Chapter 7
Activity-Based Imaging and Biochemical Profiling Tools for Analysis of the Cancer Degradome

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Abstract Proteases represent one of the largest and most well-characterized families of enzymes in the human genome. Furthermore, there are many human health conditions associated with alterations in protease activity and function, most notably cancer. Frequently, associations between specific proteases and a given disease are correlative, and there is a need to determine the significance of direct causal relationships. Unfortunately, our understanding of protease function in the context of complex proteolytic cascades involved in human biology remains in its infancy. This gap in our knowledge has to do with the high degree of complexity both at the level of expression and also at the level of posttranslational regulation of proteases involved in the pathways that regulate human physiology or disease pathology. Thus, in order to begin to decipher complex regulatory networks and to assign function to the more than 500 proteases in the human genome, tools will need to be generated that allow direct assessment of protease activity in the context of complex biological systems. In this chapter, we will focus on the recent advances in the development and application of protease probes that can be used to directly monitor levels of active proteases in biological environments ranging from whole cell lysates to whole organisms. This chapter focuses on new developments in the field of small molecule activity-based probes and the development of protease sensors based on substrates. The goal of this chapter is to give the reader an update on our ability to spy on proteases at the level of their enzymatic activity.

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Introduction: Why Monitor Protease Activity?

Proteases, like most other classes of enzymes, are rarely regulated at the level of transcription and translation. Instead, virtually all proteases are synthesized as inactive zymogens that are subsequently activated by any of a number of stimuli that can range from change of pH to direct proteolytic processing by other proteases. To further complicate the regulatory process, many classes of endogenous, protein-based inhibitors exist that serve to regulate the activity of proteases even after processing of an inactive zymogen has occurred. Thus, simple measurement of location and abundance of a protease is not sufficient to be able to define its functional roles in a given disease process. In response to this challenge, significant efforts have been made to develop tools that allow direct monitoring of protease activity in the context of their native biological environment. One of the most common ways to monitor activity of a protease is to develop substrates whose processing by a protease can be easily monitored. Thus, substrate turnover can be measured and the overall binding specificity and enzyme efficiency can be determined. Simple fluorogenic substrates have been designed for use with purified proteases, and modifications of this design have resulted in substrate-based probes that have been used to visualize proteolytic activity in living cells and tissues. The specificity of these reagents is often controlled both by the localization of the probes and by the use of extended peptide sequences that show high selectivity for a given protease target. However, these reporters often lack the required selectivity for use in complex biological samples containing hundreds of proteases. Thus, in order for reagents to be useful for profiling protease activities in complex samples they must have a high degree of selectivity or allow direct identification of the target proteases. The use of small molecule inhibitors with exquisite selectivity for specific proteases has resulted in significant advances in the development and application of activity-based reagents that make use of these highly selective reactive functional groups to limit the complexity of protease that are targeted by a single probe. Furthermore, the permanent nature of activity-based probe (ABP) labeling allows identification of targets using biochemical methods. While it remains difficult to make either ABPs or substrate-based imaging agents with absolute specificity for a given protease, these reagents can often be highly effective tools for monitoring the regulation of a defined and relatively small subset of target proteases. As we outline below, these tools have led to a number of important discoveries and have greatly increased our understanding of protease function in disease states such as cancer.

Small Molecule Activity-Based Probes for Proteases

The field of activity-based proteomics is a relatively new discipline that makes use of small molecules, termed ABPs, to tag and monitor distinct sets of proteins within a complex proteome. These activity-dependent labels facilitate analysis of system-wide changes at the level of enzyme activity rather than simple protein

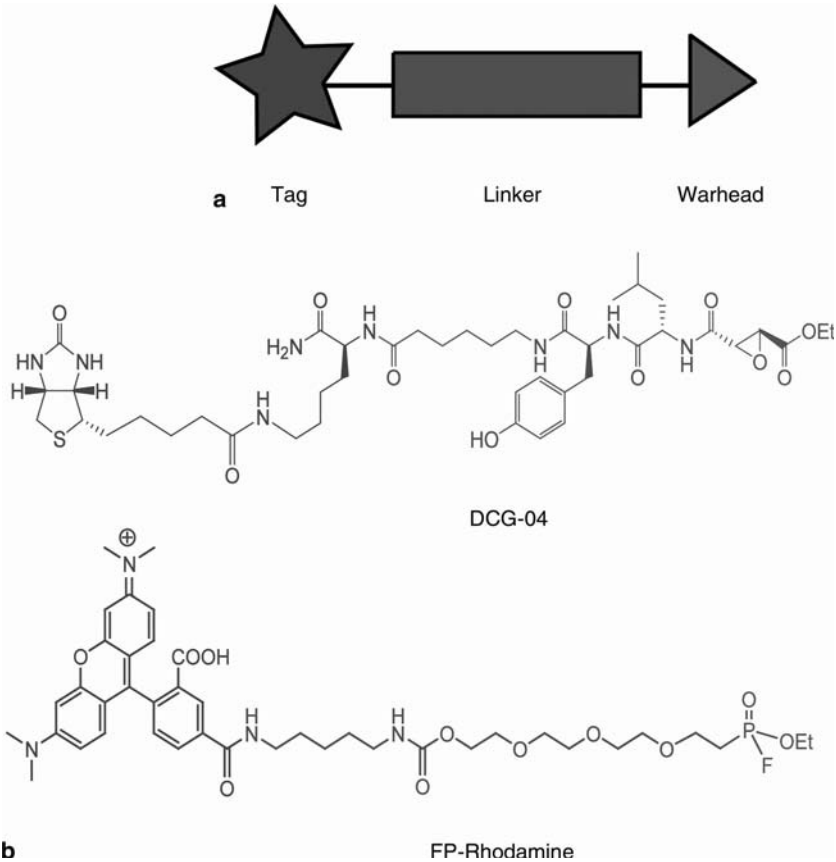


Fig. 7.1 General and specific structures of activity-based probes (*ABPs*). (**a**) General structure of an *ABP*. (**b**) DCG-04 is an *ABP* that targets cysteine proteases and contains a biotin tag (red), a dipeptide-containing linker (blue), and an epoxide as a warhead (green). Fluorophosphonate (*FP*)-rhodamine is an *ABP* that targets the serine hydrolase superfamily of enzymes and contains a rhodamine fluorophore (red), a poly (ethylene glycol) linker (blue), and a fluorophosphonate as a warhead (green). (*See also* Color Insert I)

abundance. In their most basic form, ABPs consist of three distinct functional elements (Fig. 7.1): 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 enzymes.

Perhaps the greatest challenge in the design of a chemical probe is the selection of a reactive group that provides the necessary covalent modification of a target protein. It is often difficult to select effective reactive functional groups as they must be both reactive toward a specific residue on a protein and inert toward other reactive species within the cell or cell extract. In general, the reactive groups of most of the successfully designed chemical probes have been based on the

chemistries of covalent, mechanism-based inhibitors of various enzyme families. Protease inhibitors provide a rich source of reactive groups that have been designed based on subtle differences in reaction mechanisms for the major protease families (for an extensive review, *see* Powers et al. 2002). However, only the serine, threonine, and cysteine protease families utilize a catalytic mechanism that allows direct covalent modification of the primary active-site nucleophile. For these two protease families, the majority of probes have been designed based on the prior work of medicinal and natural product chemists that have pioneered the use of suicide inhibitors (for reviews, *see* Powers et al. 2002, Jeffery and Bogyo 2003, Speers and Cravatt 2004, Evans and Cravatt 2006, Sadaghiani et al. 2007b). For the metalloprotease family, the lack of a direct acyl-enzyme intermediate in the catalytic hydrolysis reaction has required the use of alternate strategies to direct covalent modification of target proteases. All of the current examples of metalloprotease ABPs make use of a pharmacophore that binds with high specificity and affinity to the catalytic metal in the active site. This tight binding element is attached to a photocrosslinker that can be used to secure the probe in place through the formation of a nonspecific, light-activated covalent bond. Because of the limitations in developing metalloprotease ABPs, there has been a significant effort in developing substrate-based imaging probes for metalloproteases, as discussed below.

In ABPs, the linker region of a chemical probe connects the reactive group to the tag used for identification and/or purification. The linker region can serve multiple purposes. Its primary function is to 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. The linker can also incorporate specificity elements used to target the probe to a desired enzyme or family of enzymes. These specificity elements normally take the form of a peptide or a peptide-like structure, particularly for the ABPs used to target proteases.

The purpose of the tag on a chemical probe is to allow quick and simple identification and purification of probe-modified proteins. The most commonly used tags are biotin, fluorescent, and radioactive tags (for review *see* Sadaghiani et al. 2007b). Biotin facilitates detection by simple Western blot approaches using a reporter avidin molecule in place of the standard secondary antibody. Furthermore, biotin allows direct isolation of labeled targets through affinity chromatography. Fluorescent and radioactive tags can be visualized by direct scanning of gels with a fluorescence or phosphorimager scanner and typically have a greater dynamic range than do streptavidin–biotin detection methods. Fluorescent tags also have the added advantage of allowing direct microscopic imaging of proteases that have been modified by an ABP. Specific application of fluorescent ABPs for imaging applications are discussed later in this chapter.

The last 10 years have seen a significant increase in the number and types of ABPs that have been designed by chemists. While the field of activity-based protein profiling has continued to expand in the scope of enzymatic targets that can be profiled, the greatest progress has been made with probes that target proteases (for a relatively complete list, *see* Table 7.1). Profiling of the active forms of cysteine, serine, and threonine proteases in complex proteomes has been successfully

Table 7.1 List of commonly used protease activity-based probes (ABPs)

	Papain family	Probe name	References
Cysteine proteases	Biotin tags		
	Cathepsins/ Calpains	DCG-04	(Greenbaum et al. 2000)
	Cathepsin B	NS-196	(Schaschke et al. 2000)
	Legumain	Asp-AOMK	(Kato et al. 2005)
	Fluorescent tags		
	Cathespins/ Calpains	BODIPY-DCG-04	(Greenbaum et al. 2002)
	Cathepsins	GB-111, GB-123, GB-137	(Blum et al. 2005, 2007)
	Cathepsin B	SV5	(Verhelst and Bogyo 2005)
	Legumain	Fam-XPD-AOMK	(Sexton et al. 2007b)
	Radiolabels		
	Cathespins/ Calpains	Cbz-Try-Ala-N2	Enzyme Systems Products
		DCG-04	(Greenbaum et al. 2000)
		JPM-565	(Shi et al. 1992)
		JPM-OEt	(Bogyo et al. 2000)
		LHVS-PhOH	(Bogyo et al. 2000)
	Cathepsin B	MB-074	(Bogyo et al. 2000)
	Caspases		
	Biotin tags		
	General caspase	Biotin-X-VAD(OMe)-fmk	Calbiochem
		Z-VK-X-(biotin)-D (Ome)-fmk	Calbiochem
		KMB-01	(Berger et al. 2006)
	Caspase-3	b-EvaD-epoxide	(Sexton et al. 2007a)
	Caspase-8	bAB06, bAB13	(Berger et al. 2006)
	Caspase-9	bAB19	(Berger et al. 2006)
		bAB28	(Berger et al. 2006)
	Fluorescent tags		
	General caspase	SR-VAD-fmk	Immunochemistry Technologies, LLC
		FAM-VAD-fmk	
	Deubiquitinating enzymes		
	HA tags	HA-Ub-VS	(Borodovsky et al. 2002)
		HA-Ub-Cl	(Borodovsky et al. 2002
		HA-Ub-Br2	(Borodovsky et al. 2002)
		HA-Ub-Br3	(Borodovsky et al. 2002)
		HA-Ub-VME	(Borodovsky et al. 2002)
		HA-Ub-VSPh	(Borodovsky et al. 2002)
		HA-Ub-VCN	(Borodovsky et al. 2002)
	Arg-Gingipain		
	Biotin tags	BiRK	(Mikolajczyk et al. 2003)

(*continued*)

Table 7.1 (continued)

	Papain family	Probe name	References
Serine proteases	Radiolabels		
	All hydrolyases	^{3}H-DFP	PerkinElmer
	Biotin tags		
	All hydrolyases	FP-Biotin	(Liu, Patricelli and Cravatt 1999)
		FP-Peg-Biotin	(Kidd, Liu and Cravatt 2001)
	Chymotrypsin-like	Biotin-AAF-cmk	Enzyme Systems Products
	Trypsin-like	Bio-PK-DPP, Bio-NK-DPP	(Pan et al. 2006)
	Granzyme B	Bio-x-IEPD^{P}-$(OPh)_2$	(Mahrus and Craik 2005)
	Granzyme A	Bio-x-IGN(AmPhg)^{P}-$(OPh)_2$	(Mahrus and Craik 2005)
	Fluorescent tags		(Patricelli et al. 2001)
	All hydrolyases		(Liu et al. 1999)
			(Patricelli et al. 2001)
Threonine proteases	*The proteasome*		
	Radiolabels	NP-LLL-VS	(Bogyo et al. 1997)
	Biotin tags	Epoxomicin biotin	(Meng et al. 1999)
		AdaLys(Bio)AhX_3L_3-VS	(Kessler et al. 2001)
Metalloproteases	*General probes*		
	Biotin tags		
	Hydroxamates	TFMPD-K(Bio)-GGX-NHOH	(Chan et al. 2004)
	Fluorescent tags	HxBP-Rh	(Saghatelian et al. 2004)
	Hydroxamates	TFMPD-K(Cy3)-GGX-NHOH	(Chan et al. 2004)
	Radiolabel		
	Phosphinic peptides	Compound 1	(David et al. 2007)

achieved through the development of ABPs that exploit the presence of conserved active-site nucleophiles in these protease families. These probes selectively interact with the enzyme active site, free of propeptide or inhibitor, and then form specific mechanism-based covalent bonds between the active-site nucleophile and the reactive warhead group. After modification by the probe, target proteases can be isolated, biochemically monitored, or imagined by virtue of the tagging group used on the probe. For metalloproteases, the lack of corresponding conserved nucleophiles in the active site has required the use of a photochemical group which, upon light excitation, forms a stable linkage with amino acid residues of enzyme active site. Several ABPs have been successfully developed for zinc proteases, leading in some cases to the discovery of elevated zinc protease activities in pathological samples (Chan et al. 2004, Saghatelian et al. 2004). However, up to now, the ABPs developed for matrix metalloproteases (MMPs) have failed to detect endogenous

active forms of these proteases in samples known to overexpress MMPs (cancer cells or tumors). Possible reasons for this failure as well as future directions to overcome the actual limitations are discussed below.

Substrate-Based Imaging Agents

The principle of using substrate-based imaging agents as a means to detect proteolytic activity in vivo has been demonstrated by a number of investigators. The Weissleder group has made inroads into the application of near infrared (NIR) probes for proteolytic activity as a means to measure protease activity and subsequent inhibition (Weissleder 2002). NIR fluorophores have been attached to a linear copolymer either directly (Weissleder et al. 1999) or via a peptide containing a proteolytic cleavage site (Bremer et al. 2001). These probes function on the principle of fluorescence/Förster resonance energy transfer (FRET): close proximity of the fluorophores results in resonance transfer that quenches the fluorescent signal, and the fluorescence intensity increases after cleavage of the peptide linker abrogating the quenching of the fluorophore. These probes have been injected into tumor-bearing mice and have applications relevant to many diseases, including cancer (McIntyre and Matrisian 2003). For example, Cy 5.5-based reagents containing a peptide cleavable by cathepsin B have been used as a method to distinguish well-differentiated and undifferentiated breast cancers (Bremer et al. 2002) and the early detection of intestinal adenomas (Marten et al. 2002) in preclinical models.

More than three decades ago, reagents to measure intermolecular distances by FRET (Förster 1948) between donor (D) and acceptor (A) chromophores (Stryer and Haugland 1967) were incorporated into substrates designed to measure hydrolase activity based on increased fluorescence attendant on loss of FRET (Latt et al. 1972). In these kinds of FRET-based reagents, FRET reduces the apparent lifetime of the excited state of the chromophore resulting in a reduction in the amplitude of fluorescence, particularly of the D chromophore. The efficient quenching of EDANS by DABCYL, introduced in a novel fluorogenic substrate for assaying retroviral proteases by FRET (Matayoshi et al. 1990), has been used in a plethora of FRET substrates with specificity for various proteases afforded by the sequence of the peptide linking the D fluorophore (EDANS) and the A quencher (DABCYL), for example, Calbiochem, http://www.emdbiosciences.com; Sigma-Aldrich, http://www.sigmaaldrich.com. In such FRET-based protease substrates, the intrinsic fluorescence of a specific chromophore incorporated into the substrate is quenched by resonance energy transfer to an acceptor; FRET is optimized by using fluorophores with relatively long fluorescence lifetimes and acceptors with resonance absorption bands that overlap the fluorescence emissions (Haugland et al. 1969, Matayoshi et al. 1990). In the design of FRET-substrates for proteases, though, optimal FRET quenching is afforded by attachment of donor and acceptor chromophores in proximity on either side of the cleavable peptide bond; interference of the chromophores with peptide recognition and cleavage must be considered. For example, a FRET-peptide substrate for ADAMTS13 with fluorophore donor

located at P_7, the seventh amino acid from the scissile bond, and acceptor (quencher) at P_5', that is, separated by 11 amino acids, serves as an efficient and selective substrate for the protease, while a substrate with the donor located at P_4 to reduce the D–A separation was cleaved less efficiently, attributed to proximity of the modified residue to the cleavage site (Kokame et al. 2005). The design of these kinds of fluorogenic peptide substrates is particularly pertinent in the development of fluorescent triple-helical peptide collagen-like substrates (Lauer-Fields et al. 2003). In such reagents, the conformation of the substrate, known to be critical for platelet–collagen interactions (Smethurst et al. 2007), has a significant effect on protease affinity and specificity (Lauer-Fields et al. 2007b) and has resulted in the development of a new class of selective MMP inhibitors based on the structure of the triple-helical transition state (Lauer-Fields et al. 2007a).

FRET-based proteases probes have also been developed based on fluorescence homotransfer (Erijman and Weber 1993), in which the fluorescence is attenuated by resonance energy transfer to an adjacent identical chromophore. For example, the fluorescence of multiply labeled fluorescein (FL)-Dipyrromethene boron Difuoride (BODIPY) albumin is 98% quenched due to homotransfer, providing a reagent that can be used to monitor proteolytic cleavage of albumin (Reis et al. 1998). Likewise, collagen and gelatin heavily labeled with FL, referred to as dye-quenched (DQ)-collagen and DQ-gelatin, respectively, are minimally fluorescent with fluorescence being manifest following proteolytic cleavage; these kinds of reagents have been used for in vitro imaging of proteolysis by human breast cancer cells (Sameni et al. 2000). The principle of homotransfer self-quenching has been used in a number of polymer-based NIR fluorescent (NIRF) protease probes developed by Weissleder's group (Weissleder et al. 1999, Bremer et al. 2001, Weissleder 2002). A similar strategy has been used recently to measure the degradation of a NIRF-labeled polyglutamic acid by cysteine proteases (Melancon et al. 2007).

In addition to the classical fluorescent chromophores, FRET has also been demonstrated between variants of green fluorescent protein (GFP) (Heim and Tsien 1996, Zhang et al. 2002) as well as with a number of new classes of fluorophores such as nanocrystals and nanoparticles (Sapsford et al. 2006). FRET within a fusion protein of blue- and green-fluorescent proteins (BFPs–GFPs) was abrogated by proteolytic cleavage of the linker between the two domains (Heim and Tsien 1996). The development of GFPs with different spectral properties (Piston et al. 1999) has opened the possibility for generating reagents for in situ assay of various proteases. For example, a modified GFP–*Discosoma* sp. Red (DsRed) sensor with a linker cleavable by the 2A-protease encoded by enterovirus 71 (EV71) has been developed to detect EV71 virus infection manifest by increased lifetime of the GFP donor following proteolytic cleavage of the substrate sensor (Ghukasyan et al. 2007). Another recently developed GFP-based sensor uses a nonfluorescent yellow-FP as acceptor to measure FRET in living cells (Ganesan et al. 2006); in such resonance energy-accepting chromoprotein (REACh) reagents, the donor GFP is quenched by FRET to a dark nonfluorescent acceptor. Analogous to the DABCYL quenching of EDANS (Matayoshi et al. 1990) and the more recently described "DQ" reagents such as those with "black hole quencher" (Zheng et al. 2007), the REACh reagents have

only a single fluorogenic chromophore (donor fluorescence), facilitating fluorescence lifetime imaging (FLIM). The quenched GFP–REACh reagents have been used to visualize the distribution of specific biological processes, for example, ubiquitination machinery, without the attenuation of signal that accompanies the spectral selection required with reagents that contain more than one fluorophore (Ganesan et al. 2006).

ABPs for Cysteine and Serine Proteases

The development of ABPs for cysteine proteases has been particularly successful mainly due to the availability of a large number of covalently reactive functional groups and to the fact that this catalytic class is divided into relatively small subfamilies with overlapping substrate specificity. Thus, probes with optimal reactive functional groups and selective linker sequences can be used to monitor small sets of related cysteine proteases.

By far the largest family of cysteine proteases is the family of deubiquitinating proteases (DUBs) with a total of more then 60 members in the human genome (Lopez-Otin and Overall 2002). These proteases regulate the removal of ubiquitin from target proteins, thus controlling their rates of degradation by the proteasome. In addition, there are a number of small ubiquitin-like modifiers (SUMOs) that also are attached to protein substrates and eventually are removed by proteases related to the DUBs. Members of this family of cysteine proteases are somewhat unique in that they recognize a folded protein (ubiquitin) as a substrate and therefore only inefficiently process small peptide substrates. Thus, ABPs designed to target this family of enzymes have required the use of the full 76 amino acid ubiquitin chain as the linker region of the probe. A number of useful ABPs for the DUB family have been synthesized by native ligation of a range of small electrophiles to the C-terminus of epitope-tagged ubiquitin (*see* Table 7.1). These probes have been used to identify new protease families and to monitor changes in activities of DUBs in cancer cells as outlined later in this chapter.

Two other significant subfamilies of cysteine proteases that have been studied with ABPs are the primarily lysosomal cysteine proteases of the papain family (clan CA/CB) and the cytosolic caspases (clan CD) involved in the regulation of cell death. A number of different classes of probes for both papain family and caspases have been developed (*see* Table 7.1). The majority of these probes are short, tri- or tetrapeptides that carry reactive functional groups such as epoxides, acyloxymethyl ketones (AOMK), or vinyl sulfones. Since members of both of these protease families play important roles in regulation of cellular processes involved in cancer progression or response to chemotherapy, probes have already found widespread use for imaging and biomarker discovery. These applications are outlined later in this chapter.

Serine proteases, while similar in overall number of total family members to the cysteine protease family, have been somewhat more difficult to study using ABPs. This is primarily because the serine protease family is made of many highly

specialized subfamilies that have relatively few or even single members. In addition, serine proteases are part of a larger group of serine hydrolyase enzymes, thus complicating the development of protease-specific probes. Regardless of these limitations, general serine hydrolyase probes have proven to be highly valuable reagents for monitoring this larger family of enzymes (*see* Table 7.1). In particular, a general fluorophosphonate probe has been used to identify a number of significant new cancer biomarkers as outlined later in this chapter. In addition, probes containing peptide-based scaffolds in combination with a less reactive diphenyl phosphonate warhead have proven to be quite selective as ABPs for serine proteases (*see* Table 7.1). By changing the sequence of the primary peptide scaffold, probes of this family have been designed to target diverse enzymes in both trypsin and chymotrypsin family proteases and granzyme family proteases with distinct specificities and functional roles. It is clear that further development of probes for serine proteases will likely yield new tools with direct applications to cancer in the near future.

Probes for Metalloprotease Activity

Small Molecule Activity-Based Probes

Development of ABPs to covalently modify the active site of zinc metalloproteases relies first on the selection of an optimal broad-spectrum or selective synthetic inhibitor and second on the identification of a suitable photolabile group that can be incorporated into the inhibitor structure. The main classes of photolabile groups (or photophores) yielding reliable and reproducible labeling of target proteins are depicted in Fig. 7.2. These photophores fulfill a number of important criteria for photoaffinity labeling experiments: reasonable stability under ambient light, a photochemically generated

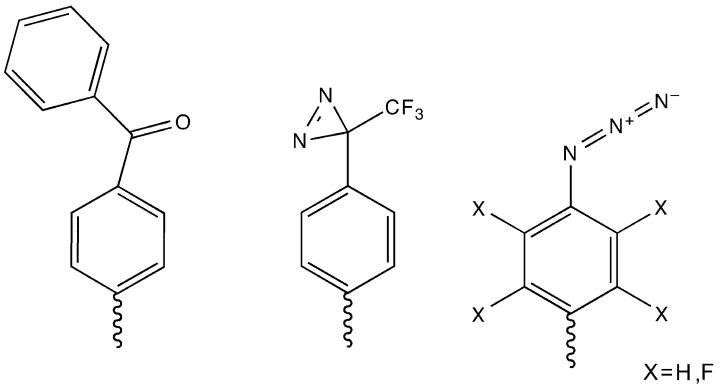


Fig. 7.2 Structures of the three major photophores incorporated in zinc metalloprotease activity-based probes (*ABPs*): benzophenone, diazirine, and phenylazide.

excited state with lifetime shorter than the dissociation of the inhibitor–enzyme complex but long enough to spend sufficient time in close proximity to the target site for covalent linkage, unambiguous photochemistry to provide a single covalent adduct, an activated form that reacts with CH groups as well as nucleophilic X–H bonds, and an activation wavelength longer than the ultraviolet absorption of protein targets (>300 nm) (Fleming 1995, Dorman and Prestwich 2000). These common photophores are hydrophobic groups and thus their incorporation into an inhibitor may compromise inhibitor solubility. In complex proteomes, potentially useful ABPs might be missed because of high nonspecific background labeling. Also, given the bulkiness of these photophores, the site of incorporation into the inhibitor should be chosen carefully in order to preserve the inhibitor affinity for its targets. Commercially available derivatives of amino acids incorporating these photophores can be used during classical peptide synthesis. Introduction of the photophore at a specific site on the inhibitor is also possible; however, this often requires significant synthetic efforts that often make such strategies impractical.

While attachment of a highly reactive photolabile group may yield the desired results for cross-linking, in some cases synthesis of multiple analogues may be required to ensure an efficient labeling of the targeted enzymes. In fact, the chemical composition of the residues surrounding the photoreactive group in the enzyme active site often varies leading to highly variable yields of covalent cross-linking among related protease targets. Efficient labeling is expected to occur when, in the enzyme–ABP complex, the reactive group points toward cavities of the enzyme active site, while weak labeling may arise if the reactive moiety is highly exposed to solvent molecules. Thus, the positioning of the photolabile group in the inhibitor structure requires significant optimization. This was illustrated in a series of probes developed to target MMP active forms. In this study, a benzophenone photolabile group was incorporated either in the P_2' or P_3' position of hydroxamate peptide inhibitors (Fig. 7.3) (Sieber et al. 2006). Whereas a strong labeling of MMP-1 was observed when the benzophenone occupied the inhibitor P_2' position, only weak labeling of MMP-1 was observed when this group was positioned at the P_3' position. The same trends were reported for MMP-9 and MMP-12. These results can be explained by the fact that the S_3' subsite of MMPs is much more solvent exposed than the S_2' subsite. Variation in the cross-linking yield was also found to depend on the identity of the target protein, even within the same protein subfamily. The presence of a very deep cavity (the S_1' subsite) in most MMP active sites (except MMP-1 and -7) was exploited to introduce a photolabile group (an azide group) in the P_1' position of a phosphinic peptide inhibitor of MMPs (Fig. 7.3) (David et al. 2007). In this MMP probe, the photolabile group was incorporated on the distal part of an unnatural side chain, placing the azido group deep inside the S_1' cavity of MMPs, in a position protected from bulk solvent. This probe was shown to selectively label only the active site of MMP-12 with a cross-linking yield of 42%. Determination of the probe efficiency in modifying other MMPs revealed some unexpected results. In fact, this probe was observed to cross-link the active site of eight MMPs (MMP-2, -3, -8, -9, -11, -12, -13, and -14), but with a 40-fold difference in efficacy, a variation that may limit the detection of some MMP active forms in complex proteomes. The

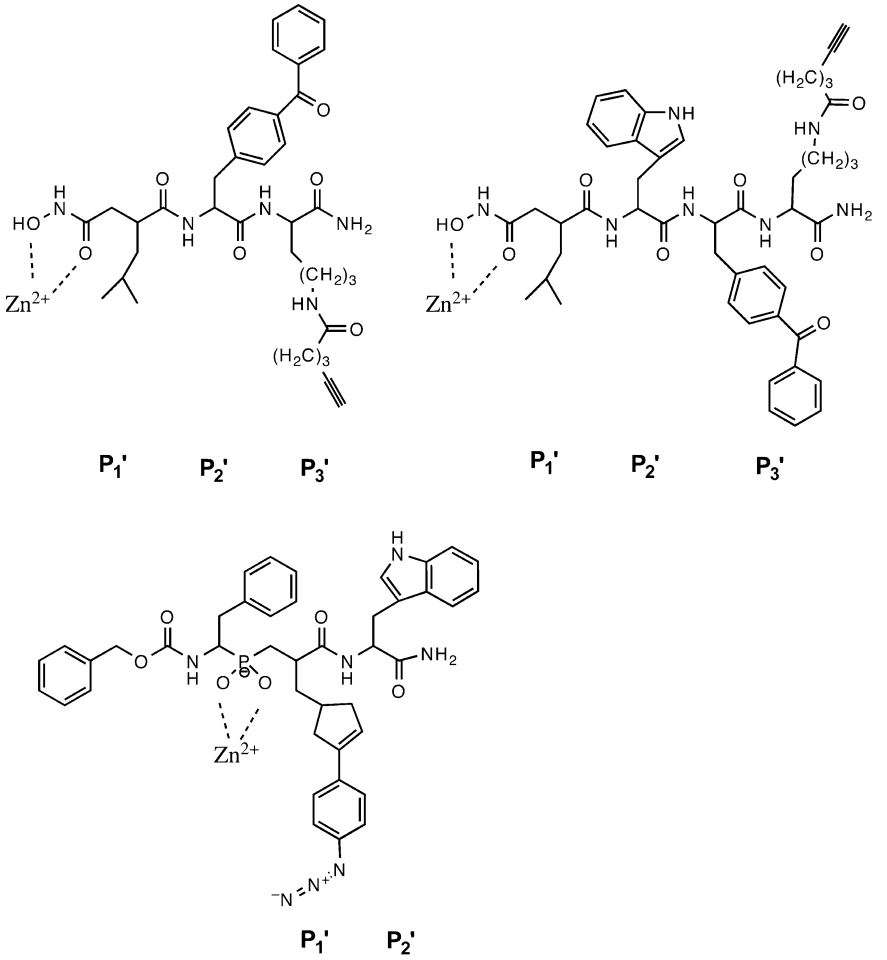


Fig. 7.3 Examples of activity-based probes (*ABPs*) developed to detect matrix metalloproteases (*MMPs*), using hydroxamate or phosphinic peptide templates. The hydroxamate *ABPs* incorporate an alkyne group in the C-terminal position making it possible to add, post-photoactivation, either a fluorescent or a biotin tag using click chemistry

lowest yield of covalent modification with this probe was observed for MMP-3 and MMP-8, suggesting that it may be difficult to detect these two MMPs with this probe. These results highlight one of the main drawbacks of using a photolabile group to cross-link ABPs to their target, namely, that even modest changes in the active sites may result in dramatic variation in cross-linking yields and thus a strong impact on the detection threshold for a given target protease.

Many ABPs developed to target the MMPs have made use of hydroxamate-containing peptides as templates (Chan et al. 2004, Saghatelian et al. 2004, Sieber

et al. 2006). Given the avidity of the hydroxamate group for the zinc atom, hydroxamate inhibitors were observed to display only modest selectivity toward zinc metalloproteases (Brown et al. 2004, Cuniasse et al. 2005). Thus, ABPs using this pharmacophore bind many classes of metalloproteases. Ilomastat (GM6001), a highly potent hydroxamate-based MMP inhibitor, was used as a template to derive one of the first generation metalloprotease ABPs (Saghatelian et al. 2004). By incorporating a benzophenone photolabile group in the P_2' position of Ilomastat, a highly potent ABP was developed, allowing efficient labeling of recombinant MMP-2. However, treatment of tumor cells known to overexpress MMPs led to strong labeling of three zinc proteases (NEP, LAP, and DPPIII), but not of any of the MMP family proteases. Labeling of these targets was rationalized by showing that Ilomastat displayed nanomolar potency toward these zinc proteases. It is worth noting that these zinc proteases display low sequence homology with MMPs and possess very different active site topologies. Thus, the use of the relatively nonselective hydroxamate inhibitor scaffold may allow broad coverage of the zinc metalloprotease family; however, it may also be possible that the lack of probe selectivity toward MMPs is responsible for the inability to detect MMPs using this class of probes. Furthermore, the potentially low abundance of MMP active forms may require the generation of extremely selective ABPs to isolate and enrich the MMPs from the proteome to facilitate their detection and identification. The use of lower affinity zinc-binding elements, such as the phosphoryl group, can be exploited to develop phosphinic peptide inhibitors exhibiting high potency and restricted selectivity profile toward MMPs (Devel et al. 2006).

The difficulty in detecting active forms of MMPs can be explained by arguing that these proteases are mostly expressed as inactive zymogen forms and that most of the activated MMP fractions are blocked by endogenous inhibitors (i.e., the tissue inhibitors of metalloproteinases, TIMPs). Thus, active forms of MMPs may exist at levels that are well below the current detection limit of ABP technologies, even when using the exceptional resolution and sensitivity of multidimensional liquid chromatography coupled to mass spectrometry (MS). Analysis of MMP expression by cells or tumor tissues by gelatin zymography allows the detection of extremely low levels of activated forms of MMP-2 and MMP-9 in complex proteomes. In fact, for MMP-9, a threshold of detection of 100 attomoles has been reported (Masure et al. 1991). Given the sensitivity of this method, the detection of MMP-9 and MMP-2 active forms in cell supernatants and tumors tissues extracts is often reported. However, these data should be interpreted with great caution since the "active" forms of MMP-2 and MMP-9 detected at 82 and 62 kDa in gelatin zymography may account for both active forms and their TIMP complexes, since the denaturing conditions of electrophoresis dissociate the MMP–TIMP complexes. Using affinity capture approaches, which allow enrichment of only active forms of MMPs, it has been reported that the conditioned media of HT1080 fibrosarcoma cells, pretreated with concanavalin A to activate pro-MMP-2, mostly contains TIMP-2/MMP-2 complexes and only trace amounts of active MMP-2 (Hesek et al. 2006). Here again, trace amounts of active MMP-2, as detected by gelatin zymography, may represent only a few femtomoles of protein, a quantity poorly

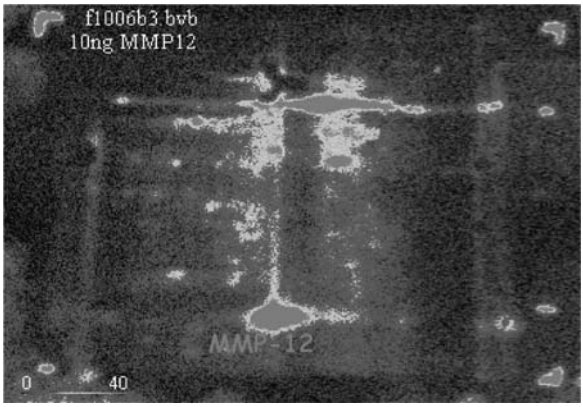


Fig. 7.4 Labeling of mice tumor extract (colon carcinoma) by the phosphinic probe displayed in scheme 2. Before photoactivation, 0.5 pmol of h-matrix metalloprotease (*MMP*)-12 were added to the sample as an internal standard for protein quantification. The sample was analyzed by 2D sodium dodecyl sulfate–polyacrylamide gel electrophoresis (*SDS–PAGE)* followed by detection. (*See also* Color Insert I)

detected even by MS. In confirmation of this finding, labeling of murine tumor extracts with a phosphinic radioactive probe led to similar conclusions. In order to quantify the amounts of MMP active forms labeled by the probe in tumor extracts, a known quantity of the catalytic domain of human MMP-12 was added to the sample, before photoactivation of the probe. The catalytic domain of h-MMP-12 was chosen due to its unique molecular weight and isoelectric point that allow it to be differentiated from murine-derived MMPs. Furthermore, sensitive detection of h-MMP-12 was achieved with the phosphinic probe; as low as 2.5 fmol of h-MMP-12 can be detected (David et al. 2007). As shown in Fig. 7.4 (unpublished results), labeling of exogenous MMP-12 (0.5 pmol) can be observed, confirming that the probe is able to find its targets, even in complex proteomes. Other labeled proteins may represent either specific labeling of endogenous MMP active forms or nonspecific labeling of proteins present in high abundance in the tumor extract. Competitive experiments with excess of MMP inhibitors suggest that several proteins detected in this experiment represent MMP active forms. Based on radioactivity counting, it can be estimated that, as a whole, ~0.5 pmol of endogenous proteins are labeled by the ABP in this sample, with the most intense spots representing about 50 fmol of MMP active forms, a level preventing direct identification of the labeled proteins by 2DE-MS. These experiments again suggest that MMP active forms are present in extremely low amounts, a result in agreement with gelatin zymography data and which may explain the failure to detect MMP active forms in previous reports. These results support the notion that MMPs are mostly present in their zymogen form and in complex with TIMPs, a situation that could be very specific to the MMP family, as compared to other classes of zinc metalloproteases. In this respect, it is worth noting that many zinc metalloproteases identified by the ABP profiling approach are expressed directly as active forms, for which no natural

inhibitors have been reported. Included in this family are the three metalloproteases (neprilysin, dipeptidylpeptidase, and leucine aminopeptidase) observed to be covalently labeled by an ABP developed to target MMPs. Using a more sensitive approach to detect labeled proteins, based on a liquid chromatography–MS platform and a series of new ABPs, Sieber et al. (2006) identified several zinc metalloproteases whose activity is not regulated by zymogen activation and natural inhibitors. In a recent report, using sepharose resin functionalized with a hydroxamate-based MMP inhibitor, detection of MMP active forms in tumor extracts was achieved (Hesek et al. 2006). However, the quantity of total protein loaded on this affinity column was not specified, thus it is not possible to estimate the percentage of MMP active forms relative to total protein levels present in these tumor extracts.

While a number of valuable new metalloprotease probes have been reported that are highly useful reagents for monitoring the activities of a number of metalloprotease families (*see* Table 7.1), optimal covalent labeling of active MMPs remains challenging. As discussed above, the yield of cross-linking by photoaffinity probes is likely to be controlled by several factors, which are not easy to explicitly take into consideration when designing a probe. Thus, the development of ABPs that are able to label all MMPs with high efficiency will require more systematic studies evaluating the influence of the photolabile group, as well as its site of incorporation on the probe scaffold. The specificity of the inhibitors selected for developing ABP probes is likely to be critical to allow successful detection of MMP active forms when these forms are present at a much lower abundance compared to other related zinc proteases. Thus, fine-tuning the yield of covalent modification and selectivity of the ABP will require dedicated efforts. Additional factors, such as tissue extraction procedures, stability of the MMP active forms in extraction buffers, storage of the sample before processing, and analysis conditions, may also be critical for detection of proteins expressed in low amounts. Thus, optimization of all these factors may prove to be critical to fill the gap in MMP detection. Finally, since the photoactivation step of the metalloprotease ABPs limits their use to ex vivo experiments, future developments in reactive moieties to incorporate in ABPs will be necessary to generate ABPs that can be used to profile the zinc metalloproteases in vivo.

Substrate-Based Imaging Agents for Metalloprotease Activity

A NIR FRET substrate-based probe containing the peptide sequence GPLGVRGK was developed and used to detect MMP-2 activity in HT1080 human fibrosarcoma xenografts, giving a fluorescence response that could be inhibited by treatment with a synthetic MMP inhibitor (Bremer et al. 2001). In more recent studies, these kinds of polymer-based protease substrates have been used to assess proteases activities in murine arthritis (Izmailova et al. 2007) and in cardiovascular disease (Jaffer et al. 2007). Interestingly, a peptide-based NIRF probe, quenched by heterotransfer to a NIR absorber and designed to detect MMP-7 activity (Pham et al. 2004), appears to

provide detection of tumor-associated MMP activity without the use of a polymer delivery vehicle (Wellington Pham, personal communication). For optical imaging, Achilefu and colleagues have prepared a number of NIR optical contrast agents designed to either bind to or be metabolized by tumors and, together with Britton Chance, have demonstrated the feasibility of detecting 2 cm-deep subsurface tumors using a metabolism-enhanced NIR fluorescent contrast agent and NIRF in vivo imaging (Achilefu et al. 2002, Chen et al. 2003, Achilefu 2004). The Tsien group have described a new strategy to use activatable cell-penetrating peptides (ACPPs), consisting of a polyarginine membrane-translocating motif linked via an MMP-cleavable peptide (PLG*LAG) to an appropriate masking polyanionic domain (a cleavable peptide hairpin), to deliver fluorescent labels to within tumor cells both in vitro and in vivo after cleavage by tumor-associated proteases (Jiang et al. 2004). Such ACPPs offer a general strategy toward both imaging and delivery of therapeutics in a variety of diseases in which extracellular proteases have been implicated.

McIntyre and Matrisian have generated dendrimer-based optical proteolytic beacons (PBs) for MMP-7 detection (McIntyre and Matrisian 2003, McIntyre et al. 2004) (Fig. 7.5). Their PBs are built on a dendrimeric polymer core, such as Generations 4 Starburst® Polyamidoamine (PAMAM) (nominal MW, 14,215). The prototype visible-range PB for MMP-7, PBvisM7, consisted of dendrimer coupled to substrate peptide previously labeled with fluorescein (FL) linked to its N-terminus (McIntyre et al. 2004). The peptide sequence is selectively cleaved by MMP-7 as compared to other MMPs (Welch et al. 1995). A caproyl linker (Ahx) is included adjacent to the N-terminal FL so as to diminish the solubility of the FL-RPLA peptide produced by proteolysis. The FL-labeled cleavable peptide serves as the

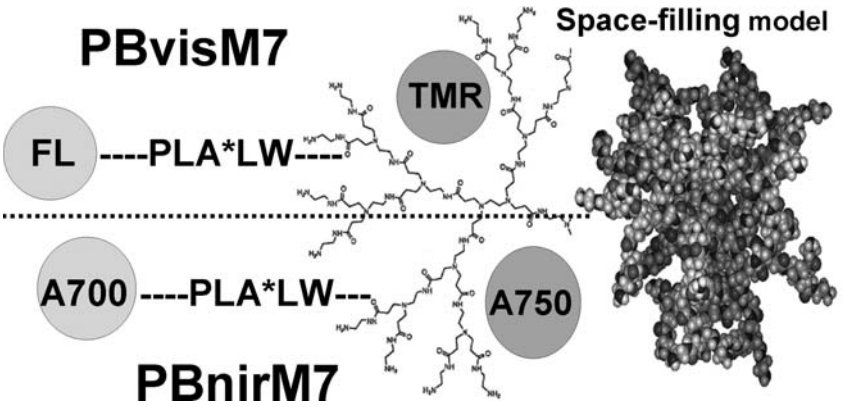


Fig. 7.5 Schematic structure of PBvis and PBnir. In PBvisM7, fluorescein (*FL*), linked at the N-terminus of the matrix metalloprotease (*MMP*)-selective cleavable peptide (cleavage site denotated by *), serves as the optical sensor. The internal reference, tetramethylrhodamine (TMR), is linked directly to the Starburst (PAMAM) dendrimer (generation 2 shown at the left, generation 4 in the space-filling model). For PBnirM7, AlexaFluor700 serves as the sensor and AlexaFluor750 as the reference

optical sensor and the PBs also include tetramethylrhodamine (TMR) linked directly to the dendrimer scaffold; the TMR serves not only to quench the fluorescence of the FL on the protease sensor, but also as an internal reference that is used to track both substrate and product. Treatment of PBvisM7 with MMP-7 results in a significant enhancement in the FL fluorescence with a minimal change in the fluorescence of TMR, with the maximal MMP-7 enhancement in green fluorescent signal ~17-fold (McIntyre et al. 2004). The more recently reported NIR version of the MMP-7-PB, PBnirM7, uses AlexaFluor700 or Cy5.5 as the sensor instead of FL and AlexaFluor750 instead of TMR for the reference (Scherer et al. 2008).

Applications of Activity-Based Probes

Once a small-molecule ABP has been designed and its targets identified by affinity purification, it is possible to use this probe in a number of diverse applications. In one of the most useful applications of an ABP, levels of active proteases can be monitored in a range of samples that differ in stages or types of disease pathology. This application for ABPs allows specific proteases to be identified that may serve as useful biomarkers of that disease. A number of elegant examples of the use of ABPs to identify proteases as cancer biomarkers are outlined below. Proteases that show altered levels during progression toward disease may also represent valid targets for drug development. ABPs also serve as potentially valuable tools to monitor inhibition of target proteases by small molecule drug leads. ABPs allow therapeutic effects of a drug to be linked with inhibition of specific protease targets, thus helping to validate that target for further drug development efforts. Finally, since ABPs form direct covalent bonds with their targets, it is possible to directly visualize the localization of active proteases in whole cells and even in whole animals. The final application of ABPs covered in this chapter is their use for in vitro and in vivo imaging. Note that the applications of ABPs are also pertinent to the use of substrate-based imaging probes, that is, as noninvasive cancer biomarkers, tools for target modulation by small molecule protease inhibitors, and in cancer detection.

Using ABPs to Identify Protease Biomarkers in Cancer

One of the potentially most promising applications for ABPs is for the discovery of new biomarkers of disease. Since members of all of the major classes of proteases have been implicated in some stage of cancer progression, it is likely that proteases will be a rich source for new markers for disease diagnosis and prognosis. Several ABPs have been developed to target enzymes implicated in cancer progression and tumorigenesis, including metalloproteases, cysteine cathepsins, and esterases (Evans and Cravatt 2006, Schmidinger et al. 2006, Fonovic and Bogyo 2007). These ABPs have been used to profile human tumors and tumor cell lines and

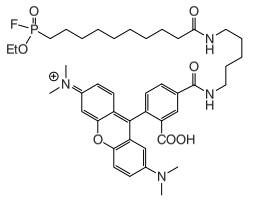
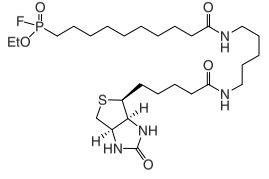
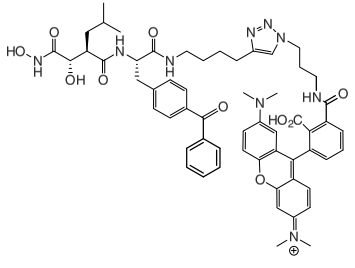
identify novel enzyme activities for the diagnosis and treatment of cancer (Table 7.2). In a typical experiment, normal and disease proteomes are labeled with an ABP and the proteins are separated and analyzed by gel electrophoresis (Fig. 7.6). Enzymes that differ in their activity levels can then be identified as potentially interesting new biomarkers.

In one example of an application of ABPs to cancer biomarker discovery, FP-rhodamine, an ABP that targets the serine hydrolase superfamily of enzymes, was used to profile the activities of these enzymes in a set of human breast and melanoma cancer cell lines (Jessani et al. 2002). This study confirmed that highly invasive cancer cells from several different tumor types upregulate a distinct set of serine hydrolase activities, including the protease urokinase and a novel integral membrane hydrolase, KIAA1363. Although urokinase was known to be involved in tumor progression, KIAA1363 had never been implicated in cancer and therefore represents a potentially important new cancer biomarker (Jessani et al. 2002). In a related study, a panel of primary human breast cancer tissues was probed with the biotin-labeled version of FP-rhodamine (Jessani et al. 2002). Probe-labeled proteins were enriched using avidin-conjugated beads, digested by trypsin, and subjected to semiquantitative MS analysis. A set of enzymes, including KIAA1363, with elevated activities in the most aggressive tumor tissues, was identified as potential breast cancer biomarkers. Recently, both FP-rhodamine and FP-biotin were used to identify enzymes that are involved in cancer cell intravasation, the process by which tumor cells enter into the vasculature (Madsen et al. 2006). The activity level of the serine protease urokinase-type plasminogen activator (uPA) was substantially elevated in the high intravasating (HT-high/diss) variants of the human fibrosarcoma cell line HT-1080. Inhibition of uPA activity significantly reduced the rate of intravasation and metastasis of HT-high/diss cells, suggesting that active uPA is a key determinant of these processes (Madsen et al. 2006).

Metalloproteases also play key roles in cancer progression events such as angiogenesis and metastasis (Deryugina and Quigley 2006). Several metalloprotease genes are overexpressed in metastatic cancers, and inhibitors of these enzymes reduce tumor angiogenesis in animal models of cancer (Egeblad and Werb 2002). A library of metalloprotease probes based on a peptide hydroxamate scaffold carrying a photocrosslinker was used to profile the activities of metalloproteases in both breast carcinoma and melanoma cell lines (Saghatelian et al. 2004, Sieber et al. 2006). Neprilysin, alanyl aminopeptidase, and ADAM10 activities were found to be elevated in invasive cells. Although neprilysin has historically been considered a negative regulator of tumorigenesis, its high activity in invasive melanoma cells suggests that this enzyme may contribute to cancer progression.

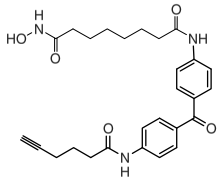
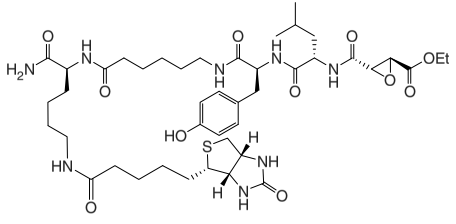
Histone deacetylases (HDACs) are enzymes that remove acetyl groups from lysine residues on histone tails and therefore are important regulators of gene expression. These enzymes have also been implicated in tumor growth and development (Minucci and Pelicci 2006). While not proteases, these enzymes carry out a hydrolysis reaction using catalytic residues that are similar to a metalloprotease. Thus, ABPs have been designed to target HDACs that are similar to ABPs that target metalloproteases. An HDAC-selective ABP has been designed based on a

Table 7.2 List of probes used for proteomic profiling of protease activity in cancer models

ABP	ABP structure	Proteome	Enzyme activity	References
FP-rhodamine		Human breast melanoma cell lines	Serine hydrolases	(Jessani et al. 2004)
		Human ER(+) and ER(−) breast tissue samples		(Jessani et al. 2002)
		HT-high/diss and HT-low/diss human fibrosarcoma cell lines		(Jessani et al. 2005)
		MDA-MB-231 breast cancer cells before and after passage in mice		(Madsen et al. 2006)
FP-biotin		Human ER(+) and ER(−) breast cancer tissue samples	Serine hydrolases	(Jessani et al. 2005)
		HT-high/diss and HT-low/diss human fibrosarcoma cell lines		(Madsen et al. 2006)
HxBP-Rh		Human melanoma cell lines	Metalloproteases	(Saghatelian et al. 2004)
		Human breast cancer cell lines		(Sieber et al. 2006)

(*continued*)

Table 7.2 (continued)

ABP	ABP structure	Proteome	Enzyme activity	References
SAHA-BPyne		Human melanoma and breast cancer cell lines	Class I/II HDACs	(Salisbury and Cravatt 2007)
HAUb-VME		Cervical carcinoma biopsies tissue samples and cell lines	USPs	(Ovaa et al. 2004)
		Human tumor cell lines		(Rolen et al. 2006)
DCG-04		PyMT/ctsb$^{+/+}$, PyMT/ctsb$^{-/-}$, and PyMT/ctsb$^{+/-}$ murine tumor cells	Cathepsins	(Vasiljeva et al. 2006)

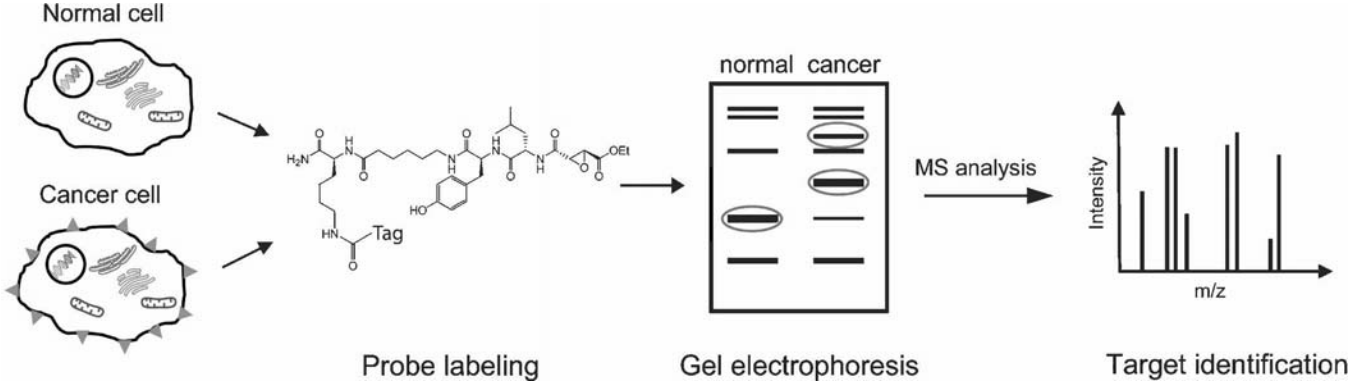


Fig. 7.6 Tumor biomarker discovery using activity-based probes (*ABPs*). Enzymes from cancer cells and normal cells are reacted with a biotin-containing *ABP* and are then separated and analyzed by gel electrophoresis. Probe-labeled enzymes are visualized, and enzymes with altered activities in normal and cancerous cells are identified. These potential tumor biomarkers can then be identified by mass spectrometry (*MS*) analysis

hydroxamic acid zinc-chelating moiety and a photoactivatible benzophenone group (Salisbury and Cravatt 2007). This probe was used to analyze HDAC activity in melanoma and ovarian cancer cell proteomes (Salisbury and Cravatt 2007). Differences in the composition and activity of HDACs were found among cancer cells indicating that the members of the HDAC enzyme family may have a variety of functional roles in cancer.

The ubiquitin-specific proteases (USPs) are a large family of proteolytic enzymes that regulate the production and recycling of ubiquitin and are involved in cell growth and differentiation (Rolen et al. 2006, Ovaa 2007). ABPs containing a reactive electrophile conjugated to the full-length ubiquitin protein have been shown to be highly selective probes of the USPs. A number of these probes were used to identify unique and tumor-specific activities in a variety of human tumor cell lines (Ovaa et al. 2004). One specific USP, UCH-L1, was highly active in numerous malignant tumor cell lines. UCH-L1 activity was also found to be upregulated in normal B cells after in vitro Epstein-Barr virus infection. This increase in activity correlated with a transition from slow to rapid proliferation of the cells, implicating UCH-L1 in this adaptation. USP-specific ABPs have also been used to profile USP activity in human cervical cancer biopsies (Rolen et al. 2006). The activities of two USPs, UCH-L3 and UCH-37, were elevated in tumor tissue when compared to normal tissue. Additionally, the activities of four USPs were upregulated in primary keratinocytes upon infection with human papilloma virus oncogenes, suggesting that the USPs are involved in growth transformation (Rolen et al. 2006).

ABPs have also been applied to functionally characterize enzyme activities in mouse models of cancer. The biotinylated ABP DCG-04 that targets the papain family of cysteine proteases was used to evaluate cysteine cathepsin activity in mammary tumor cells from PyMT;ctsb$^{-/-}$ mice, a mouse mammary cancer model deficient in cathepsin B (Vasiljeva et al. 2006). Although cathepsin B is the most active cysteine cathepsin on the surface of PyMT;ctsb$^{+/+}$ mammary cells, tumor cells lacking this protease (from PyMT;ctsb$^{-/-}$ mice) show an upregulation of active cathepsin X on their cell surfaces. Cathepsin X activity partially compensates for the deficiency of cathepsin B in these tumor cells. Data from these experiments suggest that proteases can dynamically compensate for each other, thus complicating the analysis of data from genetically deficient "knock-out" mice. In a similar study using the DCG-04 probe, the levels of multiple cysteine cathepsins were found to be highly upregulated in tumors that developed in the beta cells of the pancreas of the RIP1-Tag2 mouse, a mouse model of pancreatic cancer. Cathepsin expression was found to be linked to processes such as angiogenesis, and levels of active protease correlated with overall invasiveness of tumors (Joyce et al. 2004).

In an effort to more fully characterize the enzyme activity profiles of xenografted mouse tumors, ABPs such as FP-rhodamine have been used to characterize enzyme activities in MDA-MB-231 breast cancer cells both before and after growth as tumors in the mammary fat pad of immune-deficient mice (Jessani et al. 2004). Many serine hydrolase activities, such as uPA and tissue plasminogen activator (tPA), were highly elevated in the in vivo-derived lines of MDA-MB-231 and correlated with increased tumor growth rates and metastasis upon reintroduction into mice.

ABPs in Enzyme Inhibitor Discovery and Verification

In traditional drug discovery, libraries of small molecules are screened in vitro against purified, often recombinant, protein targets to identify inhibitors. However, in vitro assays provide only limited information regarding the in vivo potency and selectivity of an inhibitor for a related series of enzymes and provide no information about the selectivity pattern that will be observed once the compound is used in vivo. Since ABPs bind to the active sites of their enzyme targets, probes have been used to develop small molecule inhibitor screens that resolve many of the shortcomings that plague standard in vitro inhibitor assays (Greenbaum et al. 2002, Leung et al. 2003, Evans and Cravatt 2006, Fonovic and Bogyo 2007, Sadaghiani et al. 2007a). In an ABP-based screen, whole cells, cell lysates, or even whole organisms are treated with a range of concentrations of a potential inhibitor (Fig. 7.7). Total tissue or cell extracts are then reacted with an ABP and subjected to gel electrophoresis to separate the labeled enzymes. Small molecule inhibitor binding to a target is then measured as a decrease in enzyme labeling by the ABP. The resulting percent competition values can be measured by quantification of labeled proteins and used to generate IC_{50} values of the small molecule for each of the primary targets of the ABP. In contrast to standard inhibitor assays, ABP-based assays can be performed in complex proteome mixtures (including cells and whole organisms) containing multiple related enzymes, thus allowing for the evaluation of both potency and selectivity in a native cellular environment. These assays also eliminate the need for time-consuming expression and purification of drug targets and can be used to identify inhibitors for enzymes that lack known substrates. Finally, when used in vivo, ABP-based drug screens provide information regarding potency, selectivity, and biodistribution of an inhibitor in the context of a whole organism.

In one example of an ABP-based competition study, the potency and selectivity of a series of cysteine protease inhibitors was monitored in rat liver extracts (Greenbaum et al. 2002). This screen identified a small molecule that selectively targeted cathepsin B activity. Since cathepsin B is suspected of facilitating tumor invasion, this compound could potentially be used as a lead target for cancer therapy. ABP-based assays using the serine hydrolase probe FP-rhodamine have also been applied to the discovery of novel, selective inhibitors of KIAA1363, a poorly characterized enzyme with highly elevated activity in invasive cancer cells (Leung et al. 2003, Chiang et al. 2006). This screen yielded valuable lead compounds that facilitated further study of the function of KIAA1363 in the metabolism of lipids. Highly selective inhibitors of the caspases, cysteine proteases involved in apoptosis that are often dysregulated in cancer, have also been identified using an ABP-based competition assay (Berger et al. 2006).

In addition to the discovery of new inhibitors for cancer-related enzymes, ABPs have been applied to the characterization of existing drugs. Proteasome-directed ABPs have been used to evaluate the specificity of bortezomib, a clinically approved proteasome inhibitor for the treatment of multiple myeloma (Altun et al. 2005, Berkers et al. 2005). Myeloma cells were cultured in the presence or

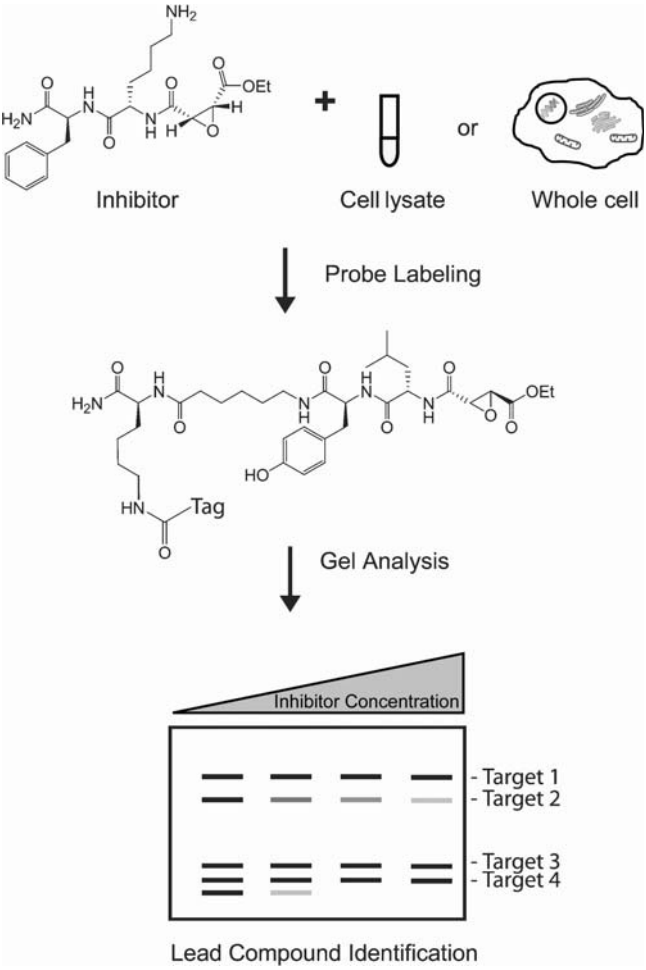


Fig. 7.7 Enzyme inhibitor discovery using an activity-based probe (*ABP*)-based assay. Cell lysates or whole cells are treated with a range of concentrations of an inhibitor. These samples are then reacted with an *ABP* and subjected to gel electrophoresis to separate active enzymes. A decrease in residual activity corresponds to more potent inhibition by the inhibitor. Additionally, the selectivity of the inhibitor for one or multiple enzymes can be determined using this assay

absence of bortezomib, incubated with a cell-permeable, proteasome-specific ABP, lysed, and then analyzed by gel electrophoresis. Results from these experiments revealed that only the activities of the β1/β1i and β5/β5i subunits of the proteasome were inhibited by bortezomib (Altun et al. 2005, Berkers et al. 2005).

ABPs have also been employed for the in vivo evaluation of inhibitors that target enzymes involved in cancer. Kraus and colleagues used a proteasome-directed ABP to identify the active human proteasomal subunits targeted by bortezomib in patients receiving this drug (Kraus et al. 2007). Blood cells obtained from a patient receiving bortezomib monotherapy for multiple myeloma were treated with a proteasome-

specific ABP. Bortezomib treatment was shown to reversibly eliminate both β1 and β5 proteasomal activities and reduce β2 proteasomal activity in human blood cells. Since proteasomal subunits in cancer cells are known to have variable activity, the preferences of bortezomib for certain subunits may explain the differences in patient sensitivity to this cancer drug (Kraus et al. 2007). The in vivo specificity and biodistribution of another proteasome inhibitor, MG262, was monitored in murine tissues using a fluorescently labeled proteasome-specific ABP (Verdoes et al. 2006).

In another example of an application of ABPs to monitor the in vivo potency and selectivity of small molecule inhibitors, the cathepsin-specific ABP DCG-04 was used to evaluate inhibition of cysteine cathepsins in the RIP1-TAG2 transgenic mice, a mouse model of pancreatic cancer (Joyce et al. 2004, Sadaghiani et al. 2007b). In these studies, inhibitors were injected into mice, and normal and tumor tissue samples were collected and analyzed for residual cathepsin activity. The inhibition of these proteases by the cathepsin-specific inhibitor JPM-OEt resulted in a reduction in invasion, angiogenesis, and tumor growth (Joyce et al. 2004). Importantly, fluorescently labeled DCG-04 enabled the biochemical identification and monitoring of the cysteine cathepsins during tumorigenesis in these mice. In a follow-up study, a panel of cathepsin inhibitors was evaluated in this same mouse model using radiolabeled DCG-04 (Sadaghiani et al. 2007b). Inhibitors that had been optimized for selectivity and potency against target proteases in crude tissue extracts were tested for overall potency, biodistribution, and selectivity in vivo. From these studies, a set of inhibitors was identified that showed optimal potency and selectivity in tumor tissues and can be used as lead compounds for cancer therapy. These studies demonstrate that ABPs can be a valuable tool for the analysis of drug specificity and pharmacodynamic properties in vivo.

The substrate-based proteolytic probes have also been used as pharmacodynamic markers to demonstrate efficacy of small molecule protease inhibitors. A NIR FRET substrate-based probe detected proteolytic activity in HT1080 human fibrosarcoma xenografts, giving a fluorescence response that could be inhibited by treatment with the synthetic MMP inhibitor prinomastat (Bremer et al. 2001). Using the dendrimer-based proteolytic beacon PBvisM7, treatment of tumor-bearing mice with the broad-spectrum MMP inhibitor BB-94 resulted in a marked reduction in sensor FL fluorescence to ~40% of that before treatment (McIntyre et al. 2004). Interestingly, this effect was observed only in MMP-7-transfected tumors, with no effect on the FL fluorescence detected over the control tumor, suggesting that the selectivity of the peptide sequence in substrate-based proteolytic probes may be a useful tool in assessing the inhibitory profile of small molecule protease inhibitors in vivo.

Imaging Protease Activity in Tumors

One of the major challenges in cancer diagnosis is the early detection of small primary tumors (Weissleder et al. 1999). Since many enzyme activities are upregulated in tumor cells, probes that report on enzymatic activity represent valuable tools

for early diagnostic imaging strategies (Weissleder et al. 1999, Mahmood and Weissleder 2003, Sloane et al. 2006). In general, optical imaging techniques are used to image protease activity in vivo. The cost, space, and time involved in optical imaging are less demanding compared to other imaging modalities. Furthermore, the advantage of optical imaging methods include the use of nonionizing low-energy radiation, high sensitivity with the possibility of detecting micron-sized objects, and continuous data acquisition in real time and in an intact environment. Optical imaging in the NIR region between 700 and 900 nm has a low absorption by intrinsic photoactive biomolecules and allows light to penetrate several centimeters into the tissue, a depth that is sufficient to image practically all small animals (Zuzak et al. 2002). Imaging in the NIR region has less tissue autofluorescence, markedly improving the target/background ratio as compared with the visible region of the spectrum (Rudin and Weissleder 2003). The detection sensitivity depends both on selection of the fluoroscent probe and optimization of imaging geometry for detection with a highly sensitive charge-coupled device (CCD) camera. These kinds of optical imaging systems are capable of detecting a small number of photons that are transmitted through living tissues permitting real-time images to be collected within a few seconds. A fast and relatively easy imaging procedure makes this modality attractive for potential clinical use. Fluorescence-mediated tomography (FMT) has recently been shown to three-dimensionally localize and quantify fluorescent probes in deep tissues at high sensitivity (Ntziachristos et al. 2002).

Current methods for imaging enzymes mainly rely on antibody labeling or on substrates that become fluorescent after enzyme cleavage (Baruch et al. 2004, Sloane et al. 2006). Although antibodies are specific for their enzyme targets, they are not cell permeable and do not give information about enzyme activity. Fluorescent substrates are useful for the activity-based imaging of proteases; however, these compounds often suffer from a lack of specificity, leading to cleavage by multiple classes of proteases (Baruch et al. 2004, Sloane et al. 2006). Furthermore, there is no way to determine which protease is responsible for substrate processing in vivo using fluorescent substrate reporters. In contrast, ABPs covalently bind to active enzymes, thus permitting assignment of imaging signals to specific enzymes (Fig. 7.8). In fact, a number of ABPs that target cysteine proteases have been used to image enzyme activity in tumor cells both in vitro and in vivo (Joyce et al. 2004, Blum et al. 2005, 2007).

A fluorescently tagged DCG-04 analogue has been used to image cysteine cathepsin activity during tumorigenesis in RIP1-TAG2 transgenic mice (Joyce et al. 2004). In this study, the ABP was administered systemically by intravenous injection into a mouse. After allowing the probe to circulate for several hours, pancreatic tumor tissue was collected and imaged using fluorescent microscopy. Cathepsin activity was found to be elevated in tumors and at the invasive edges of islet carcinomas. After imaging, tumor tissues were lysed and analyzed by gel electrophoresis, providing an activity profile that could be used to identify and quantify the levels of probe-modified cathepsins that produced the fluorescent signals. Additionally, this ABP was applied to the imaging of cathepsin activity

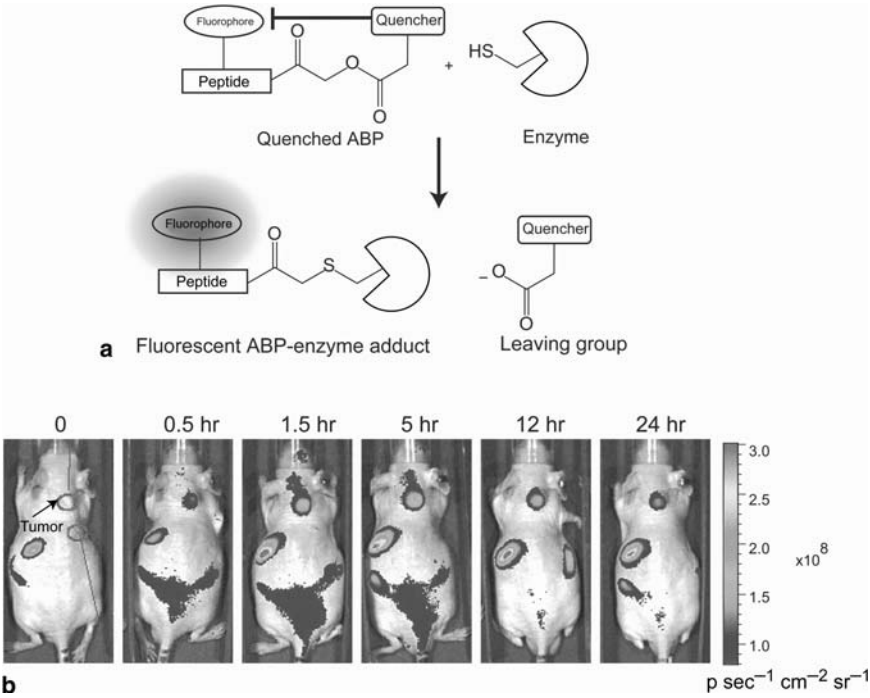


Fig. 7.8 Quenched activity-based probes (*ABPs*) for the noninvasive imaging of tumors in vivo. (**a**) Covalent labeling of a cysteine protease target by a quenched *ABP*. Activity-based labeling of the target enzyme results in the loss of the quenching group and subsequent generation of a fluorescently-labeled enzyme. (**b**) Optical imaging of MDA-MB-231 breast cancer xenograft tumors in nude mice using a quenched cysteine cathepsin-specific *ABP*. The quenched probe was injected intravenously, and fluorescent images of the mice were taken at various time points after injection. Images taken from Blum et al. (2007). (*See also* Color Insert I)

in a mouse model of cervical carcinogenesis (K14-HPV/E_2 mice) (Joyce et al. 2004). Cathepsin activity levels were also elevated in cervical tumor tissues, further confirming that cysteine cathepsin activity can serve as a useful cancer biomarker and that cathepsin-specific ABPs have potential value as imaging agents to monitor tumor progression in whole animals.

In a recent advance, a cathepsin-specific ABP that becomes fluorescent only upon binding to its enzyme target has been developed (Blum et al. 2005). Since tagged ABPs used in imaging are constitutively fluorescent, they generate a high nonspecific fluorescent background when used in living cells. The newly designed quenched ABP (qABP) makes use of the acyloxy leaving group found on the acyloxymethyl ketone warhead. By attaching a fluorescent quencher, the probe is rendered nonfluorescent when free in solution. Covalent modification of cysteine cathepsins by this probe liberates the quencher moiety, and the probe becomes fluorescent. This cell-permeable, quenched probe has been used to image cathepsin activity levels in both the murine fibroblast cell line NIH-3T3 and the human MCF-10A breast cancer cell line

(Blum et al. 2005). Cells treated with the qABP showed distinct punctuate fluorescent staining of lysosomal compartments, whereas the unquenched control probe produced bright, nonspecific intracellular fluorescence that required extensive washing to reveal specific target labeling. Importantly, the fluorescent signals could be specifically blocked by pretreatment of cells with a general cysteine protease inhibitor.

In a follow-up study, Blum et al. used a related series of quenched and nonquenched ABPs to noninvasively image cathepsin activity in a xenografted mouse model of breast cancer (Blum et al. 2007). NIR-labeled versions of the cysteine cathepsin probes produced spatially resolvable fluorescence in the tumor tissues of live mice that correlated with the levels of active cathepsins in those tissues (Fig. 7.8). Both quenched and nonquenched ABPs were able to selectively label tumor tissue and had similar signal-to-background ratios; however, the quenched probe achieved its maximum signal-to-background ratio much more rapidly than the nonquenched probe. Ex vivo analysis of tumor tissues from these mice further confirmed that the signals observed in the live animals were due to specific probe labeling of active cathepsins.

The substrate-based PBs selective for MMP-7, PBvisM7, and PBnirM7, have been used to detect MMP-7 activity in xenograft tumors in mice. In these studies, pairs of xenograft tumors were established on the rear flanks of each animal; one tumor with human colorectal tumor cells that express several MMP family members but do not express detectable amounts of endogenous MMP-7, and a second with the same cells transfected with an MMP-7 expression vector (Witty et al. 1994). Imaging was achieved following intravenous injection of a single bolus of either PBvisM7 or PBnirM7. Approximately 2–4 h following PB injection, the reference (*R*) signal was low in both the control and MMP-7-transfected tumors while the sensor (*S*) channel showed an approximately tenfold difference between the control and MMP-7-expressing tumors with a comparable difference in sensor/reference (*S*/*R*) ratio (Scherer et al. 2008). The second generation PBnirM7 has been used to detect intestinal adenomas in the multiple intestinal neoplasia (Min) mouse model of familial polyposis (Scherer et al. 2008). In those studies, the animals were sacrificed post intravenous administration of PBnirM7 revealing enhanced fluorescence of the MMP-7 sensor associated with a number of adenomas in the intestinal tract examined ex vivo (Fig. 7.9). The S/R ratio was consistently and significantly higher in Min adenomas compared to normal intestinal tissue of mice lacking the Min mutation, and in Min adenomas from MMP-7-null mice. Similar studies by the Weissleder group also demonstrated the detection of adenoma-associated protease activity in the intestines of Min mice (Marten et al. 2002). Taken together, these fluorescence imaging studies in living mice indicate that PB-M7s can be used to detect and selectively image MMP-7 activity in vivo due to the enhanced fluorescence of the sensor in the proteolyzed reagent that results in an increase in S/R ratio. The optical imaging approach using new optical reporters has the potential for highly sensitive, noninvasive, in vivo detection and imaging of tumor-associated proteolytic activity.

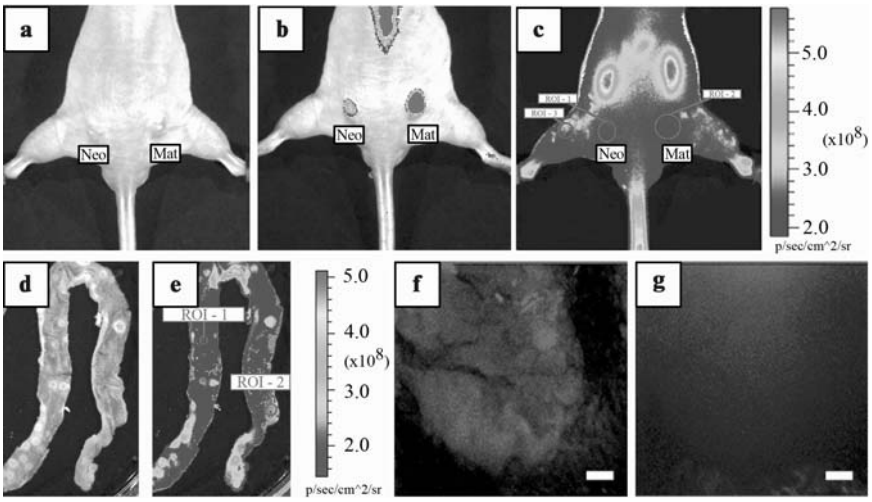


Fig. 7.9 In vivo imaging with substrate-based proteolytic beacon PB-M7NIR. (**a–c**) In vivo imaging of mouse subcutaneous xenograft tumors with PB-M7NIR. Dorsal, caudal view of a nude mouse of 4 weeks following subcutaneous injection of SW480neo (Neo) or MMP-7-expressing SW480mat (Mat) cells. Tumor areas (~57 mm^2 each) are shown in white light (**a**), the Cy5.5 sensor channel (**b**), and the reference channels (**c**) 4 h post retro-orbital intravenous injection of 1.0 nmol PB-M7NIR. Encircled areas represent regions of interest for quantitative assessment. Note accumulation of PB-M7NIR in the kidneys of the mouse as detected with the reference channel (**c**), but selective accumulation of sensor signal in the Mat tumor indicative of proteolytic activity (**b**). Sensor signal on the spine and tail are presumed to be due to low levels of circulating, activated probe that become detectable when they are close to the surface of the mouse. (**d–g**) Ex vivo imaging of PB-M7NIR in APC^{MIN} intestinal adenomas. Explanted mouse intestine from an APC^{MIN} mouse with spontaneous polyps in 60 min. Post-injection of 1 nmol of PB-M7NIR Beacon. (**d**) White light image and (**e**) NIRF image in the Cy5.5 (sensor) channel. (**e–f**). High power images of a single, intact adenoma (10×-objective) from an APC^{Min} mouse (**e**). False-red coloring in the Cy5.5 (sensor) channel (**f**) Cy7 (reference-green) channel. White line = 100 microns. Adapted fr om Scherer et al. (2008). *ROI*, region of interest. (*See also* Color Insert I)

Conclusion and Future Directions

Over the last several years, the field of activity-based proteomics has produced a wealth of new technologies for the direct biological study of enzymes. Protease probes that monitor the activity of numerous diverse enzyme classes have been synthesized, and these probes have been applied to many biologically and pathologically relevant fields. Additionally, a number of new tools, including gel-free screening systems and quenched probes, have been developed that allow rapid identification and visualization of enzyme activity in vitro and in vivo. Both ABPs and substrate-based imaging probes have been applied to the identification and evaluation of potential enzyme inhibitors in the physiologically relevant environments of a complex proteome, cell, or even whole animal. However, challenges in the field of activity-based proteomics still remain to be addressed. In order to identify new probe scaffolds that allow for greater

proteomic coverage by ABPs, structurally diverse probe libraries need to be developed. Furthermore, advances in gel-free analysis systems will be required to profile proteins with low activities or abundances and to rapidly identify large numbers of proteins targeted by ABPs. Perhaps the most important challenge facing activity-based proteomics is the need to combine the data from activity-based assays with relevant biological experiments to gain a more complete understanding of enzyme function in cancer and other biological processes and diseases. The continued use of optical imaging of proteolytic activity has exciting potential both for the understanding of cancer and in applications to cancer detection, diagnosis, and treatment. For preclinical studies, extension into the use of multiphoton fluorescence microscopy for intravital imaging of protease activities should facilitate the further delineation of specific roles of proteases in processes critical to tumor progression. New developments in NIR optical tomography research (Nioka and Chance 2005, Ntziachristos et al. 2005) are yielding promising optical approaches for imaging in clinical practice, particularly as a complementary modality for breast cancer detection (Chance et al. 2005, Zhu et al. 2005). The development of new kinds of targeted optical reagents, including those providing for both imaging and therapy (Chen et al. 2005, Zheng et al. 2007), will likely provide new paradigms for the clinician. Noninvasive imaging techniques for proteolytic activity provide an extraordinary opportunity to increase the sensitivity of detecting early-stage tumors and to identify tumors that require particularly aggressive therapy. With time and the rapid advance in technology, we are likely to see a sharp increase in the number and types of applications of protease probes to oncology.

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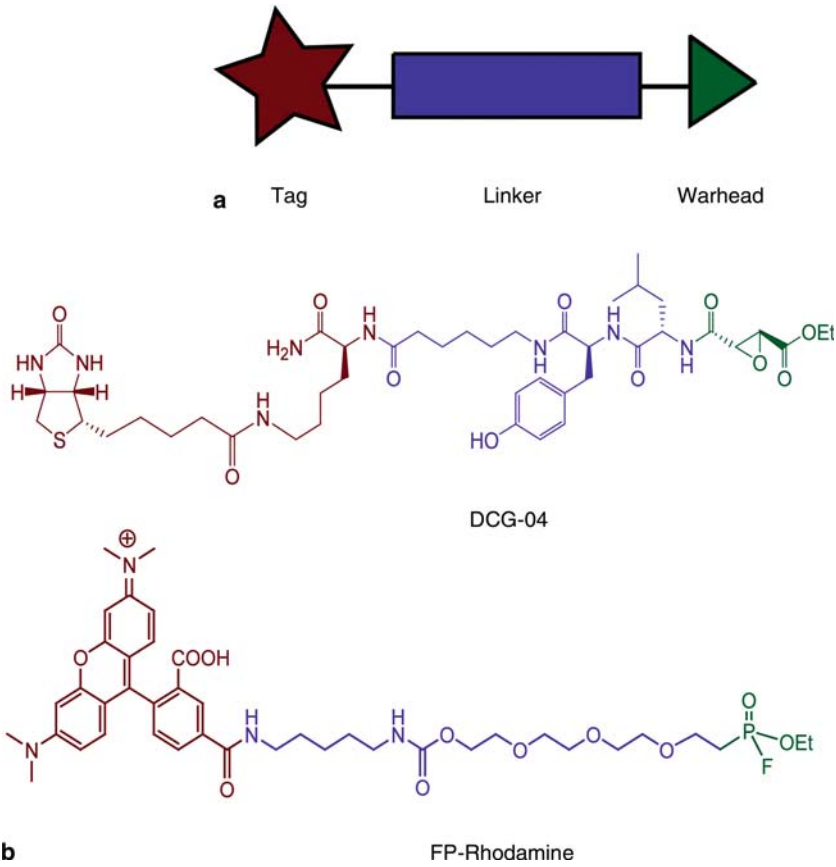

Fig. 7.1 General and specific structures of activity-based probes (*ABPs*). (**a**) General structure of an *ABP*. (**b**) DCG-04 is an *ABP* that targets cysteine proteases and contains a biotin tag (red), a dipeptide-containing linker (blue), and an epoxide as a warhead (green). Fluorescent protein (*FP*)-rhodamine is an *ABP* that targets the serine hydrolase superfamily of enzymes and contains a rhodamine fluorophore (red), a poly (ethylene glycol) linker (blue), and a fluorophosphonate as a warhead (green).

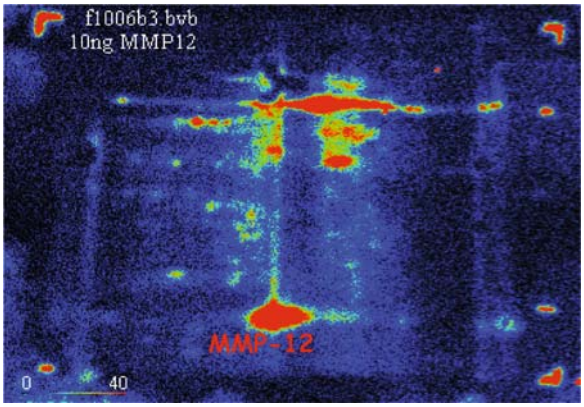

Fig. 7.4 Labeling of mice tumor extract (colon carcinoma) by the phosphinic probe displayed in scheme 2. Before photoactivation, 0.5 pmol of h-matrix metalloprotease (*MMP*)-12 were added to the sample as an internal standard for protein quantification. The sample was analyzed by 2D sodium dodecyl sulfate–polyacrylamide gel electrophoresis (*SDS–PAGE)* followed by detection.

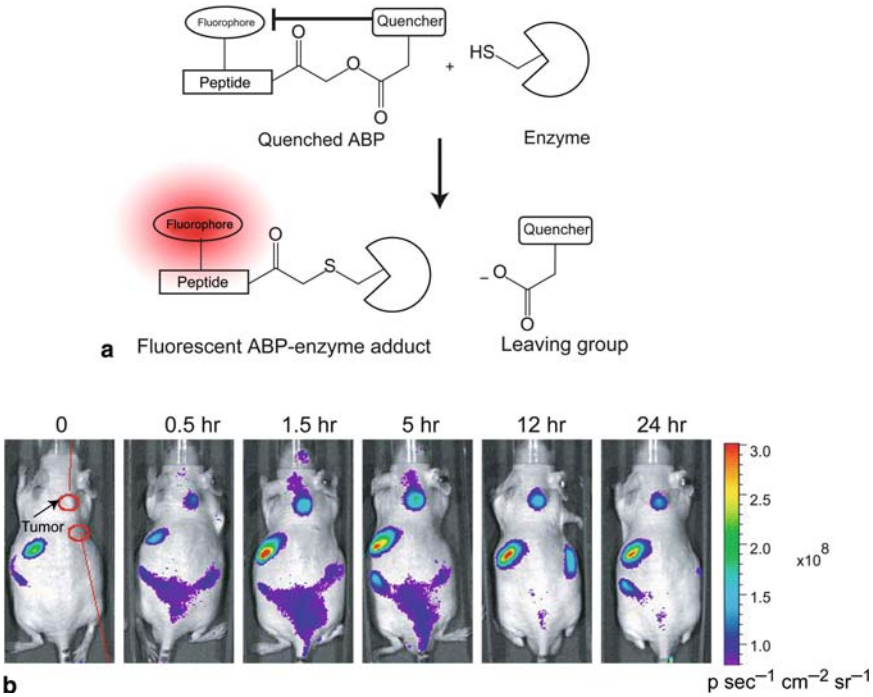

Fig. 7.8 Quenched activity-based probes (*ABPs*) for the noninvasive imaging of tumors in vivo. (**a**) Covalent labeling of a cysteine protease target by a quenched *ABP* (GB137). Activity-based labeling of the target enzyme results in the loss of the quenching group and subsequent generation of a fluorescently labeled enzyme. (**b**) Optical imaging of MDA-MB-231 breast cancer xenograft tumors in nude mice using a quenched cysteine cathepsin-specific *ABP*. The quenched probe was injected intravenously and fluorescent images of the mice were taken at various time points after injection. Images taken from Blum et al. (2007).

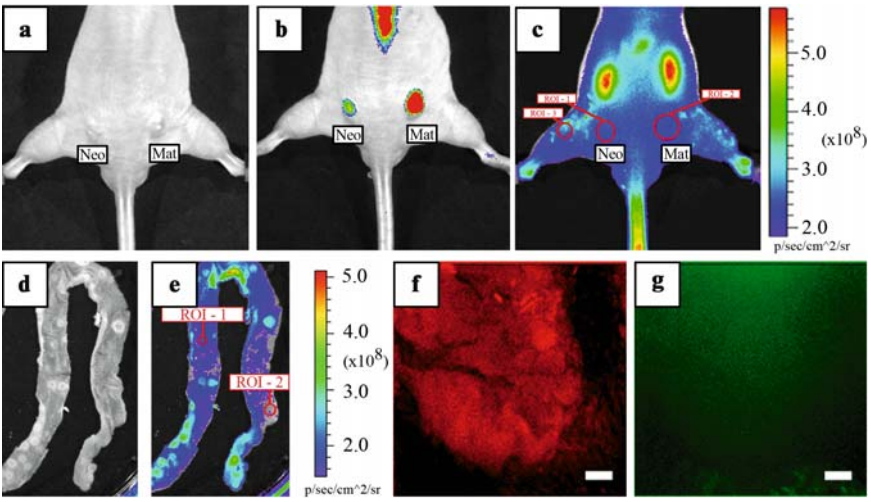

Fig. 7.9 In vivo imaging with substrate-based proteolytic beacon PB-M7NIR. (**a–c**) In vivo imaging of mouse subcutaneous xenograft tumors with PB-M7NIR. Dorsal, caudal view of a nude mouse of 4 weeks following subcutaneous injection of SW480neo (Neo) or MMP-7-expressing SW480mat (Mat) cells. Tumor areas (~57 mm^2 each) are shown in white light (**a**), the Cy5.5 sensor channel (**b**), and the reference channels (**c**) 4 h post retro-orbital intravenous injection of 1.0 nmol PB-M7NIR. Encircled areas represent regions of interest for quantitative assessment. Note accumulation of PB-M7NIR in the kidneys of the mouse as detected with the reference channel (**c**), but selective accumulation of sensor signal in the Mat tumor indicative of proteolytic activity (**b**). Sensor signal on the spine and tail are presumed to be due to low levels of circulating, activated probe that become detectable when they are close to the surface of the mouse. (**d–g**) Ex vivo imaging of PB-M7NIR in APC^{MIN} intestinal adenomas. Explanted mouse intestine from an APC^{MIN} mouse with spontaneous polyps in 60 min. Post-injection of 1 nmol of PB-M7NIR Beacon. (**d**) White light image and (**e**) NIRF image in the Cy5.5 (sensor) channel. (**e–f**). High power images of a single, intact adenoma (10×-objective) from an APC^{Min} mouse (**e**). False-red coloring in the Cy5.5 (sensor) channel (**f**) Cy7 (reference-green) channel. White line = 100 microns. Adapted fr om Scherer et al. (2008). *ROI*, region of interest.