Toxicoproteomics – a new preclinical tool

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The publication of the human genome has presented the scientific community with an unprecedented amount of genetic information with the potential to revolutionize the drug discovery process. This information could be used to identify novel drug targets and disease markers or could aid in the development of personalized medicines. The realization that genetic changes must ultimately influence protein function has pushed the field of proteomics further into the limelight. In this review the applications of proteomics to the field of toxicology will be discussed. It is anticipated that, in the future, toxicologists will apply a range of genomic and proteomic techniques to address issues in toxicity.

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▼ Scientists in the 21st century have witnessed an explosion of genetic information following the completion of the Human Genome Project. Many genome-based technologies have been used to document a plethora of potential new drug targets and diagnostic markers and this trend seems likely to continue. In the future, patients could be offered personalized medicines based on their individual genetic composition; however, this concept comes with many practical issues, which will be debated in the next decade. It has become increasingly clear that genetic alterations will ultimately influence proteins and their function; therefore, the study of protein changes or proteomics has recently received intense scientific interest. Proteomics has a wide range of potential applications in drug discovery and development and the application of proteomics to the pharmaceutical industry has been aptly named 'pharmacoproteomics' [1].

The term 'proteome' is often used to describe the full protein complement of a given cell, although this is a constantly changing entity [2,3]. Proteomics is the process of separation and identification of proteins with the potential for assigning a function to a given biological phenomenon. It should be emphasized, however, that unidentified proteins could also be of value if the 'blinded' protein profiles are predictive of a disease or a specific cellular process. A particular advantage of proteomics is that many proteins, unlike DNA or RNA, are often secreted into bodily fluids in response to a particular physiological state. As a result, proteomic analysis can be performed in large numbers of patients via a straightforward blood or urine test, rather than the collection of a biopsy sample, which is potentially more hazardous and expensive.

Traditionally, toxicologists have defined the preliminary risk of a new compound to human safety using animal studies, as recommended by the International Conference on Harmonization (ICH, http://www.mcclurenet.com/ICHsafety.html), together with histopathological and biochemical techniques [4]. Proteomics represents one of many new technologies that can aid a new generation of toxicologists to address issues in toxicity through the identification of novel proteins or protein profiles that measure sensitive cellular changes in response to xenobiotic exposure. It is a technology with the potential to change the current approach to safety assessment in animals and humans, both in the laboratory and the clinic. At present, there is a need to improve our understanding of toxicity and improve the predictability of preclinical testing. The identification of individual proteins or groups of proteins associated with the properties of a single toxicant could be reflective of a common mechanism of toxicity. New markers could be more sensitive and predictive than current markers and, therefore, more appropriate for assessing the toxicity of new drugs. Examples of the applications of proteomics to study differing mechanisms of toxicity in preclinical models will be reviewed

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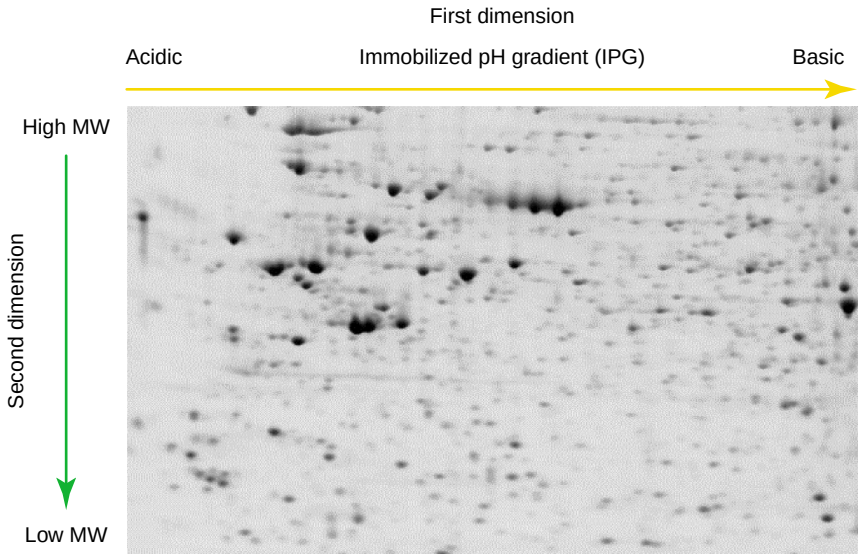

Figure 1. A typical two-dimensional gel image. Proteins are initially loaded onto an immobilized pH gradient (IPG) gel and separated in the first dimension by charge. The second dimension separates proteins according to their molecular weight (MW). Gels are treated with a fixative and stained using an appropriate protein dye. The resulting image is generated by scanning the gel using a high-resolution fluorescence scanner.

briefly. In addition, the use of proteomics in aiding toxicology and drug development will be discussed.

Proteomics technology

The most common technique for studying the proteome is two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE). It is now possible to run this process in a high-throughput format using sophisticated automation and digital imaging coupled with MS and bioinformatics techniques. Traditionally, protein samples are initially separated by electric charge in the first dimension and by molecular weight (MW) in the second dimension (Fig. 1).

Several technological developments have aided the automation of the proteomics process (an overview of this process is summarized in Fig. 2). Researchers at Oxford GlycoSciences (UK) (OGS; Oxford UK) have bound the rigid gel separation medium to a glass support to successfully overcome the difficulty of reliably manipulating the fragile gel matrix. Once the proteins have been separated, they can be visualized using a variety of staining dyes. Sensitive fluorescent dyes have also been developed at OGS that can detect proteins of low abundance [5]. The reproducibility of this process is a key feature for identifying relevant protein changes. Slight fluctuations in the gel compositions can occur, which could in turn influence the data interpretation; however, this is overcome by scanning several gels to produce a composite or master gel. Software programs incorporating appropriate algorithms are used to generate the master gel, which could comprise several-thousand protein features [5].

Because a large number of protein features are produced from a 2D gel image, additional software is necessary for mining and interpreting the information. Several groups have developed software specifically for this process; the Swiss Institute of Bioinformatics (Geneva, Switzerland), for example, has produced the software program Melanie 3. In addition, proteomics companies, such as OGS and Large Scale Proteomics Corporation (Rockville, MD, USA), have developed sophisticated in-house image analysis platforms, such as Rosetta™# (OGS) and Kepler™ (Large Scale Proteomics Corporation). By using Rosetta™, for example, the presence of protein features from different gels and samples can be compared and analyzed using a variety of statistical parameters [5]. Once specific proteins of interest have been selected, they can be excised from the gel, fragmented via protease digestion and subjected to analysis by MS. Specifically, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) can be used to assign peptide sequence. This information can then be used to search a variety of sequence databases to identify the protein and predict its full sequence. The continuing refinement of bioinformatic and MS techniques will increase the throughput of analysis and also reduce the risk of ambiguities that can arise from MS data [6,7].

Applications of proteomics in toxicology

The applications of proteomics in toxicology – 'toxicoproteomics' – can be divided into two broad overlapping areas: (1) investigative or mechanistic studies and (2) screening or predictive toxicology. Given the novelty of this technology, it is important to recognize that supporting experiments should be designed using protocols based on good toxicological principles. There is also a need for a comprehensive and careful analysis of the results and it is important that new approaches, such as proteomics, compare favourably with conventional toxicology techniques [8,9].

Proteomics has been used to elucidate mechanisms of toxic damage in several model systems. When combined with conventional methods, proteomics offers the prospect of identifying new toxic mechanisms. Such insights could

enable the recognition of a species-specific effect, which could give a more accurate prediction of toxicity in man. In addition to supporting mechanistic investigation, the real value of proteomics could reside in screening and predictive toxicology. The ability to establish relationships between toxic effects and protein markers could mean that it will be possible to screen new compounds for toxicity using a panel of predictive markers. The sensitive nature of proteomics could result in the detection of toxic effects at lower doses than is possible using conventional methods, such as histopathology and clinical chemistry. The early detection of potential toxicity and the opportunity of ranking compounds during drug development could result in significant financial savings in the latter stages of the process.

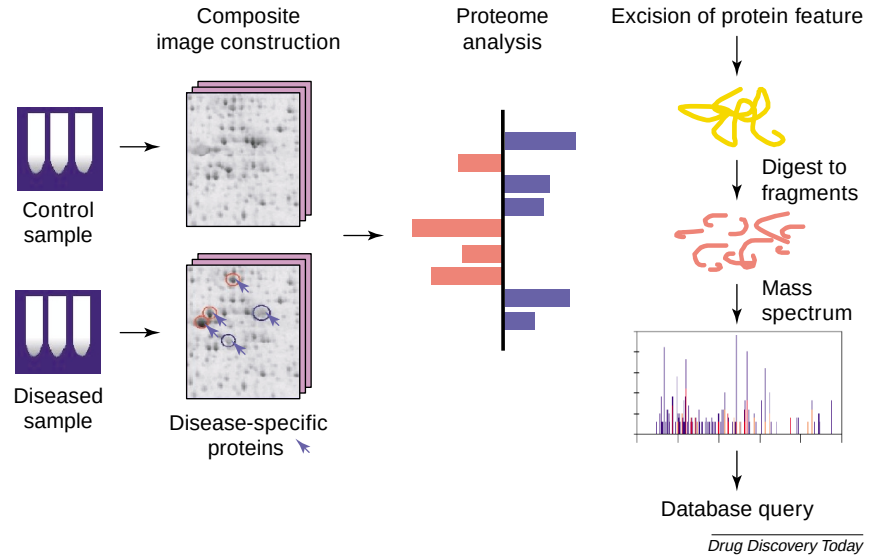

Figure 2. Summary of the different stages of the proteomic process. Protein samples from control and disease groups are run on two-dimensional gels. Multiple images are generated and the resulting information is assembled into a proteomic database. The blue arrows indicate proteins that are specifically altered in disease samples. Protein profiles can be compared using appropriate software. The columns represent proteins that are increased (red) or decreased (blue) on the gels. Protein features of interest are identified by excision from the gel, followed by protease digestion and MS analysis.

Hepatotoxicity prediction

The liver is a major site of metabolism and detoxification in the body and also a common site for toxicity. It is also well recognized that liver toxicity results in the failure of many drug candidates during development because of adverse drug reactions (ADRs) [10]. Consequently, liver function testing is a routine part of the toxicological evaluation of any new compound. In an attempt to develop a rodent liver proteomic toxicity database, Anderson and colleagues characterized the effect of a range of xenobiotics on protein expression in the liver [11]. Using this database, it was possible to detect, classify and characterize a broad range of hepatotoxins. In many cases specific proteins were identified and shown to correlate with a molecular pathway of cellular toxicity. Data on the SAR of the H1 receptor antagonist, pyrilamine, and the non-genotoxic carcinogenic analogue, methapyrilene, revealed differing proteomic profiles despite a similar chemical structure [12]. Widespread changes in liver proteins were observed for methapyrilene but not pyrilamine, emphasizing the potential role for proteomics in lead candidate selection.

The development of this type of database has considerable implications for predictive toxicology. The potential hepatotoxicity of a new compound can be studied by comparing effects on the proteome against a panel of known toxins that are already analyzed and categorized in the database. Positive correlations could serve as an early warning of toxicity for further studies. Unfortunately, this particular database was based on information from limited doses and time points that were the same for each toxin and, therefore, might not have been relevant for different mechanisms of toxicity. A larger collection of data points would be required to gain confidence in the predictive value of such a preclinical tool.

In a more focused proteomic study, the hypoglycaemic compound SDZPGU693 was evaluated extensively. SDZPGU693 is known to stimulate glucose utilization in peripheral tissues and, in this study, was shown to induce hepatocellular hypertrophy. Liver samples from treated rats were analyzed for changes in protein profiles and 29 proteins were identified that correlated to changes in liver function [13]. Major increases in several microsomal proteins were observed, strongly suggesting that SDZPGU693 induces microsomal proliferation and induction of the P450 pathway. The authors suggested that these effects were likely to be associated with the toxic effects of SDZPGU693. Additional proteins were down-regulated in response to compound treatment, suggesting that mitochondrial liver fatty acid metabolism could be inhibited [13]. It should also be noted that several of the proteins identified could not be linked to the mechanism of action of the compound.

Similar studies have been performed using the peroxisome proliferator WY14643 and paracetamol (acetaminophen).

In each case, several compound-specific proteomic changes were identified and protein annotations revealed a variety of proteins involved in liver metabolism [14–16].

Nephrotoxicity prediction

The kidney is another common site of toxicity within the body. Several proteomic studies have been performed to define changes in kidney tissues in response to damage. The antibiotic gentamicin is representative of a class of aminoglycosides with known renal toxicity. A dose- and time-dependent study was developed based on the known behaviour of this compound, which also included a treatment-free recovery phase [8]. Routine clinical biochemistry tests confirmed the presence of renal toxicity at high doses and evidence of renal regeneration was confirmed by histopathology during the recovery phase. Proteomic evaluation of kidney cortex samples revealed several treatment-specific changes, some of which were unexpected and could reflect novel aspects of gentamicin toxicity [8]. Examination of the serum samples from treated rats revealed a protein that was consistently overexpressed in all of the gentamicin groups. Interestingly, this marker was present at low treatment doses and at early time points before changes were observed by routine clinical pathology. Intriguingly, the marker also returned to control levels during the recovery phase, highlighting the sensitivity of proteomics. A marker of this type is particularly interesting because it raises the possibility of monitoring the onset of toxicity before cellular damage is evident.

The clinical use of the immunosuppressant cyclosporine A (CsA) is also limited by adverse effects, of which renal damage is most prevalent. A proteomic analysis of kidney homogenates from CsA-treated rats identified the calcium-binding protein, calbindin-D (28K), as a novel marker of CsA renal toxicity [17]. Subsequent studies in a range of species indicated that rats and humans displayed CsA-mediated renal toxicity, whereas monkeys and dogs did not. Calbindin-D was also regulated in a species-specific manner consistent with cellular toxicity [18]. The discovery of calbindin-D (28K) in CsA-mediated toxicity was exciting because it had not previously been associated with this process. The identification of this protein again emphasizes the role proteomics can have in providing essential information to understand the mechanisms of toxicity. Several other rodent and non-rodent studies have also been conducted to assess proteomic changes in response to nephrotoxins [19–21].

Cardiotoxicity prediction

Cardiovascular toxicity could occur via a wide variety of cellular and molecular mechanisms, many of which have been well documented in the literature [22,23]. However, cardiotoxicity remains a major concern during drug development, particularly because several therapeutic entities have recently been associated with this type of toxicity [24–26]. Consequently, new markers, such as cardiac troponin, have received much attention, although its value in predicting cardiac damage is questionable [27,28]. Despite keen interest in the use of proteomics to study cardiotoxicity, few studies have been performed [29,30]. Studies of protein changes that occur in cardiovascular diseases or after cardiac failure have identified several protein markers that might also be regulated in a toxic response [31,32]. In an inventive study by Holt and Sistare (G. Holt, personal communication), rats were treated with the anti-cancer drug, doxorubicin, which induces dose-dependent cardiotoxicity. Specific treatment groups also received the metal chelator ICRF187, which provides significant chemoprotection against the toxic effects of doxorubicin. Proteomic analysis of serum samples revealed 34 potential markers of toxicity that increased or decreased in response to doxorubicin treatment. In the groups that were co-administered with ICRF187, 29 of the marker proteins were fully normalized and the remaining five were partially normalized (Fig. 3). The abrogation of the cardiotoxicity markers by the ICRF187 treatment is an important observation because it confirmed the validity of the markers. Further validation was also obtained through identification of the proteins, which revealed several protein classes that are consistent with the chemoprotective effects of ICRF187.

Carcinogenicity

Assessing the carcinogenic potential of a new compound can be both costly and time-consuming. Therefore, any rapid assay that predicts this characteristic would be very valuable during drug development. Numerous experiments have been performed to identify proteomic changes that occur in cancerous cells [33–35]. It is possible that some of these markers could also be regulated in response to treatment with carcinogens and could be used as early indicators of tumourigenesis. Several experiments have been performed to assess the action of growth-promoting agents. For example, Chevalier and co-workers exposed primary rat hepatocytes to either epidermal growth factor (EGF) or the non-genotoxic hepatocarcinogen, nafenopin [36]. Both induce DNA synthesis, EGF through the EGF receptor and nafenopin via the peroxisome proliferator receptor. When compared, the protein expression patterns of cells treated with either EGF or nafenopin were different, suggesting that proteomics can be used to distinguish between different mitogenic pathways. A combined proteomic and RNA analysis has also been performed on a

pancreatic cell line treated with the cytotoxic agent daunorubicin. Surprisingly, the proteins regulated in response to daunorubicin treatment showed no change in the corresponding messenger RNA (mRNA) profile, suggesting post-translation mechanisms of control [37]. These data underscore the need to monitor both RNA and protein profiles to understand complex biological processes.

The US National Cancer Institute (Bethesda, MD, USA) has accumulated a vast array of information on the growth inhibitory effects of compounds on 60 human cancer cell lines. This information has been combined with protein expression data to create a database linking protein expression changes with the growth inhibitory activity of several anticancer compounds [38,39]. This archived information could prove valuable for future drug development if protein signature patterns can be used to predict carcinogenicity or anti-tumour efficacy.

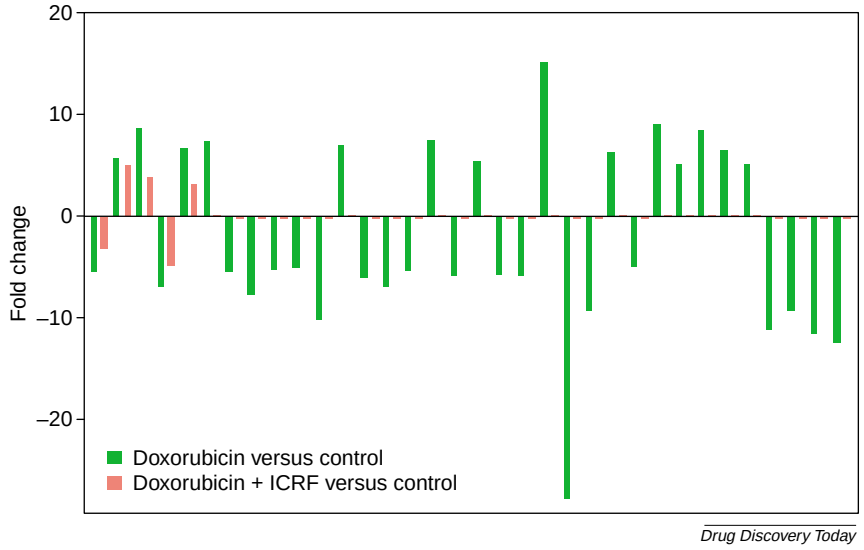

Figure 3. Examples of plasma biomarkers identified from doxorubicin-treated rats. The fold changes of 34 potential doxorubicin-induced biomarkers are shown (green). Note: 29 of the markers were normalized in rats treated with doxorubicin and ICRF187 (red), indicating a correlation between the biological effects and the marker presence.

Opportunities for proteomics in drug development

As discussed previously, a significantly large body of proteomic data has already been collected on the effects of xenobiotics in preclinical models. In many cases, protein profiles can be correlated with the mechanistic effects of the toxin in question. Such data can be combined into a reference proteomic library for comparison with new drug candidates. Early signs of toxicity could serve as a warning for further studies and might aid the prioritization of several lead compounds based on the evidence of toxicity. For such a database to be of value it would be necessary to evaluate several known classes of toxin in a time- and dose-dependent manner. Common patterns of toxicity could be elucidated together with compound-specific mechanisms.

Toxicoproteomic profiles derived from preclinical models could also be used to complement classical histopathology in a clinical setting. Protein expression patterns that are characteristic of common mechanisms of toxicity in non-human species could be a valuable aid to clinicians in monitoring drug toxicity. This would require the marker(s) identified to be relevant to toxicity in man. It could also be necessary to measure the markers using a non-2D gel format to enable multiple samples to be evaluated rapidly in a cost-effective manner. As an alternative to using animal models, human-specific markers could be identified by analyzing samples collected from patients undergoing treatment with drugs known to cause toxicity. A retrospective analysis of the samples would facilitate the identification of markers expressed before clinical manifestations are evident. In this scenario, alternative treatment regimes could be initiated before extensive tissue damage occurs to avoid future complications.

Perhaps the most interesting and challenging application of proteomics could be in identifying markers of ADRs or a predisposition to such an event. Again, proteomic data derived from preclinical research could be valuable in defining markers of an adverse reaction conserved in man. However, it is more probable that more appropriate markers will be captured from a clinical study. Although ADRs generally occur at low frequencies, it might be possible to collect samples from large clinical trials and to analyze a subset of these in which the clinical outcome has been established [40,41]. In this situation the appearance of a marker could serve as an early warning to stop drug treatment. Alternatively, markers could be used to identify patients with a predisposition to a given adverse reaction. These individuals could therefore be pre-selected to receive the most appropriate drug treatment, thereby preventing complications in the clinic. Although the application of proteomics in the research environment is well established, a role in regulatory toxicology is more complicated. Any new markers identified would need to be validated in larger patient populations and compliance with the Bradford Hill criteria would be an advantage [42]. It could

Table 1. A comparison of different proteomic platform technologies

	2D gel	Antibody-based chip	Protein affinity chip	ICAT™
Requirement for protein identification	No	Yes	No	No
Potential for discovering novel proteins	Yes	No	Yes[a]	Yes
Detection of specific protein isoforms	Yes	Yes[b]	No[a]	No
Relative assay time	Moderate	Rapid	Rapid	Rapid

ICAT, isotope-coded affinity tag.
[a]Isoforms can be detected only if the appropriate antibody is generated.
[b]Requires additional purification steps for protein identification.

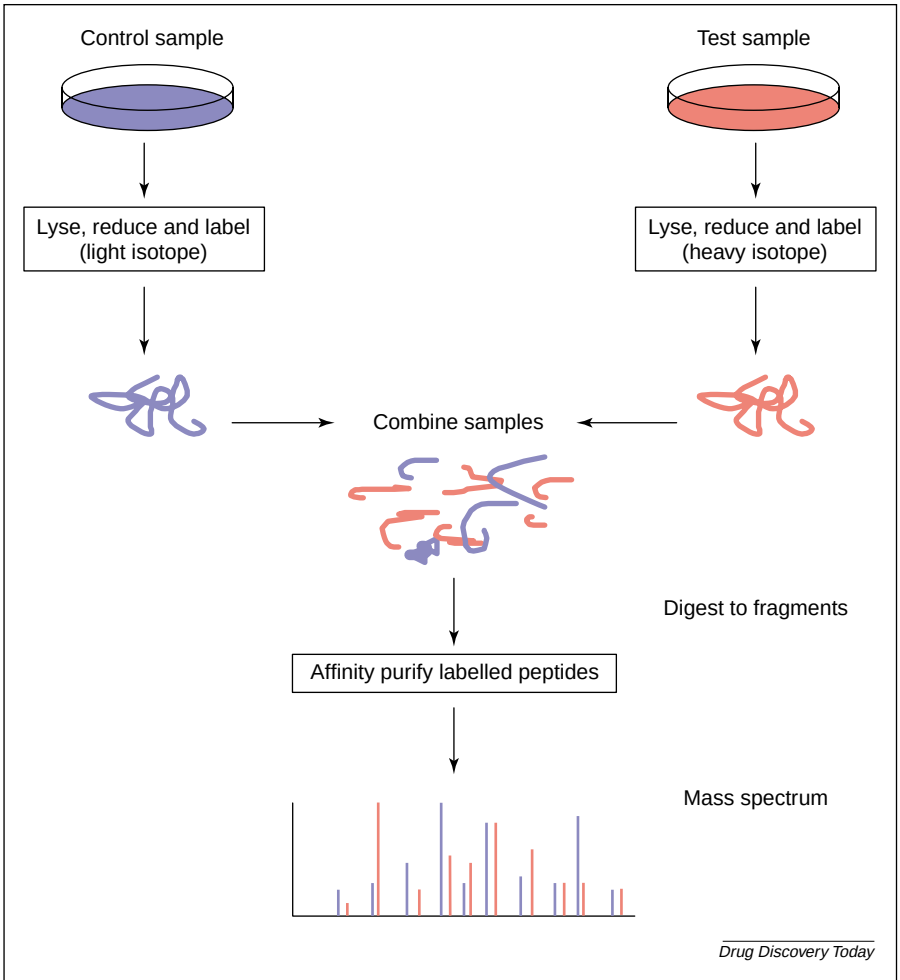

Figure 4. Overview of the isotope-coded affinity tag (ICAT™) process. Test (red) and control (blue) samples are isolated and labelled with different isotopes. The labelled samples are combined, digested with proteases to yield peptide fragments and purified to isolate the labelled peptides only. The labelled peptide mixture is subjected to MS analysis and the different isotope labels enable a parallel binary comparison of the peptides from the test and control samples.

take many years for a new marker to gain regulatory acceptance, however, it is probable that promising markers will undergo early adoption by a scientific community eager for information.

Alternative proteomic technologies

Although 2D gels offer a high-quality approach to studying the proteome, several alternative technologies have been developed that will complement this approach (Table 1). Several protein chips have been developed to bind proteins directly or indirectly [43–45]. For example, antibody-based chips have been developed to selectively capture and separate known proteins. In some cases the proteins must be labelled to facilitate detection. Alternatively, a dual antibody strategy is required using one antibody to bind the protein and the second to detect it. The advantage of this kind of approach is the high-throughput nature of the technology, making it applicable to routine testing. However, major limitations include the requirement for the proteins to be known, characterized and amenable to antibody production.

The protein chip format ProteinChip® developed by Ciphergen Biosystems (Fremont, CA, USA) uses a variety of affinity surfaces to bind proteins based on their chemical and physical characteristics. The chip can be washed to remove non-specific protein and further characterized via surface-enhanced laser desorption/ionization (SELDI)-TOF MS. An advantage of this type of chip is the ability to bind a range of proteins to different molecular surfaces without the requirement for antibody production. In addition, once binding and wash conditions have been optimized, data can be generated rapidly. A disadvantage is the need to use additional techniques to identify peptide sequences. Nevertheless, the system has already been used to identify markers of prostate cancer and changes in renal cell carcinomas, and

could also be applied to the discovery of new toxicity markers [46,47].

A recent and exciting development by Abersold and colleagues is the isotope-coded affinity tag (ICAT™*) method, which can be used to label proteins before separation [48]. The method uses a thiol-specific reactive group linker that incorporates a stable isotope and an affinity tag for purification. By using two different isotopes for labelling it is possible to perform a binary comparison in a single step. After labelling, test and control samples are pooled and digested with proteases to produce peptide fragments. ICAT-labelled peptides are separated by microcapillary liquid chromatography (LC) and analyzed by tandem MS (Fig. 4). Because the ICAT™ method is designed to combine labelling, separation and the analysis of peptides into a single automated procedure, it is possible to scan several thousand peptide pairs in a day.

Further comparative analysis of genomic and proteomic pathways using ICAT™ revealed an interesting divergence between protein and RNA expression patterns in yeast. Several genes whose protein products were regulated in response to galactose utilization were identified; however, when compared, the corresponding mRNA levels were found to be unchanged [49]. Consequently, the authors highlighted the importance of combining both protein and mRNA expression profiles to study biological systems. One could imagine that a similar comparative approach could be used for monitoring toxic effects of known compounds with the aim of understanding the action of novel drugs. A large body of information has already been collected, cataloguing mRNA changes altered in response to xenobiotic exposure, however, a major challenge will be to correlate these changes with proteomic profiles [50–52]. This information, coupled with the polymorphic analysis of drug metabolizing enzymes [53,54], will greatly enhance our understanding of molecular toxicity in the future.

Conclusions

Proteomics, in combination with genomics, will yield a large source of drug targets as well as biomarkers of disease. In addition to markers of toxicity, it is also likely that markers of drug efficacy or drug suitability will be identified. Because protein markers are available from bodily fluids, samples can be acquired by non-invasive methodologies and converted into standard immunoassays for routine screening. The implication is that the new markers identified by proteomics will significantly reduce the investment of time and money required to develop new drugs. In particular, proteomics will enable the rapid selection of the most promising drug candidates for further development, as well as enabling candidates to be eliminated earlier in development because of insufficient efficacy or excessive toxicity.

Proteomics techniques can potentially make a considerable contribution to research and regulatory toxicology. However, it is anticipated that proteomics will complement other new technologies and existing methods for regulatory testing purposes. Proteomics holds great potential for identifying biomarkers that will improve the predictivity of drug development. New protein markers will improve our understanding of preclinical models and expand our knowledge of idiosyncratic clinical responses.

*ICAT is a trademark of the University of Washington (Seattle, WA, USA), exclusively licensed to Applied Biosystems (Foster City, CA, USA).
#Rosetta is a trademark of Oxford GlycoSciences.

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