

Activity-based protein profiling for biochemical pathway discovery in cancer

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Abstract | Large-scale profiling methods have uncovered numerous gene and protein expression changes that correlate with tumorigenesis. However, determining the relevance of these expression changes and which biochemical pathways they affect has been hindered by our incomplete understanding of the proteome and its myriad functions and modes of regulation. Activity-based profiling platforms enable both the discovery of cancer-relevant enzymes and selective pharmacological probes to perturb and characterize these proteins in tumour cells. When integrated with other large-scale profiling methods, activity-based proteomics can provide insight into the metabolic and signalling pathways that support cancer pathogenesis and illuminate new strategies for disease diagnosis and treatment.

Bio-orthogonal chemical handle

A chemical handle that can be specifically modified with reporter tags within the confines of a biological environment.

Click chemistry

Chemistry that allows for quick and reliable joining of small units; the most commonly used click-chemistry reaction is the Huisgen azide-alkyne cycloaddition using a copper catalyst.

Cells with fundamental metabolic alterations commonly arise during tumorigenesis, and it is these types of changes that help to establish a biochemical foundation for disease progression and malignancy^{1,2}. A seminal example of this was discovered in the 1920s when Otto Warburg found that cancer cells consume higher levels of glucose and secrete most of the glucose carbon as lactate rather than oxidizing it completely^{3,4}. Since then, studies by multiple groups have uncovered a diverse array of metabolic changes in cancer, including alterations in glycolytic pathways^{3–6}, the citric acid cycle⁷, glutaminolysis^{8,9}, lipogenesis¹⁰, lipolysis¹¹ and proteolysis¹². These in turn modulate the levels of cellular building blocks (lipids, nucleic acids and amino acids), cellular energetics, oncogenic signalling molecules and the extracellular environment to confer pro-tumorigenic and malignant properties.

Despite these advances, our current understanding of cancer metabolism is far from complete and would probably benefit from experimental strategies that are capable of profiling enzymatic pathways on a global scale. To this end, conventional genomic^{13,14} and proteomic^{15–18} methods, which comparatively quantify the expression levels of transcripts and proteins, respectively, have yielded many useful insights. These platforms are, however, limited in their capacity to identify changes in protein activity that are caused by post-translational mechanisms¹⁹. Annotating biochemical pathways in cancer is further complicated by the potential for enzymes to carry out distinct metabolic activities in tumour cells that might not be mirrored in normal physiology. In addition, a substantial

proportion of the human proteome remains functionally uncharacterized, and it is likely that at least some of these poorly understood proteins also have roles in tumorigenesis. These challenges require new proteomic technologies that can accelerate the assignment of protein function in complex biological systems, such as cancer cells and tumours. In this Review, we discuss one such proteomic platform, termed activity-based protein profiling (ABPP)^{20–22}, and its implementation in the discovery and functional characterization of deregulated enzymatic pathways in cancer. We discuss the evidence that, when coupled with other large-scale profiling methods, such as metabolomics^{23,24} and proteomics^{15–18}, ABPP can provide a compelling, systems-level understanding of biochemical networks that are important for the development and progression of cancer.

ABPP for enzyme discovery in cancer

ABPP uses active site-directed chemical probes to directly assess the functional state of large numbers of enzymes in native biological samples (FIG. 1). Activity-based probes consist of at least two key elements: a reactive group for binding and covalently labelling the active sites of many members of a given enzyme class (or classes), and a reporter tag for the detection, enrichment and identification of probe-labelled enzymes in proteomes. Activity-based probes can be adapted for *in situ* or *in vivo* labelling by substituting the reporter tag with a bio-orthogonal chemical handle, such as an alkyne. Probe-labelled enzymes are then detected by subsequent click chemistry conjugation

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At a glance

- Activity-based protein profiling (ABPP) facilitates the discovery of deregulated enzymes in cancer.
- Competitive ABPP yields selective inhibitors for functional characterization of cancer enzymes.
- ABPP can be integrated with metabolomics to map deregulated enzymatic pathways in cancer.
- ABPP can be integrated with other proteomic methods to map proteolytic pathways in cancer.
- ABPP probes can be used to image tumour development in living animals.

to various azide-modified reporter tags^{25,26}. There are currently activity-based probes for a multitude of enzyme classes, including many that have central roles in cancer, such as hydrolases and proteases^{20,27–34}, kinases^{35–38}, phosphatases³⁹, histone deacetylases^{40,41}, glycosidases^{42,43} and various oxidoreductases^{44,45}. ABPP can be applied to virtually any cell or tissue (assuming that the genome of the parental organism has been sequenced) and can be combined with a range of analytical methods for data acquisition, including gel- and mass spectrometry (MS)-based methods²¹. Although the specificity of ABPP probes is not absolute, and these probes can be toxic and disrupt biochemical pathways when applied to living systems, they are of great value for characterizing deregulated enzymatic activities in various cancer models and specimens, as discussed below. Examples of activity-based probes that have been used in cancer studies are provided in TABLE 1.

Serine hydrolases are one of the largest and most diverse enzyme classes in mammalian proteomes and include esterases, thioesterases, lipases, amidases and proteases⁴⁶. Several serine hydrolases have been implicated in tumorigenesis, including fatty acid synthase¹⁰, protein methyl esterase 1 (REF. 47), and urokinase-type (uPA) and tissue-type (tPA) plasminogen activators⁴⁸. Fluorophosphonate probes that target the serine hydrolase superfamily^{20,27,28} (TABLE 1) have been used to discover several deregulated enzymes in cancer^{49–52}. Using ABPP, we discovered that two serine hydrolases — the uncharacterized enzyme KIAA1363 (REFS 49,52,53) and monoacylglycerol (MAG) lipase (MAGL)¹¹ — are highly expressed in aggressive human cancer cells and primary tumours. We also used ABPP to develop selective inhibitors of KIAA1363 and MAGL for the functional characterization of these enzymes in cancer (discussed below; FIG. 2). Using ABPP, Shields and colleagues⁵⁴ recently determined that the activity of the serine hydrolase retinoblastoma-binding protein 9 (RBBP9) is increased in pancreatic carcinomas, in which it promotes anchorage-independent growth and pancreatic carcinogenesis through overcoming transforming growth factor- β (TGF β)-mediated antiproliferative signalling by reducing the phosphorylation levels of SMAD2 and SMAD3.

ABPP has also contributed to our knowledge of serine protease activities in cancer. In the course of characterizing an *in vivo*-derived variant of the human breast cancer line MDA-MB-231, termed 231MFP cells, we determined that these cells possess increased uPA and tPA activity in their secreted proteome⁵¹. These cells also show increased

tumour growth *in vivo*, suggesting that deregulated proteolytic activity could contribute to their increased pathogenicity. Madsen and colleagues⁵⁵ compared serine hydrolase activities in high and low intravasating variants of the human fibrosarcoma HT-1080 cell line and found increased uPA activity in the high-intravasating variant, in which the protease controlled tumour cell intravasation. Interestingly, in these examples, alterations in protease activity occurred without significant changes in mRNA⁵¹ or protein⁵⁵ expression.

Interrogating the activities of other protease families has also provided new insights into deregulated proteolytic processes in cancer. Using epoxide-electrophile probes for cysteine proteases⁵⁶ (TABLE 1), Joyce and colleagues⁵⁷ found that cathepsin activity is higher in angiogenic vasculature and at the invasive fronts of carcinomas, and that pharmacological ablation of a wide range of cathepsins impaired the angiogenic switch, tumour growth, vascularity and invasiveness. They also found that cysteine cathepsins are increased in human papilloma virus (HPV)-induced cervical carcinomas⁵⁷. Profiling metalloproteinase activities with photoreactive, hydroxamate activity-based probes (TABLE 1) has uncovered neprilysin as a membrane-associated glycoprotein that has increased activity in aggressive human melanoma lines compared with less-aggressive counterparts³¹. Comparison of ubiquitin-specific protease activities using the haemagglutinin-tagged ubiquitin-vinyl methyl ester probe (TABLE 1) revealed that the deubiquitylases ubiquitin-carboxyl esterase-L3 (UCHL3) and UCH37 were upregulated in HPV-positive tumours compared with adjacent normal cervical tissue⁵⁸. Comparison of a heptaoma cell line that expressed a stably replicating hepatitis C virus subgenomic replicon RNA with the parental cell line using ABPP probes composed of an *N*-acetylated amino acid that mimicked the P1 position in the peptide substrates of the protease, identified several differentially regulated enzyme activities, some of which were decreased or increased during HCV replication⁵⁹.

ABPP has also been used for imaging enzyme activities. Bogoy and colleagues⁶⁰ have introduced quenched near-infrared fluorescent activity-based probes (qNIRF-ABPs) to image cysteine protease activities in tumour xenografts *in vivo* and *ex vivo*. These probes emit a fluorescent signal only after covalently modifying a specific protease target, and they can also be used to monitor small-molecule inhibition of protease targets both biochemically and by direct imaging methods. The same researchers have implemented a similar approach using a highly selective aza-peptidyl asparadiny epoxide qNIRF-ABP⁶¹ to target a specific lysosomal cysteine protease, legumain, the expression of which is increased in many human cancers. In another study, mice were treated with dexamethasone, which induced apoptosis and caspase activation in the thymus, both of which were visualized *in vivo* using a caspase-directed activity-based probe⁶² (TABLE 1). This probe could also detect apoptosis that was induced by the monoclonal antibody Apomab in mice bearing xenografted human colorectal tumours⁶². Furthermore, Blum and colleagues⁶³ demonstrated that among contrast agents for protease activities (small peptides,

Intravasating

A process in cancer metastasis in which the cancer cells invade through the basement membrane into blood vessels.

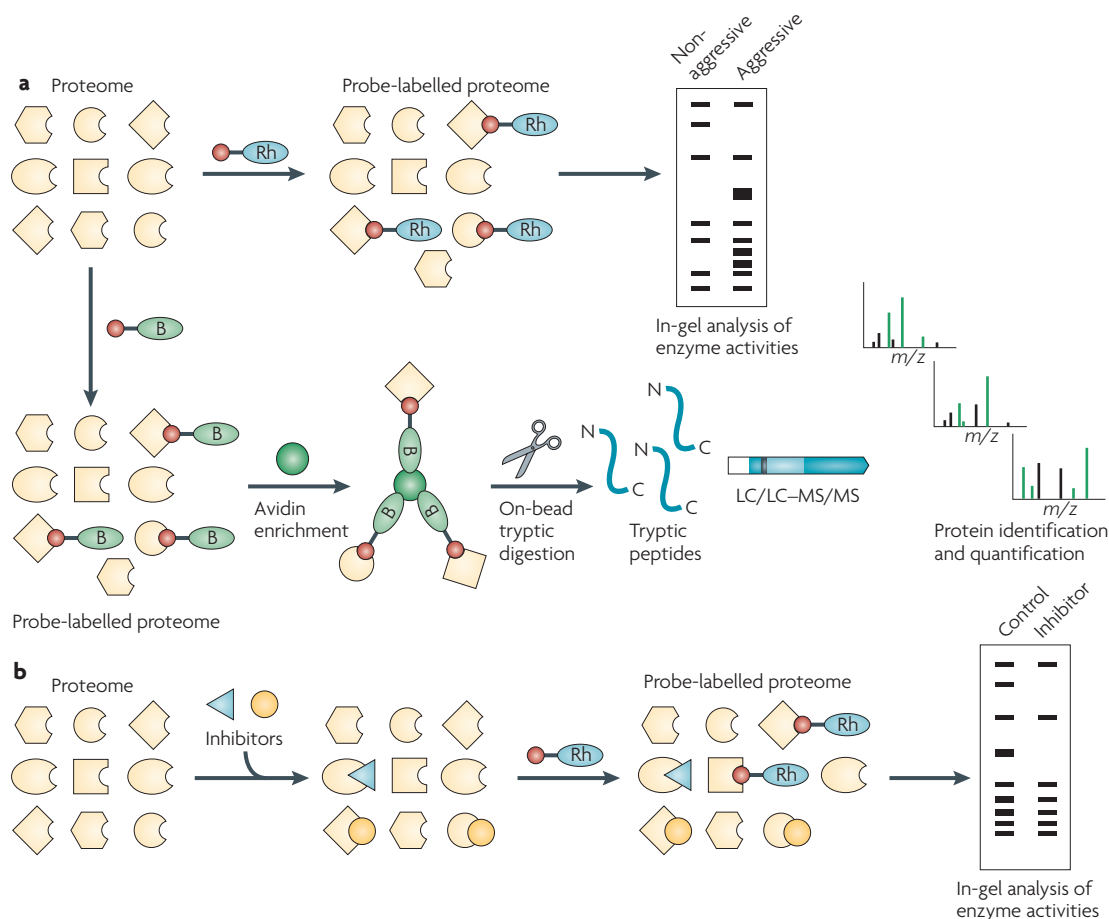


Figure 1 | Activity-based protein profiling. **a** | Activity-based protein profiling (ABPP) uses active site-directed chemical probes to assess the functional state of large numbers of enzymes in native biological systems. Activity-based probes consist of a reactive group (red ball) for targeting a specific set of enzymes and a detection handle (a fluorophore, such as a rhodamine (Rh) or biotin (B)). In a typical ABPP experiment, a proteome is reacted with the activity-based probe and probe-labelled proteins detected by either in-gel fluorescence scanning (for fluorophore-conjugated probes; top) or avidin enrichment, on-bead tryptic digest and liquid chromatography–mass spectrometry (LC–MS) analysis (for biotinylated probes; bottom). **b** | ABPP can also be used in a competitive format to evaluate the potency and selectivity of enzyme inhibitors in native biological samples. Inhibitors compete with activity-based probes for enzyme targets, and this competition is read out by loss of fluorescence (for fluorophore-conjugated probes) or MS (for biotinylated probes) signals (not shown). m/z , mass to charge ratio.

large polymer-based quenched fluorescent substrates and fluorescently labelled ABPP probes), fluorescent ABPP probes showed more rapid and selective uptake into tumours and overall brighter signal compared with substrate-based probes. These approaches can potentially be used in the clinic to define tumour margins, diagnose tumour grade and assess drug-target occupancy *in vivo*.

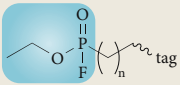
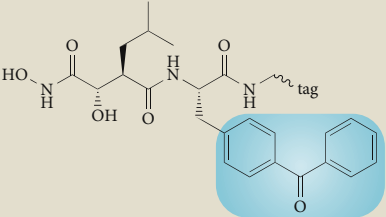
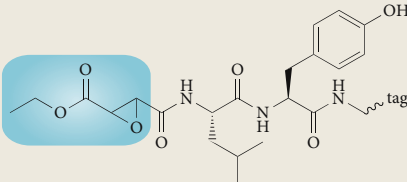
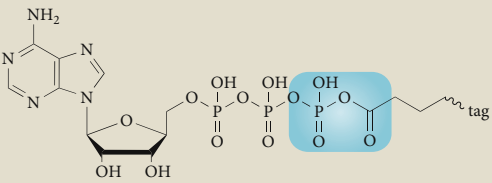
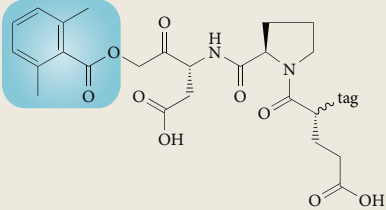
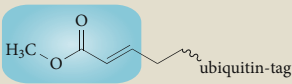
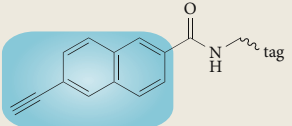
ABPP for inhibitor discovery in cancer

Because activity-based probes label the active sites of their enzyme targets, they can form the basis for a competitive screen for enzyme inhibitors^{27,64,65}. This competitive ABPP platform has several advantages compared with conventional substrate assays, as inhibitor screens can be conducted directly in complex proteomes and allow concurrent optimization of potency and selectivity against many enzymes in parallel (FIG. 1). Inhibitors can also be developed for uncharacterized enzymes that lack known substrates^{53,66}. Beyond its important role in inhibitor

discovery, competitive ABPP has been used to identify drug targets and off-targets in preclinical or clinical development to gauge mechanism of action and safety^{31,37,67,68}.

Competitive ABPP has served as a principal assay for screening directed libraries of inhibitors and for optimizing their selectivity against serine hydrolases that are expressed in cancer cell and tissue proteomes. This effort has led to the identification of two carbamate agents, AS115 (REF. 53) and JZL184 (REF. 69), which are potent and selective inhibitors of KIAA1363 and MAGL, respectively. Competitive ABPP has also been used to explore the full target profile for anti-cancer drugs. Profiling cytochrome P450 enzymes with clickable aryl-alkyne probes (TABLE 1) showed that the aromatase inhibitor anastrozole, which is approved for breast cancer therapy, significantly increases the activity (as determined by probe labelling) of CYP1A2 and decreases the activity of CYP2C19 (REF. 68). These results indicate that anastrozole interacts with multiple P450 enzymes and, in at least one case (CYP1A2), might cooperatively

Table 1 | Representative activity-based probes and their application to cancer research

Structure*	Enzyme class	Applications in cancer
	Serine hydrolases	Identified increased KIAA1363 (REFS 49,52,53) and MAGL ¹¹ activities in aggressive human cancer lines ^{11,52} and primary tumours ^{11,49} . Identified increased uPA and tPA serine protease activities in secreted proteomes of aggressive cancer cells ^{51,52} . Identified increased RBBP9 activity in pancreatic carcinomas ⁵⁴
	Metalloproteinases	Identified increased neprilysin activity in aggressive melanoma cell lines ³¹
	Cysteine proteases	Identified increased cathepsin cysteine protease activities in pancreatic islet tumours ⁵⁷ . Used for <i>in vivo</i> imaging of tumour cathepsin activity ^{57,60}
	Kinases	Inhibitor selectivity profiling of kinase inhibitors ^{35,71}
	Caspases	<i>In vivo</i> and <i>ex vivo</i> visualization of apoptosis in colon tumour-bearing mice treated with Apomab ⁶²
	Deubiquitylases	Identified increased carboxy-terminal hydrolase UCHL3 and UCH37 activity in HPV cervical carcinomas ⁵⁸
	Cytochrome P450s	Identified the aromatase inhibitor anastrozole as an inducer of CYP1A2 activity ⁶⁸

HPV, human papilloma virus; tPA, tissue plasminogen activator; uPA, urinary plasminogen activator. *Blue boxes around structures represent the portion of the activity-based probes that react with the active sites of enzymes.

enhance the binding of other drugs. Characterization of the matrix metalloproteinase (MMP)-directed inhibitor GM6001 (ilomastat) by competitive ABPP revealed that this agent also inhibits several metalloproteinases outside of the MMP family³¹, which could explain some of the toxicity issues that broad-spectrum MMP inhibitors have confronted in clinical development⁷⁰. Kinase-directed activity-based probes (TABLE 1) have been used to identify 39 kinase targets of the broad-spectrum inhibitor staurosporine in cancer cell lines³⁵. Activity-based

probes based on the structure of the PI3K inhibitor wortmannin^{37,38}, revealed that this natural product also targets members of the polo-like clan of kinases^{37,71}. The integration of competitive ABPP platforms into the pre-clinical and clinical development of cancer therapeutics has the potential to clarify the mechanism of action and reduce off-target toxicity for future drug candidates.

Finally, we should note that competitive ABPP experiments have historically been analysed using one-dimensional (1D) SDS-PAGE or MS, which are

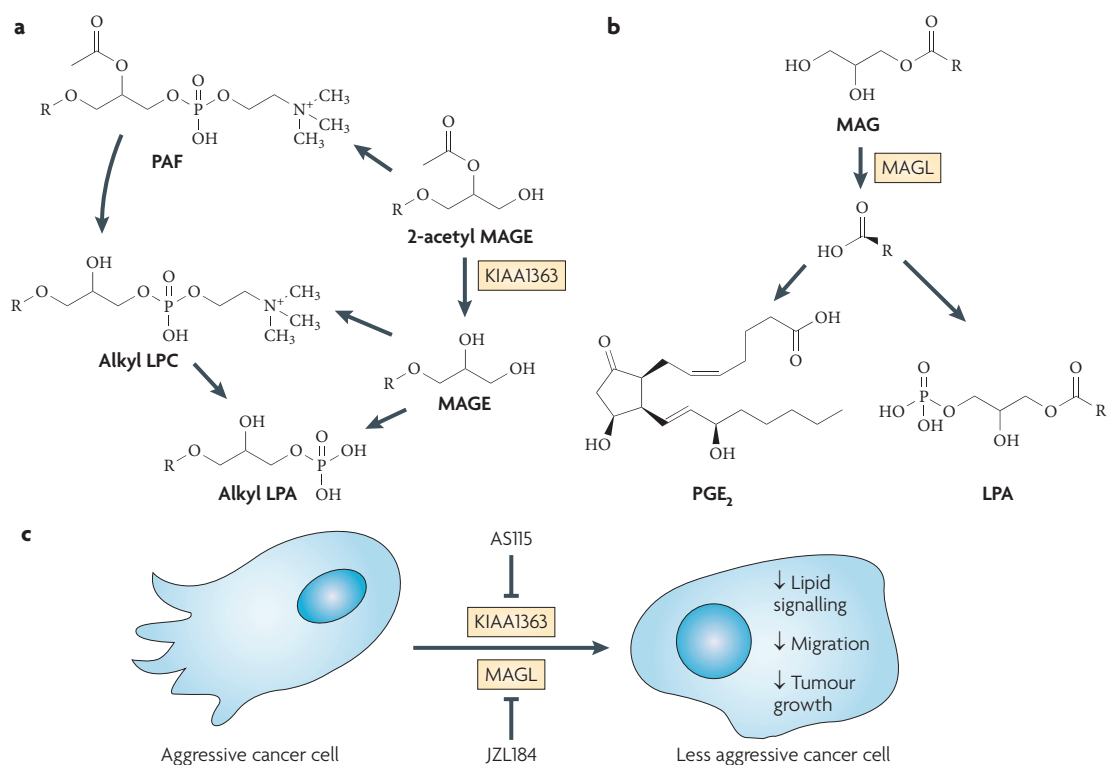


Figure 2 | Serine hydrolases KIAA1363 and MAGL regulate lipid metabolic pathways that support cancer pathogenesis. Activity-based protein profiling (ABPP) identified KIAA1363 (part **a**) and monoacylglycerol (MAG) lipase (MAGL) (part **b**) as being increased in aggressive human cancer cells from multiple tumour types. Pharmacological and/or RNA interference ablation of KIAA1363 and MAGL coupled with metabolomic analysis revealed specific roles for KIAA1363 and MAGL in cancer metabolism. Disruption of KIAA1363 by the small-molecule inhibitor AS115 lowered monoalkylglycerol ether (MAGE), alkyl lysophosphatidic acid (alkyl LPA) and alkyl lysophosphatidyl choline (alkyl LPC) levels in cancer cells. Disruption of MAGL by the small-molecule inhibitor JZL184 raised MAG levels and reduced free fatty acid, lysophosphatidic acid (LPA) and prostaglandin E₂ (PGE₂) levels in cancer cells. Disruption of KIAA1363 and MAGL leads to impairments in cancer cell aggressiveness and tumour growth (part **c**). PAF, platelet-activating factor.

limited in throughput to compound libraries of a modest size (200–300 compounds). This limitation has recently been addressed, at least in part, by coupling competitive ABPP with fluorescence polarization (fluopol-ABPP)⁷², which provides a homogeneous assay that is compatible with high-throughput screening and which can be adapted to different classes of enzymes and activity-based probes. Fluopol-ABPP has been successfully used to discover selective inhibitors for two cancer-related enzyme targets, the hydrolytic enzyme RBPP9 and the thioltransferase glutathione S-transferase omega 1 (GSTO1)^{72,73}.

Integration of ABPP with other profiling methods

Integration of ABPP with metabolomics. Metabolomics has emerged as a powerful method for broadly assessing the biochemical functions of enzymatic pathways in normal physiology and disease^{23,24} (BOX 1). When complemented with selective inhibitors developed through competitive ABPP, metabolomics can be used to not only identify endogenous substrates and products of enzymes, but also metabolites that are upstream or downstream of these immediate substrates and products, allowing the integration of individual enzymatic reactions into the larger metabolic networks of cancer cells. Two examples

of how coupling of ABPP with metabolomics has helped in defining contributions made by enzymes in cancer are discussed below.

A role for KIAA1363 in regulating pro-tumorigenic ether lipids. As mentioned above, ABPP studies identified increased KIAA1363 activity in both aggressive human cancer cell lines⁵³ and primary tumours⁴⁹, and have identified AS115 as a potent and selective inhibitor of this enzyme⁵³. Untargeted liquid chromatography (LC)–MS analysis of lipophilic metabolites from AS115-treated cancer cells revealed that KIA1363 regulates an unusual class of lipids — the monoalkylglycerol ethers (MAGEs)¹¹. Previous studies had shown that tumours contained increased levels of MAGE and other ether lipids and identified positive correlations between ether lipid content and tumorigenicity in cancer cells^{74,75}. However, the enzymes that regulate ether lipid metabolism in cancer have remained enigmatic. Additional studies showed that KIAA1363 is the principal 2-acetyl MAGE hydrolase in cancer cells, providing one potential pathway by which this enzyme could influence ether lipid content. Importantly, stable knock down of KIAA1363 by RNA interference (RNAi) also led to a reduction in MAGE levels, and this effect was found to further perturb other

Ether lipid

A lipid in which one or more of the oxygens on a glycerol backbone is bonded to an alkyl chain by an ether linkage.

pro-tumorigenic lipids, including alkyl lysophosphatidic acid (alkyl-LPA)⁵³. These metabolic changes in cancer cells correlated with reductions in migration and *in vivo* tumour growth, thus pointing to an important role for the KIAA1363-ether lipid pathway in supporting cancer pathogenesis⁵³.

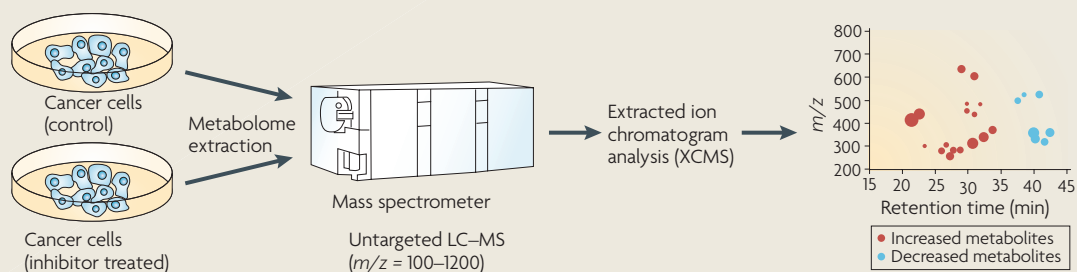
A role for MAGL in regulating fatty acid pathways in cancer cells. Competitive ABPP identified a potent and selective MAGL inhibitor, JZL184 (REF. 69), which increases MAG levels in multiple tissues in mice without concurrent changes in global free fatty acid levels⁷⁶. In contrast to this metabolic profile, MAGL inhibition in aggressive melanoma cells, and ovarian and breast cancer cells, not only raised MAGs but also lowered free fatty acid levels — results that were confirmed by RNAi knock down of MAGL¹¹. MAGL reductions also impaired cancer cell migration, invasion and serum-free cell survival *in vitro*, as well as tumour xenograft growth *in vivo*¹¹. These effects were rescued by the addition of free fatty acids *in vitro* or treatment with a high-fat diet *in vivo*, which supports a pro-tumorigenic role for MAGL-generated fatty acids. Also consistent with this premise, overexpression of wild-type, but not catalytically dead, MAGL was sufficient to increase free fatty acids and confer malignant properties on non-aggressive cancer cells¹¹. Metabolomic profiles revealed that the MAGL-fatty acid pathway feeds into a larger lipid network that includes the pro-tumorigenic signalling molecules LPA and prostaglandin E₂ (PGE₂)¹¹. These results suggest that as tumour development progresses, cancer cells with increased MAGL activity produce more cellular fatty acids, which can serve as building blocks for lipid transmitters that further drive cancer malignancy.

Additional metabolomic studies in cancer. Metabolomics has also been a useful technology for characterizing enzymes that are genetically mutated in cancer. An integrated genomic analysis, consisting of sequencing 22,661 protein-coding genes coupled with high-density oligonucleotide array analysis of 22 human glioblastoma (World Health Organization (WHO) grade IV) samples, identified mutations in the enzyme cytosolic isocitrate dehydrogenase 1 (IDH1) as a common feature of a major subset of primary human brain cancers⁷. These mutations produce a single amino acid change in the IDH1 active site, resulting in the loss of the ability of the enzyme to convert isocitrate to α -ketoglutarate. Metabolomics revealed that cancer-associated mutations also result in a new catalytic activity — the NADPH-dependent reduction of α -ketoglutarate to R(2)-2-hydroxyglutarate (2-HG)⁷. Human malignant gliomas with an IDH1 mutation exhibit markedly increased levels of 2HG compared with gliomas without this mutation⁷. These intriguing findings designate 2-HG as a potential onco-metabolite. Consistent with this hypothesis, an excess accumulation of 2-HG has been shown to lead to an increased risk of brain tumour development in patients with inborn errors of 2-HG metabolism⁷⁷.

ABPP and proteases. Proteases have long been implicated as drivers of tumorigenesis and are important for early tumour progression, as well as invasion and metastasis^{78–81}. Increased protease activity is also useful as a diagnostic marker for many cancers^{78–81}. Although proteases catalyse one of the most pervasive post-translational modifications in living systems, most of these enzymes remain incompletely characterized with respect to their endogenous substrates. Whereas technologies such as ABPP are useful for identifying deregulated proteolytic activities, additional methods are required to identify

Box 1 | Metabolomics

Metabolomics has emerged as a powerful approach for investigating enzyme function in living systems^{23,24}. Metabolomic experiments in the context of enzyme studies typically start with the extraction of metabolites from control and enzyme-disrupted biological systems, followed by metabolite detection and comparative data analysis. For example, lipophilic metabolites can be enriched from cells or tissues by organic extraction. Mass spectrometry (MS) has become a primary analytical method for surveying metabolites in complex biological samples, with upfront separation accomplished by liquid chromatography (LC–MS) or gas chromatography (GC–MS). MS experiments can be carried out using targeted⁹² or untargeted^{93,94} approaches, depending on whether the objective is to profile and quantitate known metabolites or to broadly scan for metabolites across a large mass range, respectively. As metabolomic experiments generate a large amount of data, powerful software tools are needed for identification and quantitation of ions in LC–MS data sets (see the figure; the mass to charge ratio (m/z) is indicated). One such program is XCMS⁹⁵, which aligns, quantifies and statistically ranks ions that are altered between two sets of metabolomic data. This program can be used to rapidly identify metabolomic signatures of various disease states or to assess metabolic networks that are regulated by an enzyme using pharmacological or genetic tools that modulate enzyme function. Additional databases assist in metabolite structural characterization, such as HMDB^{96,97}, METLIN^{98,99} and LIPID MAPS¹⁰⁰.



the substrates of proteases. To address this issue, several proteomic approaches have been developed that globally profile protease–substrate relationships in native biological systems^{82–86} (FIG. 3). One group of proteomic methods, N-terminal labelling techniques, relies on chemically tagging neo-N termini that are produced through proteolytic cleavage events. The chemically tagged N-terminal peptides can then be enriched and analysed using MS methods. Many variations of this strategy have been developed and have been instrumental in identifying exact sites of proteolytic cleavage for various proteases, including caspases⁸⁴ and MMPs⁸⁵. Wells and colleagues⁸⁴ have introduced an engineered enzyme (subtiligase) to tag and enrich nascent N termini in complex protein mixtures. Application of this technique to study apoptosis led to the identification of 292 caspase-cleaved proteins⁸⁴. Overall and colleagues⁸⁵ have recently developed the terminal amine isotopic labelling of substrates (TAILS) technique, which uses an amine-reactive polymer to capture internal peptides leaving only labelled neo-N termini and mature blocked N termini for MS analysis. They used this technique to extensively study the substrate profile of MMP2 in mouse fibroblast secretomes and identified 288 potential substrates for this protease⁸⁵. Van Damme *et al.*⁸² developed an N-terminal enrichment strategy, which relies on combined fractional diagonal chromatography (COFRADIC), and so identified 93 caspase-mediated cleavage events during CD95-induced apoptosis. Also

of note is the N-CLAP method developed by Jaffrey and colleagues⁸³, which uses a chemical treatment strategy to selectively label N-terminal amines on proteins.

Although N-terminal labelling methods have provided valuable insights into biological systems that are controlled by proteases, they possess some drawbacks. Perhaps most notably, the identification of a cleavage event relies on the detection of a single peptide from the C-terminal portion of a cleaved protein, and therefore does not provide information on the size or stability of the remaining protein fragments. With this consideration in mind, we developed a platform for protease substrate discovery that aims to provide a more complete topological map of proteolytic events occurring in biological systems. This approach, termed the Protein Topography and Migration Analysis Platform (PROTOMAP), consists of the fractionation of active and inactive protease samples by 1D SDS–PAGE, followed by LC–MS analysis of tryptically digested proteins from individual gel bands^{86,87} (FIG. 3). Identified proteins are subsequently assembled into two-dimensional peptographs that combine sequence coverage with gel migration information, and when coupled with spectral counting data this yields a semi-quantitative topographical map for all detectable proteins (and their cleavage products) in a sample. PROTOMAP has been used to study the intrinsic apoptotic pathway in Jurkat T cells, resulting in the discovery of more than 250 protein cleavage events in apoptotic cells, most of which had not previously been reported in the literature^{86,87}. Interestingly,

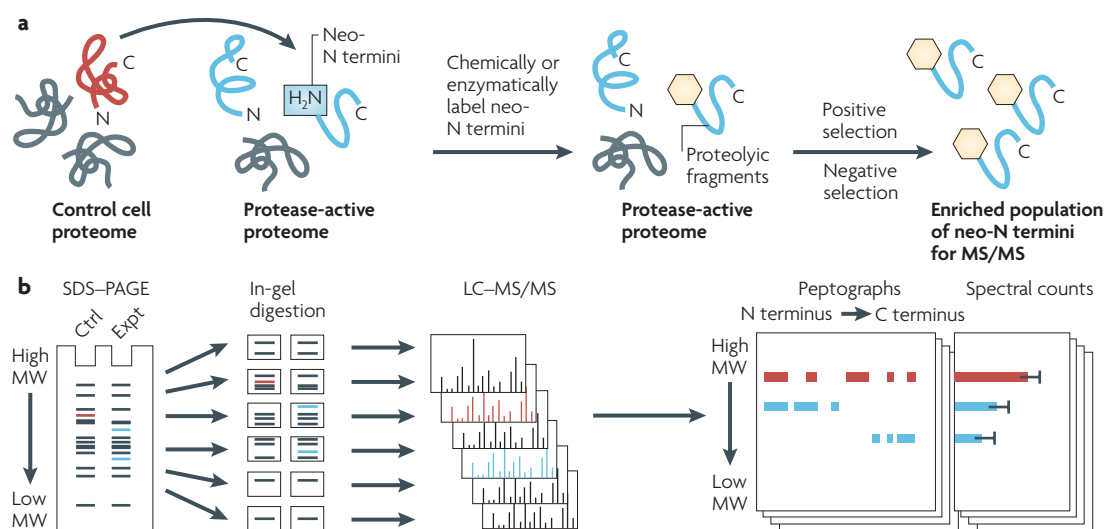


Figure 3 | Proteomic strategies for mapping protease substrates. a | Amino terminal labelling techniques use chemical or enzymatic methods to selectively label neo-N termini that are created on protease treatment. The labelled N-terminal peptides can then be enriched through positive selection methods (such as the subtiligase method⁸⁴) or, alternatively, the internal unmodified peptides can be removed through negative selection methods (such as terminal amine isotopic labelling of substrates (TAILS) methods⁸⁵). The remaining pool of enriched labelled neo-N termini can then be analysed using tandem mass spectrometry (MS) and the exact sites of proteolytic cleavage can be assigned. **b** | An alternative proteomic method for protease substrate identification, Protein Topography and Migration Analysis Platform (PROTOMAP⁸⁶) combines one-dimensional (1D) SDS–PAGE fractionation with liquid chromatography (LC)–MS analysis. In a typical PROTOMAP experiment, proteomes from control (Ctrl; red) and experimental (protease-active) (Expt; blue) systems are separated by 1D SDS–PAGE. The lanes are cut into bands at fixed intervals, digested with trypsin and analysed by LC–MS/MS to generate data that are integrated into peptographs, which plot sequence coverage for a given protein in the horizontal dimension (N to C terminus; left to right) versus gel migration in the vertical dimension. Spectral count values for each protein in each gel band provide quantitation. Cleaved proteins are identified by shifts in migration from higher to lower molecular weight (MW) in Ctrl versus Expt systems.

a meta-analysis has revealed substantial congruency in the proteolytic events identified by the subtiligase and PROTOMAP methods in apoptotic cells⁷³, indicating that these approaches provide complementary ways to characterize proteolytic pathways in biological systems. We envision that coupling protease–substrate discovery platforms with ABPP should offer a versatile and a potentially routine way to map deregulated proteolytic pathways in cancer and other pathophysiological processes. The information acquired by protease–substrate discovery platforms should also guide the development of new substrate-derived ABPP probes for specific proteases.

Conclusions

Chemical labelling methods have become centrepieces for a wide range of proteomic investigations, whether for measuring the expression⁸⁸ and the post-translational modification^{89–91} of proteins or, as we discuss above, the activity state of proteins. We have attempted to highlight how one such chemoproteomic technology, ABPP, has enabled the discovery of new enzyme activities deregulated in human cancer. Importantly, ABPP also provides a built-in assay that can be used to develop inhibitors to assess the functional role of enzymes in cancer. Activity-based probes have found additional uses as, for

example, imaging agents to visualize enzyme activities in tumours. An extension of these studies into a clinical setting could enable rapid imaging, staging and diagnosis of various types of cancers both in biopsy samples and *in vivo*. Looking forwards, we also anticipate that ABPP might have an important role in demystifying the process of target identification for chemical genomic screens in cancer cells. Lead compounds emerging from phenotypic screens could be transformed into ABPP probes by introducing, for example, photoreactive groups and clickable affinity handles to facilitate the identification of protein targets and sites of probe labelling.

With the advent of fluopol-ABPP, inhibitor discovery and optimization can now be carried out in a high-throughput manner, allowing cancer-relevant enzyme targets to be screened against extensive small-molecule libraries, such as those available as part of the US National Institutes of Health-supported Molecular Libraries Screening initiative. By coupling ABPP with other large-scale profiling methods, such as metabolomics and proteomics, important insights can be gained into how certain enzymes are used or hijacked to carry out biochemical tasks that fuel tumorigenesis. Such enzymes could be important targets for the next generation of cancer therapeutics.

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Competing interests statement

The authors declare no competing financial interests.

FURTHER INFORMATION

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