Activity-based protein profiling for the functional annotation of enzymes

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Activity-based protein profiling (ABPP), the use of active site-directed chemical probes to monitor enzyme function in complex biological systems, is emerging as a powerful post-genomic technology. ABPP probes have been developed for several enzyme classes and have been used to inventory enzyme activities *en masse* for a range of (patho)physiological processes. By presenting specific examples, we show here that ABPP provides researchers with a distinctive set of chemical tools to embark on the assignment of functions to many of the uncharacterized enzymes that populate eukaryotic and prokaryotic proteomes.

Genome sequencing projects have propelled scientists into an era in which, for the first time, the genetic composition of organisms can be viewed in its entirety. Surveys of complete genome sequences have revealed that a large fraction of predicted gene products for most organisms remains essentially uncharacterized[1,2]. New methods are thus needed to accelerate the assignment of biochemical, cellular and physiological functions to these poorly annotated genes and proteins. Here we propose that the emerging chemical proteomic technology, ABPP[3], is distinctly suited to address this problem.

ABPP uses active site–directed, small molecule–based covalent probes to report on the functional state of enzyme activities directly in native biological systems[4,5]. ABPP probes are designed or selected to target a subset of the proteome based on shared principles of binding and/or reactivity (**Box 1** and **Fig. 1**) and have been successfully developed for many enzyme classes, including serine-[3,6,7] cysteine-[8,9], aspartyl-[10], and metallo-[11–13] hydrolases, kinases[14], glycosidases[15,16], histone deacetylases[17] and oxidoreductases[18,19]. These probes have been shown to selectively label active enzymes but not their inactive precursor (zymogen) or inhibitor-bound forms[6,20], thus allowing researchers to capture functional information that is beyond the scope of standard proteomic methods. When combined with high-resolution mass spectrometry (MS) analytical platforms (**Box 2** and **Fig. 2**), ABPP has facilitated the identification of conserved catalytic residues in enzyme active sites[21–23]. ABPP also provides a competitive screening platform for the generation of selective inhibitors[6,24,25].

Here we discuss how application of ABPP has led to fundamental insights into enzymes and enzymatic pathways. These discoveries address not only enzymes with established biochemical activities but also those of previously uncharacterized function. We describe how these technologies are empowering biologists with sophisticated research tools to navigate through poorly annotated sections of the proteome, and ascribe biochemical and (patho)physiological functions to its enzymatic constituents.

Assignment of enzyme mechanism by ABPP

There are multiple levels of annotation for enzymes. The most basic level is assignment to a specific mechanistic class based on the general chemical reaction catalyzed by the enzyme (for example, hydrolase, kinase, oxidoreductase and others). Additional annotation involves determining the endogenous substrates and products for the enzyme. Finally, complete annotation requires an understanding of how the specific chemical transformation(s) catalyzed by an enzyme integrate into larger metabolic and signaling pathways to influence cell physiology and behavior.

Many of the predicted enzymes uncovered by genome sequencing projects can be assigned to a mechanistic class or ascribed a putative biochemical function based on sequence homology to well-characterized enzymes[26]. But some enzymes have insufficient sequence relatedness for class assignment or have a function different from that predicted by sequence comparisons. ABPP has facilitated class annotation for several such uncharacterized enzymes. These ABPP studies have benefited from high-resolution liquid chromotography (LC)-MS/MS analytical platforms (**Box 2**), which can be used to

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assign specific sites of probe labeling to proteins enriched from complex proteomes.

Insights into enzyme mechanism. In an early example of the value of chemical probes for enzyme characterization, Li and colleagues used biotinylated photoaffinity analogs of reversible γ-secretase inhibitors to identify presenilin 1 and 2 (PS1 and PS2) as the proteases involved in processing the amyloid precursor protein to the Aβ amyloigenic peptides causally linked to Alzheimer's disease[10] (**Fig. 3**). Interestingly, PS1 and PS2 had been linked by biochemical[27] and genetic[28] evidence to Aβ processing and early-onset Alzheimer's disease, but these integral membrane proteins bore insufficient homology with classical aspartyl proteases to confidently predict their function by sequence comparisons alone. The photoreactive γ-secretase inhibitors also selectively labeled processed (active) PS1-PS2, but not their immature (inactive) precursors, indicating that this class of reagent acts as *bona fide* ABPP probes for transmembrane aspartyl proteases.

Successful characterization of new enzyme activities also has been achieved using broader, 'class-selective' ABPP probes. Borodovsky and colleagues developed electrophilic derivatives of ubiquitin as ABPP probes for the cysteine protease family of deubiquitinating enzymes (DUBs) and used these reagents to identify the predicted protein product HSPC263 as a candidate DUB (**Fig. 3**). HSPC263 is a member of the ovarian-tumor like (OTU) domain family of proteins, which share no sequence homology with other known DUBs. HSPC263 (now called otubain 1) reacts with the C terminus of ubiquitin, indicating that OTU proteins are likely a new class of DUBs[29]. Other novel DUBs have since been characterized by ABPP, including the *UL36* gene product of herpes simplex virus 1 (ref. 30; **Fig. 3**).

Combining ABPP with high-resolution MS analysis has facilitated discovery of 'orphan' members of enzyme classes through identification of their core catalytic residues. Withers and colleagues converted an inhibitor of retaining endoglycosidases into an ABPP probe by conjugation with a cleavable biotin tag and used this reagent to identify a new β-1,4, glycanase termed Cfx from the soil bacterium *Cellulomonas fimi*[16] (**Fig. 3**). Key to the success of this method was the selective release of streptavidin-captured, probe-modified peptides, done at acidic pH to increase stability of the probe-enzyme adduct, which facilitated MS/MS analysis and assignment of the catalytic aspartate nucleophile of Cfx. Similarly, Jessani and colleagues used fluorophosphonate ABPP probes[3,20] (**Fig. 3**) to determine that the mammalian enzyme sialic acid 9-*O*-acetylesterase (SAE) is a member of the serine hydrolase superfamily[31]. SAE was first identified in 1993 (ref. 32), but the lack of sequence homology with other proteins precluded its definitive assignment to a specific mechanistic class of hydrolases. The fluorophosphonate probes were shown to specifically label Ser127 in SAE[31]. This serine is completely conserved among the SAE family of enzymes and, upon mutation to alanine, produced a catalytically inactive enzyme that no longer reacted with fluorophosphonate probes.

Insights into enzyme substrate selectivity. In addition to mining sites of reactivity in the proteome for residues important for catalysis, ABPP can also provide information about the binding specificity of enzymes. ABPP probes directed toward protease families have long been used to generate 'fingerprints' of sequence specificity for inhibitors (and, by extension, substrates) of closely related enzymes. Bogyo and colleagues first applied this strategy to the proteasome, a multisubunit protease responsible for protein degradation. Using libraries of tri- and tetrapeptide vinyl sulfones, they showed the importance of the P4 position for binding and inhibition, thus implying that the proteasome has a minimal peptide substrate length requirement[33,34]. They were also able to show differences in inhibitor selectivity for individual subunits of the proteasome, leading to the development of Z subunit–specific inhibitors that were used to confirm that this protein is fully responsible for the trypsin-like activity of the proteasome.

Libraries of peptide-based ABPP probes have since been applied to map active-site specificity in several other classes of proteases[9,13,35,36]. A provocative conclusion that can be drawn from these studies is that proteases with similar inhibitor sensitivity profiles often share very little sequence identity, indicating that primary structural homology is not a good predictor of active-site relatedness among members of protease (and likely other enzyme) superfamilies.

Insights into enzyme active-site architecture. ABPP probes with diversified binding groups have also been implemented to investigate

BOX 1 PROBE DESIGN FOR ABPP

ABPP probes (**Fig. 1**) have three main components: (i) a reactive group, typically an electrophilic or photoreactive group, for covalent labeling of protein targets; (ii) a binding group, which directs the reactive element toward specific classes of proteins; and (iii) an analytical tag, typically a fluorophore or biotin, for detection and/or enrichment of probe-labeled proteins. More recently, the development of bioorthogonal reactions, such as the copper-catalyzed [3+2] Huisgen cycloaddition (often called 'click chemistry'), has allowed the synthesis of probes with latent analytical tags such as azides and alkynes[47], permiting profiling of enzyme activities in living systems.

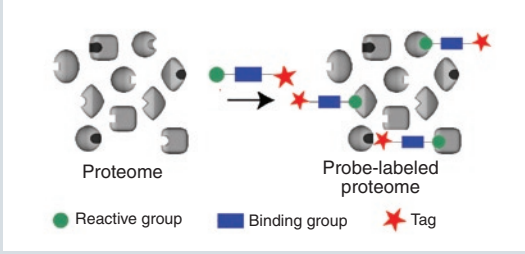

Figure 1 | Schematic of a proteome reacting with a probe.

There are two general strategies for probe design. In the directed approach, probes incorporate well-characterized affinity labels and/or binding groups to bias their reactivity toward enzymes in the proteome that share a similar catalytic mechanism and/or substrate selectivity[6–9]. Directed probes can also be adapted from reversible, tight-binding inhibitors by the addition of a photoreactive group for covalent labeling[10–13,17]. In the nondirected approach, structurally diverse libraries of candidate probes bearing electrophilic groups are synthesized and screened against the proteome for activity-dependent labeling events[18,19].

the active-site architecture of less familiar classes of enzymes. Nondirected screening of a library of dipeptide α-chloroacetamide (αCA) probes identified agents that label multiple members of the nitrilase family of enzymes in proteomes[37]. One of these nitrilases, beta-ureidopropionase (Upβ), was labeled exclusively by the Leu-Asp–αCA probe, but not closely related Leu-Glu– or Leu-Asn–αCA probes. LC-MS analysis confirmed that labeling occurred on the catalytic cysteine nucleophile conserved among all nitrilases. Upβ participates in the breakdown of uracil to β-alanine, and mutations of this enzyme in humans result in neurotoxicity as a result of accumulation of the enzyme's principal natural substrate, *N*-carbamoyl-β-alanine[38]. Notably, the Leu-Asp–αCA probe has many common structural features with *N*-carbamoyl-β-alanine, suggesting that the probe exploits substrate mimicry to specifically target Upβ (**Fig. 4**). This premise was confirmed by the synthesis of probe analogs containing the core features of *N*-carbamoyl-β-alanine, which labeled Upβ in an active site–directed manner[37]. In contrast, nitrilase 1 (Nit1), a less well-characterized mammalian nitrilase, was targeted by a wide variety of dipeptide-αCA probes (although not by the Leu-Asp–αCA probe), suggesting that this enzyme may have a broader substrate selectivity than Upβ. Realizing that it is possible to discover probes by nondirected ABPP that act as substrate mimetics, we anticipate that applications of this functional proteomic method may lead to the *de novo* assignment of endogenous substrates for uncharacterized enzymes.

Assignment of enzyme function by ABPP

Determination of enzyme mechanisms, although certainly important, is not sufficient to predict metabolic and physiological functions. Indeed, many enzymes share the same basic catalytic mechanism, but accept widely varied substrates *in vivo* (for example, proteases versus lipases, protein versus metabolic kinases, histone demethylases versus amino oxidases and others). Elucidation of the endogenous substrates and products of enzymes thus constitutes a higher-order annotation problem that requires selective genetic or pharmacological tools to perturb enzyme function in living systems. The most technically straightforward way to disrupt the function of an enzyme is to 'knock out' its expression using RNA interference (RNAi) or targeted gene disruption techniques. These approaches have the advantages of methodological generality and high target selectivity. But they suffer from some limitations, including the constitutive nature of enzyme inactivation, which can lead to compensatory systems-wide effects that mask the role of enzymes as well as, in the case of RNAi, insufficient 'knockdown' of the target of interest.

Chemical inhibitors of enzymes constitute a valuable complementary approach to genetic methods, as these pharmacological tools can be applied acutely across a large concentration range to effect the transient and complete disruption of enzyme function. The development of inhibitors has, however, historically been limited to relatively well-characterized proteins.

BOX 2 THE INTERFACE OF ABPP AND MS

MS has emerged as the analytical method of choice for detailed characterization of protein targets of ABPP probes. In ABPP-MudPIT[48] (**Fig. 2**), shotgun LC-MS methods are used to identify the targets of biotinylated ABPP probes after enrichment on (strept)avidin beads. Enriched proteins are digested on-bead with trypsin, analyzed by multidimensional LC-MS/MS and identified by searching the resulting MS/MS data using algorithms such as SEQUEST. This approach has been shown to afford enhanced resolution and sensitivity compared to more conventional gel-based methods for ABPP target analysis.

MS-based methods have also been developed to directly identify the sites of ABPP probe labeling in proteomes (**Fig. 2**). In these active-site peptide profiling methods[21,22], probe-labeled proteomes are first digested with trypsin and then subjected to enrichment to isolate probe-labeled peptides, which are then chemically eluted, analyzed by LC-MS/MS and identified using a modified version of SEQUEST. Alternately, the tandem orthogonal proteolysis–ABPP method[23] combines peptide profiling with ABPP-MudPIT by introducing of a second protease cleavage site (for example, TEV protease) into the biotin tag, which allows for gentle proteolytic elution of (strept)avidin-bound peptides and removal of the biotin tag. Chemically cleavable linkers have also recently been introduced for the elution of (strept)avidin-bound proteins or peptides in a format compatible with direct LC-MS/MS analysis[16,49,50].

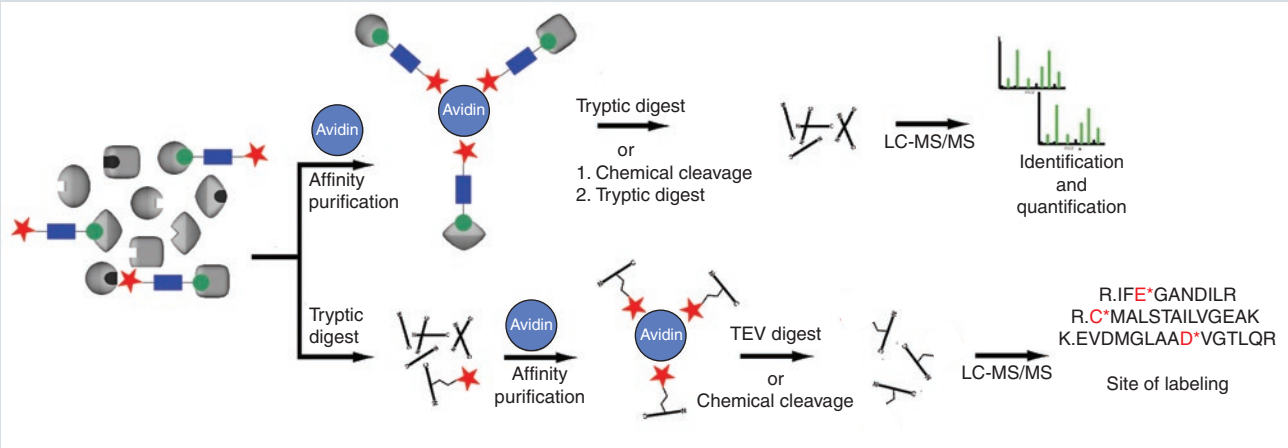

Figure 2 | Schematic of an MS experiment. Identification and quantification by ABPP–multidimensional protein identification technology (Mud-PIT) (top), and identification of sites of probe labeling (bottom).

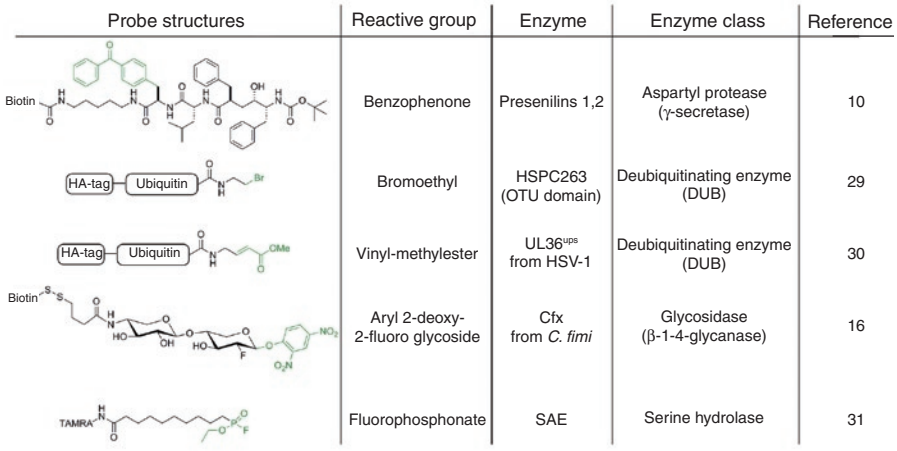

Figure 3 | Examples of enzymes assigned to specific mechanistic classes by ABPP. Reactive groups are shown in green.

Toward the goal of expanding the scope of pharmacology to unannotated portions of the proteome, a competitive version of ABPP has been introduced to develop potent and selective inhibitors for enzymes of uncharacterized function[6,25,33,35,36]. Competitive ABPP involves testing libraries of candidate inhibitors, either reversible or irreversible, for their ability to block probe labeling of an enzyme target (**Fig. 5**). Competitive ABPP has several advantages over conventional substrate assays for the discovery of enzyme inhibitors. First, inhibitors can be screened directly in native proteomes without requiring the recombinant expression or purification of enzyme targets. Second, inhibitors are assayed against many enzymes in parallel, allowing assignment of both potency and selectivity factors to compounds. Indeed, as long as enzymes in the proteome are analyzed under kinetically controlled reaction conditions, the IC_{50} values for inhibitors tested in competitive ABPP experiments have been shown to mirror accurately their inhibitor binding constant (K_i) values calculated from standard substrate assays[25]. Finally, and of particular relevance for proteome annotation, selective inhibitors can be discovered for uncharacterized enzymes[39,40]. Competitive ABPP has been successfully used to optimize the potency and selectivity of inhibitors for several enzymes, including cathepsins[5,35], caspases[36], proteosome subunits[33] and fatty acid amide hydrolase[25].

Competitive ABPP has, in at least one instance, facilitated the functional annotation of an uncharacterized enzyme. Initial ABPP studies of a panel of human cancer cell lines identified an integral membrane serine hydrolase of unknown function (KIAA1363) that was highly elevated in activity in aggressive cancer cells from multiple tumor classes[20]. Chiang and colleagues screened KIAA1363 by competitive ABPP for sensitivity to a library of hydrolase-directed inhibitors, resulting in the discovery of a carbamate agent, AS115, that potently and selectively inactivated this enzyme in human cancer cells[40]. Cancer cells treated with AS115 were then shown, using a global metabolite profiling platform[41], to display a dramatic reduction in the levels of an unusual class of lipids, the monoalkylglycerol ethers (MAGEs). Biochemical analyses revealed KIAA1363 to be a 2-acetyl MAGE hydrolase that regulates a metabolic network bridging the platelet-activating factor and the lysophospholipid classes of signaling molecules (**Fig. 6**). A key role for KIAA1363 in this network was confirmed by RNAi studies, which replicated the metabolic effects observed with AS115 and significantly ($P < 0.01$) impaired cancer cell migration and tumor growth rates in mouse xenograft models[40].

Competitive ABPP has also been used to discern a role for the *Plasmodium falciparum* protease falcipain 1 in host invasion. The genome of *P. falciparum*, the parasite responsible for malaria disease, encodes for close to 100 proteases[42] and determining the specific roles that each of these enzymes has in the parasite life cycle is a major experimental challenge. Greenbaum and colleagues used a general peptide epoxide ABPP probe for cysteine proteases to profile these enzyme activities across the parasite lifecycle. These profiles revealed a selective elevation in falcipain-1 activity coincident with the timing of host invasion. A competitive ABPP screen of a positional-scanning library of peptide epoxides identified several inhibitors that were selective for falcipain-1 over other *P. falciparum* cysteine proteases. Parasites treated with one of these inhibitors, YA29, exhibited a specific defect in red blood-cell invasion by merozoite-stage parasites, indicating a role for falcipain-1 in this important stage of the parasite lifecycle[43]. Subsequent work with falcipain-1 knockout parasites has shown that this protease is not required for red blood cell invasion[44,45], indicating that there may be a redundant pathway that can compensate for its loss. Notably, this additional pathway also appears to be sensitive to YA29, suggesting that its identity could be revealed using ABPP methods.

Future directions and challenges

Eukaryotic and prokaryotic proteomes each contain hundreds to thousands of unannotated enzymes. ABPP appears especially well-suited to tackle this challenging portion of the proteome, which is not easily addressable using more conventional methods. ABPP can provide information on the relative activity state of these enzymes in native proteomes, thereby directing researchers to specific biological systems in which the proteins may have a substantive role. ABPP can also garner insights into the mechanism and active-site architecture of enzymes, which can lead to hypotheses about endogenous substrate classes and biochemical functions. ABPP can also be implemented as a direct assay for inhibitor discovery, allowing researchers to develop potent and selective pharmacological probes for uncharacterized enzymes.

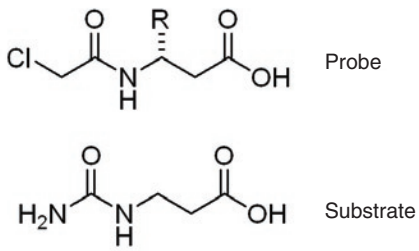

Figure 4 | Substrate mimicry of an ABPP probe. Structures of the Leu-Asp-αCA probe that selectively labeled Upβ (top) and of the endogenous Upβ substrate, *N*-carbamoyl-β-alanine (bottom).

We anticipate several considerable challenges in the application of ABPP for proteome-wide enzyme characterization. First, the fundamental currency of ABPP, namely active site–directed chemical probes, have yet to be developed for all enzyme classes in the proteome. But there has been a tremendous amount of activity in the field of chemical proteomics[4], and we expect that a sustained effort in this area will result in continued development of ABPP probes for new enzyme classes. These probes must also be judged on the breadth of coverage that they offer for their target enzyme class, especially for competitive ABPP applications. Confidence in the selectivity of an inhibitor discovered by competitive ABPP depends on the number of enzymes surveyed in these experiments. Some ABPP probes, such as fluorophosphonate[3,6,7] and acyl phosphate[14] agents, label conserved active site residues in their target enzyme classes (serine hydrolases and kinases/ATPases, respectively) and, accordingly, provide exceptional coverage. In contrast, ABPP probes that depend on binding interactions to label their enzyme targets (for example, photoreactive probes for aspartyl[10] and metalloproteases[11–13]) typically show less promiscuous class-wide reactivity. In the latter cases, libraries of structurally diverse probes can be generated and applied as a cocktail to improve proteome coverage[13].

Another challenge can be recognized by reviewing the chemical features of selective inhibitors originating from competitive ABPP studies performed to date. These inhibitors have all used class-directed binding or reactive groups to strengthen interactions with their enzyme targets. Such chemotypes (for example, epoxides for cysteine proteases[43], carbamates for serine hydrolases[39,40]) allow researchers to screen very modest-sized libraries of compounds (<100 compounds) to identify useful pharmacological agents and, at the same time, generate fingerprints of the ligand (and presumably substrate) binding specificity of enzymes. This approach, however, cannot be generically applied, as many enzyme classes lack cognate binding or reactive chemotypes. The adaptation of competitive ABPP assays for compatibility with high-throughput screening, which will require advanced gel- and MS-free platforms, such as microarrays[46], would open up new opportunities to screen uncharacterized enzymes against much larger libraries of compounds for inhibitor discovery.

Finally, it is important to stress that the complete annotation of enzymes, including deciphering their endogenous biochemical and physiological functions, requires the integrated application of ABPP with other biological methods. The determination that KIAA1363 regulates ether lipid signaling pathways in cancer, for instance, would not have been possible without the combined implementation of ABPP, RNAi and metabolomic methods[41]. Thus, ABPP should be viewed as an emerging technology with complementary value to more conventional methods for enzyme characterization. These approaches, when applied in unison, should equip enzymologists with the requisite experimental tools to systematically explore uncharacterized portions of the proteome. Such efforts should in turn lead to the discovery of new metabolic and signaling pathways that contribute to human health and disease, and provide a direct experimental conduit to develop selective chemical tools to perturb these pathways for therapeutic gain.

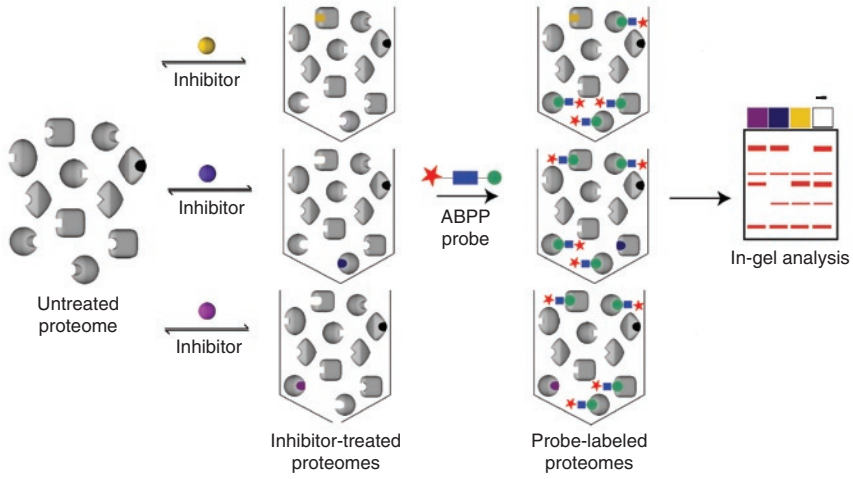

Figure 5 | Inhibitor screening by competitive ABPP. Proteomes are incubated sequentially with an inhibitor and then with an ABPP probe. Inhibitor-binding to one or more enzyme targets of the probe is read out by SDS-PAGE as a quantitative reduction in fluorescence band intensity.

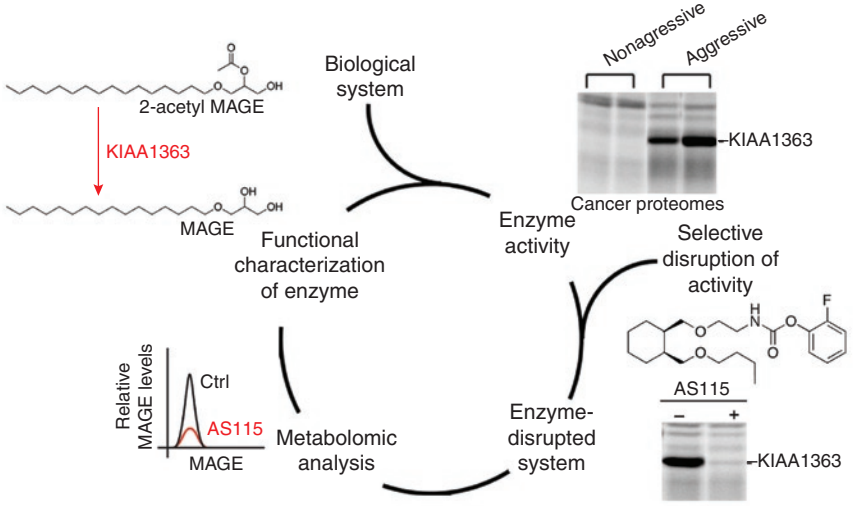

Figure 6 | Multidimensional profiling strategy for the annotation of the cancer-related enzyme KIAA1363. ABPP using fluorophosphonate probes identified KIAA1363 as a highly elevated enzyme activity in aggressive cancer cells. Competitive ABPP was then used to develop a selective KIAA1363 inhibitor (AS115). Metabolomic analysis of cancer cells treated with AS115 determined a role for this enzyme in the regulation of MAGE lipids in cancer cells. Biochemical studies confirmed that KIAA1363 acts as 2-acetyl MAGE hydrolase in a metabolic network that bridges the platelet-activating factor and lysophosphatidic acid classes of signaling lipids.

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