NIH Public Access
Author Manuscript
Curr Proteomics. Author manuscript; available in PMC 2012 February 2.

Published in final edited form as:
Curr Proteomics. 2011 October ; 8(3): 216–228. doi:10.2174/157016411797247512.

Abundance- and Activity-Based Proteomics in Platelet Biology

Stephen P. Holly[¶], **Xian Chen**[¶,‡], and **Leslie V. Parise**[¶,‡,*]

[¶]Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

[‡]Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

Abstract

Human platelets are thought to express approximately 2000–3000 proteins, but post-translational modifications, alternatively spliced variants and a rich diversity of vertebrate domain architectures likely make this a conservative estimate. Even though rapidly advancing proteomic techniques have facilitated the identification of roughly one third of the platelet proteome, a combination of abundance-based and activity-based proteomics methodologies is needed for elucidation of platelet functional characteristics including the definition of a "core proteome" and recognition of diverse enzyme activity profiles associated with various physiological states. In this review, we describe the latest mass spectrometry-based techniques capable of providing some of these physiological details required for more comprehensive evaluation of the human platelet repertoire.

Keywords

ABPP; biomarker; cancer; library; mass spectrometry; platelet; proteome; rat

1. INTRODUCTION

Platelet-mediated aggregation has long been recognized as the final event in fatal myocardial infarctions and other thrombotic disorders. Major platelet proteins were first characterized in the late 1960's and early 1970's as new gel electrophoresis and surface labeling techniques became available, with tens to hundreds of proteins being described. The ease of acquiring platelets in abundance and the lack of a nucleus also made these cells readily amenable to early protein discovery efforts. However, recent estimates indicate that platelets may contain as many as 3000 distinct proteins and many of these may exist in different post-translationally modified states. Moreover, protein contents of platelets change upon platelet activation and in response to certain disease states, not only due to loss of released proteins but also due to limited protein synthetic capability. The advent of abundance-based, quantitative proteomics and creative approaches to obtain profiles or "fingerprints" of active enzymes via activity-based protein profiling (ABPP) has opened up new avenues for further understanding how platelets function. Here we review recent advances in abundance-based proteomics that have been applied to platelets and activity profiling approaches that are just beginning to be applied to these cells.

[*]corresponding author: Dr. Leslie V. Parise, Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7260, Phone: (919) 966-2238, Fax: (919) 966-2852, parise@med.unc.edu.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

2. ABUNDANCE-BASED PLATELET PROTEOMICS

In addition to the well-characterized roles of platelets in hemostasis and thrombosis, emerging evidence suggests that platelets function in diverse biological processes including wound healing, angiogenesis, innate and adaptive immune responses to microbial pathogens, tumor metastasis and liver regeneration [1–4]. Platelets have recently been shown to express functional toll-like receptor 4 (TLR4), for instance, which recognizes lipopolysaccharides (LPS) on the surface of gram-negative bacteria [1]. This interaction elicits inflammatory signals from the platelet that stimulate other immune cells such as macrophages and neutrophils, which can lead to the rapid formation of neutrophil extracellular traps (NETs) that capture bacteria [2]. Platelets contribute to cancer metastasis via multiple mechanisms, one of which involves direct adhesion to tumor cells and the formation of a protective layer that renders tumors "invisible" to surveillance mechanisms of the immune system [3]. Furthermore, when platelets are depleted from the bloodstream of mice undergoing hepatectomy (partial liver removal), hepatocytes fail to proliferate. Deliberately loading platelets with serotonin rescues hepatocyte growth and liver architecture, suggesting that serotonin trafficking by platelets facilitates liver regeneration [4].

Despite the multi-functional nature and clinical relevance of platelets, platelets are anucleate and somewhat refractory to genomics approaches that query gene expression. However, exploration of the platelet proteome via mass spectrometry (MS)-based proteomics provides an efficient means to understand the molecular bases for the diverse physiological functions of this important cell type. Platelets are ideal for MS-based proteomic studies since these cells 1) contain only mitochondrial DNA and small pools of mRNA derived from megakaryocytes, 2) are the second most abundant primary cell type in the blood after red blood cells, which allows adequate quantities of proteins to be isolated and 3) are central to many normal and pathological human conditions such as thrombosis, making platelets extremely important therapeutic targets. The absence of a nucleus is highly advantageous for proteomic strategies, since this property allows recovery of a pure protein fraction uncontaminated by nucleic acids. In addition to providing potential therapeutic targets, comprehensive profiling of platelet proteins by abundance-based proteomics is likely to reveal clinically relevant biomarkers for diagnosis and progression of certain pathologies including cardiovascular disease and cancer [5,6]. MS-based proteomic efforts to discover novel biomarkers in plasma or serum, however, can be hampered by overwhelmingly abundant proteins such as albumins and IgG which comprise over 95% of the total protein mass of plasma or sera. Platelets are largely devoid of these MS signal-suppressing proteins, which highlights an additional reason why proteomic techniques are well-suited for the elucidation of platelet function and the potential discovery of platelet-derived biomarkers.

A variety of MS-based proteomic techniques involving two-dimensional (2D) gels, differential in-gel electrophoresis (2D-DIGE), liquid chromatography tandem mass spectrometry (LC-MS/MS), etc., have been utilized to catalog the platelet proteome. Studies focusing on platelet subproteomes such as the platelet "releasate" (the large collection of proteins secreted from intracellular vesicles by activated platelets), phosphoproteome or microparticle proteome have provided an integrated dataset that partially defines the platelet proteome. To find novel biomarkers secreted by platelets, for example, a typical proteomic strategy generates peptides by proteolytic digestion of platelet proteins separated by either 1D or 2D polyacrylamide gel electrophoresis (PAGE) followed by LC-MS/MS. Over 700 proteins were identified in this manner from the platelet releasate in thrombin receptor activating peptide (TRAP)-activated platelets isolated from three healthy donors [7], which represented a dramatic expansion over previous releasate profiling efforts. A "core releasate" defined by the expression of 225 proteins found in all three donors represents a consensus subproteome of proteins secreted from platelet α-granules, dense granules and

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

lysosomes. A similar strategy involving in-gel peptide generation and LC-MS/MS analysis of the platelet microparticle proteome identified 578 proteins. Approximately half of these proteins (55%) were redundant with the releasate data above, suggesting that platelet microparticles contain a comparable mixture of secreted, granular proteins and non-secreted, cellular proteins [8].

Microenvironmental cues and/or agonist-dependent effects on the platelet proteome can also be investigated through differential proteomic analysis. For example, glycoprotein VI (GPVI) is known to mediate platelet adhesion to exposed collagen at sites of vascular injury and generate intracellular signals that lead to activation of the αllbβ3 integrin and platelet aggregation, but the signaling pathways involved are still incompletely characterized. A recent study examined the global effects of GPVI activation on the human platelet proteome. Isolated human platelets were stimulated with an activating monoclonal antibody specific for GPVI and were comparatively analyzed by 2D-DIGE followed by LC-MS/MS. Eight differentially abundant proteins were identified and linked to GPVI-mediated changes in various cellular processes such as metabolism and signaling, but some of these proteins including aldose reductase had never been previously associated with thrombus formation, which illustrates the power of unbiased proteomics for complex pathway analysis [9].

Although these studies were successful in identifying an impressive number of novel platelet proteins from diverse subproteomes, less than 30% of the total components of the human platelet proteome estimated to contain 2000–3000 unique proteins have been identified by these methods. This is in part due to inherent trade-offs within MS platforms that occur when a single type of separation/identification scheme is employed [10]. To minimize these trade-offs, Yu and colleagues recently developed an integrated platform that incorporates multi-step protein extraction and multidimensional separation/identification approaches in combination with mass spectra interpretation algorithms [11]. This integrated platform was used to create the most extensive characterization of the rat platelet proteome to date, which was contrasted with the existing human platelet proteome to generate a core mammalian platelet dataset. These authors also used isobaric tags for relative and absolute quantification (iTRAQ) to examine agonist-related changes to the rat platelet proteome. What follows is a detailed review of the integrated design of their MS-based abundance-oriented approaches and the corresponding results of their work.

A Novel Integrated Platform for Global Platelet Proteomics

Multiple MS-based proteomic approaches are required to maximize sensitivity, accuracy, and throughput when profiling proteins with diverse physical properties. To streamline protein identification and expand the global platelet proteome, the laboratory of Xian Chen has developed a new proteomic platform that integrates multiple components, which features a comprehensive extraction method, complimentary separation methods including both gel-free and gel-based protocols and comprehensive peptide database mining. As an experimental test of this new platform, Yu and colleagues generated a high quality rat platelet proteome for the purpose of discovering common elements within the human platelet proteome and the discernment of conserved platelet functions [11].

To maximize the total number of rat platelet proteins discovered, an extraction procedure utilizing both gel-based and gel-free separation methods was designed to recover membrane and cytosolic proteomes from purified platelets via two major workflows (Fig. 1). For the first workflow, rat platelets were solubilized with CHAPS detergent to generate a membrane/cytoskeleton proteome. Whole proteins were separated by SDS-PAGE and 14 gel fractions were subjected to in-gel tryptic digestion and LC-MS/MS with either an LTQ Orbitrap (SDS-LTQ method) or QTOF mass spectrometer (Fig. 1 left). Two types of mass spectrometers were used to overcome intrinsic weaknesses of each type of instrument [10],

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

but the SDS-LTQ method was much more productive in terms of identifying the greatest number of proteins (634 vs. 83). In parallel, CHAPS solubilized proteins were fractionated according to pH with isoelectric focusing (IEF) and analyzed by LC-MS/MS, but this method identified only 220 proteins, roughly one third of the peptides identified by SDS-LTQ. IEF was also used to fractionate platelet proteins extracted in 8 M urea. Urea extracted proteins were digested with trypsin in solution, separated by IEF and analyzed by LC-MS/MS; the number of proteins identified by this method (156) was comparable to the CHAPS/IEF method mentioned above. For the second workflow, a soluble proteome was again generated by 8 M urea extraction of platelets followed by direct digestion in solution with trypsin. Peptides were separated and analyzed by multidimensional protein identification technology (MudPIT), a gel-free protocol that relies on LC fractionation of peptides (Fig. 1 right). MudPIT identified 371 proteins, the second highest number of proteins identified among the five methods used, but this number was relatively low compared to the SDS-LTQ total given that the two methods are thought to be equally sensitive.

Altogether, two major workflows composed of five different methods produced overlapping peptide data that yielded a combined total of 837 unique proteins from quiescent rat platelets [11]. A single method, SDS-PAGE fractionation combined with LC-MS/MS performed by an LTQ mass spectrometer, identified 634 proteins, by far the most efficient approach of the five described above. Having established a baseline proteome dataset, Yu and colleagues next analyzed thrombin-induced changes. Using the quantitative iTRAQ approach, 415 total proteins were identified by 2D-LC-MS/MS performed on a hybrid QSTAR Elite mass spectrometer. Thrombin stimulation of rat platelets resulted in the relative increase of 16 proteins including calpain 1(CAPN1), a cysteine protease thought to play a role in platelet activation [12]. MS detection of increased protein levels in platelets may reflect stimulus-dependent translation of preexisting mRNAs. Indeed, Bcl-3 message is actively translated in response to thrombin, resulting in elevated levels of Bcl-3 protein in human platelets [13]. Although eukaryotic translation initiation factor 4B (eIF4B) was among the upregulated proteins found in thrombin-stimulated rat platelets, Bcl-3 was not. This may reflect an interesting species divergence regarding the precise mechanisms of protein translational machinery or simply reflect the incomplete identification of the entire rat proteome. Conversely, 17 proteins were found to be down-regulated by thrombin including apolipoprotein E (ApoE), which has been found previously in the platelet releasate [7]. Decreased abundance of proteins such as ApoE clearly reinforces the role of agonist-dependent secretion as a mechanism for decreasing protein content in platelets.

Analysis of peptide data with two independent search engines provided optimal coverage of the rat platelet proteome. Of the total number of peptides, 7241 were confidently assigned to protein database entries by either MASCOT or SEQUEST search algorithms. Over half of these peptides were recognized by both search engines, but 16% were exclusively identified by SEQUEST and 29% by MASCOT, which is similar to previous approaches [10]. These search engines also differed in their ability to identify proteins according to their abundance distribution, which was calculated using the exponentially modified protein abundance index or emPAI [14]. For highly abundant proteins, more than 70% were assigned by both search engines with extremely high confidence, but this percentage dramatically decreased for low-abundance proteins, as only 43% could be confirmed by both algorithms. Furthermore, Yu et al. found that the SEQUEST algorithm matched more low-abundance proteins than MASCOT, while exclusive matches from MASCOT were significantly higher than those from SEQUEST for high-abundance proteins. Overall, the probability-based algorithm, MASCOT, may be more sensitive than the empirical and correlative measurements provided by SEQUEST. The discrepancies between search engines indicate that a single algorithm is not sufficient for coverage of protein identification at all levels of protein abundance, especially for low abundance proteins, due to the complexity and diversity of the observed

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

protein composition in platelets. Thus, a combination of two multi-faceted proteomic workflows coupled with overlapping search engine mining of peptide databases resulted in maximum coverage of a given proteome and has led to an impressive initial description of the rat proteome.

Global Analysis of the Rat and Human Platelet Proteomes

After the identification of 837 platelet proteins established a working draft of the rat proteome, a global comparison of the rat and human proteomes using bioinformatics and gene ontology (GO) tools was undertaken. This comparison revealed that 1) the top 10 cellular pathways (groups of proteins performing a similar function) in human platelets mapped via KEGG (Kyoto Encyclopedia of Genes and Genomes) [15] matched eight out of 10 pathways in rat, 2) GO annotations for protein localization and function were nearly identical between rat and human (Fig. 2) and 3) the number of human and rat orthologous proteins was almost half of the total number of rat proteins identified.

Since orthologs are genes from distinct species that likely evolved from a common parental gene, identification of orthologs in related proteomes provides a rapid assessment of gene function across species. Extraction of orthologous pairs of rat and human platelet proteins via the new rat platelet proteome generated by Yu et al. and the OrthoMCL multi-species database [16] resulted in the identification of 386 orthologous pairs [11]. Analysis of the abundance distribution of these pairs revealed that over half were categorized as highly abundant proteins. In addition, protein subcellular localization and function of these orthologous pairs were similar to nonorthologous proteins from either the rat or human proteome. Collectively, these data suggest that orthologous proteins represent a “core proteome” evident within different species that can accomplish an essential group of functions for specialized cell types including platelets, but that an equivalent number of proteins are species-specific. Whether this “core proteome” changes with each differentiated cell type or remains relatively static across cell types of different origins remains to be determined by future proteomic efforts.

3. ABPP

Unlike abundance-based proteomic strategies described above, activity-based protein profiling or ABPP is a relatively new proteomic technology based on mechanistic enzyme catalysis. The pressing demand for annotation of the vast human genome and the advent of new target discovery technologies has synergistically catalyzed the development of ABPP, a high throughput method for surveying the enzyme activity profiles of up to hundreds of enzymes simultaneously [17,18]. ABPP requires active site-directed chemical probes, which irreversibly or reversibly modify active site residues in the catalytic pocket of enzymes from a particular family and report enzyme activity levels (Figure 3). Because ABPP is a measure of enzyme activity, this method potentially yields more biologically relevant information than other proteomic or genomic techniques that are limited to the measurement of bulk protein levels or gene expression changes. Furthermore, ABPP reports the effects of post-translational modifications (if any) such as phosphorylation, acetylation, methylation, nitrosylation or ubiquitination on the relative activity of probe-labeled enzymes. Another important advantage over existing systems methods lies in the fact that ABPP can be applied to almost any biological sample in which active enzymes are present, making the profiling of hundreds of active enzymes from complex yet biologically meaningful samples feasible. Examples of potential applications include profiling of subcellular organelles (e.g. membrane fractions), panels of cell lines with varying phenotypes, normal vs. diseased human tissue (e.g. biopsies or plasma), wildtype vs. knockout mouse tissue or whatever biological process under investigation requires active enzymes. To appreciate the utility of ABPP applied to physiologically important cell types such as platelets, however, the basic

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

operating principles are explained below starting with a description of general ABPP probe structure.

ABPP Probe Structure

There are three essential structural features of an effective ABPP probe: a protein reactive group, a spacer and a reporter group. At one terminus of the probe is the protein reactive group, which physically interacts with a specific active site geometry of the target enzyme class. This group should theoretically interact with all enzymes of a given family having the same reaction mechanism. However, the selectivity of the reactive group determines how many enzymes within a given family genuinely interact with the probe and equally importantly, how many enzymes outside of the given family interact with the probe. Protein reactive groups interact either covalently or reversibly with the active site and this mode of interaction is largely dictated by the type of catalytic mechanism employed by the particular enzyme family under investigation. Chemically, these groups are either highly electrophilic, being attacked by nucleophilic amino acids such as catalytic serines found in serine hydrolases, or photoreactive, requiring UV-crosslinking for labeling active site amino acids and nearby residues. Electrophilic probes often forge covalent bonds with their target enzymes, mimicking the assembly of covalent intermediates between active site residues of enzymes and their endogenous substrates. These probe-enzyme interactions are consequently irreversible, which has implications for enzyme selectivity and drug discovery strategies. In contrast, photoreactive probes can be effective labelers of enzymes such as kinases, which have more complicated reaction mechanisms that do not involve covalent intermediates (i.e. kinase-mediated phosphotransfer reactions utilize activated water molecules).

Adjacent to the protein reactive group and spanning the middle of the probe is the spacer group. This is often an unbranched hydrocarbon chain whose length is empirically determined to yield the most favorable binding affinity of the protein reactive group with its target enzyme. While spacer groups can sometimes aid in protein-probe association, their most common function is to provide optimal separation of the other groups in the probe. At the probe's distal terminus is the reporter group, which provides a means of physical detection of the probe-protein interaction. Published examples of reporter groups are rhodamine, biotin, radioisoptopes and click chemistry “handles” [19–24]. Rhodamine is a fluorophore that emits orange-red light when excited by the appropriate wavelength and facilitates fluorescent detection of probe-labeled enzymes. Biotin reporter groups in combination with streptavidin-based affinity enrichment protocols allow LC-MS/MS identification and Western blotting of target enzymes. Biotin has the advantage of being less bulky than rhodamine, making it less likely to affect the affinity of the protein reactive group with enzyme targets. The smallest and least interfering reporter groups are the click chemistry handles. These handles are usually alkyne or azide groups that are attached directly to peripheral groups on the probe and are commonly used to label enzymes in the context of a living cell. For detection of the probe-labeled enzyme, these handles are reacted either in the cell or in cell lysates via a cycloaddition reaction (click chemistry) with a second reagent that contains a fluorescent reporter group or an affinity tag amenable to purification procedures and LC-MS/MS [23,25].

ABPP Detection Methods

The type of reporter group described above dictates the method of detection of enzymatic activities. Two general methods are currently available for detection of ABPP probe-enzyme complexes that can be distinguished on the basis of their dependence on gel electrophoresis. Fluorescent probes are amenable to detection via in-gel fluorography wherein enzyme-probe complexes are directly visualized within a gel following electrophoresis. A typical workflow

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

for these types of probes is as follows: proteomes are labeled in parallel with a given fluorescent probe and separated via 1D or 2D gel electrophoresis. Following electrophoresis and resolution of proteins based on isoelectric point and/or size, the gel is directly analyzed using a scanner/imager that both excites the reporter group at the appropriate wavelength and captures a digital image of the resultant fluorescence emission. A more time consuming yet more sensitive variation of this theme involves biotinylated probes, which can be detected by Western blotting. The advantages of these gel-based methods are the speed of processing, relatively high throughput (i.e. many proteomes can be analyzed per day) and the small amounts of sample needed to generate robust signals (micrograms of lysate). Disadvantages are intimately related to the well-known limitations of gel electrophoresis, namely weak resolving power of complex mixtures and inability to identify protein species based solely on isoelectric point or molecular weight criteria.

Gel-free MS methods circumvent limitations of gel-based ABPP and are usually employed in combination with competitive ABPP methods described later. Biotinylated or affinity-labeled probes are incubated with whole proteomes or subproteomes to create probe-enzyme complexes, which are isolated with enrichment protocols utilizing streptavidin retention or other solid state precipitation methods that generate partially purified pools of probe-labeled enzymes. These pools can be digested with trypsin and subjected to LC-MS/MS for high resolution of peptide masses and subsequent protein identification. The very act of detection results in enzyme identification and comparison of spectral counts across samples allows for semi-quantitative measurement of enzyme activities. Even though these remarkable MS techniques provide a wealth of data, they are experimentally demanding, requiring milligrams of sample, extended run times for MS equipment and tedious *in silico* peptide identification. These reasons are typically so prohibitive that higher throughput gel-based methodologies are still the platforms of choice for rapid profiling of enzymatic proteomes. In fact, identification of interesting enzymes is most swiftly achieved by a combination of gel-based profiling and MS-based identification of proteins using affinity labeled probe complexes, which synergizes the advantages of both methods [26].

The aforementioned methods do not allow identification of the exact amino acid residues covalently modified by ABPP probes, but a variation of ABPP called active site peptide profiling (ASPP) has been specifically developed for the identification of probe-reactive amino acids. In this approach, proteomes are labeled as before with probes containing affinity tags such as biotin and proteolytically digested to release probe-bound peptides *before* affinity isolation. Labeled peptides are captured by affinity enrichment techniques, which excludes peptides not bound to the probe and facilitates identification of only probe-bound peptides by MS and database fingerprinting. Because only peptides bound to ABPP probes are processed, peptide coverage of the entire enzyme is not possible and identification of the intact protein is more difficult. However, ASPP potentially provides valuable evidence that active site amino acids are being covalently targeted by ABPP probes, making interpretation of enzymatic profiling data more precise. ASPP has also been fused with ABPP-based MS protein identification protocols to provide enzyme identification and active site residue resolution simultaneously [27].

Application of ABPP to Pathological Settings

ABPP probes have been designed to react with a number of mechanistically distinct enzyme classes that are represented in highly differentiated cell types such as platelets as well as proliferating, less differentiated cell types including most tumors. Current enzyme classes amenable to ABPP include hydrolases, kinases, phosphatases, glycosidases, cytochrome P450 enzymes and nucleotide-binding proteins, which together comprise a significant fraction of the proteome. Profiling of these disparate enzyme families via ABPP is providing fascinating insights into diseases such as cancer and arthritis and is just beginning to provide

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

data concerning platelet function by linking novel and common enzyme activities to important aspects of disease and/or physiological state.

ABPP in Platelets and Megakaryocytes

To date, no ABPP studies have yet emerged regarding platelet or megakaryocyte function, but Parise and colleagues have initiated ABPP studies in platelets from a genetic animal model of human cardiovascular disease (unpublished data). These studies are designed to profile diverse enzyme activities associated with normal and potentially altered platelet behaviors associated with disease progression to a hyperlipidemic state. Hundreds of enzyme activities are currently being analyzed for their ability to discriminate between normal platelet physiology and aberrant platelet function induced by a hyperlipidemic environment in diseased animals. Identification of either single enzyme activities or combinations of activities may reveal molecular patterns that reflect the numerous changes resulting from dysregulated lipid metabolism and perhaps implicate new diagnostic or therapeutic targets for hyperlipidemic pathologies.

A technique very similar to ABPP that profiles ATP-binding proteins has already been applied to platelets. Resting and TRAP-activated platelet lysates were incubated with ATP molecules attached to polyacrylamide resins to identify ATP-binding proteins in the platelet proteome [28]. Even though a mild, anionic detergent was used to generate a soluble platelet lysate, the most abundant group of proteins identified were cytoskeletal in nature, with β-actin and myosin-9 being the most prominent. Overall, actin, actin-like proteins and myosins made up over 70% of the total peptides identified in this study, which reinforces the central role of the actin cytoskeleton in platelet biology and underscores the difficulty of identifying low abundance proteins via proteomics in platelets. Heat shock proteins represented the second most abundant class of proteins found at 15%, while the most abundant signaling proteins identified were kinases, namely c-Src and Fyn, which are thought to constitutively interact with another abundant platelet protein, αllbβ3 [29]. Interestingly, proteins intimately associated with mRNA translation such as eukaryotic initiation factor 4 (eIF4A1) and glycyl t-RNA synthetase were identified as ATP-binding proteins, which further supports the observed translational capacity of platelets [13]. Surprisingly few ATP-binding proteins (10) were altered between resting and TRAP-activated platelets [28]. Over half of these ATP-binding proteins showed decreased peptide counts upon TRAP activation, which is likely due to their secretion or translocation to the actin cytoskeleton upon platelet activation.

Despite the identification of 175 distinct ATP-binding proteins in platelets described above, application of ABPP to platelets and megakaryocytes might provide even more information that could be potentially exploited for many aspects of antiplatelet therapy in future studies. ABPP exploration of platelets and megakaryocytes could reveal novel enzymatic pathways involved in thrombosis or thrombocytopenia *in vivo*, biomarkers that predict onset of cardiovascular pathology, biomarkers that characterize latent or overt disease progression or novel drug targets, all of which could ultimately lead to new therapeutic strategies for cardiovascular medicine.

Large-scale ABPP approaches such as the one launched by the Parise laboratory may identify novel biomarkers that report the development, maintenance or cause of certain types of cardiovascular disease. Current cardiovascular biomarkers such as cardiac troponin and C reactive protein (CRP) for example, aid in the diagnosis or prognosis of disease but have major limitations that are potentially addressable by ABPP. Diagnostic and prognostic cardiovascular biomarkers are only useful *after* disease onset, which minimizes their ability to assist in the prevention of primary disease. Another limitation of biomarkers is that none of the popular biomarkers such as troponin, myoglobin, CRP or creatine kinase have been shown to *cause* vascular disease and therefore are not therapeutic targets. Even though some

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

studies have implicated CRP in the biogenesis of atherosclerotic plaques through effects on macrophage lipid uptake and endothelial vasoreactivity, these effects may simply reflect a preexisting proinflammatory environment [30,31]. There is also no direct evidence to suggest that therapeutic reduction of biomarker levels like CRP improves disease outcome. Thus, biomarkers are currently not therapeutic targets of disease and must be used in conjunction with existing treatment options such as medications (antiplatelet drugs, lipid lowering agents, beta blockers, angiotensin converting enzyme inhibitors, and thrombolytics), stenting, or bypass surgery. Furthermore, individual diagnostic and prognostic biomarkers have limited statistical power, as a single biomarker is not appropriate for all clinical settings. This is why multiple biomarker or "multimarker" strategies have been adopted [32]. While these multimarker strategies improve specificity and stratification of risk for a given patient population, they still omit a significant portion of patients who go on to develop disease, are not yet routine in clinical settings and often need to be recalibrated for diverse patient populations that represent various ethnicities, ages and risk factors.

Some of these issues may be circumvented by ABPP-mediated discovery of biomarkers. Application of ABPP to platelets, for example, from patients with cardiovascular disease may reveal individual or multiple enzymes that are differentially present or active compared to healthy control platelets. ABPP would likely be most useful for detection of biomarkers that predict future disease or that reveal gradual or chronic disease progression (e.g. atherosclerosis) since acute clinical diagnostics for cardiovascular issues are already highly effective. Since ABPP has the power to rapidly identify and correlate multiple biomarkers with any particular disease state (or normal physiological state), statistical power is greatly enhanced compared to the correlation of single biomarkers with disease. When linked to outcomes data, ABPP-based biomarkers may be able to serve prognostic roles by identifying which high risk patients are most likely to suffer recurrent thrombotic events (e.g. diabetics, patients with coronary artery disease at high risk for subsequent thrombotic events following standard treatments, such as stent thrombosis after undergoing PCI, etc.). In this way, enzymatic "signatures" can be generated for a given pathological or physiological state at the molecular level that uniquely identify and potentially discriminate among these various states. Therefore, unique platelet signatures generated by ABPP could potentially be used as novel biomarkers for multiple clinical situations to stratify patients according to high or low risk of thrombotic events.

This strategy is already being tried at the gene expression level in human aorta tissues in an attempt to generate an atherosclerotic gene signature [33]. Seo and colleagues succeeded in finding a set of 208 genes that were predictive of "minimally diseased" or "severely diseased" aorta phenotypes. Not surprisingly, the genes overrepresented in this dataset are thought to participate in cell cycle control or the inflammatory response. While these data do not address what gene expression changes, if any, are causally related to the development of atherosclerosis, they do provide potential insights as to what genes may be involved in the progression, maintenance or severity of this disease.

Not only does ABPP have the potential to generate novel biomarkers for vascular pathology, but this strategy could also lead directly to the discovery of new drug targets important for platelet activation. Probes that identify enzyme activities that correlate closely with diseases such as atherosclerosis could potentially be used as lead compounds for the design of small molecule inhibitors that reveal *causal* associations between molecular targets and vascular diseases. ABPP therefore has the potential to help uncover new therapeutic targets and novel biomarkers in human platelets that contribute to thrombosis.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

ABPP in Cancer

Several different enzyme classes have been analyzed by ABPP in cancer cells [18]. Since enzymatic changes that favor increased cell migration, invasion of surrounding tissues and/ or cell proliferation often occur in metastatic tumors relative to the primary tumor mass, Jessani and colleagues used ABPP to examine activities of the serine hydrolase superfamily in a panel of invasive human cell lines relative to less aggressive tumor lines [34]. A fluorophosphonate reactive group conjugated to rhodamine (FP-Rh) served as the ABPP probe in this study that queried soluble, cytoplasmic proteomes or membrane proteomes of metastatic breast, melanoma and ovarian carcinoma cell lines. Arylacetamide deacetylase-like 1 (AADACL1) was identified as a member of the G-D-X-G family of serine hydrolases whose enzymatic activity was increased in the membrane proteomes of invasive carcinoma cells. Consistent with this, cell fractionation experiments and computer topography models revealed that AADACL1 is an integral membrane protein having one or two predicted transmembrane domains, supporting the hypothesis that AADACL1 is a membrane hydrolase associated with metastatic breast cancer [34].

To test whether AADACL1 is causally related to or merely a reflection of a metastatic phenotype, the Cravatt laboratory synthesized a selective carbamate inhibitor of AADACL1 called AS115, which irreversibly labels serine 191 in the active site and blocks hydrolase activity [35]. AS115 was derived from trifluoromethyl ketone (TFMK) parent compounds and selectively inhibits AADACL1 with an IC_{50} of 150 nM. Treatment of human ovarian carcinoma cells with AS115 resulted in accumulation of lipids involved in platelet activating factor (PAF) and lysophosphatidic acid (LPA) synthesis, suggesting that AADACL1 contributes to the metabolism of bioactive lipids in aggressive cancer cells. More directed pharmacological experiments using AS115 and specific lipid substrates revealed that AADACL1 can deacetylate 2-acetyl monoalkylglycerol (2-acetyl MAGE), hydrolysis of which theoretically creates additional lipid substrates such as MAGE for subsequent reactions leading to the production of LPA or PAF species *in vivo*. To genetically test whether AADACL1 was important for cellular production of MAGE-like lipids, Chiang et al. reduced AADACL1 protein levels via RNA interference and showed that MAGE, alkyl-LPA and to a lesser extent alkyl-LPC were reduced in the ovarian carcinoma cell line SKOV-3. AADACL1 knockdown also reduced the migratory capacity of SKOV-3 cells and addition of alkyl-LPA in AADACL1-depleted cells rescued these migration defects. Furthermore, AADACL1 knockdown slowed SKOV-3 growth in mouse xenografts and reduced tumor volume by over 50% [35]. Thus, AADACL1 is a 2-acetyl MAGE hydrolase identified by ABPP that is required for optimal tumor migration and growth, two cellular behaviors implicated in the metastatic tumor phenotype.

In addition to serine hydrolases, cysteine proteases, ubiquitin-specific proteases (USPs) and histone deacetylases (HDACs) have also been profiled in cancer cells using tagged ABPPs. Cysteine proteases of the cathepsin class have been implicated in cancer progression and are known to be highly expressed and/or secreted at the invasive edges of breast tumors. In a mouse model of human breast cancer, cathepsins have been profiled with DCG-04, a biotinylated epoxide-containing derivative of E-64 that covalently alkylates the active sites of multiple cathepsins including the B and L isoforms [36]. DCG-04 identified cathepsin X as a protease that partially compensates for the absence of cathepsin B on cathepsin B knockout mammary cells, suggesting the presence of a redundant network that gauges surface protease activity during tumor invasion [37]. Like cathepsins, USPs are also involved in tumorigenesis and growth control. These proteases have been targeted with ubiquitin-containing probes such as hemagglutinin-tagged ubiquitin vinyl methylester (HAUb-VME) [38]. Ubiquitin C-terminal hydrolase (UCH)-L1 was identified as an upregulated USP activity in human cancer cell lines compared to primary cells [39] and similarly UCH-L3 and UCH-L7 were detected as USPs with increased activity in cervical

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

carcinoma biopsies compared to normal tissue isolated from the same patient [40]. Nuclear enzymes such as HDACs have also been analyzed by ABPP in melanoma, ovarian carcinoma and breast carcinoma cell lines using a suberoylanilide hydroxamic acid benzophenone alkyne (SAHA-BPyne) probe [41]. This unique probe can be used in living cells due to the absence of bulky reporter groups such as rhodamine or biotin. The benzophenone group allows irreversible photo-crosslinking of SAHA-BPyne to HDACs in a cellular environment and the alkyne group provides a means of detection via addition of reporter groups through click chemistry reactions. SAHA-BPyne profiling showed that HDAC activity levels did not vary between normal and tumorigenic cells, with the notable exception of HDAC6, which was found to be expressed at much greater levels in the nonaggressive melanoma cell line MUM2C versus the aggressive line MUM2B.

ABPP in Osteoarthritis

Collagen degradation mediated by matrix metalloproteases (MMPs) and serine proteases is an important early event in the development of osteoarthritis (OA). Serine proteases have been shown previously to contribute to activation of MMPs during collagen proteolysis in cartilage explants *ex vivo* [42]. These proteases are thought to originate from chondrocytes resident in living cartilage near the initial sites of collagen degradation. To identify which serine protease in cartilage might be responsible for collagen proteolysis, Milner and colleagues used ABPP and an FP-biotin probe to survey chondrocyte membranes for active serine proteases [43]. Enrichment of FP-biotin binding proteins and MS identification of tryptic peptides revealed fibroblast activation protein (FAPα), a type II membrane protease with dipeptidyl peptidase activity. In bovine cartilage explant cultures, FAPα mRNA was upregulated by proinflammatory stimuli and this occurred prior to collagen hydrolysis, consistent with a role for FAPα in collagen degradation. FAPα message was also increased in hip cartilage from OA patients and importantly, FAPα protein localized to chondrocytes isolated from OA patients. Since FAPα can cleave gelatin (denatured collagen), its primary function may be as a gelatinase in inflamed cartilage following initial collagen breakdown by MMPs, but the exact role of FAPα in OA is yet to be determined.

Drug and Target Discovery via Chemotype Library Screening

The successful examples of ABPP described thus far have relied on the existence of ABPP probes that interact with a defined class of enzymes such as proteases. This approach can be described as directed ABPP since the enzyme targets elucidated in this manner are usually related by reaction mechanism, which results in a biased interrogation of the proteome. However, using similar principles that guide ABPP probe design for enzyme families, libraries of chemically diverse compounds can be constructed that react with multiple enzyme families in a relatively unbiased or non-directed fashion. This has been accomplished using chemotypes, which are small, moderately reactive chemical groups that are not specific for one class of enzymes. Chemotype library molecules consist of a central chemotype that is typically a carbon electrophile as in the case of sulfonate esters [26] and epoxides [22,44], which is flanked by an organic scaffold designed for conjugation to distinct side groups that impart unique steric constraints, polarities or chemical reactivities to the probe. As with ABPP probes, the chemical nature of the chemotype in combination with these side groups determines which proteins interact with the library, but as mentioned above, chemotype probes are designed to react more broadly with their protein targets than ABPP probes.

Depending on the steric limitations and hydrophobicity of the side groups, some chemotype probes can be made cell permeable and screened for their ability to block cellular functions, making these molecules potential lead compounds for drug discovery efforts. To this end, a cell permeable chemotype library has been developed to probe chemical space in malignant

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

breast carcinoma cells [44]. This chemotype library features an electrophilic spiroepoxide moiety that is known to interact with aminopeptidases and other classes of enzymes [36,45], flanked by an alkyne handle and a variable amine group designed for specificity. At least 50 distinct amine groups ranging from saturated hydrocarbon chains to bulky phenyl groups were conjugated to the core spiroepoxide with the expectation that each different amine group might impose a different selectivity for protein/enzyme targets. The spiroepoxide library was introduced into human breast carcinoma cells and screened for inhibition of cell growth using a colorimetric assay. One compound, called MJE3, was identified that blocked cell growth with an IC_{50} of 19 μM. Using click chemistry, rhodamine and biotin tags were directly conjugated to MJE3 to facilitate identification of its endogenous protein target. By isolating rhodamine-labeled bands unique to lysates from MJE3-treated cells, streptavidin enrichment and MS, phosphoglycerate mutase 1 (PGAM1) was identified as a target of MJE3. MJE3 was also shown to block PGAM1 activity in cells, but with an IC_{50} almost two times higher than that needed for cell growth inhibition, suggesting that MJE3 possibly inhibits other breast cancer proteins with higher potency to block cell proliferation. In addition, no independent evidence such as pharmacological inhibition or RNA interference of PGAM1 was provided in this study to suggest that PGAM1 is important for breast cancer growth. Nonetheless, this work represents an innovative application of an efficient chemotype library that because of its versatile chemical design, was not only able to achieve a desired cellular phenotype but also able to rapidly identify an endogenous target of the relevant small molecule inhibitor.

In addition to PGAM1, other enzymes including glutathione S-transferase omega 1 (GSTO1) have been linked to malignant breast cancers via chemotype libraries. GSTO1 is an omega-class glutathione S-transferase identified by a chemotype probe containing a sulfonate ester protein reactive group coupled to a phenyl binding group and rhodamine (PS-Rh) [26]. GSTO1 was initially detected as a 30 kDa activity present in breast cancer lysates that was dramatically increased in the invasive breast cancer cell lines MDA-MB 231 and MDA-MB 435 compared to the benign breast carcinoma lines MCF-7 and T-47D. The GST substrate glutathione blocked probe labeling of MDA-MB 435 lysates and inhibited probe labeling of recombinant GSTO1, indicating that PS-Rh was indeed labeling GSTO1 in cell lysates via an interaction near the glutathione binding site. Although no evidence was provided demonstrating that GSTO1 is important for any malignant or tumorigenic properties of these breast carcinoma cells, the correlation between high enzymatic activity of GSTO1 and aggressive tumor cell behavior *in vitro* is suggestive of a potential role for this enzyme in breast cancer progression. In a related study, the same phenyl sulfonate probe minus the bulky rhodamine tag exclusively identified a novel enoyl-CoA hydratase the authors termed ECH2 in living breast cancer cells [24].

4. COMPETITIVE ABPP

Competitive ABPP is a multifunctional variation of ABPP that can be applied to many aspects of drug discovery including lead compound discovery and optimization, selectivity determination in the presence of multiple potential targets and identification of “off-target” effects through the identification of enzymes that interact with a given drug. Like ABPP, competitive ABPP requires the profiling of complex proteomes with a fluorescent or affinity-tagged probe, often the same probe used for ABPP (e.g. FP-Rh). Before labeling with such a probe, however, proteomes are preincubated with a small molecule for which selectivity has been minimally determined as in the case of an existing drug or for which target selectivity is completely unknown as in the case of an experimentally derived molecule. If these molecules block the active site of any target enzymes in the proteome that also react with the ABPP probe, then they may compete for binding with the probe and interfere with probe-dependent signals. Failure of the ABPP probe to bind a given enzyme

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

will result in reduction of fluorescent signals in 1D gels or spectral counts from gel-free LC-MS platforms. Enzymes separated by 1D gels can be identified using trifunctional probes that are both fluorescent and affinity tagged. With gel-free MS, however, competitive ABPP can simultaneously identify enzyme targets and quantify their relative activities by revealing differences in spectral counts from probe-labeled proteomes in the presence and absence of competing compounds (i.e. ABPP probe alone is the positive control). Regardless of how protein targets are identified, one of the many advantages of competitive ABPP lies in its ability to rapidly identify target enzymes in a manner that is compatible with small molecule library screening, an example of which is described below.

In Vivo Lead Compound Discovery

Drug discovery is often performed *in vitro* using large libraries of small molecule inhibitors intended to maximize coverage of chemical space and block the activities or interactions of purified proteins. Since hits from this type of screening may not reflect true *in vivo* interactions between target and drug, methods involving drug screening directly in animal models would likely circumvent these issues. Such a method has been developed to identify potent inhibitors of the cathepsin family of cysteine proteases as anticancer drugs. A library of up to 80 distinct epoxysuccinyl-based inhibitors was synthesized with the goal of creating highly selective inhibitors that target a single cathepsin [22]. The entire library was initially screened by performing competitive ABPP on isolated rat liver proteomes. Rat liver extracts were pretreated with library compounds and then radiolabeled with a broad-spectrum ABPP probe, ^{125}I-DCG-04. A subset of 14 library compounds (AMS1-14) that showed potent inhibition of ^{125}I-DCG-04 binding in rat liver and kidney tissues was used to optimize amine substituents for a final lead series of four compounds, AMS17, AMS28, AMS30 and AMS36. These compounds were subsequently injected intraperitoneally at a concentration of 50 or 100 mg/kg into RIP1-Tag2 mice, which is a model of pancreatic cancer, and after 5 days of injections, livers, kidneys and pancreatic tumors were isolated and labeled with ^{125}I-DCG-04 to evaluate cathepsin B, X, H and C activities. The nonselective compound AMS28 potently blocked all four detectable cathepsin activities but displayed high toxicity levels that led to premature sacrifice of the animals. The remaining three compounds retained degrees of selectivity in the tumors that mirrored their selectivity patterns in rat liver extracts *in vitro*. All four compounds tended to accumulate in the liver and kidney, however, which reduced the selectivity of cathepsin inhibition presumably due to the irreversible mechanism of inhibition. Thus, competitive ABPP provided a fast, informative strategy for lead inhibitor optimization and discovery *in vivo* without prior knowledge of endogenous cathepsin substrates or the added time constraints and complications of *in vitro* screening methods that depend on thousands or millions of compounds and purified proteins. A similar strategy combining competitive ABPP with *in vivo* screening of lead compounds that reversibly block target enzymes has been reported for serine hydrolases as well [46].

Iterative Drug Discovery for Known Targets

Competitive ABPP can also be used to design small molecule inhibitors in an iterative fashion for a known enzyme target. Existing drugs or novel parent compounds serve as templates for successive chemical modifications that improve a desired characteristic of the inhibitor such as affinity for target or selectivity for a single enzyme or group of highly related enzymes. This approach was recently taken to design a specific inhibitor for α/β–hydrolase domain 6 (ABHD6), an uncharacterized brain serine hydrolase [47]. Since no parent compound known to inhibit ABHD6 was available, Li and colleagues synthesized a library of 55 novel carbamate compounds and tested each compound individually for reactivity with ABHD6 in membrane proteomes using a competitive ABPP assay. The assay readout involved the previously characterized ABPP probe, FP-Rh, which reacts with a large fraction of the serine hydrolase superfamily including ABHD6 and provides a fluorescent

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

depiction of serine hydrolase activities in whole or fractionated proteomes even for enzymes with unknown physiological substrates. Of the 55 original compounds, three competed with FP-Rh binding to exogenously expressed ABHD6. These three compounds also blocked FP-Rh binding to endogenous levels of ABHD6 in native brain proteomes, but only one proved selective by failing to bind other prominent serine hydrolases in brain such as fatty acid amide hydrolase (FAAH). Because of its relative selectivity, this carbamate was chosen for further chemical modification in an effort to improve inhibition of ABHD6. One of the terminal phenyl rings of this carbamate was modified with 20 chemically distinct groups to generate a second-generation library that was screened against ABHD6 in brain proteomes. Of these 20 compounds, a carbamate containing a *para*-carboxamide group linked to a terminal phenyl group improved potency by five times over the parent compound without altering its selectivity. This compound also blocked FP-biotin binding to ABHD6 but not to 27 other serine hydrolases identified by LC-MS, providing further evidence for ABHD6 selectivity.

Drug Screening and Selectivity Determination

Perhaps a more established application of competitive ABPP is the ability to assess the selectivity profiles of existing drugs. This has enormous implications in the identification of “off-target” effects caused by unknown drug-protein interactions. On a case-by-case basis, ABPP has already identified drugs that have broader target profiles than previously appreciated. For example, ABPP probes have recently been developed for the metalloprotease (MP) family of hydrolases, which utilize a distinct mechanism for hydrolysis of endogenous substrates compared to nucleophilic enzymes such as the serine hydrolases. Taking advantage of the fact that MPs hydrolyze peptide bonds through a zinc cofactor that coordinates active site water molecules, Sagatelian and colleagues designed a photoreactive probe called HxBP that chelates zinc atoms through a hydroxamate group and covalently binds target enzymes upon ultraviolet radiation via a benzophenone group [48]. Since the molecular backbone of HxBP is derived from GM6001 and marimastat, two potent small molecule inhibitors of matrix metalloproteases (MMPs) including MMP2 and MMP9, the target specificity of HxBP was first tested on MMP2. HxBP coupled to rhodamine (HxBP-Rh) allowed visualization of binding to purified MMP2 *in vitro*, but not its inactive zymogen, pro-MMP2. Pretreatment with GM6001 severely blocked HxBP-Rh binding to MMP2 in the presence of UV light, demonstrating that probe binding was occurring at the MMP2 active site in a photoreactive manner.

Having created a novel probe for MPs, these investigators used competitive ABPP to examine the selectivity of GM6001 by HxBP-Rh interrogation of cellular proteomes. HxBP-Rh labeled metalloproteases unrelated to MMPs including neprilysin from the secreted proteome of human melanoma cells, leucine aminopeptidase (LAP) from whole kidney proteomes and dipeptidyl peptidase III (DPPIII) from breast carcinoma cells. These enzymes were identified using trifunctional HxBP, which contains a biotin group in addition to its rhodamine fluorophore; biotin enrichment of rhodamine-labeled bands allowed MS identification of probe-binding proteins. After pretreatment of the above proteomes with increasing concentrations of GM6001, HxBP-Rh failed to label neprilysin, LAP or DPPIII, indicating that all three of these MPs were sensitive to GM6001 inhibition despite their lack of sequence homology to MMPs. Inhibition of HxBP-Rh binding by GM6001 was potent for all three enzymes (e.g. IC_{50} of 17 nM for neprilysin). Thus, HxBP-Rh identified three metalloproteases as unpredicted targets of GM6001 in an activity-based manner that bypassed the limitations of sequence homology modeling.

Antiplatelet therapy could potentially benefit from a competitive ABPP approach as a means to rigorously assess the selectivity profiles of existing drugs. Drug interactions with newly identified target proteins identified by competitive ABPP may consequently explain

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

common off-target effects that plague a fraction of patients undergoing antiplatelet therapy. The two most widely used antiplatelet drugs, aspirin and clopidogrel, would be excellent candidates for selectivity profiling because significant numbers of patients are either aspirin-resistant, clopidogrel-resistant or both [49–51]. Furthermore, some patients are overly sensitive to clopidogrel and suffer from serious bleeding events. Although genetic polymorphisms in the P2Y12 gene (the protein product of which is antagonized by clopidogrel) and cytochrome P450 genes have been proposed to partially account for clopidogrel resistance in some patients, bleeding events are not accounted for by these mechanisms and may reflect unknown protein targets of these drugs. ABPP probes currently exist for cytochrome P450 enzymes [52], but unfortunately no broadly reactive probes have been constructed that profile cyclooxygenases (which are acetylated by aspirin) or ADP receptors such as P2Y12, meaning aspirin and clopidogrel cannot yet be tested by competitive ABPP strategies. Since ABPP probes are continually being synthesized, however, new probes that recognize cell surface receptors or intracellular cyclooxygenases may eventually permit the application of competitive ABPP to antiplatelet drugs.

5. SUMMARY

In the wake of the tremendous scope and ambition of the genomic era, the first steps toward comprehension of the intricate interplay between individual proteins and protein complexes are being taken. In this review, we have described integrated platforms for global abundance-based platelet proteomics as well as activity-based protein profiling as a means to index not only the complete platelet proteome but also functional changes to the proteome under different physiological circumstances. Abundance-based methods have defined global platelet proteomes and subproteomes and traced subtle differences in platelet protein quantity. Activity-based methods have allowed investigators to begin linking diverse enzyme activity signatures with higher order cellular processes beyond the realms of global gene and protein expression. Recognition of the myriad relationships between the genome and proteome should lead to an awareness of how certain pieces fit within the greater puzzle and how the defective operation or absence of these pieces manifest as specific phenotypes, disease susceptibility or pathology. Finally, the future is likely to bring exciting mergers of new proteomic advances with genomic technologies that further our understanding of these complex relationships in health and disease, which could expedite new strategies for therapeutic intervention.

Acknowledgments

The authors acknowledge grant support from the American Heart Association (0970317N, 10GRNT3870017 to LVP and SPH) and the NIH (1R01HL092544 to LVP and 1R01A1064806 to XC). The authors also wish to thank Yanbao Yu for discussions and excellent assistance with figures.

References

1. Andonegui G, Kerfoot SM, McNagny K, Ebbert KV, Patel KD, Kubes P. Platelets express functional Toll-like receptor-4. Blood. 2005; 106(7):2417–2423. [PubMed: 15961512]
2. Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, Patel KD, Chakrabarti S, McAvoy E, Sinclair GD, Keys EM, Allen-Vercoe E, Devinney R, Doig CJ, Green FH, Kubes P. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. Nat Med. 2007; 13(4):463–469. [PubMed: 17384648]
3. Trikha M, Nakada MT. Platelets and cancer: implications for antiangiogenic therapy. Semin Thromb Hemost. 2002; 28(1):39–44. [PubMed: 11885024]
4. Lesurtel M, Graf R, Aleil B, Walther DJ, Tian Y, Jochum W, Gachet C, Bader M, Clavien PA. Platelet-derived serotonin mediates liver regeneration. Science. 2006; 312(5770):104–107. [PubMed: 16601191]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

5. Gnatenko DV, Perrotta PL, Bahou WF. Proteomic approaches to dissect platelet function: Half the story. Blood. 2006; 108(13):3983–3991. [PubMed: 16926286]
6. Macaulay IC, Carr P, Gusnanto A, Ouwehand WH, Fitzgerald D, Watkins NA. Platelet genomics and proteomics in human health and disease. J Clin Invest. 2005; 115(12):3370–3377. [PubMed: 16322782]
7. Piersma SR, Broxterman HJ, Kapci M, de Haas RR, Hoekman K, Verheul HM, Jimenez CR. Proteomics of the TRAP-induced platelet releasate. J Proteomics. 2009; 72(1):91–109. [PubMed: 19049909]
8. Garcia BA, Smalley DM, Cho H, Shabanowitz J, Ley K, Hunt DF. The platelet microparticle proteome. J Proteome Res. 2005; 4(5):1516–1521. [PubMed: 16212402]
9. Schulz C, Leuschen NV, Frohlich T, Lorenz M, Pfeiler S, Gleissner CA, Kremmer E, Kessler M, Khandoga AG, Engelmann B, Ley K, Massberg S, Arnold GJ. Identification of novel downstream targets of platelet glycoprotein VI activation by differential proteome analysis: implications for thrombus formation. Blood. 2010; 115(20):4102–4110. [PubMed: 20107233]
10. Elias JE, Haas W, Faherty BK, Gygi SP. Comparative evaluation of mass spectrometry platforms used in large-scale proteomics investigations. Nat Methods. 2005; 2(9):667–675. [PubMed: 16118637]
11. Yu Y, Leng T, Yun D, Liu N, Yao J, Dai Y, Yang P, Chen X. Global analysis of the rat and human platelet proteome-the molecular blueprint for illustrating multi-functional platelets and cross-species function evolution. Proteomics. 2010; 10(13):2444–2457. [PubMed: 20443191]
12. Kuchay SM, Chishti AH. Calpain-mediated regulation of platelet signaling pathways. Curr Opin Hematol. 2007; 14(3):249–254. [PubMed: 17414215]
13. Weyrich AS, Dixon DA, Pabla R, Elstad MR, McIntyre TM, Prescott SM, Zimmerman GA. Signal-dependent translation of a regulatory protein, Bcl-3, in activated human platelets. Proc Natl Acad Sci USA. 1998; 95(10):5556–5561. [PubMed: 9576921]
14. Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, Mann M. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics. 2005; 4(9):1265–1272. [PubMed: 15958392]
15. Kanehisa M, Goto S, Hattori M, Aoki-Kinoshita KF, Itoh M, Kawashima S, Katayama T, Araki M, Hirakawa M. From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res. 2006; 34(Database issue):D354–D357. [PubMed: 16381885]
16. Chen F, Mackey AJ, Stoeckert CJ Jr, Roos DS. OrthoMCL-DB: querying a comprehensive multi-species collection of ortholog groups. Nucleic Acids Res. 2006; 34(Database issue):D363–D368. [PubMed: 16381887]
17. Cravatt BF, Wright AT, Kozarich JW. Activity-based protein profiling: from enzyme chemistry to proteomic chemistry. Annu Rev Biochem. 2008; 77:383–414. [PubMed: 18366325]
18. Paulick MG, Bogyo M. Application of activity-based probes to the study of enzymes involved in cancer progression. Curr Opin Genet Dev. 2008; 18(1):97–106. [PubMed: 18294838]
19. Adam GC, Sorensen EJ, Cravatt BF. Trifunctional chemical probes for the consolidated detection and identification of enzyme activities from complex proteomes. Mol Cell Proteomics. 2002; 1(10):828–835. [PubMed: 12438565]
20. Kidd D, Liu Y, Cravatt BF. Profiling serine hydrolase activities in complex proteomes. Biochemistry. 2001; 40(13):4005–4015. [PubMed: 11300781]
21. Liu Y, Patricelli MP, Cravatt BF. Activity-based protein profiling: the serine hydrolases. Proc Natl Acad Sci USA. 1999; 96(26):14694–14699. [PubMed: 10611275]
22. Sadaghiani AM, Verhelst SH, Gocheva V, Hill K, Majerova E, Stinson S, Joyce JA, Bogyo M. Design, synthesis, and evaluation of in vivo potency and selectivity of epoxysuccinyl-based inhibitors of papain-family cysteine proteases. Chem Biol. 2007; 14(5):499–511. [PubMed: 17524981]
23. Speers AE, Adam GC, Cravatt BF. Activity-based protein profiling in vivo using a copper(i)-catalyzed azide-alkyne [3 + 2] cycloaddition. J Am Chem Soc. 2003; 125(16):4686–4687. [PubMed: 12696868]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

24. Speers AE, Cravatt BF. Profiling enzyme activities in vivo using click chemistry methods. Chem Biol. 2004; 11(4):535–546. [PubMed: 15123248]
25. Kolb HC, Sharpless KB. The growing impact of click chemistry on drug discovery. Drug Discov Today. 2003; 8(24):1128–1137. [PubMed: 14678739]
26. Adam GC, Sorensen EJ, Cravatt BF. Proteomic profiling of mechanistically distinct enzyme classes using a common chemotype. Nat Biotechnol. 2002; 20(8):805–809. [PubMed: 12091914]
27. Speers AE, Cravatt BF. A tandem orthogonal proteolysis strategy for high-content chemical proteomics. J Am Chem Soc. 2005; 127(28):10018–10019. [PubMed: 16011363]
28. Wong JW, McRedmond JP, Cagney G. Activity profiling of platelets by chemical proteomics. Proteomics. 2009; 9(1):40–50. [PubMed: 19053083]
29. Obergfell A, Eto K, Mocsai A, Buensuceso C, Moores SL, Brugge JS, Lowell CA, Shattil SJ. Coordinate interactions of Csk, Src, and Syk kinases with [alpha]IIb[beta]3 initiate integrin signaling to the cytoskeleton. J Cell Biol. 2002; 157(2):265–275. [PubMed: 11940607]
30. Verma S, Wang CH, Li SH, Dumont AS, Fedak PW, Badiwala MV, Dhillon B, Weisel RD, Li RK, Mickle DA, Stewart DJ. A self-fulfilling prophecy: C-reactive protein attenuates nitric oxide production and inhibits angiogenesis. Circulation. 2002; 106(8):913–919. [PubMed: 12186793]
31. Zwaka TP, Hombach V, Torzewski J. C-reactive protein-mediated low density lipoprotein uptake by macrophages: implications for atherosclerosis. Circulation. 2001; 103(9):1194–1197. [PubMed: 11238260]
32. Sabatine MS, Morrow DA, de Lemos JA, Gibson CM, Murphy SA, Rifai N, McCabe C, Antman EM, Cannon CP, Braunwald E. Multimarker approach to risk stratification in non-ST elevation acute coronary syndromes: simultaneous assessment of troponin I, C-reactive protein, and B-type natriuretic peptide. Circulation. 2002; 105(15):1760–1763. [PubMed: 11956114]
33. Seo D, Wang T, Dressman H, Herderick EE, Iversen ES, Dong C, Vata K, Milano CA, Rigat F, Pittman J, Nevins JR, West M, Goldschmidt-Clermont PJ. Gene expression phenotypes of atherosclerosis. Arterioscler Thromb Vasc Biol. 2004; 24(10):1922–1927. [PubMed: 15297278]
34. Jessani N, Liu Y, Humphrey M, Cravatt BF. Enzyme activity profiles of the secreted and membrane proteome that depict cancer cell invasiveness. Proc Natl Acad Sci USA. 2002; 99(16): 10335–10340. [PubMed: 12149457]
35. Chiang KP, Niessen S, Saghatelian A, Cravatt BF. An enzyme that regulates ether lipid signaling pathways in cancer annotated by multidimensional profiling. Chem Biol. 2006; 13(10):1041–1050. [PubMed: 17052608]
36. Greenbaum D, Medzihradszky KF, Burlingame A, Bogyo M. Epoxide electrophiles as activity-dependent cysteine protease profiling and discovery tools. Chem Biol. 2000; 7(8):569–581. [PubMed: 11048948]
37. Vasiljeva O, Papazoglou A, Kruger A, Brodoefel H, Korovin M, Deussing J, Augustin N, Nielsen BS, Almholt K, Bogyo M, Peters C, Reinheckel T. Tumor cell-derived and macrophage-derived cathepsin B promotes progression and lung metastasis of mammary cancer. Cancer Res. 2006; 66(10):5242–5250. [PubMed: 16707449]
38. Borodovsky A, Ovaa H, Kolli N, Gan-Erdene T, Wilkinson KD, Ploegh HL, Kessler BM. Chemistry-based functional proteomics reveals novel members of the deubiquitinating enzyme family. Chem Biol. 2002; 9(10):1149–1159. [PubMed: 12401499]
39. Ovaa H, Kessler BM, Rolen U, Galardy PJ, Ploegh HL, Masucci MG. Activity-based ubiquitin-specific protease (USP) profiling of virus-infected and malignant human cells. Proc Natl Acad Sci USA. 2004; 101(8):2253–2258. [PubMed: 14982996]
40. Rolen U, Kobzeva V, Gasparjan N, Ovaa H, Winberg G, Kisseljov F, Masucci MG. Activity profiling of deubiquitinating enzymes in cervical carcinoma biopsies and cell lines. Mol Carcinog. 2006; 45(4):260–269. [PubMed: 16402389]
41. Salisbury CM, Cravatt BF. Activity-based probes for proteomic profiling of histone deacetylase complexes. Proc Natl Acad Sci USA. 2007; 104(4):1171–1176. [PubMed: 17227860]
42. Milner JM, Elliott SF, Cawston TE. Activation of procollagenases is a key control point in cartilage collagen degradation: interaction of serine and metalloproteinase pathways. Arthritis Rheum. 2001; 44(9):2084–2096. [PubMed: 11592371]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

43. Milner JM, Kevorkian L, Young DA, Jones D, Wait R, Donell ST, Barksby E, Patterson AM, Middleton J, Cravatt BF, Clark IM, Rowan AD, Cawston TE. Fibroblast activation protein alpha is expressed by chondrocytes following a pro-inflammatory stimulus and is elevated in osteoarthritis. Arthritis Res Ther. 2006; 8(1):R23. [PubMed: 16507127]
44. Evans MJ, Saghatelian A, Sorensen EJ, Cravatt BF. Target discovery in small-molecule cell-based screens by in situ proteome reactivity profiling. Nat Biotechnol. 2005; 23(10):1303–1307. [PubMed: 16200062]
45. Liu S, Widom J, Kemp CW, Crews CM, Clardy J. Structure of human methionine aminopeptidase-2 complexed with fumagillin. Science. 1998; 282(5392):1324–1327. [PubMed: 9812898]
46. Leung D, Hardouin C, Boger DL, Cravatt BF. Discovering potent and selective reversible inhibitors of enzymes in complex proteomes. Nat Biotechnol. 2003; 21(6):687–691. [PubMed: 12740587]
47. Li W, Blankman JL, Cravatt BF. A functional proteomic strategy to discover inhibitors for uncharacterized hydrolases. J Am Chem Soc. 2007; 129(31):9594–9595. [PubMed: 17629278]
48. Saghatelian A, Jessani N, Joseph A, Humphrey M, Cravatt BF. Activity-based probes for the proteomic profiling of metalloproteases. Proc Natl Acad Sci USA. 2004; 101(27):10000–10005. [PubMed: 15220480]
49. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Ramirez C, Sabate M, Jimenez-Quevedo P, Hernandez R, Moreno R, Escaned J, Alfonso F, Banuelos C, Costa MA, Bass TA, Macaya C. Influence of aspirin resistance on platelet function profiles in patients on long-term aspirin and clopidogrel after percutaneous coronary intervention. Am J Cardiol. 2006; 97(1):38–43. [PubMed: 16377281]
50. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Alfonso F, Macaya C, Bass TA, Costa MA. Variability in individual responsiveness to clopidogrel: clinical implications, management, and future perspectives. J Am Coll Cardiol. 2007; 49(14):1505–1516. [PubMed: 17418288]
51. Gum PA, Kottke-Marchant K, Welsh PA, White J, Topol EJ. A prospective, blinded determination of the natural history of aspirin resistance among stable patients with cardiovascular disease. J Am Coll Cardiol. 2003; 41(6):961–965. [PubMed: 12651041]
52. Wright AT, Song JD, Cravatt BF. A suite of activity-based probes for human cytochrome P450 enzymes. J Am Chem Soc. 2009; 131(30):10692–10700. [PubMed: 19583257]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

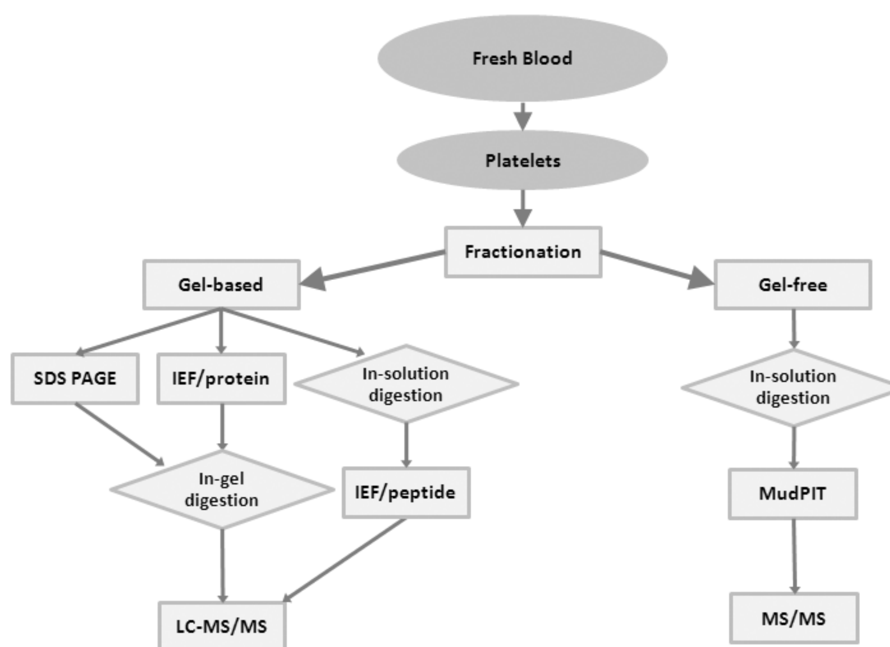

Figure 1. Integrated proteomic workflow used to define the rat platelet proteome

Rat platelet proteins were identified by two distinct proteomic workflows involving gel-based or gel-free methods [11]. Detergent solubilized proteins were subjected to gel-based fractionation (left) involving denaturing, one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE) or isoelectric focusing (IEF/protein) of whole proteins. Proteins were digested in-gel and the subsequent peptides were analyzed by LC-MS/MS with LTQ (for both SDS-PAGE and IEF resolved proteins) or QTOF (for SDS-PAGE resolved proteins only) instruments. Alternatively, urea was used to generate a soluble platelet proteome that was digested in solution, fractionated by IEF and analyzed by an LTQ mass spectrometer (in-solution digestion). The second workflow (right) also began with a urea solubilized proteome, but these proteins were subjected to gel-free multidimensional protein identification technology (MudPIT), which is typically a two phase LC separation method composed of strong cation exchange coupled to RPLC followed by MS/MS.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

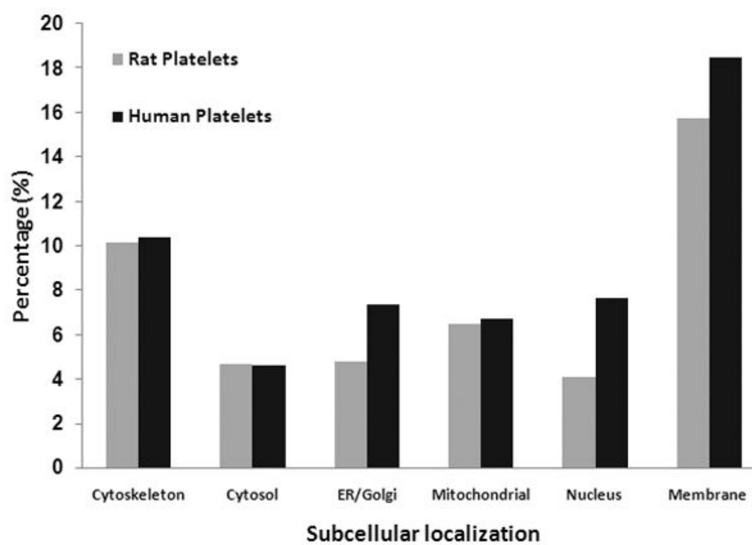

Figure 2. Comparison of the rat and human proteomes

The subcellular localization profile of rat platelet proteins identified by Yu et al. was compared to proteins from the human proteome. Data was expressed as the number of proteins having the indicated localization over the total number of proteins in that proteome (837 for rat and 1053 for human). Due to overlap, percentages do not equal 100%.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

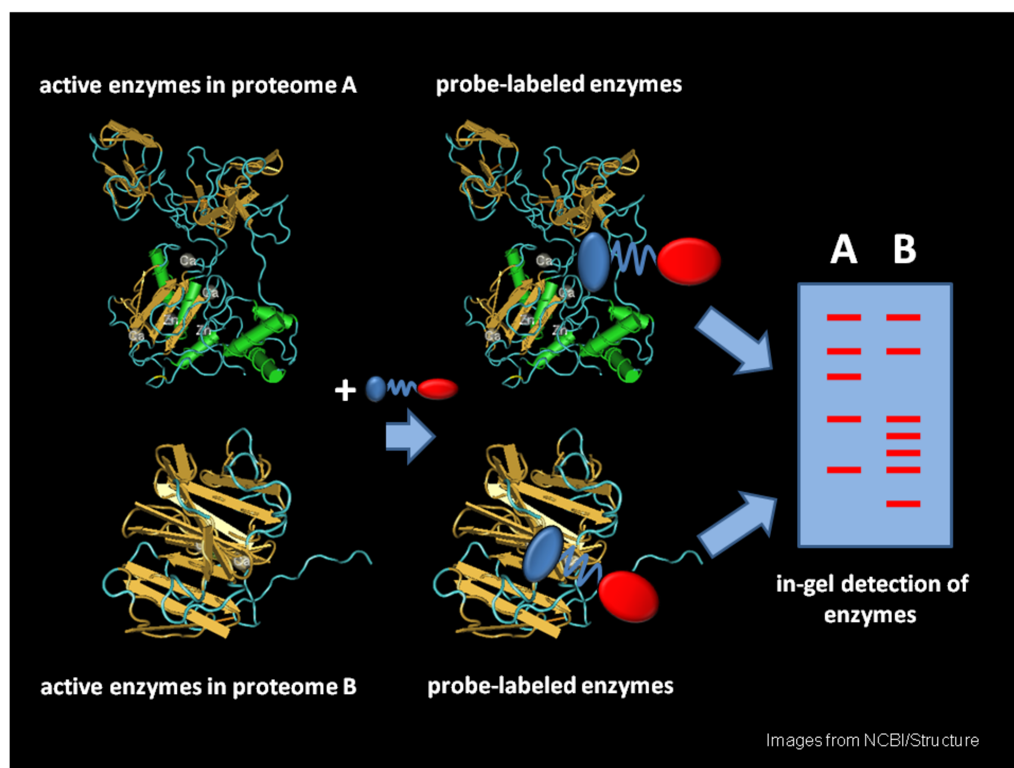

Figure 3. Overview of gel-based ABPP

Enzymes isolated from distinct proteomes can be detected using gel-based ABPP. Proteins derived from separate proteomes are prepared from a variety of sources (i.e. proteomes A and B represent platelets treated with various drugs, wildtype and knockout cells, healthy and diseased tissue, etc.) and profiled with an activity-based probe (blue and red circles) containing a broadly reactive chemotype or a specific protein reactive group that targets a particular enzyme class. Probe-labeled enzymes are subjected to SDS-PAGE and detected via in-gel fluorescence or radiography depending on the reporter group present on the probe. Biotin-labeled probes can be detected via Western blotting (not shown). Enzyme activity patterns are then quantified and compared across proteomes to identify differentially regulated enzyme activities, which could represent true changes in activity or changes in protein expression. Higher resolution gel-free methods are also compatible with ABPP (see text).