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REVIEW

The role of targeted chemical proteomics in pharmacology

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Traditionally, proteomics is the high-throughput characterization of the global complement of proteins in a biological system using cutting-edge technologies (robotics and mass spectrometry) and bioinformatics tools (Internet-based search engines and databases). As the field of proteomics has matured, a diverse range of strategies have evolved to answer specific problems. Chemical proteomics is one such direction that provides the means to enrich and detect less abundant proteins (the 'hidden' proteome) from complex mixtures of wide dynamic range (the 'deep' proteome). In pharmacology, chemical proteomics has been utilized to determine the specificity of drugs and their analogues, for anticipated known targets, only to discover other proteins that bind and could account for side effects observed in preclinical and clinical trials. As a consequence, chemical proteomics provides a valuable accessory in refinement of second- and third-generation drug design for treatment of many diseases. However, determining definitive affinity capture of proteins by a drug immobilized on soft gel chromatography matrices has highlighted some of the challenges that remain to be addressed. Examples of the different strategies that have emerged using well-established drugs against pharmaceutically important enzymes, such as protein kinases, metalloproteases, PDEs, cytochrome P450s, etc., indicate the potential opportunity to employ chemical proteomics as an early-stage screening approach in the identification of new targets.

Abbreviations

16-BAC, 16-benzyldimethyl-*n*-hexadecylammonium chloride; ABPP, activity-based protein profiling; ESI MS, electrospray ioinization mass spectrometry; FP, fluorophosphonate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iTRAQ, isobaric tags for relative and absolute quantitation; MALDI MS, matrix-assisted laser desorption/ionization mass spectrometry; Mudpit, multidimensional protein identification technology; PAC, primary affinity capture; PFF, peptide fragment fingerprint; PMF, peptide mass fingerprint; PMMA, poly methylmethacrylate; SAC, secondary affinity capture; SILAC, stable isotope labelling by/with amino acids in cell culture

Introduction to proteomics

In the early 1990s, the world of the protein biochemist was transformed by a series of technological developments. These included (i) access to commercially available, user-friendly mass spectrometers (MS) for the analysis of large biomolecules; (ii) the emergence of freely available bio-information and databases containing protein and DNA sequences on the Internet; and (iii) the creation of search engines that enable MS data content to be rapidly compared with *in silico* versions to provide a protein identification. No longer was it necessary for extensive, laborious multi-step purification to isolate a single protein in order to determine its sequence by Edman degradation chemistry (Tsugita, 1987). Now it was possible to analyse thousands of proteins with minimum sample preparation in automated high-throughput workflows (Aebersold and Mann, 2003). This revolution became known as proteomics which, in 1994, was defined by Marc Wilkins as 'the

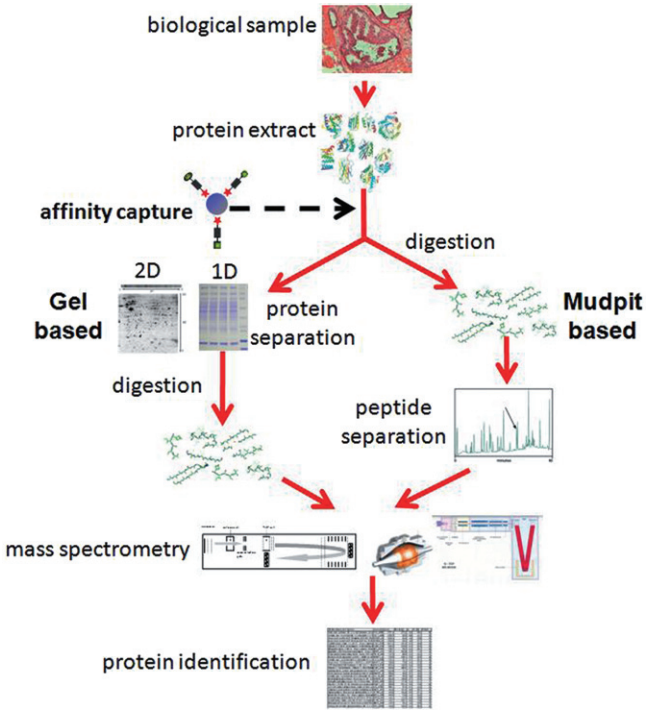

Figure 1

An outline of the conventional proteomics strategies that have developed for global protein profiling either by means of one-dimensional (1D)/2D gel separation of proteins or through 1D/2D HPLC separation of peptides (MudPIT is a shotgun proteomics technique in which the total protein mixture is digested with a protease such as trypsin. The resulting peptide mixture is separated by chromatographic and/or electrophoretic methods for analysis and identification of the protein of origin by MS/MS. The strategy enables identification of thousands of proteins in high-throughput workflows, particularly for quantitative proteomics).

examination of a complete set of proteins synthesized by a cell under a given set of physiological or developmental conditions'. This also meant that the study of proteins became more widely incorporated into other fields of study such as oncology, neurology, plant physiology, microbiology, virology, etc. In this respect, pharmacology and the pharmaceutical industry were no exception.

Initially, proteomics was used to systematically catalogue all the proteins in a model system, organism, tissue or cell line (Figure 1). Proteins were extracted from the biological source, either as a total complement, or by incorporating sub-cellular fractionation methods such as detergent solubilization of membrane proteins or ultracentrifugation for organelle isolation. Two separation approaches have been applied for the characterization of the protein mixtures, mainly (but not exclusively) driven by the choice of mass spectrometer available for analysis. If matrix-assisted laser desorption/ionization MS (MALDI MS) is the preferred analytical technique, then the separation of thousands of proteins by two-dimensional (2D) gel electrophoresis provides a powerful approach for displaying and comparing biological samples (e.g. healthy vs. normal tissues). Proteins can be digested with proteases such as trypsin, in-gel or after blotting onto membranes, to allow MS analysis. MALDI MS produce singly charged ions permitting generation of a spectrum of a complex mixture, which means that peptides from a digested protein can be analysed simultaneously as a single sample. The mass signals in the spectrum, or peptide mass fingerprint (PMF), are unique to the original protein based on the specific location of protease cleavage sites in the amino acid sequence (in the case of trypsin, at the C-terminal of lysine and arginine). The signals are searched against a database of all known protein sequences digested *in silico* under the same conditions, to identify the closest match and therefore the protein present in the 2D gel. Using robotic systems for gel spot excision and in-gel digestion, hundreds of proteins spots can be identified. However, it is not possible to identify all components of the proteome (strongly acidic, strongly basic, low molecular weight and very large proteins are frequently lost during separation), due to limitations of 2D gel separation properties. Therefore, alternative strategies that proteolytically digest the total protein mixture first and then subsequently separate the peptides have been developed. As a total pool of peptides is created from all the proteins present, MS analysis of the intact peptides cannot be employed to identify the original proteins. Instead, two separations methods (typically ion exchange and reverse-phase HPLC) are used to simplify the mixture before analysis of each peptide by MS/MS [known as multidimensional protein identification technology (Mudpit) or shotgun proteomics] (Link *et al.*, 1999). MS/MS analysis using ESI tandem (Q-TOF, ion trap, Orbitrap, Fourier Transform) or MALDI TOF-TOF MS permits isolation of peptides in the instrument, followed by their fragmentation and analysis of product ions. The MS/MS spectrum (or peptide fragment fingerprint – PFF) is unique based on the sequence of amino acids present, the mass of the intact peptide and the choice of protease cleavage. The PFF can be used to identify the original protein by comparing protein sequences treated *in silico* to the same conditions as used experimentally.

For a decade, many pharmaceutical companies invested heavily in mass spectrometers, robotics and human resources to explore proteomes that might yield new targets for drug development. Mostly this was unsuccessful due to unanticipated challenges, including (i) the absence of reliable quantitative tools to determine changes in protein expression when comparing healthy and disease tissues or control and experiment samples; (ii) protein heterogeneity (i.e. the presence of post translational modifications and splice variants resulting in multiple isoforms); and (iii) dynamic range (8 to 10 orders of magnitude variation in protein concentration). Good progress has been made in quantitative proteomics with the development of a variety of strategies. These comprise stable isotope chemistries (SILAC, iTRAQ, ICAT, ^{18}O, QconCAT) incorporated into sample-specific proteins and peptides to provide MS signature signals, and non-labelling approaches, which correlate MS signal intensity or frequency of detection (spectral counting, multiple reaction monitoring) with the amount of identified peptide, and therefore protein, present in the sample (Ong and Mann, 2005). The problems of dynamic range remain significant. Despite the generation of two-, three- and even four-dimensional separation strategies to fractionate protein and peptide mixtures into manageable packages that allow MS to detect less abun-

dant signals from low copy number components, the question remains – have the deepest levels of the proteome been reached or are there even scarcer proteins that still remain below the level of detection? In other words, of the 21 416 human protein-coding genes, approximately 8000 (38%) have so far not been detected as expressed products in any organ, tissue or cell line. A change from systematic, non-hypothesis-driven documentation of proteins in a biological sample to a more targeted hypothesis-driven approach is required to discover these elusive entities. This review covers one of the strategies, chemical proteomics, which has emerged for the analysis of individual or groups of proteins, and has particular relevance to pharmacology, as an adjunct to mainstream proteomics.

Chemical proteomics

Chemical proteomics can also be defined as bespoke affinity capture or affinity chromatography. It comprises the design of drug, co-factor, substrate or inhibitor analogues that can be immobilized on a suitable medium to trap specific proteins or subgroups of interest. Affinity chromatography has been used for nearly half a century as part of workflows to isolate proteins. With the advent of proteomics, like many traditional protein biochemistry techniques, affinity chromatography almost disappeared. However, as part of the advancement from global proteomics to hypothesis-driven investigation, affinity enrichment to access specific groups of proteins for MS characterization has re-surfaced as a valuable technique. There remains a large array of commercially available affinity media that are being used to enrich sub-proteomes based on function (Lee and Lee, 2004), including triazine dyes (albumin, dehydrogenases), nucleotide (kinases), lectin (glycoproteins), lysine/gelatin (proteases), heparin (DNA binding proteins, growth factors, coagulation factors), Protein G/Protein A (antibodies), antibody-based (immunoaffinity/immunoprecipitation) and immobilized metal affinity chromatography/IMAC (phosphopeptides). New tools in molecular biology have also emerged for the purification of recombinant proteins, which incorporate a unique functional group (e.g. His-Tag, GST-Tag) to allow efficient affinity-based separation from host proteins (Bauer and Kuster, 2003).

This review, however, describes some of the reagents that have been created in the last decade, designed to mimic naturally occurring substrates, co-factors or pharmaceuticals, to capture intact proteins of biological importance. One of the key differences between chemical proteomics and the traditional affinity methods is scale; in general, the amounts of samples and chromatography materials are substantially lower (microlitres rather than millilitres or litres) due to the availability of sensitive MS and Western blotting to analyse captured proteins.

Not all chemical proteomics is based on covalent immobilization of an active compound analogue, comprising a reactive group (e.g. primary amine) covalently immobilized on activated soft gels (e.g. epoxy-agarose) to capture proteins of interest, an approach defined here as primary affinity capture (PAC) (Figures 2A and 3). New chemistries incorporating a protein-recognition ligand, a soluble cross-linker and an affinity recognition group, such as biotin, have been designed to create soluble probes that interact with target proteins in solution. Biotinylated probes can be used in two approaches: (i) they can be immobilized on avidin or streptavidin agarose and used as described for PAC above (Figure 2C), or (ii) they can be added to the protein sample first, bind targets of interest, before capture on avidin/streptavidin agarose to remove background proteins – secondary affinity capture (SAC) (Figure 2B). Frequently, these probes incorporate a chromaphore instead of biotin. Inclusion of the affinity capture step is immediately after protein extraction and before the mainstream proteomics workflow (Figure 1). Whichever strategy is used, retaining protein functionality during protein extract/sample preparation is important for chemical proteomics. Hence, mechanical methods of protein extraction from cells and tissues (sonication, vortexing, maceration, homogenization, extrusion) are preferable to chemical methods (denaturants such as urea and guanidine, acid precipitation and detergents such as SDS).

Serine hydrolases

Activity-based protein profiling (ABPP), defined by the laboratory of Ben Cravatt (Speers *et al.*, 2003) as the bespoke synthesis of small molecule probes that can be used to identify specific proteins in a complex proteome, was one of the first examples of SAC. Initially, ABPP was used to characterize a group of enzymes broadly defined as serine hydrolases, comprising subfamilies of proteases, lipases, esterases, amidases and transacylases (Liu *et al.*, 1999; Kidd *et al.*, 2001). Many serine hydrolases have been implicated in pathophysiological processes such as drug metabolism, blood coagulation, neurotransmitter catabolism, lipid metabolism, angiogenesis and cancer. The majority of the enzymes have a common mechanism and structure, sharing an α/β hydrolase fold (parallel β sheets flanked by α helices) to create the catalytic tripeptide site – SHD or SHE. As a consequence, most serine hydrolases are irreversibly inhibited by fluorophosphonate (FP), compounds that bind stoichiometrically in the active site. An FP analogue incorporating polyethylene glycol (PEG) linked to biotin was added to protein extracts from rat testes. In the first instance, detection of modified proteins was demonstrated by blotting with avidin after separation by SDS-PAGE. In subsequent experiments, eight FP-linked serine hydrolases were identified by capture on avidin-agarose beads, separation on SDS-PAGE, in-gel digestion and MS analysis (Table 1).

The lipase and carboxylesterase subfamily of serine hydrolases play an important role in liver metabolism of lipids and detoxification of xenobiotics. A series of fluorescent (7-nitrobenz-2-oxa-1,3-diazole) and biotinylated alkylphosphonates (ethyl, triglyceride and cholesteryl) were prepared to mimic lipase and carboxyesterase substrates and characterize the enzyme complement in mouse liver cytosolic, membrane and lipid fractions (Birner-Gruenberger and Hermetter, 2007). The fluorescent probes were used to detect proteins directly in 2D gel electrophoresis for in-gel trypsin digestion and identification by ESI MS/MS without chromatography enrichment. Many spots, however, were found to be contaminated with functionally unrelated proteins. In an alternative

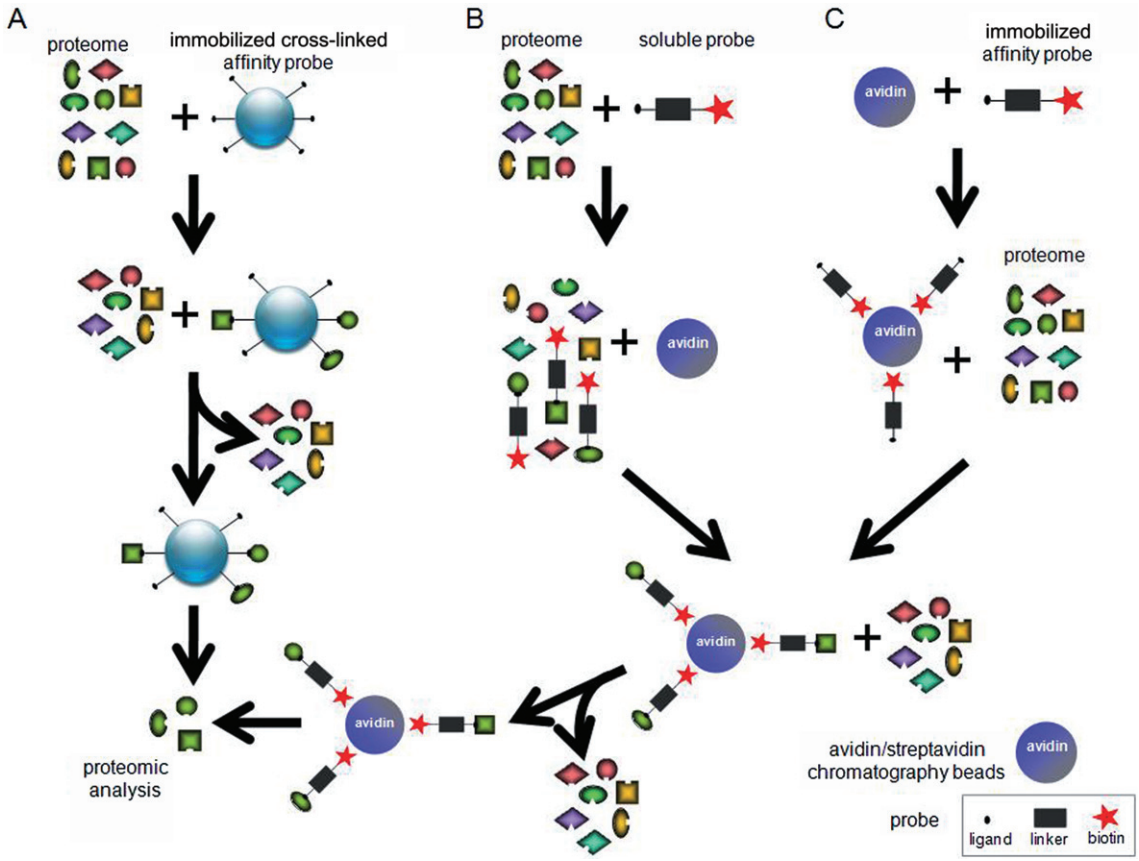

Figure 2

Affinity capture strategies. (A) Primary affinity capture in which the protein recognition ligand is covalently immobilized on soft gels or magnetic beads for conventional chromatography by binding target proteins and exclude contaminants. (B) Secondary affinity capture in which a soluble probe is combined with the protein extract before binding to avidin/streptavidin beads to isolate target proteins. (C) A hybrid approach in which the soluble probe is immobilized on avidin/streptavidin beads before addition of the protein extract to isolate the target proteins. In all cases, bound proteins are analysed by gel electrophoresis and/or MS for protein identification.

strategy, biotinylated ethyl phosphonate was used to isolate the enzymes by avidin-based affinity purification (Birner-Gruenberger *et al.*, 2008). Captured proteins were analysed by SDS-PAGE, Sypro Ruby-stained, in-gel digestion and MS identification. Using both techniques, a total of 37 lipid-binding proteins and enzymes were identified, including 11 lipases and 8 esterases.

Proteases

Proteases are a superfamily of enzymes that can be subdivided further into serine, threonine, cysteine, aspartate, metallo- and glutamic acid proteases. This classification is based on common structural requirements in their catalytic activities and on cleavage properties – exopeptidases (sequential removal of N- or C-terminal amino acids) or endoproteinases (cleaving peptide bonds within a protein). Proteases are involved in a multitude of physiological reactions including digestion of food proteins, highly regulated cascades systems (e.g. the blood clotting cascade, the complement system, apoptosis pathways) and extracellular matrix remodelling in soft tissues and joints. For many groups of proteases, protein-based inhibitors have been identified, which play important specific physiological roles in the regulation of proteolytic activity; however, it is the application of small molecule ligands and peptide mimics that are the focus of chemical proteomics.

E64 (Figure 4), which irreversibly inhibits many members of the cysteine protease family by epoxy-derivatization of the catalytic site cysteine residue, was modified to incorporate ^{125}I-iodotyrosine residues for autoradiography and a biotinylated spacer arm for affinity capture (Greenbaum *et al.*, 2000). Mouse melanoma cell line or rat kidney extracts were used as a source for enrichment using one of the probes, DCG-04, and cathepsins B, H and L identified. However, additional purification steps were required before the proteins were identified by MS.

Metalloproteases, the majority of which have zinc-dependent catalytic activity (metzincins), can be further subdivided into MMPs (23 members), adamlysins (ADAMs – a disintegrin and metalloproteinase, 19 members), ADAMTS peptidases (a disintegrin and metalloproteinase with thrombospondin motif 1, 19 members) and aminopeptidases. In

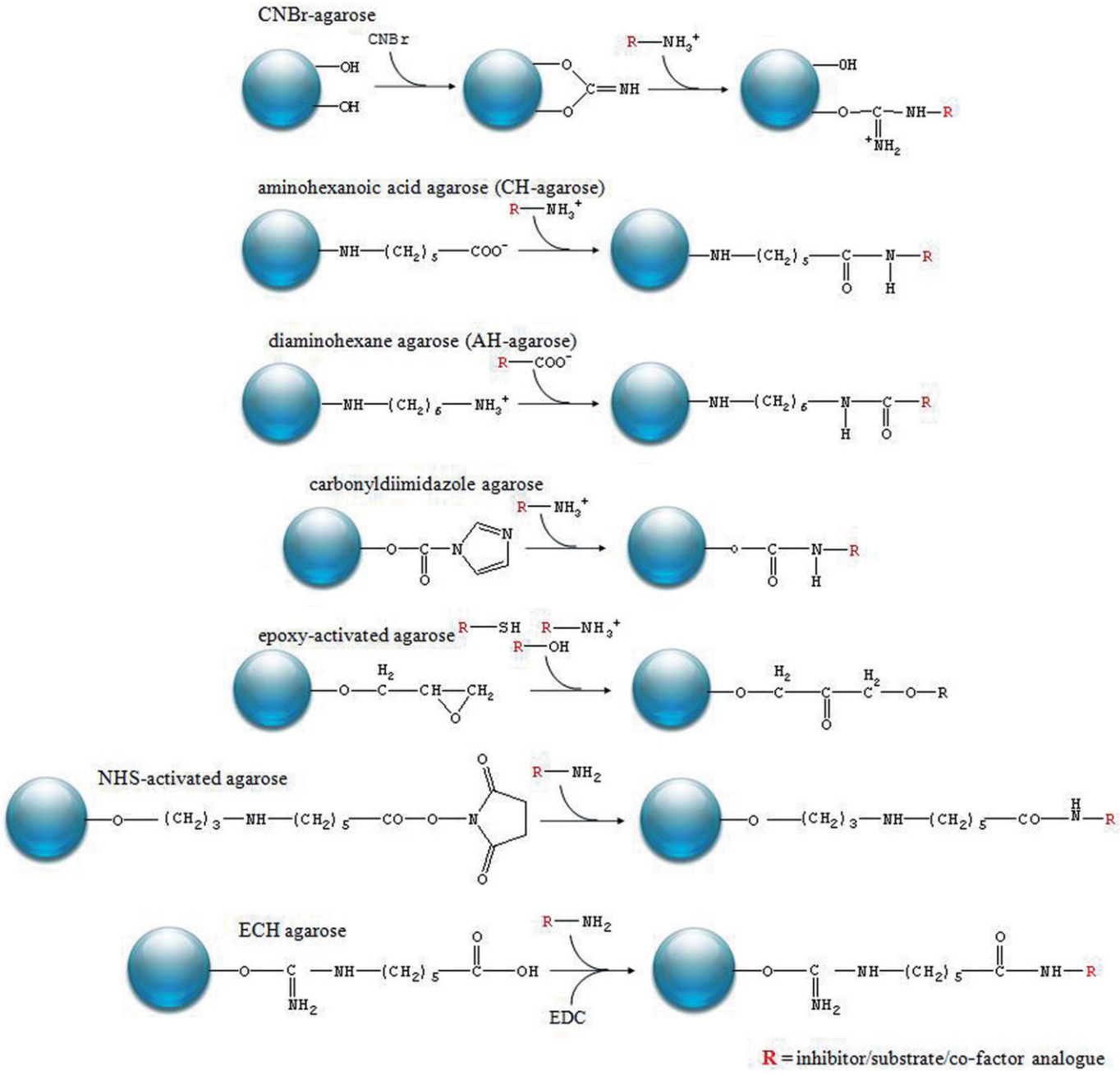

Figure 3

Coupling chemistries for immobilization of protein recognition ligand or active compound to activated soft gels or magnetic beads for PAC.

humans, MMPs mediate extracellular matrix and basement membrane degradation (Murphy and Nagase, 2008). Their activity is tightly regulated at many levels, being expressed as a prepro-MMP, initially cleaved to a latent pro-MMP and subject to further proteolytic cleavage to provide an active protease. There are four naturally occurring inhibitors, tissue inhibitors of metalloproteinases (TIMPs), with different tissue expression profiles that preferentially inhibit different repertoires of MMPs to regulate their activity. Extensive studies have provided evidence for involvement of MMPs and TIMPs in numerous physiological and pathological processes involving tissue turnover, including arthritic diseases, periodontal disease, processing of cytokines, tumour progression and metastasis. For the most part, MMPs are low abundance proteins, difficult to purify and therefore appropriate targets for affinity enrichment. Whereas cysteine proteases have a number of irreversible inhibitors ideally suited to chemical proteomics, MMPs do not use an active-site nucleophile that can be manipulated for affinity probe design and require an alternative approach.

Two hydroxamate-containing, peptide-based compounds (PLG-NHOH and TAPI-2) were prepared (Freije *et al.*, 2006)

Table 1

Summary of affinity methods – proteases, esterases, cytochrome P450s

Protein target	Drug/inhibitor analogues	Compound class	Affinity method	Separation method	Detection method/MS	Identified proteins	Reference
Proteases							
Metalloproteinases	Ilomostat	Hyroxamate	Fluorescent probe	SDS-PAGE/avidin blot	ESI LCQ MS	MMP-2, -7, -9, MME, DPP3, leucine aminopeptidase	(Saghatelian *et al.*, 2004)
Metalloproteinases	tripeptide array	Hyroxamate	SAC, fluorescent probe	Avidin chromatography, SDS-PAGE	ESI LCQ MS	ADAM and ADAMTS family, APG7, 8 aminopeptidases	(Sieber *et al.*, 2006)
MMPs	Batimastat	Hyroxamate	PAC	SDS-PAGE	Zymogram, immunoblot	MMP-2, -9, -14	(Hesek *et al.*, 2006)
MMPs	PLG-NHOH/TAPI-2	Hyroxamate	PAC	SDS-PAGE	Zymogram, enzyme activity	MMP-1, -7, -8, -9, -10, -12, -13	(Freije *et al.*, 2006)
MMPs		Phosphinic, azido		SDS-PAGE	Fluorescence imaging	MMP-2, -3, -8, -9, -11, -12, -13, -14	(David *et al.*, 2007)
MMPs		Phosphinic, azido, tritiated			Radiolabel, ESI IT MS	MMP-12	(Dabert-Gay *et al.*, 2008)
MMPs		Phosphinic, azido, biotin	SAC	SDS-PAGE/avidin blot	MALDI MS	MMP-8, -12	(Bregant *et al.*, 2009)
Cysteine proteases	E-64	Epoxy	SAC	SDS-PAGE/avidin blot	MALDI MS/ESI Q-TOF MS, autoradiography	cathepsins B, H and L	(Greenbaum *et al.*, 2000)
Serine hydrolases		Fluorophosphonate	SAC	SDS-PAGE/avidin blot	MALDI MS/ESI LCQ MS	CES1, PAFAH2, PLA2G7, carboxylesterase 10, long chain acyl CoA hydrolase 2, acylpeptidase, prolyloligopeptidase	(Kidd *et al.*, 2001)
Esterases		Ethyl, triglycerol, cholesterol phosphonates	SAC, fluorescent probe	2D gels, avidin chromatography	ESI LTQ/LCQ MS	37 lipid-binding proteins	(Birner-Gruenberger and Hermetter, 2007; Birner-Gruenberger *et al.*, 2008)
Cytochrome P450s	2-EN	Aryl alkynes	Fluorescent probe	SDS-PAGE, ESI MS/MS	Fluorescence scanning, ESI MS/MS	CYP1a1, 1a2, 2c29, 2d9, 2d10, 3a11	(Wright and Cravatt, 2007)
Cytochrome P450s	Multiple	Aryl alkynes	Fluorescent probe	SDS-PAGE	Fluorescence scanning	CYP 1A1, 1A2, 1B1, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 2J2, 3A4, 4A11, 4F2, 19A1 (aromatase)	(Wright *et al.*, 2009)

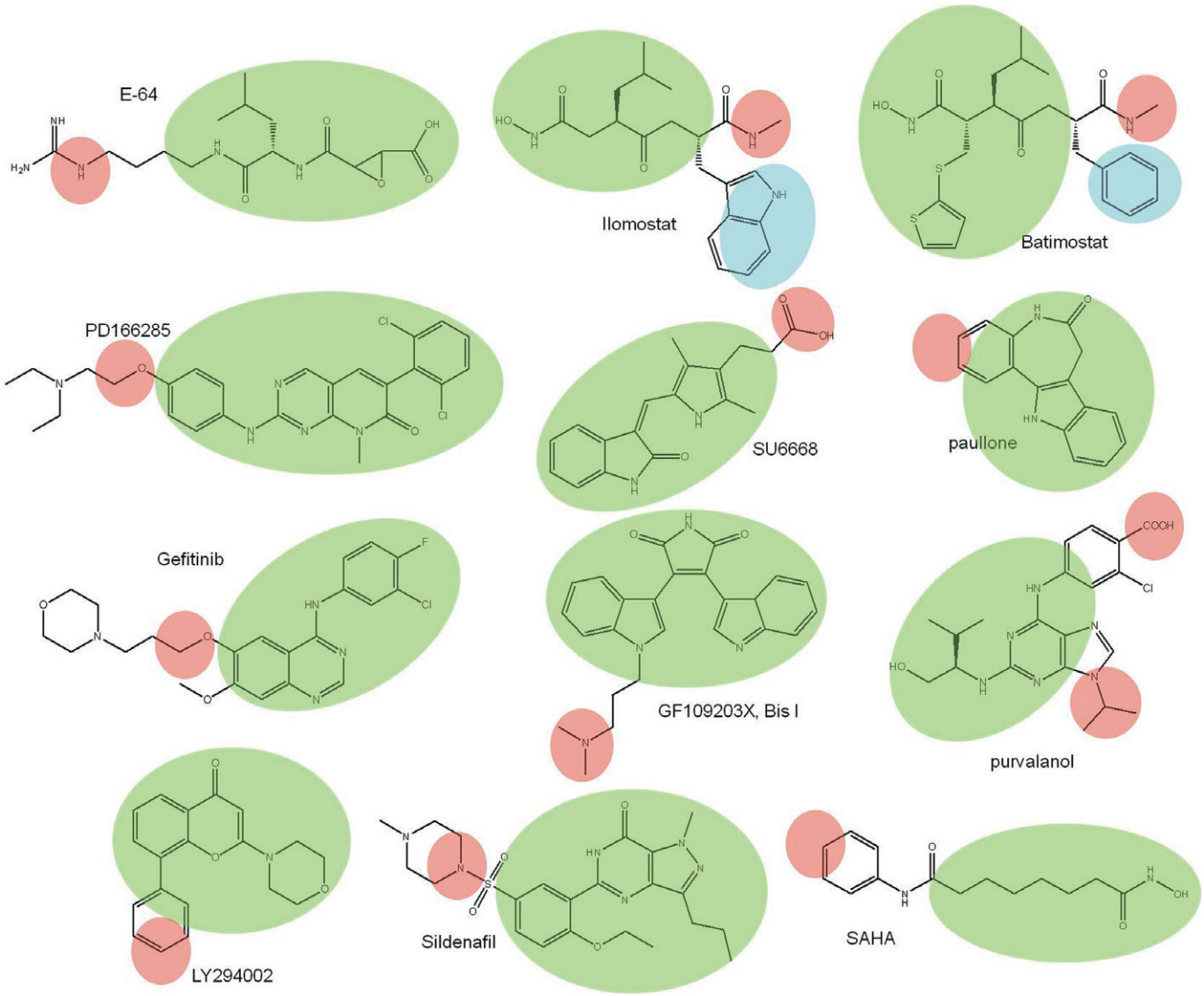

Figure 4

Drugs and inhibitors adapted for chemical proteomics. Green-shaded area represents the key structural component for recognizing the target enzyme, the red area is the moiety that can be modified or substituted for immobilization to activated beads or incorporation of biotin, and the blue area represents additional groups that can be sacrificed to include a fluorophore to produce tri-functional probes.

and immobilized on NHS-activated sepharose (Figure 3) for MMP purification from synovial fluid of patients with rheumatoid arthritis. Evaluation was based primarily on gelatine zymography for the synovial fluid or the loss of proteolysis activity in the unbound material (relative to the starting sample) rather than the bound, eluted fraction, so the effectiveness of removal of contaminating proteins remained unconfirmed.

Hesek and co-workers based their chromatographic isolation of MMPs on a well-characterized broad spectrum MMP hydroxamate inhibitor Batimastat (Hesek *et al.*, 2006). Batimastat is a synthetic, low-molecular weight MMP inhibitor, with a collagen-mimicking structure, which facilitates chelation of the active site zinc ion in MMPs and was originally developed as an anticancer drug to inhibit angiogenesis (Figure 4). Analogues of Batimastat containing reactive thiol groups were immobilized on epoxy-activated Sepharose beads (Figure 3). HT1080 cell line, a strong expresser of MMP-2, MMP-9 and TIMP-2, was initially used to demonstrate, with zymogram and immunoblot assays, that only active MMPs, and not proforms or TIMP-complexed enzymes, bound to the inhibitor beads. Batimastat-Sepharose was then used to show that (i) active MMP-2 (bound) was present in breast carcinoma and not in benign tissue; (ii) pro-MMP-9 (unbound) was present in the carcinoma and to a lesser extent in the benign sample; and (iii) active MMP-14 was present in laryngeal carcinoma but not in benign tissue. Overall, these results intimate that active MMPs may contribute to cancer-related activities such as angiogenesis or metastatic spread.

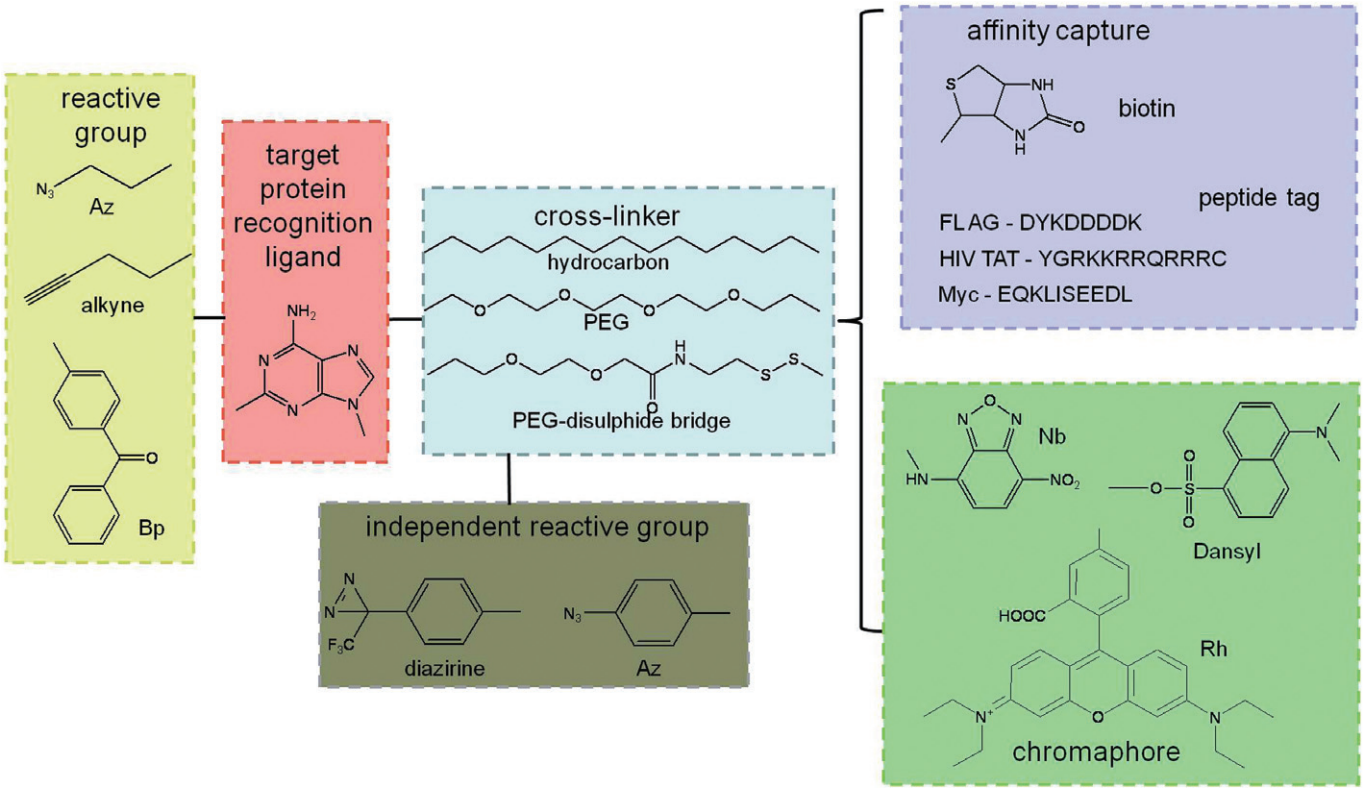

Figure 5

Key building blocks in the design of affinity probes; the *target protein recognition ligand* can include an optional *reactive* (alkyne) or *photoreactive group* (Az – azido, Bp – benzophenone) for covalent binding to the target, a *cross-linker* or spacer arm (hydrocarbon, pegylated – PEG and/or disulphide bridge) to prevent stearic hindrance between the protein and the capture surface, and a capture tag such as *biotin* for streptavidin capture or a peptide tag for immunoaffinity purification. In tri-functional probes, a *chromaphore* (Rh – rhodamine, Nb – nitrobenz-2-oxa-1,3-diazole) for detection of the labelled protein or an independent *reactive* or *photoreactive* group to bind to a different site on the target protein than the specific recognition ligand.

Rather than covalently immobilize the inhibitor probes on activated agarose, the Dive laboratory prepared solution-based photo-affinity and biotinylated probes to capture MMPs (SAC approach) (David *et al.*, 2007; Bregant *et al.*, 2009). Both types of probes incorporated phosphinic peptide chemistry, producing compounds that interacted with the zinc metalloproteinase transition state and were potent active-site inhibitors of MMPs. One probe also incorporated an azido group for photo-reactive cross-linking to the active site (Figure 5). Following incubation of the probes with isolated recombinant MMP-8 or MMP-12 or spiked into extracts of murine C_{26} colorectal tumours, bound proteins were isolated on streptavidin-coated magnetic beads and eluted in SDS-PAGE loading buffer or by on-bead trypsin digestion for MALDI MS analysis. From the MS data, PMFs consistently produced more confident identification of MMPs captured with the phosphinic affinity probe (K_i = 7 pM) than with the photoaffinity probe (K_i = 180 pM). The strong affinity of the probes enabled extensive washing of the magnetic beads to enable removal of abundant contaminating proteins that might otherwise be detected by MS analysis.

Cravatt's group extended the ABPP strategy to look at specific subgroups of metalloproteases (Saghatelian *et al.*, 2004). Initially a bifunctional analogue (HxBP-Rh) of the MMP inhibitor, Ilomostat (Figure 4) (IC_{50} = 1.1 nM – MMP-2, 34 nM – MMP-7, 0.5 nM – MMP-9), was designed incorporating a rhodamine group for fluorescence detection and a benzophenone group for photoreactively induced covalent binding of the probe (IC_{50} = 13 nM – MMP-2, 135 nM – MMP-7, 13 nM – MMP-9) to target proteins (Figure 5). HxBP-Rh was shown to bind active MMP-2, MMP-7 and MMP-9 and not to proforms or MMPs complexed with TIMP-1 when spiked into mouse kidney extracts. A biotinylated version of HxBP-Rh (or tri-functional probe) was then synthesized to enable affinity purification. A protein, which was significantly up-regulated in invasive melanomas compared with non-invasive cell lines, was identified as the zinc-dependent metallo-endopeptidase, neprilysin (also known as common acute lymphoblastic leukaemia antigen, CALLA) by ESI MS/MS. Further proteomics investigation of proteins captured with the tri-functional probe identified two additional non-MMP metalloproteases that are potentially inhibited by Ilomostat, leucine aminopeptidase (LAP) and dipeptidyl aminopeptidase 3 (DPPIII), which consequently could lead to side effects in clinical trials.

The hydroxamate strategy was extended to create a compound library based on a tripeptide-hydroxamate metalloproteinase inhibitor backbone, each incorporating a pho-

toreactive benzophenone group for covalently binding target proteins, and a reactive alkyne group to cross-link an azide-rhodamine reporter tag for fluorescent-based detection or an azide-biotin reporter tag for avidin enrichment and MS identification (Figure 5) (Sieber *et al.*, 2006). Different probes were shown to generate different metalloproteinase profiles on gels using a range of biological samples including mouse liver and brain and human breast (MDA-MB-231, MCF-7) and hepatic cancer (MUM-2B and MUM-2C) cell lines. Identified metalloproteinases included members of the ADAM and ADAMTS family, paraplegin and eight aminopeptidases. MMPs were not detected in natural sources probably due to their very low abundance; however, recombinant MMPs were spiked into mouse liver extracts to demonstrate affinity capture and recovery using the probes.

Protein kinases

The human genome contains more than 518 protein kinase genes, constituting approximately 2.4% of the total genome. The enzymes can be sub-grouped into families including AGC (containing PKA, PKG and PKC families); CAMK (e.g. Calcium/calmodulin-dependent protein kinase); CK1 (Casein kinase 1); CMGC (containing CDK, MAPK, GSK3 and CLK families); STE (e.g. MAP kinase cascade kinases); TK (Tyrosine kinases – receptors and non-receptor signalling cascades); TKL (Tyrosine kinase-like – serine-threonine protein kinases but with similar sequences to tyrosine kinases) and RGC (receptor guanylate cyclases) (Edwards, 2009). Some protein kinases are expressed explicitly in certain cell types, playing a critical role in signal transduction pathways that when de-regulated are a primary cause of tissue-specific diseases. Hence, members of the protein kinase family have become key targets for drug development. The majority are expressed at low levels and are often not detected with confidence using systematic proteomics methods. Pharmacological inhibition of protein kinases can be achieved using small molecule inhibitors that interfere with ATP-binding in the catalytic site. Chemical proteomics has contributed towards refining inhibitor design by increasing the specificity required for a particular protein kinase to reduce potential harmful side effects.

Members of the PKC family have roles in cell proliferation and differentiation, apoptosis, ion channel regulation, glycogenolysis and protein secretion and have also been implicated in various cancers and heart failure. There are a number of inhibitors of PKC inhibitors that can be divided into those that interact with the activation domain (e.g. Calphostin C) and those that reversibly compete with ATP in the nucleotide-binding pocket (e.g. bisindolylmaleimides) (Goekjian and Jirousek, 1999). Bisindolylmaleimide, GF109203X (BisI), has been used extensively in the study of PKCs and is known to inhibit other kinases and receptors (Figure 4). Chemical proteomics was used to establish the full repertoire of target proteins (Brehmer *et al.*, 2004). Three commercially available analogues (BisIII, BisVIII and BisX) of GF109203X, each with an alkylamine chain extension, were immobilized on epoxy-activated Sepharose 6B beads (Figure 3). Protein extracts of COS-7 and HeLa cells, in a high salt (1 M NaCl) buffer, supplemented with phosphatidylserine and diacylglycerol, were incubated with all three types of inhibitor beads and eluted with SDS-PAGE buffer for Western blot analysis. Subsequently, proteins bound to a BisIII column on a preparative scale (2.5×10^9 cells), were separated by 2D 16-BAC/SDS-PAGE, and individual spots were identified by in-gel digestion and MS. Of 38 proteins identified by MS, 17 were kinases and 21 were abundant cellular components (GAPDH, tubulins, myosin, heat shock proteins). In addition to previously known targets for GF109203X [PKC isozymes α and δ, ribosomal S6 protein kinase (Rsk2) and glycogen synthase kinase 3 (GSK3) isoforms α and β], further protein kinases were identified. These included Rho-dependent protein kinase 1 (ROCK1), Ste20-related kinase (SLK) and calmodulin-dependent kinase IIγ (CaMKIIγ), as well as non-protein kinase targets, adenylate kinase and quinone reductase-type 2 (NQO2). The use of three GF109203X analogues highlighted subtle variations in specificity for different protein kinases. For example, cyclin-dependent kinase 2 (CDK2), strongly interacted with BisX, weakly with BisXIII and not at all to BisIII. In order to eliminate false positives, Saxena *et al.* used a two pronged approach to identifying BisIII targets. In addition, to immobilized Bis-III chromatography, a soluble probe comprising BisIII, LC-SMCC spacer arm and FLAG-peptide (DYKDDDDK) (Figure 5) was used to bind kinases from HeLa cell lysates and the complexes captures on anti-FLAG antibody affinity beads (Saxena *et al.*, 2008). The proteins profiles for each method were determined by MS and compared to identify true BisIII interacting proteins, PKC-R, GSK3-β, CaMKII, adenosine kinase, CDK2, quinine reductase type 2, PKAC-α, prohibitin, VDAC and heme binding proteins. In an elegant variation of this approach, a BisIII probe linked to a fluorescent HIV TAT-tag peptide (YGRKKRRQRRRC), with subsequent anti-HIV TAT antibody immunoaffinity purification, was used with live HEK cells to capture proteins in their native physiological environment compared with cell lysates, thereby improving the accuracy of identifying target proteins (Saxena *et al.*, 2009) (Table 2).

Imatinib (Gleevec, ST1571), a phenylaminopyrimidine, has been used successfully to treat the early phase of chronic myeloid leukaemia (CML) by inhibiting the contributory tyrosine kinase, BCR-Abl; however, patients became resistant in advanced stages with concomitant failure of therapy. Relapse is associated with amino acid substitutions in the catalytic pocket of BCR-Abl kinase that render Imatinib ineffective. Consequently, further drug development is still required for a more effective treatment. PD180970 is a member of the pyrido[2,3-d]pyrimidine class of compounds (Figure 4) that have been shown to inhibit a number of clinically relevant mutant forms of BCR-Abl kinase as well as the wild type and therefore offers the potential for a second generation treatment for CML. However, there are a number of non-specific toxic side effects, which undermine the usefulness of PD180970 and prevent it achieving the critical selectivity required by protein kinase therapeutics. A PD180970 analogue, PP58, with a short ethylamine extension was bound to ECH-Sepharose (carbodiimide-based coupling; Figure 3) (Wissing *et al.*, 2004). PP58-sepharose was used to purify protein kinases from HeLa cell extracts, by specific elution using a combination of free PP58 and ATP in the same protocol to that used for GF109203X analogues. Of

Table 2

Summary of affinity methods – kinases

Protein target Protein kinases	Drug/inhibitor analogues	Compound class	Affinity method	Separation method	Detection method/MS	Identified proteins	Reference
CDKs (ser/thr-specific)	purvalanol	2,6,9-tri-substituted purine	PAC	SDS-PAGE	microsequencing	9 protein kinases	(Knockaert *et al.*, 2000)
CDKs/KSK-3 (ser/thr-specific)	paullones		PAC	SDS-PAGE	microsequencing	GSK-3α, GSK-3β, MDHM	(Knockaert *et al.*, 2002)
CDK2 (ser/thr-specific)	PHA-539136	cyclopropylpyrazole	PAC	SDS-PAGE/2D PAGE	MALDI MS	Cdk2, HSP-90α, 90β, 78, 70, 27, enolase, tubulin–β chain	(Lolli *et al.*, 2003)
CDK2 (ser/thr-specific)	(R)-Roscovitine	pegylated roscovitine	PAC	SDS-PAGE	MALDI MS, Western blotting		(Bach *et al.*, 2005)
p38/MAPK family	SB 203580	pyridinyl imidazole	PAC	16-BAC/SDS-PAGE	MALDI MS		(Godl *et al.*, 2003)
PKC family (ser/thr-specific)	GF109203X (BisI)	bisindolylmaleimide	PAC	16-BAC/SDS-PAGE	MALDI MS	17 protein kinases	(Brehmer *et al.*, 2004)
PKCα family	BisIII	bisindolylmaleimide	PAC /SAC	IAP, SDS-PAGE	ESI IT MS/MS	PKC-R, GSK3-β, CaMKII, adenosine kinase, CDK2	(Saxena *et al.*, 2008) (Saxena *et al.*, 2009)
Bcr-Abl (tyr-specific)	PD180970/PP58	pyrido[2,3,-*d*]pyrimidine	PAC	16-BAC/SDS-PAGE	MALDI MS/ESI Q-TOF MS	35 protein kinases	(Wissing *et al.*, 2004)
Bcr-Abl (tyr-specific)	imatinib, nilotinib, dasatinib, INNO-406	2-phenylamino pyrimidine	PAC	SDS-PAGE	ESI Q-TOF MS	multiple kinases	(Rix *et al.*, 2007) (Rix *et al.*, 2011) (Li *et al.*, 2010)
EGFR (tyr-specific)	Gefitinib/AX14596		PAC	16-BAC/SDS-PAGE	MALDI MS	20 protein kinases	(Brehmer *et al.*, 2005)
PDGFR, VEGFR2 (tyr-specific)	SU6668	indolinone	PAC	16-BAC/SDS-PAGE	MALDI MS	8 protein kinases	(Godl *et al.*, 2005)
Jurkat, HCT-116, A549 cell kinomes	multiple		PAC	SDS-PAGE	ESI Q-TOF MS	140 protein kinases	(Wissing *et al.*, 2007)
HeLa S3 S- and M- phase kinomes	multiple		PAC	SDS-PAGE, Mudpit	SILAC, LTQ-Orbitrap MS	219 protein kinases	(Daub *et al.*, 2008)
MV4–11 leukaemia, HCT116, MDA-MB-435S kinomes	VI16741/VI16743/ VI16832	pyrido[2,3,-*d*]pyrimidine	PAC	SDS-PAGE	SILAC, LTQ-Orbitrap MS	200 protein kinases	(Oppermann *et al.*, 2009)
Multiple mouse and human cell lines and primary tissues	multiple		PAC		iTRAQ, LTQ-Orbitrap MS	307 protein kinases	(Bantscheff *et al.*, 2007)
CLL cells	multiple		PAC		iTRAQ, LTQ-Orbitrap MS	multiple kinases	(Kruse *et al.*, 2011)
HepG2 hepatocarcinoma, human placenta	staurosporine	azido, biotin	SAC	Streptavidin chromatography	LTQ-Orbitrap MS	100 kinases	(Fischer *et al.*, 2010)

IAP. immuno affinity purification.

50 eluted proteins identified, 25 were protein kinases (including ERK2, Aurora, JNK2, p38a, Yes, RICK, TEK, JAK1, FAK and Fer) in addition to BCR-Abl. Direct analysis of the eluate by nano-HPLC Q-TOF MS revealed a further 10 tyrosine protein kinases (including Fyn, Lyn, BLK, DDR2 and EphA and EphB family members). Overall, the results suggest that further refinement of the pyrido[2,3-d]pyrimidine class of compounds is required to improve specificity before candidates can be presented for clinical trials. The binding profile for BCR-Abl kinase inhibitors imatinib, nilotinib, dasatinib and INNO-406 (Rix *et al.*, 2007; 2011) has been determined in CML and dasatinib in lung cancer (Li *et al.*, 2010) (Table 2).

The Daub group used the same approach to identify the array of proteins interacting with Gefitinib (Figure 4), an ATP-competitive inhibitor of epidermal growth factor tyrosine kinase (EGFR tyrosine kinase), which is currently approved for treatment of non-small cell lung cancer (Brehmer *et al.*, 2005). Gefitinib analogue, AX14596, immobilized on epoxy-activated Sepharose 6B beads was used to capture proteins from HeLa cell extracts, and more than 20 protein kinases were identified. In addition to EGFR tyrosine kinase, many have important physiological roles that could limit the effectiveness of Gefitinib in chemotherapy or produce unwanted side effects. As with previous examples using this strategy, many abundant cellular proteins were also detected including GAPDH, tubulins, myosin, heat shock proteins, hnRNPs, ribosomal proteins and importins.

A further demonstration of this strategy was described in the assessment of the specificity of anti-cancer drug SU6668 (Godl *et al.*, 2005). SU6668 (Figure 4) is known to inhibit autophosphorylation of βPDGFR and VEGFR2, resulting in disruption of tumour vasculature, but in mouse studies, prolonged therapy could not sustain tumour regression. As plasma levels were not maintained, there was the possibility that SU6668 bound to other receptors leading to low bioavailability. To test this hypothesis, the drug was modified to incorporate a primary amine linker for immobilization to epoxy-activated sepharose, and eight new protein kinases targets for SU6668, including Src-family members Yes and Lyn and serine/threonine protein kinases TBK1, Aurora A and B, RSK3, AMPKα1 and ULK3 were identified in HeLa cells. Inhibition of Aurora kinases (cell cycle arrest) and TBK1 (tumour cell apoptosis) would further enhance the anti-cancer impact of SU6668, but inhibition of AMPK would potentially produce side effects in cellular glucose homeostasis.

Kinases with an integral role in cancer have also been targeted. CDKs are a family of at least 11 protein kinases, many of which, in partnership with cyclins (a family of proteins that complex with specific CDKs to regulate their activity), control progression through different phases of the cell cycle. As a consequence, CDKs are most prominent in rapidly proliferating cells and therefore are the focus of drug development by the pharmaceutical industry as important targets to combat aggressive carcinomas. A large number of potent inhibitors to members of the CDK family have been designed. Purvalanol (Figure 4) and paullones analogues were used for affinity capture to determine their specificity repertoire. Initially, purvalanol (inhibitory) and methyl purvalanol (non-inhibitory) analogues, incorporating a pegylated cross-linker with a terminal primary amine for immobilization to ReactiGel soft gel (carbonyl-diimidazole activated) (Figure 3), were synthesized (Knockaert *et al.*, 2000; Knockaert and Meijer, 2002). The affinity media were used to capture CDKs from a range of protein extracts, which were eluted with gel loading buffer and analysed by SDS-PAGE (silver staining) and Western blotting. The pattern of proteins eluted with purvalanol and methyl purvalanol were compared in order to identify those binding specifically to the inhibitor (Figure 6A). CDK1, CDK2 and CDK5 were identified in tissues from various species along with other kinases, Erk1, Erk2, RS6K, CaMKII, p42 MAPK, p44 MAPK and creatine kinase 1. Although the IC_{50}s for non-CDK kinases were at least 1000 times higher than for CDKs, the approach illustrated the challenges of creating specific inhibitors for specific targets.

Analogous experiments, using members of the paullone family (inhibitors of CDKs and GSK-3) to capture proteins from a variety of sources (Knockaert *et al.*, 2002), were performed. Gwennpaullone has a primary amine linker for immobilization to activated beads; however, in the absence of a closely related non-inhibitory analogue, inactivated beads were used as a control (Figure 6B). Although a potent inhibitor of CDK1 (IC_{50} = 0.5 μM) and CDK5 (IC_{50} = 1 μM), the immobilized inhibitor did not capture these enzymes but was effective for GSK-3α and GSK-3β (IC_{50} = 0.04 μM) detection. Unexpectedly, immobilized Gwennpaullone captured mitochondrial malate dehydrogenase (MDH) (IC_{50} = 3.3 μM), but not cytoplasmic MDH (IC_{50} = 22 μM) from a number of protein extracts. In addition to potential pharmacological effects paullones may have, due to inhibition of MDH, an important component of the TCA cycle, the results suggest more than one mechanism of interacting with target proteins.

Cyclopropylpyrazole (PHA-539136) was used to determine its specificity for Cdk2 kinase by affinity chromatography (Lolli *et al.*, 2003). PHA-539136 cross-linked to NHS-activated sepharose (Figure 3) or Actigel B Ultraflow was compared. Coupling of PHA-539136 to the former was more efficient (75% coupled in 16 h) than the latter (90% couple in 20 days). However, in comparisons of inactivated matrices (PHA-539136 absent; Figure 6E), higher non-specific binding of partially purified Cdk2/cyclinA complex was observed with NHS-sepharose compared with Actigel B. PHA-539136-Actigel B was used to purify Cdk2 from pancreatic acinar and CHT116 cells. After extensive washing with inhibitor solution, the kinase was released from the chromatography beads with SDS-PAGE buffer. Cdk2 could only be identified by Western blotting due to the presence of other proteins that also bound specifically to PHA-539136-Actigel B beads. MS analysis (PMF) identified proteins as heat shock protein family (90α, 90β, 78, 70, 27), enolase and tubulin β chain. Isothermal titration calorimetry confirmed binding of PHA-539136 to HSP-27 and HSP-70 with similar properties (K_D) to Cdk2, but HSP-90 subunits, enolase and tubulin–β chain were 20- to 80-fold lower. Although binding to the latter group maybe due to slow thermodynamics, there may also be an element of non-specific interaction with beads.

Kinome studies

The presence of large quantities of non-specifically bound protein can prevent the detection of very low abundance

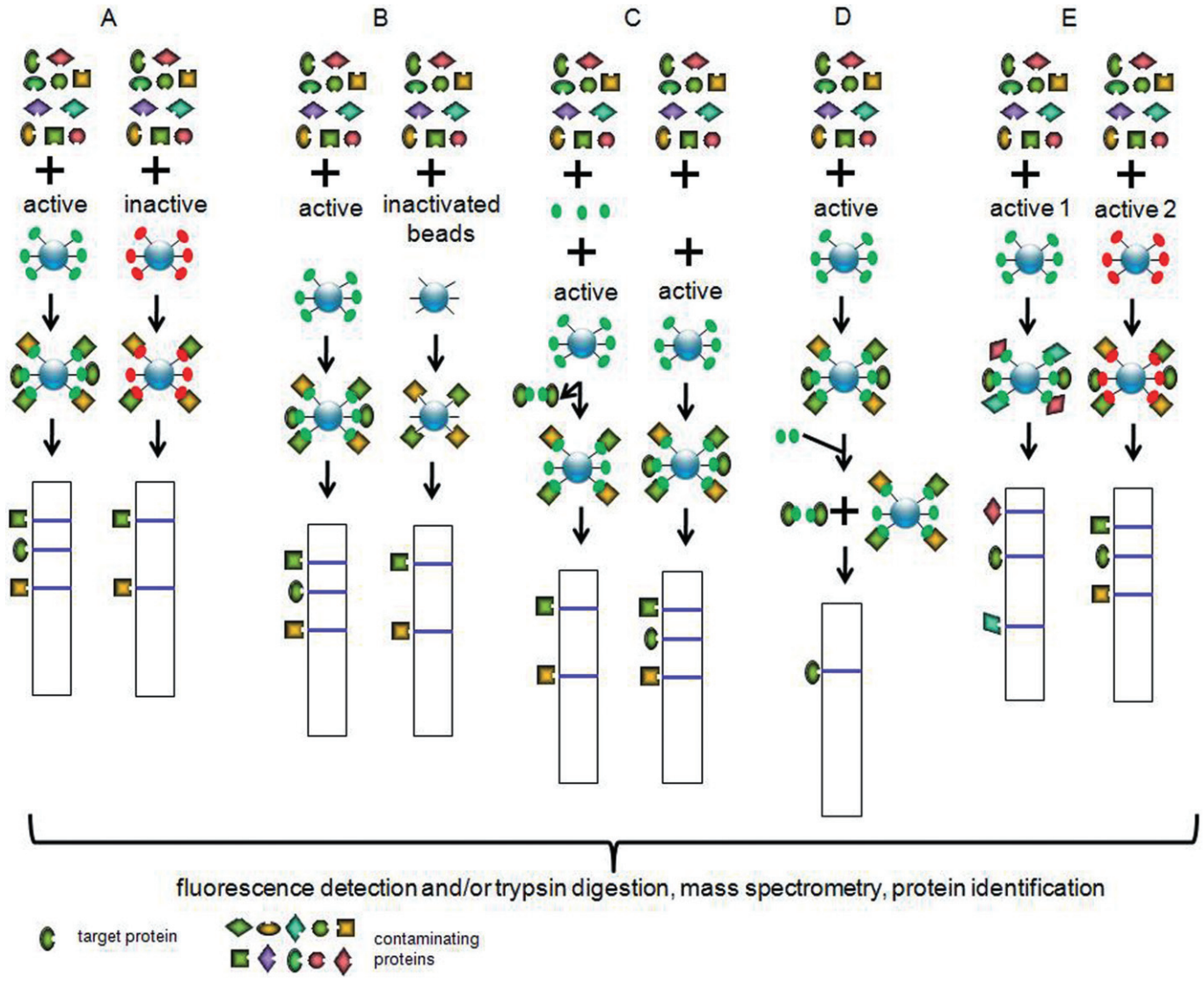

Figure 6

Strategies for identifying or removing non-specific binding proteins. (A) Comparison of the proteins captured with closely related active and inactive compounds. (B) Comparison of the proteins captured with active compound and inactivated beads (hydrolysed or modified with a blocking agent such as ethanolamine). (C) Comparison of the proteins captured in the presence or absence of competing soluble compounds including the active ligand, ADP and GDP. (D) Elution of captured proteins with soluble active compounds or analogues. (E) Comparison of proteins capture by two or more active compounds.

proteins, such as protein kinases, by suppressing weak MS signals. Wissing *et al.* proposed a solution by applying sequentially four different protein kinase inhibitor (purvalanol B, PP58, AX14596, BisX) affinity chromatography steps to profile as many protein kinases and phosphorylated proteins events (the kinome) as possible (Wissing *et al.*, 2007) (Figure 7). Total protein extracts from Jurkat, HCT-116 or A549 cells were passed through each column. The respective soluble inhibitor, ADP and non-hydrolysable ATP analogue AMP-PNP were used to elute proteins from each column for analysis by SDS-PAGE and ESI MS/MS. This approach identified 140 protein kinases and more than 200 phosphorylation events. Furthermore, the levels of non-specifically bound proteins were significantly reduced with each ordered chromatography step enhancing the ability to detect less abundant kinases. The approach was extended further using sequential entrapment of protein kinases with five different inhibitors (VI16832, BisX, AX14596, SU6668 and purvalanol B) to study changes in S- and M-phase of SILAC-labelled HeLa S3 cells (Daub *et al.*, 2008). A total of 219 protein kinases and more than 1000 phosphorylation sites were identified and quantified including many previously unidentified M-phase induced phosphorylation events. Additional exploitation of the broad-selectivity inhibitor VI16832 was described by Oppermann *et al.* to study the relative expression levels of more than 200 SILAC-labelled protein kinases and 1200 phos-

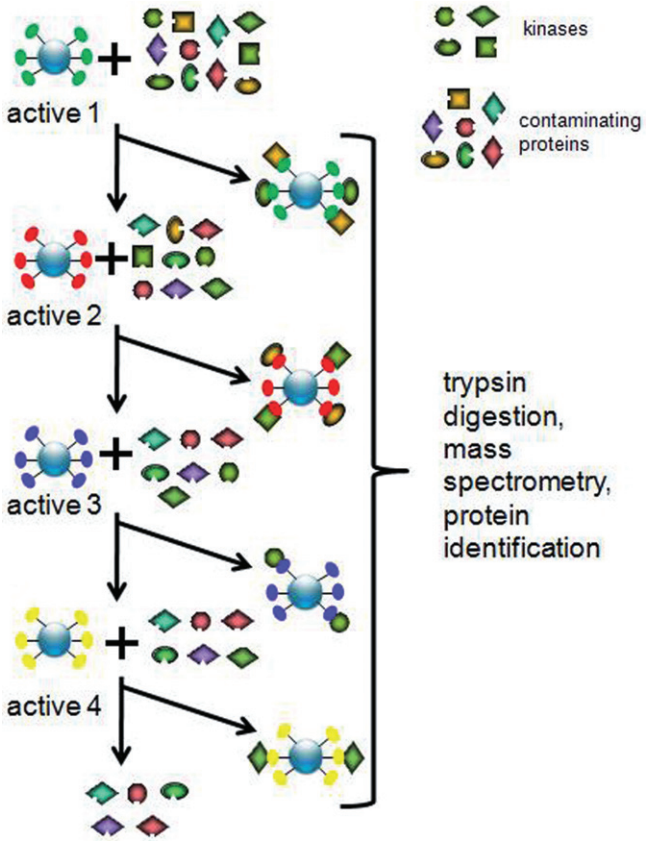

Figure 7
Workflow for profiling the kinome with serial inhibitor chromatography steps.

phorylation sites in MV4–11 leukaemia, HCT116 colon epithelial and MDA-MB-435S breast cancer cells (Oppermann *et al.*, 2009).

A demonstration of the comprehensive data gathering power of proteomics was provided by Bantscheff *et al.* using kinobeads (seven broad-range kinase inhibitors – Bis-(III) indolyl-maleimide, purvalanol B, staurosporine, CZC8004, and analogues of PD173955, sunitinib and vandetanib immobilized on NHS-activated Sepharose) to characterize the protein kinase profiles for lysates of 14 human and rodent cell lines and tissues (human HEK 293, HeLa, Jurkat, K562, Ramos, THP-1, kidney, placenta; mouse heart, liver, brain, muscle, kidney; and rat RBL-2H3) (Bantscheff *et al.*, 2007). Between 150 and 200 kinases were identified in each biological sample out of a total of 1200 to 1800 proteins that bound to the beads (13% of the protein identified, but 79% of MS signals corresponded to kinases). Competition assays using nine concentrations of imatinib, dasatinib and bosutinib pre-incubated with lysates of K562 cells before treatment with kinobeads were performed to determine which protein kinases were selectively prevented from binding (and hence specifically targeted by each inhibitor). Quantitation, to determine IC_{50} values for all the protein kinases detected, was performed by iTRAQ labelling of the tryspin-digested proteins that were bound following treatment. From the results, imatinib showed the best specificity, binding only 13 proteins (defined by 50% inhibition of binding to kinobeads at 1 μM drug), whereas dasatinib and bosutinib exhibited broad range specificity (39 and 53 proteins, respectively); however, in all cases, novel kinase targets were identified for each drug. By enriching for phosphopeptides using IMAC, it was possible to identify which phosphorylation events were inhibited and confirm the specific components of the ABL/BCR-ABL signal transduction pathway that were affected by imatinib.

Kinobeads have also been used to determine the mechanism of action, or drug deconvolution, of multi-kinase inhibitors (some of which are in clinical trials) in the apoptosis of chronic lymphocytic leukaemia (CLL) cells (Kruse *et al.*, 2011). Tyrosine kinase inhibitors dasatinib, sunitinib, vandetanib, bosutinib, TKI258, pazopanib and axitinib, and the receptor kinase inhibitors sorafenib were relatively inactive, whereas CDK inhibitors, BMS-387032 and flavopiridol and the non-specific kinase inhibitor staurosporine were potent activators of apoptosis. The CDK inhibitors were most effective against CDK9, cyclin T1, AFF3/4 and MLLT1, putative members of transcriptional elongation factor (p-TEFb) complex and CDK7 in the transcription factor BTF2/TFIIH complex, whereas staurosporine targeted BLK kinase, which is involved in regulation of B-cell proliferation. Using a trifunctional agent, comprising the protein kinase inhibitor staurosporine, a photosensitive azido group to covalently cross-link the protein at a different site from the inhibitor and a biotin group for affinity capture, Fischer and co-workers demonstrated the pan-specific nature of the inhibitor identifying 100 kinases in human HepG2 hepatocarcinoma cell line extracts and 24 kinases in the membrane fraction of human placental tissue using ESI MS/MS (Fischer *et al.*, 2010) (Table 2).

Phosphatidylinositol 3-kinases

Other kinases also play important roles in cellular mechanisms. Phosphatidylinositol 3-kinases (PI3Ks) are a group of 14 enzymes that regulate growth, metabolism and differentiation of cells, but through phosphorylation of phosphatidylinositols, which are important messengers in lipid signalling, cell signalling and membrane trafficking (Kok *et al.*, 2009). An analogue, PI828, of LY294002 (a PI3K inhibitor derived from the flavonoid quercetin, Figure 4) was synthesized incorporating an amine group for immobilization on epoxy-activated sepharose (Gharbi *et al.*, 2007). Protein extracts from HeLa or WEHI231 cells were incubated with the PI828-bead slurry and bound proteins eluted with competing inhibitors or gel loading buffer. Proteins were successful recovered using LY294002, but not free PI828 despite similar IC_{50}s for PI3Ks. From preparative gels, bands were excised, proteins digested with trypsin and the resulting peptides analysed by ESI MS. Almost 100 proteins were identified with many different cellular functions and substrate specificities, including many kinases and dehydrogenases involved in metabolic pathways (Table 2).

Pasquali *et al.* employed a series of phosphoinositide (Ptdins) probes linked to biotin, via a thiol linker (Figure 5), as a bait to pull out PI3Ks from mouse bone marrow macrophages (PI3K-dependent PtdIns signalling regulates many processes in macrophages including cytokine and radical

oxygen production, phagocytosis, and directed cell migration) (Pasquali *et al.*, 2007). Extracts were pre-treated with streptavidin beads to remove proteins that may bind non-specifically, before addition of the water soluble probe and capture on a second batch of streptavidin beads. Bound proteins were eluted with dithiothreitol and analysed by SDS-PAGE or subject to trypsin digestion and MS analysis. PI3Ks could only be identified by Western blotting probably due to low abundance; however, other PtdIns binding proteins were identified by MS, including known entities gelsolin, talin, hexokinase II and Ras GTPase-activating protein 3 and new ones such a peroxiredoxin 1 and adenylyl cyclase-associated protein 1. A combination of supplementary lipid–protein interaction analytical methods, such as thin layer chromatography with ^{32}P-labelled PtdIns and micelle-dot blots on nitrocellulose were used to confirm specific interactions.

PDEs

PDEs present another protein superfamily (11 groups) with important physiological roles in the body, such that the pharmaceutical industry has endeavoured to develop a series of inhibitors specific to particular PDE isoforms. Tissue-specific PDEs metabolize secondary messenger cAMP and cGMP regulating the localization, duration and amplitude of cyclic nucleotide signalling. Inhibition of PDEs can prolong the effects mediated by cAMP and cGMP with a potential positive therapeutic impact by increasing blood vessel relaxation in coronary heart disease, dementia, depression, Duchenne muscular dystrophy and schizophrenia (Boswell-Smith *et al.*, 2006).

PF-4540124, an inhibitor of PDE5, structurally related to male erectile dysfunction drug Sildenafil (Viagra) (Figure 4), was used to identify other proteins that may be targeted (Dadvar *et al.*, 2009a,b). Sildenafil has presented side effects during clinical trials and since becoming available to the public. Minor concerns included headaches, flushing, dyspepsia, nasal congestion and impaired vision. However, serious adverse effects include priapism, severe hypotension, myocardial infarction (heart attack), stroke, increased intraocular pressure and sudden hearing loss, which although exceptional are a concern and may be due to interaction of Sildenafil with other important physiologically important proteins including inhibition of other PDE isoforms. PF-4540124 contains an aminohexyl group for immobilization onto NHS-activated sepharose affinity beads (Figure 3). Initial pull-down experiments using PF-4540124 affinity chromatography to capture proteins from mouse lung tissue lysates showed a complex mixture of proteins on SDS-PAGE, which though different from the starting material, indicated considerable non-specific binding. To improve interpretation of specific binding, two significant steps were included: (a) cell lysates were supplemented with ADP and GDP prior to addition of the PF-4540124 affinity beads to prevent ATP and GTP binding proteins (tubulins and heat shock proteins which made up a major proportion of non-specifically bound protein) from interacting with the beads, and (b) a negative control pull-down of cell lysates, pre-treated with PF-4540124 solution, to compete with proteins binding specifically to the PF-4540124-immobilized affinity beads (Figure 6C). In so doing, the first step reduced the number of detected proteins to seven main bands and from second step, five of these were confirmed to specifically binding PF-4540124. Full-length and truncated PDA5 along with prenyl binding protein, which is a subunit of PDE6 and specific dehydrogenases and reductases (Table 3), were identified by ESI MS/MS. Verification of the specificity was demonstrated using dimethylation, stable isotope labelling of the endoproteinase lys-N/trypsin digests of normal and control pull-down proteins with formaldehyde-D_2 and formaldehyde-H_2 respectively. When the digests were combined for MS analysis, the signal intensities of paired parent ions were used to determine the relative abundance of each protein. Hence, a ratio of 1 indicated that the protein was present equally in both samples and not bound specifically, whereas a high ratio value indicated that the protein was most abundant in the normal pull down and hence bound specifically.

Cytochrome P450s

Cytochrome P450s (CYPs) present potential therapeutic targets in their own right, particularly those over-expressed in certain cancers as a focal point for pro-drug design; however, they have a more substantial bearing on the pharmaceutical industry due to their intervention in drug metabolism in the liver where they are most diverse and abundant (Ingelman-Sundberg, 2002; Nebert and Russell, 2002). CYPs in the liver can modify drugs by insertion of a hydroxyl group, which in most cases make them more hydrophilic and susceptible for excretion. However, CYP hydroxylation may also lead to metabolites that are more active (i.e. tamoxifen and cyclophosphamide) or carcinogenic (benzopyrene). Hence, the CYP profile in preclinical models or patients in clinical trials can play an important role in the destiny of drug candidates. Cytochrome P450s are a superfamily with 57 genes in humans (102 genes in mouse), which can be subdivided based on a preference for metabolism of xenobiotics (families 1,2 and 3) or endogenous compounds and can be divided further based on protein sequence similarity. There is considerable overlap in substrate and inhibitor specificity amongst CYPs that have been purified but many have not been detected as expressed proteins. The development of affinity probes, with both specific and broad spectrum binding profiles, would be valuable tools for screening CYPs in biological samples and biopsies.

Initially, Wright *et al*. using ABPP, designed a probe based on the broad-spectrum CYP inhibitor 2-ethynylnaphthalene (2EN) incorporating a rhodamine fluorescence tag (Figure 5), to show NADPH-dependent binding to mouse liver CYP1a1, 1a2, 2c29, 2d9, 2d10 and 3a11 (Wright and Cravatt, 2007). Analysis of fluorescently stained, in-gel trypsin digested bands from SDS-PAGE (48–55 kDa), by quantitative LC ESI MS/MS using spectral counting demonstrated differential relative changes in expression levels of these CYPs in β-naphthoflavone or dexamethasone-treated compared with untreated mice. This approach was extended with the design of a library of probes (Figure 6E) that integrates two aryl alkyne groups into known substrates and inhibitors (Wright *et al.*, 2009). One group is incorporated into the active moiety of the molecule for oxidation by the CYP to a reactive ketene

Table 3

Summary of affinity methods – others

Protein target	Drug/inhibitor analogues	Compound class	Affinity method	Separation method	Detection method/MS	Identified proteins	Reference
Other known targets							
Phosphatidylinositol 3-kinases	LY294002	Flavonoid	PAC	SDS-PAGE	ESI Q-TOF MS	FN3K, FN3KRP, GALK1, PFKM, PXDK	(Gharbi *et al.*, 2007)
Phosphatidylinositol 3-kinases	phosphoinositide	Thiol, biotin	SAC	SDS-PAGE	MS	GSN, TLN1, HK2, RASA1, PRDX1, CAP1	(Pasquali *et al.*, 2007)
PDEs	PF-4540124		PAC	SDS-PAGE	LTQ-FT-ICR MS	PDE5, prenyl binding protein, NDHRS4, ECH1, GLO1	(Dadvar *et al.*, 2009b)
Histone deacetylases	SAHA	Diaza cycle, biotin	SAC	Streptavidin chromatography	LTQ-Orbitrap MS	HDAC 1, 2, 3, 6, ISOC2	(Fischer *et al.*, 2011)
Post-translational modifications							
Palmitoylation	N/A	HPDP, biotin	SAC	Mudpit	ESI LTQ MS	50 palmitoylated proteins	(Wan *et al.*, 2007)
S-glutathiolation	Glutathione	Biotin		SDS-PAGE	ESI Q-TOF MS, Western blotting	TPIS, GAPDH, MYBPC, ACTN2, DES, HADHA, MYH, creatine kinase	(Brennan *et al.*, 2006)
Novel target identification							
Unknown	(+)-avrainvillamide (anti-proliferative)	Alkaloid	SAC	Avidin chromatography, SDS-PAGE	LC MS/MS, Western blotting	Nucleophosmin	(Wulff *et al.*, 2007)
Unknown	triptolide	Diterpene, tritiated		IEC, SDS-PAGE	radiolabel, MALDI MS	Ca^{2+} channel polycystin-2	(Leuenroth *et al.*, 2007)
Unknown	QS11	Purine	PAC	SDS-PAGE	ESI LCQ MS	ADP-ribosylation factor 1	(Zhang *et al.*, 2007)
Unknown	CBI-0997, KRIBB-3	Biphenyl isoxazole, biotinylated	SAC	Avidin chromatography	ESI Q TOF MS	HSP27	(Shin *et al.*, 2005)
Unknown	HUN-7293	Cyclodepsipeptide, rhodamine azido		Immunoprecipitation	Western blotting	Sec61α	(MacKinnon *et al.*, 2007)
Unknown	Quinolines (anti-malarials)	Purine	PAC	SDS-PAGE/2D gels	ESI Q-TOF MS, microsequencing	ALDH1A1, NQO2	(Graves *et al.*, 2002)

N/A, not applicable.

intermediate, which then irreversibly covalently modifies the protein (Figure 5). The second alkyne group extends on a carbon cross-linker group for reaction with rhodamine-azide, post incubation with the protein sample. Individual human CYPs (1A1, 1A2, 1B1, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 2J2, 3A4, 4A11, 4F2 and 19A1) expressed in baculovirus-infected insect cell microsomes (also known as supersomes) were incubated with probes in the presence or absence (negative control) of NADPH. Rhodamine-azide was added to the mixture to provide a fluorescent tag to those proteins complexed with the affinity probe, the samples run on SDS-PAGE and proteins identified by direct fluorescence scanning of the gel. Each probe revealed a distinctive profile of preference for binding different members of the CYP panel. Reactions were prepared primarily on a small scale enabling analysis by fluorescence but insufficient for MS confirmation. Further adaptations of the probe design would be required to facilitate their use for affinity purification. CYP19A1 (aromatase) has been of interest as a target for treatment of breast cancer because of its role in oestrogen metabolism associated with increased tumour cell proliferation. Formestane (steroidal) and anastrozole (non-steroidal) are inhibitors currently in clinical trials and were used in competition assays to confirm the probes bound CYP19A1, but also other CYP isoforms.

Histone deacetylases

Histone deacetylases (HDACs) are a small family of proteins (11 HDACs and 7 sirtuins) that remove acetyl groups from an ε-N-acetyl lysine amino acid predominantly from histones but also other target proteins. They have been the subject of inhibition due their role in DNA expression for many years. Inhibitors have been used in neurology as anti-epileptics, in treatment of neurodegenerative diseases and have potential in cancer chemotherapy. Suberoylanilide hydroxamic acid (SAHA), also known as Vorinostat (Figure 4), is a HDAC inhibitor used in treatment of T-cell lymphomas, but the mechanism of action is not fully understood. SAHA was incorporated into a trifunctional probe that also included a photosensitive cross-linking group and biotin for streptavidin affinity capture. In addition to HDACs 1, 2, 3 and 6 being captured and identified, isochorismatase domain-containing protein 2 (ISOC2), which has a possible role negative regulation of tumour suppression was also found to be a new target for the inhibitor (Fischer *et al.*, 2011).

Post-translational modifications

Although so far only applied to a yeast model, chemical proteomics was used to identify proteins containing palmitoylation or S-acylation. Palmitoylation provides target proteins with a membrane anchor via cysteine residues but so far seems unique in allowing the protein to be released by disulphide exchange. This characteristic was exploited to develop an affinity method (Wan *et al.*, 2007). Initially, free thiols were irreversibly modified with N-ethyl maleimide, then the palmitoyl group was removed by hydroxamate treatment (native cystine-disulphide bridges remain unaffected) leaving a free thiol. Biotin-HPDP (N-[6-(Biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide) was substituted for the PTM and allowed streptavidin-agarose capture of the modified proteins. The proteins were then eluted with β-mercaptoethanol for trypsin digestion and MS identification. Due to the presence of non-specifically binding proteins, genuine palmitoylated proteins were identified by comparing the quantitative MS data (spectral counts) from the experiment with a parallel control in which proteins had not been treated with hydroxamate. Of 50 palmitoylated proteins identified, 15 were known, but 35 were novel.

Protein S-glutathiolation, an important component of redox regulation in cells, was investigated using a biotinylated analogue of glutathione disulphide (Biotin-GSSG) (Brennan *et al.*, 2006). Biotin-GSSG, as a mimic of naturally occurring GSSG, undergoes disulphide interchange with cysteine residues on target proteins during oxidative stress. Protein extracts from rat heart, lung, kidney, skeletal muscle and liver were treated with biotin-GSSG, residual free disulphide groups modified with maleimide and the extracts run on non-reducing SDS-PAGE. Following Western blotting, biotinylated proteins were detected with streptavidin-horseradish peroxidase. Protein identification was performed by ESI Q-TOF MS of in-gel digests, and the key proteins undergoing S-glutathiolation were identified (Table 3).

Anti-malaria drug targets

Chemical proteomics has also been employed using anti-malarial drug analogues to identify target proteins in whole homogenized mouse and from proteomes of healthy and *Plasmodium falciparum*-infected human red blood cells (RBC). Because of the similarity of quinolines to ATP, dual-affinity approaches were used (Graves *et al.*, 2002). Protein extracts were either run through an ATP-sepharose column, and bound proteins were eluted with anti-malarial drugs (displacement affinity chromatography) or run through a hydroxychloroquine- or primaquine-Sepharose column and bound proteins again eluted with anti-malarial drugs (Figure 6D). ATP-sepharose resulted in many proteins binding and being eluted with competing nucleotides. However, when using quinoline anti-malarial drugs for competitive displacement, the approach resulted only in the elution of aldehyde dehydrogenase 1 (ALDH1) and quinine reductase 2 (QR2) from mouse extracts and healthy human RBC, but not from *P. falciparum*-infected RBC. Although not as potent as quinolines, established inhibitors of ADH1 (diethylaminobenzaldehyde) and QR2 (quercetin and chrysin) were shown to possess anti-malarial activity. The role of the two enzymes in *P. falciparum* remains unclear but could relate to control of the redox potential with RBC. By inhibiting the enzymes, oxidative stress would provide an unfavourable environment for *P. falciparum* survival.

Non-specific binding problems and solutions

Chemical proteomics has for the most part been successful (as summarized in Tables 1–3); however, throughout much of

the research, there is an understated theme of non-specific binding particularly where soft gels such as agarose and sepharose are used. Covalent binding of probes in the active site of the enzymes of interest permit more extensive washing of chromatography beads to remove contaminating proteins; however, not all proteins can be targeted with irreversibly acting agents. Appropriate controls and extensive supplementary experimentation are essential to clarify which proteins are genuinely captured by the affinity probe and some of these approaches are shown in Figure 6. A number of experiments have been described to specifically highlight the problem and present possible solutions.

In a series of studies to optimize purification of FKBP12 (peptidyl-prolyl cis-trans isomerase FKBP1A) using FK506 (also known as Fujimycin or Tacrolimus, an immunosuppressive drug mainly used after allogeneic organ transplant) affinity chromatography, Tanaka's group used new cross-linking agents or substrates to reduce non-specific binding (Shiyama *et al.*, 2004; Takahashi *et al.*, 2006; Yamamoto *et al.*, 2006). By incorporating a hydrophilic tartaric acid-based cross-linking agent into TOYOPEARL beads (PMMA resins are better suited for organic solid phase preparation of affinity matrices, but with higher non-specific binding protein properties than agaroses), levels of contaminating actin and tubulin were reduced 90% and 65%, respectively, without affecting binding of FKBP12.

The bead proteome (i.e. the proteins that agarose, sepharose and magnetic beads bind non-specifically) was defined in a study of protein–protein interactions (Trinkle-Mulcahy *et al.*, 2008). The survival motor neuron protein (SMN) complex was used as a model in which, GFP (green fluorescent protein)-tagged SMN or free GFP were expressed in SILAC-labelled HeLa or U2OS cells. The complex was purified initially by pre-clearing non-specific bound proteins through Sepharose beads and then affinity captured using immunoaffinity purification on anti-GFP antibodies coupled to Protein-G Sepharose. By comparing 'light' or 'heavy' SILAC-labelled peptide MS signal intensity corresponding to proteins co-purified with the GFP protein tag or free GFP, it was possible to determine which proteins bound specifically to the target protein and which were non-specifically binding to the Sepharose. An extensive study, using cytoplasmic, nuclear and whole cell extracts, identified more than 100 proteins binding to Sepharose non-specifically, including highly abundant cytoskeletal and structural proteins (actin, cofilin, desmin, myosin, tropomyosin, tubulin and vimentin), eukaryotic translation elongation and initiation factors, heat shock proteins, histones, human nuclear ribonucleoproteins and ribosomal proteins. Although there were individual variations in relative abundance, sepharose, agarose and magnetic beads all exhibited extensive non-specific protein binding profiles, suggesting none are ideal as affinity capture media. In addition to incorporating a chromophore into protein constructs, GFP has the advantage of very low protein–protein interactions. Despite this, some proteins (heat shock proteins, keratins and ubiquitin) were identified that may be co-purified via GFP rather than the target protein or Sepharose. The study elegantly illustrated the challenges of defining specific affinity-bound proteins when using proteomics approaches.

Conclusions

Chemical proteomics is providing a new approach to identify low abundance proteins from a range of biological sources. It represents an intermediate strategy between global profiling and the exclusivity afforded by selective or multiple reaction monitoring (SRM/MRM), which enable targeting of specific protein isoforms in a total mixture with disregard to all others present in a sample (Lange *et al.*, 2008). Due to the diverse range of compounds being examined and the availability of suitable controls, a wide range of variations on the main two main methodologies (PAC and SAC) have evolved. Various means of determining specific-binding have been developed to counter the inherent attraction of proteins to soft gel chromatography media. Alternative substrates, such as those developed for microarray technologies (glass slides, silicon chips) may provide lower non-specific interaction and will hopefully be the subject of method development for chemical proteomics in the near future.

Much of the work to date has been performed by groups with the right mix of skills – synthetic chemistry, biology, pharmacology, proteomics and MS. Chemical proteomics has proven particularly useful for refinement of drug design, to identify proteins other than the intended target that can cause adverse effects in clinical trials. In the future, it has the potential for application in many new areas such as diagnostic kit development and could provide a high-throughput approach for targeting biomarkers in clinical samples supported by MS, without the need for a large bank of expensive antibodies. In addition, chemical proteomics can be used to identify novel therapeutic targets particularly for compounds that are being identified through investigation of traditional remedies from Chinese and aboriginal sources.

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Conflict of interest

The author declares no conflict of interest.

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