IDENTIFICATION AND CHARACTERIZATION OF MOLECULAR TARGETS OF NATURAL PRODUCTS BY MASS SPECTROMETRY

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Natural products, and their derivatives and mimics, have contributed to the development of important therapeutics to combat diseases such as infections and cancers over the past decades. The value of natural products to modern drug discovery is still considerable. However, its development is hampered by a lack of a mechanistic understanding of their molecular action, as opposed to the emerging molecule-targeted therapeutics that are tailored to a specific protein target(s). Recent advances in the mass spectrometry-based proteomic approaches have the potential to offer unprecedented insights into the molecular action of natural products. Chemical proteomics is established as an invaluable tool for the identification of protein targets of natural products. Small-molecule affinity selection combined with mass spectrometry is a successful strategy to "fish" cellular targets from the entire proteome. Mass spectrometry-based profiling of protein expression is also routinely employed to elucidate molecular pathways involved in the therapeutic and possible toxicological responses upon treatment with natural products. In addition, mass spectrometry is increasingly utilized to probe structural aspects of natural products–protein interactions. Limited proteolysis, photoaffinity labeling, and hydrogen/deuterium exchange in conjunction with mass spectrometry are sensitive and high-throughput strategies that provide low-resolution structural information of non-covalent natural product–protein complexes. In this review, we provide an overview on the applications of mass spectrometry-based techniques in the identification and characterization of natural product–protein interactions, and we describe how these applications might revolutionize natural product-based drug discovery. © 2009 Wiley Periodicals, Inc., Mass Spec Rev 29:126–155, 2010

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I. INTRODUCTION

Nature has provided a myriad of compounds that inspired the development of therapeutics, and rendered an enormous contribution to the treatment of diseases. Natural products, and their derivatives and mimics, have remained the frontline drugs to combat various infections and cancers. Notable examples include antibiotics penicillin and tetracycline derived from microbes. Paclitaxel (Wani et al., 1971), vinca alkaloids (Noble, 1990), and etoposide (Liu, 1989) are plant-derived chemotherapeutic agents that are effective in the treatment of a variety of cancers (da Rocha, Lopes, & Schwartsmann, 2001). In a comprehensive survey, Newman and Cragg (2007) showed that natural products or drugs created from a natural pharmacophore accounted for half of all small-molecule drugs introduced during 1981–2006. Despite the historical importance of natural agents in drug discovery, their development has been scaled down by many pharmaceutical companies as they shift their focus towards combinatorial chemistry (Lam, 2007). Thus far, the introduction of innovative and clinically effective drugs from this chemical pipeline has yet to be realized (Newman, Cragg, & Snader, 2003). Mechanically, natural products execute their molecular actions by interacting with their cellular targets with greater potency and specificity (Rouhi, 2003; Burdine & Kodadek, 2004). It is perhaps a surprise that the identity of the cellular targets of natural product-based drugs is often unknown (Piggott & Karuso, 2004). The general lack of the information regarding molecular receptors and their mode of interaction is a potential bottleneck to the development of more effective derivatives. Equally important for target identification is to pinpoint any interaction(s) with "off-targets" that might cause adverse side effects at an early stage to avoid costly failure in clinical trials due to toxicity (Barros & Martin, 2008).

Mass spectrometry (MS) is an essential tool at many stages of drug discovery and development. Many natural products interact with protein receptors at the molecular level; thus, the development of soft-ionization methods such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) suitable for rapid protein analysis have greatly facilitated studies of natural product–protein interactions. Different chromatographic and electrophoretic methods in conjunction with MS analysis have been exploited to "fish" binding partner(s) of natural products, and to elucidate molecular pathways involved in the therapeutic and possible toxicological responses. Chemical proteomics aims to directly isolate the

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protein receptor by utilizing affinity selection of a tagged or immobilized natural product. Small molecule-immobilized affinity chromatography is a simple but powerful approach to detect unknown receptors and to evaluate target selectivity. Apart from small molecule-immobilized chromatography, chemical proteomics encompasses several alternative methods for target identification, including phage display and the yeast three-hybrid (Y3H) systems (Walsh & Chang, 2006). Although chemical proteomics approaches are usually of low-throughput and demand a careful design of the tagged or immobilized natural product probe, they afford direct determination of binding partners and offer clues on how the natural product exerts its cellular effect. A potential bottleneck that might beset the interpretation of the influence of protein–natural product binding is that about half the proteins expressed by the genome have not been ascribed to a function (Kraljevic et al., 2006). In this sense, expression proteomics is complementary because it can provide a detailed map of alternations in molecular signaling following the natural product–target binding. At present, there are two major technologies in expression proteomics: the ''gel-based'' approach, which employs two-dimensional gel electrophoresis (2-DGE) to resolve protein mixtures, followed by MALDI-MS analysis; and ''shotgun'' proteomics, which uses multi-dimensional liquid chromatography coupled with ESI-MS/MS^n (He & Chiu, 2003). Both are routinely used for the global assessment of alternation of protein expressions in drug-treated cells or tissues. Changes in post-translational modifications (PTMs) including phosphorlyation, cysteine oxidation, and acetylation also play key roles in regulating cellular response following drug treatment. These PTMs can be readily identified by characteristic mass shifts in MS and MS/MS^n spectra (Burkitt et al., 2003; Schermann, Simmons, & Konermann, 2005). With the implementation of affinity purification and immunoblotting detection strategies, quantitative global profiling of post-translationally modified peptides would uncover yet another dimension through which natural products could modulate protein functions.

Identifying the binding partner is a crucial step to unravel molecular mechanisms; however, to fully understand the impact of natural products on protein function(s), further analysis of natural product–protein complexes should be carried out. In this regard, a wide array of MS-based techniques is available. ESI-MS is usually the default choice, with many examples of preservation of non-covalent protein–ligand complexes during transfer from solution to gas-phase ions (Winston & Fitzgerald, 1997; Akashi, 2006), whereas with MALDI-MS these complexes might be disrupted or non-specific interactions might be formed when dried samples are co-crystallized with the matrix (Akashi, 2006). MS information derived from ESI-MS experiments provides a direct measure of the stoichiometry of natural product–protein complex (Ganem, Li, & Henion, 1991). The amount of energy needed to disrupt the non-covalent complex is a measure of binding affinity of a ligand (Winston & Fitzgerald, 1997). MS is also an invaluable tool to provide low-resolution structural information on the interface of the natural product–protein complexes. Although X-ray crystallography and NMR methods remain the gold standard in terms of resolution, MS possesses distinct advantages such as relative speed, sensitivity, and adaptability to different types of protein that account for its popularity to analyze protein complexes. These advantages are exemplified by a large number of publications that contribute to the study of protein–ligand complexes by MS, in particular ESI-MS (Schermann, Simmons, & Konermann, 2005). Several classes of protein-reactive natural products covalently modify target proteins. These covalent natural product–protein complexes can be studied by a strategy that involves protease digestion followed by MS and MS/MS^n to localize modified peptide and amino acid residues, respectively. A majority of natural product–protein complexes, however, involves non-covalent interactions. Chemical strategies (limited proteolysis, hydrogen–deuterium (H/D) exchange, and photoaffinity labeling, in conjunction with MS) are required to probe the interfacial regions of non-covalent complexes (Aoki et al., 2003; Hoofnagle et al., 2003; Imre, Zsila, & Szabo, 2003). These techniques can provide information on the specific amino acid residues or regions of the protein that interface with a natural product. Such information is invaluable to identify an active pharmacophore, and thus guides design towards more potent and selective ligands in the lead optimization.

In this review, we overview the recent applications of MS and MS/MS to study the molecular interactions of natural products and their protein receptors, and highlight the different studies that demonstrated the applicability of MS at multiple stages of drug development from natural products.

II. THE ROLE OF MS TO IDENTIFY MOLECULAR TARGETS OF NATURAL PRODUCTS

A. Chemical Proteomic Strategies

1. *Affinity Chromatography*

Identification of protein targets is a major bottleneck in drug development because protein targets of the majority of natural products, including those in clinical use, are poorly understood. The most widely employed approach is ''pull-down'' experiments, in which a natural product-immobilized affinity matrix is used to ''fish'' target proteins from cellular or tissue extracts. In the 1990s, several classic papers demonstrated applicability of natural product-immobilized matrix to isolate protein receptors such as FK506–FK506 binding protein and Trapoxin-histone deacetylase (Harding et al., 1989; Crews et al., 1996; Taunton et al., 1996). The recent advert of highly sensitive MS identification has greatly miniaturized the scale of the required affinity purification. Typically, separations can be performed with several milligrams of cellular extracts on a small quantity of affinity matrix. The latter is not a trivial factor—given the small quantities of natural products due to difficulties in collection of the starting materials as well as in the purification process (Harvey, 2007). Below, we highlight several affinity pull-down approaches to identify protein receptors by citing representative examples. A more comprehensive listing of its applications is compiled in Table 1.

One straightforward method is to directly immobilize a natural product onto a matrix via a linker (Fig. 1). An important consideration is a suitable strategy to derivatize parent drug without significantly altering its biological activity. Structure–

TABLE 1. Proteomics to uncover protein targets

Natural product	Origin	Protein Target(s) Identified
Affinity chromatography		
Immobilized-small molecule chromatography		
Ampicillin	Mircobial	Penicillin-binding proteins (von Rechenberg et al., 2005)
EGCG	Plant	Vimentin (Ermakova et al. 2005)
Flavopiridol	Synthetic mimic	Cytosolic aldehyde dehydrogenase 1 (Schnier& Kaur& Kaiser& Stinson& Sausville& Gardner& Nishi& Bradbury& Senderowicz, 1999)
Hymenialdisine	Marine	Glycogen synthase kinase 3, Mitogen-activated protein kinase kinase-1, p90 ribosomal S6 kinase, β-tubulin (Wan et al., 2004)
Jacalin	Plant	*Anopheles gambiae* aminopeptidase N (Dinglasan et al., 2007)
Myriocin	Microbial	Palmitoyltransferase subunits LCB1 and LCB2 (Chen et al., 1999)
Resveratrol	Plant	Dihydronicotinamide riboside, quinone reductase 2, (Wang et al., 2004), Glutathione sulfotransferase-π, Estrogen receptor-β (Hsieh et al., 2008)
Saframycin	Microbial	Glyceraldehyde 3-phosphate dehydrogenase (Xing et al., 2004)
Biotinylated small molecule-streptavidin chromatography		
Ent-15-oxokaurenoic acid	Plant	FK506 binding protein 12-rapamycin associated protein, Phosphate carrier precursor isoform 1a, p53-associated Parkin-like cytoplasmic protein, Ran-binding protein 2 (Rundle& Nelson& Flory& Joseph& Th'ng& Aebersold& Dasso& Andersen& Roberge, 2006)
Eponemycin	Microbial	Proteasomal beta-catalytic subunit LMP2 and LMP7 (Meng et al., 1999)
FR177391	Microbial	Protein phosphatase 2A (Yamaoka et al., 2005)
(-)-FR182877	Microbial	Carboxylesterase-1 (Adam& Vanderwal& Sorensen& Cravatt, 2003)
2'-hydroxyl-cinnamaldehyde	Plant	Proteasome subunit alpha type 1, Proteasome subunit beta type 4 precursor (Hong et al., 2007)
Pateamine A	Marine	Eukaryotic translation initiation factor 4A (Bordeleau& Matthews& Wojnar& Lindqvist& Novac& Jankowsky& Sonenberg& Northcote& Teesdale-Spittlet& Pelletier, 2005,Low& Dang& Schneider-Poetsch& Shi& Choi& Rzasa& Shea& Li& Park& Ma& Romo& Liu, 2007,Low& Dang& Schneider-Poetsch& Shi& Choi& Merrick& Romo& Liu, 2005)
Photoaffinity labelling		
Cyclosporine	Plant	Cyclophilin A (Lamos& Krusemark& McGee& Scalf& Smith& Belshaw, 2006)
Ovalicin	Microbial	Methionine aminopeptidase type 2 (Griffith et al., 1997)
Pladienolide	Microbial	Spliceosome-associated proteins 145 (SAP145) and SAP130 (Kotake& Sagane& Owa& Mimori-Kiyosue& Shimizu& Uesugi& Ishihama& Iwata& Mizui, 2007)
Tamoxifen	Plant	Carboxylesterase, Liver fatty acid binding protein (Mesange et al., 2002)

TABLE 1. (*Continued*)

Affinity displacement chromatography		
Quinine	Plant	Aldehyde dehydrogenase 1, Quinone reductase 2 (Graves et al., 2002)
Phage display		
Camptothecin	Plant	Topoisomerase I, hEP1 receptor, Carboxylesterase (Takakusagi et al., 2007)
Curcumin derivative	Plant	Ca^{2+}/calmodulin (Shim et al., 2004)
Demethylasterriquinone b1	Microbial	Glyceraldehyde 3-phosphate dehydrogenase (Kim et al., 2007)
FK506	Microbial	FK506 binding protein 12 (FKBP12), FKBP12.6, FKBP13 (McKenzie& Videlock& Splittgerber& Austin, 2004,Sche& McKenzie& White& Austin, 1999,Sche& McKenzie& White& Austin, 2001)
Kahalalide F	Marine	Ribosomal protein S25 (Piggott& Karuso, 2008)
Paclitaxel	Plant	B-cell CLL/ lymphoma 2 (Bcl-2) (Rodi& Agoston& Manon& Lapcevich& Green& Makowski, 2001,Rodi& Janes& Sanganee& Holton& Wallace& Makowski, 1999), Neuronal Ca^{2+} sensor 1 (Boehmerle& Splittgerber& Lazarus& McKenzie& Johnston& Austin& Ehrlich, 2006), Nuclear transcription factor, X-box binding 1 (Aoki& Morohashi& Sunoki& Kuramochi& Kobayashi& Sugawara, 2007)
Resveratrol	Plant	Breast cancer-associated antigen, Breast cancer resistance protein, Death-associated transcription factor, Human cyclin-dependent kinase (Feng et al., 2006)
mRNA display		
FK506	Microbial	FKBP12 (McPherson et al., 2002)
Yeast-three-hybrid		
FK506	Microbial	FKBP12 (Licitra& Liu, 1996)
Two-dimensional gel electrophoresis		
Bengamides	Marine	Methionine aminopeptidases (Towbin et al. 2003)
Mycophenolic acid	Microbial	ATP synthase α and β, Protein disulfide isomerase A3, Selenium binding protein (Shipkova& Beck& Voland& Armstrong& Grone& Oellerich& Wieland, 2004), Protein disulfide isomerase, Aldehyde dehydrogenase, Triosephosphate isomerase, Aminoacylase, Tropomyosin 1 and 4, Peroxiredoxin 3 and 6 (Asif& Armstrong& Voland& Wieland& Oellerich& Shipkova, 2007)
Teucrin A	Plant	46 proteins involved in the metabolism of lipids, amino acids, and drugs (Druckova& Mernaugh& Ham& Marnett, 2007)

activity relationship (SAR) and structural information on the natural product-known target complex are certainly useful to decide a tethering position; however, caution should be excised because alternative binding modes often exist through which drugs can form complexes with unanticipated target(s) (Uga et al., 2006). Polyphenols are bioactive compounds that are widely distributed in fruits, vegetables, and medicinal herbs (Aherne & O'Brien, 2002). Substantial in vitro and in vivo investigations have demonstrated cardioprotective and chemopreventive effects of this class of natural products (Scalbert,

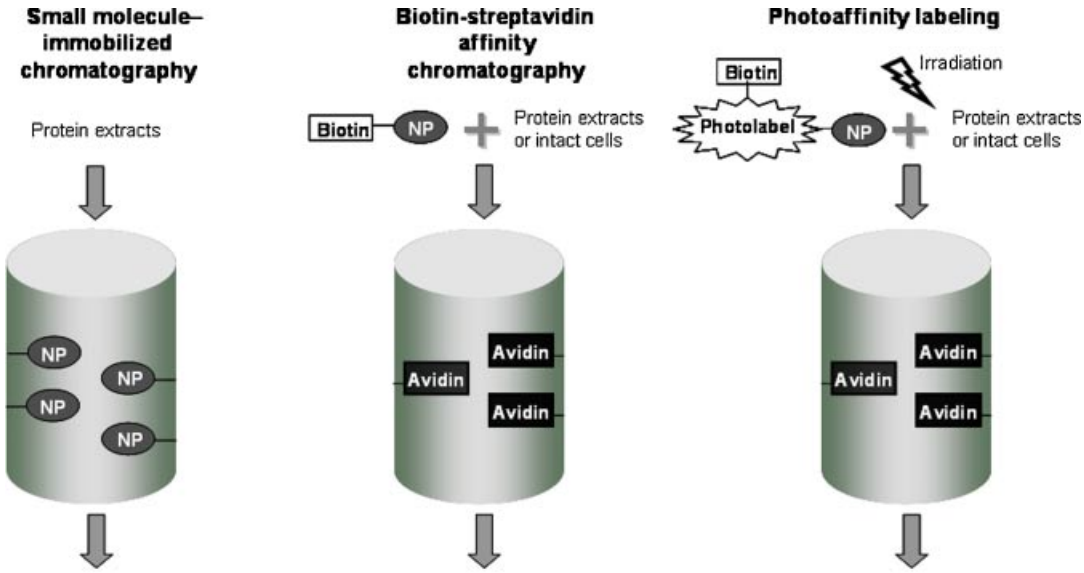

FIGURE 1. The three major small-molecule affinity chromatography approaches to identify receptor proteins of natural products. (1) In small molecule-immobilized chromatography, natural products are directly immobilized onto an affinity resin via a spacer arm to reduce steric hindrance. (2) In another popular approach, natural products are attached to a biotin moiety. The biotinylated natural products are incubated with protein extracts or intact cells, which are selectively enriched on a streptavidin-coated matrix. (3) In photoaffinity labeling, natural products are derivatized with two functional moieties, the photoreactive and biotin groups. Upon irradiation, the photoreactive group forms covalent cross-links with proteins in close proximity to stabilize the natural product–protein complex. The complex could be purified with a streptavidin-coated matrix. Target proteins isolated from these three approaches are visualized on SDS–PAGE and identified with MALDI-MS or ESI-MS analysis.

Johnson, & Saltmarsh, 2005). Affinity pull-down experiments have been employed to probe the protein targets of epigallocatechin gallate (EGCG) (Ermakova et al., 2005), flavopiridol (Schnier et al., 1999), and resveratrol (Wang et al., 2004; Hsieh et al., 2008). Chemically reactive hydroxyl groups facilitate convenient immobilization to epoxy- or CNBr-activated agarose gel via a spacer arm. An affinity matrix so produced is heterogeneous because polyphenols are attached via either of its multiple hydroxyls, and as a result, ligands are presented in different orientations to maximize the capacity of the column to capture different protein species.

Epigallocatechin gallate (EGCG), a major bioactive catechin in green tea (Lambert & Yang, 2003), was found to interact with plasma proteins and laminin (Tachibana et al., 2004; Tachibana, 2008). In search of alternative high-affinity receptors for this catechin, Ermakova et al. (2005) generated an EGCG-Sepharose matrix to capture target proteins from cell lysates. 2-DE analysis revealed consistently the presence of three spots, which were purified and identified to be vimentin by MALDI-MS. EGCG was found to bind to this intermediate filament with high affinity (K_D of 3.3 nM), and specifically inhibited its phosphorylation at serine 50 and serine 55. Importantly, the ability of EGCG to inhibit cell proliferation, which was lost after knockdown of vimentin expression, indicated a crucial role of vimentin-binding interaction to mediate the chemopreventive effect of EGCG. Flavopiridol, a semi-synthetic derivative of rohitukine isolated from *Dysoxylum binectariferum*, is a potent anticancer agent against the breast and lung cancer cell lines (Senderowicz, 2003; Cragg & Newman, 2005), with promising clinical efficacy towards chronic lymphocytic leukemia (Byrd et al., 2007; Heerema et al., 2007). It was suggested that inhibition of cyclin-dependent kinases (CDKs) could be a molecular mechanism that contributed to the anti-proliferative effect of flavopiridol; however, it is apparent that additional targets were involved because cells that did not express active CDKs were also sensitive to this drug (Brusselbach et al., 1998). Flavopiridol-immobilized agarose affinity chromatography has identified the aldehyde dehydrogenase class 1 (ALDH-1) as a receptor from NSCLC cell extracts (Schnier et al., 1999). Interestingly, the flavopiridol–ALDH-1 interaction did not have an effect on the activity of ALDH-1, and a high ALDH-1 expression protected the cells from flavopiridol-induced cytotoxicity. One possible explanation is that abundant ALDH-1 might quench flavopiridol and lower its effective concentration in cytosol; thus, ALDH-1 might be a possible factor of drug resistance against flavopiridol. In addition, it was found that CDKs, putative targets of flavopiridol, were easily displaced by ATP. The ability of ATP to displace flavopiridol from CDKs is in

accordance with another study that showed that flavopiridol binding was competitively blocked by ATP with K_i of 41 nM (Senderowicz, 2003). The displacement by ATP brought into question the in vivo relevance of CDKs–flavopiridol interaction, where the concentrations of ATP are around 5 mM. Indeed, alternative mechanisms exist that might account for the potent cell-cycle arresting effect of this drug (Brusselbach et al., 1998; Carlson et al., 1999; Melillo et al., 1999). Small molecule-immobilized chromatography is certainly a very useful tool to unravel unknown protein targets of natural products. A drawback is that it necessitates the use of cell extracts or tissue homogenates, which might increase the chances of non-specific adsorption of proteins in high abundance, and thus lower the chances to find low-abundance targets (Deibel et al., 2004). Moreover, the loss of cellular compartmentation means that those targets that are not accessible to natural products in intact cells might also be isolated, and lead to a false positive identification.

Another widely employed affinity pull-down assay utilizes natural products incorporated with a biotin moiety that is readily affinity-purified by a streptavidin matrix (Fig. 1). An advantage of this approach is that biotin-labeled natural products can be delivered to intact cells prior to purification, because the natural product–protein complex is sufficiently strong to withstand sample preparation procedures. This strategy circumvents some of the drawbacks of small molecule-immobilized chromatography. *Ent*-15-oxokaurenoic acid (EKA) is a diterpenoid isolated from plants that induces mitotic arrest in cancer cells. Rundle et al. (2006) attempted to uncover cellular targets of EKA by derivatization with a biotin group. Biotinylated EKA retained the cell-permeable function and the antimitotic activity of the parent compound. EKA-biotin and negative control-biotin were incubated with cells, followed by cell lysis and affinity precipitation with streptavidin-agarose beads. Six specifically bound proteins were identified with LC-MS/MS, including the FK506-binding protein 12-rapamycin-associated protein, phosphate carrier precursor isoform 1a, p53-associated Parkin-like cytoplasmic protein, and Ran-binding protein 2 (RBP2). Among these proteins, RBP2 is intimately involved in mitotic progression (Joseph et al., 2004), and its inactivation by EKA is reminiscent of the cell-arresting effects of RBP2-siRNA knockdown (Salina et al., 2003); those data affirm the important role of this receptor in the action mechanism of EKA. Biotin–streptavidin affinity assay was also used to search for protein targets of Pateamine A (PatA), a potent anti-proliferative agent first isolated from marine sponges (Northcote, Blunt, & Munro, 1991). Structure–activity studies have indicated its C-3 amino group as a suitable site for derivatization (Low et al., 2007). Biotin was thus incorporated into PatA via this position, and the probe was screened with RKO cell extracts. MALDI-MS analysis revealed eIF4A and the serine/threonine kinase receptor-associated protein as the targets of PatA (Low et al., 2005). eIF4A participates in eukaryotic translation initiation (Gingras, Raught, & Sonenberg, 1999). Interaction with PatA results in binding of eIF4A with eIF4B and stalling the formation of initiation complexes on mRNA in vitro, to subsequently induce apoptosis in cancer cells. PatA is an attractive anticancer lead because it preferentially targets fast-growing cancer cells with less damage to normal cells. Interestingly, another research group has also independently identified eIF4A as a protein target of PatA with a PatA-immobilized Sepharose affinity column (Bordeleau et al., 2005). Although it is somewhat fortuitous that PatA immobilization can be accomplished simply by means of its primary amino group C-3, this phenomenon actually emphasizes, in some instances, the general applicability of affinity chromatography to ''fish'' protein targets, even without any prior knowledge of bioactivity or detailed SARs.

Photoaffinity labeling (PAL) uses bifunctional probes that consist of (1) bioactive natural products; (2) a photoreactive group that forms a covalent bond with proteins upon irradiation; and (3) a tag that allows for the detection or purification of natural product–protein complexes (Fig. 1) (Dorman & Prestwich, 2000). The photoaffinity label is first incubated with a protein extract, in which it interacts irreversibly with the target proteins. Irradiation would lead to formation of a covalent complex of natural product–protein at the ligand binding site. The complex could then be purified and visualized. As discussed later in this review, PAL also provided structural aspects of natural product–target interaction. PAL has been employed to isolate the receptors for the antitumor natural product pladienolide produced by *Streptomyces platensis* (Kotake et al., 2007). The authors used ^{3}H-labeled, fluorescence-tagged, and photoaffinity/biotin bifunctional probes for this investigation. Using a ^{3}H probe, the target was found to be localized in the nucleus fraction, and could be enriched by immunoprecipitation with SAP155 antibody. To isolate the receptor(s), a photoaffinity/biotin probe was incubated with HeLa cells, immunoprecipitated with anti-SAP155 antibody, and irradiated to form cross-links. The precipitated proteins were separated with SDS–PAGE, and detected with immuoblotting. LC-MS/MS analysis confirmed their identity as spliceosome-associated proteins (SAP) 145 and 130, subunits of splicing factor SF3b. Specific binding between pladienolide and SAP130 resulted in the splicing inhibition in vivo. Splicing inhibition through SF3b is a novel antitumor target that deserves further investigation. An innovative PAL development is to incorporate affinity, photoaffinity, and mixed isotopic labels into a tri-functional probe—as proposed by Lamos et al. (2006). In this approach, the label is first cross-linked with target proteins, affinity purified, and subjected to LC-MS/MS analysis (Fig. 2). The identity of target proteins is determined by sequencing the unlabeled peptide (M); the presence of the isotope-labeled peptide (M + 11) would immediately confirm the specificity of interaction. As a proof-of-concept experiment, cyclosporin A (CsA) was derivatized with a [d_0] and [d_{11}] label, and mixed in a 1:1 molar ratio with a protein mixture that contained the known CsA target cyclophilin A. Following irradiation and avidin-column purification, the tryptic digest was analyzed with LC-MS/MS. Apart from the 11 peptides assignable to cyclophilin A, peptides of ubiquitin and keratin were also recognized. However, only two peptides that contained the isotopic fingerprint M and M + 11 were found that corresponded to cyclophilin A peptides 92–118 and 56–82. The isotopic signature enabled a rapid differentiation of specific interactions from non-specific binding or contaminants. Moreover, labeled peptides represented regions of the protein that were exposed to, or in close proximity with, the cyclosporine binding site (Weber et al., 1991). Because PAL stabilizes non-covalent interactions, it is an excellent tool to identify low-affinity and low-abundance receptors that cannot be detected with conventional affinity chromatography. In addition,

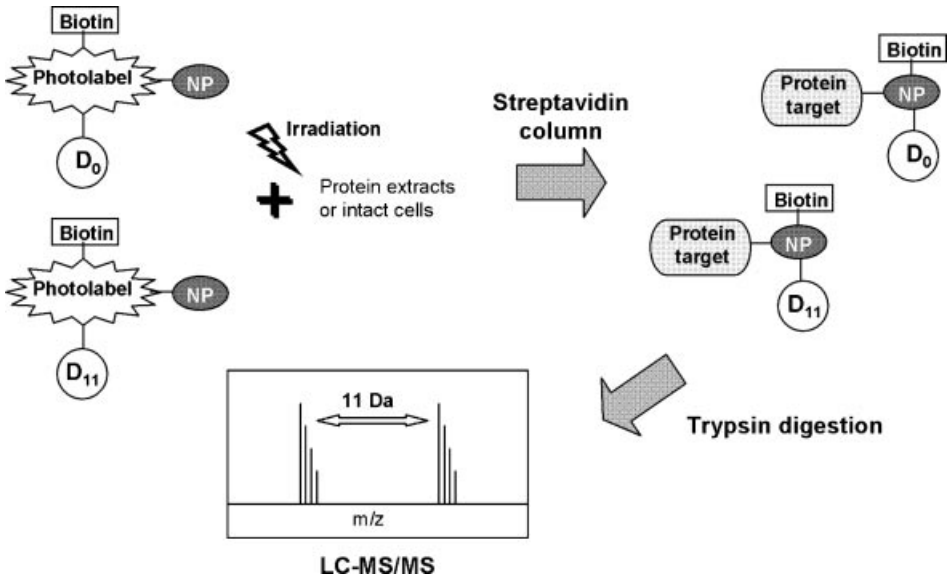

FIGURE 2. In a recent development in chemical proteomics, Bantscheff and co-workers (2007) developed a ''kinobead'' matrix to evaluate the global kinase selectivity of various drugs. The ''kinobead'' consists of seven ATP-binding site ligands that can retain protein kinases. To assess the specific binding of drugs to protein kinases, a protein extract is incubated with the kinobeads in the absence or presence of the drug. Bound proteins are isotopically labeled with iTRAQ, and analyzed with LC-MS/MS. Because the drug competes with the matrix for its kinase targets, target proteins could be identified by virtue of a decrease in iTRAQ intensity signal of the binding proteins. A major advantage over the previous affinity approaches is that native drugs are used to circumvent the need for derivatization.

PAL enables binding and cross-linking in various intact cell systems, if the probe is cell-permeable, to thus allow native-state characterization of natural product–receptor complexes.

Affinity chromatography is still a popular choice to isolate protein receptors of natural products; however, associated limitations are increasingly recognized, such as the need for a strong affinity between the ligand and the target to withstand the multiple washing steps, as well as the difficulties to detect low-abundance proteins. Emerging quantitative proteomic tools could be exploited for non-biased and highly sensitive comparison between control and affinity-purified proteomes to improve the detection of low-abundance binding receptors, which might be difficult to detect with conventional SDS–PAGE. Oda et al. (2003) employed a combination of two-dimensional difference gel electrophoresis (DIGE) and isotope code affinity tags (ICAT) to detect specifically retained proteins on an E7070-affinity matrix compared to the control matrix. This approach successfully identified over 377 potential protein targets, and suggested that the compound examined binds to various proteins in a non-specific manner. Furthermore, a ranking of the binding specificity was discerned from ratio of peaks in the control and the E7070 matrix to identify cytosolic malate dehydrogenase as the most-specific receptor. Despite these advances, natural-product derivatization and non-specific interaction with high-abundance proteins remained a rate-limiting step in routine target identification. With these issues in mind, several groups recently developed an affinity displacement chromatography approach that used an affinity column that can bind to sub-groups of proteins of interest, from which specific protein receptors were determined by the reduced affinity and hence enrichment of proteins when co-incubated with drugs (Peters & Gray, 2007). Major breakthroughs in this approach are twofold: proteins that are important for signal transduction, such as kinases, could be specifically enriched relative to non-target high abundance proteins; and natural product-derivatization is not required for the affinity assay.

Graves et al. (2002) screened several quinine derivatives against the purine-binding proteins from red blood cells and the *Plasmodium falciparum*. Quinine is an alkaloid isolated from bark of *Cinchona officinalis*, and has been the most effective drug to treat malaria for many years (Foley & Tilley, 1998). Despite its extensive use, the mechanism of action remains poorly understood. Because these quinoline drugs share several structural features with purine nucleotides, it was hypothesized that the molecular targets of quinoline are members of purine-binding proteins such as kinases and dehydrogenases. In this ''displacement'' affinity chromatography (Fig. 3), γ-phosphate-linked ATP-Sepharose was first used to capture the entire purine proteome on the affinity matrix, followed by elution with a quinoline drugs-containing buffer to displace the specific receptors that were identified with MS. A quinoline-immobilized-affinity matrix was also used to purify protein targets. Using extracts from red blood cells and *P. falciparum* parasites, it was found that chloroquine, primaquine, and mefloquine all selectively eluted ALDH-1 and quinone reductase 2 (QR2). Affinity chromatography with derivatized quinoline also selectively isolated these two receptors from red blood cells. Quinolines were found to inhibit ALDH-1 and, in particular, QR2 in vitro.

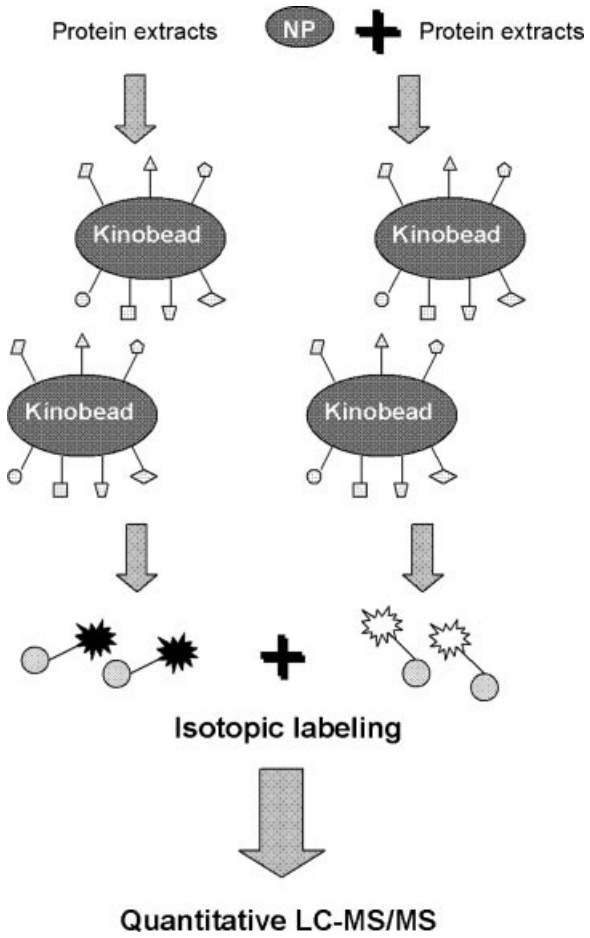

FIGURE 3. The use of tri-function probes for photoaffinity labeling. This tri-function probe consists of a natural product conjugated with a photoreactive and a biotin moieties carrying normal or D_{11} isotopes. Following cross-linking, the natural product–protein complexes are purified with a streptavidin column. Tryptic digests are analyzed with LC-MS/MS. Target proteins are validated with MS sequencing; those data can be further confirmed through the presence of isotope-labeled peptides that differ by 11 Da from the unlabeled ones.

Inhibition of QR2 might lead to an enhanced oxidative stress that is lethal to *P. falciparum* parasites. Building on a similar concept, Bantscheff et al. (2007) constructed a ''kinobead'' to capture the kinase proteome, and employed state-of-the-art quantitative proteomics technology to assess drug–protein interaction by measuring the reduced retention of targets on an affinity matrix when co-incubated with drugs. Kinases are attractive drug targets, particularly in oncology (Blume-Jensen & Hunter, 2001). To address the specificity of kinase inhibitors, recombinant kinase panels are routinely used, but these panels are neither a complete representation of the human kinome nor do they consider non-kinase targets. In Bantscheff' work, the ''kinobead'' was constructed by immobilizing seven different ATP-binding site ligands onto a single matrix, which could non-selectively enrich protein kinases from various sources and reduce the enrichment of abundant non-kinase proteins compared to ATP-Sepharose (White, 2007). To identify protein targets, cell or tissue lysates were treated with three different doses of the drug, followed by precipitation with kinobeads. The beads were washed, bound proteins were eluted and digested, and the four samples were labeled with iTRAQ reagent. Samples were mixed and analyzed with LC/MS/MS. Competition of the added drug with the kinobead for binding to its target proteins resulted in a reduced affinity and thus enrichment of these proteins and a decreased intensity in their corresponding peptides in the mass spectra compared to the control. When this procedure was repeated over a range of ligand concentrations, competition binding curves could be constructed, which allowed IC_{50} of dissociation to be determined. The method has been successfully validated with the synthetic kinase inhibitors imatinib, dasatinib, and bosutinib. As in Graves's work, only ''free'' ligand is required. Therefore, this simple but elegant approach could be applied to literally any molecule of interest, including natural products whose molecular targets are still poorly understood. Rapid advances in these chemical proteomics technologies hold the key to accelerate the rate-limiting step to unravel the molecular mechanisms and undesirable side effects of natural products.

2. *Alternative Methods*

Apart from MS-based identification of protein targets, several genetic approaches have been proposed, including display technology, Y3H, and microarrays. Some of these techniques such as mRNA display, Y3H, and microarray are relatively new developments that are reported largely in proof-of-principle studies. Currently, phage display is one of the most popular alternatives to MS-based target identification (Sergeeva et al., 2006). In phage display, polypeptides are present as fusion proteins on the surface of phages, and large libraries of up to 10^{10} unique members could be created and screened against an immobilized natural product. Enriched clones are multiplied in host bacteria, usually *E. coli*. The amplification process increases the chance to identify low-abundance protein targets of natural products. Phage display has been applied to the identification of cellular receptors of FK506, FKBP12 (Sche et al., 1999, 2001; McKenzie et al., 2004), and the novel Taxol targets Bcl-2 and neuronal Ca^{2+} sensor (Rodi et al., 1999, 2001; Boehmerle et al., 2006; Aoki et al., 2007). Phage display has also demonstrated a potential to identify epitopes that might interact with natural products (Rodi et al., 2001; Rodi, Makowski, & Kay, 2002). It is thus a potential alternative when a receptor could not be found with affinity chromatography–mass spectrometry. As mentioned previously, the major advantage of phage display is phenotype–genotype linkage that offers tremendous sensitivity via a stepwise amplification process. One concern with the use of phage display is the lack of cellular machinery to carry out the complex folding process as in eukaryotic cells that results in misfolding and their inability to select multimeric or transmembrane proteins. This problem might be partly avoided by the expression of short-length epitope-coding pieces (Makowski & Rodi, 2003); however, a loss of structural context might lead to a reduced number of ''hits'' and a reduced affinity for the ligand. Non-specific interactions could also happen with phage display, as Piggott and Karuso (2004) observed in several studies hNopp 140 was isolated as receptors despite using natural products with

different structural and biological properties. Further examples of these genetic-based approaches were summarized in another recent review (Wong et al., 2008a).

B. Expression Proteomics Strategies

1. Two-Dimensional Gel Electrophoresis (2-DGE) to Profile Differentially Expressed Proteins

In recent years, 2-DGE has been extensively utilized to analyze the molecular action of natural products (Table 2). The study of alterations of proteins expression in the untreated versus drug-treated samples could reveal molecular mechanisms and networks that contribute to the potential therapeutic and adverse effects of a natural product. Apoptosis-inducing agents are considered attractive leads for the development of novel chemotherapeutics. A large number of studies documented alternations in the protein expression levels upon treatment with apoptosis-inducing natural products (Table 2). In our laboratory, anticancer activity of dioscin on HL-60 leukemia cells has been studied with 2-DGE (Wang et al., 2006; Wang et al., 2007a). Dioscin is a saponin isolated from the root of *Polygonatum zanlanscianense* that exhibits potent anti-proliferative activity towards cancer cells (Wang et al., 2001; Cai et al., 2002; Liu et al., 2004). Whole-cell proteomes of dioscin-treated HL-60 cells and untreated control were separated with 2-DGE, and the differentially expressed proteins were identified with PMF. We observed substantial alternations of mitochondrial proteins, including the mitochondrial ATP synthase, fumarase, and chaperonins. It was thus hypothesized that the mitochondrion is the primary target of dioscin in HL-60 cells. Reaffirmation of this notion was the observation that treatment with dioscin resulted in a dissipation of the mitochondrial membrane potential and an activation of caspase-3 and caspase-9, the typical hallmarks of mitochondrion-mediated apoptosis (Affar et al., 2001). In this case, 2-DGE has successfully pinpointed an important pathway through which dioscin can exert its apoptotic effects on cancer cells. However, from our studies it was apparent that the complexity of the proteome far exceeded the analytical capabilities of 2-DGE; and of LC-MS-MS. A simple and effective approach to simplify the sample matrix is the analysis of sub-proteomes obtained by subcellular fractionations or affinity chromatographic separations (Jung et al., 2000; Dreger, 2003; Huber, Pfaller, & Vietor, 2003). To further study the mechanisms of apoptosis induction by dioscin, we prepared soluble proteins from the microsomal fractions of dioscin-treated HL-60 cells with centrifugation, followed by 2-DGE analysis. Thirty-nine differentially expressed proteins were identified, with a notable altered expression of chaperones and proteasome subunits. Such alternations were suggested to be associated with increased oxidative stress imposed upon the cell by dioscin (Wang et al., 2007a). Indeed, functional studies indicated that a substantial number of reactive oxygen species (ROS) were accumulated following dioscin treatment, with the mitochondrion being the primary source. Taken together, the mitochondrion is a primary target of dioscin, and the subsequent generation of ROS is critical to execute the cell-death signaling cascade. Despite the fact that the exact molecular receptor(s) of dioscin could not be identified in our studies, we demonstrated that, in conjunction with suitable functional studies, the major molecular mechanisms associated with this natural product can be established.

An improved variant of traditional 2-DGE is the DIGE. The most problematic aspect of the traditional 2-DGE is the lack of good reproducibility due to gel-to-gel variation, and the narrow dynamic range of detection offered by the widely used silver staining methods. In DIGE, quantitative analysis of two to three samples is performed on the same gel simultaneously (Viswanathan, Unlu, & Minden, 2006). Samples are first labeled with different fluorescent dyes, Cy3 or Cy5, mixed, and separated on the same 2-D gel (Unlu, Morgan, & Minden, 1997). In this way, protein spots from the two samples superimpose on each other, and could be easily quantified with fluorescence detection. This method is also highly sensitive, and has a linear detection range in excess of 10,000-fold (Gong et al., 2004), in contrast to an ~10-fold range with silver staining. DIGE was used to explore early apoptotic events in human hepatoma HepG2 cells treated with β-phenylethyl isothiocyanate (PEITC) (Neo et al., 2005), an effective apoptosis inducer that is derived from enzymatic hydrolysis of glucosinolates present in *Cruciferous* vegetables. In this experiment, 17 differentially expressed spots were found with DIGE; 14 were identified with PMF analysis using MALDI-MS. Three additional proteins, the hnRNP, heat shock protein 27, and macrophage migration inhibitory factor, underwent PTM and resulted in a shift in their isoelectric points. Most of the altered proteins detected are related to oxidative stress, mitogen-activated protein kinase (MAPK) signaling cascades, and apoptosis. All together, these results suggested that PEITC triggered apoptosis by the generation of ROS, which in turn activated stress-related MAPK signaling cascades, and ultimately led to apoptosis.

2. 2-DGE for Direct Target Identification

Although 2-DGE is a very useful approach to understand the downstream mechanisms of a drug, the direct identification of cellular receptors with this technique is rather limited because of the observation that a majority of the studies listed in Table 3 were not successful in identifying the cellular receptors of natural products. In rare instances, protein targets can be inferred from a careful examination of 2-DGE data. In an effort to determine the cellular targets of LAF389, a synthetic analog of the marine natural product bengamide B, Towbin et al. (2003) analyzed protein expression difference in H1299 cells caused by this drug. Drug treatment was found to result in shifts in the isoelectric point of a subset of proteins; one was identified with MALDI-MS as 14-3-3γ. Analysis of mass spectra showed that *N*-terminal processing was absent after drug treatment, whereas in normal form 14-3-3γ protein *N*-terminal methionine was cleaved by methionine aminopeptidases (MetAps), followed by acetylation. Blockade of *N*-terminal processing suggested that MetAp might be a possible target of LAF389 and bengamide B. This hypothesis was further confirmed by in vitro assays with MetAp 1 and 2. Thus, 2-DGE analysis established MetAps as a target of bengamide E and its structural analogs.

An alternative strategy for target identification is based on comparing the 2-DE expression profiles of a drug of unknown targets with the protein alternations induced by drugs with known

TABLE 2. Applications of expression proteomics

Bioactivity	Natural products
Two-dimensional gel electrophoresis	
Antimicrobials	Penicillin (Bandow et al., 2003), Daunomycin, Adriamycin (Sender et al., 2004), 30 Antibiotics (Bandow& Brotz& Leichert& Labischinski& Hecker, 2003)
Apoptosis induction	Actinomycin D (Kim et al., 2005) All-trans retinoic acid (Cimmino et al., 2007,Harris et al., 2004,Smith et al., 2007,Wang et al., 2007,Wu et al., 2006,Wu et al., 2004,Zheng et al., 2005), Dioscin (Wang& Che& Chiu& He, 2007,Wang et al., 2006), Geistein (Rowell et al., 2005; Zhang et al., 2007), Kendomycin (Elnakady et al., 2007), Leptomycin B (Tsuchiya et al., 2005), Manumycin (Hu et al., 2003) Paclitaxel, (MacKeigan et al., 2003; Lee et al., 2005; Izbicka et al., 2006; Lee et al., 2007), β-Phenylethyl isothiocyanate (Neo et al., 2005), Quercetin (Wenzel et al., 2004), Resveratrol (Lee et al., 2006), Staurosporine (Short et al., 2007), Vinca alkaloid (Verrills et al., 2003)
Anti-angiogenesis	Endostatin, Fumagillin, Thrombospondin-1 (Keezer et al., 2003), Vinblastine and Rapamycin (Campostrini et al., 2006)
Antioxidant/antiaging	EGCG (Lee et al., 2006), Genistein (Fuchs et al., 2005,Fuchs et al., 2005), Grape seed extract (Deshane et al. 2004; Kim et al., 2006)
Cardiovascular diseases and lipidemia	Genistein (Fuchs& Erhard& Rimbach& Daniel& Wenzel, 2005), Ouabain (Kesiry& Liu, 2005,Qiu et al., 2008,Qiu et al., 2007) Chitosan oligosaccharides (Rahman et al., 2008), Olive oil(Arbones-Mainar et al., 2007)
Metabolic syndrome	Proanthocyanidin (Li et al., 2008), Fungal polysaccharides (Hwang et al., 2007,Kim et al., 2006)
Immunomodulation	Cyclosporin, PSP (Lee et al., 2007), Lipopolysaccharide (Gadgil et al., 2003,Zhang et al., 2006), Pycnogenol (Wu et al., 2007)
Mass spectrometry-based proteomics	
LC-MS/MS without isotopic labeling	
Anticancer	Scutellaria barbata (Goh& Lee& Ong, 2005), Berberine (Tan& Goh& Ong, 2006)
ICAT and LC-MS/MS	
Anticancer	Camptothecin (Yu et al., 2002)
^{18}O labelling and LC-MS/MS	
Anticancer	Doxorubicin (Brown& Fenselau, 2004)
Immunomodulation	Lipolysaccharide (Rao& Palamalai& Dunlevy& Miyagi, 2005)
Functional proteomics	
Phosphoproteomics	Phorbol myristate acetate (Metodiev& Timanova& Stone, 2004), Lipopolysaccharide (Hauschildt& Schwarz& Heine& Ulmer& Flad& Rietschel& Jensen& Mann, 1997), Capsaicin (Nagumo& Han& Arimoto& Isoda& Tanaka, 2007), Okadaic acid (Hill& Callaghan& Ding& Kelly& Chakravarthy, 2006), Naringin (Larsen& Moller& Blankson& Samari& Holden& Seglen, 2002) Rapmycyin (Raught et al., 2000)
Redox proteomics	Adriamycin (Chen& Daosukho& Opii& Turner& Pierce& Klein& Vore& Butterfield& St Clair, 2006), Cytotoxic drugs (England& Driscoll& Cotter, 2006), Eptoside (England& O'Driscoll& Cotter, 2004), Antioxidants (Opii& Joshi& Head& Milgram& Muggenburg& Klein& Pierce& Cotman& Butterfield, 2008)

TABLE 3. Application of MS to study covalent natural product–protein complexes

Natural product	Origin	Target protein	Major interacting peptides/residues identified
ESI-MS/MS			
Allyl sulfides	Plant	β-tubulin	Cys-12 and 354 (Hosono& Fukao& Ogihara& Ito& Shiba& Seki& Ariga, 2005)
Cyclostreptin	Marine	β-tubulin	Thr-220, Asn-228 (Buey& Calvo& Barasoain& Pineda& Edler& Matesanz& Cerezo& Vanderwal& Day& Sorensen& Lopez& Andreu& Hamel& Diaz, 2007)
Fumagillin	Microbial	Methionine aminopeptidase	His-79 (Lowther& McMillen& Orville& Matthews, 1998)
Bicyclomycin	Microbial	Rho	Lys-181 (Riba& Gaskell& Cho& Widger& Kohn, 1998)
4-Isoavenaciolide	Microbial	Vaccinia H1 related	Cys-124 (Ueda et al., 2002)
ESI-FT-ICR-MS			
Xanthohumol, isoliquiritigenin, 10-shogao	Plant	Keap1 protein	Cys-151 (major) and 23 alternative cysteine sites (Luo& Eggler& Liu& Liu& Mesecar& van Breemen, 2007)
MALDI-MS			
Curcumin	Plant	Thioredoxin	Cys-496, Sec-497 (Fang& Lu& Holmgren, 2005)
Avicins	Plant	OxyR	Cys-199 (Haridas et al., 2005)
Penicillic acid	Microbial	GDP-mannose dehydrogenase	Cys-213, Cys-246 (Kimmel& Tipton, 2005)
Imipenem	Microbial	Peptidoglycan $_{LD}$-transpeptidase	Cys-442 (Mainardi et al., 2007)

cellular targets or molecular mechanism of action. Such an approach has been applied to unravel cellular targets of novel antibiotics (Bandow et al., 2003). Reference protein-expression maps were first generated by treating *Bacillus subtilis* with 30 antibiotics from different classes, including protein synthesis inhibitors, inhibitors of cell-wall biosynthesis, inhibitors of DNA gyrase or RNA synthesis, base analogs, and cytoplasimic disruptors. The 2-DGE maps of antibiotic-treated *B. subtilis* showed that differentially expressed proteins were consistent with their mechanisms of action; and importantly, a good correlation of protein profiles was obtained for antibiotics within the same class. These 2-DGE reference maps thus allow for the differentiation between different classes of antibiotics, and protein markers can be chosen from the overlapping alternations that define a particular class of antibiotics. Cellular targets of a novel antibiotic can thus be predicted on the basis of the degree of matching with the identified protein markers. This concept was applied to BAY 50-2369, a novel pyrimidinone antibacterial drug. It was found that this compound induced a protein-expression profile that closely resembled markers of chloramphenicol and tetracycline; both belong to the category of inhibitors of the peptidyltransferase-catalyzed step in protein synthesis. The inhibitory effect of BAY 50-2369 was confirmed independently in a cell-free translation system. With this latter approach, a novel drug could only be matched correctly provided that drugs with related mechanisms of action were included in the database. Thus, a large database and a broad coverage of different classes of drugs are crucial for its wide applicability to predict mechanisms of action of natural products.

Given the enormous chemical diversity of natural products, it is not surprising that there is a subset that possesses highly reactive electrophilic groups that covalently react with nucleophilic residues on protein targets (Drahl, Cravatt, & Sorensen, 2005). Endogenous metabolism generates reactive electrophiles from natural products that covalently modify proteins (Liebler, 2008). However, it has been recognized that these covalent natural product–protein interactions might also lead to protein damage and tissue toxicity (Evans et al., 2004; Baillie, 2006). Radioactivity and immunochemical detection methods identify covalently adducted proteins that are resolved over a 2-D gel. In recent literature, immunochemical methods were adopted to identify protein targets of mycophenolic acid and teucrin A (Shipkova et al., 2004; Asif et al., 2007; Druckova et al., 2007). Mycophenolic acid was first isolated from a culture of *Penicillium stoloniferum*, and is now commonly administered as the prodrug mycophenolate mofetil for the treatment of

autoimmune diseases and as an immunosuppressant after transplantation (Ishikawa, 1999; Sugiyama et al., 2005; Shimmura et al., 2006). In the body, mycophenolic acid is mainly metabolized by glucuronidations, with a major phenolic glucuronide and an acyl glucuroide as a minor metabolite (Budde et al., 2004). Acyl glucuronide is a reactive metabolite that covalently binds to proteins, and is considered to take part in drug toxicity such as small intestinal injury (Seitz & Boelsterli, 1998; Shipkova et al., 2003). Protein targets of this reactive acyl glucuronide were investigated in liver and colon tissues of rats fed with mycophenolate mofetil (Shipkova et al., 2004). Liver and colon proteomes were separated with 2-DGE, and protein adducts were recognized by an anti-mycophenolic acid antibody. ATPase alpha and beta subunits, protein disulfide isomerase A3, and selenium-binding protein 2 were identified with MALDI-MS as putative targets. Immunochemical blotting is a simple approach for target identification; however, the limited resolution and dynamic range of 2-DGE mean that some targets might be missed. In a subsequent study, Asif et al. (2007) investigated target proteins of mycophenolic acyl glucuronide in the rat kidney, the major organ responsible for excretion of this drug (Bullingham, Nicholls, & Kamm, 1998). 2-DGE and immunoblots revealed 12 target proteins; seven were confirmed with immunoprecipitation with an anti-mycophenolic acid antibody. Another 21 proteins were identified with immunoprecipitation, SDS–PAGE, and MALDI-MS. These proteins are involved in energy metabolism and oxidative stress. Many of them are also targets of other drug metabolites. Covalent modification of proteins involved in the energy production and redox balance of cells could compromise cell function, and might be associated with toxicity of this drug.

The ample examples of protein-expression profiling reported in the literature (Table 3) exemplified the robustness and usefulness of 2-DGE to analyze the molecular action mechanism of natural products. But, ultimately these studies were limited by the analytical capability of 2-DGE; a low dynamic range and low resolution are the major concerns (Petrak et al., 2008). Although application of DIGE improves dynamic range, resolution is still typically limited to a few hundred proteins per gel. In addition, a frequently raised concern is that high-abundance proteins would mask detection of low-abundance ones. It was recently noted by Petrak et al. (2008) that 2-DGE somehow repeatedly identified similar proteins such as enolase 1, heat shock proteins (HSPs) and peroxiredoxins, regardless of the nature of treatment and tissues or cells utilized. One likely explanation is that these are high-abundance proteins whose expression responds significantly to general cellular stress. Whereas 2-DGE remains a very useful tool to characterize molecular pathways, caution should be exercised during data interpretation. On the basis of this consideration, it is highly commendable to include pre-fractionation protocols to reduce the complexity of the proteome and to remove abundant non-target proteins (Ahmed et al., 2003; Chromy et al., 2004; Kim et al., 2007). As will be discussed in this review, enrichment of different classes of proteins or specific detection methods in conjunction with 2-DGE are currently employed for routine global profiling of protein modifications, the so-called ''functional proteomics'' profiling.

3. LC-MS/MS to Profile Differentially Expressed Proteins

Multi-dimensional protein identification technology (MudPIT) uses combined ion-exchange and reverse-phase chromatography coupled to a mass spectrometer to simultaneously identify and quantify proteins (Roe & Griffin, 2006; America & Cordewener, 2008). Quantitative analysis was performed via comparison of peak areas or more commonly, through isotope labeling techniques, such as ICAT (Gygi et al., 1999), isobaric tag for relative and absolute quantitation (iTRAQ) (Ross et al., 2004) and ^{18}O labeling. Ong and co-workers (Goh, Lee, & Ong, 2005; Tan, Goh, & Ong, 2006) used off-line comparative MudPIT to analyze the mechanism of action of berberine and a standardized *Scutellaria barbata* extract. HepG2 cells were treated with different doses of berberine, an anticancer alkaloid, and a whole-cell proteome was first partially purified with solid-phase extraction (SPE). SPE is an alternative to the SCX column; however, this extra sample preparation step might introduce experimental variables that could mask small but significant alternations. Individual fractions from SPE were subjected to RP-LC-MS/MS analysis, and relative expression of peptides was compared by a superimposition of the chromatograms from control and drug-treated samples without the use of labeling techniques. Peaks that were altered >2-fold were considered as positive hits. Mitogen-activated protein kinase kinase kinase 4 (MAPKKK4) and GTP-binding protein 2 were up-regulated, whereas expression of the 26S proteasome regulatory chain, GTP-binding protein ARD-1, cell death activator CIDE-B, and ribosomal protein S2 were suppressed following berberine treatment. Activation of MAPK signaling cascades (Dent et al., 2003) and down-regulation of the 26S proteasome (Tan et al., 2002; Poirier et al., 2003) were positively correlated to apoptosis induction, and might account for the growth inhibitory effects of berberine. Quantification with peak areas is conceptually straightforward; nevertheless, variations in run-to-run signals hamper an accurate quantitative analysis. As noted by the authors, it is difficult to ascertain alterations <2-fold with accuracy. Recent development of software tools will aid the quantitative analysis, but the key to success for this approach is to have highly controlled experimental procedures for protein extraction and separation to minimize experimental variations (America & Cordewener, 2008).

Isotope code affinity tags (ICAT) regents have been employed to identify differentially expressed proteins in cortical neurons treated with camptothecin (Yu et al., 2004, 2002a), an antitumor alkaloid first isolated from *Camptotheca acuminate* that is a potent inhibitor of topoisomerase I (Kehrer et al., 2001). Protein extracts from control and drug-treated samples were labeled with the light (ICAT-d_0) and heavy (ICAT-d_8) reagent, respectively. Following trypsin digestion and affinity purification, the peptides were analyzed with RP-LC-MS connected to a Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS). FT-ICR-MS affords high sensitivity and a wide dynamic range, and allows for precise quantitative measurements of low-abundance proteins. Using this method, Yu et al. (2002b) successfully identified 129 proteins whose expression levels were altered after camptothecin treatment. The major groups of

differentially expressed proteins are primarily involved in stress response and carbohydrate metabolism. In a follow-up study, an improved ICAT reagent was employed to investigate the molecular action of camptothecin (Yu et al., 2004). One drawback of deuterium-ICAT is that chromatographic properties of some peptides are altered by the incorporation of deuterium, and complicate subsequent analysis (Zhang et al., 2001). In the new reagent, 9 ^{12}C were replaced with ^{13}C. Unlike deuterium, substitution with ^{13}C in heavy reagent has much less effect on chromatographic behavior. In addition, the new reagent possesses an acid-cleavable bond for the removal of biotin to help suppress tag fragmentation and improve protein identification (Canas et al., 2006). A similar profile of protein alternations was found in this study, especially those proteins involved in stress response and glycolysis, to highlight the importance of these proteins in camptothecin-induced cytotoxicity. Using ICAT, it was possible to determine the expression of 1,000–2,000 proteins in a single analysis and this method has gained widespread use in quantitative MS-based proteomic analysis (Shiio & Aebersold, 2006).

In the past, ^{18}O labeling of peptides was often achieved via trypsin digestion. However, during the digestion process trypsin generates a mixture of isotopic peptides that incorporate either one or two ^{18}O that complicate spectra and increase errors in analysis of $^{18}O/^{16}O$ peptide ratios (Schnolzer, Jedrzejewski, & Lehmann, 1996). Recently, an improved method of ^{18}O labeling based on the peptidyl-Lys metalloendopeptidase (Lys-N) was described. This method involves incorporation of a single ^{18}O atom into the carboxyl terminus of proteolytically digested peptides (Rao et al., 2005b). Lys-N ^{18}O labeling was applied in proteomic analysis of cytokine/lipopolysaccharide (LPS)-treated human retinal pigment epithelium cells (ARPE-19) (Rao et al., 2005a). Proteomes from untreated and cytokine/LPS-treated ARPE-19 cells were digested with Lys-N in the presence of H_2O^{16} and H_2O^{18}, respectively. The samples were mixed in a 1:1 molar ratio, separated with a SPE-SCX column, and isolated fractions were analyzed with RP-LC-MS/MS. This method quantified 562 proteins, and revealed 49 proteins that were differentially regulated following cytokine/LPS treatment. ^{18}O labeling has yet to be commonly employed compared to ICAT or SILAC, because the proteolytic labeling generally suffers from an incomplete isotopic incorporation that results in a complex mass spectrum and probably, a false positive identification ($\sim$17%) (Roe & Griffin, 2006). The ^{18}O-labeling approach is further compounded by the current lack of computational tools for data interpretation (Miyagi & Rao, 2007). With the rapidly evolving MudPIT technique in place to address many of the limitations of the traditional 2-DGE approach, higher sensitivity, a wider dynamic range, and identification of membrane proteins not easily amenable to 2-DGE techniques can largely be realized. Therefore, MudPIT is an attractive alternative to 2-DGE to understand the molecular pathways that underlie the therapeutic or toxicological response of natural products. At the current state, a strategy that combines the use of MudPIT and 2-DGE could potentially maximize coverage of the proteome analysis. Indeed, combined 2-DE and MudPIT analyses have gained an increasing popularity in the comprehensive quantitative proteome profiling in several recent studies (Kubota, Wakabayashi, & Matsuoka, 2003; Schmidt et al., 2004, 2006; Kim et al., 2006b).

C. Functional Proteomics Analysis

1. Mass Spectrometry Identification of Protein Modifications

Apart from alternations in the protein expression, numerous protein PTMs also play a key role in signal transduction (Parekh & Rohlff, 1997). Phosphoproteomics is dedicated to the understanding of protein phosphorylation on a proteome-wide scale. To elucidate the influence of natural products on phosphoproteome, researchers often use phosphor-specific antibodies or phosphor-stains for detection of phosphorylated proteins that have been resolved with 2-DGE (Hauschildt et al., 1997; Larsen et al., 2002; Nagumo et al., 2007). Nagumo et al. (2007) demonstrated that capsaicin, a bioactive component from red pepper, triggered dephosphorylation of cofilin in Caco-2 cells. 2-D gels of the control and capsaicin-treated proteome were stained with Pro-Q diamond stain (Schulenberg, Arnold, & Patton, 2003) to selectively identify phosphorylated proteins. A spot on the gel was characterized with MALDI-MS as cofilin, whose phosphorylation was found to decrease substantially after treatment with capsaicin. The altered phosphorylation status of cofilin in capsaicin-treated cells was confirmed with Western blotting with an antibody specific to phosphocofilin. Functional studies indicated that substantial dephosphorylation of cofilin caused by capsaicin mediated the re-organization and opening of actin tight junctions in Caco-2 cells. Gel-based approaches are simple variants of the traditional 2-DGE method, but they invariably suffer from limited resolution and sensitivity of 2-DGE. Enrichment strategies have been proposed to enhance sensitivity of detection. Affinity chromatographic separation of phosphorylated peptides could be achieved based on charge properties and antibody recognition of phosphopeptides. Immobilized metal-affinity chromatography could capture the phosphopeptides via trivalent metal cation (Fe^{3+}, Ga^{3+}) chelation, but it suffers from a high level of non-specific binding of other metal-binding proteins (Sun, Chiu, & He, 2005). Antibodies specific to phosphotyrosine have been used to generate an affinity matrix for selective immuo-enrichment of phosphopeptides and phosphoproteins (Blagoev et al., 2004; Rush et al., 2005). Hill et al. (2006) identified the okadaic acid-induced phosphorylation with a combination of affinity purification and ICAT-MS. As a toxin initially characterized from the sponges *Halichondria okadai* and *H. melanodocia* (Tachibana et al., 1981), okadaic acid is a potent small-molecule inhibitor of protein phosphatase 2A (Yasumoto et al., 1987). Phosphoproteins were enriched with commercially available affinity columns based on phosphopeptide-specific antibodies (Metodiev, Timanova, & Stone, 2004). The affinity-purified control and treatment proteomes were digested and labeled with the ICAT reagent, and analyzed with nano-LC-MS/MS. The phosphorylation status of 13 proteins was altered with treatment with okadaic acid; eleven included dihydropyrimidinases, signaling, and cytoskeleton-related proteins showed elevated phosphorylation. Some of the proteins are substrates of protein phosphatase 2A that indicated that the inhibition by okadaic acid contributed to the enhanced in vivo phosphorylation. With this method, a highly specific affinity-capture matrix is required to minimize false positive identification, and altered phosphorylations should be further confirmed with phosphospecific antibodies.

Another class of PTMs that has attracted recent interest in natural product research is the proteome-wide identification of alternations in the redox state of proteins, termed ''redox proteomics.'' Many anticancer natural products induce a rapid increase in oxidative stress, which in turn mediates the progression of apoptosis (Wang et al., 2007a; Wong et al., 2008b). It is, therefore, of interest to investigate any proteome-wide effect of natural products on protein oxidation. ROS-mediated carbonylation of protein is an important biomarker for protein oxidation (Ghezzi & Bonetto, 2003). Protein carbonylation can be quantified on a 2-D gel via the conjugation of carbonyls with 2,4-dinitrophenylhydrazine (DNPH), followed by western blots with an antibody raised against the dinitrophenyl group (Shacter et al., 1994; Ghezzi and Bonetto, 2003). Characterization of global protein carbonylation has been reported for anticancer natural products (Chen et al., 2006; England, Driscoll, & Cotter, 2006; England, O'Driscoll, & Cotter, 2004). England, Driscoll, and Cotter (2006) analyzed the redox proteomics of HL-60 leukemia cells treated with doxorubicin, etoposide, and mitoxantrone. The strategy involved separation of a whole-cell proteome by isoelectric focusing, after which the gel strip was stained with DNPH before the second dimension SDS–PAGE (Conrad et al., 2001). This strategy minimized problems associated with DNPH-induced alterations of the isoelectric point of proteins and DNPH-derived contaminants. A 2-D Western blot revealed that all three natural products induced a consistent pattern of enhanced carbonylation on proteins, including several glycolytic and ER-resident proteins. It was suggested that rapid carbonylation of glycolytic enzymes through treatment with cytotoxic drugs might compromise ATP production and cell function (England, O'Driscoll, & Cotter, 2004). On the other hand, protein carbonylation in ER caused ER stress and protein unfolding that led to induction of apoptosis (England & Cotter, 2004). The metabolic enzymes triosephosphate isomerase, β-enolase, and ETF-ubiqunione oxidoreductase had an elevated level of carbonylation in the cardiac tissue of adriamycin-treated mice with DNPH staining and 2-D Western blot (Chen et al., 2006). ROS stress induced by adriamycin is a probable cause for its cardiotoxicity. It appears that ROS stress is a two-edged sword, whose induction leads to anticancer and toxicity effects. Another important therapeutic implication of protein oxidation is in aging. Oxidative stress is a major mechanism of neurodegeneration (Lovell et al., 1995; Butterfield et al., 2001). Opii et al. (2008) reported that a diet of natural antioxidants combined with behavioral enrichment significantly suppressed protein carbonylation in the brain proteome of a canine model of human aging. 2-D Western blots of a DNPH-stained proteome showed that several proteins were considerably less oxidized in antioxidant-fed group compared to the control group. Thus, treatment with antioxidants might help protect brain proteins involved in energy metabolism, antioxidant systems, and cell structure from oxidative damage. The frequent identification of proteins associated with metabolism might indicate a probable bias towards high-abundance proteins due to the limitation of 2-DGE methodology. Future studies could perhaps be improved by adopting the combination of immunopurification and MS-based proteomic analysis approaches.

III. THE USE OF MS TO ANALYZE NATURAL PRODUCT–PROTEIN COMPLEXES

The characterization of natural product–protein complexes is a critical step in the drug discovery process, and closely follows the isolation of protein targets by chemical proteomic methodology. Since the advent of soft ionization techniques, especially ESI, it has been demonstrated that the specific interaction between protein and natural products are preserved during the transition to gas-phase ions (Loo, 1997). The observation of natural product–protein complex with ESI-MS was first demonstrated with FK506- and FK506-binding protein (Ganem, Li, & Henion, 1991). Mass information derived from ESI-MS experiments provides a direct measure of the stoichiometry of complex formation (Ganem, Li, & Henion, 1991), and the stability of the non-covalent complex during collision-induced dissociation might be predictive of binding affinity (Winston & Fitzgerald, 1997). It was subsequently shown that binding constants of vancomycin to several bacterial peptides analogs in solution could be determined simultaneously with ESI-MS with good correlation through conventional spectroscopic titration methods (Heck et al., 1998; Jorgensen, Roepstorff, & Heck, 1998; Jorgensen et al., 1999). Whereas electrostatic molecular forces such as hydrogen bonding can be maintained in the gas phase, hydrophobic interactions are less well-preserved (Burkitt et al., 2003). The distinctive characteristics between these two types of molecular interactions are reflected in the marked difference between the gas-phase and solution-phase behavior of complexes maintained by hydrophobic interactions (Robinson et al., 1996). Nevertheless, ESI-MS, and to a lesser extent MALDI-MS, have demonstrated tremendous success in the detection and characterization of natural product–protein complexes. In combination with size-exclusion or affinity chromatography, mass spectrometry is increasingly being used for the high-throughput screening of drug candidates against protein targets for the rapid determination of binding constants and lead prioritization (Muckenschnabel et al., 2004; Khandekar et al., 2005). Potential applications of MS in HTS screening have been covered in detail in several excellent reviews (Schermann, Simmons, & Konermann, 2005; Deng & Sanyal, 2006; Hofstadler & Sannes-Lowery, 2006; Kaveti & Engen, 2006). Another area in which MS-based techniques hold great promise is the elucidation of mechanisms of molecular recognition that lead to the formation of natural product–protein complexes. Understanding the molecular interactions that contribute to natural product–protein complex formation offers vital clues to SARs (Geoghegan & Kelly, 2005), which might facilitate the design of more potent natural product derivatives. X-ray crystallography and NMR spectroscopy provide high-resolution structures of a ligand–protein interface, and are currently benchmark methods for the structural analysis of protein–ligand complexes. X-ray crystallography is amenable only to protein–ligand complexes that can be crystallized, whereas for NMR large quantities of protein are required. Neither of these methods affords high throughput, which is an important consideration of the fast-evolving modern drug discovery regime. In this regard, MS is an attractive alternative to provide important low-resolution structural information, but with better speed and sensitivity of analysis.

MS can monitor conformational alternations in flexible loop regions that are often not resolved by traditional methods. Another advantage of MS is that the targeted proteins can interact with their ligands in solution under native conditions, and can be monitored under a more physiological-like concentration. Analysis under native conditions circumvents artifacts such as dimerization or aggregation of proteins under the high concentration required for NMR analysis. Although atomic level of resolution is currently not achievable with MS, it can often pinpoint specific amino acid residue(s) that interface with the natural product and/or regions that are in close proximity during ligand binding (Schermann, Simmons, & Konermann, 2005).

In addition, the changes in protein conformations induced by ligand binding can also be assessed (McCammon & Robinson, 2004). In this section, emphasis is on the emerging role of MS-based techniques to provide structural information that relates to binding sites of natural products. As described below, different strategies are adopted for structural analysis of covalent natural product–protein adducts and non-covalent complexes. ESI-MS or MALDI-MS analysis is directly applicable to the analysis of irreversible binding of natural products to proteins that often follows trypsin digestion. On the other hand, for non-covalent complexes chemical strategies such as limited proteolysis, H/D exchange, and photoaffinity labeling are often used in conjunction with MS analysis to acquire structural information. Recent applications of the MS-based identification of protein binding sites are listed in Tables 3 and 4.

TABLE 4. Applications of MS to elucidate non-covalent natural product–protein complexes

Protein-ligand	Origin	Target protein	Major interacting peptides/residues identified
Limited proteolysis			
Cyclosporine A	Plant	Cyclophilin A	Residues 92-118 (Teffera& Bakhtiar, 2005)
Doxorubicin	Plant	Tetanus toxin C-fragment	Residues 299-316, and 351-316 (Shields et al., 2003)
Cis-parinaric acid	Plant	Bovine β-lactoglobulin	Residues [41-70]S-S[149-162], (Imre& Zsila& Szabo, 2003)
Photoaffinity labeling			
Coumarin	Plant	HIV-1 integrase	Residues 128-136 (Al-Mawsawi& Fikkert& Dayam& Witvrouw& Burke& Borchers& Neamati, 2006)
Phlorizin	Plant	Na^+-glucose cotransporter	Arg-602 (Raja& Tyagi& Kinne, 2003)
Lapachenole	Plant	Cytochrome P450 3A4	Residues 97-105, and 459-469 (Wen& Doneanu& Gartner& Roberts& Atkins& Nelson, 2005)
Hydrogen/deuterium (H/D) exchange			
Doxycycline	Semi-synthetic	Matrilysin	Residues 145-153, and 193-204 (Garcia et al., 2005)
Radicicol	Microbial	Heat shock protein 90	Residues 146-153, multiple conformational changes detected (Phillips& Yao& Zhang& McLaughlin& Laue& Robinson& Jackson, 2007)
Retinoic acid	Plant	Retinoid X receptor	Residues 271-278, 313-319, and 423-429 (Yan& Broderick& Leid& Schimerlik& Deinzer, 2004)
Retinoic acid	Plant	Retinoic acid binding protein I	Conformation stability of hydrophobic binding pocket is enhanced upon ligand binding (Xiao& Kaltashov, 2005,Xiao et al., 2003)
Photoaffinity labelling and H/D exchange			
11-*cis*-retinal	Plant	Cellular retinaldehyde binding protein	Photoaffinity labell: Tyr-179, Phe-197, Cys-198, Met-208, Lys-221, Met-222, Val-223 and Met-225; H/D exchange: residues 197-255 (Wu& Hasan& Liu& Teller& Crabb, 2004)

A. Characterization of Natural Product–Protein Adducts

1. MS and MS^n Analysis

Protein-reactive natural product adducts are an interesting class of biologically active molecules that can irreversibly modify the activity of their protein target(s). It is thus not surprising that these molecules often possess potent activity. These compounds are endowed with reactive electrophilic functional groups, such as an epoxide, carbonyl, or β-lactam group, which can covalently interact with nucleophilic residues on specific protein targets (Drahl, Cravatt, & Sorensen, 2005). Due to the high reactivity of thiol side chain(s), nucleophilic cysteine residues are a frequent target for such covalent modifications (Fang, Lu, & Holmgren, 2005; Hosono et al., 2005; Luo et al., 2007). Other residues involved in enzyme catalysis such as histidine, serine, threonine, and lysine are also important targets for covalent modification (Lowther et al., 1998; Riba et al., 1998; Buey et al., 2007). Although these amino acid residues are present throughout a protein sequence, often only 1–2 residues are specifically labeled; that small number reflects the importance of structural context in molecular recognition. A simplistic model to explain this observation would be that interaction begins with an initial molecular recognition step that brings reactive natural products into close proximity to specific nucleophilic amino acid residue(s), and thus subsequent chemical modification could occur favorably. A popular strategy for MS-based identification of binding residue(s) involves direct ESI- or MALDI-MS analysis of the peptides derived from digestion of natural product–protein complex of interest. A comparison of the mass spectra of a native protein versus its natural product complex could reveal readily the specific mass shift in peptides that are adducted (Fig. 4). In a similar manner, characteristic mass shifts of adducted proteins distinct from native proteins could be identified with tandem MS analysis to locate the exact residue(s) at which covalent modification occurs. MS-based methods to elucidate initial molecular recognition regions, which might involve multiple amino acid residues, will depend on the use of additional chemical methods, which will be described in a later section on non-covalent complex characterization.

An early example was the MS-based characterization of a complex between fumagillin and MetAps from *E. coli* (Lowther et al., 1998). Fumagillin and its analogs are potent anti-angiogenic molecules (Ingber et al., 1990), and they possess a key spiroepoxide motif that can covalently modify proteins. MetAp, a metalloprotease that catalyzes the removal of the *N*-terminal methionine residue, is the molecular target of fumagillin analogs (Griffith et al., 1997). Tandem MS analysis of digested MetAp-fumagillin peptide 76–86 revealed an ion with the predicted mass that corresponded to an adduct formed between His-79 and fumagillin. Although there are numerous nucleophilic amino acid residues such as Cys-78, Asp-83, and Asp-84 nearby in the sequence, covalent modification occurred uniquely on His-79. According to the crystal structure of MetAp, His-79 is situated at a position within an active site just above the essential metal center (Roderick & Matthews, 1993). Perturbation of the catalytically essential metal center could be responsible for the elimination of the peptidase activity following fumagillin incubation (Sin et al., 1997). Such inhibition could account for their anti-angiogenic activity. Indeed, the targeted deletion of the methionine aminopeptidase-2 gene in mice led to an impaired vascular development, and siRNA knockdown of this gene suppressed the proliferation of the cultured endothelial cells; those data confirmed an essential role of MetAp in the promotion of angiogenesis (Yeh et al., 2006). Thus, these studies have led to identification of MetAp as an attractive therapeutic target.

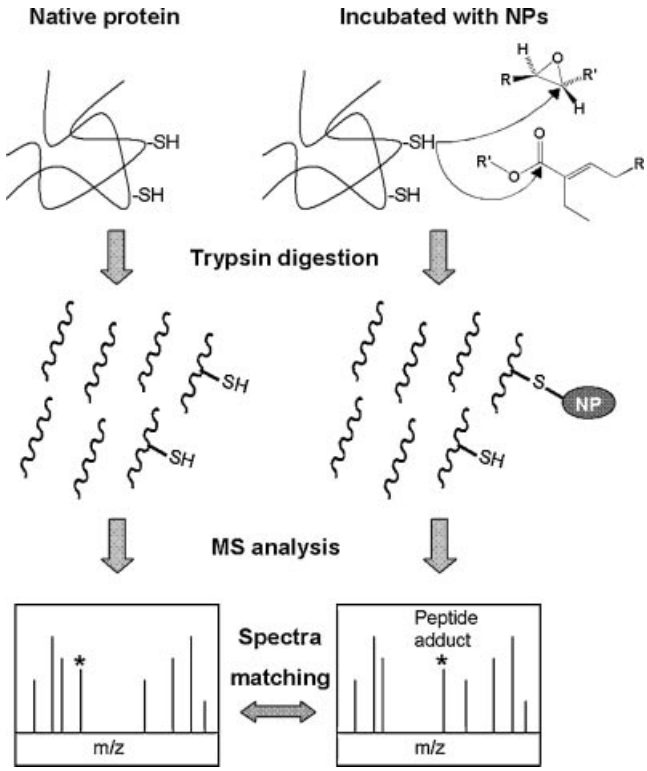

FIGURE 4. Characterization of natural product–protein adducts. Chemically reactive groups such as epoxide in natural products interact with cysteine residues in proteins, to thereby form covalent adducts. To locate the peptide(s) or residue(s) responsible for the covalent interaction, a natural product reacts with its target protein to form adducts, which are digested with trypsin, and the tryptic peptides are analyzed with MS. A comparison between the MS spectra of the native and the adducted proteins would reveal the presence of peptide(s) (*) that show a mass shift that corresponded to the molecular weight of the adducted natural product.

Cyclostreptin is a natural product from *Streptomyces* sp. that targets β-tubulin, and causes cell growth arrest and apoptosis (Edler et al., 2005). Buey et al. (2007) reported that cyclostreptin covalently interacted with polymerized tubulin in vitro to irreversibly stabilize cellular microtubules and led to potent anti-proliferative activity. Cyclostreptin is characterized by a highly strained bridgehead olefin together with lactone carbonyl that is an electrophilic moiety capable of initiating nucleophilic attack on proteins (Adam et al., 2003). To identify possible site(s) of covalent modification, untreated- or cyclostreptin-treated β-tubulins were digested with trypsin or chymotrypsin, and analyzed with nano-LC-ESI-MS. Several peaks were unique to

cyclostreptin-treated samples, and MS/MS sequencing of peptides mapped all these sequences into β-tubulin$_{219-243}$. The MS/MS spectra suggested that there were two different sites of modification at Thr-220 and Asn-228. However, the total-ion chromatogram showed that β-tubulin$_{219-243}$ was modified consistently by a single cyclostreptin, and suggested that the two modifications are mutually exclusive. To verify these interactions in living cells, the multiple-reaction monitoring (MRM) scan mode was employed successfully to specifically detect tubulin$_{219-243}$-adducted peptide in peptides generated from extracts of A549 cells treated with cyclostreptin. Although this natural product is less potent compared to paclitaxel, the irreversible nature of its protein interaction means that it can avoid being pumped out by paclitaxel-resistant cells that over-express P-glycoprotein. It also retains strong activity in cells that expressed mutant β-tubulin. Thus, covalent modification of tubulin by cyclostreptin is an attractive strategy to target cancer cells that have developed multi-drug resistance. Conversely, this type of highly reactive drug might also cause formation of non-specific protein adducts, and lead to extensive cellular damage and severe toxicity (Evans et al., 2005). A more comprehensive profiling of cyclostreptin's cellular protein targets by chemical proteomic profiling is imperative to determine its suitability as a lead compound for anticancer drug development.

Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) is a promising tool for proteomic analysis because it offers the highest mass-measurement accuracy, resolution, and sensitivity (Marshall, Hendrickson, & Jackson, 1998). Due to its superior resolution and sensitivity, FT-ICR dramatically improves the sequence coverage of the analysis of tryptic digests (Bruce et al., 1999). An improved coverage can clearly be valuable to examine most of the possible residue(s) in proteins that could be adducted by natural products. As mentioned previously, cysteine is a frequent target for natural products due to the high reactivity of its thiol group. Luo et al. (2007) developed an LC-FT-ICR-MS method to analyze alkylation of multiple cysteine residues in human Kelch-like ECH-associated protein 1 (Keap1). Keap1 resides in the cytosol and sequesters nuclear factor-E-$_2$-related factor (Nrf-2) (Itoh et al., 1999). Upon alkyation of one or more of its 27 cysteine residues by ROS or xenobiotics, Keap1 releases Nrf-2 that, in turn, up-regulates the expression of cytoprotective genes that contain antioxidant response elements (ARE) (Zhang & Hannink, 2003). Using the LC-FT-ICR-MS method, the sequence coverage for Keap1 reached 90%, and importantly all of the cysteine-containing sequences were covered. Compared to traditional LCQ analysis, additional cysteine modification sites were identified with the model electrophilic substrate, 1-biotinamido-4-(4′-[maleimidoethyl)cyclohexane]-carboxamido. This method was further applied to the comprehensive analysis of covalent cysteine alkylation of three electrophilic natural products, isoliquiritigenin, xanthohumol, and 10-shogaol with chemopreventive properties. It was found that isoliquiritigenin reacted mainly with Cys-151 and Cys-226; 10-shogaol formed adducts at Cys-151, Cys-257, and Cys-368; and xanthohumol, with least reactivity, acted on Cys-151, Cys-319, and Cys-613. Adduction at specific cysteine residues was further confirmed with MS/MS. Regardless of the natural product used, Cys-151 represents the most favorable position for natural product adduction. This study thus suggests that alkylation of Cys-151 is closely associated with the up-regulation of ARE by chemo-preventive agents (Zhang & Hannink, 2003). Caution should be exercised, however, when examining relative reactivity, because the strength of an MS signal intensity can be complicated by the differential ionization efficiency of different peptides (Liebler, 2008). Being able to rapidly provide information on residue(s) with a high propensity to modification, even with small quantities of proteins and natural products, these examples clearly illustrated the potential of MS to characterize natural product–protein adducts.

B. Characterization of Non-Covalent Complexes

1. *Limited Proteolysis*

Limited proteolysis is a commonly employed strategy to probe the higher order structures of proteins, and to predict the interfaces of protein–protein complexes (Hubbard, 1998). In most cases, proteolysis is a pre-requisite step to identify proteins with MS methods. Complete proteolysis is usually initiated under denaturing conditions in which proteins are unfolded to expose all of the peptide bonds to achieve complete digestion (Svasti et al., 2005; Zhang et al., 2007). On the other hand, to gain structural insights, partial digestion is deployed for ''limited proteolysis.'' Typically, the proteolytic process could be slowed down by digestion under the ''native'' state of proteins (Fontana et al., 2004). In this way, the degree of proteolytic cleavage is highly dependent on the accessibility of suitable peptide bonds to the protease. Whereas the solvent-exposed residues and flexible loop regions are readily digested, the portions of peptides buried in the protein core are less accessible. Partial digestion is further aided by a low protease/protein molar ratio, sub-optimal pH, or temperature. Moreover, when the protein examined is in a complex mixture that contains ligands, including natural products, the peptide bonds at the ligand-binding sites can be protected from proteolysis. Through a comparison of native and ligand-associated digested peptides, possible ligand-binding sites could be localized to those peptides that become resistant to protease action after ligand binding (Fig. 5) (McDonald & Li, 2005). Adding to the assignment of potential ligand-binding sites, limited proteolysis is also widely used to monitor any protein folding/unfolding and conformational changes following ligand binding. However, the fact that there is always a possibility that a reduced proteolytic susceptibility could arise as a result of ligand-induced alternation in protein conformation at a site remote from the ligand-binding site could actually complicate analysis (Pedigo & Shea, 1995). Despite this pitfall, limited proteolysis remains a suitable tool to probe various structural aspects of natural product–protein complexes.

In a proof-of-principle experiment, limited proteolysis in combination with MALDI-TOF-MS was employed to define binding regions of doxorubicin on tetanus toxin C-fragment (Shields & Oyeyemi, 2003). Tetanus neurotoxin is a 150 kDa molecule, that consists of 50 and 100 kDa peptides that are linked via a disulfide bridge, and doxorubicin is known to bind to the 51 kDa carboxyl terminus of the heavy chain (Cosman et al., 2002). Shields and Oyeyemi (2003) reported that, when

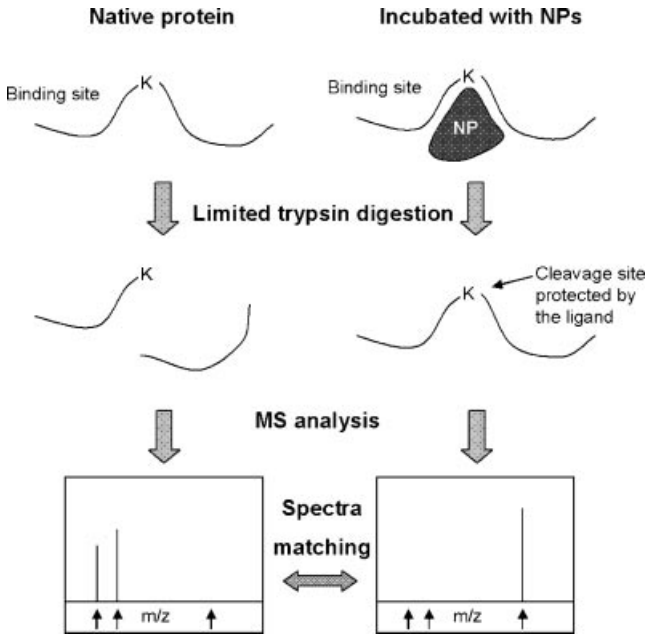

FIGURE 5. Limited proteolysis. In this approach, a protein is digested with a protease, usually trypsin, under non-denaturing conditions. When the protein is digested in the presence of a ligand, potential cleavage sites at the binding site(s) are blocked by steric hindrance introduced by the ligand. Interfacial regions can thus be identified by the absence of cleaved peptides or the presence of additional peptides that contain a missed cleavage site.

doxorubicin was added in molar excess (>10:1) to tetanus toxin, significant changes were observed in MALDI-MS spectra that indicated a significantly altered proteolysis. ESI-MS analysis showed that there was no change in the charge-state distribution of the tetanus toxin–doxorubicin complex. In other words, the binding did not appear to induce any significant conformational changes. Trypsin proteolysis in the presence of doxorubicin altered the relative ion abundance of numerous peptides. Among these altered peptides, Arg_{305}–Lys_{311} and Phe_{312}–Arg_{316} showed a reduced abundance, and the intensity of the R_{305}–R_{316} ions was increased. A cleavage site at amino acid Lys-311 on Arg_{305}–Arg_{316} was not acted on by the protease, and indicated that doxorubicin protected Arg_{305}–Arg_{316} from trypsin binding to this site. Other possible residues blocked by doxorubicin included Arg-305 and Arg-360. The blocked digestion at Lys-311 and Arg-360 was consistent with the binding mode predicted with a computational model derived from X-ray crystal structure of tetanus toxin C-fragment. Surprisingly, several peptides that corresponded to regions that flank the binding site exhibited an increased relative intensity, in contrast to a decreased intensity mostly observed for the ligand-binding site. It was suggested that ligand binding induced an increased disorder in the flanking regions, which might lead to an enhanced exposure to trypsin. Thus, limited proteolysis also provides additional information on the conformational changes upon ligand binding.

The ligand-binding site of *cis*-parinaric acid on bovine beta-lactoglobulin has been investigated with limited proteolysis (Imre, Zsila, & Szabo, 2003). *Cis*-parinaric acid is a natural fatty acid derived from the seeds of the Makita tree (Beisson et al., 1999), and it was found to possess potent cytotoxic activity in vitro (Traynelis, Ryken, & Cornelius, 1995). Bovine beta-lactoglobulin is a major whey protein in milk, and it binds hydrophobic substrates, including fatty acids, cholesterol, and retinoid that are sparsely soluble in water, to thus improve their bioavailability (Wang, Allen, & Swaisgood, 1997a,b). Initial ESI-MS analysis of the intact *cis*-parinaric-beta-lactoglobulin complex revealed a 1:1 stoichiometry in the binding of *cis*-parinaric acid to beta-lactoglobulin A and B. Limited proteolysis was performed with trypsin under non-denaturing and low protease/protein molar ratio (1:100) conditions to ensure that digestion was incomplete. Proteolysis was stopped with acetic acid over several time intervals. The analysis of the ESI-MS spectra of digested peptides revealed two ion peaks with high intensity, which corresponded to peptides of molecular masses of 2,708 and 5,200 Da, respectively. However, only the peptide with a predicted MW of 5,200 Da maintained an interaction with *cis*-parinaric acid after proteolysis, and thus gave an additional signal that corresponded to a peptide of MW 5,477 Da. Sequencing showed that this digested fragment corresponded to a disulfide-bonded dimmer of residues 41–70 and 149–162. This peptide also contains residues Lys-60 and Lys-69 that are thought to be significant in complexion with carboxyl acid through formation of hydrogen bonds. This structure–activity characteristic agreed well with information based on X-ray crystallography, which showed that the cysteine residues, Cys-66, and Cys-160, involved in disulfide bonding of the 5,200 Da peptide were localized close to the hydrophobic binding site (Brownlow et al., 1997). Although limited proteolysis requires careful optimization of the digestion procedure, it has a much higher throughput and demands a much lower concentration of proteins compared to X-ray and NMR analysis.

2. *Photoaffinity Labeling*

Chemical cross-linking is another popular strategy to obtain structural information on non-covalent natural product–protein complexes. PAL, a technique described in section I, allows the formation of a covalent complex between a small-molecule photoaffinity label and the target protein that facilitates further analysis. For identification of protein receptors, the photoaffinity label is employed to isolate the target protein(s) from a complex mixture. On the other hand, this label can also be incubated with a single target protein, irradiated, and digested with trypsin. In conjunction with ESI-MS or MALDI-MS, it enables rapid identification of peptides that lie in close proximity to the ligand in the complex, as indicated by a mass shift in the MS spectrum acquired from photolabeled protein sample relative to that from the native form (Robinette et al., 2006). Alternatively, the digested and photolabeled peptides are selectively enriched with affinity chromatography, followed by MS analysis (Fig. 6). Frequently, binding site-containing peptides are readily isolated, and in some instances exact amino acid residues could also be resolved by MS/MS to provide valuable information on the 3-D structure of natural product–receptor complexes. A critical element to success in PAL experiments, as in target identification, is the design of a suitable derivatized photolabel that does not significantly impede the protein-binding and the biological activity of the parent compound. A wide range of small molecule-

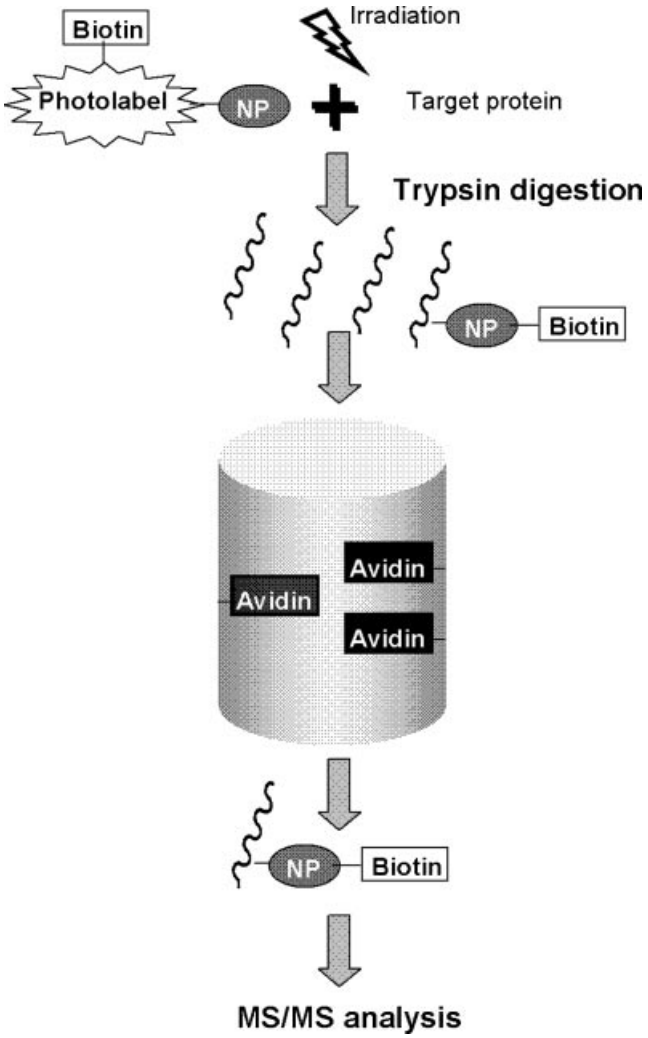

FIGURE 6. Photoaffinity labeling. To specifically identify photolabeled peptides or residues, a protein is incubated with a natural-product photolabel and cross-links are formed by irradiation. Because the yield of the photolabeling reaction can be low, after trypsin digestion, the cross-linked peptides are enriched with a streptavidin column and analyzed with MS. MS/MS sequencing also directly locates the specific residues that are cross-linked by the natural product photolabel.

protein complexes have been studied with PAL reagents, including those that involve integral membrane proteins that are notoriously difficult to analyze with X-ray crystallography and NMR (Radeke & Snapper, 1998; Wu et al., 1998; Raja, Tyagi, & Kinne, 2003; Pleban et al., 2005; Wen et al., 2005; Al-Mawsawi et al., 2006).

Photoaffinity labeling (PAL)-MS in combination with site-directed mutagenesis has been employed to determine binding sites of inhibitor–enzyme complexes. This approach was adopted for the discovery of an HIV-1 integrase inhibitor binding site (Al-Mawsawi et al., 2006). HIV-1 integrase, which mediates the insertion of viral DNA into a host genome, is an emerging drug target for the development of therapeutics against AIDS (Pommier, Johnson, & Marchand, 2005; Savarino, 2006). The inhibitor-binding site of HIV-1 integrase was studied with photoreactive coumarin derivatives. Coumarins, a group of toxins found in plants, are potent inhibitors of HIV-1 integrase (Yu et al., 2003). Coumarin-based inhibitors conjugated with benzophenone were incubated with HIV-1 integrase, and cross-linking was initiated with irradiation at 360 nm. A tryptic digest of the photolabeled protein was separated with HPLC, and fractions that exhibited differences in UV absorbance were analyzed with LC-MS/MS. A single peptide, Ala_{128}–Lys_{136}, was found to exhibit a mass shift that was consistent with photolabel incorporation. Thus, four residues in this peptide (Cys-130, Trp-131, Trp-132, and Lys-136) were chosen for site-directed mutagenesis. The substitutions at Cys-130 and Trp-132 that significantly reduced the inhibitory effects of coumarin inhibitors supported an important role of these residues in inhibitor binding. Similar methodologies have been applied to study the substrate-binding sites of membrane transporters such as P-glycoprotein, sodium–glucose co-transporter, and ion channels, which are notorious for their difficulty in crystallization (Raja, Tyagi, & Kinne, 2003; Safa, 2004; Vila-Carriles, Zhao, & Bryan, 2007).

The combined use of PAL and MS/MS sequencing of labeled peptide(s) might also directly identify the specific amino acids that are involved in ligand binding without resorting to a mutagenesis experiment. Wen et al. (2005) employed PAL and LC-MS/MS analysis to elucidate structural elements responsible for substrate recognition for cytochrome P450 3A4 (CYP3A4), a major drug-metabolizing enzyme in human liver. P450s are all integral membrane proteins that present particular difficulties for X-ray crystallography. In this study, the authors chose the natural photoaffinty label, lapachenole, a benzochromene derived from plants. Lapachenole interacts with the active site of CYP3A4, and is normally metabolized by hydroxylation. Upon UV irradiation, however, lapachenole is activated and forms adducts with CYP3A4 at cysteine residues (Gartner et al., 2005). Incubation of lapachenole with recombinant CYP3A4 with irradiation at 360 nm generated a mass shift of 240 Da in the ESI-MS spectrum of the intact lapachenole–protein complex that suggested that one molecule of lapachenole was adducted per CYP3A4 protein. To further understand the location of the active site, lapachenole-modified CYP3A4 was digested with trypsin, and peptides that exhibited fluorescence (due to the fluorochrome of lapachenole) were purified with HPLC and analyzed with MALDI-MS and LC-ESI-MS/MS. MS analyses showed that Glu_{97}–Arg_{105} and Val_{459}–Lys_{469} were covalently modified by lapachenole. MS/MS sequencing further assigned the site of modification to Cys-98 and Cys-468 on the basis of the 240 Da mass shift in the product-ion spectra. Cys-98 is located in the putative B–B′ loop as part of the active site (Williams et al., 2004), whereas Cys-468 is found near the outer surface distant from the active site. Interestingly though, multiple sequence-alignment studies have showed that both are highly conserved in CYP3A enzymes (Lewis et al., 1996); those data highlight their role in molecular recognition and catalysis of this class of enzymes.

3. Hydrogen–Deuterium Exchange

Exchangeable hydrogen atoms in a protein are exchanged with deuterium when it is dissolved in ^2H_2O. The rate of deuterium incorporation depends on its chemical environment and pH of the

solution. Whereas hydrogen atoms in hydroxyl, amino, and carboxyl groups exchange rapidly, backbone amide hydrogens exchange at rates that vary from several seconds to months in folded proteins (Wales & Engen, 2006). The key to differential deuterium incorporation of amide hydrogens is their solvent accessibility. Hydrogens that are solvent-exposed exchange relatively quickly, whereas those hydrogens that are buried in the protein interior or are involved in intramolecular hydrogen bonding will have slower exchange rates. H/D exchange can be used to monitor interfacial interactions in ligand–protein complexes. Interaction of a ligand with binding residues on a protein would lead to a corresponding decease in solvent accessibility of the hydrogens, either by hydrogen bonding or steric hindrance (Smith et al., 1997; Tang et al., 2007). As a result, amide hydrogens on these residues show an altered H/D exchange compared to the native protein. Such alterations are readily reflected in the changes in molecular mass of the protein or peptides. Hence, MS has become a major platform for rapid and sensitive detection of the rate of H/D exchange. Consequently, H/D exchange in conjunction with MS has been developed to characterize interactions between ligands and their receptors. There are two major experimental designs in H/D exchange. In on-exchange experiments, the protein–ligand complex and the control are incubated in ^2H_2O, respectively, after which the binding regions might be determined as residues with a decreased incorporation of deuterium (Fig. 7) (Zhang & Smith, 1993; Smith et al., 1997). In off-exchange settings, the protein is first deuterated after several rounds of incubation with ^2H_2O, and diluted in H_2O to off-exchange the deuterium in the presence or absence of a ligand (Sinz, 2007). The exchange reaction is quenched by adjusting the pH of the medium to 2–3, and cooling to 0°C, which slowed the exchange reaction by a factor of 10^4–10^5 in 30–90 min (Bai et al., 1993; Yan et al., 2004b). Pepsin, which is active at acidic pH, is commonly utilized to digest the peptides. Although the cleavage is non-specific, this factor actually helps to improve the spatial resolution through the generation of overlapping peptides. The digested peptides are usually analyzed with LC coupled to ESI-MS or MALDI-MS. An alternative method is to directly inject the intact protein sample into a mass spectrometer. ESI-FT-ICR-MS, with the superior mass accuracy and resolution, has been employed for the latter approach (Kaltashov & Eyles, 2002).

Hydrogen–deuterium (H/D)-exchange in conjunction with mass spectrometry has numerous applications in the analysis of binding sites of natural-product drugs. H/D-exchange-MS facilitated the identification of the ligand-binding pockets of retinoid X receptor (RXR), the protein target of 9-*cis*-retinoic acid (Yan et al., 2004a). RXR is a 27 kDa protein that mediates the transcriptional repression or activation of target genes, and is an attractive drug target for cancer and metabolic diseases (Altucci et al., 2007). Retinoic acid is an active metabolite of vitamin A that is effective against various cancer cells (Lehmann et al., 2000). In Yan's study (2004a), deuterium on-exchange was initiated with a dilution of RXR in the absence or presence of retinoic acid. At specified time points, the reaction was quenched by adjusting the pH to 2.6, followed by pepsin digestion, and LC-ESI-MS analysis of peptic peptides. In total, 65 peptic peptides were generated that covered 98% of the protein sequence; fragments 271–278 and 313–319 in the RXR-retinoic acid

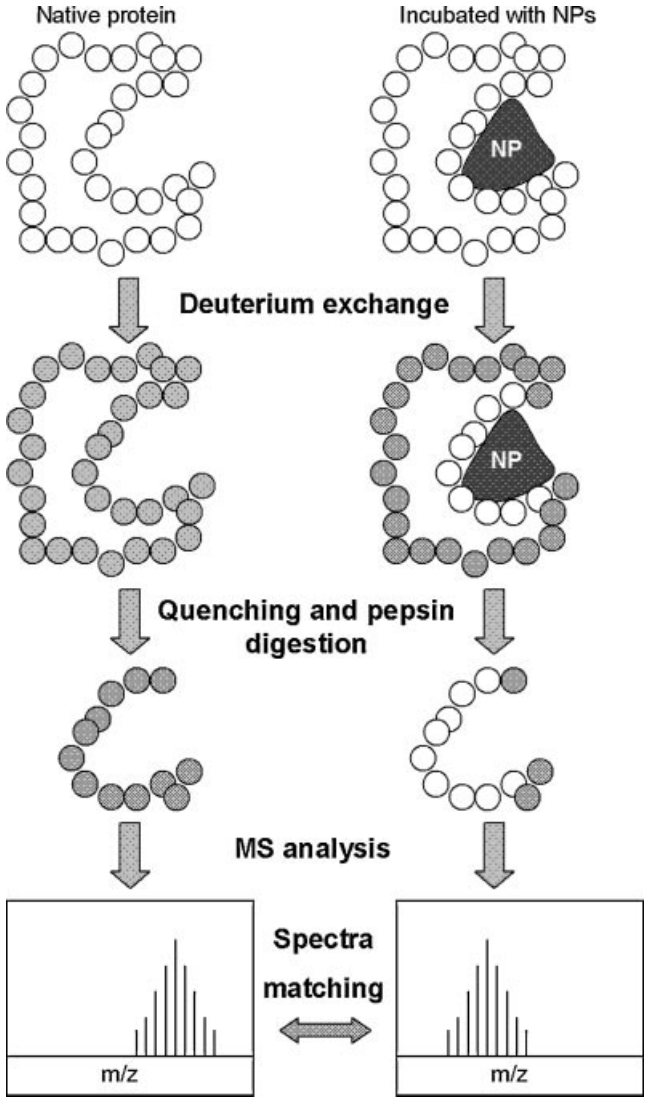

FIGURE 7. Hydrogen–deuterium exchange. The deuterium exchange of a hydrogen on the amide backbone depends on its solvent accessibility with a reaction time that varies between seconds to months. The protein is subjected to H/D exchange in the absence or the presence of a natural product. The natural product lowers the solvent accessibility of amide hydrogen of residues that reside in the binding site, and lead to a reduced deuterium incorporation. H/D exchange is quenched by adjusting the pH to 2.5, the resulting complex is immediately digested with pepsin, and the peptides are analyzed with MS. Levels of deuterium incorporation are calculated from the mass values of peptides, and can be compared with the mass spectra of the native protein to identify the potential binding sites.

complex exhibited a significantly reduced deuterium incorporation. These results are consistent with crystallographic data of retinoic acid-binding sites (Newcomer, Pappas, & Ong, 1993). Another fragment, 423–429, incorporated less deuterium. Although these fragments were not directly bound to the ligand, they were located near Cys-432 and His-435 that made contacts with 9-cis-retinoic acid. These results indicated that ligand binding restricts solvent accessibility surrounding the binding

site(s) and obstructs deuterium exchange. Deuterium content in certain amino acid residue(s) could also be determined by overlapping fragments, to thereby identify Leu-279 and Val-354 with largely reduced deuterium incorporation. Apart from the primary binding site, there were other peptides that showed an altered deuterium content that accounted for approximately 50% of the protein sequence. These alternations were attributed to conformational changes and re-arrangement induced by the ligand binding. Due to the ability of H/D exchange to determine the flexibility of protein regions upon ligand binding, it affords supplementary information to X-ray crystallography, especially with regard to conformational alternations.

The protein dynamics of cellular retinoic acid-binding protein I (CRABP I) upon retinoic acid binding has been investigated with H/D-exchange and ESI-FITCR-MS with collision-induced dissociation of protein ions (Xiao & Kaltashov, 2005). CRABP I represents an interesting case because binding of retinoic acid did not result in a significant change in its tertiary structure, despite the consequential inaccessibility of the binding site(s) (Krishnan et al., 2000). This phenomenon could be caused by a conformational flexibility of regions that surround the binding site(s). In Kaltashov's study, H/D off-exchange was carried out by incubating CRABP I with ^2H_2O to deuterate the liable hydrogens, followed by ligand binding and subsequent back exchange in a buffer. The protein–ligand complex was directly analyzed with ESI-FITCR-MS after various off-exchange intervals. Due to the high resolving power of FT-ICR-MS, LC separation was not required, and 19 peptides that span the entire sequence of the protein could be assigned readily in CID spectra. Because retinoic acid interacts mostly via hydrophobic contacts and does not involve any hydrogen bond formation with backbone amide, it was suggested that an altered deuterium incorporation resulted from an altered flexibility due to the presence of the ligand in the binding pocket. Indeed, several segments that were significantly protected from deuterium off-exchange by retinoic acid were involved in forming the hydrophobic pocket buried in the binding site. These segments were also highly flexible in the absence of a ligand, as indicated by their exchangeable nature in native deuterated proteins. In contrast, segments that contained positively charged side chains that interacted with the carboxylate group of retinoic acid at physiological pH were inflexible with and without retinoic acid. Based on these observations, it was proposed that the flexible segments provided an unobstructed assess to the ligand-binding site, and that retinoic acid entered the pocket guided by electrostatic interactions with positively charged Arg-111 and Arg-131. After ligand binding, the previously flexible hydrophobic segments were stabilized and thus effectively trapped retinoic acid in the hydrophobic pockets. These studies have demonstrated that H/D exchange-MS is a powerful tool to monitor protein conformational changes in response to ligand binding.

The more widespread use of FT-ICR-MS in conjunction with H/D exchange could reap multitude benefits (Akashi & Takio, 2000). Successful detection of isotopic distributions of peptides holds the key to the identification of altered deuterium incorporation. However, H/D exchange generates highly complex mass spectra that cannot be resolved with conventional low-resolution mass analyzers that heavily rely on an efficient chromatographic step to separate peptide ions with identical nominal *m*/*z* ratios (Chalmers et al., 2006). Back exchange is inevitable over chromatographic runs, and further back exchange occurs during the digestion process. Even after chromatographic separation, analysis of relatively large proteins might be affected by the presence of multiple overlapping peptide ions that lead to a reduced sequence coverage and thus loss of structural information. For example, even when coupled to a high-resolution nano-ESI-MS, the protein coverage of H/D-exchange spectra of heat shock protein 90 (HSP90) could reach only 68% (Phillips et al., 2007). Taking advantage of the superior accuracy and resolution of FT-ICR, chromatographic procedures could be minimized to reduce any back exchange. In addition, spatial resolution and sequence coverage could be improved. For low-molecular-weight proteins such as papain (23 kDa) and CRABP I (16 kDa) (Akashi & Takio, 2000; Wu et al., 2004a), deuteration can be determined by direct analysis of intact proteins with FT-ICR-MS without the need of prior digestion or peptide separation. Instead, collision induced-fragmentation might be used to produce fragmented peptides for analysis of the degree of deuteration. However, parameters of dissociation should be set carefully to minimize any H/D scrambling. Propensity to this undesirable phenomenon is especially high in slow-heating dissociation processes, which should thus be avoided. In this regard, rapid heating methods such as collision-activated dissociation (CAD) or gentle electron capture dissociation (ECD) methods could prove valuable to prevent any H/D scrambling (Eyles et al., 2000; Charlebois, Patrie, & Kelleher, 2003). The combined use of FT-ICR-MS and H/D exchange is a powerful approach to elucidate protein–ligand interactions, even for larger proteins in excess of 100 kDa.

Even with overlapping peptic peptides to monitor altered deuterium incorporation in certain residues, H/D exchange usually could only localize the site of binding to stretches of short peptides on a protein. In addition, in cases when there is little structural information available on a target protein, it will be very difficult to distinguish regions that are actually in close proximity to the binding sites from those distant peptides with solvent accessibility being altered upon ligand binding. Therefore, it would be of merit to combine different MS-based approaches to provide additional insights into protein–ligand complexes. One strategy is the combined use of photoaffinity labeling with H/D exchange-MS, recently exploited by Wu et al. (2004b). In this study, the complex formed between cellular retinaldehyde-binding protein (CRALBP) and 11-*cis*-retinol was characterized with photoaffinity labeling with 3-diazo-4-keto-11-*cis*-retinal (DK-11-*cis*-retinal) and H/D-exchange MS. The specific residues modified by DK-11-*cis*-retinal were analyzed with LC-MS/MS sequencing of tryptic peptides, which identified eight residues (Tyr-179, Phe-197, Cys-198, Met-208, Lys-221, Met-222, Val-223, and Met-225) as labeled. On the other hand, H/D on-exchange MS revealed a hydrophobic region that encompassed residues 197–255 that had a significantly reduced deuterium incorporation to indicate a reduced solvent accessibility in the presence of the ligand. It appears that, apart from residues that constitute the ligand-binding pocket, surrounding residues can also initiate conformational alterations upon ligand binding, probably in a manner similar to shutting of the hydrophobic pocket in the case of CRABP I. H/D exchange

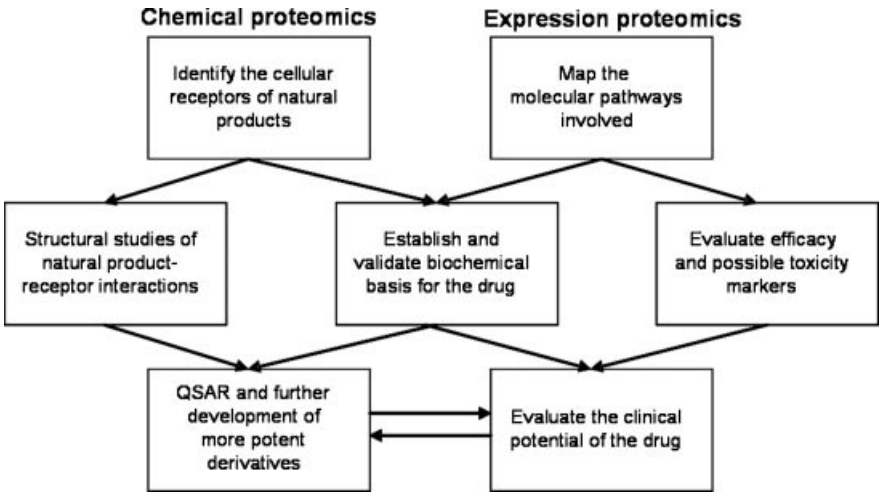

FIGURE 8. The role of MS-based proteomic approaches in drug development from natural products.

experiments also showed that *N*-terminal and *C*-terminal residues incorporated more deuterium, and suggested that ligand binding enhanced the flexibility of these regions. Thus, a more complete classification of the specific binding residues and regions with altered conformations could be obtained through an intelligent combination analysis by means of the MS-based techniques.

IV. CONCLUSION

Natural products represent a rich source of novel therapeutics. A lack of understanding of their modes of action has hampered their incorporation into modern drug discovery programs that focus on targeted therapeutics. Compared with highly selective synthetic drugs, however, multi-target natural products might possess a greater efficacy at the cost of potential toxicity. Thus, there is an urgent need to develop tools to unravel the in-depth molecular action of natural products. We believe that mass spectrometry has become an invaluable tool that will play a dominant role to accelerate our understanding of natural products' molecular mechanism of action (Fig. 8). As discussed in this review, MS-based chemical and expression proteomics analysis play critical roles to identify protein targets and to map cellular pathway(s) induced by natural products. Combination of MS with a variety of techniques such as affinity chromatography, 2-DGE, or liquid chromatography has clearly facilitated target identification. In particular, the recently developed chemical proteomic platforms that utilize unmodified drug have tremendous potential for target identification of natural products with diverse structures (Kruse et al., 2008). Despite these advances, the chemical proteomics approach to identify receptors of natural products remains a challenge. Difficulties in the isolation of low-abundance receptors and distinguishing between non-specific binding proteins remain; and low throughput is another issue yet to be resolved. However, as the technology continues to mature, we believe that chemical proteomic profiling of protein targets should be incorporated into drug discovery regimes. Chemical proteomics, in combination with the analysis of signaling pathway(s) by expression proteomics, provides a more detailed biochemical map that helps to clarify therapeutic action and toxicity of natural products. On the other hand, MS is also being increasingly used in conjunction with chemical methods, including limited proteolysis, chemical cross-linking, and H/D exchange, to obtain crucial structural information. Although it could only provide low-resolution structural information, MS is nevertheless a promising tool in structural biology complimentary to NMR spectrometry and X-ray crystallography. In comparison to these traditional structural biology tools, the true value of MS analysis lies in its speed, sensitivity, and versatility, which are properties that are ideally suited to accommodate the evolving demands of rapidly advancing drug discovery regimes. Recent developments in MS, such as the use of FT-ICR-MS, have significantly improved the mass accuracy and resolution to thus allow the analysis of highly complex spectra from H/D-exchange and photoaffinity-labeling studies. With advancements in MS technology, analysis of large protein–natural product complexes in excess of 100 kDa is also possible. Moreover, MS could additionally monitor dynamics of protein–natural product interactions that are difficult to study with other tools. Therefore, we anticipate that MS will be a valuable tool to accelerate our understanding of protein–natural product complexes, and provide critical structural information for rational drug development from natural products in the foreseeable future.

V. ABBREVIATIONS

DNPH	2,4-dinitrophenylhydrazine
ALDH-1	aldehyde dehydrogenase class 1
ARE	antioxidant response elements
CRABP I	cellular retinoic acid-binding protein I
CAD	collision-activated dissociation
CDKs	cyclin-dependent kinases
CYP3A4	cytochrome P450 3A4
LPS	cytokine/lipopolysaccharide

ECD	electron capture dissociation
ESI	electrospray ionization
FT-ICR-MS	Fourier transform ion cyclotron resonance mass spectrometer
HSP90	heat shock protein 90
HSPs	heat shock proteins
iTRAQ	isobaric stable isotope tag
ICAT	isotope code affinity tags
Keap1	Kelch-like ECH-associated protein 1
MALDI	matrix-assisted laser desorption ionization
MetAps	methionine aminopeptidases
MAPK	mitogen-activated protein kinase
MAPKKK4	mitogen-activated protein kinase kinase kinase 4
MudPIT	multi-dimensional protein identification technology
MRM	multiple-reaction monitoring
Nrf-2	nuclear factor-E-$_2$-related factor
PMF	peptide mass fingerprinting
Lys-N	peptidyl-Lys metalloendopeptidase
PAL	photoaffinity labeling
PTMs	post-translational modifications
QR2	quinone reductase 2
RBP2	Ran-binding protein 2
RXR	retinoid X receptor
RP	reverse-phase
SPE	solid-phase extraction
SAP	spliceosome-associated proteins
SILAC	stable isotope labeling of amino acids in a cell culture
SCX	strong cation exchange
SARs	structure–activity relationships
TOF	time-of-flight
DIGE	two-dimensional difference gel electrophoresis
2-DGE	two-dimensional gel electrophoresis
Y3H	yeast three-hybrid

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REFERENCES

Adam GC, Vanderwal CD, Sorensen EJ, Cravatt BF. 2003. (-)-FR182877 is a potent and selective inhibitor of carboxylesterase-1. Angew Chem Int Ed 42:5480–5484.

Affar EB, Germain M, Winstall E, Vodenicharov M, Shah RG, Salvesen GS, Poirier GG. 2001. Caspase-3-mediated processing of poly(ADP-ribose) glycohydrolase during apoptosis. J Biol Chem 276: 2935–2942.

Aherne SA, O'Brien NM. 2002. Dietary flavonols: Chemistry, food content, and metabolism. Nutrition 18:75–81.

Ahmed N, Barker G, Oliva K, Garfin D, Talmadge K, Georgiou H, Quinn M, Rice G. 2003. An approach to remove albumin for the proteomic analysis of low abundance biomarkers in human serum. Proteomics 3:1980–1987.

Akashi S. 2006. Investigation of molecular interaction within biological macromolecular complexes by mass spectrometry. Med Res Rev 26: 339–368.

Akashi S, Takio K. 2000. Characterization of the interface structure of enzyme-inhibitor complex by using hydrogen-deuterium exchange and electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. Protein Sci 9:2497–2505.

Al-Mawsawi LQ, Fikkert V, Dayam R, Witvrouw M, Burke TR Jr, Borchers CH, Neamati N. 2006. Discovery of a small-molecule HIV-1 integrase inhibitor-binding site. Proc Natl Acad Sci USA 103:10080–10085.

Altucci L, Leibowitz MD, Ogilvie KM, de Lera AR, Gronemeyer H. 2007. RAR and RXR modulation in cancer and metabolic disease. Nat Rev Drug Discov 6:793–810.

America AHP, Cordewener JHG. 2008. Comparative LC-MS: A landscape of peaks and valleys. Proteomics 8:731–749.

Aoki S, Matsui K, Takata T, Kobayashi A. 2003. In situ photoaffinity labeling of the target protein for lembehyne. A, a neuronal differentiation inducer. FEBS Lett 544:223–227.

Aoki S, Morohashi K, Sunoki T, Kuramochi K, Kobayashi S, Sugawara F. 2007. Screening of paclitaxel-binding molecules from a library of random peptides displayed on t7 phage particles using paclitaxel-photoimmobilized resin. Bioconjug Chem 18:1981–1986.

Arbones-Mainar JM, Ross K, Rucklidge GJ, Reid M, Duncan G, Arthur JR, Horgan GW, Navarro MA, Carnicer R, Arnal C, Osada J, de Roos B. 2007. Extra virgin olive oils increase hepatic fat accumulation and hepatic antioxidant protein levels in APOE−/− mice. J Proteome Res 6:4041–4054.

Asif AR, Armstrong VW, Voland A, Wieland E, Oellerich M, Shipkova M. 2007. Proteins identified as targets of the acyl glucuronide metabolite of mycophenolic acid in kidney tissue from mycophenolate mofetil treated rats. Biochimie 89:393–402.

Bai YW, Milne JS, Mayne L, Englander SW. 1993. Primary structure effects on peptide group hydrogen-exchange. Proteins Structure Funct Genet 17:75–86.

Baillie TA. 2006. Future of toxicology-metabolic activation and drug design: Challenges and opportunities in chemical toxicology. Chem Res Toxicol 19:889–893.

Bandow JE, Brotz H, Leichert LIO, Labischinski H, Hecker M. 2003. Proteomic approach to understanding antibiotic action. Antimicrob Agents Chemother 47:948–955.

Bantscheff M, Eberhard D, Abraham Y, Bastuck S, Boesche M, Hobson S, Mathieson T, Perrin J, Raida M, Rau C, Reader V, Sweetman G, Bauer A, Bouwmeester T, Hopf C, Kruse U, Neubauer G, Ramsden N, Rick J, Kuster B, Drewes G. 2007. Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. Nat Biotechnol 25:1035–1044.

Barros SA, Martin RB. 2008. Predictive toxicogenomics in preclinical discovery. Methods Mol Biol 460:89–112.

Beisson F, Ferte N, Nari J, Noat G, Arondel V, Verger R. 1999. Use of naturally fluorescent triacylglycerols from Parinari glaberrimum to detect low lipase activities from Arabidopsis thaliana seedlings. J Lipid Res 40:2313–2321.

Blagoev B, Ong SE, Kratchmarova I, Mann M. 2004. Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. Nat Biotechnol 22:1139–1145.

Blume-Jensen P, Hunter T. 2001. Oncogenic kinase signalling. Nature 411:355–365.

Boehmerle W, Splittgerber U, Lazarus MB, McKenzie KM, Johnston DG, Austin DJ, Ehrlich BE. 2006. Paclitaxel induces calcium oscillations via an inositol 1,4,5-trisphosphate receptor and neuronal calcium sensor 1-dependent mechanism. Proc Natl Acad Sci USA 103:18356–18361.

Bordeleau ME, Matthews J, Wojnar JM, Lindqvist L, Novac O, Jankowsky E, Sonenberg N, Northcote P, Teesdale-Spittlet P, Pelletier J. 2005. Stimulation of mammalian translation initiation factor eIF4A activity by a small molecule inhibitor of eukaryotic translation. Proc Natl Acad Sci USA 102:10460–10465.

Brown KJ, Fenselau C. 2004. Investigation of doxorubicin resistance in MCF-7 breast cancer cells using shot-gun comparative proteomics with proteolytic O-18 labeling. J Proteome Res 3:455–462.

Brownlow S, Cabral JHM, Cooper R, Flower DR, Yewdall SJ, Polikarpov I, North ACT, Sawyer L. 1997. Bovine beta-lactoglobulin at 1.8 angstrom resolution—Still an enigmatic lipocalin. Structure 5:481–495.

Bruce JE, Anderson GA, Wen J, Harkewicz R, Smith RD. 1999. High mass-measurement accuracy and 100% sequence coverage of enzymatically digested bovine serum albumin from an ESI-FTICR mass spectrum. Anal Chem 71:2595–2599.

Brusselbach S, Nettelbeck DM, Sedlacek HH, Muller R. 1998. Cell cycle-independent induction of apoptosis by the anti-tumor drug flavopiridol in endothelial cells. Int J Cancer 77:146–152.

Budde K, Curtis J, Knoll G, Chan L, Neumayer HH, Seifu Y, Hall M. 2004. Enteric-coated mycophenolate sodium can be safely administered in maintenance renal transplant patients: Results of a 1-year study. Am J Transplant 4:237–243.

Buey RM, Calvo E, Barasoain I, Pineda O, Edler MC, Matesanz R, Cerezo G, Vanderwal CD, Day BW, Sorensen EJ, Lopez JA, Andreu JM, Hamel E, Diaz JF. 2007. Cyclostreptin binds covalently to microtubule pores and lumenal taxoid binding sites. Nat Chem Biol 3:117–125.

Bullingham RE, Nicholls AJ, Kamm BR. 1998. Clinical pharmacokinetics of mycophenolate mofetil. Clin Pharmacokinet 34:429–455.

Burdine L, Kodadek T. 2004. Target identification in chemical genetics: The (often) missing link. Chem Biol 11:593–597.

Burkitt WI, Derrick PJ, Lafitte D, Bronstein I. 2003. Protein-ligand and protein-protein interactions studied by electrospray ionization and mass spectrometry. Biochem Soc Trans 31:985–989.

Butterfield DA, Drake J, Pocernich C, Castegna A. 2001. Evidence of oxidative damage in Alzheimer's disease brain: Central role for amyloid beta-peptide. Trends Mol Med 7:548–554.

Byrd JC, Lin TS, Dalton JT, Wu D, Phelps MA, Fischer B, Moran M, Blum KA, Rovin B, Brooker-McEldowney M, Broering S, Schaaf LJ, Johnson AJ, Lucas DM, Heerema NA, Lozanski G, Young DC, Suarez JR, Colevas AD, Grever MR. 2007. Flavopiridol administered using a pharmacologically derived schedule is associated with marked clinical efficacy in refractory, genetically high-risk chronic lymphocytic leukemia. Blood 109:399–404.

Cai J, Liu MJ, Wang Z, Ju Y. 2002. Apoptosis induced by dioscin in Hela cells. Biol Pharm Bull 25:193–196.

Canas B, Lopez-Ferrer D, Ramos-Fernandez A, Camafeita E, Calvo E. 2006. Mass spectrometry technologies for proteomics. Brief Funct Genomic Proteomic 4:295–320.

Carlson B, Lahusen T, Singh S, Loaiza-Perez A, Worland PJ, Pestell R, Albanese C, Sausville EA, Senderowicz AM. 1999. Down-regulation of cyclin D1 by transcriptional repression in MCF-7 human breast carcinoma cells induced by flavopiridol. Cancer Res 59:4634–4641.

Chalmers MJ, Busby SA, Pascal BD, He YJ, Hendrickson CL, Marshall AG, Griffin PR. 2006. Probing protein ligand interactions by automated hydrogen/deuterium exchange mass spectrometry. Anal Chem 78:1005–1014.

Charlebois JP, Patrie SM, Kelleher NL. 2003. Electron capture dissociation and C-13, N-15 depletion for deuterium localization in intact proteins after solution-phase exchange. Anal Chem 75:3263–3266.

Chen JK, Lane WS, Schreiber SL. 1999. The identification of myriocin-binding proteins. Chem Biol 6:221–235.

Chen Y, Daosukho C, Opii WO, Turner DM, Pierce WM, Klein JB, Vore M, Butterfield DA, St Clair DK. 2006. Redox proteomic identification of oxidized cardiac proteins in adriamycin-treated mice. Free Radic Biol Med 41:1470–1477.

Chromy BA, Gonzales AD, Perkins J, Choi MW, Corzett MH, Chang BC, Corzett CH, McCutchen-Maloney SL. 2004. Proteomic analysis of human serum by two-dimensional differential gel electrophoresis after depletion of high-abundant proteins. J Proteome Res 3:1120–1127.

Cimmino F, Spano D, Capasso M, Zambrano N, Russo R, Zollo M, Iolascon A. 2007. Comparative proteomic expression profile in all-trans retinoic acid differentiated neuroblastoma cell line. J Proteome Res 6:2550–2564.

Conrad CC, Choi JG, Malakowsky CA, Talent JM, Dai R, Marshall P, Gracy RW. 2001. Identification of protein carbonyls after two-dimensional electrophoresis. Proteomics 1:829–834.

Cosman M, Lightstone FC, Krishnan VV, Zeller L, Prieto MC, Roe DC, Balhorn R. 2002. Identification of novel small molecules that bind to two different sites on the surface of tetanus toxin C fragment. Chem Res Toxicol 15:1218–1228.

Cragg GM, Newman DJ. 2005. Plants as a source of anti-cancer agents. J Ethnopharmacol 100:72–79.

Crews CM, Lane WS, Schreiber SL. 1996. Didemnin binds to the protein palmitoyl thioesterase responsible for infantile neuronal ceroid lipofuscinosis. Proc Natl Acad Sci U S A 93:4316–4319.

da Rocha AB, Lopes RM, Schwartsmann G. 2001. Natural products in anticancer therapy. Curr Opin Pharmacol 1:364–369.

Deibel MR Jr, Bodnar AL, Yem AW, Wolfe CL, Heckaman CL, Bohanon MJ, Mathews WR, Sweeney MT, Zurenko GE, Marotti KR, Boyle TP, Thorarensen A. 2004. Immobilization of a novel antibacterial agent on solid phase and subsequent isolation of EF-Tu. Bioconjug Chem 15:333–343.

Deng G, Sanyal G. 2006. Applications of mass spectrometry in early stages of target based drug discovery. J Pharm Biomed Anal 40:528–538.

Dent P, Yacoub A, Fisher PB, Hagan MP, Grant S. 2003. MAPK pathways in radiation responses. Oncogene 22:5885–5896.

Dorman G, Prestwich GD. 2000. Using photolabile ligands in drug discovery and development. Trends Biotechnol 18:64–77.

Drahl C, Cravatt BF, Sorensen EJ. 2005. Protein-reactive natural products. Angew Chem Int Ed Engl 44:5788–5809.

Dreger M. 2003. Subcellular proteomics. Mass Spectrom Rev 22:27–56.

Druckova A, Mernaugh RL, Ham AJ, Marnett LJ. 2007. Identification of the protein targets of the reactive metabolite of teucrin A in vivo in the rat. Chem Res Toxicol 20:1393–1408.

Edler MC, Buey RM, Gussio R, Marcus AI, Vanderwal CD, Sorensen EJ, Diaz JF, Giannakakou P, Hamel E. 2005. Cyclostreptin (FR182877), an antitumor tubulin-polymerizing agent deficient in enhancing tubulin assembly despite its high affinity for the taxoid site. Biochemistry 44:11525–11538.

Elnakady YA, Rohde M, Sasse F, Backes C, Keller A, Lenhof HP, Weissman KJ, Muller R. 2007. Evidence for the mode of action of the highly cytotoxic Streptomyces polyketide kendomycin. Chembiochem 8:1261–1272.

England K, Cotter T. 2004. Identification of carbonylated proteins by MALDI-TOF mass spectroscopy reveals susceptibility of ER. Biochem Biophys Res Commun 320:123–130.

England K, Driscoll CO, Cotter TG. 2006. ROS and protein oxidation in early stages of cytotoxic drug induced apoptosis. Free Radic Res 40:1124–1137.

England K, O'Driscoll C, Cotter TG. 2004. Carbonylation of glycolytic proteins is a key response to drug-induced oxidative stress and apoptosis. Cell Death Differ 11:252–260.

Ermakova S, Choi BY, Choi HS, Kang BS, Bode AM, Dong Z. 2005. The intermediate filament protein vimentin is a new target for epigallocatechin gallate. J Biol Chem 280:16882–16890.

Evans DC, Watt AP, Nicoll-Griffith DA, Baillie TA. 2004. Drug-protein adducts: An industry perspective on minimizing the potential for drug

bioactivation in drug discovery and development. Chem Res Toxicol 17:3–16.

Evans DC, Watt AP, Nicoll-Griffith DA, Baillie TA. 2005. Drug-protein adducts: An industry perspective on minimizing the potential for drug bioactivation in drug discovery and development (vol 17, pg 3, 2004). Chem Res Toxicol 18:1777–1777.

Eyles SJ, Speir JP, Kruppa GH, Gierasch LM, Kaltashov IA. 2000. Protein conformational stability probed by Fourier transform ion cyclotron resonance mass spectrometry. J Am Chem Soc 122:495–500.

Fang J, Lu J, Holmgren A. 2005. Thioredoxin reductase is irreversibly modified by curcumin: A novel molecular mechanism for its anticancer activity. J Biol Chem 280:25284–25290.

Feng L, Jin J, Zhang LF, Yan T, Tao WY. 2006. Analysis of the resveratrol-binding protein using phage-displayed random peptide library. Acta Biochim Biophys Sin 38:342–348.

Foley M, Tilley L. 1998. Quinoline antimalarials: Mechanisms of action and resistance and prospects for new agents. Pharmacol Ther 79:55–87.

Fontana A, de Laureto PP, Spolaore B, Frare E, Picotti P, Zambonin M. 2004. Probing protein structure by limited proteolysis. Acta Biochim Pol 51:299–321.

Fuchs D, de Pascual-Teresa S, Rimbach G, Virgili F, Ambra R, Turner R, Daniel H, Wenzel U. 2005a. Proteome analysis for identification of target proteins of genistein in primary human endothelial cells stressed with oxidized LDL or homocysteine. Eur J Nutr 44:95–104.

Fuchs D, Erhard P, Rimbach G, Daniel H, Wenzel U. 2005b. Genistein blocks homocysteine-induced alterations in the proteome of human endothelial cells. Proteomics 5:2808–2818.

Gadgil HS, Pabst KM, Giorgianni F, Umstot ES, Desiderio DM, Beranova-Giorgianni S, Gerling IC, Pabst MJ. 2003. Proteome of monocytes primed with lipopolysaccharide: Analysis of the abundant proteins. Proteomics 3:1767–1780.

Ganem B, Li YT, Henion JD. 1991. Detection of noncovalent receptor ligand complexes by mass-spectrometry. J Am Chem Soc 113:6294–6296.

Garcia RA, Pantazatos DP, Gessner CR, Go KV, Woods VL, Villarreal FJ. 2005. Molecular interactions between matrilysin and the matrix metalloproteinase inhibitor doxycycline investigated by deuterium exchange mass spectrometry. Mol Pharmacol 67:1128–1136.

Gartner CA, Wen B, Wan J, Becker RS, Jones G, Gygi SP, Nelson SD. 2005. Photochromic agents as tools for protein structure study: Lapachenole is a photoaffinity ligand of cytochrome P450 3A4. Biochemistry 44:1846–1855.

Geoghegan KF, Kelly MA. 2005. Biochemical applications of mass spectrometry in pharmaceutical drug discovery. Mass Spectrom Rev 24:347–366.

Ghezzi P, Bonetto V. 2003. Redox proteomics: Identification of oxidatively, modified proteins. Proteomics 3:1145–1153.

Gingras AC, Raught B, Sonenberg N. 1999. eIF4 initiation factors: Effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem 68:913–963.

Goh D, Lee YH, Ong ES. 2005. Inhibitory effects of a chemically standardized extract from Scutellaria barbata in human colon cancer cell lines, LoVo. J Agric Food Chem 53:8197–8204.

Gong L, Puri M, Unlu M, Young M, Robertson K, Viswanathan S, Krishnaswamy A, Dowd SR, Minden JS. 2004. Drosophila ventral furrow morphogenesis: A proteomic analysis. Development 131:643–656.

Graves PR, Kwiek JJ, Fadden P, Ray R, Hardeman K, Coley AM, Foley M, Haystead TA. 2002. Discovery of novel targets of quinoline drugs in the human purine binding proteome. Mol Pharmacol 62:1364–1372.

Griffith EC, Su Z, Turk BE, Chen S, Chang YH, Wu Z, Biemann K, Liu JO. 1997. Methionine aminopeptidase (type 2) is the common target for angiogenesis inhibitors AGM-1470 and ovalicin. Chem Biol 4:461–471.

Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. 1999. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol 17:994–999.

Harding MW, Galat A, Uehling DE, Schreiber SL. 1989. A receptor for the immunosuppressant Fk506 is a cis-trans peptidyl-prolyl isomerase. Nature 341:758–760.

Haridas V, Kim SO, Nishimura G, Hausladen A, Stamler JS, Gutterman JU. 2005. Avicinylation (thioesterification): A protein modification that can regulate the response to oxidative and nitrosative stress. Proc Natl Acad Sci USA 102:10088–10093.

Harris MN, Ozpolat B, Abdi F, Gu S, Legler A, Mawuenyega KG, Tirado-Gomez M, Lopez-Berestein G, Chen X. 2004. Comparative proteomic analysis of all-trans-retinoic acid treatment reveals systematic post-transcriptional control mechanisms in acute promyelocytic leukemia. Blood 104:1314–1323.

Harvey AL. 2007. Natural products as a screening resource. Curr Opin Chem Biol 11:480–484.

Hauschildt S, Schwarz C, Heine H, Ulmer AJ, Flad HD, Rietschel ET, Jensen ON, Mann M. 1997. Actin: A target of lipopolysaccharide-induced phosphorylation in human monocytes. Biochem Biophys Res Commun 241:670–674.

He QY, Chiu JF. 2003. Proteomics in biomarker discovery and drug development. J Cell Biochem 89:868–886.

Heck AJR, Jorgensen TJD, O'Sullivan M, von Raumer M, Derrick PJ. 1998. Gas-phase noncovalent interactions between vancomycin-group antibiotics and bacterial cell-wall precursor peptides probed by hydrogen/deuterium exchange. J Am Soc Mass Spectrom 9:1255–1266.

Heerema NA, Byrd JC, Andritsos LA, Lozanski G, Blum K, Fischer B, Jones JA, Moran ME, Groering S, Schaaf LJ, Mahoney LS, Johnson AJ, Smith LL, Wagner AJ, Raymond CA, Phelps MA, Dalton JT, Grever MR, Lin TS. 2007. Clinical activity of flavopiridol in relapsed and refractory chronic lymphocytic leukemia (CLL) with high-risk cytogenetic abnormalities: Updated data on 89 patients (Pts). Blood 110:3107.

Hill JJ, Callaghan DA, Ding W, Kelly JF, Chakravarthy BR. 2006. Identification of okadaic acid-induced phosphorylation events by a mass spectrometry approach. Biochem Biophys Res Commun 342: 791–799.

Hofstadler SA, Sannes-Lowery KA. 2006. Applications of ESI-MS in drug discovery: Interrogation of noncovalent complexes. Nat Rev Drug Discov 5:585–595.

Hong SH, Kim J, Kim JM, Lee SY, Shin DS, Son KH, Han DC, Sung YK, Kwon BM. 2007. Apoptosis induction of 2′-hydroxycinnamaldehyde as a proteasome inhibitor is associated with ER stress and mitochondrial perturbation in cancer cells. Biochem Pharmacol 74:557–565.

Hoofnagle AN, Resing KA, Ahn NG. 2003. Protein analysis by hydrogen exchange mass spectrometry. Annu Rev Biophys Biomol Struct 32: 1–25.

Hosono T, Fukao T, Ogihara J, Ito Y, Shiba H, Seki T, Ariga T. 2005. Diallyl trisulfide suppresses the proliferation and induces apoptosis of human colon cancer cells through oxidative modification of beta-tubulin. J Biol Chem 280:41487–41493.

Hsieh TC, Wang ZR, Deng HT, Wu JM. 2008. Identification of glutathione sulfotransferase-pi (GSTP1) as a new resveratrol targeting protein (RTP) and studies of resveratrol-responsive protein changes by resveratrol affinity chromatography. Anticancer Res 28:29–36.

Hu W, Wu W, Verschraegen CF, Chen L, Mao L, Yeung SC, Kudelka AP, Freedman RS, Kavanagh JJ. 2003. Proteomic identification of heat shock protein 70 as a candidate target for enhancing apoptosis induced by farnesyl transferase inhibitor. Proteomics 3:1904–1911.

Hubbard SJ. 1998. The structural aspects of limited proteolysis of native proteins. Biochim Biophys Acta Protein Struct Mol Enzymol 1382: 191–206.

Huber LA, Pfaller K, Vietor I. 2003. Organelle proteomics—Implications for subcellular fractionation in proteomics. Circ Res 92:962–968.

Hwang HJ, Baek YM, Kim SW, Kumar GS, Cho EJ, Oh JY, Yun JW. 2007. Differential expression of kidney proteins in streptozotocin-induced diabetic rats in response to hypoglycemic fungal polysaccharides. J Microbiol Biotechnol 17:2005–2017.

Imre T, Zsila F, Szabo PT. 2003. Electrospray mass spectrometric investigation of the binding of cis-parinaric acid to bovine beta-lactoglobulin and study of the ligand-binding site of the protein using limited proteolysis. Rapid Commun Mass Spectrom 17:2464–2470.

Ingber D, Fujita T, Kishimoto S, Sudo K, Kanamaru T, Brem H, Folkman J. 1990. Synthetic analogs of fumagillin that inhibit angiogenesis and suppress tumor-growth. Nature 348:555–557.

Ishikawa H. 1999. Mizoribine and mycophenolate mofetil. Curr Med Chem 6:575–597.

Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M. 1999. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev 13:76–86.

Jorgensen TJD, Delforge D, Remacle J, Bojesen G, Roepstorff P. 1999. Collision-induced dissociation of noncovalent complexes between vancomycin antibiotics and peptide ligand stereoisomers: Evidence for molecular recognition in the gas phase. Int J Mass Spectrom 188:63–85.

Jorgensen TJD, Roepstorff P, Heck AJR. 1998. Direct determination of solution binding constants for noncovalent complexes between bacterial cell wall peptide analogues and vancomycin group antibiotics by electrospray ionization mass spectrometry. Anal Chem 70:4427–4432.

Joseph J, Liu ST, Jablonski SA, Yen TJ, Dasso M. 2004. The RanGAP1-RanBP2 complex is essential for microtubule-kinetochore interactions in vivo. Curr Biol 14:611–617.

Jung E, Heller M, Sanchez JC, Hochstrasser DF. 2000. Proteomics meets cell biology: The establishment of subcellular proteomes. Electrophoresis 21:3369–3377.

Kaltashov IA, Eyles SJ. 2002. Studies of biomolecular conformations and conformational dynamics by mass spectrometry. Mass Spectrom Rev 21:37–71.

Kaveti S, Engen JR. 2006. Protein interactions probed with mass spectrometry. Methods Mol Biol 316:179–197.

Keezer SM, Ivie SE, Krutzsch HC, Tandle A, Libutti SK, Roberts DD. 2003. Angiogenesis inhibitors target the endothelial cell cytoskeleton through altered regulation of heat shock protein 27 and cofilin. Cancer Res 63:6405–6412.

Kehrer DFS, Soepenberg O, Loos WJ, Verweij J, Sparreboom A. 2001. Modulation of camptothecin analogs in the treatment of cancer: A review. Anticancer Drugs 12:89–105.

Kesiry R, Liu J. 2005. GRP78/BIP is involved in ouabain-induced endocytosis of the Na/K-ATPase in LLC-PK1 cells. Front Biosci 10:2045–2055.

Khandekar SS, Feng BB, Yi T, Chen S, Laping N, Bramson N. 2005. A liquid chromatography/mass spectrometry-based method for the selection of ATP competitive kinase inhibitors. J Biomol Screen 10:447–455.

Kim H, Deng L, Xiong X, Hunter WD, Long MC, Pirrung MC. 2007. Glyceraldehyde 3-phosphate dehydrogenase is a cellular target of the insulin mimic demethylasterriquinone b1. J Med Chem 50:3423–3426.

Kim HK, Kong MY, Jeong MJ, Han DC, Choi JD, Kim HY, Yoon KS, Kim JM, Son KH, Kwon BM. 2005. Investigation of cell cycle arrest effects of actinomycin D at G1 phase using proteomic methods in B104-1-1 cells. Int J Biochem Cell Biol 37:1921–1929.

Kim SW, Hwang HJ, Kim HM, Lee MC, Shik Lee M, Choi JW, Yun JW. 2006a. Effect of fungal polysaccharides on the modulation of plasma proteins in streptozotocin-induced diabetic rats. Proteomics 6:5291–5302.

Kim YH, Cho K, Yun SH, Kim JY, Kwon KH, Yoo JS, Kim SI. 2006b. Analysis of aromatic catabolic pathways in Pseudomonas putida KT 2440 using a combined proteomic approach: 2-DE/MS and cleavable isotope-coded affinity tag analysis. Proteomics 6:1301–1318.

Kimmel JL, Tipton PA. 2005. Inactivation of GDP-mannose dehydrogenase from Pseudomonas aeruginosa by penicillic acid identifies a critical active site loop. Arch Biochem Biophys 441:132–140.

Kotake Y, Sagane K, Owa T, Mimori-Kiyosue Y, Shimizu H, Uesugi M, Ishihama Y, Iwata M, Mizui Y. 2007. Splicing factor SF3b as a target of the antitumor natural product pladienolide. Nat Chem Biol 3:570–575.

Kraljevic S, Sedic M, Scott M, Gehrig P, Schlapbach R, Pavelic K. 2006. Casting light on molecular events underlying anti-cancer drug treatment: What can be seen from the proteomics point of view? Cancer Treat Rev 32:619–629.

Krishnan VV, Sukumar M, Gierasch LM, Cosman M. 2000. Dynamics of cellular retinoic acid binding protein I on multiple time scales with implications for ligand binding. Biochemistry 39:9119–9129.

Kruse U, Bantscheff M, Drewes G, Hopf C. 2008. Chemical and pathway proteomics: Powerful tools for oncology drug discovery and personalized health care. Mol Cell Proteomics 7:1887–1901.

Kubota K, Wakabayashi K, Matsuoka T. 2003. Proteome analysis of secreted proteins during osteoclast differentiation using two different methods: Two-dimensional electrophoresis and isotope-coded affinity tags analysis with two-dimensional chromatography. Proteomics 3:616–626.

Lam KS. 2007. New aspects of natural products in drug discovery. Trends Microbiol 15:279–289.

Lambert JD, Yang CS. 2003. Mechanisms of cancer prevention by tea constituents. J Nutr 133:3262S–3267S.

Lamos SM, Krusemark CJ, McGee CJ, Scalf M, Smith LM, Belshaw PJ. 2006. Mixed isotope photoaffinity reagents for identification of small-molecule targets by mass spectrometry. Angew Chem Int Ed Engl 45: 4329–4333.

Larsen AK, Moller MT, Blankson H, Samari HR, Holden L, Seglen PO. 2002. Naringin-sensitive phosphorylation of plectin, a cytoskeletal cross-linking protein, in isolated rat hepatocytes. J Biol Chem 277:34826–34835.

Lee CL, Jiang PP, Sit WH, Wan JM. 2007. Proteome of human T lymphocytes with treatment of cyclosporine and polysaccharopeptide: Analysis of significant proteins that manipulate T cells proliferation and immunosuppression. Int Immunopharmacol 7:1311–1324.

Lee JH, Chung KY, Bang D, Lee KH. 2006a. Searching for aging-related proteins in human dermal microvascular endothelial cells treated with anti-aging agents. Proteomics 6:1351–1361.

Lee SC, Chan J, Clement MV, Pervaiz S. 2006b. Functional proteomics of resveratrol-induced colon cancer cell apoptosis: Caspase-6-mediated cleavage of lamin A is a major signaling loop. Proteomics 6:2386–2394.

Lehmann S, Bengtzen S, Broberg U, Paul C. 2000. Effects of retinoids on cell toxicity and apoptosis in leukemic blast cells from patients with non-M3 AML. Leuk Res 24:19–25.

Lewis DFV, Eddershaw PJ, Goldfarb PS, Tarbit MH. 1996. Molecular modelling of CYP3A4 from an alignment with CYP102: Identification of key interactions between putative active site residues and CYP3A-specific chemicals. Xenobiotica 26:1067–1086.

Li BY, Cheng M, Gao HQ, Ma YB, Xu L, Li XH, Li XL, You BA. 2008. Back-regulation of six oxidative stress proteins with grape seed proanthocyanidin extracts in rat diabetic nephropathy. J Cell Biochem 104:668–679.

Licitra EJ, Liu JO. 1996. A three-hybrid system for detecting small ligand-protein receptor interactions. Proc Natl Acad Sci USA 93:12817–12821.

Liebler DC. 2008. Protein damage by reactive electrophiles: Targets and consequences. Chem Res Toxicol 21:117–128.

Liu LF. 1989. DNA topoisomerase poisons as antitumor drugs. Annu Rev Biochem 58:351–375.

Liu MJ, Wang Z, Ju Y, Zhou JB, Wang Y, Wong RNS. 2004. The mitotic-arresting and apoptosis-inducing effects of diosgenyl saponins on human leukemia cell lines. Biol Pharm Bull 27:1059–1065.

Loo JA. 1997. Studying noncovalent protein complexes by electrospray ionization mass spectrometry. Mass Spectrom Rev 16:1–23.

Lovell MA, Ehmann WD, Butler SM, Markesbery WR. 1995. Elevated thiobarbituric acid-reactive substances and antioxidant enzyme-activity in the brain in Alzheimers-disease. Neurology 45:1594–1601.

Low WK, Dang Y, Schneider-Poetsch T, Shi ZG, Choi NS, Rzasa RM, Shea HA, Li SK, Park K, Ma G, Romo D, Liu JO. 2007. Isolation and identification of eukaryotic initiation factor 4A as a molecular target for the marine natural product Pateamine A. Methods Enzymol 431:303–324.

Low WK, Dang YJ, Schneider-Poetsch T, Shi ZG, Choi NS, Merrick WC, Romo D, Liu JO. 2005. Inhibition of eukaryotic translation initiation by the marine natural product pateamine A. Mol Cell 20:709–722.

Lowther WT, McMillen DA, Orville AM, Matthews BW. 1998. The anti-angiogenic agent fumagillin covalently modifies a conserved active-site histidine in the Escherichia coli methionine aminopeptidase. Proc Natl Acad Sci USA 95:12153–12157.

Luo Y, Eggler AL, Liu D, Liu G, Mesecar AD, van Breemen RB. 2007. Sites of alkylation of human Keap1 by natural chemoprevention agents. J Am Soc Mass Spectrom 18:2226–2232.

Mainardi JL, Hugonnet JE, Rusconi F, Fourgeaud M, Dubost L, Moumi AN, Delfosse V, Mayer C, Gutmann L, Rice LB, Arthur M. 2007. Unexpected inhibition of peptidoglycan LD-transpeptidase from Enterococcus faecium by the beta-lactam imipenem. J Biol Chem 282:30414–30422.

Makowski L, Rodi DJ. 2003. Genome-wide characterisation of the binding repertoire of small molecule drugs. Hum Genomics 1:41–51.

Marshall AG, Hendrickson CL, Jackson GS. 1998. Fourier transform ion cyclotron resonance mass spectrometry: A primer. Mass Spectrom Rev 17:1–35.

McCammon MG, Robinson CV. 2004. Structural change in response to ligand binding. Curr Opin Chem Biol 8:60–65.

McDonald C, Li L. 2005. Limited proteolysis combined with isotope labeling and quantitative LC-MALDI MS for monitoring protein conformational changes: A study on calcium-binding sites of cardiac Troponin C. Anal Chim Acta 534:3–10.

McKenzie KM, Videlock EJ, Splittgerber U, Austin DJ. 2004. Simultaneous identification of multiple protein targets by using complementary-DNA phage display and a natural-product-mimetic probe. Angew Chem Int Ed 43:4052–4055.

McPherson M, Yang YF, Hammond PW, Kreider BL. 2002. Drug receptor identification from multiple tissues using cellular-derived mRNA display libraries. Chem Biol 9:691–698.

Melillo G, Sausville EA, Cloud K, Lahusen T, Varesio L, Senderowicz AM. 1999. Flavopiridol, a protein kinase inhibitor, down-regulates hypoxic induction of vascular endothelial growth factor expression in human monocytes. Cancer Res 59:5433–5437.

Meng LH, Kwok BHB, Sin N, Crews CM. 1999. Eponemycin exerts its autitumor effect through the inhibition of proteasome function. Cancer Res 59:2798–2801.

Metodiev MV, Timanova A, Stone DE. 2004. Differential phosphoproteome profiling by affinity capture and tandem matrix-assisted laser desorption/ionization mass spectrometry. Proteomics 4:1433–1438.

Miyagi M, Rao KC. 2007. Proteolytic 18O-labeling strategies for quantitative proteomics. Mass Spectrom Rev 26:121–136.

Muckenschnabel I, Falchetto R, Mayr LM, Filipuzzi I. 2004. SpeedScreen: Label-free liquid chromatography-mass spectrometry-based high-throughput screening for the discovery of orphan protein ligands. Anal Biochem 324:241–249.

Nagumo Y, Han J, Arimoto M, Isoda H, Tanaka T. 2007. Capsaicin induces cofilin dephosphorylation in human intestinal cells: The triggering role of cofilin in tight-junction signaling. Biochem Biophys Res Commun 355:520–525.

Neo JCH, Rose P, Ong CN, Chung MCM. 2005. beta-Phenylethyl isothiocyanate mediated apoptosis: A proteomic investigation of early apoptotic protein changes. Proteomics 5:1075–1082.

Newcomer ME, Pappas RS, Ong DE. 1993. X-ray crystallographic identification of a protein-binding site for both all-trans-retinoic and 9-Cis-retinoic acid. Proc Natl Acad Sci USA 90:9223–9227.

Newman DJ, Cragg GM. 2007. Natural products as sources of new drugs over the last 25 years. J Nat Prod 70:461–477.

Newman DJ, Cragg GM, Snader KM. 2003. Natural products as sources of new drugs over the period 1981–2002. J Nat Prod 66:1022–1037.

Noble RL. 1990. The discovery of the vinca alkaloids—Chemotherapeutic agents against cancer. Biochem Cell Biol 68:1344–1351.

Northcote PT, Blunt JW, Munro MHG. 1991. Pateamine—A potent cytotoxin from the New-Zealand Marine Sponge, Mycale Sp. Tetrahedron Lett 32:6411–6414.

Oda Y, Owa T, Sato T, Boucher B, Daniels S, Yamanaka H, Shinohara Y, Yokoi A, Kuromitsu J, Nagasu T. 2003. Quantitative chemical proteomics for identifying candidate drug targets. Anal Chem 75: 2159–2165.

Opii WO, Joshi G, Head E, Milgram NW, Muggenburg BA, Klein JB, Pierce WM, Cotman CW, Butterfield DA. 2008. Proteomic identification of brain proteins in the canine model of human aging following a long-term treatment with antioxidants and a program of behavioral enrichment: Relevance to Alzheimer's disease. Neurobiol Aging 29:51–70.

Parekh RB, Rohlff C. 1997. Post-translational modification of proteins and the discovery of new medicine. Curr Opin Biotechnol 8:718–723.

Pedigo S, Shea MA. 1995. Quantitative endoproteinase gluc footprinting of cooperative Ca^{2+} binding to calmodulin—Proteolytic susceptibility of E31 and E87 indicates interdomain interactions. Biochemistry 34: 1179–1196.

Peters EC, Gray NS. 2007. Chemical proteomics identifies unanticipated targets of clinical kinase inhibitors. ACS Chem Biol 2:661–664.

Petrak J, Ivanek R, Toman O, Cmejla R, Cmejlova J, Vyoral D, Zivny J, Vulpe CD. 2008. Deja vu in proteomics. A hit parade of repeatedly identified differentially expressed proteins. Proteomics 8:1744–1749.

Phillips JJ, Yao ZP, Zhang W, McLaughlin S, Laue ED, Robinson CV, Jackson SE. 2007. Conformational dynamics of the molecular chaperone Hsp90 in complexes with a co-chaperone and anticancer drugs. J Mol Biol 372:1189–1203.

Piggott AM, Karuso P. 2004. Quality, not quantity: The role of natural products and chemical proteomics in modern drug discovery. Comb Chem High Throughput Screen 7:607–630.

Piggott AM, Karuso P. 2008. Rapid identification of a protein binding partner for the marine natural product kahalalide F by using reverse chemical proteomics. Chembiochem 9:524–530.

Pleban K, Kopp S, Csaszar E, Peer M, Hrebicek T, Rizzi A, Ecker GF, Chiba P. 2005. P-glycoprotein substrate binding domains are located at the transmembrane domain/transmembrane domain interfaces: A combined photoaffinity labeling-protein homology modeling approach. Mol Pharmacol 67:365–374.

Poirier F, Joubert-Caron R, Labas V, Caron M. 2003. Proteomic analysis of a lymphoma-derived cell line (DG75) following treatment with a demethylating drug: Modification of membrane-associated proteins. Proteomics 3:1028–1036.

Pommier Y, Johnson AA, Marchand C. 2005. Integrase inhibitors to treat HIV/AIDS. Nat Rev Drug Discov 4:236–248.

Qiu J, Gao HQ, Li BY, Shen L. 2008. Proteomics investigation of protein expression changes in ouabain induced apoptosis in human umbilical vein endothelial cells. J Cell Biochem 104:1054–1064.

Qiu J, Gao HQ, Zhou RH, Liang Y, Zhang XH, Wang XP, You BA, Cheng M. 2007. Proteomics analysis of the proliferative effect of low-dose ouabain on human endothelial cells. Biol Pharm Bull 30:247–253.

Radeke HS, Snapper ML. 1998. Photoaffinity study of the cellular interactions of ilimaquinone. Bioorg Med Chem 6:1227–1232.

Rahman MA, Kumar SG, Kim SW, Hwang HJ, Baek YM, Lee SH, Hwang HS, Shon YH, Nam KS, Yun JW. 2008. Proteomic analysis for inhibitory effect of chitosan oligosaccharides on 3T3-L1 adipocyte differentiation. Proteomics 8:569–581.

Raja MM, Tyagi NK, Kinne RK. 2003. Phlorizin recognition in a C-terminal fragment of SGLT1 studied by tryptophan scanning and affinity labeling. J Biol Chem 278:49154–49163.

Rao KC, Palamalai V, Dunlevy JR, Miyagi M. 2005a. Peptidyl-Lys metalloendopeptidase-catalyzed 18O labeling for comparative proteomics: Application to cytokine/lipolysaccharide-treated human retinal pigment epithelium cell line. Mol Cell Proteomics 4:1550–1557.

Rao KCS, Carruth RT, Miyagi M. 2005b. Proteolytic O-18 labeling by peptidyl-Lys metalloendopeptidase for comparative proteomics. J Proteome Res 4:507–514.

Raught B, Gingras AC, Gygi SP, Imataka H, Morino S, Gradi A, Aebersold R, Sonenberg N. 2000. Serum-stimulated, rapamycin-sensitive phosphorylation sites in the eukaryotic translation initiation factor 4GI. EMBO J 19:434–444.

Riba I, Gaskell SJ, Cho H, Widger WR, Kohn H. 1998. Evidence for the location of bicyclomycin binding to the Escherichia coli transcription termination factor Rho. J Biol Chem 273:34033–34041.

Robinette D, Neamati N, Tomer KB, Borchers CH. 2006. Photoaffinity labeling combined with mass spectrometric approaches as a tool for structural proteomics. Expert Rev Proteomics 3:399–408.

Robinson CV, Chung EW, Kragelund BB, Knudsen J, Aplin RT, Poulsen FM, Dobson CM. 1996. Probing the nature of noncovalent interactions by mass spectrometry. A study of protein-CoA ligand binding and assembly. J Am Chem Soc 118:8646–8653.

Roderick SL, Matthews BW. 1993. Structure of the cobalt-dependent methionine aminopeptidase from Escherichia-coli—A new type of proteolytic-enzyme. Biochemistry 32:3907–3912.

Rodi DJ, Agoston GE, Manon R, Lapcevich R, Green SJ, Makowski L. 2001. Identification of small molecule binding sites within proteins using phage display technology. Comb Chem High Throughput Screen 4: 553–572.

Rodi DJ, Janes RW, Sanganee HJ, Holton RA, Wallace BA, Makowski L. 1999. Screening of a library of phage-displayed peptides identifies human Bcl-2 as a taxol binding protein. J Mol Biol 285:197–203.

Rodi DJ, Makowski L, Kay BK. 2002. One from column A and two from column B: The benefits of phage display in molecular-recognition studies. Curr Opin Chem Biol 6:92–96.

Roe MR, Griffin TJ. 2006. Gel-free mass spectrometry-based high throughput proteomics: Tools for studying biological response of proteins and proteomes. Proteomics 6:4678–4687.

Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ. 2004. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 3:1154–1169.

Rouhi AM. 2003. Rediscovering natural products. Chem Eng News 81:77–78.

Rundle NT, Nelson J, Flory MR, Joseph J, Th'ng J, Aebersold R, Dasso M, Andersen RJ, Roberge M. 2006. An ent-kaurene that inhibits mitotic chromosome movement and binds the kinetochore protein ran-binding protein 2. ACS Chem Biol 1:443–450.

Rush J, Moritz A, Lee KA, Guo A, Goss VL, Spek EJ, Zhang H, Zha XM, Polakiewicz RD, Comb MJ. 2005. Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. Nat Biotechnol 23:94–101.

Safa AR. 2004. Identification and characterization of the binding sites of P-glycoprotein for multidrug resistance-related drugs and modulators. Curr Med Chem Anticancer Agents 4:1–17.

Salina D, Enarson P, Rattner JB, Burke B. 2003. Nup358 integrates nuclear envelope breakdown with kinetochore assembly. J Cell Biol 162:991–1001.

Savarino A. 2006. A historical sketch of the discovery and development of HIV-1 integrase inhibitors. Expert Opin Invest Drugs 15:1507–1522.

Scalbert A, Johnson IT, Saltmarsh M. 2005. Polyphenols: Antioxidants and beyond. Am J Clin Nutr 81:215s–217s.

Sche PP, McKenzie KM, White JD, Austin DJ. 1999. Display cloning: Functional identification of natural product receptors using cDNA-phage display. Chem Biol 6:707–716.

Sche PP, McKenzie KM, White JD, Austin DJ. 2001. Display cloning: Functional identification of natural product receptors using cDNA-phage display (vol 6, pg 707, 1999). Chem Biol 8:399–400.

Schermann SM, Simmons DA, Konermann L. 2005. Mass spectrometry-based approaches to protein-ligand interactions. Expert Rev Proteomics 2:475–485.

Schmidt F, Dahlmann B, Janek K, Kloss A, Wacker M, Ackermann R, Thiede B, Jungblut PR. 2006. Comprehensive quantitative proteome analysis of 20S proteasome subtypes from rat liver by isotope coded affinity tag and 2-D gel-based approaches. Proteomics 6:4622–4632.

Schmidt F, Donahoe S, Hagens K, Mattow J, Schaible UE, Kaufmann SH, Aebersold R, Jungblut PR. 2004. Complementary analysis of the Mycobacterium tuberculosis proteome by two-dimensional electrophoresis and isotope-coded affinity tag technology. Mol Cell Proteomics 3:24–42.

Schnier JB, Kaur G, Kaiser A, Stinson SF, Sausville EA, Gardner J, Nishi K, Bradbury EM, Senderowicz AM. 1999. Identification of cytosolic aldehyde dehydrogenase 1 from non-small cell lung carcinomas as a flavopiridol-binding protein. FEBS Lett 454:100–104.

Schnolzer M, Jedrzejewski P, Lehmann WD. 1996. Protease-catalyzed incorporation of O-18 into peptide fragments and its application for protein sequencing by electrospray and matrix-assisted laser desorption/ionization mass spectrometry. Electrophoresis 17:945–953.

Schulenberg B, Arnold B, Patton WF. 2003. An improved mechanically durable electrophoresis gel matrix that is fully compatible with fluorescence-based protein detection technologies. Proteomics 3: 1196–1205.

Seitz S, Boelsterli UA. 1998. Diclofenac acyl glucuronide, a major biliary metabolite, is directly involved in small intestinal injury in rats. Gastroenterology 115:1476–1482.

Sender U, Bandow J, Engelmann S, Lindequist U, Hecker M. 2004. Proteomic signatures for daunomycin and adriamycin in Bacillus subtilis. Pharmazie 59:65–70.

Senderowicz AM. 2003. Novel direct and indirect cyclin-dependent kinase modulators for the prevention and treatment of human neoplasms. Cancer Chemother Pharmacol 52:S61–S73.

Shacter E, Williams JA, Lim M, Levine RL. 1994. Differential susceptibility of plasma-proteins to oxidative modification—Examination by Western-blot immunoassay. Free Rad Biol Med 17:429–437.

Shields SJ, Oyeyemi O, Lightstone FC, Balhorn R. 2003. Mass spectrometry and non-covalent protein-ligand complexes: Confirmation of binding sites and changes in tertiary structure. J Am Soc Mass Spectrom 14: 460–470.

Shiio Y, Aebersold R. 2006. Quantitative proteome analysis using isotope-coded affinity tags and mass spectrometry. Nat Protoc 1:139–145.

Shim JS, Lee J, Park HJ, Park SJ, Kwon HJ. 2004. A new curcumin derivative, HBC, interferes with the cell cycle progression of colon cancer cells via antagonization of the Ca2+/calmodulin function. Chem Biol 11:1455–1463.

Shimmura H, Tanabe K, Habiro K, Abe R, Toma H. 2006. Combination effect of mycophenolate mofetil with mizoribine on cell proliferation assays and in a mouse heart transplantation model. Transplantation 82:175–179.

Shipkova M, Armstrong VW, Oellerich M, Wieland E. 2003. Acyl glucuronide drug metabolites: Toxicological and analytical implications. Ther Drug Monit 25:1–16.

Shipkova M, Beck H, Voland A, Armstrong VW, Grone HJ, Oellerich M, Wieland E. 2004. Identification of protein targets for mycophenolic acid acyl glucuronide in rat liver and colon tissue. Proteomics 4:2728–2738.

Short DM, Heron ID, Birse-Archbold JL, Kerr LE, Sharkey J, McCulloch J. 2007. Apoptosis induced by staurosporine alters chaperone and endoplasmic reticulum proteins: Identification by quantitative proteomics. Proteomics 7:3085–3096.

Sin N, Meng LH, Wang MQW, Wen JJ, Bornmann WG, Crews CM. 1997. The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAP-2. Proc Natl Acad Sci USA 94:6099–6103.

Sinz A. 2007. Investigation of protein-ligand interactions by mass spectrometry. Chemmedchem 2:425–431.

Smith DL, Deng YZ, Zhang ZQ. 1997. Probing the non-covalent structure of proteins by amide hydrogen exchange and mass spectrometry. J Mass Spectrom 32:135–146.

Smith JC, Duchesne MA, Tozzi P, Ethier M, Figeys D. 2007. A differential phosphoproteomic analysis of retinoic acid-treated p19 cells. J Proteome Res 6:3174–3186.

Sugiyama K, Satoh H, Saito K, Takahashi K, Saito N, Hirano T. 2005. Immunosuppressive efficacy of mycophenolate mofetil when compared with azathioprine and mizoribine against peripheral lymphocytes from renal transplant recipients. Transpl Int 18:590–595.

Sun XS, Chiu JF, He QY. 2005. Application of immobilized metal affinity chromatography in proteomics. Expert Rev Proteomics 2:649–657.

Svasti J, Srisomsap C, Subhasitanont P, Keeratichamroen S, Chokchaichamnankit D, Ngiwsara L, Chimnoi N, Pisutjaroenpong S, Techasakul S, Chen ST. 2005. Proteomic profiling of cholangiocarcinoma cell line treated with pomiferin from Derris malaccensis. Proteomics 5:4504–4509.

Tachibana H. 2008. Green tea polyphenol EGCG signaling pathway through 67 kDa laminin receptor. J Pharmacol Sci 106:25p–25S.

Tachibana H, Koga K, Fujimura Y, Yamada K. 2004. A receptor for green tea polyphenol EGCG. Nat Struct Mol Biol 11:380–381.

Tachibana K, Scheuer PJ, Tsukitani Y, Kikuchi H, Vanengen D, Clardy J, Gopichand Y, Schmitz FJ. 1981. Okadaic acid, a cyto-toxic polyether from 2 marine sponges of the genus halichondria. J Am Chem Soc 103:2469–2471.

Takakusagi Y, Takakusagi K, Kuramochi K, Kobayashi S, Sugawara F, Sakaguchi K. 2007. Identification of C10 biotinylated camptothecin (CPT-10-B) binding peptides using T7 phage display screen on a QCM device. Bioorg Med Chem 15:7590–7598.

Tan S, Seow TK, Liang RCMY, Koh S, Lee CPC, Chung MCM, Hooi SC. 2002. Proteome analysis of butyrate-treated human colon cancer cells (HT-29). Int J Cancer 98:523–531.

Tan YL, Goh D, Ong ES. 2006. Investigation of differentially expressed proteins due to the inhibitory effects of berberine in human liver cancer cell line HepG2. Mol Biosyst 2:250–258.

Tang L, Hopper ED, Tong Y, Sadowsky JD, Peterson KJ, Gellman SH, Fitzgerald MC. 2007. H/D exchange- and mass spectrometry-based strategy for the thermodynamic analysis of protein-ligand binding. Anal Chem 79:5869–5877.

Taunton J, Hassig CA, Schreiber SL. 1996. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science 272:408–411.

Teffera Y, Bakhtiar R. 2005. Confirmation of the binding site of cyclophilin-cyclosporin non-covalent complex using limited proteolysis and liquid chromatography-quadrupole-time-of-flight mass spectrometry. J Liquid Chromatogr Relat Technol 28:1497–1508.

Towbin H, Bair KW, DeCaprio JA, Eck MJ, Kim S, Kinder FR, Morollo A, Mueller DR, Schindler P, Song HK, van Oostrum J, Versace RW, Voshol H, Wood J, Zabludoff S, Phillips PE. 2003. Proteomics-based target identification—Bengamides as a new class of methionine aminopeptidase inhibitors. J Biol Chem 278:52964–52971.

Traynelis VC, Ryken TC, Cornelius AS. 1995. Cytotoxicity of cis-parinaric acid in cultured malignant gliomas. Neurosurgery 37:484–489.

Tsuchiya A, Tashiro E, Yoshida M, Imoto M. 2005. Involvement of nuclear accumulation of heat shock protein 27 in leptomycin B-induced apoptosis in HeLa cells. J Antibiot (Tokyo) 58:810–816.

Ueda K, Usui T, Nakayama H, Ueki M, Takio K, Ubukata M, Osada H. 2002. 4-isoavenaciolide covalently binds and inhibits VHR, a dual-specificity phosphatase. FEBS Lett 525:48–52.

Uga H, Kuramori C, Ohta A, Tsuboi Y, Tanaka H, Hatakeyama M, Yamaguchi Y, Takahashi T, Kizaki M, Handa H. 2006. A new mechanism of methotrexate action revealed by target screening with affinity beads. Mol Pharmacol 70:1832–1839.

Unlu M, Morgan ME, Minden JS. 1997. Difference gel electrophoresis: A single gel method for detecting changes in protein extracts. Electrophoresis 18:2071–2077.

Vila-Carriles WH, Zhao GL, Bryan J. 2007. Defining a binding pocket for sulfonylureas in ATP-sensitive potassium channels. FASEB J 21:18–25.

Viswanathan S, Unlu M, Minden JS. 2006. Two-dimensional difference gel electrophoresis. Nat Protoc 1:1351–1358.

von Rechenberg M, Blake BK, Ho YS, Zhen Y, Chepanoske CL, Richardson BE, Xu N, Kery V. 2005. Ampicillin/penicillin-binding protein interactions as a model drug-target system to optimize affinity pull-down and mass spectrometric strategies for target and pathway identification. Proteomics 5:1764–1773.

Wales TE, Engen JR. 2006. Hydrogen exchange mass spectrometry for the analysis of protein dynamics. Mass Spectrom Rev 25:158–170.

Walsh DP, Chang YT. 2006. Chemical genetics. Chem Rev 106:2476–2530.

Wan YQ, Hur WY, Cho CY, Liu Y, Adrian FJ, Lozach O, Bach S, Mayer T, Fabbro D, Meijer L, Gray NS. 2004. Synthesis and target identification of hymenialdisine analogs. Chem Biol 11:247–259.

Wang QW, Allen JC, Swaisgood HE. 1997a. Binding of retinoids to beta-lactoglobulin isolated by bioselective adsorption. J Dairy Sci 80:1047–1053.

Wang QW, Allen JC, Swaisgood HE. 1997b. Binding of vitamin D and cholesterol to beta-lactoglobulin. J Dairy Sci 80:1054–1059.

Wang Y, Cheung YH, Yang Z, Chiu JF, Che CM, He QY. 2006. Proteomic approach to study the cytotoxicity of dioscin (saponin). Proteomics 6:2422–2432.

Wang Y, Che CM, Chiu JF, He QY. 2007a. Dioscin (saponin)-induced generation of reactive oxygen species through mitochondria dysfunction: A proteomic-based study. J Proteome Res 6:4703–4710.

Wang Y, He QY, Chen H, Chiu JF. 2007b. Synergistic effects of retinoic acid and tamoxifen on human breast cancer cells: Proteomic characterization. Exp Cell Res 313:357–368.

Wang Z, Zhou JB, Ju Y, Zhang HJ, Liu MJ, Li X. 2001. Effects of two saponins extracted from the Polygonatum Zanlanscianense Pamp on the human leukemia (HL-60) cells. Biol Pharm Bull 24:159–162.

Wang ZR, Hsieh TC, Zhang ZT, Ma YL, Wu JM. 2004. Identification and purification of resveratrol targeting proteins using immobilized resveratrol affinity chromatography. Biochem Biophys Res Commun 323:743–749.

Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT. 1971. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. J Am Chem Soc 93:2325–2327.

Weber C, Wider G, Vonfreyberg B, Traber R, Braun W, Widmer H, Wuthrich K. 1991. The Nmr structure of cyclosporine—A bound to cyclophilin in aqueous-solution. Biochemistry 30:6563–6574.

Wen B, Doneanu CE, Gartner CA, Roberts AG, Atkins WM, Nelson SD. 2005. Fluorescent photoaffinity labeling of cytochrome P450 3A4 by lapachenole: Identification of modification sites by mass spectrometry. Biochemistry 44:1833–1845.

Wenzel U, Herzog A, Kuntz S, Daniel H. 2004. Protein expression profiling identifies molecular targets of quercetin as a major dietary flavonoid in human colon cancer cells. Proteomics 4:2160–2174.

White FM. 2007. On the iTRAQ of kinase inhibitors. Nat Biotechnol 25:994–996.

Williams PA, Cosme J, Vinkovic DM, Ward A, Angove HC, Day PJ, Vonrhein C, Tickle IJ, Jhoti H. 2004. Crystal structures of human cytochrome P450 3A4 bound to metyrapone and progesterone. Science 305:683–686.

Winston RL, Fitzgerald MC. 1997. Mass spectrometry as a readout of protein structure and function. Mass Spectrom Rev 16:165–179.

Wong CC, Cheng KW, He QY, Chen F. 2008a. Unraveling the molecular targets of natural products: Insights from genomic and proteomic analyses. Proteomics Clin Appl 2:338–354.

Wong CC, Wang Y, Cheng KW, Chiu JF, He QY, Chen F. 2008b. Comparative proteomic analysis of indioside D-triggered cell death in HeLa cells. J Proteome Res 7:2050–2058.

Wu N, Zhang W, Yang Y, Liang YL, Wang LY, Jin JW, Cai XM, Zha XL. 2006. Profilin 1 obtained by proteomic analysis in all-trans retinoic acid-treated hepatocarcinoma cell lines is involved in inhibition of cell proliferation and migration. Proteomics 6:6095–6106.

Wu Q, Bounaud PY, Kuduk SD, Yang CPH, Ojima I, Horwitz SB, Orr GA. 1998. Identification of the domains of photoincorporation of the 3′- and 7-benzophenone analogues of taxol in the carboxyl-terminal half of murine mdr1b P-glycoprotein. Biochemistry 37:11272–11279.

Wu TF, Hsu CY, Huang HS, Chou SP, Wu H. 2007. Proteomic analysis of pycnogenol effects in RAW 264.7 macrophage reveals induction of cathepsin D expression and enhancement of phagocytosis. J Agric Food Chem 55:9784–9791.

Wu Y, Mou Z, Li J, Zhou W, Wei B, Zou L. 2004a. Identification of a S100 calcium-binding protein expressed in HL-60 cells treated with all-trans retinoic acid by two-dimensional electrophoresis and mass spectrometry. Leuk Res 28:203–207.

Wu Z, Hasan A, Liu T, Teller DC, Crabb JW. 2004b. Identification of CRALBP ligand interactions by photoaffinity labeling, hydrogen/deuterium exchange, and structural modeling. J Biol Chem 279:27357–27364.

Xiao H, Kaltashov IA. 2005. Transient structural disorder as a facilitator of protein-ligand binding: Native H/D exchange-mass spectrometry study of cellular retinoic acid binding protein I. J Am Soc Mass Spectrom 16:869–879.

Xiao H, Kaltashov IA, Eyles SJ. 2003. Indirect assessment of small hydrophobic ligand binding to a model protein using a combination of ESI MS and HDX/ESI MS. J Am Soc Mass Spectrom 14:506–515.

Xing CG, LaPorte JR, Barbay JK, Myers AG. 2004. Identification of GAPDH as a protein target of the saframycin antiproliferative agents. Proc Natl Acad Sci USA 101:5862–5866.

Yamaoka M, Sato K, Kobayashi M, Nishio N, Ohkubo M, Fujii T, Nakajima H. 2005. FR177391, a new anti-hyperlipidemic agent from Serratia IV. Target identification and validation by chemical genetic approaches. J Antibiot 58:654–662.

Yan X, Broderick D, Leid ME, Schimerlik MI, Deinzer ML. 2004a. Dynamics and ligand-induced solvent accessibility changes in human retinoid X receptor homodimer determined by hydrogen deuterium exchange and mass spectrometry. Biochemistry 43:909–917.

Yan XG, Watson J, Ho PS, Deinzer ML. 2004b. Mass spectrometric approaches using electrospray ionization charge states and hydrogen-deuterium exchange for determining protein structures and their conformational changes. Mol Cell Proteomics 3:10–23.

Yasumoto T, Seino N, Murakami Y, Murata M. 1987. Toxins produced by benthic dinoflagellates. Biol Bull 172:128–131.

Yeh JR, Ju R, Brdlik CM, Zhang W, Zhang Y, Matyskiela ME, Shotwell JD, Crews CM. 2006. Targeted gene disruption of methionine aminopeptidase 2 results in an embryonic gastrulation defect and endothelial cell growth arrest. Proc Natl Acad Sci USA 103:10379–10384.

Yu DL, Suzuki M, Xie L, Morris-Natschke SL, Lee KH. 2003. Recent progress in the development of coumarin derivatives as potent anti-HIV agents. Med Res Rev 23:322–345.

Yu LR, Conrads TP, Uo T, Issaq HJ, Morrison RS, Veenstra TD. 2004. Evaluation of the acid-cleavable isotope-coded affinity tag reagents: Application to camptothecin-treated cortical neurons. J Proteome Res 3:469–477.

Yu LR, Johnson MD, Conrads TP, Morrison RS, Veenstra TD. 2002a. Proteome analysis of camptothecin-treated cortical neurons using isotope-coded affinity tags (vol 23, pg 1591, 2002). Electrophoresis 23:3252–3252.

Yu LR, Johnson MD, Conrads TP, Smith RD, Morrison RS, Veenstra TD. 2002b. Proteome analysis of camptothecin-treated cortical neurons using isotope-coded affinity tags. Electrophoresis 23:1591–1598.

Zhang D, Tai YC, Wong CH, Tai LK, Koay ES, Chen CS. 2007. Molecular response of leukemia HL-60 cells to genistein treatment, a proteomics study. Leuk Res 31:75–82.

Zhang DD, Hannink M. 2003. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. Mol Cell Biol 23:8137–8151.

Zhang RJ, Sioma CS, Wang SH, Regnier FE. 2001. Fractionation of isotopically labeled peptides in quantitative proteomics. Anal Chem 73:5142–5149.

Zhang X, Kuramitsu Y, Fujimoto M, Hayashi E, Yuan X, Nakamura K. 2006. Proteomic analysis of macrophages stimulated by lipopolysaccharide: Lipopolysaccharide inhibits the cleavage of nucleophosmin. Electrophoresis 27:1659–1668.

Zhang ZQ, Smith DL. 1993. Determination of amide hydrogen-exchange by mass-spectrometry—A new tool for protein-structure elucidation. Protein Sci 2:522–531.

Zheng PZ, Wang KK, Zhang QY, Huang QH, Du YZ, Zhang QH, Xiao DK, Shen SH, Imbeaud S, Eveno E, Zhao CJ, Chen YL, Fan HY, Waxman S, Auffray C, Jin G, Chen SJ, Chen Z, Zhang J. 2005. Systems analysis of transcriptome and proteome in retinoic acid/arsenic trioxide-induced cell differentiation/apoptosis of promyelocytic leukemia. Proc Natl Acad Sci USA 102:7653–7658.