New Technologies and Screening Strategies for Hepatotoxicity: Use of In Vitro Models

Donna M. Dambach a, Barbara A. Andrews a, Frederic Moulin a
a Discovery Toxicology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey, USA

First Published on: 01 June 2005
To cite this Article: Dambach, Donna M., Andrews, Barbara A. and Moulin, Frederic (2005) 'New Technologies and Screening Strategies for Hepatotoxicity: Use of In Vitro Models', Toxicologic Pathology, 33:1, 17 - 26
To link to this article: DOI: 10.1080/01926230590522284
URL: http://dx.doi.org/10.1080/01926230590522284

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article maybe used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.
New Technologies and Screening Strategies for Hepatotoxicity: Use of In Vitro Models

DONNA M. DAMBACH, BARBARA A. ANDREWS, AND FREDERIC MOULIN

Discovery Toxicology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543, USA

ABSTRACT

Hepatotoxicity remains a significant cause for drug failures during clinical trials. This is due, in part, to the idiosyncratic nature of toxicity in humans and inherent physiological differences between humans and preclinical species leading to limited correct prediction of adverse responses in humans. To address this issue, robust screening assays are being developed, which have heightened predictive capacity for human hepatotoxicity, and may be utilized throughout the discovery and development phases in conjunction with traditional in vivo methods, for decision making during drug selection and risk assessment. This manuscript describes an example application of in vitro-based strategies using human hepatocyte cultures in lead optimization screening in conjunction with ADME profiling, for evaluation of compound-associated CYP450 induction potential, and the identification of potentially useful biomarkers as predictors of hepatotoxicity for use in vitro, and in preclinical species and humans.

Keywords. Hepatotoxicity; in vitro cultures; CYP450 induction; biomarker; hepatocytes; screening.

INTRODUCTION

Drug-induced hepatotoxicity represents a major clinical problem accounting for 50% of all cases of acute liver failure. Although the majority of cases of acute liver failure are due to intentional or unintentional misuse, 16% are idiosyncratic (Bissell et al., 2001; Lee, 2003). At issue is not intrinsic toxicity, which is well correlated across species (Zimmerman, 1999a; Bissell et al., 2001), but idiosyncratic toxicity. Contributing to the difficulty in predicting idiosyncratic events is the rare incidence in humans, 1 in 10,000 to 1 in 100,000, and the diverse mechanisms of toxicity, which are multifactorial and related to individual-specific responses (Lee, 2003). Further, the number of animals used in toxicology studies make it difficult to detect hepatotoxicity occurring at a low incidence, as it is impractical to appropriately power such studies. Although a few retrospective studies examining the correlation between the occurrence of hepatotoxicity in humans and in animal studies have been performed, sufficient data are not available to reliably assess the value of preclinical animal studies to predict hepatotoxicity in humans (Ballet et al., 1997; Olson et al., 2000). As such, it is understandable that preclinical animal toxicity studies may not be sufficient as the only modeling systems used to predict idiosyncratic toxicity.

Therefore, in continuing efforts to improve the identification of potential hepatotoxicity issues during lead optimization, the addition of new, predictive assays to the discovery armamentarium is necessary. In vitro systems are an important component of these new assay system applications. Use of in vitro systems is not new to investigative toxicology or lead optimization processes. Systems such as primary cell cultures, immortalized cell lines, liver slices and whole, perfused livers are well-established models used for investigative work (Acosta et al., 1985; Butterworth et al., 1989; Ulrich et al., 1995; Guillouzo et al., 1997; Groneberg et al., 2002). Likewise, the use of HepG2 cell lines and primary hepatocyte cell cultures to assess potential cyotoxicity are promoted by several commercial laboratories. It is the development of updated screening paradigms, which combine analysis of data generated by new or modified in vitro assays with established in vivo methods, that may generate additional insights to help guide the risk assessment/decision-making process. In combination, these assays provide a more well-rounded assessment of potential risk.

This manuscript will present an example of a multi-tier paradigm using in vitro cultures of immortalized cell lines and primary hepatocyte cultures to assess potential risk for acute clinical liabilities, and demonstrate how the results from these systems are used to help make recommendations regarding lead optimization and the potential for clinical hepatotoxicity (acute liver failure). Further, the use of these in vitro models to assess CYP450 enzyme induction potential and biomarker identification will also be described.

In Vitro Cell Systems

The cell-based systems described herein include immortalized cell lines and primary hepatocyte cultures. The immortalized cell system is comprised of a series of five cell lines utilized together as an assay system. These cells were originally derived from normal human hepatocytes (Pfiefer et al., 1993). The parent cell line has no significant phase I and phase II metabolizing capabilities based upon in-house characterization.
studies that examined mRNA expression levels, protein levels and protein activities for the major CYP450 enzymes and uridine phosphogluconuronyltransferase (UDGFT) I and II isoforms as compared to primary human hepatocyte expression (Andrews et al., 2003). Four “daughter” cell lines were derived from the parent cell line. Each of the daughter cell lines has stable expression of an individual human CYP450 enzyme under the control of a cytomegalovirus (CMV) promoter (Mace et al., 1997). The CYP450 enzyme expression and activity of these four cell lines are markedly elevated (25- to 90-fold) when compared to primary human hepatocytes (Figure 1). Therefore, high CYP450 activity in combination with the low rate of conjugation results in a maximized ability to produce reactive intermediates from test compounds. These CYP450-transfected cell lines have also been shown to metabolize specific substrates and generate metabolite profiles qualitatively similar to those produced by isolated human microsome and supersome preparations (Andrews et al., 2003). As such, each of these 5 cell lines represent an exaggerated case of metabolizing enzyme disruption, e.g., over- or underexpression of 1 or more enzymes, which may predict a rare situation in which this would exist clinically, e.g., as a consequence of genetic polymorphism and/or environmental factors.

The primary cell culture system is well-established and consists of confluent cultures of freshly isolated hepatocytes from humans or preclinical species (Acosta et al., 1985; Butterworth et al., 1989; Ulrich et al., 1995; Guillouzo et al., 1997; Groneberg et al., 2002). Primary hepatocyte cultures have the full complement of metabolizing enzymes, albeit with inherent donor-to-donor variability, and thus represent an assay system that is more representative of hepatocytes in vivo as compared to immortalized cells (Guillouzo et al., 1993).

The immortalized cell line series consisting of CYP450 enzyme-transfected cells and the non-metabolizing parental cell line is currently routinely used in-house during the early discovery process (i.e., prior to in vivo testing) to predict the potential for clinical acute hepatotoxicity, i.e., acute liver failure. Specifically, this 5-cell-line set is used as a first-tier screen to assess whether toxicity may occur, whether toxicity is due to the parent compound or an active metabolite, and whether, in the instance of a toxic parent compound, there is CYP450 detoxification of this toxic compound. Based upon historical information, 90% of all drugs are metabolized by CYP450 enzymes (Watkins et al., 1990). Of these, 6 isoforms, 3A4, 2C9, 2D6, 2C19, 2E1 and 1A1/2, account for 95% of all CYP450-mediated reactions with 3A4-mediated metabolism accounting for 65% percent of this total (Zimmerman, 1999b). Clinically relevant polymorphisms have been identified for the 2C9, 2C19, and 2D6 isoforms (Fromm et al., 1997; Nebert, 1997; Tanaka, 1999; Poosup et al., 2000; Roses, 2000). The 4 CYP450 enzyme isoforms, 3A4, 2C9, 2C19, and 2D6, were chosen for use in the immortalized cell cytotoxicity assay because these enzymes represent 1 that is largely responsible for metabolism of drugs (3A4) and 3 with relevant polymorphisms that may contribute to toxicity.

The assay was specifically developed to predict the potential for acute direct or metabolism-mediated hepatic necrosis and not immune-mediated or cholestatic injury. The rationale for this endpoint was due to the fact that nearly 50% of all drug-related hepatic damage is hepatic necrosis (Zimmerman, 1999a), and that modeling of immune-mediated and cholestatic injury is complex and may require coculture systems that are not conducive to medium- or high-throughput screening (Guillouzo et al., 1997). In addition, these assessments require minimal compound, i.e., 2 mg, which is an important consideration during the discovery process when resources to allow substantial compound synthesis are not present.

The basis of the assay is determination of an IC50 value for cytotoxicity using a 12-point dose-response curve. The endpoint evaluated is a decrease in energy status of the cell through mitochondrial function, i.e., impaired tetrazolium salt reduction (MTS) or decreased ATP levels. This is a sensitive, early indicator of toxicity that is common to many mechanisms of cellular injury (Marshall et al., 1995; Sun et al., 1997). Confluent cultures are exposed for 20 hours to compound and dimethyl sulfoxide (DMSO) vehicle. In

**Applications During Lead Optimization**

The following section describes 3 examples of the uses of these in vitro systems during the discovery lead optimization process. The data from these assays are used to help guide decision making with regard to compound ranking early in the discovery process, to predict the safety “profile” of these compounds and generate recommendations for clinical monitoring, and to optimize or develop additional in vitro or in vivo preclinical screening assays. These assay systems are also used in a more traditional manner for investigational and mechanistic studies. This latter topic has been extensively covered elsewhere (Acosta et al., 1985; Butterworth et al., 1989; Ulrich et al., 1995; Guillouzo et al., 1997; Groneberg et al., 2002).

**Cytotoxicity as a Predictor of Clinical Acute Hepatotoxicity:** The immortalized cell line series consisting of CYP450 enzyme-transfected cells and the non-metabolizing parental cell line is currently routinely used in-house during the early discovery process (i.e., prior to in vivo testing) to predict the potential for clinical acute hepatotoxicity, i.e., acute liver failure. Specifically, this 5-cell-line set is used as a first-tier screen to assess whether toxicity may occur, whether toxicity is due to the parent compound or an active metabolite, and whether, in the instance of a toxic parent compound, there is CYP450 detoxification of this toxic compound. Based upon historical information, 90% of all drugs are metabolized by CYP450 enzymes (Watkins et al., 1990). Of these, 6 isoforms, 3A4, 2C9, 2D6, 2C19, 2E1 and 1A1/2, account for 95% of all CYP450-mediated reactions with 3A4-mediated metabolism accounting for 65% percent of this total (Zimmerman, 1999b). Clinically relevant polymorphisms have been identified for the 2C9, 2C19, and 2D6 isoforms (Fromm et al., 1997; Nebert, 1997; Tanaka, 1999; Poosup et al., 2000; Roses, 2000). The 4 CYP450 enzyme isoforms, 3A4, 2C9, 2C19, and 2D6, were chosen for use in the immortalized cell cytotoxicity assay because these enzymes represent 1 that is largely responsible for metabolism of drugs (3A4) and 3 with relevant polymorphisms that may contribute to toxicity.

The assay was specifically developed to predict the potential for acute direct or metabolism-mediated hepatic necrosis and not immune-mediated or cholestatic injury. The rationale for this endpoint was due to the fact that nearly 50% of all drug-related hepatic damage is hepatic necrosis (Zimmerman, 1999a), and that modeling of immune-mediated and cholestatic injury is complex and may require coculture systems that are not conducive to medium- or high-throughput screening (Guillouzo et al., 1997). In addition, these assessments require minimal compound, i.e., 2 mg, which is an important consideration during the discovery process when resources to allow substantial compound synthesis are not present.

The basis of the assay is determination of an IC50 value for cytotoxicity using a 12-point dose-response curve. The endpoint evaluated is a decrease in energy status of the cell measured by loss of mitochondrial function, i.e., impaired tetrazolium salt reduction (MTS) or decreased ATP levels. This is a sensitive, early indicator of toxicity that is common to many mechanisms of cellular injury (Marshall et al., 1995; Sun et al., 1997). Confluent cultures are exposed for 20 hours to compound and dimethyl sulfoxide (DMSO) vehicle. In

**Figure 1:** The steady-state mRNA expression of cytochrome P450 isozymes 3A4, 2C9, 2C19, and 2D6 is greatly elevated in CYP450-transfected, immortalized human hepatocyte cell lines (THLE-3A4, THLE-2C9, THLE-2C19, THLE-2D6) as compared to primary human hepatocyte (PHH) cultures. Data is expressed as fold-increases in expression over primary human hepatocyte expression levels (black bar).
addition, a known human hepatotoxic drug, perhexiline, and nonhepatotoxic drug, theophylline, are used as positive and negative controls, respectively.

To evaluate and validate the ability of this assay system to predict the potential for clinical hepatotoxicity, 679 marketed pharmaceuticals were tested. Structure, pharmacology, human toxicity, and pharmacokinetic data were obtained for all of these drugs to achieve chemical and therapeutic target diversity and to relate the in vitro results to clinically relevant exposures and outcomes (Andrews et al., 2003). Overall, the results of the validation study correlated well with clinical outcomes. The assay correctly predicted 585/587 nonhepatotoxic drugs, 15/21 severely hepatotoxic drugs, i.e., “black-box warnings” or withdrawn drugs, and 51/71 variably hepatotoxic drugs (Andrews et al., 2003). With regard to the 6 severe hepatotoxicants not identified in the assay, in all instances, the false-negative results were linked to the absence in the assay of the enzyme necessary for metabolism to the reported reactive metabolite. The 20 false-negative predictions in the “variable hepatotoxicant” group all represented drugs that primarily caused cholestasis in humans.

Based on the findings of the validation set, a test article with an IC$_{50}$ value of 50 μM or less in any of the 5 cell lines is interpreted to have an increased potential risk for clinical hepatic liabilities (Table 1).

In this case, the IC$_{50}$ values for each of the 5 cell lines are evaluated together to further interpret the contribution of metabolism by CYP450 enzymes to the development of toxicity. Examples of three potential outcomes related to toxicity are shown in Figure 2. The first example is perhexiline maleate, a drug for which toxicity is thought to be mediated by the cationic amphiphilic structure of the molecule and unaffected by CYP450 metabolism (Halliwell, 1997). Danazol is a drug which is metabolized by CYP3A4 leading to the formation of an active metabolite and is an example of bioactivation. The mechanism of danazol hepatotoxicity is not clearly understood at this time, but the selective inactivation of the CYP3A4 enzyme by its metabolite has been well documented and may contribute toward toxicity (Betz et al., 1981; Konishi et al., 2001). Finally, felbamate metabolism is an example of a CYP450-mediated detoxification pathway. Felbamate toxicity is linked to the formation of a toxic metabolite (atropaldehyde) by esterases (Dieckhaus et al. 2002), and the particular sensitivity of humans to this effect has been attributed, in part, to a decrease in the CYP450-mediated metabolic pathways when compared to preclinical species (Dieckhaus et al., 2000).

**TABLE 1.—Immortalized cell line cytotoxicity assay interpretation criteria and recommended follow-up action.**

<table>
<thead>
<tr>
<th>IC$_{50}$ value (μM)</th>
<th>Interpretation</th>
<th>Comment</th>
<th>Second-tier primary human hepatocyte cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;50</td>
<td>Increased risk for clinical hepatic liability</td>
<td>Liability unless compound is very potent or poorly soluble</td>
<td>Tests compounds of interest for importance of metabolizing pathway</td>
</tr>
<tr>
<td>&gt;50</td>
<td>Reduced risk for clinical hepatic liability</td>
<td>Tests compounds for non-P450-mediated toxicity</td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 2.—Example potential outcomes of the immortalized hepatocyte cell line system with respect to hepatic toxicity.** Perhexiline maleate, an inherently toxic compound that is not detoxified by cytochrome P450 enzymes (A). Felbamate, a toxic compound that is detoxified by all 4 cytochrome P450 enzymes in the assay system (B), and cytochrome P450-3A4-mediated bioactivation of danazol (C).
The limitations of this assay are considered when evaluating the results. These limitations include predictive value limited to a single endpoint (hepatocellular necrosis), difficulty in separating major and minor metabolic pathways, and lack of sensitivity due to the lack of a specific enzyme necessary for metabolism to the reactive metabolite. Recognizing these limitations, the use of this assay in early discovery is restricted to 2 approaches. In the first instance, it is used early in the discovery process to screen several compounds, e.g., > 10, that may be in the same or different chemical class. In this capacity the assay serves as a screen to evaluate the potential for hepatotoxicity across candidate compounds for lead optimization ranking, and across chemical classes for evaluation of potential structure activity relationships (SAR) to toxicity. This screening allows a first assessment of the potential for hepatic liability and for selection of lead compounds; it is not meant to result in a “go/no-go” decision. In instances in which a compound is cytotoxic, the IC50 value is compared to ED50 values for in vitro efficacy studies as well as in vivo toxicology, and toxicokinetic data, which may have been obtained in preclinical species during efficacy testing. Secondly, this assay is part of a larger profiling paradigm, which takes into account absorption/distribution/metabolism/elimination (ADME) properties and traditional in vivo animal toxicology studies to develop a well-rounded assessment of the potential for hepatotoxicity for a lead candidate compound. In this paradigm, primary human hepatocyte cultures from multiple donors are used in a manner identical to the immortalized cell assay, i.e., IC50 interpretation, as a second tier assessment of lead compounds, because primary culture cells have a more complete set of phase 1 and phase 2 metabolizing enzymes (Guillouzo et al., 1993). The use of primary hepatocytes would therefore give a better assessment of the metabolic route of the drug in a human. In addition to assessment in primary human hepatocytes, ADME properties are also taken into account, such as whether the compound is a CYP450 substrate or inhibitor and whether reactive metabolites are formed (Riley and Kenna, 2004). These data are all evaluated in a manner similar to that outlined in the algorithm shown in Figure 3.

The utility of this assessment is to offer guidance both preclinically and clinically regarding the potential for hepatic liability, and focus attention on potential hepatic adverse effects that may occur at very low frequencies. The results of these in vitro assays are related to both in vitro and in vivo efficacy exposures, and the projected human dose to formulate recommendations and follow-up monitoring or investigative plans as necessary.

**CYP450 Enzyme Induction:** Drug–drug interactions are a significant issue not only related to effects on efficacious exposure levels, but also as related to the potential to cause toxicity (Ajayi et al., 2000; Fuhr, 2000; Rodrigues et al., 2001; Riley and Kenna, 2004). Two classical scenarios are associated with toxicity derived from drug–drug interactions (Li et al., 1997; Ajayi and Perry, 2000). The first involves competitive or noncompetitive inhibition of a CYP450 enzyme that is the principal route of metabolism of a drug with inherent toxicity. The result is accumulation of a toxic drug, e.g., concurrent treatment of terfenadine with other CYP3A4 substrates leading to prolonged QT intervals and development of serious ventricular arrhythmias (Ajayi et al., 2000). The second scenario is typically associated with drug-mediated induction of a CYP450, most typically 3A4, leading to enhanced enzymatic activity and thus increased production of reactive metabolites, of which there are numerous examples (Li et al., 1997; Fuhr, 2000).

With regard to enzyme induction, the common triggers for examination of this include a known class liability, characteristic changes in exposure and/or an increase in liver weight

---

**Figure 3:** In vitro multi-tier hepatotoxicity screening paradigm illustrating the use of an immortalized human hepatocyte cell line assay system followed by assessment in primary human hepatocytes and evaluation of potential metabolites.
with histological changes suggestive of microsomal induction during in vivo animal studies (Haschek and Rousseaux, 1998), or as a result of clinical trial toxicokinetic data suggestive of enzyme induction. Primary hepatocyte cell cultures have proven valuable as a screening modality since these cells express phase I enzymes that are capable of being induced by the appropriate substrate (Guillouzo et al., 1993; Li et al., 1997; Riley and Kenna, 2004). In addition, comparing results from in vitro primary cultures from preclinical species to results from preclinical in vivo toxicokinetic studies allows evaluation of in vitro–in vivo correlations. If a correlation is present, it can then be used for screening purposes, so that cross-species comparisons can be made using primary hepatocyte cell cultures, e.g., rat versus human, and therefore improve the risk assessment decisions regarding the potential issues in humans (Ballet, 1997).

The culture methods for CYP450 enzyme induction in primary hepatocyte cultures has been described previously (Guillouzo et al., 1993; Maurel, 1996; Li et al., 1997; LeCluyse, 2001; Venkatakrishnan et al., 2001). With regard to endpoint measurements, evaluation of a combination of changes in CYP450 enzyme mRNA levels and enzyme activity will give the most useful information for interpretation (Wrighton et al., 1993; Rodriguez et al., 2001; Perez et al., 2003). The major limitations of these assays are availability of acceptable human donor material and the inherent polymorphic variability in enzymatic activity across donors. Therefore, assays are typically conducted using cells from at least 2, but preferably 3, separate donors along with a known CYP450 isoform-inducing drug as a positive control.

**Biomarker Identification Using In Vitro Systems:** Useful biomarkers may include not only traditional biofluid endpoints, i.e., clinical pathology parameters, but also in vitro predictive assays, such as those described above, that signal the potential for a liability and that have been validated using clinical data. In vitro assay systems may also be useful in biomarker identification using transcriptional and proteomic profiling, not only for target-specific investigative application, but on a more global predictive level (Waring et al., 2001; Kramer et al., 2002, 2003). Thus, biomarkers identified during the discovery process can be used to develop specific in vitro screening systems, to optimize or enhance current in vitro screening systems, and in vivo in preclinical species or as bridging biomarkers.

In the example described here, the immortalized cell line overexpressing CYP450-3A4 was used to identify more sensitive and/or specific markers of cytotoxicity. These identified biomarkers potentially could be applied to enhance the accuracy of prediction by further delineating the categories of hepatotoxicants, i.e., severe versus variable, as well as suggest modes of action for toxicity.

The CYP450-3A4 overexpressing cell line was chosen, as mentioned earlier, because this CYP450 enzyme is responsible for greater than 60% of all drug metabolism. In brief, the study design was to identify a diverse group of classes of structurally and pharmacologically related compound pairs that consisted of a known human hepatotoxic drug and a known nonhepatotoxic drug. The rationale was that such a comparison would allow the pharmacologically-mediated gene changes to be subtracted from the changes associated with the hepatotoxin. A decrease in cellular ATP level was used as an indicator of early cytotoxicity in the selection of exposure levels. As a consequence, morphological changes associated with necrosis were never achieved. In the initial experiments, cultures were exposed to drugs for 3 time points, 4, 8, and 20 hours, and the dose used was the IC50 value determined in the cytotoxicity model. All nonhepatotoxic compounds were dosed at 50 µM. The endpoints for biomarker assessment included both changes in transcriptional profiles and protein levels in the conditioned media (Figure 4). This dual platform approach

![Figure 4](image-url) -- An algorithm for the use of in vitro cell lines to identify potential biomarkers of hepatotoxicity illustrating concurrent sampling for transcriptional and protein profiling.
allowed for evaluation of transcriptional and translational relationships, as well as to give a broader palette of potentially useful biomarker candidates. The results presented here are from a test-set comparison of the thiazolidinedione (TZD) PPARγ agonist compounds pioglitazone and troglitazone. Pharmacologically, pioglitazone is approximately 100-fold more potent than troglitazone; however only troglitazone has been associated with significant hepatotoxicity and death (Lebovitz, 2002; Lee, 2003). These two compounds have greater than 90% structural similarity. In the in vitro immortalized hepatocyte cell line assay for cytotoxicity, pioglitazone never achieved an IC₅₀ (maximal dose 350 µM) and so is interpreted as nontoxic, while the IC₅₀ for troglitazone was ~34 µM in the nonmetabolizing parental cell line and in each of the CYP450 overexpressing cell lines, i.e., interpreted as potentially hepatotoxic.

Changes in transcriptional expression were examined using unsupervised and supervised methods. In the unsupervised method, the overall change in the entire expression profile for each compound was compared to the overall expression changes induced by the vehicle, DMSO, using regression analysis. In addition to pioglitazone and troglitazone, 2 other TZD drugs, cigitazone and rosiglitazone, were also compared. From this global gene expression analysis, which does not discriminate pharmacologically induced gene changes from those associated with injury, compounds can be ranked by which overall gene profiles look most like DMSO, i.e., R² value near 1.0 (Figure 5). Based upon this assessment, the safety ranking order for the predicted development of hepatotoxicity would be DMSO, rosiglitazone, pioglitazone, cigitazone, and troglitazone. Thus, an unsupervised assessment of the entire expression profile of compounds in a similar structural and/or pharmacological class may be helpful in ranking compounds, e.g., when attempting to identify optimal lead compounds. This approach can be used on any cell line or from organs obtained from preclinical samples. However, this approach is not useful alone and is best considered with all other in vitro and in vivo efficacy/toxicity data and any other literature related to a drug class, if that information exists.

Supervised assessment of gene expression changes can be performed in a variety of ways. In this example, a t-test was performed, which compared troglitazone-induced gene changes versus combined gene changes associated with both pioglitazone and DMSO treatment. Such a comparison should separate background and pharmacologically induced gene changes from those associated with troglitazone-induced toxicity. Using the t-test comparison (p < 0.001), 40 genes were identified which were significantly associated with troglitazone treatment after 8 hours. Principal component analysis (PCA) using these 40 genes clearly segregated the 3 treatments (Figure 6). Further, comparison of additional TZD drugs, cigitazone and rosiglitazone, and 3 proprietary compounds (A, B, C), showed a clear segregation of compounds and demonstrated which compounds were more “like” DMSO or troglitazone (Figure 6). Comparison of the supervised gene expression ranking to DMSO and the known clinical outcomes shows good concordance (Gale, 2001) (Table 2).

Thus, with regard to utilizing these results for risk assessment decisions, they may be used to help rank compounds, to identify specific gene changes to further evaluate the mechanistic potential of off-target or exaggerated pharmacological activity, and for biomarker identification. As with unsupervised analysis data, these findings are used to help guide decision-making and further investigative work and not as a “go/no-go” determinant.

Concurrent identification of potential protein biomarkers of hepatotoxicity from the same experimental system used for transcriptional profiling allows for efficient use of resources, reduces inherent interexperiment variability, and, most importantly, is a more appropriate comparison of compound-induced changes at both the transcriptional and translational levels. The use of multidimensional packed capillary high performance liquid chromatography coupled to tandem mass spectrometry (LC/LC/MS/MS) allows for simultaneous identification of large numbers of soluble proteins from a relatively small volume of culture medium (Gao et al., 2004). Using this technology, 2 proteins, 14-3-3 zeta and macrophage migration inhibitory factor (MIF), were highly associated with troglitazone treatment versus pioglitazone and DMSO treatment in both immortalized and primary hepatocyte cultures. The identity of these proteins was confirmed using Western blot and enzyme-linked immunosorbent assay (ELISA) analyses (Gao et al., 2004). Changes in 14-3-3 zeta protein levels did not correlate with changes in transcriptional expression levels, thus demonstrating the usefulness of combined protein and transcriptional profiling for efficient identification of biomarkers.

The use of in vitro systems for biomarker identification has several major limitations. This is especially true for the use of immortalized cell lines, which are genotypically and phenotypically different from organ systems (Riley and Kenna, 2004). Likewise, these cultures represent single-cell systems, so can only model events that directly affect the cells being evaluated. Finally, the assay conditions are highly unnatural when compared to in vivo conditions, and the type of assay, e.g., cytotoxicity, biases all other results. However, in vitro systems are used for reasons of practicality and reproducibility, and when the limitations of these systems are taken into consideration with regard to interpretation, and post hoc analyses are used to verify the results, such systems can be utilized. In the example described here, further characterization of the identified biomarkers has included evaluation in primary human hepatocytes and in nonhepatocyte cell culture systems, as well as evaluation in animal models of hepatic injury and human biofluids. These technologies offer great power and promise for the identification of biomarker genes and proteins. However, the characterization and validation of these potentially new biomarkers requires extensive time and resources, and this should be a consideration when conceptualizing the “goal” of biomarker identification.

---

**Table 2.—Comparison of unsupervised transcriptional profile ranking to clinical outcomes.**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Clinical outcome</th>
<th>Gene profile rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosiglitazone</td>
<td>No/rare hepatotoxicity</td>
<td>1</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>No/rare hepatotoxicity</td>
<td>2</td>
</tr>
<tr>
<td>Ciglitazone</td>
<td>Elevated liver function tests</td>
<td>3</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>Hepatotoxicity</td>
<td>4</td>
</tr>
</tbody>
</table>

*Gale (2001); Leibowitz (2002); Lee (2003).*
FIGURE 5.—Unsupervised regression analysis of transcriptional profiles from immortalized cell lines exposed to the thiazolidinedione (TZD) drugs, pioglitazone, rosiglitazone, ciglitazone, and troglitazone, compared to dimethyl sulfoxide (DMSO) vehicle (A) shows quantitative differences among the 4 drugs that can be used to rank compounds for safety, i.e., profiles most “like” DMSO (B).
FIGURE 6.—Principal component analysis (PCA) using a troglitazone-induced hepatotoxicity gene profile, made up of 40 genes significantly associated with troglitazone treatment, demonstrates a distinct separation from pioglitazone- and DMSO-related changes (A). Further comparison using the troglitazone-induced hepatotoxicity gene profile to additional thiazolidinedione drugs and proprietary compounds demonstrates ability to segregate compounds for safety ranking purposes (B).

CONCLUSIONS
Experimental in vitro modeling systems have utility in predictive toxicology profiling, identification of potential biomarkers and in mechanistic studies as long as the assumptions and limitations of the systems are realized. The purpose of the modeling system should be rigorously defined and the model endpoints validated based on real clinical outcomes for predictive assays or outcomes that have a mechanistic basis.

To maximize the predictive value, data obtained from these systems should not stand alone, but should be viewed in the context of a multitiered approach and assessed in combination with other toxicology, efficacy, and toxicokinetic parameters including traditional in vivo studies. Finally, care must be taken to relate these results to in-life or projected human exposures for efficacy and toxicity. The combination of results from well-validated in vitro systems, new technologies and more traditional toxicity studies will likely result in more robust risk assessment and decision-making. Specifically, these systems are used to identify potential issues and thereby attempt to minimize these risks during the discovery lead optimization process, as well as understand the relevance of this
risk to humans. By “flagging” the potential liabilities early on in discovery and attempting to understand their relevance, more useful recommendations and guidance with regard to follow-up studies or monitoring during the development process may be possible.

ACKNOWLEDGMENTS

Appreciation is expressed to Dr. Michael Neubauer for performing the unsupervised and supervised methods for the analysis of the transcriptional profiles and to Drs. Jun-Hsiang Lin and Shen-Jue Chen for their work in overseeing and completing the expression profiling experiments.

REFERENCES


