INTRODUCTION TO LIVER TOXICITY

The liver is the largest internal organ in the human body and it is the main organ for the metabolism and detoxification of drugs and environmental chemicals (Klaassen, 2007). Other main functions of the liver include glucose storage and synthesis, decomposition of red blood cells, plasma protein synthesis, hormone production, and bile formation. Anatomically, the liver lies slightly below the diaphragm and anterior to the stomach, a position that facilitates maintaining metabolic homeostasis of the body. Two distinct blood supplies feed the liver: the portal vein and the hepatic artery. The portal vein carries blood containing digested nutrients from the gastrointestinal tract, spleen and pancreas, while the hepatic artery carries oxygenated blood from the lungs. The human liver consists of four lobes, and each lobe is made up of many lobules, which is defined at the microscopic scale. The classical lobule is a hexagonal-shaped unit centered around a central vein. In each functional unit, blood enters the lobules from the portal vein and hepatic artery, and then flows down past the cords of hepatocytes. The lobule is divided into three regions (Figure 13.1): (1) periportal (Zone 1) is the closest to the entering blood supply with the highest oxygen tension; (2) centrilobular (Zone 3) abuts the central vein and has the poorest oxygenation; and (3) midzonal (Zone 2) is intermediate.

Due to the blood flows from the stomach and intestine, the liver is the first internal organ to encounter a number of insults including ingested metals, drugs, and environmental toxicants (Klaassen, 2007). As a consequence, liver cells are exposed to significant concentrations of these chemicals, and liver functions can be adversely affected by the acute or chronic exposure. For example, acetaminophen (APAP) is a widely used over-the-counter analgesic and antipyretic in the United States (Hinson et al., 2010). When used at recommended therapeutic doses, APAP is rarely associated with liver injury. Unfortunately, APAP can cause fatal acute liver failure when therapeutic doses are exceeded due to the production of a highly reactive hepatotoxic metabolite in the liver (Lee, 2004). Since the liver is often exposed to the highest concentrations of orally consumed drugs, it is not surprising that the liver is often the target organ with ensuing drug-induced liver injury (DILI). DILI is a major challenge for the pharmaceutical industry and public health, since DILI is a common cause of drug development termination, drug restrictions, and post-marketing drug withdrawal (Kaplowitz, 2005). Currently, more than 1000 drugs have been reported to be associated with DILI (Shi et al., 2010). In addition, the increasing popularity of dietary supplements (DS) may contribute to the high incidence of DILI since some DS have various toxicological effects or alter the toxicity of concomitantly administered drugs (Abebe, 2002; Estes et al., 2003; Zhou et al., 2007; Dara et al., 2008; Salminen et al., 2012).

Typically, DILI has been classified in terms of the clinical liver disease which is hepatocellular, cholestatic, or mixed hepatocellular/cholestatic. Hepatocellular often involves cellular damage of the hepatocytes such as the centrilobular necrosis caused by APAP. This type of damage is often associated with elevated serum alanine aminotransferase (ALT) levels due to leakage from damaged hepatocytes. Cholestatic injury often involves damage to some part of the bile processing or excretion apparatus, resulting in impaired bile processing or excretion.
FIGURE 13.1 Microscopic structure of the liver lobule. Blood, supplied by portal vein (PV), or hepatic artery (HA), enters the liver lobule through the portal triad, which encompasses a PV, HA, and bile duct (BD). Blood flows from Zone 1 (best oxygenation) through Zone 2 and out of the central vein (CV) in Zone 3 (poorest oxygenation).

This type of injury is often associated with elevated serum bilirubin and alkaline phosphatase (ALP), indicating alterations in bile homeostasis and/or bile duct injury. Mixed injury presents with a mixture of both types of effects. Unfortunately, drugs rarely produce a single clear clinical picture, making the diagnosis of DILI difficult. For example, amoxicillin/clavulanic acid usually causes cholestatic injury but can also produce acute hepatocellular injury or a mixed type injury (Kaplowitz, 2005; Stirmann et al., 2010). The histopathological analysis of liver biopsies is the most definitive way to diagnose and confirm various types of liver diseases; however, the biopsies are invasive and not routinely performed. Because currently available clinical laboratory tests are less than ideal, there is a pressing need for new biomarkers that are not just sensitive but are also specific and prognostic.

Serum ALT activity has been historically used as a major biomarker for liver injury in humans and in preclinical studies. Damaged hepatocytes release ALT into the extracellular space with subsequent passage into the blood; however, ALT elevations can reflect nonhepatic injury, particularly skeletal muscle injury. Moreover, the concentrations of ALT do not discriminate between different etiologies of liver injury and ALT elevations can occur after a critical therapeutic window has passed. Another weakness is that ALT levels do not provide any insight into disease prognosis.

Adaptation during continued exposure to DILI drugs has been observed in preclinical and clinical studies. For example, it is noted that ~25% of Alzheimer’s disease patients receiving tacrine experience transient, asymptomatic increases in serum ALT. The majority of these patients adapt to the drug as indicated by a return of serum ALT to baseline levels despite continued treatment. Most statins can also cause serum ALT elevations in a subset of treated patients, but they often occur in the absence of histological evidence of injury. Therefore, elevated ALT levels do not necessarily indicate the severity of the liver injury and whether or not a patient will adapt to the stressor or develop fulminant liver injury.

Total bilirubin (TBL) is a biomarker associated with altered bile homeostasis and/or hepatobiliary injury. The functional reserve of the liver for processing bilirubin is large; therefore, substantial hepatic injury often occurs before alterations in TBL are observed, making TBL an insensitive biomarker. By the time TBL is elevated, there may already be substantial loss of liver function, placing the patient in danger of liver failure. Therefore, there is a need for new biomarkers that can identify the risk to patients prior to the occurrence of serious DILI.

Hy Zimmerman first noted that “drug-induced hepatocellular jaundice is a serious lesion. The mortality rate ranges from 10 to 50 percent” (Zimmerman, 1968). He realized that when drugs cause substantial hepatocyte injury that affects overall liver function and, in particular, causes jaundice as the result of impaired bilirubin processing and transport, the hepatotoxicity is likely to lead to life-threatening events (Senior, 2006). The combination of two biomarkers, ALT and bilirubin, which was later defined as “Hy’s Law,” indicates more severe injury than serum enzyme elevations alone. It is of interest that this observation has been confirmed in recent reports (Bjornsson and Olsson, 2005; Andrade et al., 2006). The significance of “Hy’s Law” is that the combination of ALT and bilirubin, neither of which by itself is sufficiently specific, seems to be highly specific for serious liver injury. Based on “Hy’s Law,” the FDA recommends a combination of tests, including serum ALT, aspartate aminotransferase (AST), and ALP activities and TBL concentration, to identify potential DILI.

In addition to the biomarkers described above, there are symptoms commonly associated with liver injury. Abdominal pain, enlargement of the liver and spleen, distended belly full of fluid, or enlarged breasts in men are common signs of liver injury (Chopra, 2001). In combination with biomarkers of liver injury, these nonspecific and specific symptoms can help a physician identify liver injury and its seriousness. Unfortunately, there are limited tools at the physician’s disposal for determining the patient’s prognosis (i.e., will the patient recover with supportive care or will the patient develop fulminant liver injury and require a transplant).

OVERVIEW OF LIVER PHYSIOLOGY, TOXICITY, AND PATHOLOGY

Bioactivation and detoxification

The liver is the main metabolic organ in the body and is considered a viable defense against environmental
transporters (OCTs/SLC22A), the multidrug and toxin extrusion transporters (MATE transporters/SLC47A), the organic anion transporters (OATs/SLC21A), and the organic anion transporting polypeptides (OATPs/SLCO). Members of ABC superfamily are important in drug toxicity, and these include P-glycoprotein (MDR1/ABCB1), multidrug resistance-associated protein (MRP/ABCC), and breast cancer resistance protein (BCRP/ABCG2) (DeGorter et al., 2012).

Many Phase I and Phase II enzymes show significant interindividual variability, which leads to various levels of exposure to the reactive metabolites. There are two primary causes of this variation: polymorphism and environmental exposure. Polymorphisms in drug metabolizing enzymes may cause dramatic differences in drug detoxification (Sim et al., 2013). Several recent genome-wide association studies (GWAS) have been conducted based on the hypothesis that polymorphisms might play a role in determining the risk of DILI (Sim et al., 2015).

Protein synthesis and catabolism

The liver plays a vital role in protein metabolism, including deamination and transamination of amino acids, plasma protein synthesis, and removal of ammonia to urea in the urine. Several of the enzymes used in the amino acid metabolism pathways (for example, ALT/AST) are commonly assayed in serum to assess liver damage since they are present at much higher concentrations in the liver than in other organs (Dejong et al., 2007). Almost all blood proteins, except gamma globulins, are synthesized in the liver (Miller et al., 1951). Albumin, which is the main protein in human blood, plays a major role in maintaining plasma osmotic pressure as well as transportation of lipids and hormones. Some liver injury can affect the concentrations of plasma albumin, and the clinical presentations are hypoalbuminemia and hyperglobulinemia (compensatory rise to offset the fall in albumin) (Farrugia, 2010). Fibrinogen and blood clotting factors (e.g. Factor XIII) are another group of plasma proteins synthesized by the liver. During blood clot formation, soluble fibrinogen is ultimately converted into insoluble fibrin strands through a catalytic activation cascade of a series of blood clotting factors. When liver function is impaired, it can lead to excess bleeding due to impaired synthesis of the blood clotting factors. Ammonia is a metabolic product of amino acid deamination and is quickly converted by the liver to urea, which is much less toxic. In humans, if ammonia is not rapidly and efficiently removed from the circulation, it will result in central nervous system disease (e.g. hyperammonemia-related coma) (Klaassen, 2007).
Bilirubin processing

Unconjugated bilirubin is the yellow product produced by the degradation of heme, which is derived mostly from the breakdown of erythrocyte hemoglobin. Unconjugated bilirubin is very hydrophobic and is circulated in the blood by albumin. Sometimes, the high concentration of hydrophobic drugs or fatty acids can cause elevation of unconjugated bilirubin due to displacement from albumin. The liver is responsible for clearing the blood of unconjugated bilirubin by conjugating it with glucuronic acid in hepatocytes. The conjugated bilirubin is then secreted into bile for further metabolism and elimination. Increased total bilirubin (TBL) causes jaundice, which is presented as yellowing of the skin and whites of the eyes. Because the metabolic capacity of the infant liver is still developing, jaundice is a common problem seen in infants due to increased serum unconjugated bilirubin levels (Cuperus et al., 2009; Cohen et al., 2010). Occasionally, jaundice may be a symptom of other health problems; for example, hemolysis generally induces an elevation in plasma levels of unconjugated bilirubin.

Covalent binding

A large body of evidence has shown that reactive metabolites play an important role in the pathogenesis of DILI (Jaeschke et al., 2002; Evans et al., 2003; Park et al., 2005). Biotransformation of drugs results principally in detoxification. However, in certain instances, reactive and highly toxic intermediates are generated, typically during Phase I metabolism (Park et al., 2005; Omiecinski et al., 2012). Such metabolites are short-lived; however, if not detoxified, they can form covalent modifications of biological macromolecules and thereby damage proteins and nucleic acids (Park et al., 2005). Some early investigations have revealed the link between reactive metabolites and chemical carcinogenesis (Miller et al., 1948; Miller et al., 1966). Reactive metabolites have also been identified from DILI drugs, such as APAP (Hinson et al., 2004), tamoxifen (Lim et al., 1994), diclofenac (Masubuchi et al., 2002), and troglitazone (Kassahun et al., 2000). In the case of APAP, at therapeutic doses APAP is predominantly metabolized by the Phase II metabolic pathways of glucuronidation and sulfation. A small portion of APAP is metabolized by Phase I enzymes to the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), which is subsequently detoxified by conjugation with glutathione (GSH) (Bessems and Vermeulen, 2001; Hinson et al., 2010). After a toxic dose of APAP, the glucuronidation and sulfation pathways are saturated with a larger amount of APAP being metabolized by the Phase I pathways to NAPQI. NAPQI is detoxified by conjugation with GSH; however, once GSH stores become depleted, NAPQI covalently binds cellular macromolecules such as proteins. Covalent binding occurs in the hepatocytes resulting in disruption of cellular function and eventual necrosis (Roberts et al., 1991; Yang et al., 2012a). Interestingly, the protein adducts can be detected in the serum from APAP overdose patients, most likely due to release from necrotic hepatocytes (Muldrew et al., 2002).

Quantification of the protein adducts may be useful in the diagnosis of the APAP overdosed patients. Although covalent binding often occurs during hepatotoxic injury, it is not necessarily sufficient by itself to cause hepatotoxicity (Liebler and Guengerich, 2005). For instance, 3-hydroxyacetanilide (regioisomer of APAP) and APAP show similar levels of covalent binding in vivo, but AMAP is not hepatotoxic, even at very high doses (Roberts et al., 1990). Troglitazone is known to be converted into reactive metabolites that generate a relatively high level of covalent binding (Yamazaki et al., 1999), although a causal relationship with hepatotoxicity has not been further established. Furthermore, the utility of protein adducts as DILI biomarkers has been limited by the fact that many protein adducts are found in the liver but not blood (Shi et al., 2010).

Covalent binding, in general, equates poorly with hepatotoxicity, but the cellular stress responses activated by reactive metabolites may play a decisive role in the toxicity. GSH is a critical cellular antioxidant, which is important in combating cellular oxidative stress that can damage cellular macromolecules. In APAP overdose, NAPQI reacts with GSH leading to depletion of the cytoprotective molecule. NAPQI covalently binds to mitochondrial proteins (Cohen et al., 1997), increases reactive oxygen species (ROS) production, impairs mitochondrial respiration, causes ATP depletion, opens the mitochondrial permeability transition pore, and makes the mitochondrial inner membrane abruptly permeable to solutes up to 1500 Da (James et al., 2003; Jaeschke and Bajt, 2006). These events lead to onset of the mitochondrial permeability transition (MPT), which is a common pathway leading to both necrotic and apoptotic cell death. Induction of the MPT causes mitochondrial depolarization, uncoupling of oxidative phosphorylation, and organelle swelling, which all result in decreased ATP synthesis and cell death (Kim et al., 2003). Many in vitro assays have been developed to measure GSH, ATP, ROS, mitochondrial functions, and cell viability (e.g. lactate dehydrogenase release), which might be useful as in vitro DILI biomarkers in predicting hepatotoxicity.
Necrosis and apoptosis

Death of liver cells is a characteristic feature of many liver diseases, such as cholestasis, hepatitis, or ischemia/reperfusion (Malhi et al., 2006). There are two patterns of cell death: necrosis (large groups of cells die) or apoptosis (individual cells die and is ATP-dependent). Damage may be restricted to certain cell types such as hepatocytes, cholangiocytes (bile duct cells), endothelial cells, stellate cells, or Kupffer cells (Ramachandran and Kakar, 2009). The acute hepatocellular necrosis is the most common expression of drug-induced hepatotoxicity (Chalasani et al., 2008). The pathological expression of hepatotoxicity may manifest itself in a zonal pattern or it may produce more diffuse damage. Often, hepatotoxicants that cause damage primarily through a reactive metabolite will produce hepatic necrosis in a zonal pattern since, even though drug concentrations are highest in Zone 1, most CYPs, necessary for bioactivation, are highest in Zone 3. For a hepatotoxicant such as APAP, CYPs in Zone 1 are at negligible levels and cannot form the reactive metabolite. In contrast, in Zone 3, CYPs are at much higher levels and form the reactive metabolite NAPQI, restricting covalent binding to this zone (Bessems and Vermeulen, 2001).

Inflammation (immune) hepatitis

Sometimes, reactive metabolites can act as haptens, which bind to protein and lead to immune responses (Uetrecht, 2008). Emerging evidence supports the hypothesis that the Kupffer, sinusoidal endothelial, and stellate cells, and newly recruited leukocytes, play critical roles in liver injury (Adams et al., 2010). The main function of the phagocytes is to remove dead cells and cell debris in preparation for liver regeneration; however, toxic mediators generated by these phagocytes can attack stressed hepatocytes (Jaeschke et al., 2002). Hepatic inflammation is a common histopathology finding associated with a wide range of liver diseases (Reddy and Sambasiva Rao, 2006). DILI is frequently associated with lymphocytic infiltrate, and the extent of the inflammation determines the progression and severity of liver injury. Halothane is a classic example that links reactive metabolites with immunoallergic hepatitis. It has all the clinical hallmarks of an immunological perturbation, which include time of presentation, general clinical features, and greatly enhanced reaction on re-exposure to the drug (Martin et al., 1993; Zimmerman, 1999).

Steatosis

Steatosis (fatty liver) is commonly caused by insulin resistance due to obesity (Saadeh, 2007). Histologically, it is characterized as hepatocytes containing excess fat and appearing to have multiple round, empty vacuoles in the cytoplasm. Steatosis can be induced by acute exposure to many chemicals, e.g. carbon tetrachloride. Often, drug-induced steatosis is reversible and does not lead to cell death. However, it may make the liver more susceptible to other insults (Sato et al., 1981) or can develop into steatohepatitis (Saito et al., 2007), which is associated with significant liver injury.

REVIEW OF EXISTING BIOMARKERS OF LIVER TOXICITY

According to the recent version of FDA guidance for industry drug-induced liver injury: Premarketing clinical evaluation, it is recommended to use a combination of four tests as DILI biomarkers (FDA, 2009). Table 13.1 (adapted from Ozer et al., 2008) lists these four biomarkers and other commonly measured serum biomarkers used in preclinical and clinical screening for hepatotoxicity.

Alanine aminotransferase

Clinical chemistry data are routinely used for noninvasive monitoring of liver disease in preclinical species and humans, and alanine aminotransferase (ALT) is the most widely used clinical biomarker (Ozer et al., 2008). ALT is responsible for the metabolism (transamination) of alanine and is found at much higher concentrations in the liver compared to other organs. When hepatocellular injury occurs, the liver-abundant enzyme ALT will leak into the extracellular space and enter the blood, wherein it shows a slow clearance rate with a half-life of approximately 42 h (Ozer et al., 2008). The typical reference range is 7–35 IU/L in females and 10–40 IU/L for males (WebMD, 2013). An elevation of serum ALT activity is often reflective of liver cell damage. Unfortunately, extrahepatic injury, such as muscle injury, can also lead to elevations in ALT, making ALT not entirely hepato-specific. In addition, fenofibrate was found to increase ALT gene expression in the absence of apparent liver injury (false positive) (Edgar et al., 1998), and hepatotoxin microcystin-LR was reported to suppress ALT gene expression (false negative) (Solter et al., 2000; Shi et al., 2010). Despite the fact that extrahepatic injury, such as muscle damage or cardiac injury, can lead to increases in ALT, serum ALT remains the most widely used and universally accepted biomarker for DILI. It is deemed to be the clinical chemistry gold standard for DILI detection and has long been used at the FDA to facilitate regulatory decision-making (FDA,
2009). Periodic monitoring of serum ALT is also a common recommendation given to clinical practice for attempting to reduce the risks of liver injury when patients are taking drugs with known DILI potential. Recent studies have suggested that measuring the ALT isozymes, ALT1 and ALT2, may aid in differentiating the source of injury (Ozer et al., 2008; Yang et al., 2009). ALT1 has been noted to be localized in human hepatocytes, in renal tubular epithelial cells, and in salivary gland epithelial cells. ALT2, on the other hand, is localized to human adrenal gland cortex, neuronal cells bodies, cardiac myocytes, skeletal muscle fibers, and endocrine pancreas (Lindblom et al., 2007). Compared to ALT1, ALT2 was found to contribute less to the total serum ALT activity and was probably a reflection of mitochondrial damage (Yang et al., 2009). A novel immunoassay has been developed to discriminate human ALT1 and ALT2 activities and might improve the ALT assay (Lindblom et al., 2007; Ozer et al., 2008). Compared to ALT1, ALT2 was found to contribute less to the total serum ALT activity and was probably a reflection of mitochondrial damage (Yang et al., 2009). A novel immunoassay has been developed to discriminate human ALT1 and ALT2 activities and might improve the ALT assay (Lindblom et al., 2007; Ozer et al., 2008).

### Aspartate aminotransferase

Based on the same rationale as ALT, aspartate aminotransferase (AST) has also been introduced as a standard biomarker for DILI and is well accepted by clinicians (Ozer et al., 2008; Shi et al., 2010). Similar to ALT, AST is responsible for the metabolism (transamination) of aspartate. Even though the sensitivity of the AST test is believed to be lower than that of ALT, it is still a widely used liver biomarker. Owing to its more ubiquitous expression in extrahepatic organs, such as the heart and muscle, AST is significantly less specific than ALT in detecting DILI (Ozer et al., 2008). It appears that the ratio between serum ALT and AST activity is useful in differentiating DILI from extrahepatic organ injury (Ozer et al., 2008), as well as to help in diagnosing acute alcoholic hepatitis and cirrhosis with an AST/ALT ratio at 2:1. At least two isoenzymes of AST have been found; one is cytosolic AST and another is mitochondrial AST (mAST). The relative contributions of cytosolic AST or mAST to serum AST elevation have not been critically assessed. In addition, it remains unknown whether AST isoenzymes are susceptible to drug-driven induction or inhibition.

### Alkaline phosphatase

Alkaline phosphatase (ALP) is an enzyme located in the liver, and its concentration in serum increases when the
bile ducts are blocked (Burtis and Ashwood, 1999). ALP is another diagnostic biomarker recommended in the FDA guidance and is widely adopted by clinicians (FDA, 2009; Shi et al., 2010). The Council for International Organizations of Medical Sciences consensus criteria consider a more than twofold isolated elevation of serum ALP, or an ALT/ALP ratio of no more than 2, as a key biomarker of cholestatic DILI (Ramachandran and Kakar, 2009). It is noteworthy that conditions other than DILI, such as bone disease and pregnancy, are also associated with ALP elevation (Reust and Hall, 2001). Therefore, ALP should not be regarded as a specific biomarker of cholestatic DILI. The unique advantage of ALP is that it is at least partially predictive of biliary obstructive types of liver injury when used together with other DILI biomarkers.

**Total bilirubin**

Total bilirubin (TBL) is a composite of unconjugated (extrahepatic) and conjugated (hepatic) bilirubin. Increased TBL causes jaundice and can indicate metabolism problems in the liver, for example reduced hepatocyte uptake, impaired bilirubin conjugation, or reduced bilirubin secretion (Wintrobe and Greer, 2009). Therefore, serum bilirubin concentration is a real liver function biomarker, which measures the ability of liver to clear bilirubin from the blood as it circulates through the liver. In contrast, serum transaminase levels indicate the rate of enzyme release from injured cells and the Total bilirubin rates of enzyme degradation (mainly by the mononuclear phagocyte system) (Senior, 2006).

**Other existing hepatotoxicity biomarkers**

Some liver function tests are not sensitive or specific enough to be used as diagnostic biomarkers of hepatotoxicity, but are elevated in severe liver diseases. These biomarkers are used primarily to confirm the liver toxicity and indicate the extent of damage to liver function. Conventional biomarkers falling into this category are gamma-glutamyltransferase (GGT), serum total protein (albumin), ammonia, cholesterol/triglycerides, fibrinogen, prothrombin time (prothrombin ratio and international normalized ratio), and urobilinogen (Burtis and Ashwood, 1999; Cahill, 1999; Wintrobe and Greer, 2009).

Elevated serum gamma-glutamyltransferase (GGT) activity can be found in liver disease and it has a similar profile as ALP in detecting disease of the biliary system. Generally speaking, ALP is the first test for biliary disease and GGT provides a value to verify that the ALP elevations are due to biliary injury (Betro et al., 1973b). Large quantities of alcohol intake can significantly increase the serum GGT level. Slightly elevated GGT has also been found to be associated with myocardial infarction and heart failure (Betro et al., 1973a).

For both necrotic and obstructive liver diseases, serum total protein electrophoresis patterns will be abnormal. In the acute stages of hepatitis, albumin will be low and the gamma globulin fraction will be elevated due to a large increase in the production of antibodies. The globulin fractions will be elevated owing to production of acute phase proteins. In addition, biliary cirrhosis may cause beta globulin elevation.

Ammonia is normally converted by the liver to urea, which is less toxic. When liver dysfunction happens, failure of the conversion results in elevated blood ammonia; however, the liver has a high functional reserve for ammonia conversion so it often takes a significant amount of injury to alter ammonia levels. Increasing ammonia indicates end-stage liver disease and a high risk of hepatic coma.

Following hepatic uptake of lipoprotein cholesterol (insoluble), a portion is enzymatically converted to bile salt (soluble). Only hepatocytes have cholesterol 7α-hydroxylase, which is the rate-limiting enzyme for the multiple process conversion (Berkowitz et al., 1995). In acute hepatic necrosis, triglycerides may be elevated due to hepatic lipase deficiency. When the bile cannot be eliminated, cholesterol and triglycerides may accumulate in the blood as low-density lipoprotein cholesterol. Because the liver is responsible for the production of blood coagulation factors, the clotting time will be increased due to the impaired synthesis in the liver. However, it is not a sensitive biomarker because it only happens at the late stage of liver disease. Urobilinogen in urine is a colorless product of bilirubin reduction (Burtis and Ashwood, 1999). In this respect, urobilinogen level has a similar role as bilirubin to indicate liver dysfunction. Low urine urobilinogen may result from biliary obstruction or complete obstructive jaundice. Because urobilinogen is formed in the intestine by bacteria, broad-spectrum antibiotic treatment can significantly decrease its level due to the damage of intestinal bacterial flora (Wintrobe and Greer, 2009).

**REVIEW OF EMERGING BIOMARKERS**

There is a clear need to find additional biomarkers in serum and/or urine that can be measured in conjunction with ALT or outperform ALT with respect to specificity for liver injury. The omics methods (Table 13.2, adapted from Yang et al., 2012c) are well-suited to identify novel biomarkers (Table 13.3, adapted from
Ozer et al. (2008) of hepatotoxicity that have the desired specificity and sensitivity and potentially indicate injury earlier than the existing serum biomarkers (Xiong et al., 2012; Bailey et al., 2012).

Genetics biomarkers of hepatotoxicity

In the past 20 years, many genome wide association studies (GWAS) have been conducted to test the association between genetic polymorphisms and DILI. Human leukocyte antigen (HLA) genotype has been established as a risk factor for DILI, including injury with some drugs where immune-related toxicity was not suspected previously (Daly and Day, 2012). For example, in a GWAS using DILI patients and control subjects, it was found that the HLA-B*5701 genotype is a major risk factor and that ST6GAL1 is a possible cofactor of the individual vulnerability to fluvoxacillin-induced DILI (Daly et al., 2009). As fluvoxacillin-induced DILI occurs in fewer than 1 in 10,000 patients taking this drug, it is estimated that screening for HLA-B*5701 would provide a positive predictive value at 0.12%, whereas the negative predictive value was estimated to be 99% (Alfirevic and Pirmohamed, 2012). At present, GWAS on DILI have focused either on drugs that are very widely prescribed or newly licensed; therefore, it is unclear if HLA genotype will be the strongest risk factor for DILI linked to a range of different drugs. Another concern is that GWAS can reveal genetic determinants of susceptibility to DILI, but do not yet establish the mechanism by which the injury is caused or identify individual people at imminent danger.

Genomics biomarkers of hepatotoxicity

Over the past decade, the use of a genomics approach to identify patterns of changes in mRNA transcripts, referred as toxicogenomics, has gained popularity for identification of DILI biomarkers (Mendrick and Schnackenberg, 2009; Shi et al., 2010). Most studies have employed microarray analysis of the rodent liver to identify unique gene expression profiles as biomarkers and to elucidate the molecular mechanisms of DILI drugs. These genomics datasets, not one single biomarker, can be used to generate gene expression signatures associated with different drugs or chemicals that cause liver injury. However, the use of liver tissue-based genomics biomarkers is not optimal since biomarkers requiring liver biopsy have limited value in a clinical setting, especially for monitoring DILI progress in patients. Thus, genomics biomarkers from a minimally invasive source, such as the blood, have been explored in preclinical animals and humans. Interestingly, applying blood transcriptomic signatures, generated from rodents, to human blood data enabled differentiation of APAP overdose patients from healthy controls (Bushel et al., 2007). This study indicated that

### Table 13.2 Common platforms used for omics biomarkers discovery and technical challenges associated with these platforms

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<th>Technological Platforms</th>
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<td>• Concentrations range over nine orders of magnitude</td>
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<td>Proteomics</td>
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<td>Metabolomics</td>
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<td>• Protein expression in constant flux</td>
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<tr>
<td></td>
<td>• Large diversity and heterogeneity of proteome</td>
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<tr>
<td></td>
<td>• Difficult to automate</td>
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<tr>
<td></td>
<td>• Wide dynamic range of cellular protein expression</td>
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<td></td>
<td>• Limited dynamic range and detection of MS platforms</td>
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<tr>
<td></td>
<td>• Relatively low throughput</td>
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<td></td>
<td>• Metabolites altered by diet and environment</td>
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<tr>
<td></td>
<td>• Large diversity in chemical and physical properties</td>
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<tr>
<td></td>
<td>• Wide range of chemical species requires multiple analytical platforms</td>
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<tr>
<td></td>
<td>• Concentrations range over nine orders of magnitude</td>
</tr>
</tbody>
</table>

Adapted from Yang et al. (2012c).
alterations in genes involved in an inflammatory response were the best discriminators between sub-toxic/nontoxic and toxic exposure to APAP. In another study, a downregulation of mitochondrial genes involved in complex I of the oxidative phosphorylation pathway was noted in blood from humans after APAP exposure consistent with earlier rat studies (Fannin et al., 2010). In a recent study, a more comprehensive

<table>
<thead>
<tr>
<th>Biomarker Candidate</th>
<th>Biofluid Evaluated</th>
<th>Origin</th>
<th>Proposed Indication</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines</td>
<td>Plasma</td>
<td>Produced by all liver cells but primarily Kupffer cells</td>
<td>Cellular stress in the liver</td>
<td>Balkwill and Burke, 1989; Andres et al., 2003; Cameron and Kelvin, 2003; Ding et al., 2003a, b; Pfeffer, 2003</td>
</tr>
<tr>
<td>Interleukin-1</td>
<td>Plasma</td>
<td>Produced by a variety of cells</td>
<td>Cellular response to tissue damage</td>
<td>Akbay et al., 1999; Lacour et al., 2005</td>
</tr>
<tr>
<td>Glutathione S-transferase P-form</td>
<td>Serum</td>
<td>Present in the hepatocytes</td>
<td>Hepatocellular injury</td>
<td>Fella et al., 2005; Gluckmann et al., 2007</td>
</tr>
<tr>
<td>Cytokeratin-18</td>
<td>Serum</td>
<td>Expressed by epithelial cells</td>
<td>Apoptosis or necrosis</td>
<td>Cummings et al., 2008</td>
</tr>
<tr>
<td>High mobility group box protein 1</td>
<td>Serum</td>
<td>Found in a wide range of tissues</td>
<td>Necrosis and inflammation</td>
<td>Scaffidi et al., 2002</td>
</tr>
<tr>
<td>Glutamate dehydrogenase (GLDH)</td>
<td>Serum</td>
<td>Primarily found in the liver and to a lesser degree in the kidney and skeletal muscle</td>
<td>Necrotic and inflammation</td>
<td>Shi et al., 2010; Harrill et al., 2012; Schomaker et al., 2013</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>Serum</td>
<td>Localized in mitochondria and extra-mitochondrial compartment; found primarily in liver but also in skeletal muscle, heart, and brain</td>
<td>Necrotic and inflammation</td>
<td>Amacher et al., 2005; Ozer et al., 2008; Schomaker et al., 2013</td>
</tr>
<tr>
<td>Purine nucleoside phosphorylase</td>
<td>Serum</td>
<td>Primarily in the liver but also present in heart muscle and brain; mainly in cytoplasm of endothelial cells, Kupffer cells, and hepatocytes</td>
<td>Hepatocellular necrosis</td>
<td>Amacher et al., 2005; Schomaker et al., 2013</td>
</tr>
<tr>
<td>Paraoxanase 1</td>
<td>Serum</td>
<td>Produced primarily in the liver but also found in kidney, brain, and lung</td>
<td>Hepatocellular necrosis</td>
<td>Amacher et al., 2005; Schomaker et al., 2013</td>
</tr>
<tr>
<td>Glutathione S-transferase alpha</td>
<td>Serum</td>
<td>Liver specific</td>
<td>Hepatocellular necrosis</td>
<td>Giffen et al., 2002</td>
</tr>
<tr>
<td>Apolipoprotein E</td>
<td>Serum</td>
<td>Produced in the liver but also found in brain and kidney</td>
<td>Hepatocellular necrosis</td>
<td>Ozer et al., 2008; Andersson et al., 2009; Boll et al., 2012</td>
</tr>
<tr>
<td>Bile acids</td>
<td>Urine/Serum</td>
<td>Synthesized primarily in the liver</td>
<td>Liver dysfunction including intrahepatic cholestasis</td>
<td>Yang et al., 2008; Coen, 2010; Want et al., 2010; Xiong et al., 2012</td>
</tr>
<tr>
<td>Ophthalmic acid</td>
<td>Serum</td>
<td>Analog of glutathione produced along a similar biosynthetic route as glutathione</td>
<td>Oxidative stress and glutathione depletion following hepatotoxic insult</td>
<td>Kombu et al., 2009; Geenen et al., 2011b; Xiong et al., 2012</td>
</tr>
<tr>
<td>5-Oxoproline</td>
<td>Urine/Serum</td>
<td>Intermediate in the synthesis of glutathione</td>
<td>Oxidative stress and glutathione status</td>
<td>Kumar et al., 2010; Xiong et al., 2012</td>
</tr>
<tr>
<td>Steroids</td>
<td>Urine/Serum</td>
<td>Metabolites of cholesterol</td>
<td>Oxidative stress and liver damage</td>
<td>Chen et al., 2009; Kumar et al., 2011; Xiong et al., 2012</td>
</tr>
<tr>
<td>Acylcarnitines</td>
<td>Urine/Serum</td>
<td>Located in heart, muscle, brain, liver, and kidney</td>
<td>Failure of fatty acid oxidation</td>
<td>Chen et al., 2009; Zhang et al., 2011</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>Serum</td>
<td>Nontissue specific</td>
<td>Disruption of fatty acid β-oxidation</td>
<td>Zhang et al., 2011</td>
</tr>
<tr>
<td>miRNA-122</td>
<td>Plasma/Serum</td>
<td>Liver specific expression</td>
<td>Viral-, alcohol- and chemical-induced liver injury; hepatocarcinoma</td>
<td>Wang et al., 2009; Starkey Lewis et al., 2011; Ding et al., 2012</td>
</tr>
<tr>
<td>miRNA-192</td>
<td>Plasma/Serum</td>
<td>Liver specific expression</td>
<td>Chemical-induced liver injury</td>
<td>Wang et al., 2009</td>
</tr>
<tr>
<td>miR-291a-5p</td>
<td>Urine</td>
<td>Unknown</td>
<td>Chemical-induced liver injury</td>
<td>Yang et al., 2012b</td>
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</tbody>
</table>

Adapted from Ozer et al. (2008).
approach was employed to demonstrate that transcriptomic signatures extracted from blood can predict liver injury caused by a wide variety of hepatotoxicants (Huang et al., 2010).

Recently, next-generation sequencing (NGS) technologies have become available for fast, inexpensive sequencing of whole static genomes and dynamic transcriptomes (Su et al., 2011b). While the microarray platform monitors the expression levels of most annotated genes within the cell, the powerful and rapidly evolving NGS technology allows for precise quantification of gene expression including transcripts that have not been sequenced previously. Initially, there was concern regarding the comparability of NGS to microarray platforms in terms of gene expression and biological variability in a real-life toxicological study design. The MicroArray Quality Control (MAQC) group evaluated the robustness of NGS for detecting differentially expressed genes and it was reported that consistent biological interpretation was generated from both the NGS platform and microarrays (Su et al., 2011a). The NGS data are fundamentally different from microarray data, and translating these short sequences to genomic biomarkers must overcome several obstacles. One of the major challenges lies in the handling of immense volumes of sequence data generated from NGS (Wilhelm et al., 2010; Su et al., 2011b). Powerful bioinformatics tools are needed to assure sequence quality, conduct sequence alignment to the relevant genome and/or transcriptome, and provide biological interpretation from the complex datasets. Over the past five years, NGS has provided a more comprehensive understanding of complex transcriptomes, with turnaround time and cost that is comparable with that for microarrays (Rowley et al., 2011). NGS technologies are anticipated to accelerate toxicogenomics research and play a pivotal role in identifying new DILI biomarkers.

Proteomics biomarkers of hepatotoxicity

Proteomics evaluates the differential protein expression between groups to determine novel protein biomarkers in studies of toxicity and disease. Analyses involve a separation step, either gel-based or gel free, followed by tandem mass spectrometry to identify proteins. To date, proteomics has successfully discovered several promising biomarkers of acute kidney injury (AKI) including neutrophil gelatinase-associated lipocalin (NGAL), cystatin C (Cys C), kidney injury molecule-1 (KIM-1), liver-type fatty acids binding protein (L-FABP), and interleukin-18 (IL-18) (Tsigou et al., 2013). These new markers show promise as diagnostic markers and are being validated. The successful application of proteomics to determine more accurate biomarkers of AKI indicates the potential to also provide novel biomarkers of hepatotoxicity. There have been multiple literature reports of potential classes of protein biomarkers of hepatotoxicity.

One class of protein biomarkers includes the cytokines. The cytokines are generally associated with inflammation, immune reactivity, tissue injury or repair, and organ dysfunction and include interleukins, growth factors, interferons, tumor necrosis factors, and chemokines (Balkwill and Burke, 1989; Cameron and Kelvin, 2003; Pfeffer, 2003). All liver cells are capable of producing cytokines and the plasma levels may be indicative of cellular response (Andres et al., 2003; Ding et al., 2003a; Ding et al., 2003b). Their production may be related to an initial toxic injury that activates Kupffer cells, which are the major source of cytokine production in the liver. The cytokine networks have been shown to mediate the hepatic response to diverse xenobiotics including acetaminophen and PPAR ligands (Lacour et al., 2005). However, the increase in cytokines is transient and declines rapidly, making it necessary to establish a timeline over which they should be evaluated following dosing (Lacour et al., 2005). IL-1 has been proposed to be a biomarker for liver toxicity (Akbay et al., 1999; Lacour et al., 2005). IL-1 is a pro-inflammatory cytokine that can induce apoptosis, proliferation, and inflammatory processes. The activation of such proinflammatory cytokines can ultimately lead to activation of other processes, leading to generalized liver damage.

The cellular stress response pathways are also activated in response to a toxic response and mount either a homeostatic response or make cell fate/death decisions (Simmons et al., 2009). Amacher (2010) summarizes reported proteomic biomarkers associated with cellular stress response or toxicity pathways. Potential protein biomarkers of cellular stress response include those of the annexin family and those involved in anaerobic and catabolic functions as well as drug metabolism (Fella et al., 2005). The annexin family of proteins act as Ca2+ sensors and promote plasma membrane repair (Draeger et al., 2011). Additionally, carbonic anhydrase III, aflatoxin B1 aldehyde reductase, and GST-P, which play important roles in hepatocarcinogenicity, have been reported in a study of chemically induced hepatocarcinogenesis (Fella et al., 2005). Other potential protein markers of hepatocellular stress include the keratins and high mobility group box protein 1 (HMGB-1). Keratins are responsible for cell structure and integrity; the blood level of cytokertatin-18 (CK18) has been used to monitor apoptosis and necrosis (Cummings et al., 2008). HMGB-1 has been proposed to be a marker of inflammation as well as necrosis but not apoptosis (Scaffidi et al., 2002).

A variety of alternative serum biomarkers has been investigated for assessing liver injury. Similar to ALT and AST, these biomarkers are present in serum once
hepatocytes have been damaged, thus resulting in leakage of the target protein into circulation (Amacher et al., 2005). However, all of these biomarkers are at early stages of development and it remains to be determined whether they will be more selective than ALT and AST. These emerging serum biomarkers include glutamate dehydrogenase (GLDH), purine nucleoside phosphorylase (PNP), malate dehydrogenase (MDH), paraoxonase 1 (PON1), sorbitol dehydrogenase, serum F protein, glutathione-S-transferase alpha (GST\textsubscript{\textalpha}), and arginase I (Ozer et al., 2008; Schomaker et al., 2013).

GLDH is a mitochondrial enzyme found primarily in the centrilobular region of the liver (O’Brien et al., 2002). GLDH plays a role in amino acid oxidation and serum activity increases with hepatocellular injury (Shi et al., 2010). GLDH activity is more liver-specific than ALT and AST (Mastorodemos et al., 2005). In several recent studies, elevations of GLDH, similar to those observed with ALT, were reported in APAP-overdose patients and subjects receiving heparin (Harrill et al., 2012). MDH catalyzes the reversible conversion of malate into oxaloacetate and is a constitutive enzyme in the citric acid cycle (Ozer et al., 2008). MDH is a perportal enzyme whose release into the serum indicates tissue damage. Both GLDH and MDH levels are consistent in healthy populations, and they are strongly associated with elevated ALT in a broad range of liver injuries (Schomaker et al., 2013). However, MDH has been used as a biomarker for cardiac disease and hypertension (Zieve et al., 1985). PNP is an enzyme involved in purine metabolism and it is located primarily in the cytoplasm of endothelial cells, Kupffer cells, and hepatocytes. Several animal studies reported that PNP is released into hepatic sinusoids during liver damage in rodents. However, the value of PNP as a human liver injury biomarker has not been confirmed. PON1 is primarily produced in the liver and released into the circulation bound to high-density lipoprotein. Unlike other serum markers that indicate leakage, a decrease in PON1 is noted in serum after liver damage. Therefore, it is likely that liver damage reduces PON1 synthesis and secretion. PON1 has been shown to be decreased after dosing with phenobarbital and APAP (Amacher et al., 2005) and in humans with chronic liver disease (Ferre et al., 2002; Kilic et al., 2005). In a separate study evaluating the baseline level of emerging serum biomarkers, PON1 levels were found to be higher in African Americans compared with Caucasians (Schomaker et al., 2013). SDH is primarily located in the cytoplasm and mitochondria of liver, kidney, and seminal vesicles and is a marker of acute hepatocellular injury in rodents. Serum F protein has been shown to be a sensitive and specific marker of liver damage with a strong correlation to histopathology in humans (Foster et al., 1989); however, it has not been fully investigated in preclinical animal models. GST\textsubscript{\textalpha} expression is restricted to the liver and kidney and may serve as a region-specific marker of liver injury with a high concentration in centrilobular cells (Giffen et al., 2002; Ozer et al., 2008). These proposed biomarkers of liver injury must be rigorously tested in order to be qualified for clinical use. Furthermore, it may be determined that a panel of these markers provides the desired specificity for liver injury. An additional protein, serum arginase I, is highly liver specific and has been shown to have strong correlations to AST and ALT activities (Ashamiss et al., 2004). Finally, apolipoprotein E (APOE) expression has been linked to hepatotoxicity in two separate studies. APOE is a protein synthesized and secreted by hepatocytes. The major function of APOE is to transport and distribute lipids and cholesterol to cells. Therefore, it has a major role in determining the metabolic fate of lipids and proteins (Kolovou et al., 2009).

Recently, a hepatotoxicity protein biomarker panel based upon a targeted proteomics approach was reported for use in pharmaceutical toxicology assessment (Collins et al., 2012). The selected reaction monitoring (SRM) proteomics panel was developed in part based upon the hepatotoxicity biomarkers reported within the review by Amacher (2010). In total, the panel included 48 biomarker candidates from multiple pathways including but not limited to xenobiotic metabolism, PPAR\textsubscript{\alpha}/PXR activation, fatty acid metabolism and oxidative stress. This proof of principle study demonstrated the ability to develop a robust, high throughput, customizable SRM assay to evaluate putative protein markers of hepatotoxicity.

Metabolomics biomarkers in hepatotoxicity

Metabolomics involves the measurement of the metabolite pool that exists within a cell or tissue under a particular set of conditions (Fiehn, 2001). The metabolic profile is greatly influenced by both genetic and environmental factors, thereby providing phenotype-specific data that can be evaluated in a longitudinal manner. Metabolomics analyses focus on the discovery of novel, clinically relevant biomarkers in easily obtained biofluids such as urine and serum. The major analytical platforms for metabolomics include nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography (LC)-mass spectrometry (MS). As hepatotoxicity is the major cause for drug-related adverse events, metabolomics has been employed in multiple preclinical studies to identify more selective markers of drug-induced liver injury. Metabolites from several major pathways have been reported in multiple studies.

Synthesis, secretion, and recycling of bile acids are critical functions of the liver. The bile acids are
important signaling molecules in the liver and intestine; however, build-up of bile acids (BAs) due to impaired secretion or biliary obstruction can cause cellular damage to hepatocytes. Multiple studies of hepatotoxicity have noted altered profiles of BAs. Since galactosamine is a known hepatotoxin, a targeted LC-MS method was employed to investigate the metabolic effects of galactosamine by rapidly profiling BAs (Want et al., 2010). It is reported that galactosamine administration significantly increased taurine- and glycine-conjugated BAs in the rat serum (Want et al., 2010). Other hepatotoxicants have also been shown to alter BA profiles and have shown distinct mechanism-based patterns of changes in the BAs (Coen, 2010). Want et al. (2010) also evaluated the effects of galactosamine on bile acids in serum extracts. Glycine and taurine conjugates were elevated markedly with a strong correlation with the liver histopathology data. APAP, carbon tetrachloride (CCl₄), and alpha-naphthylisothiocyanate (ANIT) are classical hepatotoxicants that induce altered BA profiles after a toxic dose. APAP and CCl₄ increased urinary cholic acid and lithocholic acid (Kumar et al., 2011). CCl₄ and ANIT-induced liver failure elevated the serum levels of BAs compared to control (Yang et al., 2008). These two compounds cause distinct types of liver injury and showed different patterns of altered BAs. Isoniazid, which is used in the prevention and treatment of tuberculosis, is also hepatotoxic. A study of serum metabolome in wild-type and Cyp2e1-null mice indicated that accumulation of BAs and free fatty acids may play a role in the isoniazid-induced hepatotoxicity (Cheng et al., 2013). In a recent study of APAP-induced toxicity, increases in serum bile acids concurrent with decreases in the expression of bile acid synthesis-related genes (Cyp7a1 and Cyp8b1) and cholesterol transporter gene (Abcg8) and increases in the expression of bile acid transporter genes (Mrp3 and Mrp4) suggested a mild form of intrahepatic cholestasis (Sun et al., 2012). The aforementioned results indicate that bile acids may be a sensitive marker of DILI. Additionally, the specific bile acids altered may be able to differentiate specific types of liver injury.

Hepatotoxicants that form reactive metabolites generally induce an oxidative stress response, which can ultimately contribute to cell death. GSH is a protective molecule that is consumed as it scavenges reactive oxygen species to prevent cellular damage. GSH production proceeds by the folate-dependent transmethylation and transsulfuration pathways and intermediaries in these and analogous pathways may serve as biomarkers of hepatotoxicants-induced oxidative stress. One such molecule is ophthalmic acid (OA), an analog of GSH in which the cysteine residue is replaced by 2-aminobutyrate (2-AB); OA is synthesized in a similar route as that for GSH. The synthesis of OA is catalyzed by the same enzymes that synthesize GSH, γ-glutamylcysteine synthetase (Gcl), and glutathione synthetase (Gss). The depletion of GSH may activate Gcl and correspondingly increase the synthesis of OA (Soga et al., 2006). In a study of APAP hepatotoxicity in a mouse model, serum OA was linked to APAP-induced oxidative stress and GSH depletion (Soga et al., 2006). A depletion of GSH was noted with a concomitant increase in OA, suggesting that the OA biosynthetic pathway had been upregulated. Other methods have been developed to further assess this marker with inconsistent results (New and Chan, 2008; Kombu et al., 2009; Geenen et al., 2011b). More studies are needed to fully understand the relationship between OA and the multiple stages of liver damage. Furthermore, the multiple biosynthetic pathways involved in the biosynthesis of GSH and OA make it difficult to clearly understand the relationship between the two molecules and the oxidative stress response.

5-Oxoproline (5-OP; pyroglutamate) is an intermediate in the GSH biosynthesis pathway and may be more directly related to GSH content and cell status than OA. Hepatotoxicants such as APAP (Ghauri et al., 1993a; Sun et al., 2012), bromobenzene (Waters et al., 2006), and ethionine (Skordi et al., 2007) have induced elevations in 5-OP in biofluids and tissues in animal studies. These three compounds are all known to induce oxidative stress and deplete GSH. Increased 5-OP has been noted in humans with inborn errors of metabolism that affect GSH synthesis (Wellner et al., 1974; Creer et al., 1989; Ghauri et al., 1993b; Ristoff and Larsson, 1998; Tokatli et al., 2007) indicating its direct relation with GSH. Chronic use of APAP (Duewall et al., 2010) can also elevate 5-OP due to metabolic acidosis. It has been reported that 5-OP was increased in human liver epithelial cells following exposure to APAP (Geenen et al., 2011a) while cellular GSH content was decreased in a HPLC-MS/MS study. GSH consumption also occurs in hydrazine-induced hepatotoxicity and one study reported that 5-OP was increased in a dose-dependent manner in urine, plasma, and liver tissue (Bando et al., 2010). Based upon available literature, 5-OP appears to be directly coupled to GSH depletion and, therefore, may be more reflective of GSH status than OA.

BA metabolism and GSH biosynthesis pathways have been reported to be altered in numerous studies of hepatotoxicity. Kumar et al. (2011) employed a global profiling method for the initial discovery of potential urinary biomarkers of APAP and CCl₄ and methotrexate-induced hepatotoxicity. BAs, steroids, and amino acids were selected as liver toxicity biomarkers that could be evaluated in future drug toxicity studies. In the hydrazine-induced hepatotoxicity study
discussed above, multiple amino acids were also elevated in urine and plasma (Bando et al., 2010). These included the amino acid precursors of GSH, cysteine, glutamine and glycine. The amino acid changes were associated with hydrazine-induced fatty degeneration and glycogen depletion in the liver. Amino acid metabolism was also altered in response to bromobenzene- and galactosamine-induced hepatic necrosis (Heijne et al., 2005; Gonzalez et al., 2012). A metabolic profiling study of changes in rat urine related to dosing with the compound atorvastatin identified estrone, cortisone, proline, cystine, 3-ureidopropionic acid, and histidine as markers of liver toxicity (Kumar et al., 2010). Metabolic profiling identified 3-hydroxy-2-deoxyguanosine and octanoylcarnitine as urinary markers of valproic acid-induced hepatotoxicity in rats (Lee et al., 2009). APAP is known to inhibit fatty acid beta-oxidation; therefore, Chen et al. (2009) evaluated metabolites specifically related to this pathway in mouse serum. The results were consistent with a disruption of fatty acid beta-oxidation with an accumulation of long-chain acylcarnitines and free fatty acids in serum. The pattern of accumulation of acylcarnitines in the above study indicated that they might be useful as complementary biomarkers for monitoring APAP-induced hepatotoxicity or other compounds that disrupt fatty acid beta-oxidation. Other studies of APAP hepatotoxicity have also reported altered fatty acids and acylcarnitines (Xiong et al., 2012; Bi et al., 2013). Multiple biological matrices were evaluated by NMR after exposure of rats to aflatoxin-B1 (AFB1) (Zhang et al., 2011). Significant elevations in amino acids in plasma and liver tissue indicated that AFB1 altered protein biosynthesis. Bile acids, 5-OP, and OA, fatty acids, amino acids, and steroids are promising potential markers of hepatotoxicity that warrant further investigation to determine if they are general markers or mechanism-specific markers.

The aforementioned studies focused on biomarker discovery or targeted analysis of previously identified metabolite markers. A subset of metabolomics referred to as metabolomics flux analysis evaluates real-time synthesis and turnover rates in specific pathways through use of a 13C-labeled precursor. Stable isotope 13C labeled glucose was used to investigate the toxic effects of valproic acid (VPA) on the plasma, urine, liver, brain, and kidney metabolites (Beger et al., 2009). Results indicated that VPA disrupted the flux of acetate and its disposal via plasma cholesterol, causing liver toxicity. Usnic acid, a dietary supplement promoted for weight loss, was shown to be cytotoxic to rat primary hepatocytes in a time- and concentration-dependent manner and isotopomer distributions from flux analysis indicated a reduction in oxidative phosphorylation and gluconeogenesis at the high dose. The results from flux analysis of 13C labeled glucose were consistent with cytotoxicity and ATP depletion in the cells. Recently, Fan et al. (2012) reviewed stable isotope-resolved metabolomics and potential clinical applications.

MicroRNAs as biomarkers of hepatotoxicity

MicroRNAs (miRNAs) are ~22 nucleotides long, single-stranded, noncoding RNA that have recently been recognized as novel agents exercising post-transcriptional control over most eukaryotic genomes (Filipowicz et al., 2008). miRNAs are highly conserved among species, ranging from worms to humans, revealing their very ancient ancestry. The human genome has been predicted to encode over 2000 miRNAs (released miRBase v19), which are predicted to regulate the activity of ~60% of human genes (Friedman et al., 2009). Similarly to mRNA, some miRNAs are produced in a cell- or tissue-specific manner (Wang et al., 2009; Weber et al., 2010). Owing to its minimally invasive nature and unique stability, miRNA in biofluids holds a unique position for use as a preclinical and clinical DILI biomarker. Unlike mRNA, miRNA has been shown to be remarkably stable in many different body fluids, including blood and urine (Chen et al., 2008; Mitchell et al., 2008; Baraniskin et al., 2011). This stability greatly facilitates its use as a clinical biomarker of disease and injury since sample handling and processing are much less problematic compared to mRNA.

Using an APAP-induced mouse model of DILI, Wang et al. (2009) reported that the level of many plasma miRNAs inversely correlated with the level of hepatic miRNAs, indicating that for these miRNAs, hepatic injury caused the release of miRNAs into the circulation. Specifically, miRNA-122 and miRNA-192, which are predominantly expressed in the liver, increased in the plasma with concurrent decreases in the liver. The increases in both miRNAs were detected earlier than the increase of ALT. The increase of serum miRNA-122 and miRNA-192 was confirmed recently in patients with APAP poisoning (Starkey Lewis et al., 2011). Furthermore, miRNA-122 was also evaluated in serum from patients with liver injury due to hepatitis or hepatocarcinoma; the change was much smaller than after drug-induced injury (Ding et al., 2012). Recently, Yang et al. (2012) reported that urinary miRNA profiles were altered in rats after administration of hepatotoxic doses of APAP or CCl4. The levels of the same 10 urinary miRNAs were increased in APAP- and CCl4-treated rats. Although the functions of the 10 common miRNAs remain unknown, the possible target genes of these miRNAs are related to cell death, cell-to-cell signaling, and major metabolic pathways.
Biomarker Qualification and Validation

Applying omics-based technologies in rodents treated with a variety of hepatotoxic drugs, together with a better understanding of the mechanisms associated with hepatotoxicity, may facilitate the identification of novel DILI biomarkers. However, it is worthwhile to point out that none of the new DILI biomarkers has been qualified for preclinical or clinical use from a regulatory perspective. Furthermore, many of the potential biomarkers discussed in this review are not necessarily specific to the liver and may be the result of other types of tissue injury. True qualification of new biomarkers will ultimately require large numbers of samples obtained from animals and patients treated with many different drugs. A stringent qualification process is required to validate their specificity and sensitivity for DILI before they can supplement and/or replace existing biomarkers. Qualification has been described as “the process of linking a biomarker to a preclinical or clinical end point or to a biological process in a specific context” (Goodsaid and Frueh, 2007; Wagner, 2008). The U.S. Food and Drug Administration (FDA) and International Conference on Harmonisation (ICH) have issued guidance on biomarker qualification and the content of data submissions (ICH, 2008; USFDA, 2010; ICH, 2011). These guidance documents provide a foundation for qualifying a biomarker for a given context of use, such as preclinical versus clinical. Although specific testing and qualification plans are not provided in the guidance documents, they do highlight the fact that robust data are required to qualify a new biomarker. A biomarker will require a clearly defined context of use as well as sufficient data to support a full review of its performance characteristics within that context. Furthermore, it is required that the biomarker be measured reliably on multiple analytical platforms. Finally, in order to successfully translate a biomarker from the preclinical to the clinical setting, the marker must be qualified for its intended use and should correlate with lesions observed by histopathology. As part of the qualification process, it will be necessary to quantify a marker and provide a range of normal values in a control state. Metabolomics biomarkers can be quantified by mass spectrometry based upon a calibration curve or comparison of intensities after spiking a sample with an isotope labeled standard for the compound of interest. NMR metabolite data are quantified based upon the concentration of an internal chemical shift standard. Protein biomarkers can also be quantified by mass spectrometry methods that include a labeling procedure and tandem MS analysis. A Bradford assay can be used for quantification of a protein of interest. Gene or miRNA biomarkers can be quantified using real-time PCR, microarray, and NGS platforms based on the fluorescence signals.

Concluding Remarks and Future Directions

DILI is a major cause for limiting the use of a drug or its removal from the market. At present, the current DILI biomarkers sometimes fail to identify a toxic compound in the preclinical development stages and in even clinical trials. Among the existing biomarkers, ALT and AST are general indicators of hepatocellular injury; ALP is reflective of the cholestatic DILI pattern and elevated TBL is associated with increased DILI severity. The omics technologies are well-suited to identify novel biomarkers of DILI that can be measured in easily obtained biofluids. Genomics, proteomics, and metabolomics methodologies have produced many candidate DILI biomarkers for future investigations. In order for any of the proposed markers to move forward, they must be qualified for their intended use. In many cases, the same markers have been noted in multiple studies with overlapping pathways of interest including GSH depletion as an initial response to a cellular stress. Recently, the measurement of circulating miRNAs has appeared to be promising in identifying new biomarkers of liver injury. Further studies are needed to evaluate the sensitivity and specificity of the emerging biomarkers and it is also important to validate the omics biomarkers to develop tests that are both clinically useful and cost-effective.

Please note that the opinions expressed in this chapter do not reflect the official positions or policies of the U.S. Food and Drug Administration.

References

REFERENCES


13. HEPATIC TOXICITY BIOMARKERS


