Use of Proteomic Methods to Identify Serum Biomarkers Associated with Rat Liver Toxicity or Hypertrophy

DAVID E. AMACHER,1* RICK ADLER,2 ATHULA HERATH,3 and R. REID TOWNSEND4

Background: Our objectives were to identify serum marker proteins in rats that might serve as sensitive indicators of hepatomegaly, hepatocellular necrosis, or hepatobiliary injury and to use them to analyze data from a collaborative proteomics project.

Methods: In each of 4 studies comprising the collaborative project, rats were given 1 of 4 compounds that target the liver through different mechanisms. Sera and liver samples were collected by terminal bleeds at 1 of 3 postdose time points. Sera were depleted of major secretory proteins and then separated into protein features by 2-dimensional gel electrophoresis (2DGE). Liver specimens were also processed and subjected to 2DGE. Protein spots that significantly increased or decreased in quantity after drug treatment were recovered, digested, analyzed by mass spectroscopy, and compared with available databases for identification. Criteria for further consideration were (a) temporal expression (i.e., increase or decrease at early, fulminant, or recovery periods), (b) known biological function, (c) probable hepatic origin, and (d) any previous association with toxicity in published studies. Markers that changed significantly at the early time point were important because of their potential sensitivity for signaling minimal damage.

Results: Vitamin D–binding protein, paraoxonase, cellular retinol-binding protein, malate dehydrogenase, F-protein, and purine nucleoside phosphorylase were identified as empirically confirmed serum markers for hepatic effects in drug-treated rats.

Conclusion: Proteomics can be applied for the identification and confirmation of peripheral biomarkers for altered liver function after toxicant exposure.

© 2005 American Association for Clinical Chemistry

Hepatic responses to chemical toxicants can be acute or chronic. Histologically they can be characterized as hepatocellular or hepatobiliary, and they can be of intrinsic or secondary origin. Tissue injury by hepatotoxicants depends on the dose and duration of exposure, the principal cell population affected, and the mechanism of action. Conventional diagnostic monitoring makes use of noninvasive serum chemistry and hematology markers that are often associated with known cellular damage or functional changes, and histopathologic examination, which requires either biopsy or necropsy specimens. We used a series of archetypal liver-targeting agents to model specific hepatic injuries or changes often encountered in safety evaluation studies and then searched for chemically induced alterations in the expression of highly specific gene products. The latter may serve as particularly sensitive indicators of various hepatic effects under experimental conditions not necessarily producing frank tissue damage or functional alteration. Thus, these gene products may function in restoration, detoxification, or protection or may be involved in cell-signaling pathways leading to the selective death of damaged cells or compensatory proliferation of surviving cells.

Two-dimensional gel electrophoresis (2DGE)5 permits the resolution and analysis of up to several thousand proteins from complex mixtures such as liver homoge-
Proteomic analysis by 2DGE can determine the steady-state condition of the proteome, including posttranslational modification, turnover/stability, subcellular compartmentalization, and possibly drug-protein binding. Analysis results are easily evaluated by comparison with conventional toxicity data. Of particular interest in preclinical and clinical safety assessment are biomarkers in the peripheral circulation that are quantitatively altered functionally or morphologically after exposure to liver toxicants. Previous rat studies have clearly established the value of tissue proteomic profiling for examples that include a hypoglycemic agent and a statin hydroxymethylglutaryl-CoA reductase inhibitor for both efficacy and toxicity and an antidiabetic candidate for drug-induced liver steatosis (1). Even more important would be applications involving proteomic profiling that include both liver and sera.

As part of a Pfizer/Oxford Glycosciences collaboration, 4 drugs differing in their mechanism for producing hepatic damage or functional change were used to induce hepatotoxicity or hepatomegaly in male CD\(^\text{\textsuperscript{\textdegree}}\) rats. Acetaminophen has been associated with hepatocellular necrosis or hepatotoxicity or hepatomegaly in male CD\(^\text{\textsuperscript{\textdegree}}\) rats. Acetylation, 4 drugs differing in their mechanism for producing

**Proteomics Procedures**

Venous blood was collected at the time of necropsy. Serum was obtained by centrifugation, and 100 μL was added to 15 μL of protease inhibitor and frozen at -80°C. At the same time, a 2-g section of the median lobe was removed to a conical tube and quick-frozen in liquid nitrogen. The frozen liver section was later pulverized for 1 min with a freezer mill, after which 10-20 mg of pulverized liver was solubilized with 10 μL of protease inhibitor and 1.5 mL of lysis solution and mixed. The sample was allowed to remain at room temperature for 10 min and then was mixed again and pelleted with an Eppendorf 5417R Centrifuge for 10 min at 13,000 rpm and 4°C. The resulting supernatant was frozen at -80°C. All samples were coded to conceal their identities and then were shipped to the United Kingdom where 2-dimensional gradient gels were spotted and run by the Oxford Glycosciences staff. A detailed description of the 2DGE procedure, analysis of gel images, and methods for protein identification by mass spectrometry (MS) have been reported previously (6). Before electrophoresis, serum samples were passed through an affinity depletion column described previously for cerebrospinal fluid (7) to remove substantial proportions of albumin, transferrin, haptoglobin, α\(_1\)-antitrypsin, and IgG to increase the resolution of the system. After electrophoresis, gel features were visualized by use of a non denaturing fluorescent marker (OGT MP 17) and assigned pl and molecular weight values, and the volume (i.e., spot size and intensity) and percentage volume were calculated. In reference to a single master gel for each study and using Rosetta software, each feature was assigned a master cluster index (MCI) number denoting a unique protein within a tissue type. This procedure has been described in detail elsewhere (7). A representative liver master gel from the ANIT study is shown as Fig. 1. A total of 12,919 MCI (liver) or 22,002 MCI (serum) were included in the master group. Each MCI within a study was subjected to a visual.

---

**Materials and Methods**

**ANIMAL STUDIES**

For each study, male Sprague–Dawley rats [Crl:CD(S-D)BR] obtained from Charles River Laboratories were distributed into 3 groups (n = 5 per group) corresponding to 3 scheduled necropsy dates. Animals of similar weight distribution were selected by a computer-based randomization procedure. In a similar manner, rats were selected and distributed into 3 concurrent vehicle control groups (n = 5 per group) for each of the 4 studies. All studies were conducted in compliance with the US Animal Welfare Act and the ILAR Guide (1996). Rats in the 4 studies received one of the following treatments: (a) a single 1000 mg/kg dose of acetaminophen by oral intubation (20 mL/kg) or, for control animals, an equivalent dose volume of 5 g/L methylcellulose (in deionized water) vehicle on all dosing days; (b) ANIT in 1 dose by oral gavage (10 mL/kg) at 100 mg/kg or, for control animals, an equivalent dose volume of corn oil vehicle on the day of dosing; (c) either 1 or 4 daily doses of phenobarbital (100 mg · kg\(^{-1}\) · day\(^{-1}\)) by oral gavage (10 mL/kg) or, for control animals, an equivalent dose volume of deionized water vehicle on all dosing days; (d) either 1 or 3 daily doses of Wy-14, 643 (45 mg · kg\(^{-1}\) · day\(^{-1}\)) by subcutaneous injection (0.2 mL/kg) or, for control animals, an equivalent dose volume of corn oil vehicle on all dosing days. Animals were killed and tissue samples taken at the following time points: acetaminophen, 8 h, 18 h, and 3 days; ANIT, 12 h, 2 days, and 5 days; phenobarbital, 2 h, 5 h, and 12 days; and Wy-14, 643, 2 h, 4 h, and 15 days. These discrete time points were individually selected for each compound based on published data and internal experience with these same chemicals, but included 4 categories for each drug: (a) an early time point, before manifestation of toxicity (controls); (b) concurrent with the early appearance of lesions or clinical chemistry markers; (c) late stage when hepatic injury is fully manifest or fulminant; and (d) post recovery after cessation of treatment.
quality-control inspection to confirm its validity. Only MCI that were significantly increased or decreased ($P < 0.05$) compared with a control master gel were evaluated further. Gel pieces (MCIs) were subjected to trypsinolysis, and the supernatant was extracted. A small portion (~10%) was directly assayed via matrix-assisted laser desorption/ionization (MALDI). The MALDI spectrum was used as a first quality-control tool and also to give a list of masses for the next step. The remainder of the sample was then submitted for tandem MS analysis (by liquid chromatography quadruple ion trap if the peaks were high intensity or by quadruple time of flight if the peaks were low intensity). The masses obtained from MALDI guided the operators concerning which ions to analyze further. The tandem spectra were used for protein identification by comparison of a peptide sequence tag with a sequence in a rodent public domain database. Those not identified in the former were then compared with a human database for possible matches to human homologs.

### STATISTICAL ANALYSES

For the conventional clinical pathology and liver weight data, statistical analyses were performed separately for each treatment group vs the corresponding control group as follows. For each collection period, each treated group mean was compared with the control group mean. The Dunnett multiple comparison procedure was used if a preliminary Bartlett test for variance homogeneity was not significant at the $\alpha = 0.05$ level. If there was significant variance heterogeneity, the Cochran–Cox modified $t$-test was used for comparison between treated and control group means. Statistical significance of the comparisons was indicated at both the $\alpha = 0.05$ and 0.01 levels. Tests were 2-tailed. For the proteomics data, $P$ values were obtained by both the Wilcoxon rank-sum test (with substitution for missing values) and the Student $t$-test. With the Wilcoxon rank-sum test, we characterized MCIs by a significant difference between treated (a minimum of 4 of 5 animals were required for consideration) vs control at $P < 0.05$. Results were clustered according to a double Venn diagram into early, full, and recovery categories, which are illustrated in Fig. 2, with the overlap showing cofrequency. Because of resource limitations, only features falling into categories 1, 4, and 5 were annotated and are described here.

### Results

#### ANIMAL STUDIES

A detailed description of the gross pathology observations and histopathology findings for each of the 4 studies is given in file 1 of the Data Supplement that accompanies the online version of this article at http://wwwclinchemorg/content/vol51issue10/. The clinical chemistry changes for these studies are summarized in Table 1 of the online Data Supplement.

#### PROTEOMICS RESULTS

For each of the 12,919 or 22,002 MCIs in the master groups, a differential analysis between control and treated samples was performed at 3 time points, early, peak (full), toxicity, and recovery. In addition to the comparisons by the Wilcoxon rank-sum test and $t$-tests based on the null hypothesis that the treated and control samples have equal means, fold changes were calculated. These were the ratios of the mean of the treated samples to that of the control and were used to measure up- or down-regulation. On the basis of statistical significance, the MCIs were examined for possible errors in feature quantification and mismatches. Subsequently, MS-based identification of gel features was performed as described in the Materials and Methods. For the liver tissues, 180 gel images and 220,781 features were analyzed by use of 6 master groups, yielding 12,919 MCIs and 184 cut features (post–quality-control analysis), of which 124 were annotated. For the serum samples, 179 gel images and 187,377 features were analyzed by use of 6 master groups, yielding 22,002 MCIs and 243 cut features (post–quality-control analysis), of which 101 were annotated.

In the same study animals, 61% of the serum proteins that were significantly altered after drug treatment were down-regulated, whereas 84% of the altered liver proteins were up-regulated. The liver and sera profiles for each study are shown in Figs. 1 and 2 of the online Data Supplement. In sera, the largest proportion of proteins that were altered (33%) was affected in the early time period. The identification of those annotated proteins with likely toxicologic significance that were most affected by drug treatment are tabulated for liver and sera, respectively (see Tables 2 and 3 of the online Data Supplement). A summary analysis of 19 significantly altered serum proteins from the 4 studies can be found in Table 1. These were selected from a total of annotated protein features based on criteria that included (a) probable or exclusive liver origin, (b) evidence of a human homolog, and (c) evidence of a statistically significant ($P < 0.05$) change with treatment. All 19 proteins in Table 1...
have been reported previously to be present in human sera or plasma and thus may be relevant clinically if altered by drug treatment with liver toxicants in preclinical studies.

Significant increases in alanine aminotransferase (ALT) occurred 18 h after treatment with acetaminophen, which causes hepatocellular death at high acute doses, but not at 8 or 72 h postdose. The principal treatment-related histopathologic finding was centrilobular necrosis evident in 5 of 5 rats at 18 h postdose. Thus, acute administration of acetaminophen produced progressive hepatocellular necrosis, an effect that subsided after a 54-h recovery period. The proteomic results indicated that acetaminophen treatment caused significant increases in 10 and decreases in 11 proteins, excluding the conventional clinical chemistry endpoints. At the fulminant time point, F-protein (hydroxyphenylpyruvate dioxygenase) was increased >5-fold in treated compared with control animals. Purine nucleoside phosphorylase (PNP) was also significantly increased, whereas paraoxonase was significantly decreased at this time point as well as at the early time point. These changes were not found at 54 h postdose, suggesting recovery or restoration. Serum F-protein has previously been cited as offering advantages over ALT for detection of minor degrees of acute liver dysfunction, particularly when only centrilobular damage is involved (9). Baseline and stimulated serum paraoxonase activities are decreased in chronic hepatitis and liver cirrhosis (10). In rats, PNP activity was demonstrated to increase 5 h after the administration of endotoxin (11).

ANIT is a hepatobiliary toxicant in rats. In this study, numerous conventional serum markers were significantly increased at the midpoint [ALT and aspartate aminotransferase (AST), alkaline phosphatase, 5′ nucleotide, total bilirubin, and bile acids], with recovery noted for most at the later time point, as expected. In the fulminant dose group, 4 of 5 treated rats had single-cell hepatocyte necrosis, and all 5 treated rats had pericholangial inflammation. In the proteomics analysis, 5 serum proteins were increased and 8 were decreased. At the middle time point, cytosolic malate dehydrogenase (MDH) was increased >5-fold in some rats. At the final time point, serum hemopexin was increased 5-fold. For enolase (phosphopyruvate hydratase) and methionine adenosyltrans-
Table 1. Summary of serum proteins characterized by possible liver origin and significantly \((P < 0.05)\) altered expression after exposure of male rats to chemicals that affect the liver.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Biological function</th>
<th>How expressed in this study</th>
<th>Known indication</th>
<th>Previous findings</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelsolin</td>
<td>Extracellular actin scavenger system</td>
<td>Decreased early (C)</td>
<td>Acts as a regulator and effector of apoptosis; muscle is the primary source</td>
<td>Valproic acid is known to cause redistribution of gelsolin</td>
<td>(26, 27)</td>
</tr>
<tr>
<td>Transthyretin</td>
<td>Transthyretin–retinol binding complex prevents glomerular loss of retinol</td>
<td>Increased in recovery stage (F)</td>
<td>Negative acute-phase protein</td>
<td>Transthyretin–retinol binding complex is affected by some toxicants</td>
<td>(28–30)</td>
</tr>
<tr>
<td>Complement C3</td>
<td>Involved in the release of the inflammatory mediators, anaphylatoxins; acute-phase reactant</td>
<td>Decreased early (F) and in fulminant stage (C)</td>
<td>May be associated with interstitial nephritis and proteinuria</td>
<td>Azathioprine and CCl sub liver toxicity causes an atypical decrease in serum C3</td>
<td>(31, 32)</td>
</tr>
<tr>
<td>α1-Macroglobulin</td>
<td>An acute-phase protein expressed in response to inflammation or injury</td>
<td>Decreased early (C, D, E) and in fulminant stage (C, E, F)</td>
<td>Localized in the hepatocyte sinusoids of rat liver</td>
<td>May participate in a negative acute-phase response</td>
<td>(33)</td>
</tr>
<tr>
<td>Paroxonase (PON1)</td>
<td>An HDL-associated esterase that detoxifies organophosphates; may prevent LDL lipid peroxidation</td>
<td>Decreased early and in fulminant stage (C)</td>
<td>Some drugs decrease activities of peripheral esterases</td>
<td>PON1 is significantly decreased in chronic liver disease</td>
<td>(10, 34, 35)</td>
</tr>
<tr>
<td>Vitamin D-binding protein (Gc)</td>
<td>Controls actin homeostasis during massive tissue necrosis</td>
<td>Increased early (C)</td>
<td>In humans, actin release caused by fulminant hepatic necrosis is complex by Gc</td>
<td>Acetaminophen-induced liver damage in hamsters alters plasma Gc concentrations</td>
<td>(36–38)</td>
</tr>
<tr>
<td>Contrapsin-like protease inhibitor 3</td>
<td>Plasma serine protease inhibitor</td>
<td>Increased early (C)</td>
<td>A glycoprotein that can be secreted from adipose tissue</td>
<td>Not typically associated with liver toxicity</td>
<td>(39, 40)</td>
</tr>
<tr>
<td>Apo E</td>
<td>Component of serum lipoprotein in rat and humans</td>
<td>Increased early (C) and in fulminant (E) and recovery (F) stages; decreased early (D, E, and F)</td>
<td>A normal serum component associated with VLDL and LDL in humans</td>
<td>Galactosamine- and CCl sub-induced liver failure suppresses expression</td>
<td>(41, 42)</td>
</tr>
<tr>
<td>Nonneural α-enolase</td>
<td>A glycolytic housekeeping enzyme; not specific to liver</td>
<td>Decreased early (D) 5-fold</td>
<td>Possible association with vasoconstrictors</td>
<td>Liver α-enolase is increased by alcohol consumption</td>
<td>(12, 13)</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Cellular iron homeostasis; major serum iron transport protein</td>
<td>Decreased early (E, 5-fold) and in recovery (E); decreased early (D) and in fulminant stage (F)</td>
<td>A negative acute-phase protein</td>
<td>Liver transferrin mRNA is decreased by aflatoxin B sub 1 toxicity</td>
<td>(17–19)</td>
</tr>
<tr>
<td>Hemopexin</td>
<td>Transports heme to liver parenchymal cells; heme scavenging</td>
<td>Increased early (F) and in the fulminant and recovery (D, 5-fold) stages; decreased early (C, D)</td>
<td>Induced after inflammation; depleted by hemolysis</td>
<td>Liver function indicator (acute-phase reactant); acts as an antioxidant</td>
<td>(15, 16)</td>
</tr>
<tr>
<td>MAT</td>
<td>MAT is necessary for methylation reactions and for synthesis of polyamines and glutathione</td>
<td>Decreased early (D) 5-fold</td>
<td>Hepatic oxygen concentrations control MAT at the mRNA level; not specific to liver</td>
<td>Hepatic MAT is down-regulated by hypoxia and thioacetamide exposure</td>
<td>(14, 43–45)</td>
</tr>
<tr>
<td>Apo A-I</td>
<td>Lipoprotein metabolism</td>
<td>Decreased in fulminant stage (C, E, and F, 5-fold)</td>
<td>Controlled primarily at the posttranscriptional level; liver is the major site of synthesis</td>
<td>Apo A-I is inducible by phenobarbital; decreased in chronic liver failure</td>
<td>(20, 21, 46)</td>
</tr>
<tr>
<td>MDH</td>
<td>A periportal marker enzyme like ALT</td>
<td>Increased in fulminant stage (D) 5-fold</td>
<td>Previously established biomarker for hepatic necrosis</td>
<td>In rats, serum MDH is increased by thioacetamide, aflatoxin B sub 1, and other toxicants</td>
<td>(47, 48)</td>
</tr>
<tr>
<td>PNP</td>
<td>Hepatic sinusoidal release during necrosis</td>
<td>Increased in fulminant stage (C)</td>
<td>Hepatocellular necrosis</td>
<td>Serum PNP is increased after endotoxin treatment in rats</td>
<td>(10, 11)</td>
</tr>
<tr>
<td>Apo H</td>
<td>A serum glycoprotein that may be involved in lipid metabolism and coagulative pathways</td>
<td>Decreased in recovery stage (F)</td>
<td>Acts as a negative acute-phase protein</td>
<td>Apo H RNA is down-regulated by inflammatory mediators</td>
<td>(43, 49)</td>
</tr>
<tr>
<td>F-Protein/HPPD</td>
<td>Involved in tyrosine catabolism</td>
<td>Increased in fulminant stage (C) 5-fold</td>
<td>Is an indicator of hepatocellular dysfunction associated with anticonvulsant therapy</td>
<td>Acetaminophen poisoning increases serum concentrations</td>
<td>(9, 50)</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>A secreted, trimeric glycoprotein that mediates interactions between cells and extracellular matrix and exhibits effects on migration and proliferation</td>
<td>Increased early (C)</td>
<td>Found in hepatocytes and bile ducts, but is not specific to liver</td>
<td>May play a role in liver fibrosis</td>
<td>(51, 52)</td>
</tr>
<tr>
<td>Retinol-binding protein</td>
<td>An α2-globulin that transports vitamin A from the liver</td>
<td>Decreased early (C)</td>
<td>Known marker for hepatocellular necrosis</td>
<td>Known to be increased by acetaminophen, but decreased by galactosamine and CCl sub administration</td>
<td>(53–55)</td>
</tr>
</tbody>
</table>

\(a\) These proteins were selected from a total of 101 annotated proteins.

\(b\) C, acetaminophen; D, ANIT; E, phenobarbital; F, Wy-14, 643; HPPD, 4-hydroxyphenylpyruvate dioxygenase.
apolipoprotein E (Apo E) and a decrease in the middle time point include a significant increase in concentration but no change in hemoglobin or plasma iron reported a marked decrease in hepatic ferritin iron concentration but no change in hemoglobin or plasma iron. Transferrin protein was decreased >5-fold at the early time point. Transferrin is the iron transport protein of vertebrate serum that is primarily, although not exclusively, synthesized in the liver (17). Transferrin mRNA concentrations are diminished by aflatoxin B-1 exposure (18). Using the isolated perfused rat liver model and phenobarbitone treatment, Morton and Tavill (19) reported a marked decrease in hepatic ferritin iron concentration but no change in hemoglobin or plasma iron concentrations accompanied by the specific enhancement of transferrin synthesis in both control and iron-deficient livers. The results here appear similar. Other changes at the middle time point include a significant increase in apolipoprotein E (Apo E) and a decrease in \( \alpha_1 \)-macroglobulin and Apo A-1. The Apo A-1 and Apo E genes are independently regulated (20). These results differ from the observation of Chao et al. (21), who found that phenobarbital increases the concentration of rat liver apo-A1 RNA, but this discrepancy is most likely attributable to the considerable time differences (16 h vs 5 days postdose in this study).

Treatment with Wy-14, 643, which is known to cause hepatic cell proliferation and peroxisome proliferation in rodents, produced significant increases in absolute and relative liver weight, particularly at the middle time point. In the proteomics analysis, 6 serum proteins were significantly increased and 7 were decreased. At the early time point, hemopexin was increased, whereas Apo E and complement C3 were decreased. Changes at the other time points generally involved other apolipoproteins or acute-phase reactants with no clear pattern.

**Discussion**

Two-dimensional electrophoresis is an open separation system in which the separation of proteins takes place on hypothesis-free dimensions of the isoelectric point and the molecular weight. Proteins are often modified posttranslationally, and proteomic analysis provides a unique expression map that includes these modifications (5). Specific biological activity is created by glycosylated transcripts and other posttranscriptional modifications and shown by specific protein fragments. Two-dimensional electrophoresis is the most convenient and straightforward method for identifying such forms, and no other complex separation mechanism or assay exists. Proteomic analysis of rat liver homogenates has been applied to the identification of protein changes corresponding to xenobiotically induced hepatomegaly (22) and is particularly useful for the detection of drug-induced posttranslational protein modifications after drug-associated liver injury that would not be detectable via mRNA profiling (23). Other workers have recently characterized all of the major protein components in serum from healthy CD rats and noted changes in rats treated with hypolipidemic or antiinflammatory drugs (24). The present study is unique in that the proteome from both liver and serum from CD rats was analyzed after treatment with 1 of 4 different xenobiotics.

In this study we critically evaluated the summary data produced by the Pfizer/OGS proteomics collaboration and identified peripheral proteins that might serve as sensitive, early indicators of dose-dependent liver toxicity involving hepatocellular necrosis, hepatomegaly, or hepatobiliary injury. Although a major limitation was the significant underrepresentation of rodent sequences in the public domain databases, the study yielded some potential biomarker candidates for further consideration. Of 101 protein features that met the quality-control and statistical relevance criteria, we identified 19 possible biomarkers for altered liver function after in vivo administration of 3 categories of hepatic agents. A few markers that were observed to both increase and decrease in the same study, perhaps because of identification errors, were eliminated from consideration. It should be noted that some of the serum proteins that were significantly affected by drug treatment are generally considered to be nonhepatic in origin, are not specific to liver (e.g., gelsolin), or are of questionable relevance (e.g., serum protease inhibitor). Of particular interest because of their potential sensitivity were hepatic markers that changed significantly at the early time point. These serum marker proteins were not consistently and proportionally expressed over the 3 time periods for a single treatment and were not accompanied by corresponding increases or decreases in the liver within the context of the proteins whose definitive identity has been established by MS. Proteins with functions associated with detoxification or repair and proteins whose expression had previously been reported in toxicant-dosed animals were of particular interest. Some, such as serum F-protein (4-hydroxyphenylpyruvate dioxygenase) and MDH, have been documented previously as serum indicators of toxicity but are not necessarily in common use today in preclinical or clinical drug development studies.

Except for an up-regulation of hepatic ALT protein (proteomic data) that followed an increase in serum ALT activity (clinical chemistry data) 54 h later in the acetamin-
ophen recovery group, there was no correlation between the liver and serum protein changes for any of the 4 test groups. For example, decreases in the liver mitochondrial AST protein in the Wy-14, 643 animals at 2 days were not matched by a corresponding change in AST clinical chemistry activities or serum protein data for any of the 3 time periods. In general, treatment with these chemicals agents led to the up-regulation of liver proteins involved in detoxification or response to stress, such as catalase, glutathione peroxidase, sulfotransferases, serine proteinase inhibitor, heat shock proteins or chaperonins, and glutathione S-transferase. Serum protein changes were more likely to be characteristic of down-regulation, with some acute-phase reactants or previously identified biomarkers such as F-protein being altered in the expected manner.

In general, changes in individual protein content in treated vs control animals for these selected proteins were small (<3-fold) and not correlated with any significant changes in similar liver proteins. A few serum proteins (α-enolase, transferrin, hemopexin, MDH, and F-protein) were altered 5-fold or greater in treated vs control animals at some time points with some treatments. Further considerations, such as whether the protein was considered exclusively or predominantly of liver origin and the availability of established clinical assays, suggests that 5 proteins are of special interest as serum markers for hepatic toxicity or functional alterations in rats. These are vitamin D-binding protein, PNP, and MDH, which increased under drug treatment, and paraoxonase and cellular retinol-binding protein, which decreased under drug treatment conditions. Except for PNP, these significant changes occurred in the early treatment period, before extensive morphologic changes, which suggests the desired characteristic of early manifestation and, hence, sensitivity. Further evaluation by this laboratory of 3 of these markers has recently been reported in a study that included both male and female rats (25).

In summary, serum proteomic analysis data revealed the altered expression of early markers that may have future application in clinical chemistry analyses. These protein changes were statistically significant (P <0.05), and MS-based sequencing of peptides derived from these proteins in 4 rat serum studies has identified both protein markers previously cited as possible indicators and known proteins with potentially novel associations with hepatic effects. As markers of early and mechanism-specific drug hepatotoxicity, these proteins may be valuable for predicting toxicity and/or revealing toxic effects of novel drug candidates earlier than conventional methods.

We wish to express our thanks for the support provided by the following: Thomas R. Steiger, Lisa Fasulo, William Kluwe, Michael Silber, Daniel Lettieri, Anthony Perretta, Pamela Destremps, Angelo Cacciatore, Thomas Shutsky, Timothy Ferris, the histopathology and clinical pathology staffs of Pfizer, and Jim Bruce and Gordon Holt, formerly of Oxford Glycosciences.

References


