates MAGE levels in cancer cells by hydrolyzing the precursor lipid 2-acetyl MAGE (Figure 7). Interestingly, MAGEs were further converted by cancer cells into alkyl-lysophospholipids, including lysophosphatidic acid (LPA) (Figure 7), a bioactive lipid known to promote the pathogenic properties of cancer cells (70). Disruption of KIAA1363 expression by RNA interference impaired this ether lipid network, leading to reductions in cancer cell migration and tumor growth. These studies thus indicate that the KIAA1363-ether lipid network supports the aggressive behavior of cancer cells. Concurrent with these studies, Nomura and colleagues (67) used FP probes to identify KIAA1363 as a principal degradative enzyme for organophosphorus nerve toxins in rodent brains, indicating that the enzyme may play important functions in xenobiotic metabolism in normal tissues.

In addition to analyzing human cancer cell lines in culture, ABPP has been used to profile these cells grown in vivo as xenograft tumors in immune-deficient mice (61). These studies enabled identification of both tumor- and host-derived enzyme activities that were altered during tumor progression. This investigation also uncovered a hyperaggressive variant of the human breast cancer line MDA-MB-231, which was isolated from xenograft tumors grown in the mammary fat pad (mfp) and found to display greatly enhanced tumor-forming and metastatic potential in vivo.

ABPP of these “231mf” cells revealed that they possess dramatically elevated levels of multiple secreted serine protease activities, including uPA and tissue-plasminogen activator (tPA). Interestingly, transcript levels of uPA and tPA were essentially unaltered compared to parental MDA-MB-231 cells, thus providing a compelling case wherein ABPP garnered functional proteomic information that was not reflected in gene expression profiles.

Profiling serine protease activities in immune cell activation. Serine proteases of the granzyme subclass have been postulated to play major roles in cytotoxic lymphocyte [natural killer (NK) cell]-mediated cell death. However, the relative contributions made by individual granzymes to NK cell function remain unclear owing in large part to a lack of selective chemical inhibitors for these enzymes. Mahrus & Craik (64) set out to address this problem by first screening five human granzymes for their ability to cleave members of a combinatorial library of peptide substrates. Distinct substrate selectivity profiles were observed for each granzyme, and this information was used to create substrate-mimetic diphenylphosphonate inhibitors that selectively target granzyme A and B. Biotinylated versions of the phosphonates confirmed their target selectivity in NK cell proteomes. Interestingly, the granzyme B inhibitor blocked NK cell-mediated lysis of target cells by more than 75%; in contrast, the granzyme A inhibitor displayed little efficacy. These data thus argue that granzyme B is the dominant protease involved in effecting target cell lysis by NK cells.

Determining the proteome-wide selectivity of serine hydrolase-directed inhibitors. Competitive ABPP has been used to evaluate the selectivity of several serine hydrolase inhibitors. In an early example of the value of this approach, Leung and colleagues (54) tested inhibitors of the integral membrane enzyme fatty acid amide hydrolase (FAAH) for their selectivity in mouse tissue

proteomes. FAAH is responsible for degrading the fatty acid amide family of signaling lipids, which includes the endocannabinoid anandamide (71). Genetic (72) or pharmacological (73) inactivation of FAAH leads to analgesic, anti-inflammatory, anxiolytic, and antidepressant effects, suggesting that this enzyme may represent a novel target for several nervous system disorders. All inhibitors of FAAH reported to date possess an electrophilic carbonyl element that engages the conserved serine nucleophile in the enzyme's active site. This mode of inhibition may target other members of the SH superfamily. Indeed, competitive ABPP using reporter-tagged FP probes identified several “off targets” for first-generation FAAH inhibitors, including other lipases, as well as enzymes of uncharacterized function (54). Interestingly, none of these enzymes shared any sequence similarity with FAAH, indicating that sequence homology is not necessarily a good predictor of active-site relatedness in enzyme superfamilies. Competitive ABPP has since become an integral component of nearly all FAAH inhibitor development efforts, permitting the concurrent optimization of potency and selectivity. These efforts have culminated in the generation of multiple classes of reversible (74) and irreversible (75) FAAH inhibitors that are highly specific for this enzyme. Conversely, inhibitors that show broad activity against multiple SHs have also been exposed (43). Irreversible FAAH inhibitors have themselves been converted into ABPP probes by incorporation of a “clickable” alkyne group into their structures (56). These agents have been used to identify the direct targets of FAAH inhibitors *in vivo*.

Inhibitor development programs for other serine hydrolases have also benefited from competitive ABPP. For example, Nomanbhoy and colleagues (76) examined the proteome-wide selectivity of a panel of inhibitors of dipeptidylpeptidase IV (DPP4). DPP4 regulates the levels of insulin-promoting hormone glucagon-like peptide 1 (GLP1) and, therefore, has become an attractive therapeutic

target for type II diabetes. DPP4 inhibitors must, however, selectively target this enzyme over several closely related peptidase homologs. Competitive ABPP has provided a useful platform to rapidly assess the selectivity of DPP4 inhibitors in native proteomes and has facilitated the development of selective inhibitors for other members of the DPP class (e.g., DPP7) (77).

Cysteine Proteases

Like serine hydrolases (SHs), cysteine proteases are a huge enzyme class whose members perform myriad critical functions in prokaryotic and eukaryotic organisms. The distinct catalytic mechanisms employed by SHs and cysteine proteases, however, make them susceptible to inactivation by different electrophilic chemotypes (i.e., cysteine proteases are not labeled by a FP probe). Multiple reactive groups, including epoxides, vinyl sulfones, diazomethyl ketones, α -halo ketones, and acyloxymethyl ketones (78), have been incorporated into ABPP probes developed for cysteine proteases. Here, we highlight the most mature versions of these probes, the specific subsets of cysteine proteases that they target, and examples of their biological applications.

Epoxide probes that target the papain family of cysteine proteases. Papains are members of the CA clan of cysteine proteases that include the cathepsins, which play key roles

in a variety of physiological and pathological processes (78). Cathepsins are nearly universally inactivated by the natural product E-64 (79) (**Figure 8a**), which contains an activated epoxide that reacts with the cysteine nucleophile of these enzymes. E-64 shows very limited cross-reactivity with other cysteine proteases, which has made it an excellent pharmacological tool for biologists interested in investigating the function of cathepsins. Bogyo and colleagues (80) have appended a range of reporter tags onto E-64, including radioisotopes, fluorophores (40), and biotin (80) (**Figure 8b**), to create a series of ABPP probes with broad utility for the functional proteomic analysis of cathepsins. More recently, the researchers have shown that cathepsins can also be targeted by peptidic acyloxymethyl ketone probes that contain hydrophobic PI substituents (81).

Electrophilic ketone probes that target the caspase family of cysteine proteases.

Clan CD is another large group of cysteine proteases that includes the caspases, which play key roles in apoptosis-mediated cell death. Active-site-directed probes have a rich history of application in the field of caspase biology, including serving as key tools for the discovery of the first caspase (β -interleukin-converting enzyme) (82). Caspases have since been targeted by a number of ABPP probes, including peptidic α -halomethyl and acyloxymethyl ketones (81, 83, 84) (**Figure 9**). These probes

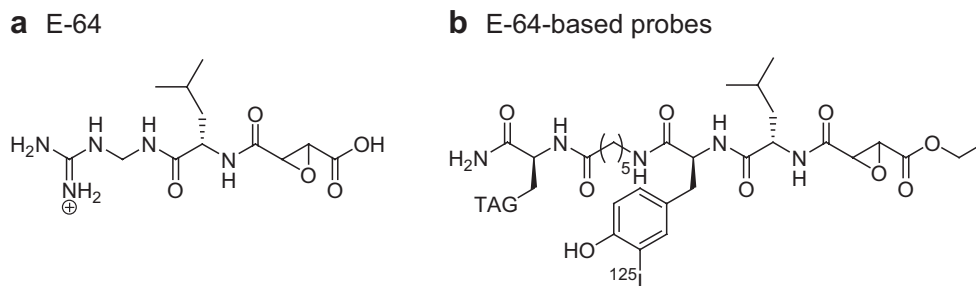


Figure 8

(a) Structures of the broad-spectrum cathepsin inhibitor E-64 and (b) activity-based protein profiling probes derived from this natural product (53, 80).

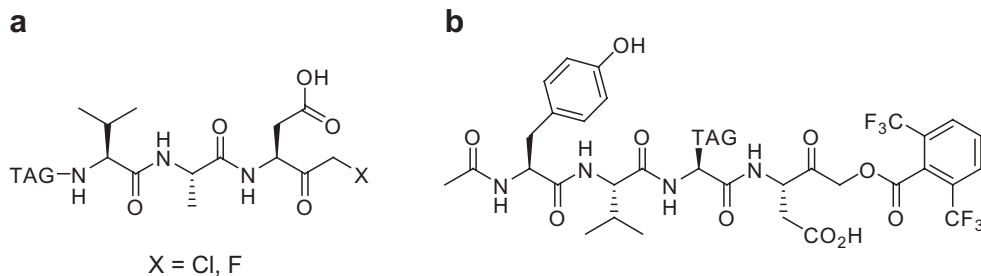


Figure 9

Representative electrophilic ketone probes that target the caspase family of cysteine proteases.

(a) Peptidic α -halomethyl ketones (84). (b) Peptidic acyloxymethyl ketones (81, 83).

achieve selectivity for caspases over other cysteine proteases by incorporating a negatively charged substituent in the P1 position. Recently, acyloxymethyl ketone probes were used to provide evidence for an activated full-length form of caspase-7 that occurs early during the apoptotic cascade (85), suggesting that certain caspases can be converted to functional proteases without requiring zymogen processing.

Electrophilic ubiquitin probes that target the deubiquitinating family of cysteine proteases.

Multiple subfamilies of cysteine proteases are involved in cleaving ubiquitin and ubiquitin-like modifications. These proteases are often referred to as ubiquitin-specific proteases (USPs) or deubiquitinating enzymes (DUBs) (86). The wide sequence divergence among these proteases has inspired the development of functional proteomic methods for their identification and characterization. Borodovsky and colleagues (57) have addressed this problem by generating electrophilic ubiquitin derivatives as ABPP probes for DUBs (Figure 10). Taking advantage of intein-based chemical ligation methods, the researchers synthesized a set of ubiquitin probes containing various Michael acceptors and alkyl halides and showed that each probe targets a distinct set of DUBs in the proteome. These probes have been used to identify novel classes of DUBs, including the ovarian-tumor like domain family (57) and the

UL36 gene product of herpes simplex virus type 1 (87).

Biological applications of ABPP probes that target cysteine proteases.

The aforementioned sets of ABPP probes have gained widespread use for the functional characterization of cysteine proteases in biological systems. Here, we highlight two prominent examples in the areas of parasitology and cancer.

Identification of cysteine proteases that contribute to the host cell invasion by the malaria parasite *Plasmodium falciparum*.

Proteases have long been suspected to play key roles at various stages of the *Plasmodium falciparum* life cycle. Nonetheless, the daunting number of proteases (>100)

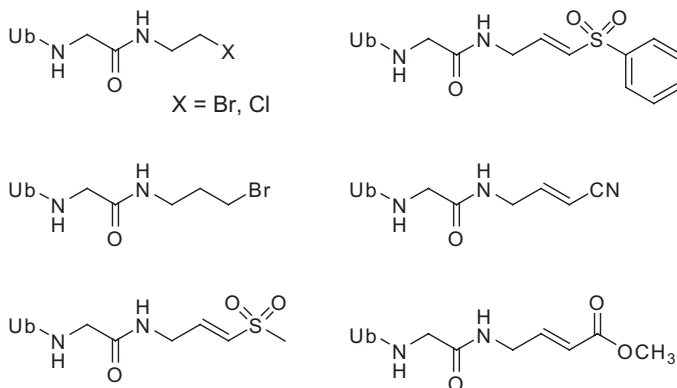


Figure 10

Representative electrophilic ubiquitin derivatives that serve as activity-based protein profiling probes for deubiquitinating enzymes (57, 86).

Abbreviation: Ub, ubiquitin.

encoded by the *P. falciparum* genome presents a major challenge for researchers interested in discerning the functions of individual proteases in this parasite system. Greenbaum and colleagues (88) utilized E-64-based probes to profile these papain activities across the parasite life cycle, revealing a selective elevation in falcipain 1 activity at the time of host invasion. Although falcipain 1 had been of interest to *Malaria* researchers for several years, the protease has proven difficult to characterize in recombinant form. Taking advantage of competitive ABPP methods, which can be applied to natively expressed enzymes, Greenbaum and colleagues developed a selective inhibitor of falcipain 1 and showed that this agent blocked red blood cell invasion by merozoite-stage parasites. The researchers have since applied competitive ABPP to generate selective inhibitors for other cathepsins, including cathepsin B (40) and legumain (89).

Identification of cysteine proteases that contribute to angiogenic switching and tumor growth in pancreatic cancer. Interested in understanding the molecular pathways that support tumorigenesis, Joyce and colleagues (55) initially performed global gene expression analyses of progressive stages of tumor development in the RIP1-Tag2 transgenic mouse model of pancreatic cancer. Among the gene products that showed elevated expression in tumors were several cathepsins. ABPP probes were then used to confirm that specific cathepsins, such as cathepsins Z, B/L, and C, were heightened in activity in tumors relative to normal or angiogenic islets. The researchers then applied a fluorescent ABPP probe to localize cathepsin activities within tumors and found that these proteases resided predominantly in infiltrating immune cells as well as in tumor cells at invasive fronts of carcinomas. Broad-spectrum cathepsin inhibitors (with E-64 as a basis) affected multiple stages of tumor development, including angiogenic switching, tumor vascularity, and invasive growth/proliferation.

These data collectively point to cathepsins as key proteases involved in tumorigenesis and a potentially new set of drug targets for the treatment of cancer.

Metallohydrolases

A third major class of hydrolytic enzymes is the metallohydrolases, which includes proteases, peptidases, and deacetylases. Unlike serine or cysteine hydrolases, which use enzyme-bound nucleophiles for catalysis, the metallohydrolases accomplish substrate hydrolysis by a zinc-activated water molecule. This difference in mechanism complicates the design of electrophilic ABPP probes for metallohydrolases. As an alternative strategy, photoreactive variants of reversible inhibitors of metallohydrolases have been introduced (42, 90, 91). These photoreactive ABPP probes thus achieve target selectivity through binding affinity, rather than “mechanism-based” reactivity. Covalent labeling is accomplished by exposure to UV light, which induces the photoreactive group to modify proteins in close spatial proximity to the probes. Next, we review the development and biological application of photoreactive ABPP probes for two major classes of metallohydrolases—the metalloproteases (MPs) and histone deacetylases (HDACs).

Photoreactive probes that target MPs.

MPs are regulated by multiple posttranslational mechanisms *in vivo*, including zymogen activation and inhibition by endogenous protein-binding partners (e.g., TIMPs). These factors complicate the analysis of MPs using conventional genomic or proteomic methods. MPs and the matrix metalloproteinases (MMPs), in particular, have been the subject of intense study by the pharmaceutical industry for more than a decade. These efforts have produced several broad-spectrum, tight-binding reversible inhibitors of MMPs, most of which contain a hydroxamic acid (hydroxamate) group that coordinates the conserved active-site zinc atom in

a bidentate manner (92) (**Figure 11a**). The hydroxamate-based inhibitor GM6001 has served as a scaffold for first-generation ABPP probes of MPs. Specifically, Saghatelian and colleagues (90) replaced the hydrophobic P2 group of GM6001 with a photoreactive benzophenone and appended a rhodamine reporter tag onto the molecule. The resulting probe, dubbed HxBP-Rh (**Figure 11b**), was found to selectively label active, but not zymogen or inhibitor-bound, forms of MMPs. Interestingly, the probe, as well as its parent inhibitor GM6001, also targeted multiple MPs outside the MMP class, including the metalloproteinases neprilysin and leucine aminopeptidases. These peptidases share very low sequence homology with MMPs. Thus, as was observed for SHs, MPs that are distantly related by sequence have been found to display significant overlap in their inhibitor sensitivity profiles. More recently, advanced versions of HxBP probes have been developed that contain an alkyne group in place of the rhodamine reporter tag (42). These probes were found to display higher-labeling efficiencies than the original HxBP-Rh probe for several MMPs and have been used to identify peptide deformylase as a potential target mediating the antibacterial effects of MP inhibitors in *Chlamydia trachomatis* (93). Additional classes of MP-directed ABPP probes have been introduced with a direct peptide scaffold as a basis and used to generate active-site fingerprints for a panel of yeast MPs (91).

Photoreactive probes that target HDACs.

The reversible acetylation of lysine residues on histone proteins plays a critical role in transcriptional activation and repression. Lysine deacetylation is catalyzed by a family of enzymes, the HDACs, which function as parts of larger protein complexes. Class I and II HDACs are zinc-dependent metallohydrolases. Inhibitors of class I/II HDACs have been shown to induce differentiation in cancer cell lines and reduce tumor volume in vivo (94, 95). Recently, one of these inhibitors, suberoylanilide hydrox-

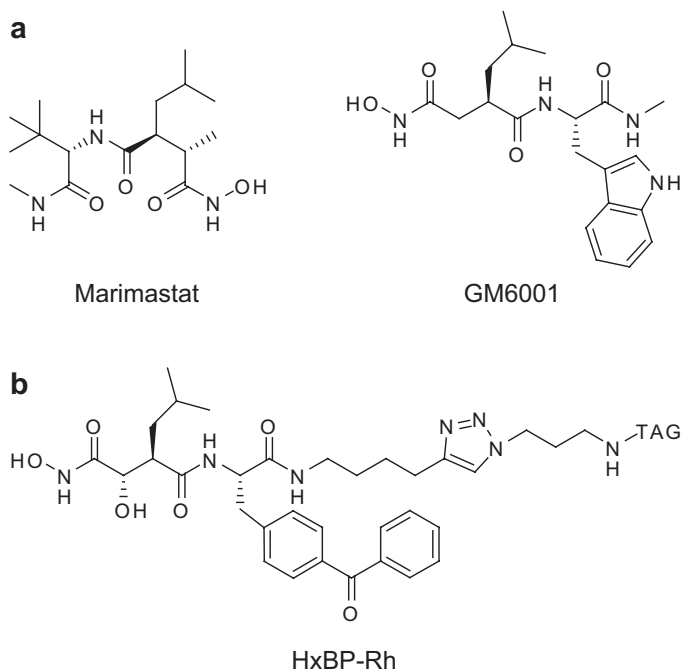
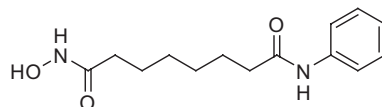


Figure 11

(a) Structures of the broad-spectrum matrix metalloproteinase inhibitors marimastat and GM6001 and (b) the derived activity-based protein profiling probe, HxBP-Rh (90).

amic acid (SAHA) (**Figure 12a**), was approved by the FDA for the treatment of cutaneous T cell lymphoma (95), and several other HDAC inhibitors are in clinical development. Salisbury & Cravatt (96) sought to develop an ABPP probe for class I/II

a SAHA



b SAHA-BPyn

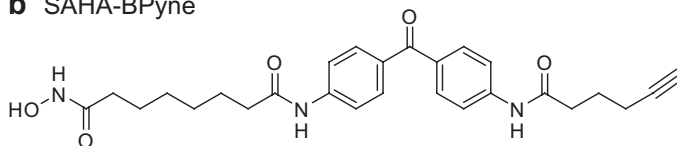


Figure 12

(a) Structures of the broad-spectrum histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) and (b) the derived activity-based protein profiling probe, SAHA-BPyn (96).

HDACs by introducing benzophenone and alkyne groups into the SAHA structure to effect (UV irradiation-induced) covalent labeling and (click chemistry-based) tagging and enrichment, respectively. The resulting probe, dubbed SAHA-BP_{yne} (**Figure 12b**), was found to target multiple class I and II HDACs in proteomes. Interestingly, several HDAC-associated proteins were also labeled by SAHA-BP_{yne}, suggesting that these proteins are in close proximity to HDAC active sites, where they could presumably regulate substrate recognition and activity. These findings thus indicate that ABPP probes have the potential to profile not only the activity state of enzymes, but also the binding proteins that regulate their function.

Additional Classes of Hydrolases

ABPP probes have also been developed for two other major classes of hydrolases—the aspartyl proteases and the proteasome. Aspartyl proteases are a small clade of proteases that include the integral membrane proteins β - and γ -secretase, which regulate processing of the amyloid precursor protein to the A β amyloidogenic peptides causally linked to Alzheimer's disease. To facilitate the molecular characterization of γ -secretase, Li and colleagues (97) developed biotinylated, benzophenone analogues of reversible inhibitors of this protease. These probes, which contained an α -hydroxy-amino acid motif that served as a transition state analogue for aspartyl protease inhibition, were used to label, affinity purify, and identify presenilin 1 and 2 as γ -secretases. Interestingly, presenilin 1 and 2 are multipass integral membrane proteins that lack sequence homology with classical aspartyl proteases, possibly explaining why more conventional sequence-guided searches to identify γ -secretases met with little success.

The proteasome is composed of multiple proteolytic subunits that utilize an N-terminal threonine nucleophile for catalysis. Nazif and Bogoy (98) have generated ABPP probes for proteasomal subunits that con-

tain a peptide-binding group and vinyl sulfone reactive group. By varying the peptide portion of the probes, the researchers gained insights into the substrate recognition properties of specific proteasomal subunits, culminating in the development of Z-subunit-specific inhibitors that were used to identify this subunit as the principal trypsin-like activity of the proteasome. More recently, Ovaa and colleagues (99) have created azide versions of vinyl sulfone probes and shown that these agents can be used to profile proteasomal activities in living cells (with detection accomplished by Staudinger ligation with a phosphine reporter tag).

Kinases and Nucleotide-Binding Proteins

The nucleotide-binding proteome is arguably the largest and richest potential area for the development of ABPP probes. Well over half of the human proteome binds some form of nucleotide or nucleotide-containing cofactor, and these proteins span most cellular processes. It is not surprising that the key role of ATP in the cell ensures that the lion's share of nucleotide-binding space is devoted to ATP binding. Approximately 60 ATP-binding protein families, each employing structurally distinct binding motifs, have been estimated in the proteome (100). The protein kinases constitute one ATP-binding family.

The human kinome has been determined to contain at least 518 kinases clustered into 17 groups and 134 families (101). Despite the sequence and structural diversity within the kinome, protein kinases all recognize a common substrate, ATP, the key phosphate donor in the phosphoryl transfer to protein or peptide substrates. Not unexpectedly, the ATP-binding site is the region with the highest sequence homology among kinases and contains two consensus motifs that serve as signature sequences for nearly all protein kinases.

Protein kinases, as regulators of many cellular pathways, have emerged as major drug targets for oncology and other therapeutic

indications. Thus far, seven kinase inhibitors have been approved as drugs. Recent estimates suggest that clinically relevant kinase target space consists of less than 40 proteins, but this is certainly destined to increase as our biological understanding of the kinome grows (102, 103). Understandably, the development of tools to interrogate the kinome has been an important focus.

The kinases present a considerable challenge to the design of ABPP probes. In the case of the serine and cysteine hydrolases discussed above, the eponymous amino acid residues are hypernucleophilic and are catalytically essential to the formation of covalent enzyme-bound intermediates leading to products. Such nucleophilic residues are ideally suited for specific covalent modification by electrophilic probes. Kinases catalyze phosphate transfer from ATP to substrate by a direct transfer mechanism that does not involve covalent enzyme intermediates. Thus, the kinase active site does not contain any unusually nucleophilic residues. Conserved active-site lysines do, however, present a possible target for ABPP probes. Sequence comparisons have shown that virtually all protein kinases have at least one conserved lysine residue within their active sites (101, 104). One lysine residue, in the ATP-binding loop region of the kinase primary sequence, is conserved in all typical protein kinases with few exceptions. A second lysine, which resides two residues to the C terminus of the catalytic aspartic acid, is conserved in the majority of serine/threonine kinases. Cocystal structures of protein kinase catalytic domains bound to ATP reveal that these lysine residues are positioned in close proximity to the β - and γ -phosphates of bound ATP, suggesting that the positively charged ϵ -amino groups of these lysines provide electrostatic interactions with the phosphate backbone of ATP (105). The possibility that an appropriately placed electrophile could efficiently react with the equilibrating deprotonated lysine through elevated effective concentration thus presented itself as a viable strategy.

Kinase probes based on 5'-p-fluorosulfonylbenzoyl adenosine. The strategic underpinning for the design of kinase probes useful for proteomic analysis lies in the extensive work that has focused on the development of nucleotide-based affinity labels. Roberta Colman (106–108) and coworkers have been instrumental over the past 30 years in creating a wide array of chemical tools for the analysis of nucleotide-binding proteins. The reactive groups incorporated into these probes are usually alkylating, acylating (sulfonylating), or photolytic groups. The literature is extensive and has been the subject of a number of reviews (106–108). The nucleotide-based affinity labels that have been reported may be roughly divided into three categories reflecting where the labeling group is incorporated: modifications on the nucleoside base, modifications of the 5'-hydroxyl of the ribose, and other modifications of the ribose sugar. In general, incorporation of the labeling moiety on the 5'-hydroxyl has offered the best possibility of creating a reasonably close facsimile of ATP without structural perturbation of the adenosine moiety that would affect its broad recognition by kinases and other ATP-binding proteins.

The prototypic nucleotide probe that has emerged from these efforts is 5'-p-fluorosulfonylbenzoyl adenosine (5'-FSBA) (106). The fluorosulfonyl moiety of 5'-FSBA, reminiscent of the fluorophos(pho)nates discussed above as serine hydrolase probes, is an electrophile that can react with nucleophilic amino acid residues with elimination of the fluoride to form a covalent adduct and that occupies, in principle, the position of the γ -phosphate of ATP (**Figure 13**). Thus, a specific reaction with the lysine amino groups in protein kinases is possible. 5'-FSBA reacts with a variety of nucleotide-binding proteins by sulfonylation of predominantly lysine amino groups and tyrosine hydroxyls. The reaction is not broadly based and favors certain proteins, thereby lacking the generality of an ideal ABPP process. Several unfavorable characteristics appear to

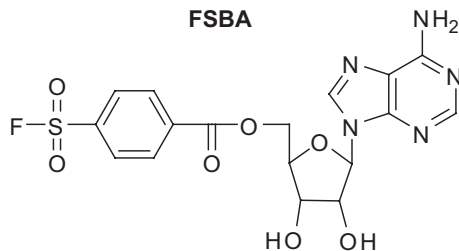


Figure 13

Structure of 5'-p-fluorosulfonylbenzoyl adenosine (FSBA), an original affinity label for ATP-binding enzymes (106).

contribute to this. 5'-FSBA is uncharged, making it a marginal ATP analog. It exhibits relatively low affinity for nucleotide-binding sites in proteins, related no doubt to the lack of charged phosphates. Finally, the molecule does not possess a straightforward site for appending a reporter tag, such as biotin, to permit target enrichment and identification from proteomes.

Despite these shortcomings, some recent work has appeared on the application of LC/MS-based methods for the development of 5'-FSBA as a proteomic tool for kinase profiling. Thus far, these studies have been limited to model systems of purified kinases, such as ALK5 and CDK2, in order to map peptide labeling sites (109–111).

Kinase probes with ATP as a basis. A recent report by Patricelli and colleagues (112) on the use of nucleotide acyl phosphates as ABPP probes for kinases and nucleotide-binding space appears to circumvent the problems of 5'-FSBA and promises to provide broad proteome coverage, simplified analysis, and high sensitivity. The finding is based on the idea that ATP itself would serve as an obvious choice for a scaffold. In addition, no modifications of the adenosine moiety should be used to ensure universal recognition of the nucleoside. Therefore, placing an electrophile at the terminal phosphate of the nucleotide was considered. Cocrystal structures of pro-

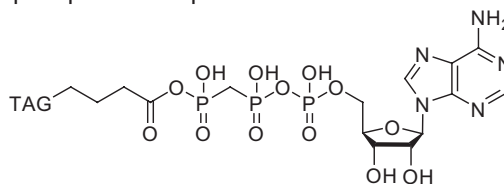
tein kinase catalytic domains bound to ATP reveal that, in addition to the close proximity of the conserved lysine residues to the β - and γ -phosphates of bound ATP, the terminal phosphate can accommodate an additional substituent owing to its solvent accessibility. Thus, by using ATP or ADP as a scaffold, the probe could bind to all protein kinases in an orientation that would place a portion of the probe in the proximity of a lysine amine. The attachment of an electrophilic acyl group directly to the terminal phosphate of the nucleotide was explored as a possible solution. Among the number of chemically reactive phosphates, acyl phosphates have the most appropriate reactivity with amines and, at the same time, sufficient stability in an aqueous environment. Acyl phosphates have been known for more than 50 years; the parent congener acetyl phosphate was extensively studied by Di Sabato & Jencks (113, 114) and acyl phosphates of amino acids and AMP are well-studied intermediates in the aminoacylation of tRNAs and other biological acyl transfer reactions (115). Most notably, acetyl phosphate is particularly reactive toward primary amines via addition of the amine nitrogen to the carbonyl group and facile elimination of the phosphate by C–O bond cleavage, resulting in the formation of the corresponding stable acetamide.

Remarkably little research has been reported for acyl phosphates of di- and triphosphates given the importance of the corresponding monophosphates. Kluger & Huang (116) synthesized acyl pyrophosphates as mechanism-based inhibitors of pyrophosphate-binding enzymes. They demonstrated that isopentenoyl pyrophosphate but not acetyl pyrophosphate efficiently inactivated farnesyl synthetase, presumably by acyl transfer to an active-site nucleophile. The acyl phosphates of nucleotide di- and triphosphates have likewise been virtually unexplored since their first synthetic report in 1976 (117). Huynh-Dinh and colleagues (118–120) have published the only other reports of these compounds. They synthesized acyl phosphates

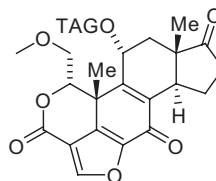
with a fatty acid side chain, such as a myristoyl or cholesteryl group, in an attempt to enhance the lipophilicity and cell permeability of nucleotides of therapeutic interest (120–122). Although they demonstrated that cholesteryloxycarbonyl-ATP could transport ~6% of the ATP across a model lipid bilayer (122), the rapid aminolysis of these acyl phosphates precluded any demonstration of the phenomenon in cell culture (121).

The ABPP method of Patricelli and colleagues (112) relies on acyl phosphate-containing nucleotides, prepared from a biotin derivative and ATP or ADP (**Figure 14a**). The acyl phosphate probes have been shown to bind selectively and react covalently at the ATP-binding sites of at least 75% (~400) of the known human protein kinases in cell lysates. As predicted, the acyl phosphate reactive group on these probes showed selective reactivity with one of the two conserved lysine amines in kinase active sites. The elevated effective concentration of the active-site lysine(s) accelerated reaction with the activated acyl phosphate to form a stable amide bond with the biotin tag, releasing ATP or ADP. The biotinylated kinases could then be subjected to proteolytic digestion with trypsin and purification of the probe-labeled peptides, accomplished with streptavidin-agarose beads. The biotinylated peptides were then analyzed by LC-MS/MS to determine the identity of the labeled protein as well as the site of labeling (45). This platform can thus be used to profile the functional state of many kinases in parallel directly in native proteomes. Competitive ABPP studies, using broad (e.g., staurosporine) and selective kinase inhibitors, can also be performed to determine inhibitor potency and selectivity against native protein kinases as well as against hundreds of other ATPases. The ability to broadly profile kinase activities in native proteomes is an exciting prospect for both target discovery and inhibitor selectivity profiling.

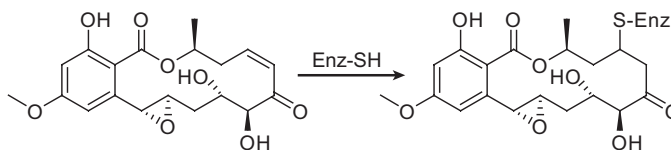
a Acyl phosphate ATP probe



b Wortmannin probes



c Hypothemycin



d fmk-pa

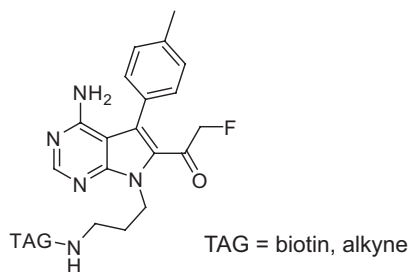


Figure 14

Structures of different classes of kinase-directed activity-based protein profiling probes. (a) An acyl phosphate ATP probe (112). (b) Wortmannin-derived probes (125, 126). (c) Hypothemycin, a resorcyclic acid lactone polyketide, which reacts with protein kinases via Michael addition (128). (d) The Rsk kinase-selective probe, fmk-pa (131, 132).

Kinase probes with covalent inhibitors as a basis. Covalent inhibitors of kinases represent a complementary, targeted approach to the more general ATP probe strategy. Wortmannin, a steroidal metabolite of the fungus *Penicillium funiculosum*, is a potent inhibitor of members of the phosphoinositide 3-kinase

(PI 3-kinase) and the PI 3-kinase-related kinase (PIKK) families and has been shown to inactivate PI 3-kinase by covalent modification of a lysine in the active site (123). This lysine is conserved in both families. X-ray crystallographic studies have provided a detailed picture of the wortmannin/PI 3-kinase interaction (124). On the basis of this covalent modification, Yee et al. (125) synthesized biotin- and fluorophore-based probes of wortmannin and studied the scope of their reaction (**Figure 14b**). They found that wortmannin probes inactivated PI 3-kinase and PIKK family members, consistent with the presence of the conserved active-site lysine. The biotinylated probe permitted isolation of the sensitive kinases. They also demonstrated that, in nuclear extracts, the probe, as expected, pulled down three important members of the PIKK family—ATM, ATR, and DNA-PKcs. The fluorescent probes were useful in quantifying activity and expression patterns of relevant kinases. The BODIPY derivative was also shown to be cell permeable. Using a related fluorophore-based wortmannin probe, Liu and colleagues (126) unexpectedly found that polo-like kinase 1 (PLK1) is potently inhibited in a number of breast cancer cell lines. The PLKs are conserved regulators of several stages of cell cycle progression and are unrelated to the PI 3-K and PIKK families. The *in vitro* IC₅₀ for wortmannin of 24 nM is 1000-fold lower than scytone-min, a known PLK1 inhibitor. Subsequent work (127) established that wortmannin inhibits both PLK1 and PLK3 by covalent modification of a conserved active-site lysine. Inhibition in live cells occurs at wortmannin concentrations commonly used to inhibit the PI 3-kinases. Inhibition of PLK3 by wortmannin in GM00637 cells significantly impaired the upregulation of p53 serine phosphorylation, thereby demonstrating a downstream effect. The wortmannin probe studies highlight the value of an ABPP probe that targets a select number of enzymes from a much larger superfamily. The high sensitivity and ability to resolve proteins in complex proteomes by sim-

ple gel-based methods allow accurate quantitation of known protein targets, as well as the identification of new targets.

Resorcylic acid lactones (RALs), polyketide natural products (**Figure 14c**), have recently shown potential as covalent modifiers of a specific fraction of the kinome. Schirmer and colleagues (128) demonstrated that RALs are susceptible to Michael addition by a cysteine thiolate, found on some protein kinases, which results in time-dependent inactivation of the kinase that is competitive with ATP. Bioinformatics analysis revealed that 46 protein kinases (~9% of the kinome) contain a conserved cysteine necessary for this reaction. The RAL-sensitive kinases were well distributed throughout the kinome. In an *in vitro* panel of 124 kinases containing 19 putative RAL-sensitive kinases, all but one, GSK3 α , showed the predicted RAL inhibition. Three kinases, cSRC, TRKA, and TRKB, which lack the target cysteine residue, were also inhibited. Subsequent analysis revealed that these three kinases were inhibited reversibly, consistent with a lack of the cysteine required for covalent modification. Furthermore, cell lines dependent on the activation of tyrosine kinase mitogen receptor targets of the RALs were unusually sensitive to the compounds and showed the predicted inhibition of kinase phosphorylation owing to inhibition of the mitogen receptors. The RAL and wortmannin studies suggest that natural products that irreversibly inactivate kinases in predictable ways can be useful as ABPP probes to interrogate well-defined fractions of the kinome and possibly identify unexpected targets.

Recent studies have reported on the design of synthetic, irreversible kinase inhibitors that selectively target subfamilies of the kinome. Klutchko and colleagues (129) have extensively explored the structure-activity relationship (SAR) of alkynamides of 4-anilinoquinazolines and 4-anilinopyrido-[3,4-d]pyrimidines as irreversible inhibitors of the erbB family of tyrosine kinase receptors. Previously, Fry and colleagues (130)

had shown that 6- or 7-acrylamido-4-anilinoquinazolines were potent, irreversible inhibitors of epidermal growth factor receptor (erbB1) and erbB2 kinase activities. The acrylamido moiety acted as a Michael acceptor to covalently react with a cysteine conserved in the ATP-binding site of both kinases. Efficient covalent modification required that the acrylamido moiety be unsubstituted, and there was a kinetic preference for the 6-acrylamido position. Klutchko further refined and expanded the SAR of these kinase inhibitors (129). It was found that analogs bearing a 5-dialkylamino-2-pentynamide class of Michael acceptor had the best potency as pan-erbB inhibitors. This was likely due to the ability of the dialkylamino substituent to function as an intramolecular general acid/base to assist in the cysteine thiol addition to the alkynamide. Although the erbB inhibitor work was purely directed toward the design of inhibitors as therapeutics, the compounds may have value as warheads for selective kinase-targeting ABPP probes.

Expanding on the exploitation of specific cysteines in kinases for irreversible inhibition, Cohen and coworkers (131) have taken an intriguing bioinformatics-based approach to identify potentially related kinase subsets. Starting with the known selectivity filter in the ATP-binding site, the “gatekeeper,” they used a kinome-wide sequence alignment to search for kinases that had the simultaneous occurrence of a threonine as gatekeeper (to permit access to a large hydrophobic binding pocket) and a solvent-exposed cysteine on a nearby conserved glycine-rich loop that could form a covalent bond with an inhibitor. Three kinases, RSK1, RSK2 and RSK4, members of the p90 ribosomal protein S6 kinase family, fulfilled the criteria. RSK3 and eight other kinases had the cysteine but had larger amino acids at the gatekeeper position, thereby limiting access to the binding pocket. Guided by crystallographic information, a series of p-tolyl-pyrazolopyrimidines that bore a halomethyl ketone at the C-8 po-

sition were synthesized as adenine analogs. The p-tolyl moiety provided the threonine gatekeeper selectivity. The fluoromethyl ketone derivative and its biotinylated analog (**Figure 14d**) were found to covalently modify the predicted kinases by reaction with the conserved cysteine with high specificity. Exchange of the biotin group with an alkyne has created clickable versions of these kinase probes (**Figure 14d**) that permit profiling of RSK activities in living cells (132). Because it is estimated that 20% (~100) of the protein kinases have a solvent-exposed cysteine in the ATP pocket, the prospect of identifying other exploitable motifs for covalent bond formation in the kinome is encouraging. Additionally, cysteines can be engineered into kinase active sites to create orthogonal kinase-ABPP probe pairs for chemical genetics studies (133).

Glycosidases

Glycosidases are a large class of enzymes found in all branches of life that play key roles in cellular metabolism and in the regulation of carbohydrate modifications to lipids and proteins. Several experimental strategies have been put forth for the functional proteomic analysis of glycosidases. Tsai and colleagues (134) synthesized candidate ABPP probes for glycosidases that utilized a latent quinone methide electrophile to promote labeling. Although effective with purified glycosidases, this approach led to cross-reactivity with other nonglycosidase proteins in complex mixtures owing to the diffusible nature of the released quinone methide group. Vocadlo & Bertozzi (135) created an azide-modified fluorosugar as an ABPP probe for retaining β -glycosidases (**Figure 15a**). Fluorosugars act as mechanism-based inhibitors of retaining β -glycosidases by trapping the covalent glycosyl-enzyme intermediate (**Figure 15a**). The azide group permitted detection of probe-labeled glycosidases by Staudinger ligation with reporter-tagged phosphines. Most recently, Hekmat and

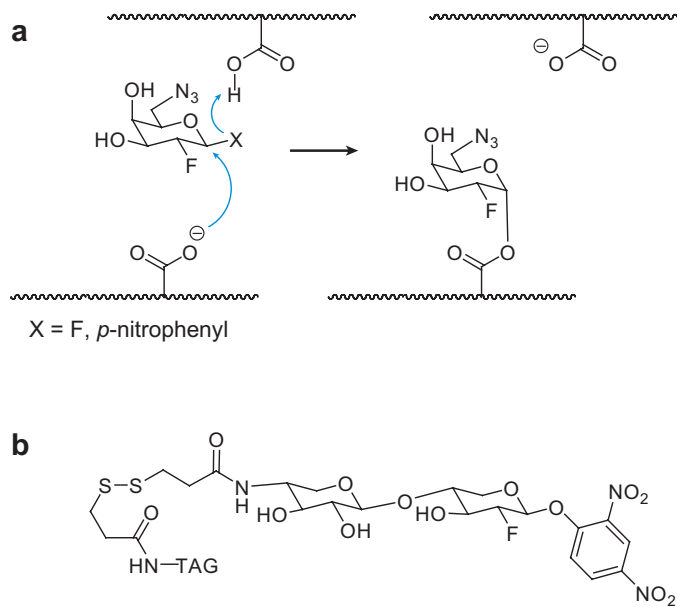


Figure 15

Activity-based protein profiling (ABPP) probes for β -retaining glycosidases. (a) Representative ABPP probes for exo-glycosidases (135). The active-site nucleophile of the glycosidase reacts to form a covalent probe-enzyme adduct. (b) Structure of a cleavable probe for β -retaining glycosidases that permits identification of probe labeling sites.

colleagues generated an advanced 1-(2,4-dinitrophenyl)-2-fluorosugar probe with a biotin tag separated by a cleavable disulfide linker (**Figure 15b**). This probe allowed the authors to profile both retaining β -glycosidases and their specific sites of labeling, resulting in the discovery of a new β -1,4-glycanase from the soil bacterium *Cellulomonas fimi*.

Cytochrome P450s

The cytochrome P450 (P450) monooxygenase enzyme family metabolizes a large number of endogenous signaling molecules, xenobiotics, and drugs. The human genome encodes 57 distinct P450 enzymes (136), many of which remain uncharacterized. The activity of P450 enzymes is regulated by multiple factors in vivo: membrane com-

position and localization; protein-binding partners, e.g., Dap1/PGRMC1 (137); post-translational modification (138); endogenous concentrations of cofactors (e.g., NADPH); and regulatory enzymes (e.g., NADPH-P450 reductases). Wright & Cravatt (139) have developed ABPP probes for P450 enzymes on the basis of mechanism-based inhibitors of the aryl acetylene class. The inactivation of P450 enzymes by aryl acetylenes, such as 2-ethynyl naphthalene (2EN) (**Figure 16a**), has been well studied (140). The hydrophobic naphthalene group directs inhibitor binding toward P450 active sites, wherein enzyme-catalyzed oxidation of the conjugated 2-acetylene generates a highly reactive ketene (**Figure 16b**). The ketene intermediate can then acylate nucleophilic residues within the P450 active site. Modification of 2EN with an alkyl (unconjugated) acetylene group converted this inhibitor into an ABPP probe (2EN-ABP) (**Figure 16a**) capable of labeling multiple P450 enzymes in vitro and in vivo. Labeling of P450 enzymes was NADPH dependent and blocked by P450 inhibitors. Notably, the 2EN-based probe was used to monitor induction and inhibition of P450 enzymes in living mice, thus providing a potentially powerful assay to characterize P450-drug interactions in vivo.

Phosphatases

Protein phosphorylation is a reversible post-translational modification. Two major classes of phosphatases, the serine/threonine and tyrosine phosphatases, are responsible for removing phosphate groups from proteins. Serine/threonine phosphatases are metal-dependent enzymes that function as part of multisubunit complexes. Several natural products have been identified that potently inhibit serine/threonine phosphatases, including the cyanobacterial hepatotoxin microcystin, which covalently modifies a non-catalytic cysteine residue in the active sites of these enzymes (141). Shreder and

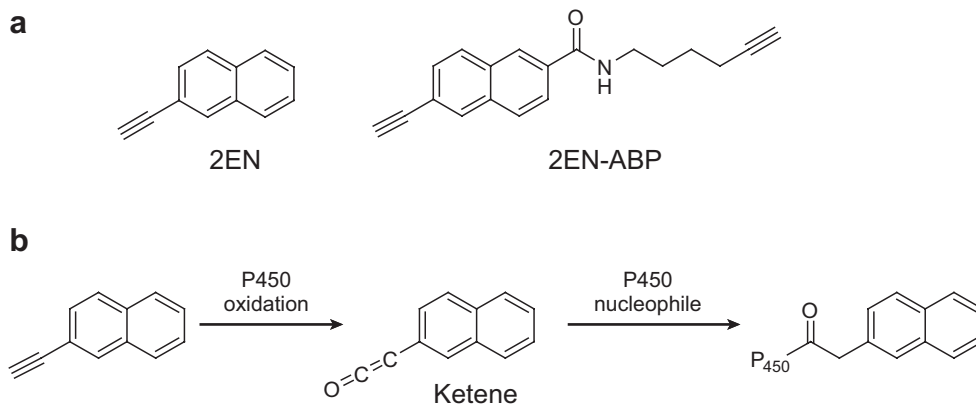


Figure 16

Activity-based protein profiling probes for cytochrome P450 enzymes. (a) Structures of the broad-spectrum cytochrome P450 (P450) inhibitor 2-ethynynaphthalene (2EN) (140) and the derived probe, 2EN-ABP (139). (b) Mechanism of inactivation of P450s by aryl acetylene inhibitors.

colleagues (142) synthesized a rhodamine-tagged derivative of microcystin and used this agent to profile serine/threonine phosphatase activities in proteomes.

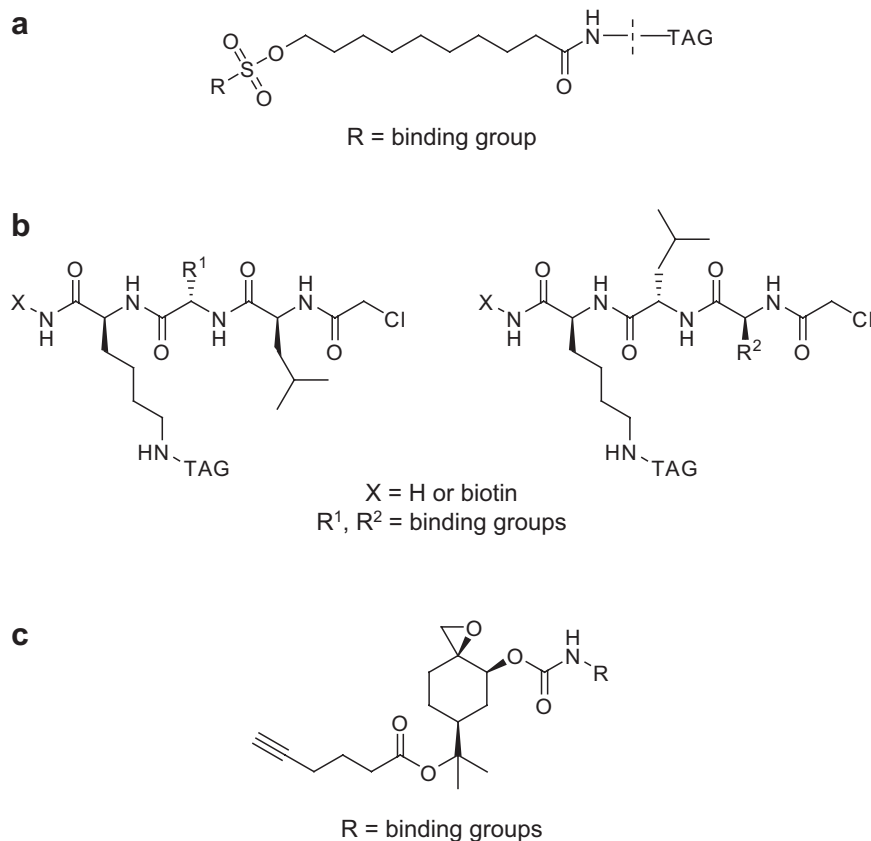
Tyrosine phosphatases utilize a cysteine nucleophile for catalysis, which initially might suggest that these enzymes would represent facile targets for ABPP. However, the cysteine nucleophile of tyrosine phosphatases appears to be less reactive toward electrophiles than the nucleophile of cysteine proteases. In this regard, mechanistic analysis of tyrosine phosphatases indicates that these enzymes operate by a predominantly dissociative mechanism whereby proton transfer to the leaving group oxygen precedes nucleophilic attack by the catalytic cysteine (143). These factors have complicated the development of ABPP probes for tyrosine phosphatases. Nonetheless, some progress has been made. Zhang and colleagues (144) have generated reporter-tagged bromobenzylphosphonate probes that label several purified tyrosine phosphatases in an active-site-directed manner and that are currently under examination for their ability to target these enzymes in proteomes.

Nondirected Probes that Target Multiple Enzyme Classes

Each of the aforementioned enzyme classes was targeted by ABPP probes that incorporated known binding groups and/or affinity labels. These chemotypes were thus used to direct probe labeling to specific enzyme classes. Ongoing directed ABPP efforts continue to provide probes for new enzyme classes, including, for example, arginine deiminases (145) and methionine aminopeptidases (146). However, many enzymes lack cognate inhibitors or affinity labels; to extend ABPP to these proteins, a nondirected strategy for probe discovery has been introduced (147, 148). Nondirected ABPP involves the synthesis and proteomic screening of libraries of probes that bear a common reactive group and a variable binding group. Specific protein targets are identified in proteomes on the basis of activity-dependent (e.g., heat-sensitive) labeling and/or selective reactivity with a subset of the probe library. Several probe libraries have been synthesized, including those that bear sulfonate ester (147, 148) (**Figure 17a**), α -chloroacetamide

Figure 17

Representative nondirected libraries of ABPP probes. (a) A library of sulfonate ester probes (147, 148). (b) A library of α -chloroacetamide probes, where R^1 and R^2 correspond to natural and unnatural amino acid side chains (149). (c) A library of spiroepoxide probes made in clickable format for cell-based screening (150).



(149) (**Figure 17b**), and spiroepoxide (150) (**Figure 17c**) reactive groups. Enzyme targets of these probes include aldehyde/alcohol dehydrogenases (36, 44, 47, 147, 148), enoyl-CoA hydratases (148), glutathione S-transferases (148, 149), nitrilases (149), and

transglutaminases (151). A clickable spiroepoxide library was recently screened for antiproliferative effects in breast cancer cells, resulting in the identification of a bioactive probe that targeted the glycolytic enzyme phosphoglycerate mutase (150).

SUMMARY POINTS

The field of ABPP has quickly reached an inflection point toward making important contributions to the overarching goals of systems biology. The ability of ABPP to integrate the classical disciplines of organic synthesis and mechanistic enzymology with contemporary analytical methods sets this technology apart as a highly interdisciplinary endeavor. Indeed, by interrogating fractions of the proteome on the basis of shared functional properties, rather than mere abundance, ABPP analyzes portions of biomolecular space that are inaccessible to other large-scale profiling methods.

In conjunction with novel cell and animal models of disease, ABPP has already established a track record of identifying enzyme activities associated with a range of diseases, including cancer (60, 61), malaria (88), and metabolic disorders (149). From a pharmaceutical perspective, ABPP is also contributing to the design and evaluation of selective

inhibitors for disease-linked enzymes, including enzymes of uncharacterized function (68, 152). This duality of ABPP as a discovery tool to identify enzymes associated with specific physiological and/or pathological processes and as an evaluative tool for the design of selective inhibitors, and potentially drugs, that target these enzymes is the hallmark of a robust technology that can serve as both a hypothesis generator and a hypothesis tester.

FUTURE ISSUES

A number of challenges lie ahead. Most obvious from the perspective of this review is the expansion of the proteome coverage of ABPP. Thus far, the field has taken a narrow, albeit pragmatic, focus on protein activity as specified by enzymatic catalysis. Given the natural affinity of ABPP to enzyme and medicinal chemistry as a source of small-molecule probes, this is understandable, and clearly, there is much more to be done here. However, enzymes need not be the only component of the proteome addressed by ABPP. Future directions may include the development of probes to interrogate receptors (e.g., G protein-coupled receptors), ion channels, and structural proteins. In these cases, chemical and biological tools to interrogate specific proteins do exist; conversion of these agents into ABPP probes to capture specific fractions of proteomic space remains to be implemented. This orthogonal expansion beyond enzymes, in conjunction with advances in the sensitivity, resolution, and throughput of analytical platforms, would complete and extend the transition from enzyme chemistry to a new, multifaceted proteomic chemistry.

We view as an ultimate goal the integration of ABPP with other large-scale profiling technologies and targeted experimental approaches to expand our understanding of the biochemical mechanisms of health and disease. For instance, ABPP is already being united with complementary systems biology methods, such as metabolomics (68), a portion of biomolecular space that constitutes the major biochemical output of enzyme activity *in vivo*. By perturbing enzyme activity in living systems and then profiling the metabolic consequences, researchers may succeed in integrating both known and uncharacterized enzymes into the higher-order biochemical networks of cells and tissues. In this manner, a new experimental platform could be established for rapidly moving from the discovery of enzyme activities associated with biological processes to elucidation of the mechanistic basis and functional significance of these relationships. This networking of systems biology platforms united and enriched by an ever-expanding repertoire of proteomic chemistry would lead a higher-order understanding of the biology of life and the pathology of disease. The next generation of novel therapeutics should certainly benefit from such advances.

DISCLOSURE STATEMENT

Dr. Kozarich is Chairman and President of Activx Biosciences, a company that specializes in the use of activity-based proteomics technologies for drug discovery, and Dr. Cravatt is a consultant for Activx Biosciences.

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