CHAPTER 2

Applications for Activity-based Probes in Drug Discovery

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2.1 Background

2.1.1 Introduction

The path to a new therapeutic drug is long and difficult and involves many stages including validation of a target, design and selection of a lead compound and finally development of the lead into a drug. This chapter will discuss a relatively new technology that makes use of small molecules termed activity-based probes (ABPs). These probes bind in the active site of a target enzyme or class of enzymes in an activity-dependent fashion. Thus probe labeling serves as an indirect readout of enzyme activity, allowing the dynamic regulation of the target enzyme to be monitored using a number of biochemical and cell biological methods. In addition, labeled targets can be directly isolated by affinity methods, thereby allowing identification of potentially valuable drug targets based solely on their ability to bind a small molecule. Finally, because of the high degree of selectivity of ABPs for a given target protein class, they can be used for studies of drug binding and efficacy in complex cellular mixtures, intact

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cells and even in whole animals. These attributes of ABPs make them extremely valuable reagents for use at multiple points in the drug discovery process.

2.1.2 Activity-based Probes

2.1.2.1 The Need for Chemical Probes

While the genomics revolution of the past decade has had a dramatic impact on research science, it is clear that analysis of DNA or RNA content alone is not sufficient to understand cell biology and disease. For example, most classes of enzymes are regulated by a complex set of post-translational mechanisms that make simple assessment of protein abundance or gene expression of limited value for understanding enzyme function. For the drug discovery process, it is essential to understand how enzymatic proteins are regulated in disease pathology in order to be able to predict how modulation of activity with a small molecule drug is likely to impact therapy outcome. Since many of the primary so-called ''druggable'' classes of targets are enzymes, the development of new methods to dynamically monitor enzyme activity has great potential to impact the drug discovery process.

Activity-based probes are one such technology that has made significant advances in the past decade (for additional reviews see Cravatt *et al.*,[1] Evans and Cravatt,[2] Fonovic and Bogyo[3] and Schmitinger *et al.*[4]). This technology is centered around the development of small molecule probes that bind to a target enzyme using a mechanism that requires enzymatic activity. Thus, an ABP binds to its target only when it is active and labeling can therefore be used as a way to assess levels of activity in a dynamic way. Depending on the desired application for an ABP, these reagents can often be built using previously validated chemistries and knowledge about a particular target protein of interest. In the case where a target is not already established, it is possible to generate diverse sets of probes and use these reagents to find previously poorly characterized enzymes that may play important roles in disease progression. Once a highly selective probe and target pair has been identified, it is possible to use the probe not only to monitor the normal physiologically relevant regulation of the target but also to monitor inhibition by small molecule drug leads. Thus, a suitably designed ABP can aid not only in the identification of novel targets, but also can be applied to the later stages of the drug discovery process to assess the overall efficacy and selectivity of lead compounds.

2.1.2.2 Anatomy of the Chemical Probe

In their most basic form, activity-based probes consist of three distinct functional elements (Figure 2.1): a reactive group for covalent attachment to the enzyme, a linker region that can modulate reactivity and specificity of the reactive group, and a tag for identification and purification of modified

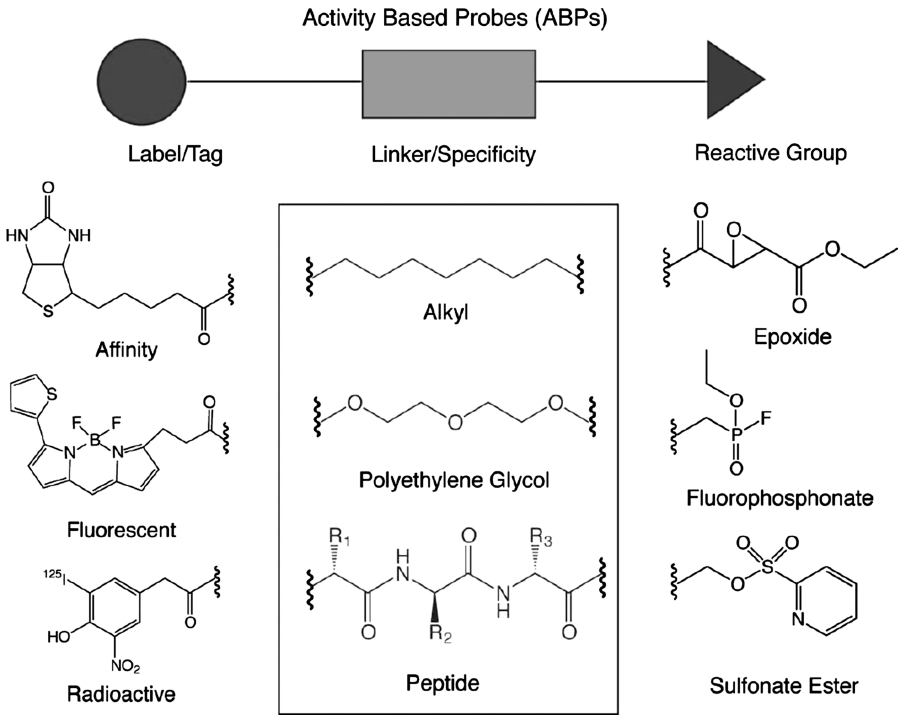

Figure 2.1 Structure of a chemical probe. A chemical probe has three basic components: a reactive group for covalent attachment to the enzyme of interest; a linker region to provide spacing and specificity; and a tag to allow for identification and/or purification. Specific examples of each are shown.

enzymes. Each of these elements must be chosen based on the type of application required.

2.1.2.2.1 Structure of the Reactive Group. The most significant challenge in the design of ABPs is the selection of a reactive group that provides the necessary covalent modification of a target protein. The reactive group must have sufficient reactivity to allow modification of the target enzyme while not reacting with other non-specific proteins inside the cell. Most of the reactive groups currently in use in ABPs have been designed based on covalent, mechanism-based inhibitors of various enzyme families. Of the many new classes of ABPs that have been developed in the past decade, the majority of the reported agents have been designed to target proteases.[5–13] This is at least partially due to the wide range of covalent reactive groups that have been designed by medicinal chemists as a means to inhibit proteases (for an extensive review see Powers *et al.*[14]). Proteases are also one of the primary families of enzymes that are currently the focus of a number of drug

discovery efforts in the pharmaceutical industry. Therefore, there is the potential for ABPs that have already been developed to find immediate use in drug discovery projects.

2.1.2.2.2 Structure of the Linker Region. The linker region is generally used to describe the region of the probe that connects the reactive group to the tag used for identification and/or purification. While the linker can have multiple purposes, it often contains binding elements to control the selectivity of the probe for a given target. In addition, it must provide enough space between the reactive group and the tag to prevent steric hindrance that could block access of the reactive group or accessibility of the tag for the purpose of purification. In the case of ABPs that target proteases, this linker region often contains basic peptide or peptide-like elements that bind in the various substrate recognition elements on a target protease. Recent efforts have also focused on the use of the linker to release the resulting labeled proteins after affinity purification. There have been several reports of cleavable linkers that can facilitate such purification methods.[15–17]

2.1.2.2.3 Structure of the Tag. The choice of tag for a given activity-based probe depends heavily on the desired application for the probe (for an extensive review see Sadaghiani *et al.*[18]). Some of the more common tags include biotin, radioisotopes and fluorescent tags. Biotin is often used because it is simple, cheap and can be used both for detection by western blot approaches and for direct purification by affinity chromatography. Fluorescent and radioactive tags are generally used for imaging applications (see Section 2.2.3) and fluorescent tags also allow for biochemical analysis of labeled proteins in SDS-PAGE gels using simple laser scanning methods that are much faster and easier than standard western blotting. Fluorescent tags also have the added advantage of allowing direct, microscopic imaging of targets that have been modified by an ABP. Thus the spatial and temporal regulation of enzymes can be monitored *in situ*[19] and *in vivo*.[20] Finally, the use of dyes that emit near infrared fluorescent light allows the use of probes for whole body, non-invasive imaging in living organisms (for more information see Section 2.2.3).

2.1.2.3 Classes of Activity-based Probes

The past decade and a half has seen extensive growth in the development of new ABPs. While there still remains many classes of enzymatic proteins for which no ABPs currently exist, the rapid development of new synthesis and screening methods coupled with advances in analytical methods that allow rapid identification of labeled targets has greatly expanded the list of validated probes. Initially, the majority of efforts in probe design were focused on proteases and hydrolases. This is due to the fact that these enzymes use a nucleophilic amino

acid to mediate a direct attack on a substrate. This fact, coupled with a wealth of published inhibitors that form covalent bonds with target proteins, has accelerated the development of activity-based probes for proteases. However, the past few years have seen the development of probes for many other classes of enzymes as well as some non-enzymatic receptor proteins. This section will outline some of the major advances in probe design for each of the primary target classes.

2.1.2.3.1 Proteases. By far the largest body of work on ABPs has been focused on protease targets. In particular, there have been a number of probes developed for the cysteine proteases that have found widespread use in biological studies of disease[5,7,10,11,21–25] (Figure 2.2). While the concept of covalent inhibition of a protease is not new, the idea of using covalent inhibitors to label proteases with tags that allow isolation and biochemical monitoring of the target enzymes is relatively recent. Some of the earliest examples of ABPs include probes for caspases,[9,13] cathepsins,[6,23] the proteasome[6] and serine hydrolases.[12] All of these probes were originally designed with a specific target protease in mind and made use of either knowledge of substrates or a well-characterized selective inhibitor as a starting point.

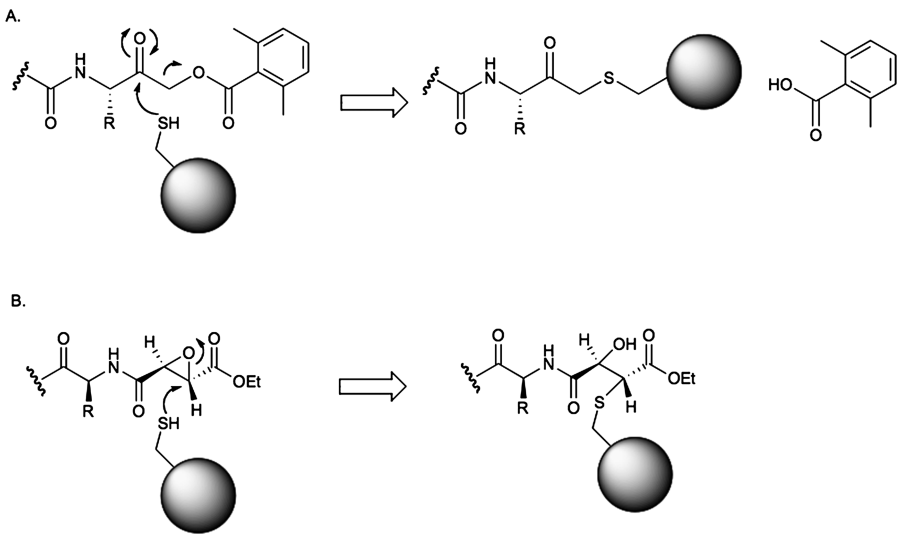

Figure 2.2 Cysteine protease ABPs. Examples of two of the most commonly used classes of probes that target cysteine proteases. (A) A peptide acyloxymethyl ketone (AOMK) reacts with the active site thiol to produce a stable thio-ether bond with loss of the acyl leaving group. This scaffold has been used to target a number of cysteine protease families including caspases, cathepsins and legumain.[5,8,10,11,21] (B) A peptide epoxide based on the natural product E-64 reacts with the active site cysteine to form a stable adduct upon epoxide ring opening. This class of compounds has been used exclusively to target cysteine cathepsins.[7,23]

Interestingly, several of these original probes continue to find new applications in a wide range of biology. The general cathepsin probe DCG-04,[23] for example, has been used to study cathepsin function in a large number of biological systems. For example, this epoxide-containing probe has been applied to functional studies of the roles of papain family proteases in processes such as tumor progression,[20] angiogenesis,[20] cataract formation,[26] pro-hormone processing,[27] malarial infections,[28] bacterial growth,[29] and plant response to pathogens.[30] In addition, a number of probes containing an acyloxymethyl ketone (AOMK) reactive group have been developed.[5,8,10,21,22] This reactive functional group has proven to be highly selective when used in complex mixtures and even *in vivo*. Thus, it is currently the electrophile of choice for use in imaging probes for proteases (see Section 2.2.3). Probes with the AOMK reactive electrophile have been successfully designed to target cathepsins,[21,22] legumain,[31] caspases[5,8,10] and separase.[32]

There have also been a number of elegant examples of the use of ABPs to target serine proteases and serine hydrolases[12,33,34] (Figure 2.3). Because of the broad reactivity of the first serine hydrolyase probe containing a fluorophosphinate (FP) electrophile, FP-Biotin[12] has been applied in a diverse range of applications and very recently has facilitated the identification of previously uncharacterized target enzymes that have direct links to human diseases such as cancer.[35–39] In addition, there have been a number probes described that can be used to selectively target serine proteases.[33] All of these protease-directed probes make use of the less reactive diphenyl phosphonate (DPP) electrophile to target the active site serine. By attaching a peptide scaffold to the probes, it becomes possible to avoid labeling of general hydrolases and lipases that are the target of the FP probes. Thus, while much less reactive than the FP probes, the DPP probes can be used for more selective studies of serine proteases.

Metalloproteases (MPs), like serine and cysteine proteases, play key roles in peptide hormone processing, tissue remodeling, and cancer.[40–42] However, this protease family uses a tightly bound water molecule to initiate attack of substrate, thereby circumventing the acyl enzyme intermediate. As a result, design of activity-based probes for this class of proteases is substantially more challenging. However, activity-based probes targeting MPs have been reported[43,44] (Figure 2.4). This new class of ABPs contains a zinc-chelating hydroxamate coupled to a peptide backbone containing a photo-cross-linking group. These probes can be used to selectively label MPs after irradiation with UV light. The high affinity of the hydroxamic acid group for the active site zinc allows the use of low probe concentrations thereby producing low background of probe labeling. While this is a big leap forward for MP-specific probes, the need for UV light to facilitate probe binding limits their use to *ex vivo* applications.

2.1.2.3.2 Kinases and Phosphatases. Due to the rapid growth in interest in kinases as drug targets in conditions such as cancer, there has been a push to develop new methods to study kinase function. In addition, the kinase family is large and it is necessary to understand the overall selectivity of a given

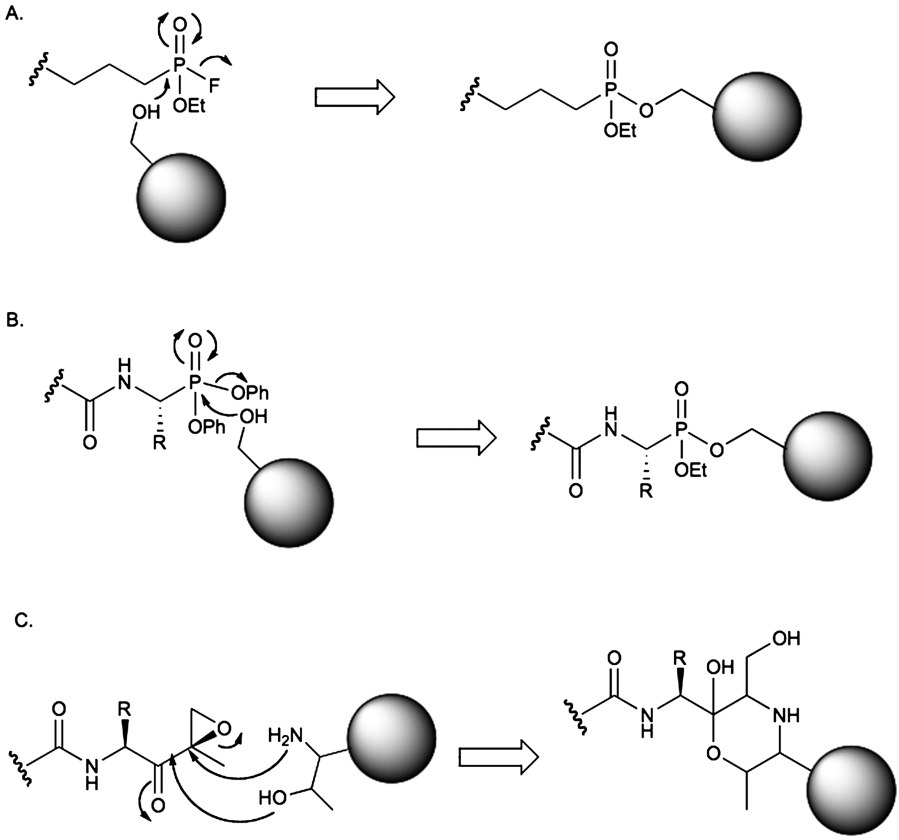

Figure 2.3 Serine protease and hydrolase ABPs. (A) Reaction of a general serine hydrolase probe containing a fluorophosphonate (FP) reactive electrophile. This class of probes has been used extensively to label various classes of serine hydrolases including proteases, esterases, lipases and others.[12] (B) The peptide diphenyl phosphonate (DPP) reacts with the serine nucleophile in the active site of serine proteases. This probe is much less reactive than the FP class of probes but is more selective towards serine proteases over other types of serine hydrolases.[33] (C) The natural product epoxomicin contains a keto-epoxide that selectively reacts with the catalytic N-terminal threonine of the proteasome β-subunit. This reaction results in the formation of a stable six-membered ring. This class of electrophile has been used in probes of the proteasome.[101]

kinase drug lead. This information becomes even more important for kinase inhibitors since most are designed to bind in the highly conserved ATP binding pocket of these enzymes. Because virtually all kinases as well as other ATP binding enzymes have similar ATP binding sites, there is a significant potential for unwanted off-target reactivity. Thus, a number of groups have focused on the design of probes for kinases. These probes have focused either on scaffolds based on the structure of ATP or on synthetic small

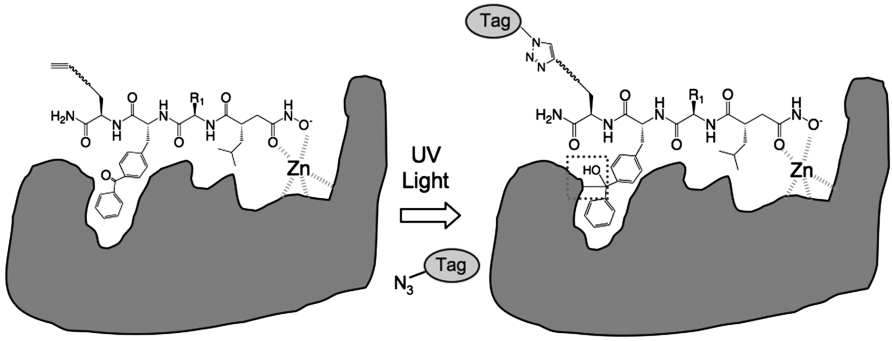

Figure 2.4 Mechanism of action of ABPs for metallo proteases. Current metallo-protease probes bind in the active site of a target enzyme using a hydroxamic acid group that coordinates the active site zinc. Once bound in the active site the probe can be permanently cross-linked into the active site using a photo-cross-linking group that forms a reactive oxygen radical upon irradiation of the probe with UV light.[43,44] This secondary reaction allows permanent modification of the target protease.

molecule inhibitors that bind in the ATP binding pocket of a target kinase (Figure 2.5).

There have been two primary reports of ATP analogs that can be used as general ABPs for kinases. The first probe is based on an analog of ATP that contains an *O*-biotinoyl group linked to the terminal nucleotide phosphate *via* a reactive acyl phosphate.[45] This probe functions by binding the ATP site and then facilitating the transfer of the *O*-acyl biotin group to a conserved lysine found in most kinases. Since this lysine residue forms close contacts with the γ-phosphate, it is ideally situated to form a covalent bond resulting in tagging of the target kinase. Amazingly, this general probe was shown to label nearly 75% of the known kinases and it is therefore a very useful reagent for applications to determine the overall inhibitor specificity of kinase drug leads using proteomic methods (see Section 2.2.1). A second probe is a biotin labeled analog of 5′-fluorosulfonylbenzoyl 5′-adenosine (FSBA).[46,47] This probe has a similar mode of action as the acyl phosphate probe in that it covalently binds to the conserved lysine residues near the ATP binding pocket. However, unlike the acyl phosphate probe that transfers only the acyl biotin group, this probe remains intact when modifying the conserved lysine residue.

The other major class of kinase probes is based on the structures of synthetic kinase inhibitors. The advantage of these probes is that it is possible to generate probes with a much narrower profile of targets. For example, the use of the natural fungal metabolite Wortmannin as a starting point yields a probe that is selective for a subset of protein and lipid kinases.[48,49] Other probes based on inhibitor scaffolds have made use of photoaffinity tags,[50,51] acrylamide electrophiles,[52] and a fluoromethyl ketone electrophile.[53,54] The compounds that use photoaffinity tags can be used to target virtually any kinase since the

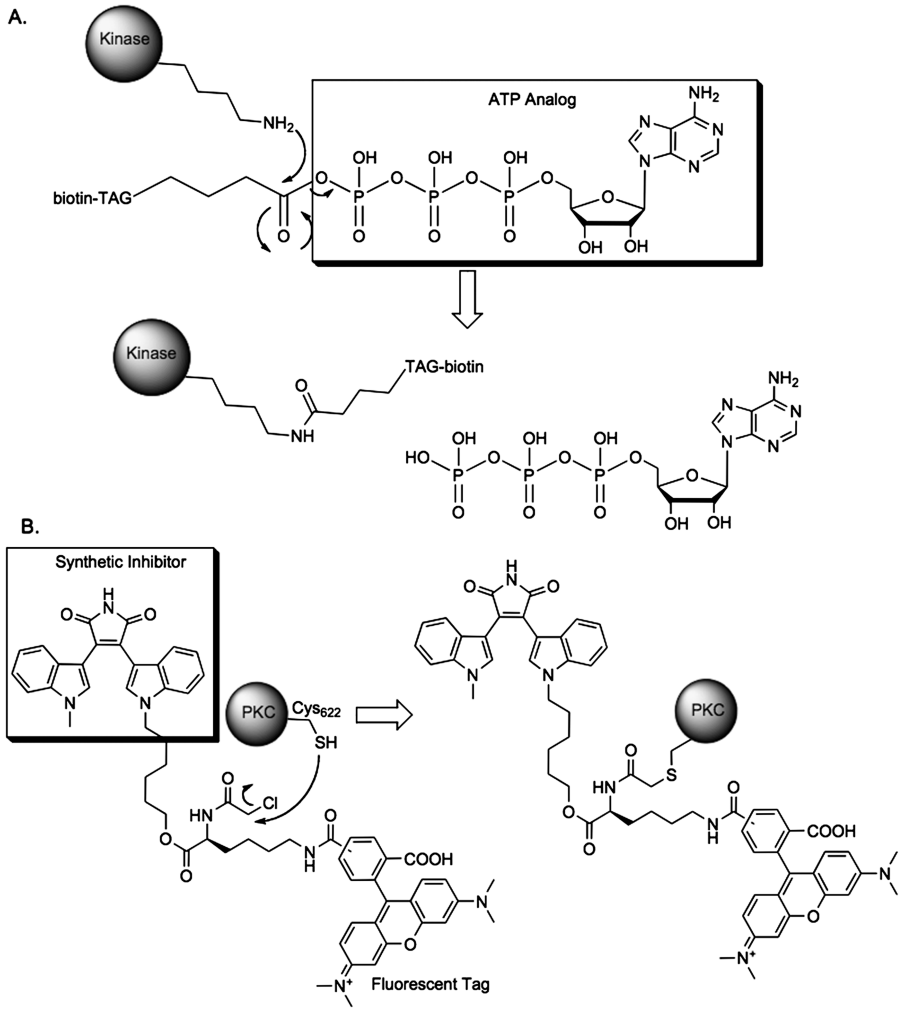

Figure 2.5 ABPs for kinase targets. Examples of two different probe classes that have been designed to target kinases. (A) Structure and mechanism of action of an acyl phosphate ATP mimetic. This probe binds in the conserved ATP binding pocket of kinases and other ATP dependent enzymes and forms a permanent covalent linkage to the kinase target by acylation of a lysine residue near the γ-phosphate of ATP. This general probe was found to label nearly 75% of all known kinases.[45] (B) Structure and mechanism of action of the protein kinase C (PKC) probe AX4697.[91] This probe uses a quinazoline-based inhibitor to facilitate selective binding to PKC. It contains a reactive chloroacteamide to covalently modify a distal cysteine residue near the ATP binding pocket.

linkage of the probe to the kinase is mediated by UV light. The probes with electrophiles have been designed to specifically target kinases that contain a cysteine nucelophile in the ATP site. These probes can be tuned to be highly selective for a small subset of kinases based on the need for a reactive cysteine coupled with structural elements of the inhibitor that only bind kinases with a small ''gate-keeper'' residue. Thus, kinase probes have made significant advances in the past five years and these probes are now proving useful for drug discovery efforts as reagents for screens of compound selectivity (see Section 2.2.1).

The flip side of protein phosphorylation by kinases is controlled by protein phosphatases. Phosphatases are another family of enzymes whose study would benefit from the development of ABPs. They make ideal targets for ABP design as their catalytic site includes a cysteine residue that acts as a nucleophilic thiolate for attack of a substrate. Previously, suicide substrates that contain a masked electrophile have been reported as probes for phosphatases.[55,56] These reagents, while potentially exciting, have yet to be shown effective for labeling of endogenous phosphatases in biologically relevant samples. Additionally, the design of cell permeable probes for phosphatases is likely to be difficult due to the need for a highly charged phosphate mimetic.

2.1.2.3.3 Other Classes of Enzymes. In addition to proteases and kinases, there have been a number of new probes reported for other families of enzymes. This includes probes that target a number of specific target proteins as well as probes that are more general for an enzyme class. For example, recent efforts in the design of probes of the histone deacetylases (HDACs) have generated general reagents that can be used for profiling of the activity of this class of metallo enzymes.[57,58] The first generation probe was designed based on the clinical candidate suberoylanilide hydroxamic acid (SAHA) by simple attachment of a photo-cross-linking agent. Due to the prominent role of these enzymes in the regulation of gene expression in cancer, a number of companies have initiated clinical trials with HDAC inhibitors. Thus, HDAC probes are particularly valuable for drug discovery efforts. More recently, a probe for HDAC enzymes was designed based on a lead series of pimelic diphenylamide inhibitors.[59] By converting the lead compound into a probe by the addition of a benzophenone linked to an alkyne tag, it was possible to identify the primary target of the lead compound as HDAC3 and define a link between this enzyme and Friedreich's Ataxia Gene Silencing.

The past 5 years have also seen a rapid increase in the number of new probe classes. This has included the development of probes that target non-enzymatic proteins such as GABA receptors[60] and acetylcholine receptors.[61] In addition, there have been recent reports of probes that target other diverse enzyme classes including sulfatases,[62] deaminases,[35] dimethylaminohydrolases,[63] lipases,[64,65] esterases,[65,66] nitrilases,[67] cytochrome P450s,[68] gylcosidases,[69] gylcanases[70] and beta galactosidase.[71] This ever-expanding list of enzyme families

and receptors that can be targeted by ABPs suggests that the field continues to advance.

In addition to the directed proteomic profiling strategies outlined above, Cravatt and co workers have pioneered an approach using non-directed probes to target multiple enzyme classes concurrently (Figure 2.6). In the first example of this approach, a library of reactive sulfonate esters was used to profile various complex proteomes, including extracts from cancer cell lines.[35,72,73] In addition to labeling class I aldehyde dehydrogenase (ALDH-1), a number of the probes in the libraries labeled mechanistically different enzyme classes that had not been previously identified or characterized in activity-based studies. These sulfonate probes were later shown to react with a diverse range of nucleophiles in an enzyme active site including cysteine, aspartic acid, glutamic acid and tryptophan.[74] Interestingly, a more recent study has made use of the same type of sulfonate ester electrophile to facilitate covalent transfer of a probe from a reversible binding ligand to protein target.[75] These studies also demonstrated that the sulfonate ester electrophile is capable of reacting with histadine as well. Using a similar strategy, Cravatt and co-workers also developed a library of probes containing a reactive chloroacetamide group and showed that they could select individual probes from the library that labeled a set of targets that are differentially expressed in obese mice.[76]

2.1.2.4 Tagging and Detection Methods

Because ABPs are used for a number of different applications ranging from *in vivo* imaging to affinity purification of labeled target proteins, there are a number of different labels that are generally used on an ABP. This section will focus on the primary types of tags used in ABPs. This includes isotope tags for biochemical and imaging applications, affinity tags for biochemical purification, stable isotope tags for mass spectrometry quantification and fluorescent tags for cell biological and whole body imaging applications. For a more extensive review see Sadaghiani *et al.*[18]

2.1.2.4.1 Isotope Tags

2.1.2.4.1.1 Radioisotopes. Radioactive isotopes are commonly used in various aspects of biology. They can also be used as tags on activity-based probes. The most commonly used isotope is ^{125}I, which has been incorporated into many classes of ABPs. This isotope can easily be introduced by simple iodination methods designed for proteins and peptides (for specific protocols for iodination of ABPs see Bogyo *et al.*[77]). In addition, some probes have been designed to incorporate ^{3}H as it can be added without significant alteration of the probe structure (for example see Fenteany *et al.*[78]). However, the use of tritium is generally not optimal since its specific activity is extremely low, thereby requiring long exposure times to analyze labeling patterns.

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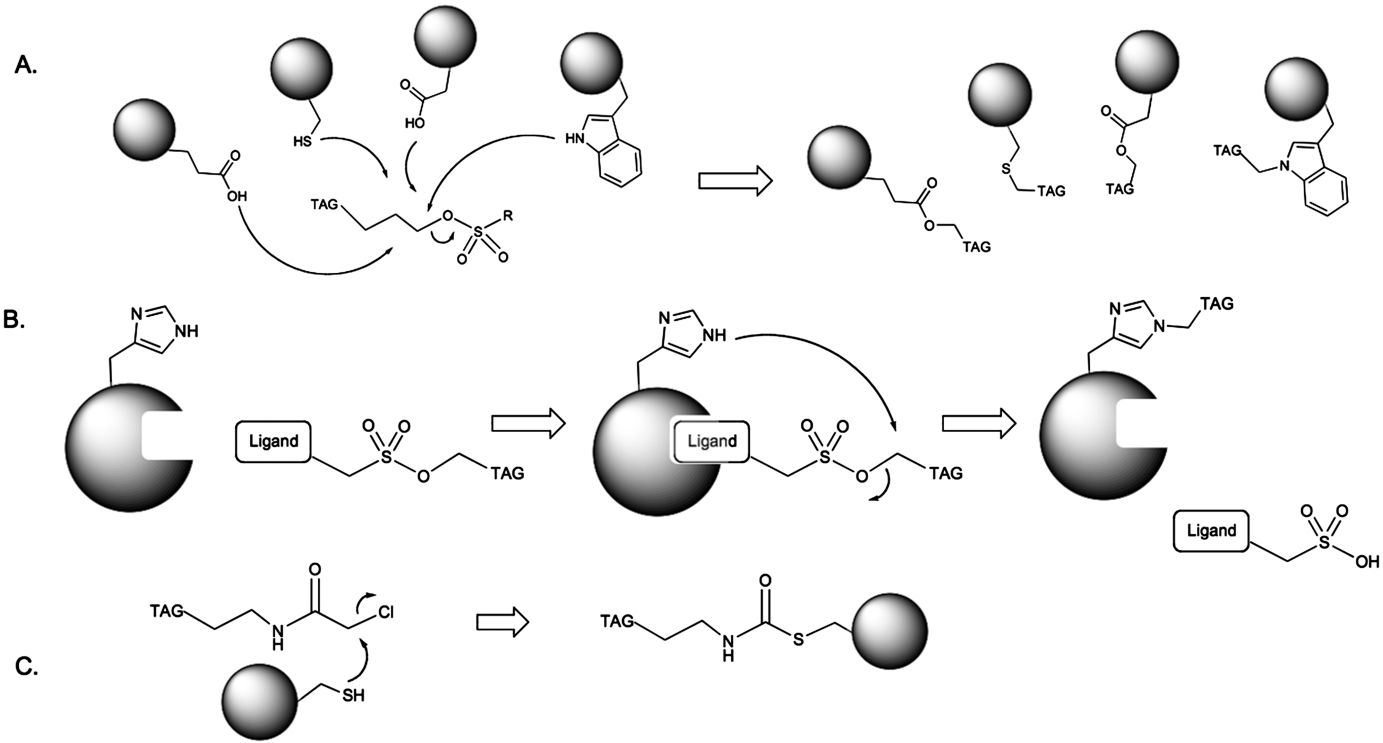

Figure 2.6 ABPs that target multiple classes of enzyme targets. (A) Reactivity of a general sulfonate ester probe. This probe has been shown to react with mechanistically distinct targets and is capable of modifying cysteine, aspartic acid, glutamic acid and tryptophan residues in the active site.[72,74] (B) The sulfonate ester reactive group has also been used to generate probes that transfer a tag to the target enzyme after binding of a high affinity ligand.[75] In this example, a high affinity ligand is linked to the sulfonate ester and tag. Binding of the ligand induces the transfer of the tag to a nearby histidine. (C) General probes containing the chloroacetamide electrophile have been used to label cysteine containing enzymes such as cysteine proteases.[76] This electrophile has been shown to be highly selective for cysteine residues.[74]

There are a number of positive and negative sides to the use of small radioisotopes as tags for ABPs. The small size of iodine makes it possible to generate an ABP based on a well-characterized inhibitor without needing to change the structure significantly by the addition of a bulky tag. However, this advantage is somewhat less important with the advent of multi functional tags that allow attachment of the tag after the probe has bound its target. The other major benefit of radioactive tags is that they can be used in very low concentrations and therefore yield high signal to noise in complex proteomes. These labeling patterns tend to be easier to analyze and provide less background noise. The major drawback of using an isotope label is that there is no direct way to affinity purify the labeled target. Thus, probes must be converted back to an affinity tagged version. However, if the target identities have already been established, it is possible to use the radiolabeled ABPs in screens of compound selectivity using SDS-PAGE analysis in a competition assay (see Section 2.2.1).

2.1.2.4.1.2 Stable Isotopes. As an alternative to the use of radioisotopes, it is possible to use stable isotopes on ABPs for applications to mass spectrometry. Isotope tags have recently been used for a number of quantitative applications in mass spectrometry. This includes the use of isotope coded affinity tags such as ICAT,[79] iTRAC[80] and metabolic isotope labeling SILAC[81] and AQUA.[82] These heavy and light reporters can be distinguished by differences in mass using mass spectrometry. For the technique of ICAT, a general cysteine reactive probe is used in heavy and light labeled form to covalently modify cysteine containing proteins in two samples of different origin. After combination and digestion, relative abundance can be quantified by measuring the abundance of heavy and light labeled peptides by mass spectrometry. An isotope coded activity-based probe has been reported by Overkleeft and co-workers, who synthesized DCG-04, a general papain probe, in a light and heavy version.[83] Alternatively, an ABP can be combined with stable isotope labeling of proteins to yield a highly quantitative method for enzyme activity monitoring.[84] It is also possible that spectral counting methods can be used for relative quantification without the need for isotope labels.[85] In fact, Cravatt and co-workers have demonstrated the use of non-isotopically labeled ABPs to quantify changes in enzyme activity levels by direct mass spectrometry methods, suggesting that the use of such stable isotope labels in ABPs may only be required for monitoring subtle changes in enzyme activity.[86]

2.1.2.4.2 Affinity Tags. One of the primary applications for activity-based probes is the direct isolation of labeled target proteins. Therefore, many of the commonly used activity-based probes contain tags for enrichment of labeled proteins using affinity resins. The most commonly used affinity tag is biotin, which binds to avadin resins with diffusion-limited kinetics. This high affinity is particularly important when probes are used to label low

abundance targets. The direct attachment of biotin to an ABP is often the most efficient method of tagging; however, the main drawback is the overall lack of cell permeability of the biotin tag. Thus, biotin-labeled probes are mainly used for labeling applications in cell and tissue extracts rather than for *in vivo* or cell based labeling studies. To get around this problem of cell permeability, a number of groups have developed orthogonal tagging methods in which the biotin or fluorescent tag is added after the probe has already modified the target enzyme (see tandem labeling methods). Thus, it is possible to use ABPs that have small, cell permeable tags that are suitable for subsequent chemical ligation with a biotin affinity tag.

As an alternative to biotin, it is also possible to use short peptide tags such as a HA or FLAG tag on ABP. This approach has been mainly used for larger protein-based probes such as those designed to target ubiquitin-specific proteases.[87–90] These probes are synthesized as recombinant proteins and the reactive electrophile is added to the C-terminus after the protein is purified. This approach allows one to genetically encode the tag into the primary peptide scaffold. Another recent development has been the use of antibodies that are specific for a small organic fluorophore such as TAMRA or BODIPY.[91,92] This allows the use of a relatively small, cell permeable fluorophore for imaging and biochemical applications and then subsequently for direct immunoprecipitation of labeled protein targets.

Another example of an affinity tag for ABPs is short stretches of peptide nucleic acids (PNAs). This method was used to create probes in which the structure of the probe is "coded" by the PNA tag. The tag also serves as a way to purify the probe labeled targets through direct hybridization to a DNA containing chip.[93,94] Finally, there have been a number of examples of the use of small molecule probes that have been directly attached to a solid support. In this case, the tag is the resin bead. While this is not optimal for covalent probes as it would require a method to cleave the probe from the resin, it has proven to be valuable for use in isolation of kinase targets using reversible probe bound resins.[95,96]

2.1.2.4.3 Fluorophores. The biggest advance in probe technology has been the addition of a wide range of fluorescent tags on ABPs. While it is obvious that fluorescent tags can facilitate imaging applications, these tags are also valuable for direct biochemical profiling studies. Since the fluorescent tags can be quantitatively detected in SDS-PAGE gels using a simple flatbed laser scanner, it has become possible to label target enzymes in intact cells or even whole organisms and then rapidly analyze the profile of labeled proteins by SDS-PAGE followed by gel scanning. This also allows quantitative profiling without the need to blot gels or even remove the gel from the gel plates. This advance has greatly improved workflow. In addition, many of the small organic dyes are highly fluorescent, cell permeable and have proven to be as sensitive as ^{125}I-labeled probes. In addition, there are a wide variety of small organic fluorophores that can be purchased from commercial sources.

By using dyes that emit light in the near infrared (NIR) region of the spectrum, it is possible to generate probes that can be used for non-invasive *in vivo* imaging applications (see Section 2.2.3).

A wide variety of fluorophores have now been used for the development of activity-based probes. The most commonly used classes include fluorescein and rhodamine,[97] dansyl,[98] NBD (nitrobenz-2-oxa-1,3-diazole),[65] BODIPY[19] and the Cy-dyes.[22,99] The first class is relatively inexpensive, but suffers from rapid photobleaching, making them less suitable for most imaging applications. BODIPY and Cy-dyes display high absorption coefficients, high quantum yields, narrow absorption peaks and relatively large Stokes' shifts. Furthermore, these fluorescent tags are hydrophobic and freely penetrate cell membranes. These combined properties make them suitable for a variety of biological applications. However, the commercially available activated ester forms of these fluorophores are extremely expensive and therefore have prevented large scale production of fluorescent ABPs.

2.1.2.4.4 Tandem Labeling Strategies. Another significant advance in probe development has been the generation of tandem labeling methods. This allows a probe to be synthesized with a relatively small tag that can serve as a site for a chemo-selective ligation reaction at a later point in time (Figure 2.7). Ideally, the probe will be cell permeable and can be used in cells and in whole organisms. This so-called tandem labeling process has been developed recently and has been successfully demonstrated for two different ligation chemistries.

The first method makes use of a modified Staudinger reduction in which a probe containing an azide functional group is reduced and subsequently

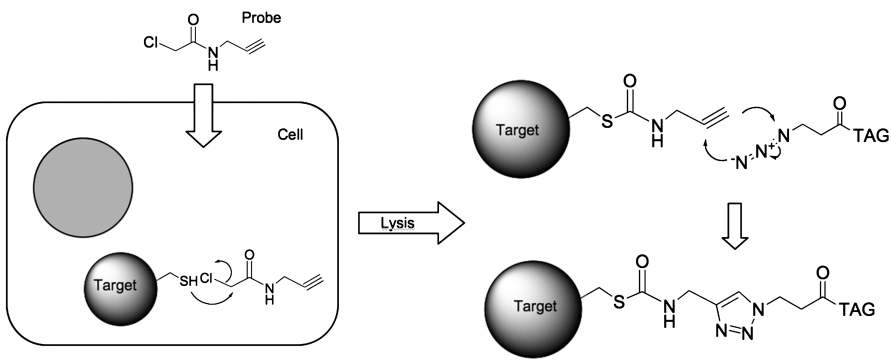

Figure 2.7 Tandem labeling methods. In this example, a general chloroacetamide probe containing an alkyne tag is used to label a target enzyme inside the cell. Once the probe labels the target, the cell is lysed and the probe labeling is visualized by a secondary CLICK reaction with an azide containing tag. The result is the formation of a stable triazole between the tag and the probe. Using this method it is possible to label targets with small, cell-permeable probes and then attach a label after cells have been lysed.[120]

reacted to form a stable amide with an appropriately derivatized phosphene carrying a reporter tag. This strategy was first developed by Bertozzi[100] to tag modified carbohydrates on the cell surface and also proved to be useful for ABPs. In particular, this two-step labeling strategy has been employed for detection of active proteasomes in intact cells using an azide-containing probe that was then used for labeling with a biotin-derivitized phosphine.[101] Interestingly, more recent work by the same group has demonstrated that direct *in vivo* labeling of proteasomes is possible using cell permeable fluorescent probes thus obviating the need for the tandem labeling reaction.[98]

A second and more common way for tandem labeling makes use of the so-called "click" chemistry, in which the 2 + 3 cycloaddition of an alkyne and azide functional group is facilitated by addition of a copper catalyst. Originally developed by Sharpless and co-workers, this strategy was modified by Cravatt and co-workers for use in activity-based proteomics.[102] The two reaction partners are both fully biocompatible and cyclizations can be initiated in aqueous solutions that even contain strong denaturants such as SDS and urea. Thus, CLICK probes have become the method of choice for tandem labeling applications and a number of probes containing either an azide or alkyne tag have been recently reported.[44,86,99] Overall, both of these tandem labeling methods have had a significant impact on the field of activity-based proteomics and will likely be extensively used in future generations of ABPs.

2.2 Applications of Activity-based Probes to Drug Discovery

Perhaps the greatest potential impact for activity-based probes is in the area of drug discovery. Since ABPs can be used to assess the activity of a given target enzyme in the context of complex proteomic samples and even in whole organisms, they can be used effectively to assess such important parameters as efficacy, pharmacodynamic properties and overall target selectivity of drug leads. These are parameters that are often difficult to measure using standard techniques yet the ability to make these measurements is often key to the success of a drug discovery program. In this section we will outline some of the most valuable applications of ABPs that are most relevant to the drug discovery progress.

2.2.1 Identification and Validation of Drug Targets Using Activity-based Probes

The drug discovery process begins with selection of a "validated" target. Once this target is selected, efforts can begin to identify small molecule lead compounds for advancement into clinical trials. In many cases, the targets of small molecule drugs are enzymes and the small molecule lead acts as an inhibitor of its enzymatic activity. Before the process of lead identification can begin, one must be certain the target enzyme is relevant for the given disease indication

and that it can be selectively inhibited to produce a positive therapeutic outcome. ABPs are extremely valuable reagents for the early validation phases of the drug discovery process. Using broad-spectrum probes, it is possible to begin to assess the repertoire of related target enzymes in a given disease model (Figure 2.8). The relevance of a specific enzyme or family of enzymes can then be assessed by monitoring changes in activity levels during disease pathogenesis. Finally, by using selective small molecule inhibitors coupled with an ABP, it is possible to correlate inhibition of specific targets with effects on disease progression in order to validate specific targets for drug discovery efforts.

There have been a number of specific examples of this type of application of ABPs over the past few years. One prime example is the application of a general cysteine protease ABP to validate the cysteine cathepsins in the regulation of cancer pathogenesis.[20] This study demonstrated that several cysteine cathepsins were up-regulated during multiple stages of tumorigenesis in a mouse model of pancreatic cancer.[20] By using a fluorescently labeled, broad-spectrum ABP, it was possible to monitor changes in cathepsin activity and furthermore to determine which cell types were producing these enzyme in the growing tumors. By coupling the use of a small molecule inhibitor with the ABP, it was also possible to assess the extent of inhibition of the target cathepsins and subsequently the effects of target inhibition on tumor growth, angiogenesis and tumor invasiveness. Thus, it was possible to directly correlate cathepsin activities with specific disease pathologies. These results suggest that the cysteine cathepsins are valuable targets for the development of new anti-cancer drugs and that ABPs are powerful reagents for validating specific cathepsin targets *in vivo*.

In several more recent examples, a general ABP for the serine hydrolyase family of enzymes was used to identify enzymes whose increase in activity is correlated with various aspects of cancer pathogenesis.[38,39] In both studies a general fluorophosphonate probe (FP) was used to profile total cell extracts from cancer cells. In the first study, the probe identified a monoacylglycerol lipase that is over-expressed specifically in invasive cancer cell lines. This enzyme was then validated by forced expression in non-invasive cell lines resulting in the transformation of these lines to more aggressive cancers.[38] This study demonstrates that by identifying enzymes whose activities are linked to a given pathology (*i.e.* invasiveness) it is possible to identify new targets that are likely to be key regulators of disease. In the second example, the same probe was used to profile primary human ductal adenocarcinomas. The probe identified the retinoblastoma binding protein 9 (RBBP9) as a previously uncharacterized serine hydrolyase that promotes anchorage independent growth and pancreatic carcinogenesis *in vivo*. This study is a particularly interesting example because the target that was identified by the probe was not known to have serine hydrolyase activity and thus was not considered to be a viable drug target. By using an activity-based probe, it was possible to demonstrate that, not only did this protein have enzymatic activity, but that this activity was required in order to regulate tumor growth. Thus, inhibitors of this activity are likely to have therapeutic benefits for cancer treatment.

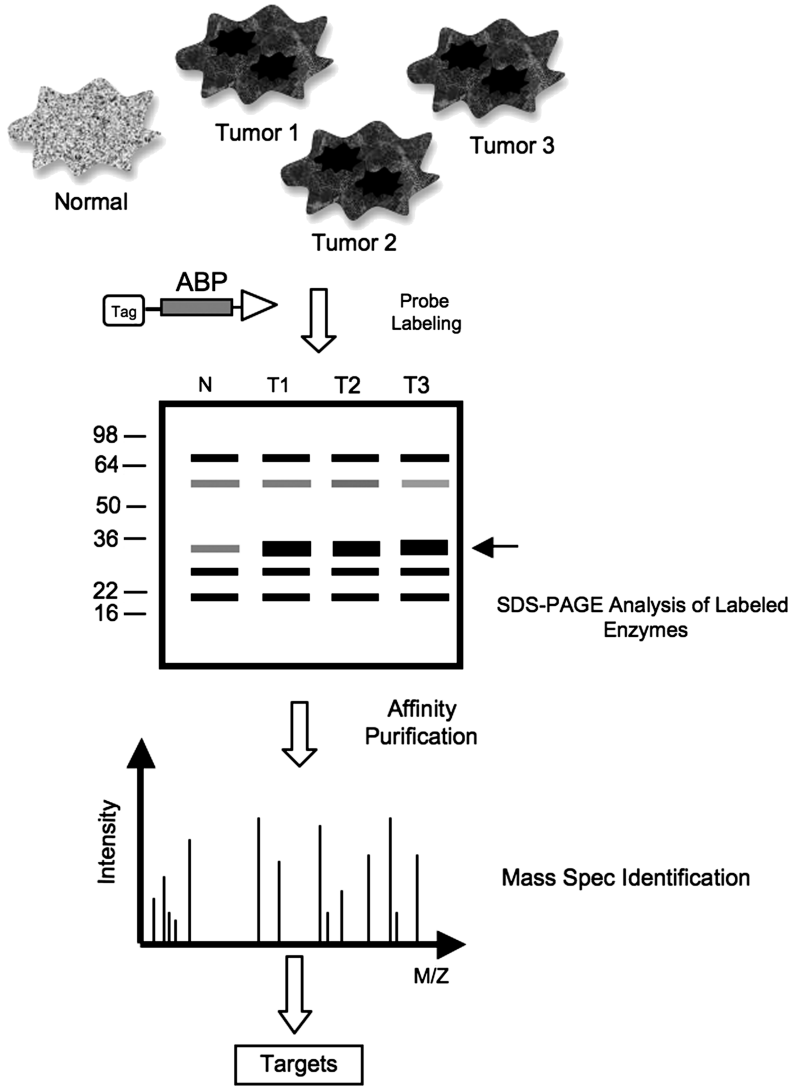

Figure 2.8 Using ABPs to identify novel drug targets associated with human disease. A general ABP can be used to profile the levels of active enzymes in normal and disease tissues. In this example, a general probe is used to label normal tissues and tissues derived from tumors. The resulting labeled enzymes can then be visualized by SDS-PAGE followed by scanning for the tag on the probe (often a fluorescent tag). Since labeling requires activity, the intensity of labeled proteins is an indication of the levels of active enzymes in the tissues. In this example, there is one protein that has higher levels of activity specifically in tumor tissues (shown with arrow). This protein can be identify by affinity purification of probe labeled proteins followed by mass spectrometry based identification of the target. Upon further analysis of the biological role of this enzyme in disease progression, it can be validated as a target for drug discovery efforts.

There have been many other examples of the use of ABPs to study the basic biology of enzymes. For example, ABPs have been used to study the function of proteases in the parasite pathogen that is the causative agent of malaria.[28,103] By using activity-based probes, a number of both host and parasite proteases that are key regulators of processes such as host cell invasion and host cell rupture have been identified. Once validated by ABPs as essential players in parasite pathogenesis, they become viable targets for development of new drug leads.

2.2.2 Use of Activity-based Probes for Drug Lead Identification and Assessment of Selectivity

As outlined above, ABPs are often valuable reagents for the identification and validation of a potential drug lead. Another major advantage of using an ABP for selection of a target is that probe labeling can be used as an assay to assess both potency and selectivity of inhibitor leads (Figure 2.9). Since the probe binds in the active site of the target enzyme, it can be used as an indirect measure of inhibition by a small molecule. Thus a competition method involves treating a target enzyme with a range of concentrations of a lead compound followed by labeling with the ABP. Since inhibition by the lead compound blocks labeling, it is possible to quantify the amount of inhibition by measuring residual labeling by the probe. This assay typically makes use of SDS-PAGE as

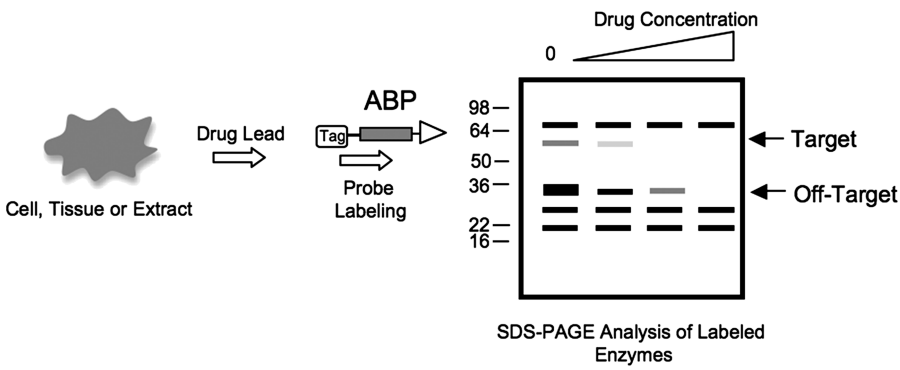

Figure 2.9 Profiling the selectivity of a drug lead using ABPs, which are valuable tools for assessing the selectivity of a given drug lead for a target enzyme of interest. This method is often referred to as a competition assay. In this assay, a tissue extract or intact cell is treated with a range of concentrations of a drug lead. The sample is then treated with a broad-spectrum probe that labels multiple related enzyme targets. Samples are analyzed by SDS-PAGE and labeled proteins are detected by scanning the gel to detect the probe tag. When the drug target binds in the active site of a target, it blocks labeling by the probe and signal is lost. In this example the drug lead is selective for the target but also shows off-target activity at higher drug concentrations.

a readout of residual probe labeling. There are several major benefits of this approach. First, the assay can be performed without any *a priori* knowledge of the native enzyme substrates. Second, if the competition assay is performed in complex mixtures that contain other related off target enzymes, the final SDS-PAGE analysis provides information regarding overall selectivity of the lead compound. Finally, compound potency and selectivity can be assessed without the need to purify and isolate an active form of the desired target enzyme since screening can be performed in crude cell extracts, in intact cells or even in whole organisms (see Section 2.2.1). It should also be noted that the ABP competition assay can be used to assess inhibition of a target enzyme by both reversible and irreversible inhibitors, thus making it suitable for most common classes of drug leads.[28,104,105]

One of the main drawbacks of using an ABP competition assay to assess inhibitor selectivity and potency is that the assay is generally not high throughput and therefore is not suitable for use as a primary screen of a large library of possible lead compounds. However, if the probe is used with purified enzyme or if the probe is highly selective for a given target enzyme, it is possible to switch to a readout that can be adapted to a HTS format. In a recent example, a competition assay was developed by measuring changes in fluorescence polarization of a fluorescent ABP upon target binding. Using this method it was possible to screen a large library of diverse small molecule and identify inhibitor leads of multiple enzymes including RBBP9, an enzyme for which no known substrates have been reported.[106]

2.2.3 Use of Activity-based Probes for Assessment of *In vivo* Pharmacodynamic Properties and Efficacy of Lead Compounds

Another beneficial application of ABPs in the drug discovery process is their use for assessment of *in vivo* properties of drug leads. In most cases, the process of identification and optimization of an inhibitor of a given target enzyme is straightforward and makes use of a simple *in vitro* assays using the purified target enzyme. This process usually results in a lead compound that is then advanced into animal models of disease to confirm therapeutic value. However, it is often difficult to predict how compounds will behave *in vivo* and therefore, results from studies in animal models of a disease can be difficult to interpret. For example, if a lead compound fails to produce the desired effect in the disease model, it is often difficult to determine if this is due to a poor choice of target enzymes or the lack of ability of the lead compound to reach the target enzyme in the tissue of interest (*i.e.* within a tumor). Furthermore, the compound may have altered specificity once introduced *in vivo* as the result of accumulation in distinct tissues and cells within the organism. ABPs can provide valuable information about the overall *in vivo* selectivity, potency and pharmacodynamic properties of a lead inhibitor (Figure 2.10). This information is generally obtained by treating animals with a candidate compound

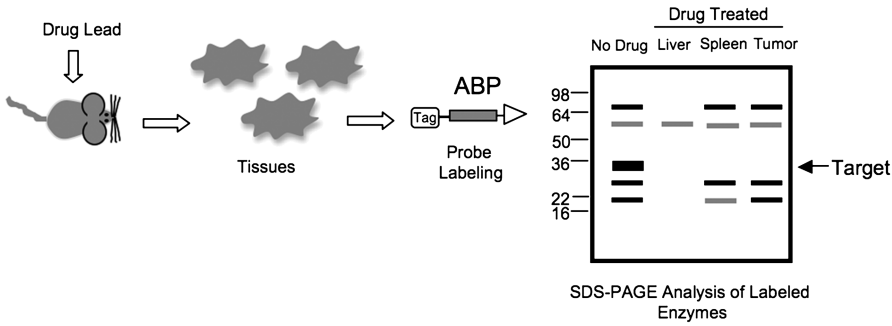

Figure 2.10 Using ABPs to monitor pharmacodynamics and *in vivo* selectivity of drug lead. ABPs can be used to perform *in vivo* competition analysis to determine the selectivity and tissue distribution of a drug lead. In this example, a mouse is treated with a drug lead. Tissues from primary organs and disease tissues are isolated and lysates labeled with a general ABP. Analysis of labeled proteins by SDS-PAGE shows how much of each target enzyme has been inhibited in each tissue. The drug shows highly selective inhibition of single target in the tumor tissue but show slight cross-reactivity in the spleen and strong cross-reactivity in the liver. This data suggests that the drug may clear through the liver and accumulate in multiple organs leading to a loss of selectivity in those organs.

followed by *in vivo* administration of an ABP and assessment of residual labeling in tissues by SDS-PAGE. If probes are sufficiently selective and stable *in vivo,* it is possible to perform such *in vivo* competition studies and obtain valuable information about the distribution, potency and selectivity of a drug lead.

In a recent example of this approach, a small library of epoxide-based inhibitors was screened for selectivity against the cysteine cathepsins.[107] Compounds that were identified as being selective for a given cathepsin were then injected into mice and overall potency and selectivity in various organs were assessed by labeling of tissue extracts with a general radiolabeled probe ^{125}I-DCG-04. These results indicated that, while the overall selectivity that was observed *in vitro* was also observed *in vivo*, the inhibitors accumulated in certain locations resulting in loss of overall selectivity. These results demonstrate the power of using ABPs to determine how compounds behave *in vivo* and to better understand the targets of the compounds *in vivo*. This information is essential to understanding how therapeutic effects correlate with inhibition of specific targets. It also can provide critical information about the value of further optimization of compound selectivity.

2.2.4 *In vivo* Imaging Applications

One of the more recent applications for ABPs has been to apply them for imaging applications *in vivo* (Figure 2.11). While ABPs that carry a cell

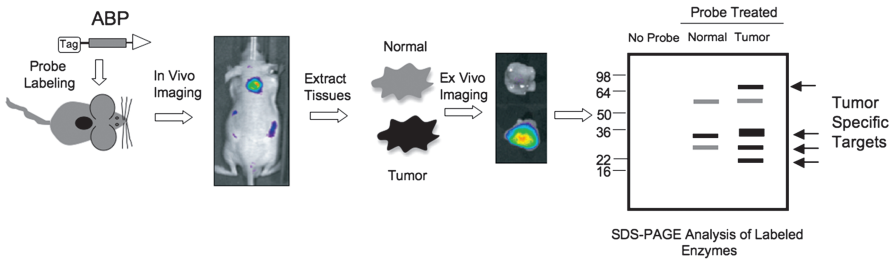

Figure 2.11 Applications for ABPs in non-invasive imaging. ABPs containing near infrared fluorophores (NIRFs) can be used to image probe labeling of target enzymes using non-invasive, whole body imaging methods. In this example, a mouse bearing a small tumor on its back is treated with a NIRF-ABP. After the probe is allowed to clear the animal it is imaged using a non-invasive imaging system. Probe accumulates in the tumor as the result of elevated levels of active target enzymes. Tumor and normal tissue is then removed, imaged *ex vivo* and analyzed by SDS-PAGE. This allows specific signals observed in the animal to be correlated with tumor specific targets that are labeled by the probe. This method can be used in early detection of disease tissue but can also be used for real-time, non-invasive measurement of target inhibition using the *in vivo* competition assay outlined in Figure 2.10.

permeable tag have been in use since the early days of probe development, they have only recently found applications in whole body, non-invasive imaging applications.[8,11,22] Specific advances include the use of near infrared (NIR) fluorophores that emit light that can be visualized through skin and other tissues. Thus, it is possible to apply NIRF-labeled probes *in vivo* and then assess levels of enzyme activity in the whole organism. This section will outline some of the most recent advances in the use of ABPs for *in vivo* imaging applications with a focus on applications that are relevant to the drug discovery process.

2.2.4.1 Non-invasive Monitoring of Drug Efficacy

With the development of new classes of NIRF-labeled ABPs has come a number of possible applications for this technology. First and foremost, probes that label targets that are highly up-regulated in disease tissues such as tumors can be used to assess critical parameters of pathogenesis such as tumor location, growth rate, *etc*. Recent examples of probes that target cysteine cathepsins and legumain demonstrate the utility of detecting tumor tissues using non-invasive methods.[11,22] Because of the potential roles of cathepsins and legumain in a number of conditions that involve inflammation, these probes are likely to find use for diagnostic purposes in the early detection and monitoring of diseases such as atherosclerosis.

In addition, these recent studies have demonstrated the potential to make a fluorescently quenched version of an ABP that produces signal only after it has

bound to the target enzyme.[22] These quenched probes therefore can be used for real-time imaging applications in whole cells and also provide a more rapid readout of specific signal when used for *in vivo* imaging. Quenched ABPs also have potential value for topical applications where the unbound probe cannot be washed away prior to imaging.

NIRF-labeled ABPs can also be used to non-invasively assess the efficacy of a small molecule drug lead. In a recent example with a cysteine cathepsin ABP, the effects of inhibition of target proteases using a general inhibitor were assessed by direct non-invasive imaging methods.[22] Since the probes bind covalently, it was possible to measure effects of the drug using non-invasive methods and then collect tumor tissues and confirm that *in vivo* signals correlated with target labeling by SDS-PAGE analysis. Therefore, it was possible to interpret the non-invasive imaging data with confidence due to link between the imaging data and the *ex vivo* biochemical analysis.

In another example of the use of non-invasive imaging of probe labeling, Edgington *et al*. used NIRF-labeled ABPs that label caspases to monitor the rates of apoptosis *in vivo*.[8] In this study, the ABP was used to assess the efficacy of a clinical antibody that induces apoptosis in tumors by ligation of death receptors on the tumor cell surface. This study demonstrated that the NIRF-ABP could be used to monitor changes in the rate of apoptosis of tumors using non-invasive imaging methods. Furthermore, accumulation of probe in apoptotic tissues could be linked to the amount of labeled caspase proteases that regulate the process of cell death. These results provide evidence that ABPs could be used to continuously monitor the effects of chemotherapy agents on tumors. This could allow assessment of patient response without the need to perform invasive analysis such as biopsy. Furthermore, such imaging-based monitoring of tumor response could greatly reduce the time required to make decisions about treatment strategies, thus reducing the amount of time patients are given drugs that are not effective against their disease.

2.2.4.2 Activity-based Probes versus Substrates

ABPs are finding increasing use for imaging applications. However, it should be noted that there are alternative methods for monitoring enzyme activity. The most commonly used methods for monitoring enzyme activity have focused on the use of reporter substrates as probes. These types of substrate probes can range from small molecules and short peptides to large proteins containing multiple reporter tags. Over the past decade, significant efforts have focused on protease reporters for use in imaging applications. Unlike ABPs that covalently bind and inhibit target proteases, reporter substrates are usually small peptide or peptide-like molecules that, when processed by the protease, produce an optical signal.

Although proteases were originally thought to completely degrade proteins in order to maintain homeostasis of overall proteins levels in the cell, it is now clear that proteases perform limited proteolysis of substrates at defined cleavage sites. This allows proteases to regulate structure, function and localization

of substrates. Although the ability to cleave a specific site on a protein substrate can be controlled by a number of factors including secondary structure and localization of target and protease, in many cases, substrate cleavage is controlled by the primary amino acid sequences surrounding the scissile amide bond. Therefore, it is possible to use optimal recognition sequences to generate fluorescent substrate probes, whose spectral properties change in the presence of active proteases. Several examples of this type of probe exist. The simplest and perhaps most widely used fluorogenic substrate probes consist of a peptide sequence attached at the C-terminus to a fluorophore, such as AFC (7-amino-4-trifluoromethyl coumarin). In the presence of the active protease, the AFC is cleaved from the peptide, leading to a detectable shift in its fluorescent spectrum.

Other examples of substrate probes include peptide recognition sequences that are flanked by a fluorophore and quencher pair, such as Cy5 and QSY21. In these probes, the quencher absorbs the photons emitted by the fluorophore, preventing fluorescence until the quencher is released by cleavage of the substrate. Specific recent examples include probes for caspase proteases as markers of apoptosis.[108–110] It is also possible to replace the fluorophore/quencher pair with a fluorescent resonance energy transfer (FRET) pair. Because FRET can be performed using genetically encodable fluorecent proteins, a number of examples of protease responsive reporters have been developed using this approach.[111–113]

Another important class of substrate-based probes for proteases uses two or more fluorophores, that are self-quenched when in close proximity.[114] Multiple fluorophores can be linked to graft polymers containing peptide substrate sequences. When these linkers are cleaved by the protease, free fluorescent monomer can be detected. This class of probes has been widely used to study the activity of the cysteine cathepsin family of proteases across many diverse disease models.

While the natural substrate sequence often serves as a starting point for probe development, these sites may be engineered to enhance potency and sensitivity towards the protease target of choice using positional scanning libraries. Proteolytic cleavage sites are denoted by P4-P3-P2-P1/P1′-P2′, with the scissile bond occurring between P1 and P1′ sites. A significant number of substrate libraries use a positional scanning approach in which pools of fluorogenic peptide substrates are generated with one position, P1 for example, held constant as a fixed amino acid, while the other positions are mixtures of all possible natural amino acids. Each pool contains a different fixed amino acid, and the pools that yield the highest fluorescence in the presence of the enzyme identify the most optimal residues for that site. Subsequently, if multiple positions are scanned, the results can be combined to produce optimal substrate recognition sequences for various proteases.[104,115,116] This approach is also useful for finding sequences that are less potent towards off-target proteases, allowing for the development of more selective reagents. However, the use of positional scanning methods has its drawbacks since it fails to provide information on the cooperativity of multiple substrate positions for binding in the

active site of the protease target. Therefore, some have chosen to focus on smaller libraries of individual protease substrates.[117,118] Once optimal substrate scaffolds are identified, these structures can be used to generate substrate based probes or they can be converted to covalent inhibitors for use as ABPs.

The addition of a number of new substrate-based probes to the imaging field has helped to advance progress in the use of molecular imaging agents. While substrates and ABPs make use of two different methods to assess activity, both methods have their strengths and weaknesses. The major strength of the ABP is that it binds covalently to the target protease resulting in retention of the label at the site of enzyme activity. In addition, the covalent modification allows the subsequent biochemical analysis of labeled targets as described above. Finally, the ABPs tend to be small molecules that readily diffuse into cells. Substrate probes, on the other hand, are often large peptide or polymer-based scaffolds that have long retention times in blood but have slow diffusion into tissues. On the flip side, substrates have the potential to provide signal amplification due to the fact that the target enzyme is not inhibited by the probe and can therefore process multiple substrate molecules. Interestingly, a recent study comparing large polymer-based substrates to ABPs suggests that this amplification process may not be a significant component of signal intensity.[119] Thus, both methods have their advantages for imaging and these advantages and disadvantages depend on the type of probe scaffold being used.

2.3 Outlook

The field of activity-based proteomics is progressing at a rapid rate. The past decade has seen a significant growth in the number of new ABPs reported and also a growth in the possible applications for ABPs. This chapter outlined the growth in both of these areas with a specific focus on applications for drug discovery. If the field continues to progress on the same trajectory, it is likely that ABPs will find applications in virtually all aspects of basic biology research and drug discovery. This chapter has outlined some of the major benefits of using ABPs in drug discovery efforts. ABPs have the potential to be applied to each and every step of the discovery process beginning with the identification and validation of new targets to use in clinical studies of drug efficacy. This chapter provides specific examples of how ABPs have been applied to target identification, HTS screening for lead compounds, medicinal chemistry efforts and selectivity profiling, *in vivo* pharmacodynamic/selectivity studies and finally *in vivo* efficacy assessment. While most of the examples presented here have focused on probes for proteases, it is clear that as the number of new probe families continues to expand, there will be examples of ABPs being applied to a more diverse range of enzyme targets. Looking toward the future for activity-based probes, it is likely that additional applications in drug discovery will be developed. In particular, the use of ABPs for monitoring clinical endpoints and for direct assessment of drug dynamics in human patients will be the next big step forward. Most of these applications are limited by the lack of probes that

have advanced through FDA approval for use in humans. Similarly, there are a large number of potentially valuable clinical imaging applications for ABPs. However, these will also require significant effort and financial resources to obtain regulatory approval for use as systemic agents in humans. A number of small start-up companies are already beginning to develop optical imaging systems for applications during surgery and for early stage diagnostic monitoring. These companies will likely rely on new developments in optical contrast agents, such as ABPs, to make a significant impact on medicine. Hopefully, an interest in contrast agents such as ABPs will help advance the more late stage applications of ABPs in the drug discovery process.

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