10 Utility of Molecular-Based *In Vitro* Assays in Metabolic Liability of Drug Candidates

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10.1 INTRODUCTION

Advances in molecular-based technologies and increased knowledge of genomics and proteomics have resulted in a greater understanding of the metabolic pathways and in particular, the polymorphisms that may cause less than optimal drug pharmacology, unexpected pharmacokinetics, and potential toxicities. The success of the Human Genome Project and improved *in vitro* systems derived from human and preclinical species provide more relevant *in vitro–in vivo* extrapolations, helping in the early attrition of potentially problematic drug candidates. These evaluations aid in the planning and design of clinical trials and selection of appropriate subjects for clinical therapies, particularly in oncology, and for drugs with a narrow therapeutic index. Cellular and subcellular fractions from specific organs including the liver, kidneys, and intestines are routinely utilized to assess metabolic and transporter influences on the bioavailability and pharmacokinetics of drug candidates. This chapter briefly describes systems used to understand the metabolic liability of drug candidates at the molecular level and reviews microarray analysis.

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The nucleic acid arrays described in this chapter have a common origin in the DNA blotting methods pioneered by Southern in the early 1970s [1]. Majority of the current methodologies continue to use immobilized or tethered DNA or RNA that is hybridized with a second nucleic acid, and the unknown targets are identified by decoding hundreds of thousands of sequences in parallel analyses on a single sample and in a single assay. Direct comparisons of parallel tests on multiple samples (different tissues, individuals, or treatment groups) provide relevant data that can be used to assess the safety and efficacy of drug candidates and drugs.

In drug development, microarrays that can identify from tens to thousands of unique proteins or nucleic acid sequences are used to assess changes in levels of multiple proteins or transcripts after *in vitro* treatment over two or more days or *in vivo* treatments upward of one week. Arrays are typically printed on multiwell plates or glass slides with specific protein or DNA binding sequences that serve to capture target proteins or DNA. Samples from control and treatment cohorts can be compared to determine their gene profile parameters and potential differences due to treatment.

Data mined from the Human Genome Project for targets of therapeutic agents in combination with combinatorial chemistry improved structure–activity relationships and from high throughput screening have lowered later stage attrition of drug candidates and yielded candidates with a higher safety profile and improved efficacy. Owing to this, microarray technology has been incorporated into preclinical assessment programs to prioritize and further understand the potential liabilities of drug candidates early in the drug discovery and development [2]. Microarrays are also used to understand mechanisms of toxicity, identify biomarkers, and accurately diagnose tumor status based on gene expression alone. While these assays have many additional uses such as understanding coordination of biological pathways, this chapter focuses on measuring changes in gene expression to provide critical information regarding the pharmacokinetics, dynamics, metabolism, and safety profiles of compounds.

10.2 PROTEIN MICROARRAYS

10.2.1 Functional Analysis

Over the past decade, a variety of functional proteomics methods have been used to understand protein–protein interactions. Many of these assays are now conducted in high throughput, for example, the yeast two-hybrid assay [3,4], the protein complementation assay [5], affinity purification coupled with mass spectrometry [6,7], peptide phage display [8], oriented peptide library screening [9], and protein microarrays [10,11].

The yeast two-hybrid and protein complementation assays entail co-expressing a "bait" and "prey" protein in the same cell. If the two proteins interact, a reporter gene is expressed or a reporter protein becomes active. Major advantages of these assays are that they are conducted within living cells and no biochemical purification steps are required. The methods allow for efficient assessment of large numbers of potential interactions and have been used to conduct numerous genome-wide investigations [12–18]. Drawbacks of these techniques are that there is limited control over the assay conditions, integrity of the proteins cannot be assessed, and concentrations and posttranslational modification are difficult to determine and manipulate.

Methods based on affinity purification/mass spectrometry are powerful and compatible with genome-wide investigations [6,7,19]. The limitation is the restricted ability to control the environment or state of the interacting proteins. For these experiments, an epitope-tagged target protein is expressed in a cell of interest, where it interacts with endogenous proteins or complexes. Following affinity purification of the bait protein, interaction partners are identified by tandem mass spectrometry. This technique is well suited to identifying physiologically relevant complexes, making the choice of the type and state of the cells used important in defining the outcome. This assay works well when the interaction partners are highly expressed in the cells used but has the potential to provide false negatives for proteins that are not expressed or are present at low concentrations.

The methods described above provide primarily binary data, that is, proteins are reported either to "interact" or "not interact." Genomic and proteomic investigations coupled with computational analyses would provide more predictive and reliable extrapolations if the data were quantitative and could be incorporated into predictive models of cellular behavior. Protein microarray technology can complement these approaches and provide a way to study protein–ligand interactions *in vitro* in a noncompetitive format.

In a typical protein microarray experiment, the target proteins are spotted in a regular pattern at high spatial density on a solid support, usually a chemically derivatized glass substrate or a glass-supported nitrocellulose membrane (Fig. 10.1). The spotted proteins become immobilized on the surface and are incubated with a labeled probe (e.g., a protein, peptide, nucleic acid, or small molecule). Then, protein–ligand interactions are identified using the label on the probe. For example, if the probe has been labeled with a fluorophore, the array is simply scanned for fluorescence. To date, this approach has been used primarily to investigate Src-homology 2 (SH2), phosphotyrosine binding (PTB), and PDZ-domain-containing proteins [20–22], many of which are critical to signaling in the liver. Going forward, investigations of other domain families (e.g., BH3-domain-containing protein, which are critical for mitochondrial apoptotic signaling, or RING-domain-containing proteins essential for ubiquitin-mediated protein degradation) will greatly enhance our understanding of fundamental liver biology.

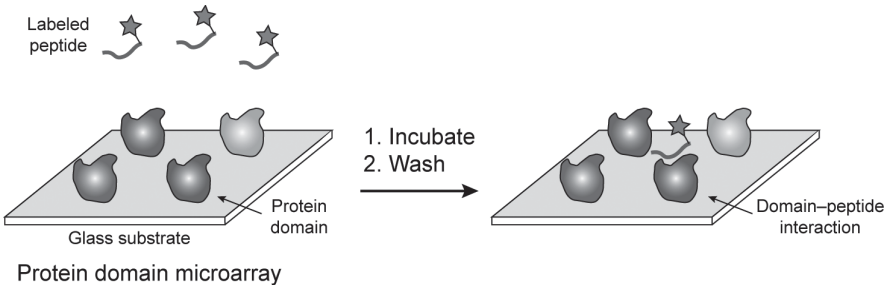

Figure 10.1 Protein domains are immobilized on a solid support (glass substrate) and probed with solution-phase, fluorescently labeled peptides. After an incubation step, the arrays are washed and scanned for fluorescence. Spots comprising domains that recognize the peptide become fluorescent. Figure and related text are adapted from Kaushansky *et al*. Ref 11. (See color insert.)

10.2.2 Analysis of Changes in Cellular Signaling

Although protein domain microarrays provide valuable data with regard to molecular affinities and interactions that occur in a cell, a broader view of cellular pathways impacted by drug treatment, for example, is necessary. A number of new technologies have made analyses that were previously only possible by traditional Western blotting, possible on a smaller, more efficient scale. This is particularly useful in the context of analyses that require the testing of hepatocyte-derived samples since primary human hepatocytes are difficult and costly to obtain in large numbers.

Antibody microarray technology uses a very small amount of total cell lysate per measurement. In an antibody array, a wide variety of antibodies are attached to a glass slide and lysates, which have been modified to be labeled on either the N-terminus or the C-terminus with a fluorescent tag, are incubated with the slide. These arrays are commercially available and able to monitor differences among a wide range of signaling proteins. Alternatively, arrays can be fabricated from lysates. In a lysate microarray, each sample is printed on a glass slide coated with nitrocellulose. The slide is then treated similar to the membranes used in Western blots. Preliminary assessments of antibody microarrays are time and resource consuming, as it is critical to validate each antibody used for a particular cell line by comparing data obtained on control arrays to that obtained by Western immunoblotting. Although antibody validation is a challenge, the ability to obtain Western-blot-like information from a very small number of cells (thousands, instead of the millions used in Western blots) is a significant advantage and provides researchers with the capacity to monitor a large number of cellular signals at the protein level while using a small number of cells, such as primary hepatocytes.

10.2.3 Advantages of Protein Lysate Array Technologies in Absorption, Distribution, Metabolism, and Excretion (ADME)

Protein lysate and antibody array technology are viable options for assessing functionality of proteins expressed in tissues, such as the liver or kidneys, and molecular changes that take place on a protein level after perturbations to liver or renal biology (e.g., drug treatment). New technologies that allow for the monitoring of binding events and/or signaling changes with very small sample size allow for unparalleled insights into organ biology.

10.3 DNA MICROARRAYS

10.3.1 Background

The microarray was forged from the convergence of disparate technologies. Advances in the semiconductor industry have yielded "on-chip synthesis," allowing for high density, *in situ* production of short oligonucleotide sequences that encompass individual probes from the genome. These oligonucleotide expression chip platforms may be produced with tens to tens of thousands of oligonucleotides, depending on the experimental design [23]. Another high throughput platform consists of spotting prefabricated polymerase chain reaction (PCR) products on a matrix [24]. Both platforms provide a stage by which sample RNA may be hybridized to the associated probe sequence and quantified to assess the functioning genome, also known as a *microarray expression analysis* [25].

In general, DNA-based microarrays are analysis tools that use nucleic acid fragments for the simultaneous and parallel examination of the changes in the levels of gene expression for individual genes and/or groups of related genes in response to environmental or biological factors. In addition, this array-based technology can assess individual genetic variations derived from polymorphisms, mutations, and genomic abnormalities associated with disease states [26–29].

Traditional solid-phase arrays use matrices made of materials such as glass, silicon, or membranes to which the nucleic acid probes are affixed. The hybridizing targets are RNA transcripts that are extracted from biological samples and converted to cDNA. In the dual color strategy, these are then converted to cDNA in conjunction with differing fluorescent labels of green (control sample) or red (test sample). The amount of labeled cDNA binding to a specific stabilized sequence is directly related to the intensity of the fluorescent signal. If the intensity of the red target is greater than the green target for a specific probe, then this indicates that the associated gene has been upregulated in the test sample as compared to the control sample. Yellow represents equal binding of the control and the sample, that is, labeled cDNA, and no change in gene expression [30–32]. Therefore, incorporation of dissimilar fluorescent dyes in the reference and experimental cDNA pools allows for differential gene expression analysis. Single color schemes provide absolute quantification of gene expression on a single matrix and may be more amenable to comparative studies that are temporally distinct [33]. In both scenarios, mRNA representing the steady-state balance between active transcriptional and degradation events within the biological source are embodied in the cDNA population [34], allowing for an expression pattern comparison of different genes among samples. Various detection methods, computational and integration software, and databases are available for subsequent data analysis and storage [35–39] and are detailed later in the chapter.

Limitations of gene expression microarrays include “noise” in the signal, leading to a higher than optimal background signal from the system. In some cases, regions of sequence homology can lead to target cross-hybridization, requiring additional confirmation using techniques such as Northern blots or S1 nuclease protection assays. Other causes of variation are the same as in all other systems, for example, human errors in pipetting, RNA extraction and labeling efficiency, microarray production, and differences in response to treatment among individual samples. Furthermore, posttranscriptional and posttranslational events necessary for appropriate gene expression are not captured.

Because of these potential complications, the microarray experimental setup should be guided by predetermined parameters and the actual design should be dictated by the questions that need to be answered. Ideally, biological and technical replicates should be included to account for variability and allow for distinction between background noise and actual signal, and data analysis and interpretation should be straightforward [40,41]. Technical replicates are useful in accounting for variations in sample preparation and labeling; biological replicates are necessary to address issues such as inherent basal variation between the reference samples. The distinction between biological and technical replicates can be somewhat vague and is determined to a great extent by sample size and availability. However, as the process progresses from starting sample to labeled sample to scanned image, background noise accumulates as a consequence of biological, biochemical, and physical effects. Standardized microarray methods can minimize technical variation, making biological variation the most relevant parameter

to be addressed [42]. In addition, these allow comparison of data from different platforms. Both cDNA- and oligonucleotide-based platforms have been used concurrently for mutual multiplexed verification and data integration using standardized methods [43,44].

10.3.2 Microarrays in ADME

Traditionally, microarrays have been used in drug discovery predominantly for therapeutic target identification and validation [45,46]. The data are used to follow the dynamic progression of disease states within a complex biological network and measure changes with drug exposure [47,48]. Literature is replete with examples of microarray gene expression studies that demonstrate the utility of microarrays in gene discovery programs. For example, much investigation has delineated gene expression signatures for tumor diagnosis, prognosis, or classification [49–52] and additionally, has explored drug response and drug resistance in tumors [53]. In fact, responses to a variety of drugs have been reported using microarrays, for example, 15 genes were found to be similarly involved and affected in cell growth, signaling, and trafficking in the cerebral cortex of mice chronically treated with lovastatin, pravastatin, and simvastatin [54]. Simvastatin modified expression of 23 genes in addition to those changed by all three drugs, including some involved in apoptosis. In another study, gene chip analysis of human duodenal biopsies and blood samples in subjects treated with valacyclovir was used to correlate drug pharmacokinetics with multiple intestinal genes [55]. The data from this study helped identify 4F2hc, an activator of the human oligopeptide transporter (HPT1), as being highly relevant to valacyclovir transport. Furthermore, microarray analysis was used to assess the changes in drug-metabolizing enzymes and transporters by the endothelin receptor-type A antagonist CI-1034 and the atherosclerosis drug avasimibe. Data revealed up- and down-regulation of several ADME genes, indicating the potential for multiple drug–drug interactions in the clinic [56,57]. More recently, the technique has been applied to determine individual variability in baseline expression of clinically relevant drug-metabolizing genes among a large population of human liver samples [58]. Establishing this foundation is crucial for the subsequent analysis of putative inductive and inhibitive roles of a potential drug compound.

Microarray analysis can be used to determine drug-mediated changes at the level of genes associated with absorption, distribution, metabolism, and excretion. Monitoring these global alterations in gene expression can help to predict potential drug interactions and cytotoxicity. Formats based on tissue or cell platforms are beneficial, but they are not necessarily amenable to an array design. Tissue arrays deal primarily with formalin-fixed, paraffin-embedded tissue blocks for testing antibodies against various normal tissue and tumor panels to screen antibodies, identify disease biomarkers, and assess antibody or biomarker specificity. These arrays are not used to assess activity. Cell arrays involve the creation of microfluidic devices or platforms of various cell types, such as bacterial, mammalian, and either transiently or stably transfected cells, for a range of cell-specific, gene-specific studies. The technology is not very applicable to or validated for ADME assays and does not offer a distinct advantage over the existing methods. Enzyme arrays can be performed in plates or slides and may use hydrogels or other biological matrices. For example, in oncological research, a protein array containing 170 breast cancers was profiled for 21 cytochrome P450 (CYP) enzymes to assess the expression profile of these proteins in breast cancer in order to

identify potential biomarkers and aid decisions regarding optimal adjuvant hormonal therapy [59]. For the most part, however, these protein arrays are not comprehensive or widely available for all enzymes and suffer from attenuation of enzyme activity when immobilized in gels or on a surface. In contrast, use of DNA microarrays is well established, and products are commercially available [35,37].

These assays can be an initial or adjunct screen to indicate which *in vitro* cell-based, gene-specific, or enzyme-specific assay should be performed to define the potential effects of drug treatment. The microarray results may be the first-line indicator of potential drug–drug interactions, particularly if the drug induces gene expression of multiple drug-metabolizing enzymes, nuclear xenobiotic receptors (NXRs), or transporters. These gene expression analyses can also be performed with quantitative polymerase chain reaction (qPCR) or reverse transcriptase polymerase chain reaction (RT-PCR). Microarrays simply permit the concurrent, convenient screening of one or more compounds against many targets. These arrays decrease assay time and generate a more complete assessment of possible off-target effects and potential for drug–drug interactions or adverse drug effects.

10.3.3 Biological Systems in ADME

Microarray advances are permitting high throughput and global transcriptome analysis of ADME-associated proteins and subsequent effects engendered by pharmaceutical agents. *In vivo* studies entail dosing animals with exogenous compounds and RNA extraction directly from the tissue to determine changes in transcriptional profiling. Well-characterized xenobiotics have been used to determine ADME gene expression patterns in rodents [60], and these correlate with existing data [34,61]. In addition, the effects of a compound may be compared to existing reference databases to define its ADME gene expression signature [62].

Laboratory rodent models are more genetically and environmentally uniform than human systems and do not include the intrinsic heterogeneity of the human population. In a study comparing transporter expression in 75 human donors with expression in 27 rhesus monkey donors [63], human transporter expression showed comparatively small variations when evaluated against human CYP3A4 transcripts. However, transporter variability was consistently lower in the monkey livers examined. A parallel study examining basal expression levels of other ADME genes demonstrated similar results, with higher variability in humans relative to the rhesus population [58]. Human gene expression data did not exhibit normal distribution patterns, indicating that the mean data could not illustrate the wide range of potential expression profiles.

Human hepatic cell lines have been used as the biological paradigm for measuring metabolic liabilities, as they have relatively limited variability [64–67]. However, these cell lines have functional disadvantages that include potentially aberrant nuclear receptor pathways, atypical metabolic enzyme expression, and inability to maintain other appropriate hepatocyte phenotypes [68,69]. The cell lines deviate phenotypically among laboratories and across passages [70], perhaps as an effect of differing culturing and subculturing conditions. Wilkening and Bader quantified gene expression of various drug-metabolizing enzymes during 10 consecutive intralaboratory passages of HepG2 cells and observed significant fluctuations in expression profiles with succeeding passage number [71]. The culturing environment was consistent at each passage. Therefore, the alterations were most likely due to inherent instability of the cell line.

Differences in drug-metabolizing activities have also been noted while monitoring within a single passage of HegG2 cells [71]. Similar modifications in nuclear receptor and transporter pathways are likely [72].

There is much greater variability among human individuals, and the quantitatively more limited variations in the cell lines do not represent the natural diversity of the human population. Primary human hepatocytes from different donor livers are the most acceptable model for determining the ADME properties of a drug candidate, and the resultant data have been shown to correlate with the *in vivo* human response. Use of multiple donors addresses potentially divergent individual responses to xenobiotics. Historically, catalytic enzyme activity has been the principal end point to detect induction instigated by a xenobiotic although mRNA has been shown to be a more sensitive assay that is not affected by potentially concomitant inhibition [73]. The U.S. food and drug administration (USFDA) has recently recognized this fact, and the 2012 drug interaction guidance recommends using mRNA analysis with three or more preparations of human hepatocytes as the end point for predicting interactions of drug-metabolizing enzymes and transporters. Extending customary PCR methods to microarray assessment of drug interactions in cultured hepatocytes creates a more complete profile for a drug candidate.

10.3.4 Strategy

The vast amount of data derived from a microarray analysis can be a bottleneck since commercial arrays can have thousands of genes. Investigation of the specific question of induction benefits from a simplified system that focuses only the relevant drug-metabolizing networks. Gene expression alterations in response to archetypal inducers have been examined reliably in cultured primary human hepatocytes using a platform with a condensed number of drug-metabolizing proteins, including some transporters [74]. Low density DNA microarrays have also been used effectively to validate the response to classic inducers in cultured cynomolgus monkey hepatocytes [75]. In addition, custom microarray slide chambers have been used to assess success of transporter inhibition mediated by small interfering RNA (siRNA) [76]. In this study, generated data were normalized to a control gene that expressed regularly across all samples, and fold change was determined by comparing the normalized values from treated and reference samples.

These types of custom microarrays in which all probe sequences are involved in drug metabolism, conjugation, or transport permit interrogation for both reported and unreported drug effects on gene expression and the investigation of coordinate regulation of gene expression following drug treatment. Since all genes on these microarrays are members of integrated regulatory gene expression pathways, these can serve as a marker or internal control for induction or suppression of gene expression as well as for drug selectivity or specificity in the modulation of gene expression (Fig. 10.2).

If the response is specific in this simplified system, then all members of the pathway should be affected. It can offer a measurement of potential off-target effects if multiple genes are affected or genes common to multiple pathways are affected. Furthermore, it permits the interrogation of expression levels for genes and pathways for which no *in vitro* or cell-based assay exists. The microarray format also lends itself quite easily to the analysis of multiple cell lines or hepatocyte lots versus a single drug or the analysis of multiple drugs versus a single cell line or hepatocyte lot, or tissues harvested from

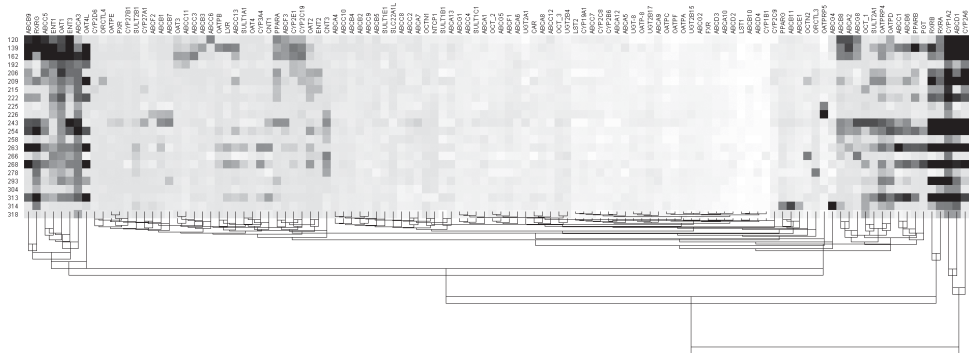

Figure 10.2 Basal level ADME-associated gene expression in 22 untreated human hepatocyte lots. Total RNA isolated from 22 untreated cryopreserved human hepatocyte samples was labeled and hybridized to individual DTEx™ microarrays (NoAb BioDiscoveries, Mississauga, ON). Gene expression values represent the normalized, mean value (relative to a constitutively expressed control gene, GAPDH) from three individual microarray hybridizations ($n = 12$ for each gene). Fold change data was generated by comparing normalized gene expression levels between treated and untreated samples. *White squares* indicate the lowest level of fold change in gene expression ($<1\times$). *Black squares* indicate the highest level of fold change in gene expression ($>10\times$). Basal variations were noted among individuals. (See color insert.)

in vivo studies. A complete gene expression profile can be established for any combination of drug/cell line/hepatocyte lot and gene/regulatory pathway (Fig. 10.3).

10.3.5 Microarrays in Toxicogenomics

Toxicity is a complex interaction of a toxin and a biological system. When a toxin is delivered to the site of action, it interacts with a target molecule or alters the biological environment. This can cause cellular dysfunction and disrepair, resulting in toxicological response such as necrosis, cancer, fibrosis, or disease. The number of mechanisms by which a drug elicits a toxicological response is proportional to the number of mechanisms maintaining homeostasis, such as mitochondrial function, cellular activity, signal transduction, and gene expression. Therefore, monitoring a single marker to assess the toxicological response will neither accurately nor adequately predict a drug's toxicological mechanism. A holistic approach must be employed to capture the specifics of a complex process. To do so, Systems Toxicology integrates technologies and information from several "-omics" disciplines to profile a toxicological event [77]. One aspect is the monitoring of gene regulation in response to exposure to toxicants, known as *toxicogenomics*.

Toxicogenomics developed in the 1990s from the availability of genome-wide DNA sequencing and the converging technology of microarrays or "chips." This allowed for measuring the expression levels of thousands of genes from the exposure to a toxicant. The sequencing of the genome powers microarray expression analysis with candidate oligonucleotide sequences covering multiple mechanisms, signal pathways, and polymorphic deviations that may influence a toxicant's response. The mRNA is quantified to form a transcript profile for a toxic insult. This in turn may be compared to similar profiles with known outcomes to better predict the toxicity potential of a test article and potential mechanism of action [78,79]. This is an oversimplification of the complexity in determining toxicity. Concentration, time course, species differences, target tissue, and target cells are a few of the variables that add to the layers of complexities in toxicogenomics. In addition, toxicogenomics cannot be viewed alone but needs to be integrated with other facets of toxicology, such as metabolomics, proteomics, pathology, biochemistry, and other disciplines, depending on the mechanism of action. This section will solely focus on the aspects associated with toxicogenomics.

10.3.6 Biological Systems in Toxicogenomics

In vivo studies have traditionally dominated toxicology. Preclinical evaluations on a rodent (typically rat) and a nonrodent species (in most cases, dog or monkey), followed by escalating dose studies in phase I clinical trials, are used to evaluate the safety profile of a drug candidate. The *in vivo* interactions among organs, tissues, and cells allow for a thorough investigation of the drug candidate in the preclinical species as tissues are harvested for biochemical, pathological, and mRNA analyses. A typical animal study consists of several dosing concentrations, including a vehicle control, to assess acute versus chronic toxicity over time [77], and observations are made on parameters such as weight, behavior, and organ function.

For toxicogenomic evaluations, biological samples are harvested and gene profile analysis is performed, comparing the dose time variables to the control treatment. The data are combined, and significant outcomes are recorded [80]. For example, brown

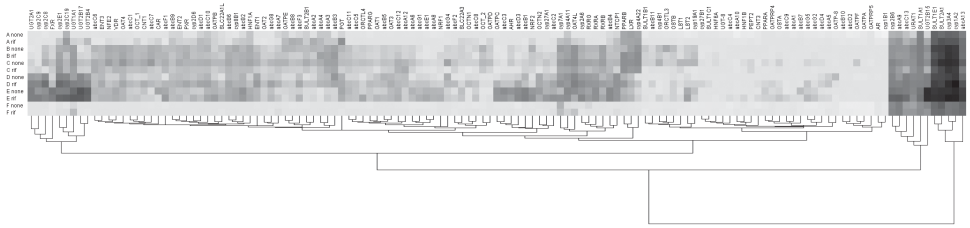

Figure 10.3 Induction of ADME-associated gene expression in six human hepatocyte lots treated with rifampicin for 24 h. Total RNA isolated from six human hepatocyte lots treated with either rifampicin (*rif*) or vehicle (*none*) were labeled and hybridized to individual DTEx™ microarrays. Gene expression values represent the normalized, mean value (relative to a constitutively expressed control gene, GAPDH) from three individual microarray hybridizations ($n = 12$ for each gene). *White squares* indicate the lowest level of ADME-associated gene expression ($<1\times$) relative to GAPDH. *Black squares* indicate the highest level of ADME-associated gene expression ($>10\times$) relative to GAPDH. (See color insert.)

Norway rats were exposed to oral dosing of hexachlorobenzene, and microarray samples were prepared from blood, thymus, liver, spleen, and mesenteric lymph nodes [81]. Results indicated that the spleen and mesenteric lymph nodes exhibited significant changes for genes associated with granulocytes, chemokines, cytokines, and immunoglobulins [82]. A limitation of the preclinical evaluations is identifying the appropriate animal model, as these in many cases do not reflect the human response, particularly with regard to metabolites formed and toxicological responses. A large number of drugs fail in the clinic because of unexpected toxicities that were not observed in the preclinical species. In many cases, human *in vitro* systems better reflect, at least qualitatively, the clinical response. Furthermore, efforts have been put forth to limit animal testing and bridge the information gap using *in vitro* evaluations [83].

In vitro testing utilizes immortalized or transformed cell lines or primary cells as the biological source. Generally, homogeneous cell culture allows for a specific view into the interaction of a compound and a single cell type, such as using hepatocytes to study the potential for hepatotoxicity [84] or isolated CD4 T cells and B cells to assess immunotoxicity [31]. *In vitro* systems are amenable to efficient testing of complex regimens of concentration and time. Interspecies comparisons can be made under similar conditions in order to reduce experimental bias. In a paper presented by Kienhuis *et al*., acetaminophen-induced hepatotoxicity in rat and human primary hepatocyte cultures was compared by using toxicogenomic data, as well as comparison of *in vitro* and *in vivo* rat data, from which relevant common pathways were identified [85]. However, *in vitro* systems have caveats, such as appropriate cell model and conditions and lack of support cells to retain *in vivo* function. Relying on a single cell type or even a mixed culture from a single organ may not provide the appropriate conditions in order to capture a toxic event. In the case of terfenadine, cardiotoxicity occurs when cardiomyocytes are exposed to high concentrations of the parental drug. Clinically, this may occur from an overdose of terfenadine or from inhibition of its intestinal CYP3A4-mediated metabolism, allowing accumulation in the blood to overdose levels. *In vitro* studies employing myocytes confirmed terfenadine effects on cardiotoxicity, while its metabolite terfenadine oxalate had no effect. A screen solely utilizing cardiomyocytes would implicate terfenadine as a cardiotoxin, as is the case; however, this would not account for the common clinical outcome of being safe because of metabolic clearance [86].

Conversely, cells lacking metabolic capacity may underestimate potential cytotoxicities when the metabolite is cytotoxic compared to its parental form. Therefore, cells retaining metabolic pathways may be required to accurately predict metabolically dependent cytotoxicity, as in the case of aflatoxin B1. When metabolically competent cells, such as primary hepatocytes or HepaRG, were employed, microarray analysis provided evidence supporting previously reported involvement of p53, as well as several novel genes FHIT, BCAS3, and SMYD3 in association with alfatoxin B1 genotoxicity [87].

Variations among cell systems and in the case of primary hepatocytes, among individual donors must be defined. Harris *et al*. [88] investigated three genotoxic hepatocarcinogens and one nongenotoxic hepatotoxin in primary hepatocytes from multiple donors and the HepG2 cell line. The results showed that both primary hepatocytes and HepG2 provided similar gene expression profiles; however, there were significant differences among the individual human donors. Although 2172 genes were modulated from all donors, individual donors expressed a portion of these. One donor expressed

only 29% of the affected genes, and 40% of the commonly identified genes were measured in all the donors. This illustrates the variation in idiosyncratic responses among humans and the difficulty extrapolating a single mechanism of action given the inherent variation. *In vitro* systems with appropriate constitutive activities must be vetted with *in vivo* responses within and between species to ensure clinical relevance and relevant extrapolations.

10.3.7 Bioinformatics

Toxicogenomic evaluations generate vast amounts of data since a DNA chip may have tens of thousands of individual genes, and multiple samples (time and dose–dependence) from different species add to the complexity. Bioinformatics has been very useful in melding statistics, computer science, database management, and analytical approaches in order to organize, visualize, and prioritize biological information [89]. The first line of analysis is to normalize the data to remove systematic bias and artifacts due to experimental conditions [31]. The resulting values are then processed for comparison among conditions or among experiments.

The standard approach for toxicogenomics studies is to determine which genes are differentially expressed, that is, which genes are induced or suppressed compared to the control. A common output is a tree-based visualization or heat map that is a graphic representation of a data matrix with a variable hierarchy of gene expression and associated sample. This tree is a simple representation of data for a single gene. It may be organized to bin up- or down-regulated genes in rank order by clustering the row vectors or relationships between samples by clustering the column vectors [32,90,91].

A statistical comparison of classes attempts to separate the gene expression into two or more groups according to specific exposure conditions. This is an effort to connect genes to an event. A *volcano plot* is a type of scatter plot made by graphing significance versus fold change [25]. The regions of interest are the points toward the top at the far right or far left of the graph. To visualize more complex relationships among multiple classes, Venn diagram may be employed to integrate the size of the information with cardinality of the sets and the intersection of their relationships [92,93].

In order to attempt mechanistic analysis, the abstract relationships among the data may be annotated by linking the genes with biological databases in order to include biological information such as proteomics, metabolomics, and phylogenetics. Together, the information may give insight into a gene's function, tissue expression, localization, and clinical ramifications from which signal transduction pathways may be identified or developmental-dependent phenotype is implicated [31].

Toxicogenomic databases provide a repository for gene expression as pathway and mechanistic information from both *in vitro* and *in vivo* testing. Toxicogenomic databases are available both commercially and as open access. The Chemical Effects in Biological Systems (CEBS) database maintained by the National Institute of Environmental Health Services (NIEHS) is "designed to display in the context of biology and study design, and to permit data integration across studies for novel meta-analysis" [94]. The EDGE database developed at the McArdle Laboratory for Cancer Research, University of Wisconsin (Madison, WI) is organized to address the differences in study design and platforms [90], while the Comparative Toxicogenomics database focuses

on cross-species comparative studies of gene expression [95]. From the "Minimum Information About Microarray Experiment (MIAME)" guidelines [31], a database was produced by the Functional Genomics Data Society (FGED) to advocate "for open access to genomic data sets" [96]. The Human Toxicogenomics Initiative (HTGI) consists of both public and private entities is working toward organizing this vast amount of data and publishing it in a public database [97].

10.4 SUMMARY

DNA microarray technology can be used efficiently to measure gene expression alterations with exposure to a compound, such as the xenobiotic induction of transporter and drug metabolism genes. Results obtained from these platforms correspond well to responses measured with activity and RT-PCR. Yet, the capability of simultaneous quantitation of many transcripts provides high throughput analysis of multiple end points. Custom cDNA microarrays can be used to examine the modulation of gene expression levels of a defined set of ADME-associated genes, focusing on the relevant proteins. Furthermore, the emphasis on transcript analysis for induction in the recent regulatory guidance creates higher relevance for these models and underscores the importance of microarrays in future drug discovery programs. Protein microarrays based on either purified proteins or cellular lysates allow for a more thorough and fundamental understanding of the signaling events that are at play during drug treatment. As these platforms are further developed, monitoring transcriptional as well as translational and posttranslational changes in response to drug treatment will become standard.

The widespread use of various microarray-based methodologies has also bolstered new disciplines, such as computational biology, bioinformatics, and toxicogenomics. More work on standardization is necessary to fulfill the expectations of toxicogenomics to adequately assess safety risks of drugs and chemicals. Standards for exposure assessment and hazard screening will need to be identified and implemented. This will require that the scientific community and policy regulators agree on acceptable screening methods, integrated databases, and reasonable decision trees. To move toward this end, variability in susceptibility of distinct populations must be determined as well as mechanistic information with clinical relevance. Given the historical reliance on animal studies, cross-species extrapolation will need to be refined and redefined in the context of gene expression and mechanism of action. Dose–response relationships may increase our understanding of low level exposures, as well as exposure to multiple therapeutics, chemicals, and behavioral influences, but will require validation with clinical outcomes. Little is known about the health impact of fetal and neonatal exposures and their effects, and this area still requires extensive work.

The microarray field has been moving toward standardization in assay design and data analysis. This will allow interpretation of results that can be understood in the context of preexisting data. As microarray tools become more sophisticated, the ability to incorporate and measure concurrent effects on multiple biological pathways will permit integration of entire networks in order to understand the global responses to a xenobiotic.

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