Chapter 9

Proteomics

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INTRODUCTION

The pharmaceutical industry has witnessed a steadily declining R&D efficiency resulting in fewer drugs reaching the market despite increased investment. A major cause for this low efficiency is the failure of drug candidates in late-stage development owing to safety issues or previously undiscovered side effects. Omics technologies provide some insights in predicting the risk of drug failure. Gene expression profiles offer clues in understanding the observed biological effects of drugs across disease areas, therapeutic targets and chemical scaffolds, and, in turn, de-risk drug development in early phases. Gene expression profiling can detect adverse effects of compounds, and is a valuable tool in early-stage drug discovery decision making (Verbist et al., 2015). Gene expression data has successfully been used to support go/no-go decisions for selected drug discovery projects within a global pharmaceutical company.

Recent advances in omics sciences, such as genomics, transcriptomics, proteomics, and metabolomics, have been used to provide alternate perspectives on quality, safety, and in understanding the mode of action (MOA) of drugs at systems level (Joshi et al., 2010; Mohd Fauzi et al., 2013). This central dogma of molecular biology forms the basis of omics trilogy (genomics, transcriptomics, and proteomics) with the addition of metabolomics and epigenomics (Joshi et al., 2012). Proteomics includes identification and quantification of proteins and also their localization, modifications, interactions, activities, and, ultimately, defining their function. Unlike DNA, proteins undergo complex biochemical modifications at the cotranslational or posttranslational level. A single gene can encode multiple proteins by means of alternative splicing of the messenger RNA or by multiples genes that encode for a single protein. Such possibilities result in a proteome that is more complex than the genome.

Innovative Approaches in Drug Discovery. DOI: http://dx.doi.org/10.1016/B978-0-12-801814-9.00009-X

RECENT ADVANCES IN PROTEOMICS TECHNOLOGIES

Proteomics, through techniques including isotope-coded affinity tags, stable isotopic labeling by amino acids in cell cultures, isobaric tags for relative and absolute quantification, multidirectional protein-identification technology, activity-based probes, protein/peptide arrays, phage displays, and two-hybrid systems are used in multiple areas throughout the drug development pipeline including target and lead identification, compound optimization, and clinical trials.

Currently, two approaches based on mass spectrometry are the most frequently used for global quantitative protein profiling: (1) two-dimensional electrophoresis (2-DE) followed by staining, selection, and identification by mass spectrometry and (2) isotope tags to label proteins, separation by multidimensional liquid chromatography, and mass spectrometry analysis (Cho, 2007).

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis (2-DE) is a key tool for comparative proteomics research. In 2-DE, mixtures of proteins are separated by charge (isoelectric point, pI) in the first dimension and further separated by mass in the second dimension on 2-D gels. Coupling 2-DE with immobilized pH gradients, IPG-Dalt, has provided higher resolution, improved reproducibility, and higher loading capacity for preparative purposes (O'Farrell, 1975). The 2-DE can achieve the separation of several thousand different proteins in one gel. Stains such as Coomassie Brilliant Blue, silver, SYPRO Ruby, and Deep Purple can be employed to visualize the proteins (Nilsson et al., 2000). Unfortunately, 2-DE technique is a time-consuming and labor-intensive process. Conventional 2-DE is restricted to the detection of denatured proteins in the size range of 10 ~ 200 kDa at pH 3.5 ~ 11.5. Traditionally, vertical and horizontal streaking of proteins can obscure analysis, and membrane proteins are usually under-represented due to extraction and insolubility problems. Furthermore, 2-DE is ineffective at distinguishing low-abundant proteins and small molecular weight proteins ($<$10 kDa). In recent years, some modified 2-DE platforms have been developed to detect nondenatured proteins in extreme size and pI. Moreover, significant improvements have been made in 2-DE technology with the development of two-dimensional fluorescence difference gel electrophoresis, which can be used to reduce gel-to-gel variations. Proteins are first labeled with one of three spectrally resolvable fluorescent cyanine dyes before being separated over the first and second dimensions according to their charge and size, respectively. It builds on 2-DE by adding a highly accurate quantitative dimension, which enables multiple protein extracts to be separated on the same 2-D gel. When used in conjunction with automated analysis packages, this multiplexing approach can accurately and reproducibly quantify protein expression for control and

experimental groups. Differentially expressed proteins can be subsequently identified by mass spectrometric methods (Marouga et al., 2005).

ELECTROSPRAY IONIZATION

Electrospray ionization (ESI) involves the release of ions achieved by spraying the sample using an electrical field so that charged droplets are formed. As the solvent gradually evaporates from these droplets, freely hovering stark-naked protein molecules remain. Because the molecules take on strong positive charges, the mass/charge ratio becomes small enough to allow the substances to be analyzed in ordinary mass spectrometers. Another advantage is that the same molecule causes a series of peaks since each can take up a varying number of charges, which gives information that makes identification easier. In recent years, a novel linear ion trap (LIT) mass spectrometer with ESI and matrix-assisted laser desorption/ionization (MALDI) has been built in the MALDI-LIT-ESI configuration. The design features two independent ion source/ion optical channels connected to opposite ends of a single mass analyzer (Smith et al., 2007).

MATRIX-ASSISTED LASER DESORPTION/IONIZATION

Ionization by MALDI involves a laser pulse striking the sample which, unlike in the spray method, is in a solid or viscous phase. When the sample takes up the energy from the 914 W.C.-S. CHO laser pulse, it is blasted into small bits. The molecules let go of one another, released as intact hovering ions with a low charge, which are then accelerated by an electrical field and detected as described above by recording their time-of-flight (TOF). The technology is able to analyze proteins down to attomole quantities. It can tolerate small amounts of contaminants. The information obtained from MALDI analysis can be automatically submitted to a database search for further examination. Currently, efforts are underway for the direct analysis and MALDI imaging of formalin-fixed, paraffin-embedded tissue sections using the strategy based on in situ enzymatic digestion of the tissue section after paraffin removal. This approach provides access to massive amounts of archived samples in the clinical pathology setting (Lemaire et al., 2007).

SURFACE-ENHANCED LASER DESORPTION/IONIZATION

The surface-enhanced laser desorption/ionization (SELDI)-TOF MS is a technological breakthrough combining chromatographic active surfaces with an interface chip for MALDI. Using as little as one microliter per sample, a high-resolution mass spectrum following a complete chromatographic separation can be performed. The development of SELDI technology holds much promise for future protein analysis. It can be used for protein purification, expression

profiling, or protein interaction profiling. There are many types of substances bound to the protein arrays, including antibodies, receptors, ligands, nucleic acids, carbohydrates, or chromatographic surfaces (e.g., cationic, anionic, hydrophobic, or hydrophilic). Some surfaces have a broad specificity that binds the whole classes of proteins, while others are highly specific in which only a few proteins from a complex sample are bound. After the capture step, the array is washed to reduce nonspecific binding. When subjected to short bursts of a laser beam, the retained proteins are uncoupled from the array surface and analyzed by laser desorption/ionization TOF MS.

Some protein arrays contain antibodies covalently immobilized onto the array surface that capture corresponding antigens from a complex mixture. Many analyses can be followed, e.g., analysis of proteolytic digests of the proteins bound to the array can disclose the antigenic determinant, other proteins of interest can be immobilized on the array, bound receptors can reveal ligands, and binding domains for protein−protein interactions can be detected. The proteins must often remain folded in the correct conformation during the preparation and incubation with the array for protein−protein interactions to occur (Cho, 2006).

PROTEIN MICROARRAY TECHNOLOGY

The microarray format provides a robust and convenient platform for the simultaneous analysis of thousands of individual protein samples, facilitating the design of sophisticated and reproducible biochemical experiments under highly specific conditions (Gouriet et al., 2008). Protein chips similar to DNA chips are likely be the next major evolution in proteomics and offer another solution for high-throughput proteomic analyses and detection of low-abundant proteins (Zhu et al., 2006). Protein chips can only provide data on a set of proteins selected by the investigator (Jones et al., 2006). These protein chips are used to study the biochemical activities of an entire proteome in a single experiment. They are used to study numerous protein interactions, such as protein−protein, protein−DNA, protein−RNA, protein−phospholipid, and protein−small molecule interactions (Zhu et al., 2001).

Antibody arrays are proteins captured on an antibody microarray substrates such as nylon membranes, plastic microwells, planar glass slides, gel-based arrays, and beads are detected by a cocktail of detection antibodies. High-throughput multiplex antibody arrays are immunoassays that quantitatively measure hundreds of known proteins in complex biological matrices for diagnostic discovery and biomarker-assisted drug development.

APPLICATIONS OF PROTEOMICS IN DRUG DISCOVERY

Proteomic technologies have advanced various areas of drug discovery and development through the comparative assessment of normal and diseased-state

tissues, transcription and/or expression profiling, side effect profiling, pharmacogenomics, and the identification of biomarkers. The majority of small molecule drugs and biologics act on protein targets. These proteins do not act in isolation but are embedded in cellular pathways and networks and are thus tightly interconnected with many other proteins and subcellular components. Given this complexity, it seems natural to apply proteomics in the drug discovery process. Target-based approaches start with the selection of a protein target based on its presumed or validated role in the relevant disease. Biochemical or biophysical assays, typically using purified protein, are developed to monitor modulation of target activity and to identify hits in high-throughput screens using large libraries of small molecules. After a hit validation, lead compounds are selected and further optimized with regard to potency, selectivity, pharmacodynamics, and pharmacokinetic properties, and are then tested for in vivo efficacy in the respective-disease model (Schirle et al., 2012). Proteomics technologies have successfully been used in biomarker discovery, target identification and validation, lead optimization, and MOA to toxicity prediction (Fig. 9.1).

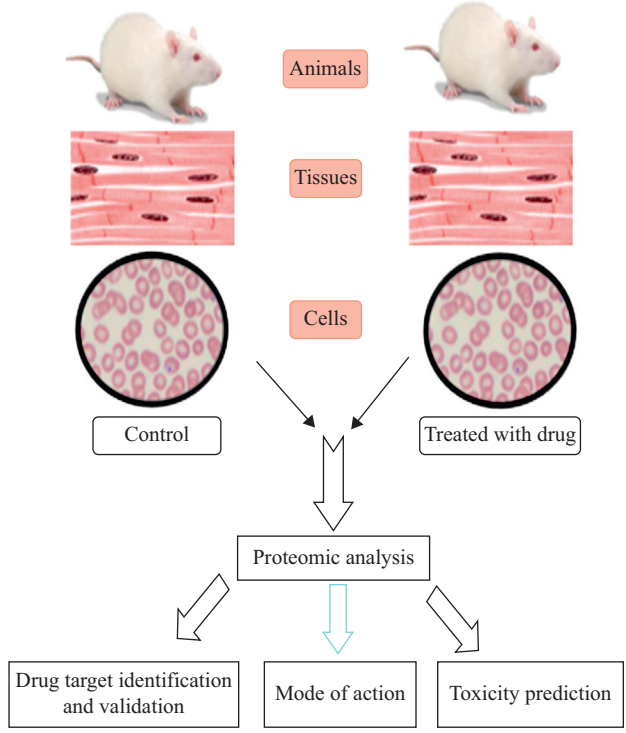

FIGURE 9.1 Protemics workflow in drug discovery.

BIOMARKER DISCOVERY AND IDENTIFICATION OF POTENTIAL THERAPEUTIC TARGETS

According to the US Food and Drug Administration (USFDA), a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic or pathogenic processes or pharmacological responses to a therapeutic intervention. It may also be defined as an in vivo derived molecule present at levels deviating significantly from the average in association with specific conditions of health (Atkinson et al., 2001; Zhang et al., 2007).

Some Key Examples

Increased levels of liver transaminases in blood indicates destruction of liver cells, the prostate specific antigen (PSA) for prostate cancer (Wang et al., 1981), or the troponin I and T for acute myocardial infarction (Mair et al., 1992; Antman et al., 1996). However, such markers exist in limited numbers and in quite low concentrations, and it can be hoped that a wide search for tissue-leakage markers should provide some new and interesting candidates (Amacher, 1998; Hsich et al., 1996; Ahmed et al., 2004). Proteomics has facilitated the cataloging of protein profiles in different tissues and biological fluids (Honda et al., 2013); however, identification of clinical biomarkers remains one of the most challenging applications (Altelaar et al., 2013). Current biomarkers or biomarker candidates struggle with limited reliability and proper validation as well as with limited sensitivity and specificity (Barbosa et al., 2012). This limitation is mainly due to the dynamic nature of proteome where proteins are continually undergoing changes, e.g., binding to the cell membrane, partnering with other proteins to form complexes, or undergoing synthesis and degradation.

In cancer, proteomics enabled the identification of several promising candidates from tissue, blood, cerebrospinal fluid, cell lines, or even animal models using 2-DE and MS, SELDI-TOF, protein microarrays, LC-MS/MS, ELISA, and so forth (Hudler et al., 2014). Signature proteins like PSA and CA-125, are the current best markers for prostate and ovarian cancer, respectively (Velonas et al., 2013; Mai et al., 2011).

Proteome analysis of normal human vitreous humor using high-resolution Fourier transform mass spectrometry should facilitate biomedical research into pathological conditions of the eye including diabetic retinopathy, retinal detachment, and cataract (Murthy et al., 2014). A quantitative proteomic profiling of synovial fluid obtained from rheumatoid arthritis (RA) and osteoarthritis (OA), using iTRAQ labeling followed by high-resolution mass spectrometry analysis, a total of 575 proteins were identified out of which 135 proteins were found to be differentially expressed by ≥ 3-fold in RA and OA synovial fluid.

Proteins not previously reported to be associated with RA including, coronin-1A (CORO1A), fibrinogen like-2 (FGL2), and macrophage-capping protein (CAPG) were found to be upregulated in RA. Proteins such as CD5 molecule-like protein (CD5L), soluble scavenger receptor cysteine-rich domain-containing protein (SSC5D), and TTK protein kinase (TTK) were found to be upregulated in the synovial fluid of osteoarthritis patients. Pathway analysis of differentially expressed proteins revealed a significant enrichment of genes involved in glycolytic pathway in RA which, in turn, might aid in early diagnosis and prognosis as well as in the evaluation of the disease progression of RA and OA. These novel proteins need to be explored further for their role in the disease pathogenesis of RA and osteoarthritis (Balakrishnan et al., 2014).

Despite the substantial advances in our understanding of the molecular basis of disease, there is a paucity of approved biomarkers. Protein biomarkers in biological fluids in particular have the potential to inform regarding risk of disease or to allow early detection for more effective treatment. There is an equally appalling lack of other types of biomarkers, whether for disease classification for individualized therapy or for other applications. The challenge for the next decade is to implement road maps that fast track the development of biomarkers, whether protein, nucleic acid, or metabolite-based, to reach the clinic in an efficient manner (Hanash, 2011).

Drug targets are proteins or signal-transduction pathways in which proteins are involved. Therapeutic relevancy of the chosen target must be proven first prior to initiating other processes in drug discovery. Tyrosine kinase receptor (PDGFR, VEGFR2, FGFR1), Aurora kinases and TANK-binding kinase-1 are identified as targets for tumor vascularization. Aldehyde dehydrogenase-1 and quinine reductase-2 in malaria, RICK (Rip-like interacting kinase), CLARP (caspase-like apoptosis-regulatory protein kinase), GAK (cyclin-G associated kinase), and CK1α are some of the targets used for drug discovery (Kopec et al., 2005; Katayama and Oda, 2007). A new drug molecule is being searched against the chosen target that usually involves high-throughput screening, wherein large libraries of chemicals are tested to determine their ability to modify the target.

UNDERSTANDING DISEASE MECHANISMS—MOA (MODE OF ACTION)

Comparative proteomics or protein−protein interaction studies can be used to elucidate mechanisms of action by which a drug can modulate target activity. Differential protein expressions observed with and without a compound treatment, either on cells or tissues or animals, allow the identification of compound-sensitive proteins and their interactions (Fig. 9.2). Proteomic analysis of SAHA treated and untreated cancer cells was used for target identification. Using tagged subunits of the SIN3A HDAC complex, Smith

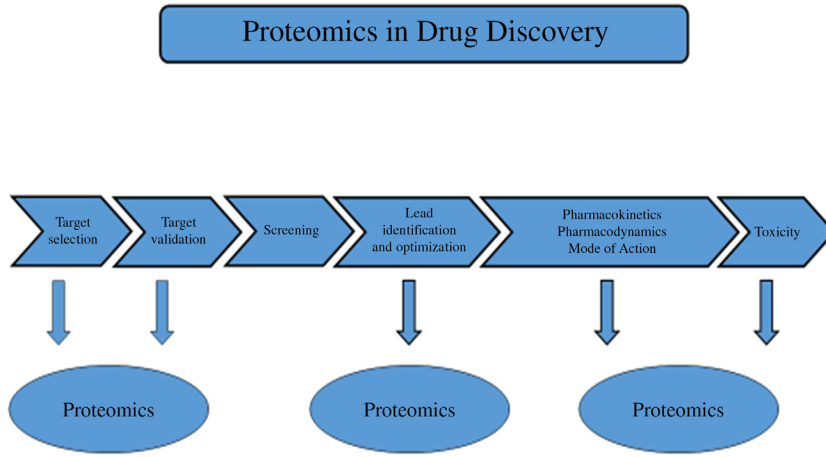

FIGURE 9.2 Proteomics in drug discovery pipeline.

and colleagues found that SAHA, the first FDA-approved HDAC inhibitor for the treatment of cancer, causes dissociation of the ING2 subunit from this complex. Absence of ING2 leads to loss of binding of the SIN3A complex to the p21 promoter and, thus, directly contributes to the growth inhibitory effect of SAHA (Smith et al., 2010).

Another example is the identification of the interaction of tagged cAMP-dependent protein kinase (PKA) subunit Cb1 and CAP1, which was shown to be sensitive to an ATP-competitive PKA inhibitor (Erlbruch et al., 2010). Developing large-scale protein interaction maps of complete disease-related signaling pathways could also be challenging but useful in the identification of druggable targets. Protein−protein interaction mapping of around 32 members of the pro-inflammatory TNF-a-induced NKkB pathway was done, resulting in 80 novel protein interactions; 10 of these proteins were confirmed to have a modulatory role in TNF-a signaling (Bouwmeester et al., 2004). Selective binding of the HSP90 inhibitor PU-H71 to cancer-specific oncoprotein-HSP90 complexes was identified using an immobilized PU-H71 matrix (Moulick et al., 2011). Differential phosphoproteomic analyses using selective small molecule inhibitors of particular kinases such as MAPK inhibitors U0126 and SB202190, the clinical BCR-ABL inhibitor Dasatinib, and inhibitors of Aurora and Polo-like kinases have been used to identify substrates in human cell lines and to characterize the effect of inhibition on signaling events (Pan et al., 2009; Kettenbach et al., 2011).

Quantitative proteomics has been used successfully in many cases to study the effect of small molecule inhibitors by monitoring protein acetylation and methylation. Quantitative effects of the HDAC inhibitor trichostatin A was studied in a similar manner on the histone modification state in a murine model of systemic lupus erythematosus (Garcia et al., 2005). Lee and

colleagues used label-free mass spectrometry to quantify the effect of HDAC inhibitors of varying degrees of selectivity on histone acetylation (Lee et al., 2008). Proteomic approaches have been used to study the effect of inhibition of the histone demethylase JMJD2A by pyridine-2,4-dicarboxylic acid derivatives (MacKeen et al., 2010) as well as the histone methyltransferases G9A and GLP by the small molecule inhibitor UNC0638 (Vedadi et al., 2011). The potential value of proteomics in drug development, especially in elucidating MOA, has been demonstrated in many successful examples. Proteomic approaches have been recognized as promising techniques that can facilitate the systematic characterization of a drug action and side effect prediction, thereby helping to reduce the typically high attrition rates in discovery projects.

PROTEOMICS IN LEAD OPTIMIZATION

Pharmacokinetics (PK) and pharmacodynamics (PD) are an integral part of drug development. Drug efficacy, drug toxicity, and the therapeutic index of a drug are important criteria for lead optimization. A panel of in vitro and in vivo assays are applied to shortlist compounds post discovery. Proteomics technologies have potential to monitor global protein expressions as a surrogate for the effect of the applied treatment (Kraus et al., 2007). It is also possible to map the pharmacological loss of function of one protein that causes the cell to express increased levels of another protein with a redundant function (Kraus et al., 2007). Global expression proteomics mainly captures the alterations in effect or pairs it with a specific cellular treatment.

Phosphoproteomics is used for selective applications such as assessing the selectivity of compounds in discovery of kinase inhibitors. For protein kinases the conserved ATP-binding site has been used to generate a nonselective protein kinase panel that provide selectivity assessments for up to 150 kinase targets in a single experiment (Schirle et al., 2012). Such selectivity matrices have been successfully used to profile clinical BCR-ABL inhibitors in the chronic myeloid leukemia cell line K562 (Bantscheff et al., 2007), EGFR inhibitors in HeLa cells (Sharma et al., 2009), and a range of investigational and clinical multikinase inhibitors in patient-derived primary chronic lymphocytic leukemia cells (Kruse et al., 2011). Immobilized kinase inhibitors have also been successfully used to identify targets in head and neck cancers by analyzing the kinase complement across 34 squamous cell carcinoma lines established from patients (Wu et al., 2011).

The lead optimization process aims at improving the hit/lead molecules properties for ADME. Lead optimization process is the balancing between multiple properties such as PK/PD without compromising efficacy, although complicated proteomics has been shown to have a potential for ex vivo lead optimization.

PROTEOMICS FOR EVALUATING DRUG TOXICITY

Drug safety is an utmost important consideration during the process of drug discovery and development. A large proportion of failures in drug discovery and development projects are not due to limited efficacy but result from toxicity: many approved drugs are later withdrawn from the market because of issues of toxicity (Scannell et al., 2012). Prediction of toxicity at an early stage of drug development is crucial for the loss due to drugs failure at later stages. Attempts have been made to compare genomics and proteomics of safe drugs with that of drugs under investigation to predict activity and toxicity (Searfoss et al., 2005). Few findings have been published on how gene expression facilitates go/no-go decisions during lead optimization (van der Veen et al., 2013; Magkoufopoulou et al., 2012; Jiang et al., 2007). Drug induced renal toxicity, prediction of genotoxicity, and prediction of skin sensitizing potential are some of the examples of use of transcriptomic technology in lead optimization. Baum et al. investigated off-target effects and were able to prioritize compounds based on transcriptional profiles (Baum et al., 2010). Although transcriptomics data have been shown to support decision making in a number of projects, they also have their limitations. Conceptually, transcriptional profiling is limited in its nature because it cannot detect changes at the metabolite or protein level.

Cancer therapeutic agents such as anthracycline and doxorubicin are reported to have cardiotoxicity in 14% to 49% of patients treated for lymphoma. Novel targeted anticancer agents such as trastuzumab, imatinib, and sunitinib, often induce adverse effects on the heart in a small population of patients. The successful use of anthracyclines like doxorubicin in chemotherapy is limited by their severe cardiotoxicity. Despite decades of clinical application, a satisfying description of the molecular mechanisms involved and a preventive treatment have not yet been achieved. Doxorubicin-induced changes in cell signaling as a novel potential mediator of doxorubicin toxicity was addressed by applying a non-biased screen of the cardiac phosphoproteome. Two-dimensional gel electrophoresis, phosphorspecific staining, quantitative image analysis, and MALDI-TOF/TOF mass spectrometry were combined to identify (de)phosphorylation events occurring in the isolated rat heart upon Langendorff-perfusion with clinically relevant (5 μM) and supraclinical concentrations (25 μM) of doxorubicin. This approach identified 22 proteins with a significantly changed phosphorylation status and these results were validated by immunoblotting for selected phosphosites. Overrepresentation of mitochondrial proteins ($>40\%$) identified this compartment as a prime target of doxorubicin. Identified proteins were mainly involved in energy metabolism (e.g., pyruvate dehydrogenase and acyl-CoA dehydrogenase), sarcomere structure and function (e.g., desmin), or chaperone-like activities (e.g., α-crystallin B chain and prohibitin). Changes in phosphorylation of pyruvate dehydrogenase, regulating pyruvate entry into the Krebs cycle, and

desmin, maintaining myofibrillar array, are relevant for the main symptoms of cardiac dysfunction related to doxorubicin treatment, namely energy imbalance and myofibrillar disorganization (Gratia et al., 2012).

Complex proteomic signature of chronic anthracycline cardiotoxicity was revealed through translational proteomics approach. In addition to mitochondrial proteins, a marked drop in myosin light-chain isoforms, activation of proteolytic machinery (including the proteasome system), increased abundance of chaperones and proteins involved in chaperone-mediated autophagy, membrane repair as well as apoptosis were found. Dramatic changes in proteins of basement membrane and extracellular matrix were documented. In conclusion, and for the first time, this enhances our understanding of the basis for this phenomenon and it may enhance efforts in targeting its reduction (Štěrba et al., 2011).

Serum Proteomic Pattern Diagnostics is a new type of proteomic platform in which patterns of proteomic signatures from high-dimensional mass spectrometry data are used as a diagnostic classifier at the critical initial stages of toxicity (Petricoin et al., 2004).

A drug targets the desired protein, but the induced loss or gain of function exhibits undesirable biological effects limiting the usefulness of the treatment. Well-known examples for this category include drugs targeting the p38 MAP kinase in inflammatory diseases (Hammaker and Firestein, 2009). Toxicoproteomic studies on drug selectivity and MOA can, therefore, often highlight potential toxicity issues early on and, thus, provide a valuable source for identifying lead molecules (Kennedy, 2002). Liver toxicity is a particularly problematic issue and is indeed frequently observed.

Global proteome profiling of human hepatocytes or rodent livers exposed to a drug can be employed to obtain an appreciation of the effects the treatment may impose. Troglitazone, a once-marketed first-generation thiazolidinedione used for the treatment of type-II diabetes mellitus, was withdrawn from the market owing to unacceptable idiosyncratic hepatotoxicity risks even though troglitazone did not cause hepatotoxicity in normal healthy rodents and monkeys in preclinical drug safety assessments and long-term studies.

To understand idiosyncratic hepatotoxicity mechanistically, Lee et al. used MS-based proteomics to characterize mitochondrial protein changes to track the involvement of specific mitochondrial proteins in troglitazone-induced hepatotoxicity in a mouse model (Lee et al., 2013). By combining high-throughput, MS-based, mitoproteome-wide profiling, biochemical endpoints, and network biology, the authors demonstrated that the hepatic mitochondrial proteome followed a two-phase response to a repeated troglitazone administration that culminated in liver injuries by the fourth week.

Meierhofer et al. demonstrated the power of protein set analysis to gain insights into the regulation of cell and tissue homeostasis during a high-fat diet feeding and medication with two antidiabetic compounds (Meierhofer et al., 2013). GSEA allowed for more sensitive detection of low-level but

coordinated protein-expression changes, and the functional modules showed a higher correlation than individual genes/proteins when comparing proteomics and transcriptomics data. Suter et al. characterized the effect of 16 test compounds using conventional toxicological parameters in the integrated EU Framework 6 Project: Predictive toxicology (PredTox) (Suter et al., 2011).

Toxicoproteomics using proteomic pattern technology can have important direct applications within the drug development pipeline as well as potentially powerful bedside applications. We can envision a future in which the specific serum/urine/plasma mass spectral proteomic portraits of a variety of major organ toxicities such as hepatotoxicity, nephro-toxicity, cardiotoxicity, and reprotoxicity, are used to rapidly screen against experimental compounds, either for toxic liability or for protective-intervention efficacy.

MS-based proteomics is maturing into a robust technology for the measurement of proteome-wide exposure effects. The benefits of including proteomic data to understand exposure effects have already been demonstrated in several case studies. Although some challenges still exist to make full use of the richness of proteomic datasets (van Vliet, 2011; Merrick and Witzmann, 2009; Martin et al., 2013), there is overall a great opportunity for proteomics to contribute to an improved understanding of toxicant action, the linkages to accompanying dysfunction and pathology, and the development of predictive biomarkers and signatures of toxicity (Titz et al., 2014).

PROTEOMICS IN ETHNOPHARMACOLOGY RESEARCH

Investigating MOA Botanical Drugs

Traditional botanical medicine preparations have been used for centuries for their health and therapeutic benefits. However, the molecular mechanisms of underlying their efficacies remain largely unclear. Resveratrol (RVT), a polyphenolic compound, has been used extensively for decades as a potential therapy or as a preventive agent for various chronic conditions such as cancer, cardiovascular atherosclerosis, hypertension, and diabetes. The underlying biological processes and molecular pathways by which RVT induces these beneficial effects remains largely undefined. Recently, few studies using proteomics approaches (2-DE combined with MS/MS) have been undertaken to explore the molecular mechanisms of RVT in the amelioration of cancer and endothelial dysfunction in human ovarian cancer cell lines and human umbilical vein endothelial cells, respectively. The cancer cell proteomic analysis found a down regulation of the protein cyclin D1 and the phosphorylation levels of protein kinase B (Akt) and glycogen synthase kinase-3b (GSK-3b) targeting signaling pathways involved in cell proliferation and drug resistance (Vergara et al., 2012). Another study on human umbilical vein endothelial proteomics found the down regulation of elongation factor 2 (EEF2), carboxymethyl-cofilin-1 (cofilin-1), acetyl-eukaryotic translation initiation factor 5A-1 (acetyl-EIF5A) and barrier-to-autointegration factor, and

upregulation of heat shock protein beta-1 (HSP27), phospho-HSP27, phosphostathmin, Nicotinate-nucleotidepyrophosphorylase, and 1, 2-dihydroxy-3-keto-5-methylthiopentene dioxygenase after RVT exposure (Shao et al., 2012). The study also demonstrated that several protein species with posttranscriptional modification (carboxymethyl, acetyl, and phospho) were found to be altered following exposure to RVT. These findings of cancer and endothelial proteome analysis could help in our understanding of the molecular mechanisms underlying the pleiotropic effects of RVT. Quercetin, a flavonoid abundantly present in plants, is widely used as a phytotherapy in prostatitis and prostate cancer. Quercetin has been reported to have a number of therapeutic effects; the cellular target(s) responsible for its anticancer action has not yet been clearly elucidated, but it is understood that anticancer effects of quercetin are mediated, in part, by impairing functions of hnRNPA1, insights that were obtained using a chemical proteomics strategy (Ko et al., 2014).

Withania somnifera, a popular Ayurvedic rasayana botanical (a medicinal plant having immunomodulatory activity) has been used as an immunomodulator and in the management of cancer. Several cellular studies on bioactive compound; withaferin A (WA) from *W. somnifera* has demonstrated that the anticancer potential is due to the modulation of processes such as apoptosis, inflammation, angiogenesis, and cell proliferation either by upregulation or downregulation of numerous proteins (Patil et al., 2013). The underline mechanistic study using proteomics with 2-DE followed by MALDITOF/TOF technologies demonstrated that WA could regulate total of 65 proteins including downregulation of many glycolysis-related proteins such as M2-type pyruvate kinase, phosphoglycerate kinase, and fructose-bisphosphate aldolase A isoform 2 in mammary tumor tissue samples (Hahm et al., 2013). The proteomic analysis suggested that the possible MOA WA-mediated mammary cancer prevention could be due to the suppression of glycolysis process in the cancer cells.

Guggulsterone (GS) is a natural hypolipidemic drug extracted from the gum resin of tree *Commiphora mukul*. The gum resin guggul has been used since ancient times for the treatment of various ailments including obesity, arthritis, inflammation and lipid disorders as an Ayurvedic medicine. Extensive molecular mechanism studies on GS showed apoptosis inducing and antiinflammatory activities have a role in obesity, arterial thrombosis, inflammatory bowel diseases, and different types of cancers. Recent proteomics analysis studies on 3T3-L1 preadipocytes treated with GS using 2-DE with MALDITOF/TOF technologies showed the upregulation of Annexin 5, marker protein of apoptosis (Pal et al., 2013).

Siwu decoction is an ancient traditional Chinese herbal medicine (*Rehmannia glutinosa*, *Angelica sinensis*, *Paeonia lacitflora*, *Ligusticum chuanxiong*) used to replenish blood, stimulate the hemopoiesis of the bone marrow for a blood-deficient subject as well as increase the peripheral blood count. With proteomics technologies including 2-DE, image analysis, in-gel

digestion, MALDI-TOF MS, and bioinformatics, has shown that Siwu decoction could regulate the protein expression of the bone marrow of blood-deficient mice, including lymphocyte specific protein 1, proteasome 26S ATPase subunit 4, hematopoietic cell protein-tyrosine phosphatase, glyceraldehyde-3-phosphate dehydrogenase, growth factor receptor binding protein 14, and lgals12. The proteome analysis provided a possible explanation of the mechanism underlying Siwu decoction in the hemopoiesis process (Guo et al., 2004).

Diabetes mellitus is a chronic progressive disease with metabolic disorder of the endocrine system. *Panax ginseng* has been used to treat diabetes mellitus since ancient time. Studies on ginsenoside Re, an active compound of *Panax ginseng* has demonstrated significant antidiabetic actions mainly as an antiinflammatory and through the reduction in insulin-resistance activities. Recent proteomics studies employing high-throughput SELDI-TOF MS and bioinformatics technologies used to explore the possible proteins involved in the antidiabetic actions of ginsenoside Re (Cho et al., 2006; Gao et al., 2013). The proteomic analysis of Cho et al. showed the presence of 293 potential biomarkers differentiating between diabetes and control normal rats. C-reactive protein, a marker protein, was found to be altered in ginsenoside Re-treated diabetic rats and was validated by ELISA. Studies by Gao et al., using a genomics and proteomics approach, demonstrated a reduction in insulin-resistance activity of ginsenoside Re through activation of PPAR-γ pathway and inhibition of TNF-α production. These findings indicate that ginsenoside Re might be beneficial to patients suffering from diabetes mellitus and its complications by alleviating inflammation and insulin resistance.

These examples have shed light on connecting the exploration of traditional medicines with powerful proteomics tools and serve as an ideal integration for discovering new treasures within herbal medicine and thereby bringing traditional medicine research to a new horizon.

QUALITY CONTROL AND STANDARDIZATION

Quality control and standardization of herbal drugs remains one of the bottlenecks in herbal drug development (Warude and Patwardhan, 2005). There are several approaches based on morphology, microscopy, preliminary qualitative phytochemistry. Phytochemical investigations based on total chromatographic fingerprint analysis or quantitative estimations that target secondary metabolites in crude, processed extracts and final formulations as well as the use of gene expressions studies using microarrays have been used for monitoring the quality of herbal drugs (Chitlange et al., 2009; Joshi et al., 2004; Patil et al., 2009). Monitoring the quality of herbal drugs based on the spectrum analysis of only secondary metabolites is not enough to reflect the quality of an entire product. Therefore, there is a need to monitor the quality of the product based on primary metabolites as well secondary metabolites. There are reports on

monitoring the quality based on primary metabolites such as polysaccharides, proteins and glycoproteins, all of which have been documented. *Panax ginseng* and *Panax quinquefolius* are two widely used valuable TCMs. However, conventional separation methods cannot distinguish different parts (main root, lateral roots, rhizome head, and skin) of the two species. The 2-DE maps have been applied to identify different ginseng samples containing distinct and common protein spots to permit easy discrimination. The use of these potential biomarkers might help to speed up the identification process of herbal drug development (Lum et al., 2002).

TOXICOKINETICS AND HERB–DRUG INTERACTIONS

There are increasing incidences of herb–drug interactions affecting the safety and efficacy of treatment mainly though pharmacokinetic and/or pharmacodynamic modulation. The concomitant administration of herbs with conventional therapeutics has shown a modulatory effect (inhibitory or inducing) on metabolizing enzymes, such as cytochrome P450s (CYPs), or transporter proteins like P glycoprotein (p-gp), organic anionic transporter peptides, or organic cationic transporter polypeptides that leads to pharmacokinetic interactions. Assessment of these interactions in the early stage of herbal drug development is an important step recommended by various international regulatory agencies such as the USFDA and the European Medicines Agency (EMA) (USFDA, 2004; EMA, 2010). There are reports available on traditional Chinese herbal medicines and Japanese herbal medicines on the early prediction of clinically relevant herb–drug interactions based on CYP modulation and transport proteins using proteomics with the aid of genomics and metabolomics approaches (Mrozikiewicz et al., 2010; Shord et al., 2009). *Ginkgo biloba* extract is one of the most popular herbal ingredients used for improvement of cognitive function and peripheral arterial disease. Bilobalide has been identified as major constituent responsible for induction of CYPs suggesting potential for HDIs. Western blot analysis and a real-time PCR study demonstrated a dose-dependent induction of CYP2B protein (Taki et al., 2009). Another study using CYP activity mediated through probe drug substrate metabolite formation showed induction of multiple CYPs such as CYP1A1 CYP2C and demonstrates significant reduction of plasma warfarin (substrate of CYP 2C9) concentration affecting its anticoagulant efficacy through pharmacokinetic interaction (Taki et al., 2012).

So far CYP enzymes have been studied using a variety of different bioanalytical methods including immunoblotting, PCR, and enzyme-activity assays. The studies performed have not provided definitive data on the protein levels of individual CYP isoforms. Although the application of RT-PCR to quantify, e.g., CYP2E1 mRNA, was successful, the result does not necessarily translate to the protein level (Haufroid et al., 2001). Western blot analysis can be performed at the protein level, but is dependent on the

availability of specific antibodies, and may not assure 100% selectivity (Guengerich and Turvy, 1991). Additionally, only one CYP-isoform can be quantified per analysis. CYP protein levels have also been investigated indirectly by studying their enzymatic activity, e.g., CYP2E1 via metabolism of chlorzoxazon (Tanaka et al., 2003). However, there remains the problem that more than one CYP-isoform can catalyze a given reaction. In recent years LC-MS/MS has gained popularity in the qualitative and quantitative determination of proteins. For CYP analysis, LC-MS/MS has been mainly used in a discovery mode, where the goal is one of protein identification. However, quantitative analysis is also possible (Wang et al., 2008).

A recent proteomic study using 2-DE coupled with MS/MS identified and quantified a total of 18 major CYP isoenzymes responsible for metabolism of more than 90% of pharmaceuticals in human liver samples (Seibert and Davidson, 2008). These findings suggest that the recent advancement in quantitative proteomics and mass spectrometry could provide useful information on absolute estimation of proteins and more accurate prediction of HDIs. Since proteomics aids in understanding the complex mechanisms of traditional herbal medicines at the cellular and molecular levels, it has great meanings to the modernization and internationalization of traditional herbal medicines.

LIMITATIONS AND FUTURE PROSPECTIVE

Despite of the rapid advancements acquired by proteomics in past years, there are also some disadvantages: low reproducibility and a difficult separation of proteins of a big or small isoelectric point. Moreover, some hydrophobic proteins, insoluble membrane proteins, and large molecular weight, low-abundance proteins may be ignored in the process of examination. It is noteworthy that the proteomics community has identified four major areas for strengthening the proteomics. These include (1) the need to provide high-quality standardized, sensitive, specific, quantitative, and readily accessible protein, peptide, or other biomarkers of health, disease, response to therapy into the approval processes of regulatory agencies (e.g., FDA), and obtain approval from the relevant agencies for their use in a clinical or other test settings; (2) implement standard processes for collecting, processing, and storing human clinical samples in biorepositories and enforcement of measures to ensure subject integrity including informed consent for the downstream use of samples and in registrations of subject identities within study databases; (3) test and validate mass spectrometry technology platforms that hold much promise for creating opportunities for obtaining new and important knowledge at levels of detection previously not achievable; and (4) organize clinical discovery operations and activities in an intuitive manner to meet the challenges of increased interests in the science and diminishing levels of centrally financed resources and infrastructure support (Fehniger et al., 2014).

Successful use of key proteomic technologies will provide in-depth understanding of the molecular and cellular mechanisms of drugs. How these proteins interact with each other at cellular level and express the phenotype remains complex. Mapping of such "interactome networks" and the transition of omic data, including proteomics to interactomics, will require ongoing continued development to understand health, disease, and drug action (Vidal et al., 2011).

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