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Jack Uetrecht
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Adverse Drug Reactions

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Preface

Adverse drug reactions (ADRs) remain a major health issue. A recent study in the United Kingdom found that 6.5% of hospital admissions were precipitated by an ADR (Pirmohamed et al. 2004). In addition, they markedly increase the cost and uncertainty of drug development. Two decades ago, the major problem leading to drug candidate failure involved problems with metabolism and pharmacokinetics; now, the major problems are lack of efficacy and toxicity, and from 1975 to 2000, over 10% of the drugs approved by the FDA either had to be withdrawn or achieved a "black box" warning because of unexpected adverse reactions (Lasser et al. 2002). The basic mechanisms of the most common "type A" ADRs such as gastrointestinal bleeding caused by nonsteroidal antiinflammatory drugs and bleeding caused by warfarin are well known. However, the mechanisms of most ADRs, especially idiosyncratic drug reactions, are not understood, and that makes it impossible to predict which drug candidates will cause such reactions and which patients are at high risk. This is unlikely to change rapidly because the unpredictable nature and virtual lack of animal models makes mechanistic studies very difficult. It is worrisome that, despite thousands of person/years of work and the availability of good animal models, our understanding of the mechanism of acetaminophen liver toxicity is not complete, and this is touched on in several chapters in this book. It is likely that advances in understanding basic mechanisms of ADRs will depend on advances in other fields of biomedical sciences, especially immunology.

In this book, we attempt to describe the current state of knowledge in this field with a focus on idiosyncratic drug reactions because they are the most difficult to deal with. It starts with a general description of the major targets for ADRs followed by a description of what are presently believed to be mediators and biochemical pathways involved in idiosyncratic IDRs. There is also a description of several examples of ADRs that serve to illustrate specific aspects of ADR mechanisms. Ultimately, better methods are needed to predict which drug candidates are likely to cause ADRs and which patients are at increased risk, but as mentioned above, we are far from this goal. There are a few examples where specific genotypes have been linked to specific ADRs, and the number of cases where this will prove useful is

likely to markedly increase in the future; however, it is unlikely that genotype alone will predict all, or even most, ADRs. With respect to screening drug candidates for ADR potential, it is my opinion that screening out candidates that form large amounts of reactive metabolite and making drugs more potent has made drugs safer, but there is no clear evidence to support this opinion, and this strategy will not eliminate ADRs. At present, there are no general biomarkers that predict ADR risk.

Despite the magnitude of human and financial costs of ADRs, the amount of basic research in this area is very limited. This field certainly has its challenges but the potential rewards are great.

Toronto, Canada Jack Uetrecht

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Part I
Target Organ Toxicity

Drug-Induced Liver Injury

Michael Holt and Cynthia Ju

Contents

Abstract Many drugs and environmental chemicals are capable of evoking some degree of liver injury. The liver represents a primary target for adverse drug reactions due to its central role in biotransformation and excretion of foreign compounds, its portal location within the circulation exposing it to a wide variety of substances, and its anatomic and physiologic structure. Drug-induced liver injury (DILI) remains the single most common adverse indication leading to drug candidate failure or withdrawal from the market. However, the absolute incidence of DILI is low, and this presents a challenge to mechanistic studies. DILI remains unpredictable making prevention very difficult. In this chapter, we focus on the current understanding of DILI. We begin with an overview regarding the significance and epidemiology of DILI and then examine the clinical presentation and susceptibility factors related to DILI. This is followed by a review of the current literature regarding the proposed pathogenesis of DILI, which involves the participation of a drug, or most often a reactive metabolite of the drug, that either directly affects cellular function or elicits an immune response. It is our hope that this chapter will shed light on the major problems associated with DILI in regards to the

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pharmaceutical industry, drug regulatory agencies, physicians and pharmacists, and patients.

Keywords Liver · Drug · Metabolite · Adduct · Immune response

Abbreviations

DILI	Drug-induced liver injury
US	United States
FDA	Food and Drug Administration
APAP	Acetaminophen
NSAIDs	Nonsteroidal antiinflammatory drugs
PPAR	Peroxisome proliferator activated receptor
ALT	Alanine aminotransferase
ULN	Upper limit of normal
AP	Alkaline phosphatase
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
CYP450	Cytochrome P450
p-i	concept Direct pharmacological interaction of drugs with immune receptors
APC	Antigen presenting cell
SMX	Sulfamethoxazole
SMX-NHOH	Hydroxylamine metabolite of SMX
SMX-NO	Nitroso-SMX
NK	Natural killer
NKT	NK cell with T cell receptor
DAMP	Damage-associated molecular pattern
TNF	Tumor necrosis factor
IL	Interleukin
IFN	Interferon
COX	Cyclooxygenase
TFA	Trifluoroacetic acid
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
polyI:C	Polyinosinic-polycytidylic-acid
TLR	Toll-like receptor
NKG2D	A lectin-like stimulatory receptor originally identified on NK cells

1 Significance and Epidemiology

Although the occurrence of adverse hepatic reactions to a drug are rare, ranging from 1 in 10,000 to 1 in 100,000 (Pessayre et al. 1999; Zimmerman 1999b), drug-induced liver injury (DILI) contributes significantly to patient morbidity and mortality (Hartleb et al. 2002; Lazarou et al. 1998; Lazerow et al. 2005; Lee and Senior 2005). A recent population-based study in France concluded that the annual incidence of hepatic adverse drug reactions is approximately 14 in 100,000 (Sgro et al. 2002). Only 20% of patients presenting with acute liver failure due to DILI survive with supportive care (Ostapowicz and Lee 2000). Liver injury with at least a possible casual relationship to drugs was reported in 77 of 1,164 cases (6.6% incidence) between 1995 and 2005 from a Swedish University's out-patient hepatology clinic (De Valle et al. 2006). Within the US alone, drugs account for more than 50% of cases of acute liver failure, with approximately 39% due to acetaminophen (APAP) and 13% the result of idiosyncratic drug reactions (Ostapowicz et al. 2002). The incidence of APAP toxicity in the US is markedly lower compared with the United Kingdom (73%) (Bernal 2003) and varies greatly among other nations. In terms of acute liver failure worldwide, the drugs most often held responsible include antituberculosis agents, other antibiotics, anesthetic drugs belonging to the halothane family, and nonsteroidal antiinflammatory drugs (NSAIDs). A report of the United Network for Organ Sharing liver transplant database identified drugs as the cause for acute liver failure in 15% of patients undergoing liver transplantation in the US between 1990 and 2002, for which APAP-induced hepatotoxicity represented 49% of those cases (Russo et al. 2004). In the non-APAP group, the most frequently implicated drugs were isoniazid (17.5%), propylthiouracil (9.5%), and phenytoin and valproate (7.3% each).

DILI is the most frequent basis for drug-related regulatory actions (Watkins and Seeff 2006), including termination of clinical drug trials (Ballet 1997), failure to obtain US Food and Drug Administration (FDA) approval, restriction of use, as well as the major reason for postmarket withdrawal of drugs (Bakke et al. 1995; Kaplowitz 2001; Larrey 2002; Temple and Himmel 2002). Drugs withdrawn from the US market since 1950 due to hepatotoxicity include pemoline, nefazodone, troglitazone, bromfenac, benoxaprofen, tienilic acid, and iproniazid. Pemoline (Cylert) was first marketed in 1975 for use in the treatment of attention-deficit hyperactivity disorder and narcolepsy. Following reports of liver failure (Abbiati et al. 2002; Safer et al. 2001; Shevell and Schreiber 1997) and the FDA recommendation that the overall risk of hepatotoxicity associated with pemoline outweighed the potential benefit, it was withdrawn by its manufacturer in May 2005 (Hogan 2000). Nefazodone (Serzone), an antidepressant, was first marketed in 1994 and was subsequently removed from the US market in May 2004 following 55 reports of liver failure and 20 deaths worldwide (Carvajal Garcia-Pando et al. 2002; Stewart 2002). Troglitazone (Rezulin) was the first of a new class of thiazolidinedione compounds, peroxisome proliferator activated receptor (PPAR)-γ agonists, approved by the FDA in January 1997 to modulate glucose levels in patients with

insulin-resistant diabetes. At least 90 cases of liver failure, including 70 that resulted in liver transplantation or death, led to the withdrawal of troglitazone in March 2000 (Gale 2001). The decision to remove the drug from market was in part due to the approval of two new thiazolidinediones, rosiglitazone (Avandia) in May 1999 and pioglitazone (Actos) in July 1999. Although these new PPAR-γ agonists do not appear to have the same degree of toxicity, there have been reports of severe liver toxicity (Al Salman et al. 2000; Forman et al. 2000; May et al. 2002). Bromfenac (Duract), a NSAID, was launched in July 1997 as a short-term analgesic for orthopedic pain. In June 1998, with less than a year on the market, it was withdrawn following more than 50 cases of severe liver injury, including 4 deaths and 8 liver transplants (Fontana et al. 1999; Meadows 2001; Rabkin et al. 1999). Benoxaprofen (Oraflex), another NSAID, first reached market in April 1982 and was subsequently removed in August of the same year due to a number of cases of fatal cholestatic jaundice (Tolman 1998). Tienilic acid (Ticrynafen), a diuretic drug developed to treat hypertension was withdrawn in 1982 following reports of hepatic injury, including 25 deaths (Zimmerman et al. 1984). Iproniazid (Marsilid), an irreversible inhibitor of monoamine oxidase B, was primarily used as an antidepressant. It was withdrawn in 1961 due to hepatotoxicity. Instead of removing a drug from market, the FDA may issue a black box warning, which severely limits a drug's usage by alerting physicians and pharmacists to the potential serious adverse effects of the drug. Recent drugs to have received a black box warning for hepatotoxicity include trovafloxacin (Trovan), tolcapone (Tasmar), felbamate (Felbatol), and zileuton (Zyflo).

2 Challenges Facing the Prediction and Prevention of DILI

A major concern associated with DILI is the failure of the drug development system to more accurately identify potentially hepatotoxic drugs. The case of troglitazone demonstrates the need to recognize signs of hepatotoxicity during drug development. During clinical trials, 12 of the 2,510 patients treated with troglitazone presented with alanine aminotransferase (ALT) levels of more than 10 times the upper limit of normal (ULN) and five had levels of more than 20 times the ULN. Furthermore, a biopsy was performed in two patients, of which one developed jaundice (Watkins and Whitcomb 1998). Although no liver failure was attributed to troglitazone during clinical trials, these signs were predictive of liver injury, as evident once the drug reached market by reports of severe and fatal liver injury in 94 of the nearly 2 million patients (Graham et al. 2003).

The difficulty in the recognizing and predicting potentially hepatotoxic drugs is due to a number of confounding factors. One of the paramount challenges remains the lack of specific diagnostic markers or tests to verify an episode of DILI. Another difficulty associated with the prediction and prevention of DILI is the generally underpowered nature of clinical trials. The limited number of participants in clinical trials, generally ranging from several hundred up to 8,000 (Larrey 2000),

enables the detection of only somewhat frequent events during drug development. The ability to identify, with at least 95% confidence, a single instance of clinically significant drug-induced hepatotoxicity would require that the number of patients in clinical trials be at least three times the frequency of the reaction (Lee 2003). Therefore, the detection of merely one case of DILI, with an incidence of 1 in 10,000, would necessitate a clinical trial consisting of approximately 30,000 patients. The majority of drugs therefore receive FDA approval prior to the advent of such an adverse event, as there is a minimal chance of observing such a reaction within the limited cohort of clinical trials. This low incident rate has further contributed to the limited number of epidemiological studies that have addressed the risks of DILI. The vast majority of published data with regard to DILI has been attained from voluntary reporting and retrospective case report studies.

An additional confounding factor in the prediction and prevention of DILI is the idiosyncratic nature of these reactions. While a universally accepted definition and characterization of such reactions remains a point of contention, these types of reactions are unique to the patient, lack a definitive correlation to the drug's known pharmacological effects, and vary in temporal patterns with regard to drug exposure (Park et al. 2000; Roth et al. 2003). A few reports have revealed a potential relationship between the daily dose of prescription medications and their propensity to cause serious hepatotoxicity (Lammert et al. 2008). Examination of commonly prescribed oral medications in the US in 2005 revealed that a significantly higher proportion of drugs with a daily dosage of 50 mg or greater were reported to cause liver failure, and liver failure leading to liver transplantation or death, than those with either a dosage of 10 mg or less/day or between 22 and 49 mg/day. Furthermore, of the 111 out of 137 non-APAP cases of DILI that required liver transplantation in the US between 1990 and 2002, 101 were reportedly caused by compounds with a daily recommended dosage of 50 mg or greater. In addition to the US analysis, this study (Lammert et al. 2008) also examined suspected hepatic adverse drug reactions submitted to the Swedish Adverse Drug Reactions Advisory Committee between 1970 and 2004. These results revealed that drugs prescribed at or greater than 50 mg/day accounted for 77% of all serious DILI cases. Although additional studies are required to confirm these initial observations, this apparent relationship between the doses of medications and DILI may assist in our understanding and potential evasion of DILI during drug development and patient usage.

3 Clinical Spectrum of DILI

The spectrum of DILI is highly variable, ranging from abnormalities in liver enzyme levels to fulminant hepatic failure resulting in liver transplantation or death. The predominant form of DILI is acute hepatocellular injury, representing up to 90% of cases (Larrey 2000). This type of hepatic damage, which is usually characterized by an initial early increase in ALT levels, manifests itself as cell death, characterized as either zonal or nonzonal, in the form of necrosis and/or

apoptosis, steatosis, and other types of hepatocyte degeneration. Drugs known to cause hepatocellular necrosis include sulfonamides, APAP, diclofenac, and pemoline. Submassive hepatocellular injury may be followed by hepatic fibrosis and cirrhosis, and examples of culprit drugs include methotrexate, isoniazid, and valproic acid. Cholestatic injury is usually associated with an early elevation in alkaline phosphatase (AP) levels, with or without hyperbilirubinemia, and represents the second most frequent type of DILI. Amoxicillin/clavulanate is one of the most common causes of acute cholestatic injury that resembles biliary obstruction. Anabolic steroids (methyl testosterone and fluoxymesterone), contraceptive steroids, and cyclosporine have been associated with pure cholestatic damage, and chlorpromazine and carbamazepine have been linked with cholestasis with hepatocellular injury. A mixed pattern of liver injury is characterized by elevations of both ALT and AP levels, and the clinicopathological manifestations include those present in both hepatocellular and cholestatic injury, as well as granulomatous reactions. A mixed liver injury has been associated with carbamazepine, flutamide, phenobarbital, sulfonamides, and verapamil. The combination of cellular injury and hepatic dysfunction as evidenced by jaundice was reported by Hyman Zimmerman to result in a mortality rate ranging from 10 to 50% (Zimmerman 1999b). These observations have subsequently been referred to as "Hy's Law," and a modified Hy's Law was defined as ALT levels $\geq 3 \times$ ULN plus serum bilirubin levels $\geq 2 \times$ ULN. Hy's Law is currently employed by the FDA in their assessment of the hepatotoxicity of newly developed drugs. This rule has been validated for the first time by two recent surveys from Sweden and Spain that reported that the combination of elevated aminotransferase levels and clinically evident jaundice was associated with a heightened risk of severe hepatic injury resulting in mortality or the need for liver transplantation (Andrade et al. 2005; Bjornsson and Olsson 2005).

4 Risk Factors

A multitude of factors may influence a patient's susceptibility to DILI, including gender, age, nutritional status, preexisting liver disease, concomitant drug use, and genetic background. For reasons that remain uncertain, the female gender is generally more susceptible to DILI, as evident from a recent study in which 79% of reactions due to APAP and 73% of idiosyncratic drug reactions were attributed to women (Ostapowicz et al. 2002). Women are also more susceptible to hepatotoxicity from halothane, chlorpromazine, and diclofenac (AYD 1963; Banks et al. 1995). The effect of age on an individual's susceptibility to DILI may be dependent in part on the role of age in metabolism, although this remains unclear. In general, the susceptibility to DILI is greater in adults than in children, which may be the result of decreased clearance, reduced hepatic blood flow, and compounding drug–drug interactions. Adults have an increased risk for hepatic injury from halothane, nitrofurantoin, floxacillin, and in particular isoniazid, for which few reactions have been reported in patients younger than 20 years of age (Black et al. 1975; Fairley et al.

1993; Farell 1994; Stricker 1992; Zimmerman 1999b). There are a few agents, among them aspirin and erythromycin estolate, for which adverse hepatic reactions are predominant in children (Fairley et al. 1993; Farell 1994; Zimmerman 1999b). Although the incidence of hepatotoxicity in regards to valproic acid is 1 in 37,000 (Bryant and Dreifuss 1996), this risk is much more pronounced (1 per 500 exposed) in certain groups, such as children younger than 3 years of age. Nutritional status may also represent a risk factor with respect to DILI. Fasting and malnutrition have been shown to enhance the toxicity of APAP, possibly by depletion of hepatic glutathione stores (Farell 1994; Pessayre et al. 1999; Stricker 1992). Patients with preexisting liver conditions are not uniformly at increased risk for DILI. Patients infected with the human immunodeficiency virus (HIV) receiving antiretroviral drug therapy have been reported to be at an increased risk for hepatotoxicity when they are coinfected with chronic hepatitis B virus and C virus (den Brinker et al. 2000; Pol et al. 2002; Sulkowski et al. 2000). However, the incidence of hepatotoxicity has been demonstrated to be comparable between patients with preexisting liver disease that did and did not receive statins (Chalasani et al. 2004). A more recent study observed no difference in the incidence of death or liver transplantation or in the degree of abnormality of liver tests among patients with disulfiram-associated hepatitis, regardless of the patient's pretreatment enzyme levels (Bjornsson et al. 2006). Liver test abnormalities have been shown to occur more frequently in patients treated with disulfiram who have preexisting hepatitis C infection (Saxon et al. 1998). Concomitant drug usage may be an additional risk factor in the assessment of an individual's susceptibility to DILI. Isoniazid has also been observed to enhance APAP toxicity (Murphy et al. 1990). The induction of particular enzymes by one drug may facilitate the increased formation of toxic metabolites of a second drug, as evident with the rifampicin-isoniazid combination, in which rifampicin facilitates the biotransformation of isoniazid into toxic metabolites (Farell 1994; Stricker 1992). On the other hand, inhibition of an enzyme pathway may influence important detoxification pathways. This effect is demonstrated by the interaction of troleandomycin and estrogen, as troleandomycin inhibits estrogen metabolism, thereby creating an overdose of estrogen resulting in cholestatic injury (Claudel et al. 1979). Genetic factors may further influence an individual's susceptibility to adverse drug reactions. There are reports of a relationship between adverse reactions and human leukocyte antigen (HLA) polymorphisms, particularly immune-mediated hypersensitivity reactions in the skin upon exposure to abacavir and carbamazepine (Chung et al. 2004; Mallal et al. 2002). Although genetic factors have the potential to influence an individual's ability to metabolize or eliminate drugs or modulate their immune response to a drug or reactive metabolite, and therefore influence susceptibility to DILI, evidence to support such a relationship remains scarce and inconclusive (Aithal et al. 2004; O'Donohue et al. 2000). On the other hand, patients with polymorphic expression of glutathione-S-transferase μ, which is important in the detoxification of electrophilic metabolites resulting in deficiency of the enzyme, have shown no increase in the risk of tacrine-induced liver damage (Green et al. 1995). Although the collection of these various risk factors may contribute to susceptibility, they do not fully account for an individual's susceptibility to severe hepatotoxicity.

5 Pathogenesis of DILI

Two major types of mechanisms have been proposed to account for DILI. The first mechanism involves the intrinsic hepatotoxicity of a particular drug itself or, more frequently, a result of the toxic effects of its metabolites on vital cellular targets of the liver. The second mechanism reflects an immune-mediated reaction culminating in hepatic inflammation and injury.

5.1 Biochemical Mechanism of Liver Injury

Although the mechanisms of toxicity for many of the drugs removed from the market due to hepatic injury remain unknown, it is possible that the formation of potentially hepatotoxic metabolites plays a pathogenic role. While other biotransformation pathways exist in drug metabolism, the primary metabolic pathway for the majority of drugs entails the cytochrome P450 (CYP450) system, a supergene family of heme-containing, mixed-function oxidase enzymes. Nefazodone is extensively metabolized by CYP3A4, and metabolites include *p*-hydroxynefazodone and *m*-cholorophenylpiperazine (Kalgutkar et al. 2005). It has been postulated that these metabolites are oxidatively bioactivated to the corresponding quinone-imine species, and it is these species that may play a role in nefazodone-induced hepatic necrosis. Troglitazone has been shown to be metabolized via the CYP450 system to a number of species, including a *p*-benzoquinone and a hydroxymethyl intermediate (Kassahun et al. 2001; Loi et al. 1997). The formation of a quinone metabolite raises the potential for covalent modification of hepatic proteins and induction of oxidative stress (He et al. 2001; Kassahun et al. 2001; Tettey et al. 2001). The sulfate metabolite of troglitazone has been demonstrated to inhibit hepatobiliary export of bile acids by competing with the bile salt export pump (Funk et al. 2001). Bromfenac and benoxaprofen both contain a carboxylic acid moiety which, similar to other NSAIDs, form reactive acyl glucuronide conjugates (Dahms and Spahn-Langguth 1996). Covalent protein adducts of benoxaprofen have been observed from in vitro incubations with rat liver microsomes as well as from in vivo studies of rats exposed to benoxaprofen (Dahms and Spahn-Langguth 1996). The formation of thiophene epoxide and/or thiophene sulfoxide intermediates have been proposed for adduct formation with tienilic acid (Koenigs et al. 1999). Reactive metabolites of tienilic acid have been observed to covalently adduct to human liver microsomal proteins (Bonierbale et al. 1999). The hydrolysis of iproniazid to isopropylhydrazine has been demonstrated to be critical in the formation of hepatic lesions in rats (Nelson et al. 1978). Isoniazid, one of the most widely used drugs in the treatment of tuberculosis, causes a dose- and duration-dependent hepatic injury. The metabolism of isoniazid has been shown to involve acetylation to the N-acetylisoniazid which, following hydrolysis, undergoes bioactivation by the CYP450 system to an acetyl radical (Huang et al. 2003).

Highly electrophilic intermediates have the potential to induce cellular injury via several mechanisms of toxicity. It remains necessary to identify the targets of covalent binding and the biochemical role of these macromolecules that will, in turn, influence the pathogenesis of hepatic injury. The covalent adduction of reactive metabolites to critical cellular macromolecules may disrupt and inhibit calcium gradients and ionic homeostasis leading to a decline in ATP levels. This process may also disrupt the endoplasmic reticulum, microtubules, and cytoskeleton resulting in cell swelling or lysis. The disruption of subcellular actin filaments and interruption of transport pumps at the canalicular membrane may generate abnormal bile flow, thereby preventing the excretion of bilirubin leading to cholestasis and jaundice. Certain drugs may induce mitochondrial dysfunction through inhibition of fatty acid oxidation and energy production resulting in a decrease in ATP production. Inhibition of mitochondrial function and lack of aerobic respiration may further generate free radical intermediates, which can directly damage cell membranes via lipid peroxidation, target nucleophilic DNA residues, or increase oxidative stress.

Efforts to further understand the involvement of metabolic activation of a drug and subsequent covalent binding to cellular macromolecules in adverse drug reactions are increasing. A recent study has incorporated both in vitro and in vivo covalent binding techniques for a variety of drugs associated with hepatotoxicity, including amodiaquin, benzbromarone, erythromycin, flutamide, nevirapine, tacrine, valproic acid, zafirlukast, and zomepirac in an attempt to assess the relationship between the covalent binding profile and occurrence of adverse hepatic events (Takakusa et al. 2008). The study revealed that drugs that were withdrawn from the market or given black box warnings demonstrated higher human liver microsome in vitro covalent binding compared to relatively safer drugs. In addition, the tissue distribution/retention of these drugs appeared to correlate with rat in vivo covalent binding data, as observed by the long-term retention of some drugs in the bone marrow, measured by rat autoradiography, which has been associated with severe agranulocytosis. Taken together, the covalent binding and tissue retention/distribution of various hepatotoxic drugs may assist in the development and risk assessment of a drug's toxicity profile. In addition to analysis of a drug's metabolic profile, further mechanistic mitochondrial damage, oxidative stress, and intracellular glutathione levels may improve our ability to accurately predict and confirm a drug's hepatotoxic potential. A recent report (Xu et al. 2008) has evaluated these three features with over 300 hepatotoxic drugs, including nimesulide, telithromycin, nefazodone, troglitazone, tetracycline, sulindac, zileuton, labetalol, diclofenac, chlorzoxazone, and dantrolene. Treatment of primary cultures of human hepatocytes, which have previously been demonstrated to maintain the differentiated functions of liver metabolism and transport (Gross-Steinmeyer et al. 2005; Hoffmaster et al. 2004; LeCluyse et al. 2005), with these various drugs, and subsequent multi-spectral live-cell imaging provided insight into key mechanisms of DILI, which were consistent with mechanistic findings previously reported in the literature regarding these various drugs. For example, analysis of hepatocytes following treatment with perhexiline demonstrated mitochondrial dysfunction and lipid

accumulation, mechanisms that have previously been proposed to participate in perhexiline-induced nonalcoholic steatohepatitis (Berson et al. 1998). Use of this high content cellular imaging was further able to point to potential new mechanisms associated with previously unidentified DILI. Mitochondrial abnormality, oxidative stress, lipid accumulation, and glutathione depletion were observed with nefazodone, all of which may contribute to nefazodone-induced liver injury. Support for such a role of mitochondrial damage in the pathogenesis of nefazodone hepatotoxicity has been recently demonstrated (Dykens et al. 2008). Overall, this high content cellular imaging technology provides an in vitro technique capable of identifying many DILI positive drugs. It should be noted that, while mitochondrial energetics and cellular redox states are important mechanisms of DILI, these factors may not be objective or exclusive biomarkers of such events because additional genetic or environmental factors may be necessary to induce or reach the threshold of hepatotoxicity, particularly in the cases of idiosyncratic liver injury. In these terms, this study provides a new approach for the identification of initiating signals of hepatic damage that may further contribute to our overall understanding of the mechanisms of DILI.

5.2 Role of Immune Reactions in Liver Injury

A number of experimental and clinical reports have suggested that a variety of factors unrelated to drug metabolism and direct hepatotoxicity may also influence susceptibility to DILI. In addition, the nature of idiosyncratic liver injuries suggests that a majority of these reactions involve an immune mechanism. Hepatic cellular dysfunction and death have the ability to initiate immunological reactions, including both adaptive and innate immune responses. This inflammatory process has been implicated in the development of liver injury induced by such drugs as APAP, dihydralazine, and halothane (Laskin and Gardner 2003; Liu and Kaplowitz 2002; Luster et al. 2001).

5.2.1 The Adaptive Immune Response in DILI

A number of DILI cases have clinical manifestations that are indicative of hypersensitivity and an immune response. These cases usually occur within 1–4 weeks after initial drug treatment and are often accompanied by symptoms of an allergic drug reaction, such as skin rash, fever, and biopsy specimens revealing evidence of monocytic or eosinophilic infiltration (Gunawan and Kaplowitz 2004). In addition, these reactions tend to occur only upon reexposure and the presence of antibodies directed against native or drug-modified hepatic proteins are frequently observed. Drugs such as halothane, tienilic acid, dihydralazine, diclofenac, and carbamazepine have been implicated in the initiation of adaptive immunity (Zimmerman 1999a). A fundamental issue remains as to how drugs, which are relatively small

molecules (< 1,000 Da) and therefore nonimmunogenic, are capable of inducing immune responses. Current theory based on the hapten hypothesis (Park et al. 1998; Uetrecht 1999) implies that drugs, or more often their reactive metabolites, act as haptens and irreversibly bind to and modify proteins to form drug–protein adducts (neoantigens), which are perceived as foreign by the immune system and induce a hapten-specific immune response. The idiosyncratic hepatotoxicity of NSAIDs such as diclofenac have been linked to such a mechanism (Boelsterli et al. 1995). The potential for hepatotoxicity among three structurally similar inhalation anesthetics, i.e., halothane, isoflurane and desflurane, relates to their degree of metabolism. The extent of metabolism after uptake ranges from 25 to 50% for halothane, 0.2 to 2% for isoflurane and 0.02 to 0.2% for desflurane (Kharasch 2008; Stachnik 2006). Halothane, with the greatest degree of metabolism is associated with the highest incidence of fatal, fulminant hepatic necrosis, with 1 case in 35,000 patients exposed, compared with <1 in 1,000,000 for isoflurane and <1 in 10,000,000 for desflurane (Eger 2004; Fee and Thompson 1997). All these volatile anesthetics undergo oxidation to trifluoroacetyl chloride, which may trigger the formation of trifluoroacetylated protein neoantigens (Njoku et al. 1997). Antibodies directed against trifluroacetylated protein neoantigens have been detected in the sera of patients with halothane hepatitis (Njoku et al. 1997; Pohl et al. 1989; Satoh et al. 1989; Vergani et al. 1980), suggesting individual host susceptibility to liver injury. A majority of the antibodies detected as a result of DILI are directed against neoantigens that include adducts with proteins in the endoplasmic reticulum and the pyruvate dehydrogenase enzyme complex of the mitochondria (Bourdi et al., 1994; Christen et al. 1994; Lecoeur et al. 1996; Satoh et al. 1985; 1989). Upon subsequent exposure to halothane or other structurally related anesthetics, these antibodies mediate an immune response causing hepatic necrosis.

As an alternative to the hapten hypothesis, the p-i concept (direct pharmacological interaction of drugs with immune receptors) has been proposed. The p-i concept postulates that drugs are directly able to stimulate T cells via interaction with the T cell receptor without the requirement for metabolism or hapten–protein formation and antigen presentation by antigen presenting cells (APCs) (Pichler 2002, 2005). Immune responses towards the drug sulfamethoxazole (SMX) were originally proposed to be the result of bioactivation to the hydroxylamine metabolite (SMX-NHOH) and ultimate oxidation to the reactive metabolite, nitroso-SMX (SMX-NO). The p-i concept was developed following the observation that some T cells in SMX-sensitive patients recognized SMX itself, rather than proteins covalently modified by SMX-NO presented in the context of the MHC (Burkhart et al. 2001; Schnyder et al. 2000). Similar drug-specific T cells have been detected in patients who developed lamotrigine and carbamazepine-induced systemic reactions (Naisbitt et al. 2003a, b; Schnyder et al. 1997).

Despite the detection of antibodies and drug-specific T cells, the role of the adaptive immune system in DILI remains inconclusive. An important reason for this uncertainty remains the fact that the liver is an immunologically privileged organ (Ju and Pohl 2005), such that the default response of the liver to antigen is tolerance rather than a pathogenic immune response. There are a number of

mechanisms that may contribute to this liver-induced tolerance. One such factor is likely due to the unique composition of tolerogenic APCs within the liver, including liver sinusoidal endothelial cells, Kupffer cells, and hepatic dendritic cells. Although it has been demonstrated that these various populations are capable of presenting antigen to naïve T cells, they are recognized as ineffective stimulators of antigen-specific T cell responses, thereby facilitating tolerance (Ju et al. 2003; Knolle et al. 1999; Limmer et al. 2000; Pillarisetty et al. 2004; You et al. 2008). This evidence suggests that the majority of patients develop tolerance towards a drug, as evident from the low incidence of DILI, while the failure of these mechanisms, as well as environmental and genetic factors, may contribute to the increased susceptibility to DILI in certain individuals. This tolerogenic nature of the liver further contributes to the lack of validated animal models that could be used to examine the role of adaptive immunity in DILI.

5.2.2 The Innate Immune Response in DILI

Cellular mediators of innate immunity include tissue macrophages, polymorphonuclear leukocytes (neutrophils, eosinophils and basophils), natural killer (NK) cells, and NK cells with T cell receptors (NKT cells). These cells provide an array of nonspecific responses to infection, such as recognition of microbial molecular patterns and generation of antimicrobial peptides, cytokine elaboration, activation of complement and mediation of opsonization, phagocytosis of infected cells and microbes, and direct killing of virus-infected cells (Janeway and Medzhitov 2002). While it is clear that hepatic innate immune cells play a key role in the progression and severity of tissue injury in some cases of DILI, it is not known what factors trigger the recruitment and activation of these cells within the liver. It is possible that damaged hepatocytes release damage-associated molecular pattern (DAMP) molecules, which induce a proinflammatory activation of innate immune cells, thereby contributing to the pathogenesis of DILI. Numerous studies have demonstrated that apoptotic and necrotic cells release DAMP molecules such as high mobility group box 1 protein, heat shock proteins, hyaluronan, surfactant protein, β-defensin, and cardiolipin, which act as endogenous stimulators of immune responses following the initial tissue damage (Asea et al. 2002; Biragyn et al. 2002; Peitsch et al. 1988; Seong and Matzinger 2004; Shi et al. 2003; Termeer et al. 2002; Wallin et al. 2002). At present, there are a few animal models investigating the role of innate immunity in DILI, including the models of APAP-induced liver injury and halothane hepatitis.

APAP-Induced Liver Injury

APAP is one of the few drugs that provides an experimental animal model of DILI; therefore, a large amount of research has focused on the role of the innate immune system in APAP-induced liver injury. Following APAP overdose, the initial

hepatocyte damage caused by the reactive metabolite can lead to the activation of innate immune cells within the liver, such as hepatic macrophages (Ju et al. 2002; Laskin et al. 1995; Michael et al. 1999), neutrophils (Cover et al. 2006; Ishida et al. 2002, 2006; Lawson et al. 2000; Liu et al. 2004, 2006; Smith et al. 1998), and NK and NKT cells (Liu et al. 2004; Masson et al. 2008). The precise role of each of these cells in the pathogenesis of APAP-induced liver injury remains controversial. Susceptibility to DILI may further result from an imbalance in protoxicant and hepatoprotective mediators that favor hepatic damage following activation of inflammatory responses. These factors include various protoxicant mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interferon (IFN)-γ (Blazka et al. 1995, 1996; Ishida et al. 2002), as well as hepatoprotective mediators, including IL-6, IL-10, IL-13, and cyclooxygenase (COX)-2 (Bourdi et al. 2002; Masubuchi et al. 2003; Reilly et al. 2001; Yee et al. 2007). A more detailed and extensive examination of the role of the innate immune system in APAP-induced liver injury is reviewed elsewhere in this book.

Halothane hepatitis

The lack of specific laboratory tests impedes the understanding of the mechanism(s) associated with DILI. Given the limitations associated with the use and analysis of cell culture systems, animal models represent a vital tool in the investigation of a wide variety of mechanistic and pathologic conditions. Although the animal model of APAP-induced liver injury is one of the most widely studied models of DILI, it does not replicate all the clinical characteristics observed in patients. Furthermore, the rare incidence of DILI observed in human patients makes these reactions just as difficult to stimulate in animals. One study revealed that in 16 out of 24 (67%) cases in which toxicity during clinical trials led to the termination of drug development, these incidences were not predicted by animal studies (Lumley 1990). A study of 139 drugs approved in Japan between 1987 and 1991 demonstrated that 39 out of 91 (43%) clinical toxicity cases were also not predicted from animal studies (Igarashi 1994). As such, additional animal models are necessary to explore the role of innate immunity in DILI, and the inhalation anesthetic halothane provides another animal model of DILI.

Halothane was first introduced into use as an anesthetic in 1956, and within 2 years, isolated case reports of liver complications and severe hepatitis were reported (Brody and Sweet 1963; Lindenbaum and Leifer 1963). A 1969 epidemiological study by the National Institutes of Health revealed an incidence of fatal hepatic necrosis of about 1 in 35,000 exposures. While concern for hepatotoxicity has virtually eliminated the use of halothane in adults in the US, halothane continues to be used as an anesthetic in children in the US because the incidence of halothane-associated hepatitis in children is between 1 in 82,000 and 1 in 200,000 (Carney and Van Dyke 1972; Kenna et al. 1987; Warner et al. 1984). Over the years, a variety of evidence has accumulated to spawn a range of theories and laboratory models. However, the exact mechanism leading to halothane

hepatotoxicity remains inconclusive and controversial because it exhibits characteristics of both a metabolic- and immunologically-mediated toxicity (Neuberger and Kenna 1987; Pohl et al. 1988).

Following halothane treatment, approximately 20% of patients present with transient elevations in serum aminotransferase levels and may experience symptoms of mild clinical hepatitis characterized by nausea, lethargy, and fever. This type of hepatotoxicity has been attributed to conditions of relative hypoxia, which favor insertion of electrons into the molecule via the CYP450 system, known as the reductive pathway, and which generate various hepatotoxic free radical intermediates. These reactive intermediates are capable of binding to and disrupting cellular macromolecules, which may be responsible for the acute injury after a single dose of halothane. Early attempts to reproduce an animal model of halothane hepatotoxicity concentrated on the reductive pathway of halothane metabolism. These studies demonstrated that treatment of rats with phenobarbital to induce biotransformation enzymes prior to halothane administration under hypoxic conditions could produce transient hepatic necrosis in rats (McLain et al. 1979; Ross et al. 1979). This reductive pathway of halothane metabolism may generate free radical intermediates, which in turn may induce lipid peroxidation and target other types of cellular macromolecules such as proteins and nucleic acids. Since halothane reduces hepatic blood flow to an extent that is sufficient to cause regions of hepatocellular hypoxia, hypoxia may be an additional important factor leading to cellular degeneration and hepatic injury (de Groot and Noll 1983; Jee et al. 1980). Although this model did produce halothane hepatotoxicity, the experimental conditions required to produce such injury were not specific to halothane; therefore, other animal models were sought out. Depending on the oxygen tension present, halothane may also undergo oxidative biotransformation by the CYP450 system to a reactive acyl halide metabolite, trifluoroacetic acid (TFA) (Gandolfi et al. 1980; Spracklin et al. 1997), which is capable of binding to and acetylating several hepatic-proteins (TFA-adducts) rendering them antigenic (Kenna et al. 1988). The guinea pig model of halothane-induced liver injury was able to demonstrate the consistent development of extensive acute centrilobular necrosis without any manipulations of the animal or the exposure conditions (Lunam et al. 1985) via the participation of halothane metabolism and TFA-adduct formation (Lind et al. 1989b; 1990). Pathological characteristics of the liver injury in guinea pigs exposed to halothane resemble the clinical spectrum of hepatotoxicity that occurs in approximately 20% of patients with nonfatal hepatitis upon halothane exposure (Lind et al. 1990; Wright et al. 1975).

Although higher levels of TFA-adducts have been observed within susceptible guinea pigs (Bourdi et al. 2001), these findings are unable to account for the interstrain- and intrastrain-dependent susceptibility of guinea pigs to halothane hepatitis (Bourdi et al. 2001; Lind et al. 1989a). The guinea pig model is further unable to explain the clinical scenario following response time to multiple exposures, as the onset of massive cellular necrosis after a single administration of halothane takes approximately 7 days and occurs in 1 in 35,000 patients, while 1 in 3,700 patients present with liver injury on secondary exposure and onset may take

only 3 days (Neuberger 1990). These observations imply that additional mechanisms, such as immunological responses, are involved in and dictate the development of halothane hepatitis (Gut et al. 1992; Kenna et al. 1987; Neuberger and Kenna 1987).

Recently, a strain-dependent susceptibility to halothane has been revealed using the first mouse model of halothane-induced liver injury (You et al. 2006). Among three common strains of mice, Balb/c mice were observed to be the most susceptible strain in response to halothane administration, followed by DBA/1, and with no significant liver injury observed in C57Bl/6 mice. The pattern and levels of TFA-adducts formed among the three strains of mice did not vary significantly, suggesting that the strain-dependent susceptibility may be independent of halothane metabolism. Upon further mechanistic analysis, this strain-dependent susceptibility was attributed to differential activation of the innate immune system within the liver. Following halothane treatment, induction of an inflammatory response was evident in susceptible mice due to increases in pro-inflammatory cytokines and mediators, including TNF-α, IL-1β, IL-6, and inducible nitric oxide synthase (iNOS). Activation of an innate immune response was also apparent due to the increase in IL-8, a neutrophil-specific cytokine, and associated increase in the number of infiltrating neutrophils within the liver of susceptible mice. A crucial pathogenic role for neutrophils in halothane-induced liver injury was confirmed using neutrophil depletion. These observations suggest that subsequent to the initial hepatocyte damage induced via halothane bioactivation and formation of TFA-adducts, the inflammatory response is a critical factor in the degree and overall hepatic injury. Furthermore, similar inflammatory events may contribute to the variability in susceptibility observed among patients with DILI.

The role of underlying inflammation in DILI

Drug metabolism and drug–protein adduct formation may not be sufficient to elicit DILI. A variety of cellular stresses may exacerbate DILI, including direct cellular injury by a drug or metabolite, temperature stress, oxidative stress, activation of cell death-inducing pathways, as well as viral or bacterial infection (Ganey and Roth 2001; Park et al. 2000). Clinical observations have suggested that episodes of inflammation, such as during bacterial or viral infection, concurrent with drug treatment, may augment the toxic response, and as such, represent key factors in determining patient susceptibility. The use of dapsone, carbamazepine, quinolones, and penicillins among patients with an HIV infection has increased the risk of drug-induced hypersensitivity (Ackerman and Levy 1987; Coopman et al. 1993; Park et al. 2000). In addition, the risk of developing extensive maculopapular pruritic rash has been observed to increase dramatically in patients with acute infectious mononucleosis following treatment with ampicillin (McKenzie et al. 1976; Pullen et al. 1967). This evidence suggests that episodes of inflammation during drug treatment may act as a key risk factor for idiosyncratic drug toxicity.

Several studies have demonstrated that co-treatment of rats with lipopolysaccharide (LPS) and ranitidine (Deng et al. 2007), diclofenac (Deng et al. 2006), or trovafloxacin (Waring et al. 2006) could augment hepatotoxicity caused by either LPS or the drug alone. A recent report has evaluated the effect of viral infection on a mouse model of halothane-induced liver injury, utilizing the viral RNA mimetic polyinosinic-polycytidylic-acid (polyI:C) (Cheng et al. 2009). Via its recognition by Toll-like receptors (TLRs) expressed on a variety of innate immune cells, including macrophages, dendritic cells, neutrophils, and NK cells (Muzio et al. 2000; Visintin et al. 2001), poly I:C triggers an immune response similar to viral infection and initiates an inflammatory cascade (Wang et al. 1998). In this study, administration of polyI:C following halothane challenge dramatically augmented hepatotoxicity (Cheng et al. 2009). The study demonstrated that the increased caspase-3 activities within the liver and increased hepatocyte apoptosis contributed to the exacerbation of liver injury. In addition, the observed activation of hepatic Kupffer cells and NK cells and their subsequent expression of multiple pro-apoptotic factors, including TNF-α, NKG2D (a lectin-like stimulatory receptor originally identified on NK cells) and FasL were proposed to mediate the poly I:C-induced hepatocyte apoptosis. These findings provide evidence that concurrent infection, through the activation of the hepatic innate immune system and subsequent inflammation, increases a patient's risk of developing DILI.

6 Conclusions

Over the past years, we have witnessed significant advances in our understanding of the mechanisms responsible for DILI. The central role of the liver in metabolism presumably accounts for its susceptibility to drug-induced injury. The parent drug or, more often, reactive metabolites may cause glutathione depletion, covalent binding, and disruption of cellular macromolecules, as well as oxidative stress and lipid peroxidation. The consequences of such events include hepatocellular damage and the initiation of immunological reactions, including both adaptive and innate immune responses.

Although the incidence of DILI is relatively low, it is this particular rarity and the lack of definitive prognostic markers that contribute to the difficulty in accurately predicting and preventing the adverse reactions. Therefore, it is necessary to gain a more thorough understanding of the sequence of events related to the initiation, propagation, and termination of DILI. Furthermore, risk factors for developing DILI include an individual's genetic profile and environmental influences. This requires incorporation of both a metabalomic and genomic approach into the investigation of the mechanisms of DILI. The future of this research may enable individualization of drug therapy, thereby eliminating the occurrence of DILI in susceptible patient populations.

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Drug Hypersensitivity Reactions Involving Skin

Oliver Hausmann, Benno Schnyder, and Werner J. Pichler

Contents

Abstract Immune reactions to drugs can cause a variety of diseases involving the skin, liver, kidney, lungs, and other organs. Beside immediate, IgE-mediated reactions of varying degrees (urticaria to anaphylactic shock), many drug hypersensitivity reactions appear delayed, namely hours to days after starting drug treatment, showing a variety of clinical manifestations from solely skin involvement to fulminant systemic diseases which may be fatal. Immunohistochemical and functional studies of drug-specific T cells in patients with delayed reactions confirmed a predominant role for T cells in the onset and maintenance of immune-mediated delayed drug hypersensitivity reactions (type IV reactions). In these reactions, drug-specific CD4+ and CD8+ T cells are stimulated by drugs through

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their T cell receptors (TCR). Drugs can stimulate T cells in two ways: they can act as haptens and bind covalently to larger protein structures (*hapten-carrier model*), inducing a specific immune response. In addition, they may accidentally bind in a labile, noncovalent way to a particular TCR of the whole TCR repertoire and possibly also major histocompatibility complex (MHC)-molecules – similar to their pharmacologic action. This seems to be sufficient to reactivate certain, probably in vivo preactivated T cells, if an additional interaction of the drug-stimulated TCR with MHC molecules occurs. The mechanism was named pharmacological interaction of a drug with (*i*mmune) receptor and thus termed the *p-i concept*. This new concept may explain the frequent skin symptoms in drug hypersensitivity to oral or parenteral drugs. Furthermore, the various clinical manifestations of T cell-mediated drug hypersensitivity may be explained by distinct T cell functions leading to different clinical phenotypes. These data allowed a subclassification of the delayed hypersensitivity reactions (type IV) into T cell reactions which, by releasing certain cytokines and chemokines, preferentially activate and recruit monocytes (type IVa), eosinophils (type IVb), or neutrophils (type IVd).

Keywords Drug hypersensitivity · T cells · p-i concept (pharmacological interaction of drugs with immune receptors) · Mechanism · Exanthema

1 Introduction

Adverse drug reactions (ADR) can be classified into those that are predictable, common, dose-dependent, and related to the pharmacologic actions of the drug (type A reactions) and those that are unpredictable, uncommon, more or less dose-independent within the therapeutic range, and usually not related to the pharmacologic actions of the drug (type B reactions) (Rawlins and Thompson 1991). It is estimated that approximately 80% of ADRs belong to the type A category, and typical examples are gastrointestinal bleeding after treatment with NSAIDs, or bradycardia with β blocker treatment. Immune-mediated or allergic drug reactions (hypersensitivity reactions) are type B reactions. They account for 15–20% of all ADR (Lazarou et al. 1998). Unlike type A reactions, type B reactions are difficult to predict and are often not recognized until a drug is marketed and widely used in humans. It may then only affect a small number of patients, implying an individual susceptibility or predisposition. Indeed, the development of hypersensitivity reactions appears to depend on both genetic and environmental factors. Reactions in this category include *drug intolerance* (an undesired drug effect produced by the drug at therapeutic or subtherapeutic doses), *idiosyncratic reactions* (reactions that are not explainable in terms of the known pharmacologic actions of the drug, but are probably due to some individual genetic predisposition), and *allergic* or *hypersensitivity reactions* (reactions that are dependent on one or more immunological mechanisms).

The effector phase of allergic reactions can be classified according to Coombs and Gell (Coombs and Gell 1968) into reactions that are mediated by drug-specific IgE antibodies (type I reactions), which are mostly immediate-type hypersensitivity reactions, cytotoxic and immune complex reactions, which are mediated by drug-specific IgG or IgM antibodies (type II and III reactions), or by drug-specific T lymphocytes (type IV), which are mostly delayed-type hypersensitivity reactions. This classification system has proven to be helpful in daily clinical practice to guide diagnostic and therapeutic decisions.

In this review, data and concepts of the role of T cells in drug hypersensitivity are presented, which evolved, on one hand, from in vitro studies of human, drug-specific T cells derived from blood and tissue of patients with various drug-induced hypersensitivity diseases (Fig. 1). These T cells were analyzed functionally regarding cytokine production and cytotoxicity after stimulation with the respective drug. On the other hand, the same functions were also evaluated by immunohistology of the organs affected by the drug allergy. The results led to new concepts in drug hypersensitivity: (1) a novel mechanism of drug stimulation of T cells could be defined, extending the hapten concept (Schnyder et al. 1997; Zanni et al. 1998; Pichler 2002; Schmid et al. 2006b), (2) the type IV reaction was further

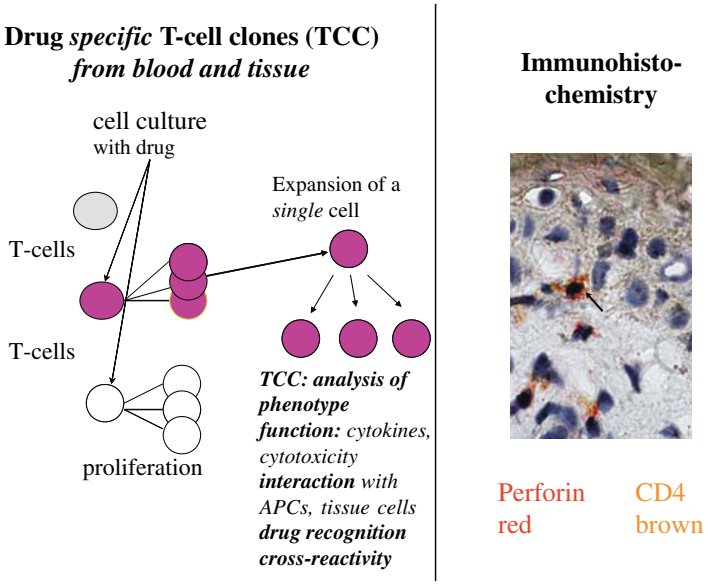


Fig. 1 Methods to study delayed drug hypersensitivity reactions in humans. (**a**) T cells – mainly from the peripheral blood, but also from tissue sections (skin, kidney), proliferate when exposed to drugs in cell culture. The proliferating cells can be expanded and then cloned by limiting dilutions, and functional and phenotypic analysis performed to evaluate how drugs are stimulatory for T cells and which functions they perform. (**b**) Immunhistology of skin (and kidney) using antibodies to cell surface and cytokines, etc.: the example shows that in a maculopapular exanthema both CD4+ and CD8+ T cells also express perforin

subclassified into four subtypes based on the clinical, immunohistochemical, and functional heterogeneity of certain drug allergies (Pichler 2003); and (3) applying these two new concepts to the clinical situation provided an explanation of how and why skin symptoms are so predominant in drug hypersensitivity although most drugs are administered orally or parentally (Posadas and Pichler 2007).

2 T Cell Stimulation by Drugs

2.1 Stimulation of naïve T Cells (Sensitization Phase)

A classical immune response starts by stimulating the innate immune system, in particular dendritic cells. The antigen (bacterium, virus, etc.) activates pattern recognition receptors, e.g., Toll-like receptors (TLR) on dendritic cells and thereby provides an initial "danger signal". The activated dendritic cells represent antigen-presenting cells (APC), which take up and process large and complex antigens. These are subsequently cleaved and presented as peptides to T cells via the MHC molecules in a suitable environment, mainly the secondary lymphoid organs such as lymph nodes. The ensuing immune response is directed against the original stimulus and is highly efficient; different antigens such as soluble or cell bound viral antigens elicit a distinct immune response, capable of eliminating the infectious agent without substantially harming the surrounding tissues.

Some therapeutic proteins such as mouse antibodies are intrinsically immunogenic because they represent foreign macromolecules. Many drugs, however, are neither proteins nor peptides, but are novel, unique structures and have a molecular mass of less than 1 kD. According to classical immunology (Landsteiner and Jacobs 1935), small molecules are not complete antigens and were considered to be incapable of directly inducing an immune response by priming of naïve T cells in their native state. For these agents to become immunogenic they must be chemically reactive (=haptens) and must be able to covalently bind to high molecular mass proteins (*hapten-carrier complexes*), which then undergo antigen processing and presentation, and are able to induce a novel immune response. Therefore, haptens need two essential features to elicit an immune response: activation of the innate immune system (=immunogenicity) and provision of novel antigenic determinants for the adaptive immune system (=antigenicity). An immune response to small molecular mass compounds is explained primarily by this *hapten hypothesis* (Landsteiner and Jacobs 1935; Griem et al. 1998; Aiba et al. 2003), which is well documented for certain chemicals such as *p*-phenylenediamine.

Some drugs, such as penicillin, are directly chemically reactive and bind spontaneously in a covalent way to other molecules, mostly proteins. Other drugs (prohaptens), however, must be metabolized or bioactivated to a chemically reactive form before they are able to covalently bind to proteins or other (intracellular) structures (Griem et al. 1998; Naisbitt et al. 2000; Aiba et al. 2003). Such

bioactivation is often accompanied by some toxic effect because the chemically reactive metabolite might bind and functionally disturb distinct proteins and metabolic or signaling pathways if it is not rapidly inactivated (e.g., by glutathione, etc.). Although bioactivation (transformation of a prohapten into a hapten) is typically mediated by cytochrome P450 enzymes in hepatocytes, it may, albeit in a reduced amount, also occur at other sites, such as skin keratinocytes or dendritic cells (Kimber and Cumberbatch 1992; Reilly et al. 2000; Janmohamed et al. 2001; Swanson 2004; Roychowdhury and Svensson 2005).

2.1.1 Generation of Reactive Compounds Inside or Outside the Liver

Interestingly, metabolism in the liver and formation of neoantigenic determinants may not necessarily induce an immune response but rather immune tolerance (Crispe 2003). This might explain the observation that many drugs are converted to reactive compounds without causing an immune response. Consequently, it is difficult to infer that the generation of an intracellular hapten of a novel compound in the liver will lead to general immunogenicity. For example, paracetamol (acetaminophen) is metabolized in the liver to many metabolites, some of which are reactive and can act as haptens; however, true hypersensitivity reactions to paracetamol occur only very rarely. The mechanism of this hepatic tolerance is not yet fully understood, but it is very important from a clinical point of view because, for example, the immunosuppression needed for a liver allograft is moderate compared to other allografts, and it is even more important for daily living because most foods with their huge diversity of (novel) antigens are usually well tolerated when processed by the liver (Crispe 2003; Bowen et al. 2004).

It may be possible, that – particularly if high doses are given – the reactive drug compound generated in the liver may escape the tolerogenic hepatic environment via the lymphatic drainage to the local lymph nodes, where it can induce immune-stimulation, or it may enter the circulation and other tissues (Bowen et al. 2004). For instance, sulfamethoxazole (SMX) is metabolized by cytochrome P450 enzymes to a hydroxylamine-derivative (SMX-NHOH), which is not yet highly chemically reactive (Knowles et al. 2000). It may, however, be easily further metabolized (oxidized) by enzymes such as myeloperoxidase (e.g., in neutrophilic granulocytes) to SMX-NO, a potent hapten. Bioactivation is usually followed by a bioinactivating process, which abolishes the toxic and immunogenic properties of some metabolites, mainly via the glutathione pathway. In some cases, however, genetic or environmental factors may disturb the balance between these two processes leading to increased formation or decreased elimination of reactive drug metabolites.

2.1.2 Immunogenicity: Drug Stimulation of the Innate Immune System

How drugs might stimulate the innate immune system is presently being intensively investigated (Girolomoni et al. 1990; Becker et al. 1992; Neisius et al. 1999; Lipper

et al. 2000; Uetrecht 2001). Rarely, the drug may *directly* stimulate the innate immune system by binding to Toll-like receptors (such as binding of imiquimod to TLR 7 on dendritic cells) (Enk and Katz 1992). More frequently an indirect pathway is taken: the intracellular processing of a drug generates a hapten-like compound, which may bind to various intracellular structures and disturb their function. This may somehow "activate" the cells and upregulate certain molecules, some acting as "danger signals" (Lipper et al. 2000; Uetrecht 2001). If this metabolism occurs inside a professional APC such as a dendritic cell, these toxic signals may lead to upregulation of costimulatory molecules such as CD86 or CD40 on the cell surface. This feature of prohapten/hapten-like drugs is already used in the screening of a potential immunogenicity of novel dermatologic compounds for treatment of skin diseases or skin care products (contact sensitizing potential) (Verrier et al. 1999; Hulette et al. 2005).

Some systemically applied drugs may also have such features; incubation of dendritic cells with SMX itself resulted in upregulation of CD40 molecules, and inhibitors of metabolism abrogated this stimulation (Sanderson et al. 2007). Albeit this effect was shown only for very high SMX-concentrations, it revealed that metabolism of a drug causing systemic immune reactions can occur inside APCs, and this may lead to enhanced antigen presenting capacity of the metabolizing cells. This could initiate an immune response to the drug, which is well documented if the reactive metabolite is given (SMX-NO) (Naisbitt et al. 2001).

2.1.3 Antigenicity: Drug Stimulation of the Adaptive Immune System

A second cardinal feature of haptens is the stimulation of the adaptive immune system ("antigenicity"): the hapten-binding to an autologous protein may modify it in such a way that it becomes a neo-antigen, which is processed and presented as hapten-modified peptides to T cells. A relatively large number of T and B cells are able to bind to hapten carrier complexes, and a vigorous immune response may arise. A predominant humoral immune response may occur if the hapten modification affects soluble and cell bound proteins, but exclusive T cell responses can be seen if the hapten binds directly to the MHC-peptide complexes. Thus, the type and target structure of the chemical modification of proteins already determines which type of immune reaction might develop (Pichler 2003).

2.2 Effector Phase in Immune-Mediated Drug Hypersensitivity

2.2.1 Antibody-Mediated Drug Hypersensitivity

Drug-carrier complexes may be antigenic for both T and B cells. Stimulation and proliferation of drug-specific T cell clones (sensitization) may provide help for the generation of a separate, B cell immune response to the hapten-carrier compound.

In a T helper cell type 2 (Th2)-cytokine milieu [interleukin (IL)-4, IL-5, IL-13], the B cell–Th2 contact (via CD40/CD40L) may allow a class switch to IgE, and drug (hapten)-specific IgE may be generated. The Fc-ε-RI-bound IgE on mast cells and basophils are the essential mediators for immediate type reactions such as urticaria and anaphylaxis (*type I reaction*). In a T helper cell type 1 (Th1)-cytokine environment (INF-γ, IL-12), IgG production is favored. Often no symptoms are seen, but under certain circumstances IgG- (and complement-) mediated cytotoxic effects may occur, which can lead to cell destruction such as in haemolytic anemia (*type II reaction*). Occasionally, an immune-complex-mediated disease with fever, urticaria, erythema multiforme, arthralgias, and lymphadenopathy may develop (*type III reaction*), for which in general a high cumulative dose of the respective drug is needed.

2.2.2 T Cell-Mediated Drug Hypersensitivity

Restimulation of Primed T Cells by Drug-Carrier Complexes (Hapten Model)

Upon reexposure to a drug acting as hapten or becoming a hapten after metabolism, the hapten-carrier-compounds are formed again and can be recognized by primed effector T cells. Recognition and the ensuing inflammation will take place in the involved tissue or in the corresponding draining lymph nodes. The tissue can be the skin, but also other organs where the drug or hapten-carrier compounds are present (Fig. 3a).

Stimulation of Primed T Cells by Native Drugs (p-i Concept)

Primary sensitization by drugs and protein allergens is asymptomatic. It has thus been a paradigm that in immune-meditated drug hypersensitivity, clinical signs or symptoms can be observed only on reexposure or longer lasting exposure (>3 days), allowing a clinically silent sensitization (Naisbitt et al. 2001). However, drug hypersensitivity reactions are rather frequently observed on first contact or after only a short period of time (hours). It has been assumed that such reactions on primary exposure are not mediated by the adaptive immune system and were therefore classified as “pseudoallergic”. However, more recent data in patients with iodinated contrast media hypersensitivity and without previous contact to the incriminated contrast medium revealed positive results in skin and cellular testing (lymphocyte transformation test, LTT). This suggests that clinically manifest hypersensitivity reactions to contrast media and other drugs (in particular neuromuscular blocking agents, NMBA) can occur on first exposure. Such reactions are best explained by a type of cross-reactivity, i.e., the T cells were originally primed by other drugs (such as pholcodine; Florvaag et al. 2006) or peptides, and happen to react with the drug as well. This would explain the rapidity of the reaction.

The p-i-Type of Stimulation of Peptide-specific TCR by Native Drugs

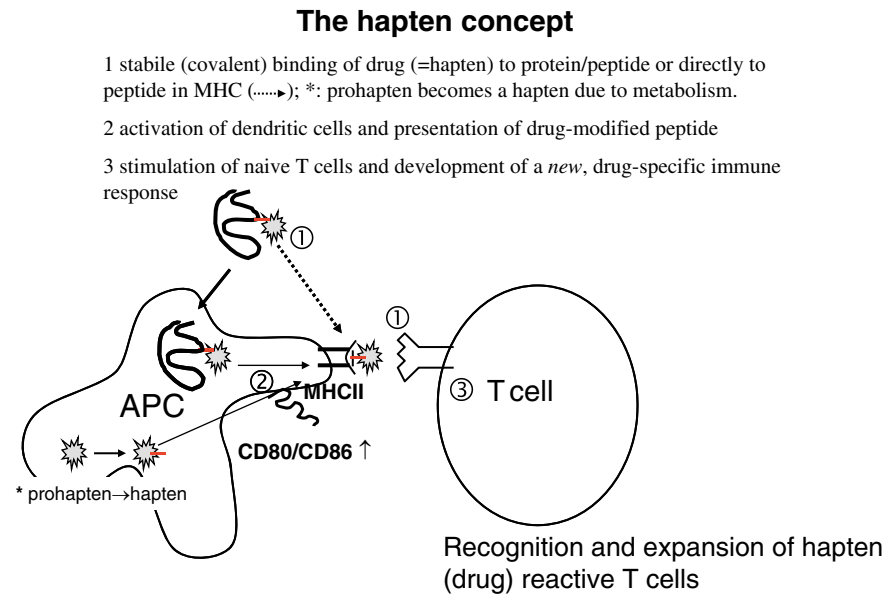
A detailed analysis of drug-reactive T cells revealed the surprising finding that most T cells do not react with the hapten (metabolite) but directly with the parent compound. This reactivity differed from hapten recognition; it occurred within minutes, before metabolism and presentation by the APC could take place. The drug binding was labile and could be washed away – in contrast to haptens, which bind covalently. Fixed APC, unable to take up and metabolize and process the drug were still able to provide interaction with the reactive T cell (Schnyder et al. 1997; Zanni et al. 1998; Pichler 2002; Schmid et al. 2006b). All these data (rapidity of reaction, independence of metabolism) led to the new p-i concept (pharmacological interaction of a drug with immune receptors) postulating that a drug might directly bind to TCR and stimulate the T cell. The interaction of the TCR with the MHC-molecule itself on APCs is well documented for TCR-MHC interactions and supplements this signal (Wu et al. 2002). This concept explains many clinical features of drug allergy better than the hapten concept, which is of particular importance for contact dermatitis but most likely not a suitable explanation for many generalized reactions to systemically administered drugs. This is particularly true for the frequent skin symptoms in drug allergy, which are explained in more detail (Fig. 3b, see Table 1 for differences between the p-i and hapten models).

The p-i Concept and Skin Symptoms

The systemic drug hypersensitivity reactions involving the skin are difficult to reconcile with the development of a classical immune response, where T cells primed in the lymph nodes home to the area where the antigen has entered and caused "danger signals". Skin symptoms in systemic drug hypersensitivity reactions appear all over; there is no danger signal from the outside. One has to take into account (1) that drugs are ubiquitously distributed in the body, (2) the lack of extensive metabolism in the skin, (3) the well described sentinel function of some resident T cells in the skin, and (4) the p-i concept (Fig. 2) (Chung et al. 2004). As an explanation for the predominance of skin reaction we recently formulated the following hypothesis (Posadas and Pichler 2007):

(a) Oral or parenteral uptake of drugs leads rapidly to a distribution throughout the body. This is particularly well documented for the skin, where, for example, antihistaminic drugs given orally can reach tissue levels in the nanomolar range within 45–60 min (Devillier 2006) and completely block H1 receptors and abolish skin test responses to allergens.
(b) According to the p-i concept, a drug may not only interact with their appropriate pharmacological receptor (e.g., H1-receptor for antihistaminics), but also with T cell receptors (TCR) of certain T cells. Of note, no metabolism is needed to induce such an interaction. The stimulatory potential of this drug-TCR interaction may decisively depend on the readiness of the T cell to react to a "minor" signal such as a drug. In this context it is interesting that

A



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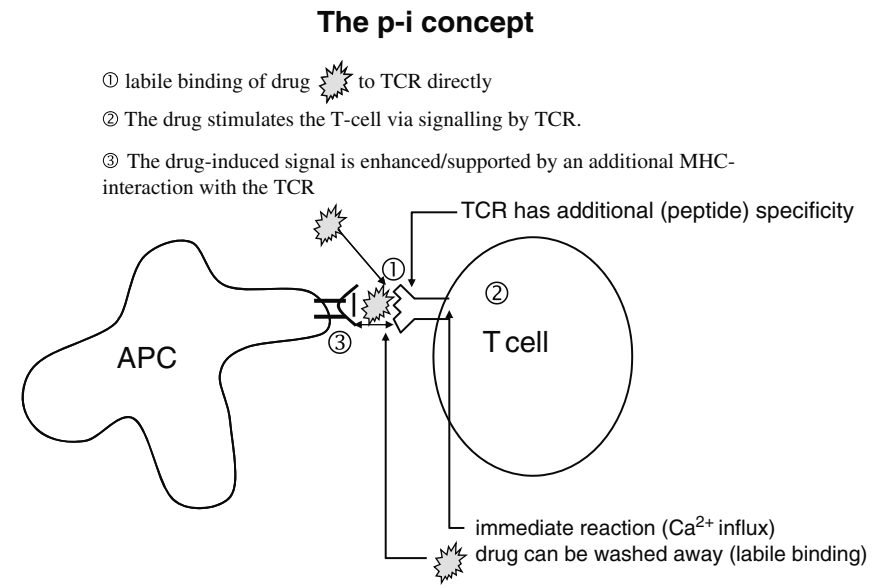


Fig. 2 Hapten/peptide and p–i concept of T cell stimulations by drugs. (**a**) Haptens: haptens are chemically reactive compounds. Drugs like penicillin are haptens: they bind to lysine amino acids in proteins or by the SH-group formed by opening the thiazolidine ring. This covalent binding can modify soluble or cell bound molecules. They can even bind directly to the immunogenic major histocompatibiliy complex (MHC)/peptide complex on antigen presenting cells (APC), either to the embedded peptide or to the MHC molecule itself. Thus, the chemical reactivity of haptens can lead to

Table 1 Important differences between hapten and p-i concept

Hapten concept	p-i concept
Activation of innate immunity required	Activation of innate immunity not required
Hapten-modified peptide is presented (stable covalent protein/petide modification)	Direct (partial) interaction with TCR without presentation on MHC (no covalent binding)
Chemical modification (hapten)	Structural similarity (fitting into TCR)
Processing and metabolism	No processing/no metabolism
Directed to MHC-APC: serves to present the hapten-modified peptide	Directed to TCR-T cell: MHC interact with TCR, which is supplementing the drug stimulation of T cells
Activation of naïve and memory T cells	Activation of memory T cells only (?)
Strictly controlled and well-coordinated immune response	Uncoordinated and overlapping immune responses (e.g., Th1 & Th2 and cytotoxic)
Often combined immune responses (Ig, T cells)	Only T cell reactions of different types (exanthema, DRESS, AGEP)

(c) The skin is a border region with an enormously dense network of APC and T cells, ready to mount an immune response (Keller et al. 2005; Clark et al. 2006). These "resident" T cells would fulfill sentinel functions and represent preactivated T cells with a high degree of reactivity. They can be characterized as chemokine ligand (CCL) 1 responders and express chemokine receptor (CCR) 8, while the immigrating inflammatory T cells are CCL20 responders and CCR6+. The frequent manifestation of drug hypersensitivity in the skin might be due to the readiness of such skin sentinel CCR8+, CD4+, TCR αβ+ T cells to be stimulated by drugs via the p-i mechanism. This initial activation provides stimulatory signals to local epidermal and endothelial cells and thus steers the immune reaction to the skin.

The possible steps of this new concept are outlined in Fig. 3b: In drug hypersensitivity, the local concentration of drugs in the skin may lead to a successful TCR engagement by drugs directly interacting with the TCR (p-i concept), supplemented by MHC interaction (through the close contact to the abundantly available

Fig. 2 (continued) the formation of many distinct antigenic epitopes, which can elicit both humoral and cellular immune responses. Other drugs are "prohaptens": they require metabolic activation to become haptens (=chemically reactive), which happens mainly via cytochrome P450 enzymes. The metabolism leads to the formation of a chemically reactive compound (e.g., from sulfamethoxazole [SMX] to the chemically reactive form SMX-NO). The metabolite may then modify cell bound or soluble proteins, as described above for haptens. If this metabolite is available in the liver or kidney only, a localized immune response may develop. If it is available all over, a generalized reaction might ensue. (**b**) The p-i concept (pharmacological interaction with immune receptors): Drugs are often designed to fit into certain proteins/enzymes to block their function. Some drugs may also happen to bind into or onto some of the available and highly polymorphic T cell receptors for antigen. Under certain conditions (see text), this drug-T cell receptor interaction may lead to stimulation of the T cell. For full T cell stimulation by such a chemically inert drug (no hapten-like features), an additional interaction of the T cell receptor with the MHC-molecule is required. This type of drug stimulation would result in an exclusive T cell stimulation. A similar model is conceivable for the hypervariable antigen-binding sites of immunoglobulins, but has never been formally demonstrated

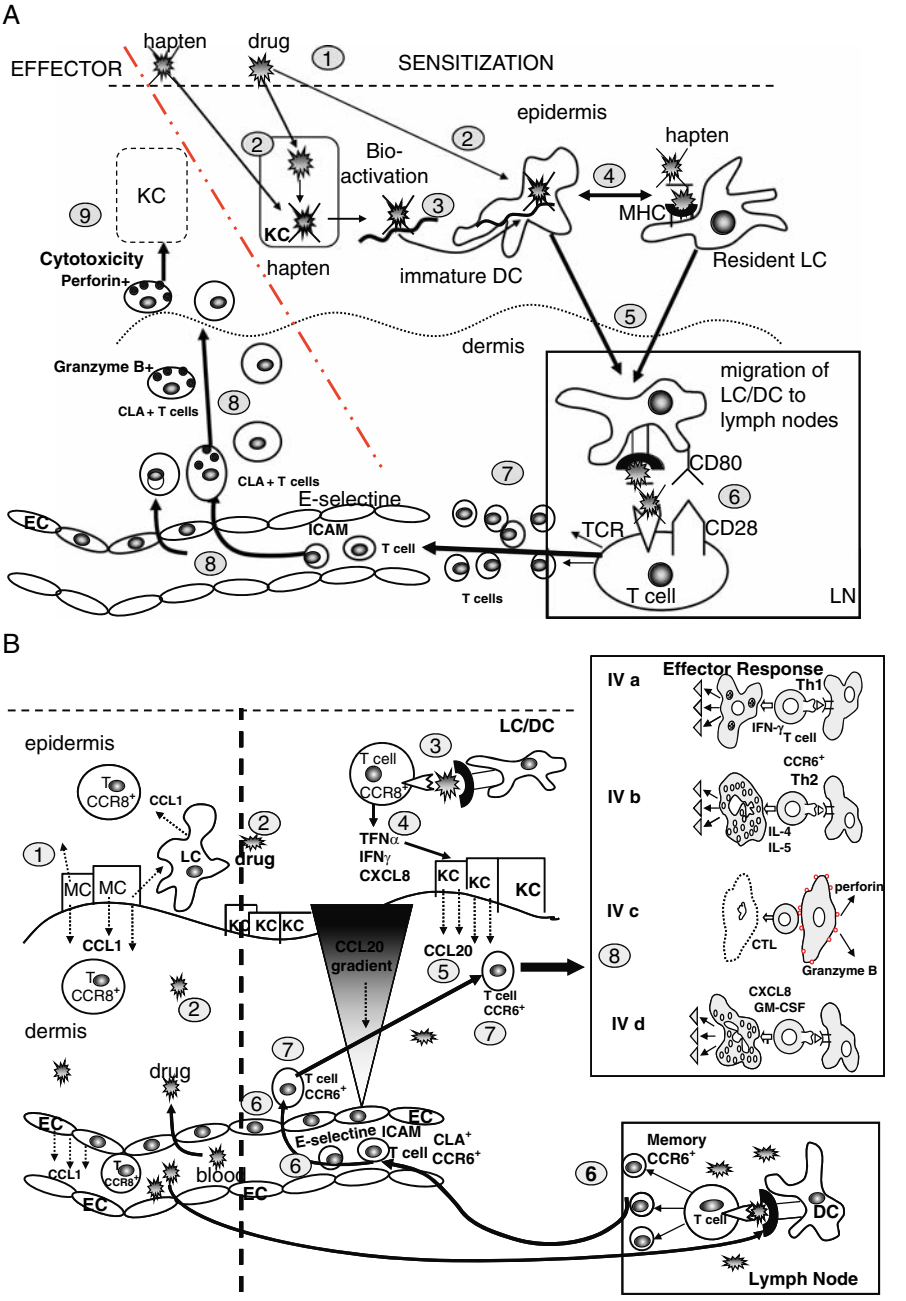


Fig. 3 Schematic representation of critical events involved in the frequent manifestation of drug hypersensitivity in the skin based on (**a**) hapten concept (contact dermatitis) or (**b**) p-i concept (generalized exanthema). Skin symptoms according to hapten/prohapten concept (a): contact dermatitis, drug applied on skin. Drugs, other chemicals or metal-ions enter the epidermis through the

epidermal antigen presenting cells (dendritic cells, DC and Langerhans cells, LC). The drug-stimulated CCR8+ T cells initiate an immune defense program by secretion of tumor necrosis factor (TNF)-α, interferon (IFN)-γ etc., which leads to an acute phase response via induction of inflammatory chemokines such as CXCL8 or CCL20 (= chemokine for CCR6+ T cells) in keratinocytes, upregulation of vascular adhesion receptors, and consequently recruitment of more leukocytes to the skin (steps 1–5; Fig. 3b). Since the majority of T cells in inflammatory conditions are CCR6-positive, they appear to be recruited and might have been activated outside the skin. Therefore, simultaneously with these local events in the skin, T cells in the lymph nodes may be activated by drugs, which are also found there because drugs are ubiquitously distributed in the body and do not need transport by the lymph or within DC/LC to the lymph nodes (like normal antigens). These T cells in the lymph nodes may be particularly responsive to the drug stimulation if they have been activated, e.g., by an infection. They will enter the circulation. Many may express CCR6 and other skin homing markers (e.g., cutaneous lymphocyte antigen, CLA), and therefore, upon arrival in the skin vasculature, they may adhere to the locally activated endothelium (due to the above described T cell stimulation in the skin) and emigrate from the vasculature to the skin following the CCL20 gradient (steps 6 and 7; Fig. 3b).

Fig. 3 (continued) stratum corneum or via the systemic circulation (1). This may already make a big difference. The hapten/prohapten concept may apply mainly to the contact dermatitis: the prohapten may undergo bioactivation in keratinocytes (KC) (2), but also in dendritic/Langerhans cells (DC/LC) (2), followed by intracellular protein haptenation. Haptens or reactive metabolites released from KC may haptenate extracellular proteins (3). These events may provide some local stimulation ("danger signal"), and in particular stimulate DC/LC (4), which process and present the hapten-protein complexes and emigrate (5) to the lymph nodes (LN), where they present the hapten-peptide complex to T cells (6). There, the engagement of the TCR of hapten-specific, naïve T cells in the presence of costimulatory signals (CD80/CD28) results in the generation of a hapten-specific immune response and clonal expansion of hapten-reactive T-effector cells (7). The activated T cells express skin homing receptors, which allow them to be recruited to the skin (8), where the local inflammation has already induced chemokine expression, which attracts these activated T cells. Local hapten presentation results in T cell (granzymeB/perforin)-mediated cytotoxicity and inflammation (9). Numbers refer to numbers in (**a**). (**b**) Skin symptoms according to p-i concept: drug-induced exanthema, drug coming from circulation. In the skin, many sentinel T cells reside (attracted by CCL1 released by melanocytes and Langerhans cells (1) and are in close apposition to Langerhans and dendritic cells (*left side*). The drug is ubiquitously distributed in the body and found in sufficiently high concentrations in the skin as well (2). Skin-resident CCR8+ CD4+ TCRαβ+sentinel T cells are stimulated by the drug directly (p-i concept, *right side*) (3), and secrete diverse cytokines (4). This drug-induced activation of the local T cells occurs better the higher the molar concentration of the drug and is facilitated by the close contact of T cells to DC/LC in the skin, and this initial T cell stimulation provides signals to local epidermal and endothelial cells and thus sets an alarm signal (3) secrete diverse cytokines (4). The keratinocytes activated thereby secrete CCL20 (5). Simultaneously, T cells with a low activation threshold may be activated by the drug in lymph nodes, where they expand and then emigrate expressing the homing receptor CCR6 (6). The local CCR8+, but mainly the recruited CCR6+ T cells (7), evoke a *generalized* inflammatory skin response because the inflammation is not restricted to a localized area where an antigen penetrated the skin (like in contact dermatitis). This generalized reaction is typical for exanthema. The cytokine pattern released by the T cells (4) leads to different forms of the exanthem (8), which can be subclassified into IVa, IVb, Ivc, and IVd type reactions. Numbers refer to numbers in (**b**)

Finally, the local sentinel CCR8+ and the recruited CCR6+ T cells (which represent the majority of T cells in inflamed areas) would evoke an inflammatory skin response. Its type might be determined by the profile of cytokines produced by the CCR6+ T cells, whereby the different responses (IVa, IVb, IVc, and IVd) described below might evolve. Why the immune response leads to a more IVa or IVb, etc., response is at present unknown (step 8; Fig. 3b).

This concept does *not* postulate drug metabolism in the skin and does *not* involve the hypothetical generation of a new immune response, but stimulates and expands *preexisting* effector or memory T cells, which already reside in the skin and lymph nodes (Pichler 2005). These T cells also already have peptide specificity. It is possible that a drug preferentially activates T cells with a particular peptide specificity, which might explain the puzzling finding that certain drugs elicit rather consistently similar clinical pictures (Peyriere et al. 2006).

This p-i concept adapted to the skin could explain:

(a) The preferential localization of drug hypersensitivity to the skin following oral or parental drug uptake. Other organs do not have such highly reactive sentinel T cells – and therefore the tissue is not "alarmed", and activated T cells, even if they would express the appropriate homing receptors, cannot migrate there so easily.

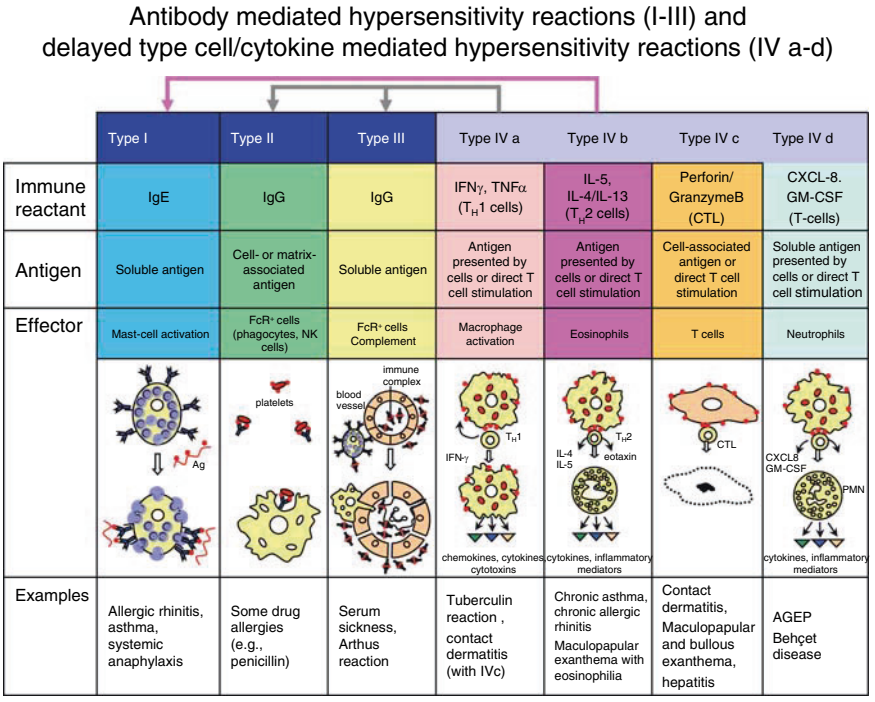
(b) The dose dependency of many drug hypersensitivity reactions. Higher concentrations of drugs are more often associated with delayed drug hypersensitivity reactions (Uetrecht 2001), and higher doses would lead to higher tissue concentrations and thus a better stimulation of T cells.

(c) The observation that generalized viral diseases such as HIV or herpes virus infections are risk factors for drug hypersensitivity. In such diseases, lymphocytes (and DC/LC) are already activated, which may lower the threshold of reactivity to a minor signal like drug binding.

3 Different Types of T Cell-Mediated Drug Hypersensitivity Reactions

A detailed analysis of T cell subsets and functions over the last 20 years resulted in a better understanding of the heterogeneity of cellular immune responses, which should also be incorporated in the well-known Gell and Coombs classification. Therefore T cell-meditated type IV reactions were further subclassified into IVa–IVd reactions as proposed in Fig. 4 (Pichler 2003). This subclassification considers the distinct cytokine production by T cells and thus incorporates the well-accepted Th1/Th2 distinction of T cells. It includes the cytotoxic activity of both CD4+ and CD8+ T cells (IVc), and it emphasizes the participation of different effector cells such as monocytes (IVa), eosinophils (IVb), or neutrophils (IVd), which are the cells that mediate the inflammation and tissue damage.

Type IVa. Reactions correspond to Th1-type immune reactions. Th1-type T cells activate macrophages by secreting large amounts of IFN-γ, drive the production of



Pichler W.J. Delayed drug hypersensitivity reactions, ***Ann. Int. Med****. 2003*

Fig. 4 Revised Gell and Coombs classification of drug reactions. Drugs can elicit all types of immune reactions. In fact, all reactions are T cell regulated, but the effector function relies mainly on antibodies (Type I-III) or more commonly T cells/cytokine secretion (type IVa to IVd). *Type I* are IgE-mediated reactions. Cross-linking IgE molecules on high affinitiy IgE receptors (FcεRI) on mast cells and basophils leads to degranulation and release of mediators, which cause a variety of symptoms (vasodilatation, increased permeability, bronchoconstriction, itch, etc.). *Type II* reactions are IgG-mediated, and cause cell destruction due to complement activation or interaction with Fcγ receptor bearing killer cells. *Type III* reactions are also IgG-mediated: Complement deposition and activation in small vessels and recruitment of neutrophilic granulocytes via Fcγ receptor interaction leads to local vascular inflammation. *Type IVa* corresponds to Th1-driven reactions with high IFNγ/TNFα secretion and involves *monocyte/macrophage* activation. Often, one can also see CD8+ T cell recruitment (type IVc reaction). *Type IVb* reactions correspond to *eosinophilic* inflammation and to a Th2 response with high IL-4/IL-5/IL-13 secretion; they are often associated with an IgE-mediated type I reaction. *Type IVc*: the cytotoxic reactions (both by CD4+ and CD8+ T cells) rely on *cytotoxic T cells* as effector cells. They seem to occur in all drug-related delayed hypersensitivity reactions. *Type IVd* corresponds to a T cell-dependent, sterile *neutrophilic inflammatory* reaction. It is clearly distinct from the rapid influx of neutrophils in bacterial infections. It seems to be related to high CXCL-8/GM-CSF production by T cells (and tissue cells). The role of IL-17 in these reactions is not yet defined

complement-fixing antibody isotypes involved in type II and III reactions (IgG1, IgG3), and are costimulatory for proinflammatory responses (TNF-α, IL-12) and CD8+ T cell responses. The T cells promote these reactions by IFN-γ secretion and possibly other cytokines (TNF-α, IL-18, etc.). An in vivo correlate would be, on the

one hand, monocyte activation, e.g., in skin tests to tuberculin or even granuloma formation as seen in sarcoidosis. On the other hand, these Th1 cells are known to activate CD8+ T cells, which might explain the common combination of IVa and IVc reactions (e.g., in contact dermatitis).

Type IVb. Corresponds to the Th2-type immune response. Th2 T cells secrete the cytokines IL-4, IL-13, and IL-5, which promote B cell production of IgE and IgG4, macrophage deactivation as well as mast cell and eosinophil responses. The high production of IL-5 leads to an eosinophilic inflammation, which is the characteristic inflammatory cell type in many drug hypersensitivity reactions (Pichler 2003). In addition, there is a link to type I reactions because Th2 cells boost IgE production by IL-4/IL-13 secretion. Thus, type IVb reactions may actually be involved in the late phase allergic inflammation of the bronchi or nasal mucosa (asthma and rhinitis), which were initiated by IgE on mast cells or basophils. Another in vivo correlate might be infestations with nematodes, eosinophil-rich maculopapular exanthema, or other T cell-dependent diseases with hypereosinophilia.

Type IVc. T cells themselves can also act as effector cells. They migrate to the tissue and can kill tissue cells such as hepatocytes or keratinocytes in a perforin/granzymeB- and FasL-dependent manner (Schnyder et al. 1998; Nassif et al. 2002, 2004; Kuechler et al. 2004). Such reactions occur in most drug-induced delayed hypersensitivity reactions, mostly together with other type IV reactions (monocyte, eosinophil, or neutrophil recruitment and activation). Cytotoxic T cells thus play an important role in maculopapular or bullous skin diseases as well as in conditions characterized by neutrophilic inflammation (acute generalized exanthematous pustulosis, AGEP), and in contact dermatitis. Type IVc reactions appear to be dominant in bullous skin reactions such as Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), where activated CD8+ T cells kill keratinocytes (Schnyder et al. 1998; Nassif et al. 2002, 2004). They may also be the dominant cell type in hepatitis or interstitial nephritis.

Type IVd. The possibility that T cells could also coordinate (sterile) neutrophilic inflammation has been rather neglected. Typical examples would be sterile neutrophilic inflammations of the skin, in particular acute generalized exanthematous pustulosis (AGEP). In this disease, T cells recruit neutrophils via CXCL8 release and prevent their apoptosis via GM-CSF release (Britschgi et al. 2001). In addition to AGEP, such T cell reactions are also found in Behçet's disease and pustular psoriasis (Keller et al. 2005). The relationship of these T cells to the recently described IL-17-producing T cells (Th17) requires clarification.

4 Clinical Features and Pathophysiology of Cutaneous Reactions

The most frequent manifestations of drug allergies are delayed appearing *cutaneous reactions*, so-called "rashes". They comprise a broad spectrum of clinical and distinct histopathological features which appear from 6 h to 10 days after drug

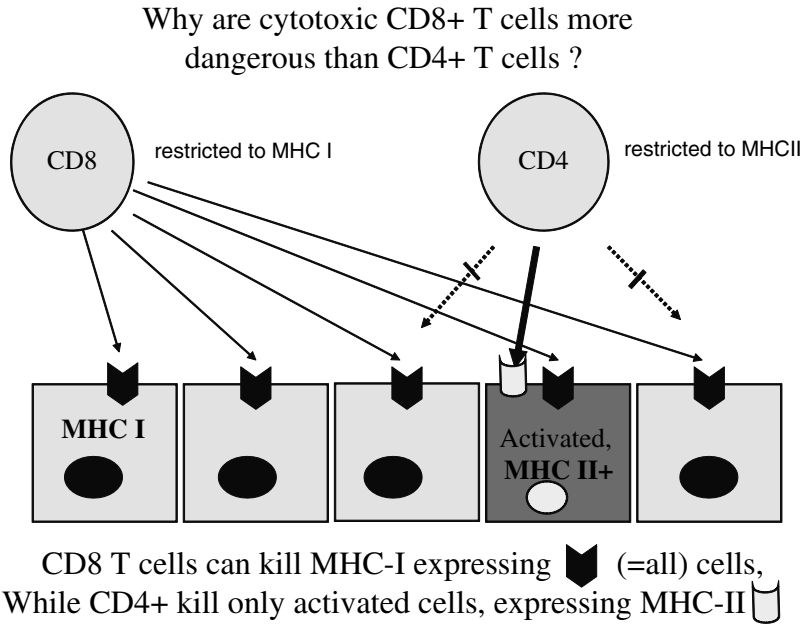


Fig. 5 Cytotoxicity of CD8+ versus CD4+ T cells: cytotoxic CD4+/CD8+ T cells recognize the drug in association with MHC and kill the respective target cell; if the killing is restricted to MHC class II (CD4+ T cells), only activated cells will be killed. If the killing process is targeted to MHC class I (CD8+ T cells), all nucleated cells, and in particular all keratinocytes (or hepatocytes), can be killed. This might explain the difference between the milder maculopapular exanthema and the more severe bullous exanthema

intake. In *all forms* of delayed drug-induced reactions, such as exanthema, interstitial nephritis, and probably hepatitis, cytotoxic mechanisms seem to play an important role. The clinical picture is determined by the *strength of cytotoxicity* (amount of drug-specific cytotoxic T cells and tissue destruction), which is also related to the proportion of cytotoxic CD8+ versus cytotoxic CD4+ T cells, because cytotoxic CD8+ T cells have a broader target cell repertoire (all nucleated cells express MHC I, which is recognized by CD8+ T cells, while CD4+ T cells require expression of MHC II which is only found on immunocompetent cells) (Fig. 5). Also important for the clinical picture is the type of associated effector mechanism (monocyte involvement or activation of eosinophilic and neutrophilic granulocytes).

4.1 Maculopapular Exanthem (MPE)

MPE is the most frequent drug hypersensitivity reaction, affecting 2–8% of hospitalized patients, especially after treatment with ß-lactams, sulfamethoxazole,

quinolones, diuretics, and many more. It most commonly appears 8–11 days after the start of treatment – sometimes even 1–2 days after the end of treatment. In previously sensitized individuals it can appear on the first day. It is clearly a dose-dependent reaction. A study investigating exanthema after treatment with gemifloxacin (a quinolone) showed that females of childbearing age had a higher risk of exanthema, suggesting the influence of oestrogens (Schmid et al. 2006a). Most MPE – particularly if caused by ß-lactams or gemifloxacin – are rather mild diseases, and treatment with an emollient cream, and possibly topical corticosteroids or systemic antihistamines for pruritus, is sufficient. Some patients can still be treated with the eliciting drug if needed without aggravation. The exanthema often heals with desquamation within 2–10 days after stopping the incriminated drug. It is rather unlikely that SJS/TEN will develop from a MPE because the involved cell types are different. On the other hand, some drugs may induce a mixed CD4+/CD8+ T cell activation. In such cases, prolonged treatment may lead to confluence of the papules; the patient may complain about malaise and fever, and liver injury tests indicate hepatitis (alanine aminotransferase >3x increase). In the blood, eosinophilia (>0.5 cells/l) as well as activated CD8+ T cells are found (Hari et al. 2001). This illustrates that even "mild" drug hypersensitivity reactions are *systemic diseases*, and that cutaneous manifestations may often be only the tip of the iceberg.

Immunohistology reveals a drug-specific CD4+ T cell infiltration (CLA+, CCR6+) in perivascular areas of the dermis (Yawalkar et al. 2000; Pichler 2003). Some T cells migrate into the dermoepidermal junction and epidermis, probably due to a gradient of chemokines produced by keratinocytes and melanocytes (e.g., CCL20 binding to CCR6). In the dermis and epidermis they encounter the drug-antigen again, and they are reactivated (they express MHC class II and CD25+) and may kill activated keratinocytes directly in a contact-dependent way by releasing perforin/granzymeB (Schnyder et al. 1998). Some keratinocytes undergo hydropic degeneration, but this apoptosis is not as extensive as with CD8+ T cell-mediated killing because not all keratinocytes express MHC class II. In addition, the immigrating CD4+ T cells exhibit a heterogeneous cytokine profile, including type 1 (IFN-γ) and type 2 (IL-4, IL-5) cytokines, which suggests that both Th1 and Th2 cells infiltrate the skin. The cytokine IL-5 is also detectable, both by immunohistochemistry and even in the serum (Hari et al. 1999). Tissue as well as blood eosinophilia can be found (Hari et al. 2001). The recruitment of eosinophils is also enhanced by the expression of the chemokines, eotaxin and RANTES, in MPE-lesions (Gerber et al. 1997).

4.2 Acute Generalized Exanthematous Pustulosis (AGEP)

Acute generalized exanthematous pustulosis is a rare disease (about 1:100,000 treatments) with an estimated incidence equal to severe bullous skin diseases (SJS and TEN combined) (Sidoroff et al. 2001). It is caused by drugs in >90% of cases (Table 2). Its clinical hallmark is the presence of myriads of disseminated,

Table 2 Drugs eliciting severe cutaneous or systemic reactions[a]

Acute generalized exanthematous pustulosis (AGEP)	Stevens–Johnson Syndrome (SJS) and toxic epidermal necrolysis (TEN)	Drug induced hypersensitivity syndrome/Drug rash with eosinophilia and systemic symptoms (DiHS/DRESS)[b]
	Nevirapine	
Aminopenicillins	Allopurinol[c]	Carbamazepin[c]
Cephalosporins	Phenytoin	Phenytoin
Pristinamycin	Carbamazepin[c]	Lamotrigin[e]
Celecoxib	Lamotrigin[e]	Minocyclin[e]
Quinolone	Cotrimoxazole	Allopurinol[c]
Diltiazem	Barbiturate	Dapsone
Terbinafin[e]	NSAID (oxicams)	Sulfasalazin
Macrolides	Sertraline	Cotrimoxazole
	Pantoprazole	Abacavir[c]
	Tramadol	

[a]List incomplete, only the most frequent elicitors are named

[b]Abacavir and lamotrigine induced systemic reactions often lack eosinophilia, abacavir affects preferentially the respiratory and GI-tract, minocycline induces lymphadenopathy more frequently, etc.; compare (Peyriere et al. 2006)

[c]The type of reaction might be determined by the presence of a certain HLA-B phenotype (Hung et al. 2005)

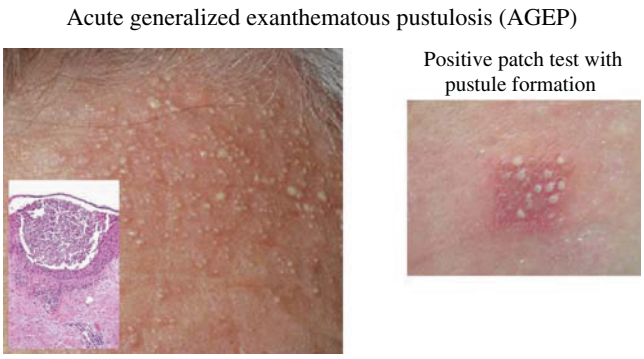


Fig. 6 Pustular drug eruption (AGEP): disseminated pustules cover the skin. Histologically, the pustules are intraepidermal and nonfollicular. A patch test reaction to the eliciting drugs can lead to a pustular reaction as well; for details see text

sterile pustules in the skin (Fig. 6), which appear rather rapidly, namely about 3–5 days after the initiation of treatment. The patients have fever and massive leukocytosis in the blood, sometimes with eosinophilia, but no involvement of mucosal surfaces. Epicutaneous patch test reactions during diagnostic work-up can cause a similar pustular reaction locally (Fig. 6). The latter feature allowed a study of the sequence of events in the development of pustules in sensitized patients using sequential biopsies of affected patients (Fig. 6).

Immunohistology of the acute lesion reveals subcorneal or intraepidermal pustules, which are filled with neutrophilic granulocytes and are surrounded by activated, HLA-DR-expressing CD4+ and CD8+ T cells. Keratinocytes show an elevated expression of the neutrophil-attracting chemokine, IL-8 (CXCL-8), and even the T cells migrating into the epidermis express CXCL-8 and GM-CSF. Analysis of sequential patch test reactions at 48–96 h suggests that drug-specific cytotoxic T cells migrate first and cause formation of vesicles by killing keratinocytes. Subsequently T cells and keratinocytes (stimulated by IFN-γ/TNF-α) release CXCL8, which recruits granulocytes into the vesicles, which are then transformed into pustules (Britschgi et al. 2001) (Fig. 6). Some pustules coalesce and may form bullae. The mortality rate is about 2–4% and older people are at the greatest risk. Healing occurs within 5 days after stopping the drug. This disease and the underlying T cell reaction seem to be a model for sterile neutrophilic inflammations (type IVd) such as pustular psoriasis and Behcet's disease (Keller et al. 2005). The relationship of these T cells to the recently described IL-17 secreting T-helper subset Th17 is unclear. Analysis of CXCL8-producing T cell clones isolated from skin lesions did not reveal elevated IL-17 production (Keller M, personal communication).

4.3 Bullous Exanthema, Stevens Johnson Syndrome (SJS) and Toxic Epidermal Necrolysis (TEN)

The most severe bullous skin diseases are SJS and TEN. TEN and SJS are rare (incidence 1:1,000,000 for TEN, ca. 1:100,000 for SJS) (Mockenhaupt 2007). Nowadays, they are considered to be the milder and the more severe form of the same disease (SJS $<10\%$, TEN $>30\%$ skin detachment), respectively. They are graded according to SCORTEN (Guegan et al. 2006), whereby age, underlying disease, and the amount of maximal skin detachment are the most important prognostic factors. According to the European Study Group of Severe Cutaneous Drug Reactions (EuroSCAR), SJS and TEN have mortalities of 13 and 39%, respectively. The intermediate form with 10–30% skin detachment is called SJS/TEN overlap syndrome and has a mortality rate of about 21%.

SJS/TEN are clearly different from erythema exsudativum multiforme, which is mainly caused by viral infections (Mockenhaupt 2007), is often recurrent, and affects younger persons (mean age 24 years). In about 6% of SJS/TEN cases, no drug treatment was reported the week before SJS/TEN started, and an infectious origin (*Mycoplasma* sp., *Klebsiella* sp.) is suspected. SJS/TEN can also be due to a graft versus host disease.

Most reactions start within the first 8 weeks of treatment (mean of first symptoms on the 17th day of treatment), with some differences according to the responsible drug (e.g., with sulfonamide antibiotics it may appear late). Once established, the disease can develop quite rapidly: initially often a macular, purple–red exanthema

is observed, which can become painful – an ominous sign. Within 12–24 h, bullae may already be seen, and the Nikolsky sign is positive. Stopping the eliciting drug at this time point may not prevent SJS from developing, but it might prevent an even more severe reaction (TEN). Mucous membranes (mouth and genitalia) are involved with blister formation as well as a purulent keratoconjunctivitis. The formation of synechiae may result in permanent eye damage.

The main causes for SJS/TEN are drugs (Table 2), which might differ in frequency in various regions due to genetic and ethnic background. The majority of cases are still due to some older drugs, and only a few newer drugs have a higher risk for SJS/TEN (Mockenhaupt et al. 2008). These include sertraline [RR=11 (2.7–46)], pantoprazole [RR=18 (3.9–85)], and tramadol [RR=20 (4.4–93)]. The most important risk factors are an HIV infection (low CD4, high CD8 counts), renal diseases, and active systemic autoimmune diseases such as systemic lupus erythematosus, Still's syndrome, Sjogren's disease, and rheumatoid arthritis, all being associated with immune stimulation.

In TEN, many dead keratinocytes are found, but cell infiltration is only rarely seen (Alanore and Roujeau 2007). However, the bullae – empty in histology – are actually filled with many cells, mainly cytotoxic CD8+ T cells, expressing CD56 and alpha/beta-T cell receptors, which may have migrated through the skin and have killed keratinocytes via perforin/granzyme B but not via the Fas-mediated pathway at this stage of the disease (Nassif et al. 2002, 2004). On the other hand, the massive cell death of keratinocytes is hard to reconcile with a cell-contact-dependent killing process. It has been proposed that the apoptosis of keratinocytes is due to Fas ligand (FasL), a soluble molecule of the TNF-family, which binds to keratinocytes via Fas and functions as a so-called death receptor (Viard et al. 1998). This FasL may also be expressed on or bound to keratinocytes, leading to a suicidal interaction between Fas and FasL. FasL bound to keratinocytes may also derive from mononuclear cells infiltrating/migrating through the skin in the early phase of the disease (Abe et al. 2003). Since blocking anti-Fas antibodies are found in immunoglobulin preparations, it has been proposed that patients with TEN should be treated with immunoglobulins. However, the efficiency of this treatment is controversial, and the content of such anti-Fas antibodies in immunglobulin preparations is highly variable (Bachot et al. 2003).

Extensive research has not revealed a pharmacogenetic predisposition or low glutathione levels in most affected persons. However, recent data have shown striking HLA-associations with severe, CD8+ T cell-mediated drug hypersensitivity reactions (Mallal et al. 2002, 2008; Chung et al. 2004; Hung et al. 2005; Yang et al. 2007). Three factors play a role: (1) the type of drug; (2) the type of reaction – as it was found for cytotoxic CD8+ T cell mediated reactions only; and (3) possibly ethnic background; some associations were exclusively found in Han Chinese. The HLA-B allele, which is the most polymorphic HLA-allele, seems to be involved: in carbamazepine-induced SJS/TEN, it is HLA-B*1502, for allopurinol it is HLA-B*5801, and for abacavir it is HLA-B*5701 together with heat shock protein70 (Mallal et al. 2002; Chung et al. 2004; Hung et al. 2005). It seems that in these CD8+ T cell-mediated reactions a certain HLA-B allele favors the presentation of certain

peptides that are able to optimally present the drug acting as a hapten. Alternatively, certain MHC-class B alleles might supplement the direct T cell stimulation via these drugs (p-i concept) better than others, while absence of these alleles renders the T cell insufficiently responsive to the drugs (Yang et al. 2007).

The extremely high association of certain HLA-B alleles with hypersensitivity reactions to certain drugs may be used to prevent such side effects in the future. Specifically, HLA-typing may identify patients at risk, and their exclusion from treatment might reduce the incidence of side effects (Mallal et al. 2008).

4.4 Drug (-Induced) Hypersensitivity Syndrome (DHS or DiHS) or Drug Rash with Eosinophilia and Systemic Symptoms (DRESS)

Some drugs are known to cause a severe systemic disease with fever, lymph-node swelling, massive hepatitis, and various forms of exanthema (Table 2). Occasionally, patients develop colitis, pancreatitis, or interstitial lung disease (Knowles et al. 1999; Shiohara et al. 2007). Over 70% of these cases have marked eosinophilia (often $>10^9$/l), and activated lymphocytes are often found in the circulation similar to acute HIV or generalized herpes infections. This syndrome has many names; the most frequently used are drug (-induced) hypersensitivity syndrome (DHS or DiHS) or drug rash with eosinophilia and systemic symptoms (DRESS). Importantly, the symptoms can start up to 12 weeks after start of treatment, often after increasing the dose, and may also persist and recur for many weeks even after cessation of drug treatment. The clinical picture resembles a generalized viral infection, e.g., an acute Epstein-Barr virus infection, but it is distinguished by prominent eosinophilia. Many patients have facial swelling, and some have signs of a capillary leak syndrome (a side effect also observed in high dose IL-2 therapy), namely enhanced vascular permeability of small vessels with generalized edema, also affecting the lungs – similar to patients with a cytokine release syndrome or patients with a "cytokine storm" (Pichler and Campi 2007). Indeed, various cytokines are massively increased in the serum of these patients – not as high, but still similar to the incident with TGN1412 (a peculiar anti-CD28 antibody able to stimulate T cells to cytokine production and proliferation) – which has certain clinical similarities to this syndrome and supports the idea that certain drugs can act in a superagonistic/superantigen-like way (Pichler 2002).

A recent careful analysis of reported cases caused by antiepileptics, minocycline, allopurinol, and abacavir revealed quite consistent clinical pictures according to the eliciting drug, but also quite striking differences in the symptoms (Peyriere et al. 2006). For example, abacavir-induced reactions lack eosinophilia and hepatitis, but cause mainly gastrointestinal and respiratory symptoms, lamotrigine does (rarely) cause eosinophilia, while allopurinol-induced DRESS often involves the kidneys, etc.

The clinical picture is quite dramatic, and because the disease tends to persist in spite of stopping the drug, many patients are not diagnosed timely and correctly. However, every doctor using anticonvulsants should know about this syndrome because it occurs in up to 1:3,000 treated patients! The mortality is about 10%, and some patients only survive after an acute liver transplantation. This disease likely causes more deaths (mainly due to fulminant hepatitis) than anaphylaxis.

Patients with DiHS/DRESS have many activated T cells in their circulation. These drug-specific T cells are directly stimulated by the respective drug (p-i concept) and they secrete large amounts of IL-5 and IFN-γ (Naisbitt et al. 2003). A peculiar feature of this syndrome is its long-lasting clinical course despite withdrawal of the causative drug. There may also be a persistent intolerance to other, chemically distinct, rather innocuous drugs (e.g., acetaminophen), leading to flare up reactions weeks after stopping the initial drug therapy and further confusing the picture (see below). Sometimes the exposure to a new drug, e.g., for the treatment of a suspected complication such as a superinfection, might precipitate fulminant liver failure (observed for vancomycin after a DRESS developed due to sulfasalazine). Treatment often requires high doses of corticosteroids and careful and slow tapering, in particular if the hepatitis is severe.

Recently, it has been shown that, in many patients with this syndrome, human herpes simplex virus 6 (HSV-6), and/or cytomegalovirus (CMV) and/or Epstein-Barr virus (EBV) DNA can be detected in serum samples during the 3rd or 4th week of the disease (but not before), followed by a rise of antibodies to these herpes viruses (Hashimoto et al. 2003). Thus, similar to HIV infections where T cell activation can also enhance virus production, the drug-induced massive immune stimulation may reactivate these latent lymphotropic herpes-viruses, which subsequently replicate and possibly contribute to the chronic course and persistent drug-intolerance in the affected patients.

While in DRESS/DiHS the exanthema might help to suggest a drug hypersensitivity reaction, there are other drug hypersensitivity reactions without exanthema. Many drugs can induce isolated *hepatitis,* and some (penicillins, proton pump inhibitors, quinolones, disulfiram, etc.) *(interstitial) nephritis* (Spanou et al. 2006). The symptoms may start insidiously and cause substantial tissue damage and even death. In drug-induced interstitial nephritis, eosinophils can sometimes be detected in the urine (even in the absence of eosinophilia in the blood) (Spanou et al. 2006; Pichler 2007). Rarer are *interstitial lung diseases* (furadantin), *pancreatitis, isolated fever or eosinophilia* as the only symptom of a drug allergy.

4.5 Multiple Drug Hypersensitivity Syndrome

The term multiple drug hypersensitivity is used for different forms of side effects to various drugs. Some use it to characterize patients with multiple drug intolerance (“pseudoallergy” to various NSAID, etc.), others reserve this term for well-documented, repeated, and clearly immune-mediated reactions to structurally

unrelated drugs (Pichler 2007). Cross-reactivity due to structural similarity is therefore not included.

According to our experience, about 10% of patients with well-documented drug hypersensitivity (skin and/or lymphocyte transformation test positive) have multiple drug allergies (Neukomm et al. 2001; Gex-Collet et al. 2005). For example, a patient may react to subcutaneously injected lidocaine with massive angioedema, then years later develop a contact allergy to corticosteroids. Alternatively, a patient may react to amoxicillin, phenytoin, and sulfamethoxazole within a few months, but with different symptoms (MPE, DiHS/DRESS, erythrodermia). Most patients have had rather severe reactions to at least one drug. An IgE-mediated reaction can be followed by a T cell-mediated reaction. The reason for this accumulation of drug hypersensitivities in one individual is unknown. One explanation might be a deficient tolerance mechanism against small chemical compounds/xenobiotics. An immune reaction to a drug, be it via hapten or p-i mechanism, can be seen as a failure of tolerance, and the same patient might not only be prone to develop other drug allergies but also autoimmune diseases. Preliminary data suggest, indeed, that a previous drug allergy might be a risk factor for the development of a delayed hypersensitivity reaction to contrast media.

4.6 Flare Up Reactions

Multiple drug hypersensitivity should be differentiated from *flare up reactions* (Pichler 2007). In patients with systemic drug allergies such as severe MPE or DRESS/DiHS, the T cell part of the immune system is massively activated, similar to acute viral infections. Like in the latter, these patients seem to have a lower threshold to react to a new drug and might show a flare up of their rash to a new drug, which is often an antibiotic. The patient is then labeled as allergic to the first and second antibiotic, but testing after remission might reveal only a sensitization to the first antibiotic, and the second is well tolerated in provocation (personal observation). The T cell reactivity to the second antibiotic would require a massive T cell stimulation, which is absent under normal circumstances.

The clinical observation of a flare up reaction, and the previously mentioned cofactors for drug hypersensitivity reactions such as viral infections, etc., suggest that an efficient stimulation of T cells by a drug is the sum of drug-TCR affinity and readiness of the cell to react. Quite a few TCRs may have the ability to interact with drugs, probably with different affinities. If the immune system is resting, only drug-TCR interactions of high affinity may be able to stimulate T cells sufficiently to cause T cell expansion. If sufficient cells react, symptoms might arise. But even such a high affinity interaction may remain unnoticed if too few cells are stimulated. If the immune system is activated and the readiness to react is increased (lower threshold), even relatively low affinity drug-TCR interactions might suffice to activate many T cells thus resulting in a symptomatic reaction. After the cofactor is eliminated, the low affinity binding drug remains negative in skin tests and is later

well tolerated if the costimulatory conditions are no longer present. This hypothesis might also explain the transient nature of many exanthema caused by antibiotics in childhood; the viral cofactors are missing during later testing and provocation.

5 Conclusion

Drug hypersensitivity reactions often have a bizarre clinical picture, possibly due to an unusual and not well-coordinated stimulation of the immune system, bypassing many rules and immunological "checkpoints". This situation is reminiscent of transplantation medicine, another "radical" intervention of modern medicine, where the allo-response caused unforeseen immunological problems. In both situations, the immune system is faced with a man-made challenge for which it was not trained during evolution – as neither transplantation nor the extensive use of xenobiotics had previously occurred. The better understanding of these puzzling diseases over recent years opens up the possibility of preventing many of them in the near future, as has been well demonstrated for abacavir and carbamazepine-induced reactions, where typing for immunogenetic markers dramatically reduced the incidence.

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Adverse Drug Reactions Affecting Blood Cells

Richard H. Aster

Contents

Abstract Numerous medications and other xenobiotics are capable of producing adverse reactions (ADRs) affecting red cells, platelets or neutrophils. Occasionally, more than one blood element is affected simultaneously. As with all drug reactions,

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some side effects are a direct consequence of a known pharmacologic action of the drug and are dose-dependent; others occur sporadically and relatively independent of dose. The latter ("idiosyncratic") reactions are unpredictable and, in general, have no known underlying genetic basis. Many are antibody-mediated, as would be expected since cellular immune effector cells have little direct access to circulating blood cells. In this chapter, we will discuss idiosyncratic drug reactions affecting blood and blood forming tissues with an emphasis on those thought to be immune-mediated.

Keywords Drug sensitivity, Drug-induced cytrpennia

Abbreviations

ADR	Adverse drug reaction
AITP	Autoimmune thrombocytopenic purpura
DDAb	Drug-dependent antibody
DIHA	Drug-induced immune hemolytic anemia
DITP	Drug-induced immune thrombocytopenia
G-CSF	Granulocyte colony simulating factor
HIT	Heparin-induced thrombocytopenia
HUS	Hemolytic uremic sundrome
NSAID	Non-steroidal anti-inflammatory drug
RGD	Arginine-glycine-aspartic acid

1 ADRs Affecting Red Blood Cells

1.1 Pathogenesis

A wide range of drugs is capable of inducing antibodies that cause red cell destruction. At least three distinct mechanisms are involved (Table 1).

Table 1 Drugs shown to cause immune hemolytic anemia[a]

Mechanism	Examples
Hapten-dependent antibody	Penicillin, cephalosporins?
Drug-dependent antibody	Cefotetin, ceftriaxone, ceftizoxime, tolmetin, etodolac, chlorpropamide, carboplatin, cisplatin, ibuprofen, oxaliplatin, piperacillin, probenecid, quinine, quinidine
Autoantibody induction	Alpha-methydopa, l-dopa, procainamide

[a]For a more extensive listing see Arndt and Garratty (2005) and Johnson et al. (2007)

1.1.1 Hapten-Specific Antibodies

In past years, when intravenous penicillin was given in massive doses to treat bacterial infections, it was found that 10–30% of patients so treated developed a positive direct antiglobulin test and that a few experienced frank hemolytic anemia (Garratty 1993). Penicillin and penicillin derivatives contain a beta lactam structural element that enables them to link covalently to amine groups on proteins, leading to the formation of drug-protein adducts capable of inducing drug (hapten)-specific antibodies. In patients receiving massive doses of penicillin, such antibodies may bind to red cell-associated drug in quantities sufficient to cause hemolysis. With the advent of newer penicillin derivatives that are effective at lower doses, hemolytic anemia mediated by this mechanism is now rare. Nonetheless, some patients with probable drug-induced immune hemolytic anemia (DIHA) have antibodies that recognize red cells that have been pretreated with the suspect drug and then washed (Garratty 1993; Arndt and Garratty 2005; Garratty and Arndt 2007). These patients often also have a second, "drug-dependent" antibody (see below) that may be the actual cause of red cell destruction.

1.1.2 Drug-Dependent Antibodies

A second type of drug-induced hemolysis is mediated by drug-induced antibodies that require soluble drugs to be present in order to bind to specific red cell membrane glycoproteins and cause red cell destruction. These antibodies can be distinguished from the hapten-specific antibodies described above by their inability to recognize drug-coated cells and by failure of soluble drugs at high concentrations to inhibit binding to their targets. It was formerly thought that this type of antibody caused red cell destruction by reacting with drugs to produce "immune complexes" which, in turn, reacted with red cells (Shulman 1964; Shulman and Reid 1993), but the putative complexes were never identified and no plausible mechanism was advanced to explain why they might be reactive with specific cell membrane glycoproteins. Moreover, drug-dependent binding of this type of antibody to its cellular target was later shown to occur by the Fab domain, rather than by Fc as would be expected of an immune complex (Christie et al. 1985; Smith et al. 1987). Although a classical "immune complex mechanism" is not involved in the destruction of cells by this type of antibody, exactly how drug promotes binding of the antibody to its target without linking covalently to either of the two interacting macromolecules is not well understood. What is known about the process will be discussed below under "thrombocytopenia." Virtually nothing is known about the mechanisms responsible for induction of drug-dependent, red cell-specific antibodies. A possible clue is provided by reports of patients whose antibodies reacted preferentially with red cells carrying specific alloantigens of the Rh, Kell, or Kidd blood group systems (Sosler et al. 1984; Salama and Mueller-Eckhardt 1987), suggesting that in some cases the immune response may involve recognition by B cells of a complex consisting of a drug bound to a polymorphic determinant on a cell membrane glycoprotein.

1.1.3 Drug-Induced Autoantibodies

Certain drugs induce red cell-reactive antibodies that bind to their targets in the absence of exogenous drug and have other characteristics of antibodies found in patients with idiopathic autoimmune hemolytic anemia. The prototype drug of this type is alpha-methyldopa, an agent formerly used widely for the treatment of hypertension. Approximately 20–30% of individuals treated with this drug for 4–6 months developed a positive direct antiglobulin test and a few of them experienced overt hemolysis, which was often mild (Petz 1993). Although the link between alpha-methyldopa and other drugs, such as levodopa and procainamide, and development of autoimmune hemolytic anemia is well established on epidemiologic grounds, how drugs induce this type of antibody is unknown. One possibility suggested is that the responsible drugs interact with cell membrane proteins and alter their structure in such a way that they become immunogenic (Green et al. 1980), but there is little experimental evidence for this. An early suggestion that alpha-methyldopa blocks the function of immune suppressor cells, leading to a state of immune hyper-responsiveness was not confirmed by later investigators (Garratty et al. 1993).

1.2 Implicated Drugs

Representative drugs that can trigger immune hemolytic anemia are listed in Table 1, but many others have been implicated in reviews and in individual case reports. For additional detail, the reader is referred to Arndt and Garratty (2005), Garratty and Arndt (2007), and Johnson et al. (2007). In a particularly useful recent review, Arndt and Garratty listed 125 drugs for which they believe there is a "reasonable" likelihood of a cause-and-effect relationship between drug exposure and immune hemolysis, and noted that the spectrum of implicated drugs has changed progressively over the years as new treatment regimens have come into use (Arndt and Garratty 2005). Drugs identified as causes of DIHA with relatively high frequency in recent years include second and third generation cephalosporin antibiotics, especially cefotetin and ceftriaxone, non-steroidal anti-inflammatory drugs (NSAIDs), including ibuprofen and diclofenac, and the chemotherapeutic drug, oxaliplatin.

1.3 Clinical Findings

Patients developing DIHA by any of the mechanisms described above will almost invariably have had prior exposure to the immunizing drug for at least 5–7 days. Mildly affected individuals may experience only a modest decrease in hematocrit; those more seriously affected may develop anemia, hemoglobinuria, and jaundice over a period of days. In an unfortunate subset of patients with high titer drug-dependent antibodies, exposure to the sensitizing medication produces

shaking chills, nausea, vomiting, and lower back pain followed by hypotension and vascular collapse, sometimes leading to a fatal outcome. Survivors may have renal failure requiring hemodialysis and other supportive measures. This extreme presentation is most likely to occur in patients sensitive to cefotetan, ceftriaxone, or NSAIDs (Garratty 1993; Arndt and Garratty 2005; Johnson et al. 2007).

1.4 Diagnosis

The hallmark of drug-induced immune hemolytic anemia is a positive antiglobulin test (Garratty and Arndt 2007; Johnson et al. 2007), indicating that the patient's red cells are coated with immunoglobulins, usually IgG, and sometimes complement. Hapten-specific antibodies can often be detected by performing an indirect antiglobulin test using patient serum and washed target red cells pretreated with the implicated drug (Garratty 1993; Garratty and Arndt 2007; Johnson et al. 2007). An eluate prepared from the patient's red cells may behave similarly. The haptenic character of the antibodies can sometimes be documented by showing that reactions with drug-coated red cells are inhibited by a soluble drug at high concentration.

Drug-dependent red cell antibodies can often be detected by showing that a soluble drug promotes binding to washed red cells of an IgG or IgM immunoglobulin present in the patient's serum. Because a soluble drug drives the reaction between antibody and target, the suspect drug should be kept in the reaction mixture throughout the test procedure for optimum sensitivity. Although these patients commonly have a positive direct antiglobulin test, eluates prepared from their red cells fail to react positively with red cells unless the drug is added to the reaction mixture. If the blood sample tested was collected soon after the last exposure to the sensitizing medication, residual drug present in the sample may cause the indirect antiglobulin test to be positive without adding the drug (Johnson et al. 2007). It is important to appreciate that some patients appear to have both hapten-specific (as defined above) and drug-dependent antibodies (Garratty 1993; Garratty and Arndt 2007). Before assuming that a drug-dependent antibody is not present, such samples should be dialyzed to remove any soluble drug and then re-tested with and without the drug.

It is not uncommon for negative test results to be obtained in patients with a history strongly suggestive of DIHA. One reason for this is that the sensitizing drug is sometimes virtually insoluble in water, making it difficult to work with in vitro. A second is that a metabolite of the drug, rather than the primary drug itself, can be the sensitizing agent (Salama and Mueller-Eckhardt 1985; Salama et al. 1991; Bougie et al. 1997; Johnson et al. 2007). In such cases, it is sometimes possible to identify an antibody by using urine from an individual taking the sensitizing medication as the source of "drug" for serologic testing. The range of drug metabolites that can trigger DIHA is not well defined, but a few studies have shown that products of both primary and secondary drug metabolism can be sensitizing agents (Bougie et al. 1997; Cunha et al. 2000).

Drug-induced autoantibodies behave serologically like those seen in patients with autoimmune hemolytic disease not associated with drug exposure. Red cell eluates often react preferentially with certain antigens of the Rh system (Salama et al. 1991). Unfortunately, there is no way to confirm that a pharmacologic agent was the trigger for the autoantibody in a particular patient.

1.5 Treatment

As with all drug-induced disorders, a high index of suspicion, leading to discontinuation of the sensitizing medication, is of paramount importance. Most patients with hapten-specific or drug-dependent antibodies recover within a few days of stopping the responsible agent. Unfortunately, it is not rare for the diagnosis to be overlooked and for a patient to be given corticosteroids for the assumed diagnosis of idiopathic autoimmune hemolytic anemia. This can lead to re-exposure to the responsible agent at a later date with serious consequences (Cunha et al. 2000). Severely affected patients may require red cell transfusions and treatment for acute renal failure. Kidney function usually returns to normal eventually, although weeks or months may be required. Patients with drug-induced autoantibodies may not recover spontaneously. When treatment is necessary, they should be managed as if they had idiopathic autoimmune hemolytic anemia.

2 ADRs Affecting Blood Platelets

2.1 Pathogenesis

For unknown reasons, platelets are targeted by drug-induced antibodies much more often than red cells, neutrophils, or hematopoietic stem cells. The etiology of drug-induced immune thrombocytopenia is complex in that, in addition to the three mechanisms described above for DIHA, at least three other distinct pathologic entities have been identified. Each of these will be briefly discussed here.

2.1.1 Hapten-Induced Antibodies

Although hapten-specific antibodies have been shown to be a cause of DIHA, it is much less certain whether this mechanism ever applies to drug-induced immune thrombocytopenia. Possible exceptions are rare patients who appear to have developed acute thrombocytopenia after being given penicillin or penicillin derivatives (Salamon et al. 2004), which as noted above, can spontaneously link to cell membrane proteins through beta-lactam structural elements. However, studies to

document the existence of hapten-specific antibodies have not been performed in such patients. Accordingly, it is uncertain whether classical, hapten-specific antibodies ever cause immune thrombocytopenia.

2.1.2 Drug-Dependent Antibodies

Drug-dependent antibodies of the type already mentioned in connection with DIHA are a much more common cause of immune thrombocytopenia (Aster and Bougie 2007). The favored targets for these antibodies on platelets are the glycoprotein IIb/IIIa complex (αIIb/β3 integrin) and the GPIb/IX complex, the receptors for fibrinogen and von Willebrand factor, respectively. In the presence of soluble drug, drug-dependent antibodies react with platelet membrane glycoproteins through their Fab domains (Christie et al. 1985; Smith et al. 1987). Binding of the antibody to its target immobilizes the drug itself, suggesting that the drug becomes "trapped" in the course of antibody binding (Christie and Aster 1982). One mechanism proposed to explain this process is that the drug reacts first with a site on the target glycoprotein to produce an epitope consisting of both drug and amino acid residues adjacent to its binding site for which antibody is specific. A second is that the drug reacts first with the antigen-combining site of the antibody and modifies it in such a way that it acquires specificity for an epitope on the target glycoprotein. Recently, a unifying hypothesis (Bougie et al. 2006; Aster and Bougie 2007) has been proposed suggesting that drug-dependent antibodies are derived from a pool of naturally occurring immunoglobulins weakly reactive with epitopes on autologous proteins, including membrane glycoproteins, and that certain drugs contain structural elements enabling them to react weakly with both antibody and its target, markedly increasing the affinity of the interaction (Fig. 1). When the drug triggers the proliferation of B cells expressing this type of receptor, antibodies having the characteristics of those seen in patients with drug-induced immune thrombocytopenia (DITP) are produced. Whether the drug reacts first with antibody or first with the

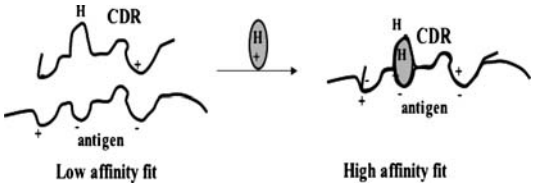

Fig. 1 A model for drug-dependent antibody binding. *Left*: Antibodies capable of causing drug-dependent thrombocytopenia recognize an epitope on a platelet glycoprotein but the reaction is too weak for significant numbers of antibody molecules to bind in the absence of drug. *Right*: The sensitizing drug contains structural elements that are complementary to charged (+/−) or hydrophobic domains (H) on the glycoprotein epitope and the complementarity determining region (CDR) of the antibody and improves the "fit" between the two proteins, increasing the K_A to a value that permits binding to occur at levels of antibody, antigen and drug achieved in the circulation after ingestion of the drug (from Bougie et al. 2006)

target protein is relatively unimportant and would depend simply on its relative affinity for one macromolecule or the other. This proposal does not, of course, provide an explanation for why platelet glycoproteins are the preferred targets for drug-dependent antibodies or shed light on how these antibodies are induced.

2.1.3 Antibodies Specific for RGD-Mimetic Platelet Inhibitors

"Fibans" are a class of drugs that react with the arginine-glycine-aspartic acid (RGD) recognition site on platelet glycoprotein IIb/IIIa and prevent this receptor from binding fibrinogen following platelet activation. Two of these "RGD-mimetic" compounds (eptifibatide and tirofiban) are widely used to prevent posttreatment thrombotic events in patients undergoing coronary angioplasty. Between 0.1 and 2% of patients given one of these drugs develop acute, sometimes severe, thrombocytopenia after their first exposure. In such patients, platelet destruction is caused by naturally occurring antibodies that recognize GPIIb/IIIa complexed with the ligand-mimetic drug (Bougie et al. 2002). It is generally thought that these antibodies are specific for conformational changes induced in GPIIb/IIIa by drugs of this class. However, antibodies induced by tirofiban rarely cross-react with those induced by eptifibatide and do not bind to GPIIb/IIIa complexed with the natural ligand, RGD. If this hypothesis is correct, then it follows that each ligand mimetic drug must induce a slightly different set of conformational determinants. Why antibodies of this type occur naturally in some individuals is an interesting, unresolved question.

2.1.4 Antibodies Reactive with Abciximab

Abciximab, the first "humanized" murine monoclonal antibody approved for human use, is a chimeric (human/mouse) Fab fragment specific for an epitope on the GPIIIa component of the GPIIb/IIIa complex close to the RGD recognition site (Artoni et al. 2004). Like the fiban drugs, abciximab inhibits binding of fibrinogen to activated GPIIb/IIIa. Roughly 1–2% of patients given abciximab for the first time and about 10% reexposed to the drug experience thrombocytopenia, often severe, within 6–12 h of starting an infusion. Thrombocytopenia after first exposure to abciximab appears to be caused by naturally occurring antibodies specific for murine sequences in the chimeric abciximab molecule (Curtis et al. 2002, 2004). Thrombocytopenia following a second exposure is caused by antibodies stimulated to a high titer by a prior treatment. A subset of patients given abciximab for the first time have normal platelet levels for 6–8 days, after which the count plummets to extremely low values. This complication is caused by high titer antibodies made in response to the initial 12–24 h infusion of the drug, which are able to cause thrombocytopenia because abciximab-coated platelets remain detectable in the circulation for 7–14 days after treatment (Mascelli et al. 1998).

2.1.5 Drug-Induced Platelet-Specific Autoantibodies

Autoimmune thrombocytopenia (AITP) is considerably more common than autoimmune hemolytic anemia and is not generally associated with drug sensitivity. Anecdotal clinical and laboratory findings suggest, however, that some medications do induce platelet-reactive autoantibodies and produce a clinical picture very similar to AITP (Aster 2000). An AITP-like disorder developed in 1–2% of patients given gold salts for rheumatoid arthritis, a treatment rarely used at this time (von dem Borne AE et al. 1986; Adachi et al. 1987). Levodopa, procainamide, penicillamine, and sulfamethoxazole (Aster 2000) (Fig. 2) have been implicated as causes of AITP. It has been suggested that these agents may perturb the processing of platelet glycoproteins by phagocytic cells in such a way that "cryptic peptides" not ordinarily seen by the immune system occasionally trigger an autoimmune response (Aster 2000), but there is no experimental evidence for this. In recent years, numerous reports have described patients who developed a clinical picture similar to AITP following treatment with the chimeric monoclonal antibodies rituximab (anti-CD20), infliximab (anti-TNF alpha),

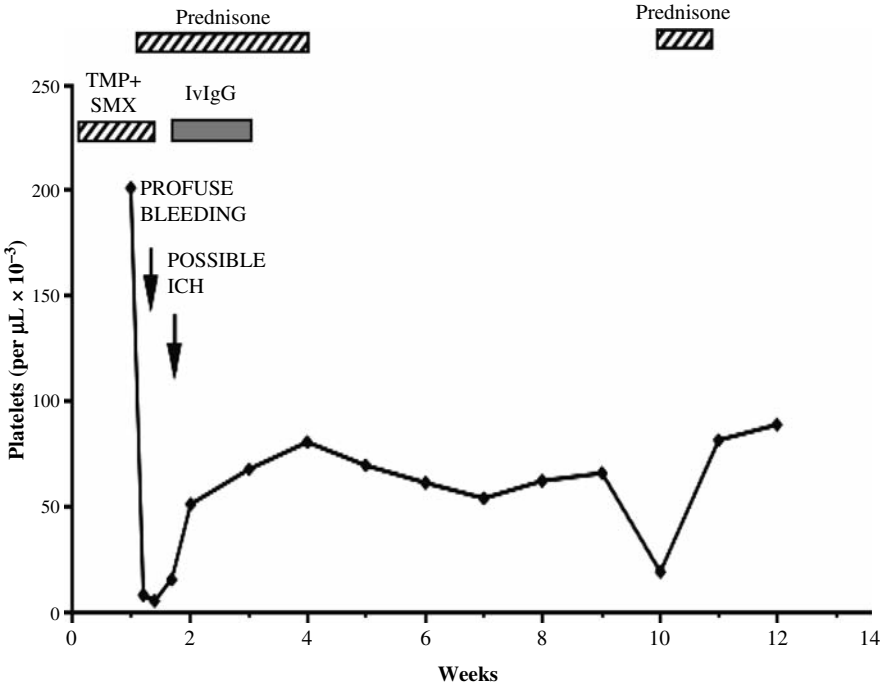

Fig. 2 Development of chronic autoimmne thrombocytopenia in a patient who presented initially with thrombocytopenia caused by sulfamethoxazole (SMX)-dependent, platelet-reactive antibodies. SMX-dependent antibodies were identified in acute phase serum together with GPIIb/IIIa-specific nondrug-dependent autoantibodies. Persistent nondrug-dependent antibodies reactive with autologous platelets were identified during weeks 1, 5, and 9. *SMX* Sulfamethoxazole; *ICH* intracranial hemorrhage; *IVIg G* intravenous gamma globulin (from Aster 2000)

etanercept (anti-TNF alpha receptor), and efalizumab (anti-CD11a) (Vidal et al. 2003; McMinn et al. 2003; Otrock et al. 2005; Warkentin and Kwon 2005) used for treatment of malignant and autoimmune conditions. It seems possible that the immunomodulatory effects of these agents somehow trigger an autoimmune response, but why platelets should be targeted rather than some other tissue is unknown.

2.1.6 Heparin-Induced Thrombocytopenia (HIT)

Up to 5% of patients given unfractionated heparin experience thrombocytopenia, usually quite mild, after 7 days or more of treatment. The thrombocytopenia itself is rarely symptomatic, but some affected patients develop arterial and/or venous thrombosis, which can be fatal or lead to loss of a limb (Warkentin 2004; Davoren and Aster 2006). HIT is caused by heparin-induced antibodies specific for complexes of heparin and platelet factor 4, a 32-kDa basic protein found in platelet alpha storage granules. Thrombocytopenia appears to be caused by immune complexes consisting of PF4 and heparin together with complex-specific antibodies, which are formed on or close to the platelet surface and interact with platelet Fc receptors to produce platelet activation and fragmentation. Thrombosis appears to be caused by the procoagulant action of activated platelets and platelet-derived microparticles, but other mechanisms may also operate. HIT is the only well-documented example of a drug-induced, immune complex-mediated thrombocytopenia. A further description of HIT is beyond the scope of this review. For further detail, the reader is referred to several recent publications, e.g., Davoren and Aster (2006), and Warkentin (2007).

2.2 Implicated Drugs

Representative drugs implicated as triggers for DITP through several different mechanisms are listed in Table 2. Many others have been documented in various reviews and individual case reports (Aster and Bougie 2007). Classes of drugs implicated include antibiotics of all types, non-steroidal antiinflammatory drugs, the cinchona alkaloid compounds quinidine and quinine, platelet function inhibitors, sedatives, and anticonvulsants. The most common cause of immune thrombocytopenia is heparin, but, as noted above, the decrease in platelets associated with heparin sensitivity is relatively modest and the condition has a unique pathogenesis. The most common triggers for drug-dependent antibodies appear to be quinine, sulfamethoxazole, and vancomycin. Whether all the anecdotal reports of DITP actually reflect cause-and-effect relationships is uncertain. George and coworkers undertook a review of all English language reports of DITP and identified about 50 drugs they considered to be “definitely established” as causative on the basis of specific criteria. Fifteen other drugs thought to be “probable” causes of DITP were also identified. A website containing reference

Table 2 Mechanisms of drug-induced immune thrombocytopenia and representative implicated drugs[a]

Designation	Mechanism	Examples
Hapten-dependent antibody	Drug (hapten) links covalently to membrane protein and induces a drug-specific immune response	Penicillin, cephalosporin antibiotics?
Drug-dependent antibody	Drug induces antibody that binds to membrane protein only in the presence of soluble drug	Quinine, many antibiotics, non-steroidal antiinflammatory drugs, anticonvulsants
Fiban-induced thrombocytopenia	Drug (ligand) reacts with membrane glycoprotein IIb/IIIa and induces a conformational change recognized by antibody	Epitifibatide, tirofiban
Drug-specific antibody	Antibody is specific for the murine component of a chimeric Fab fragment that binds to platelet GPIIb/IIIa	Abciximab
Autoantibody induction	Drug induces antibody that reacts with platelets in the absence of drug.	Gold salts, L-Dopa, procainamide
Immune complex	Drug binds to platelet factor 4 (PF4) to produce a complex for which antibody is specific. The resulting immune complex activates platelets via Fc receptors	Heparin

[a]For more extensive listings, see Aster and Bougie (2007) and the website maintained by J.N. George et al., University of Oklahoma (http://moon.ouhs3.edu\jgeorge)

material compiled by George et al. can be accessed at http://moon.ouhs3.edu/jgeorge. A recent finding of interest is that chemotherapeutic and immunosuppressive drugs, usually thought to cause thrombocytopenia through immunosuppression, can also induce drug-dependent antibodies capable of causing acute, severe thrombocytopenia (Curtis et al. 2006).

2.3 Clinical Findings

In general, most patients developing DITP will have been exposed to the sensitizing medication for at least 6–8 days prior to experiencing a drop in platelet counts. The platelet inhibitors, eptifibatide, tirofiban, and abciximab, are exceptions to this rule since, as noted above, antibodies specific for these drugs can be naturally occurring, leading to acute thrombocytopenia within hours of the first exposure. When high titer, drug-dependent antibodies are present, it is not uncommon for drug

exposure to be followed by systemic symptoms such as chills, fever, nausea and vomiting, and hypotension. In general, severity of bleeding is inversely proportional to the platelet count. Patients with very low counts (platelets 0–10,000 per μL) often present with widespread purpuric lesions on the skin and mucosal surfaces and bleeding from various body orifices. However, others with equally severe thrombocytopenia are sometimes almost asymptomatic. A subset of patients with quinine-induced immune thrombocytopenia present with renal failure and microangiopathic hemolytic anemia typical of the hemolytic uremic syndrome (HUS) (Gottschall et al. 1991; Kojouri et al. 2001). Rarely, other drugs have been implicated as triggers for immune thrombocytopenia and HUS (Wolf et al. 1989; Juang et al. 1992). It has been speculated but not established that this presentation may be the result of immune injury to endothelial cells (Dlott et al. 2004). Certain chemotherapeutic and immunosuppressive drugs can trigger HUS without accompanying immune thrombocytopenia (Dlott et al. 2004), but it is uncertain whether this condition has an immune etiology.

After discontinuing the sensitizing medication, bleeding symptoms, if present, usually subside within 1 or 2 days and platelet counts return to normal within 1 week. Catastrophic bleeding is quite rare but fatal intracranial and intrapulmonary hemorrhages have been described. Patients with immune thrombocytopenia and HUS may require hemodialysis and other supportive measures, but they usually regain normal renal function after weeks or months.

2.4 Diagnosis

A high index of suspicion is critically important when a patient presents with acute, severe thrombocytopenia of uncertain etiology. Such patients should always be asked about drug exposure, especially to quinine, antibiotics, and NSAIDs. Unfortunately, DITP is sometimes confused with acute, autoimmune thrombocytopenia. In such cases, platelet recovery resulting from discontinuation of the drug may be wrongly attributed to the effects of short-term corticosteroid therapy, placing the patient at risk for a recurrence when the sensitizing drug is taken again at a later time. Examples of patients with DITP who were hospitalized multiple times and subjected to inappropriate treatments such as splenectomy have been graphically described (Reddy et al. 2004). Surreptitious ingestion of a sensitizing drug to produce thrombocytopenia has also been reported (Reid and Shulman 1988).

Demonstration of a drug-dependent, platelet-reactive antibody (DDAb) can also be helpful. However, competent testing for DDAbs is not widely available and the antibodies are more difficult to detect in a clinical laboratory than red cell-specific antibodies. It is often possible to detect DDAbs by incubating normal washed platelets with patient serum and the suspect drug followed by washing and detection of platelet-bound immunoglobulins by flow cytometry (Visentin et al. 1990; Aster and Bougie 2007). However, it is not uncommon to obtain negative test results in patients with a clinical history strongly suggestive of DITP. As with drug-induced

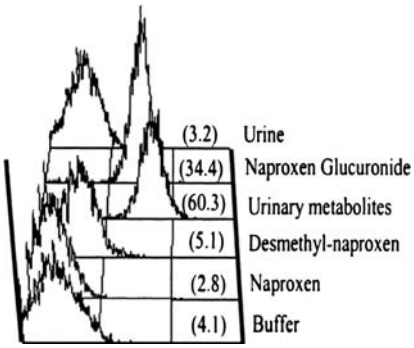

Fig. 3 Demonstration of antibodies specific for drug metabolite in a patient who developed acute thrombocytopenia after taking the non-steroidal antiinflammatory drug naproxen. Serum from the patient contained an IgG antibody that recognized normal platelets in the presence of naproxen glucuronide in a flow cytometric assay. Antibody binding was not promoted by naproxen. However, positive reactions were obtained with drug metabolites isolated from the same urine ("urinary metabolites") and with purified naproxen glucuronide. No reaction was obtained with 6-0-desmethyl naproxen, another metabolite of naproxen. Mean fluorescence intensity values are shown in parentheses (from Bougie and Aster 2001)

red cell antibodies, relative insolubility of many drugs can complicate in vitro testing for a DDAb. A second, well-documented reason for negative test results is that drug metabolites can be the sensitizing agents (Salama and Mueller-Eckhardt 1985; Bougie and Aster 2001) (Fig. 3). For reasons unknown, glucuronide conjugates of NSAIDs and other medications appear to be especially prone to trigger drug-dependent antibodies leading to DITP (Bougie et al. 2007).

2.5 Treatment

Discontinuation of the sensitizing medication is of paramount importance.

Patients with serious bleeding may require platelet transfusions, which can be transiently effective even though the transfused cells may be rapidly destroyed. IV gamma globulin and even plasma exchange have been used in severely affected individuals, but their effectiveness is uncertain (Aster and Bougie 2007). Patients with drug-induced autoimmune thrombocytopenia behave similarly to patients with autoimmune thrombocytopenia (AITP) unrelated to drug exposure and should be treated accordingly.

3 ADRs Affecting Neutrophils

3.1 Pathogenesis

Chemotherapeutic drugs used for treatment of cancer suppress hematopoiesis and can reduce neutrophil levels to a point at which the risk of infection is increased. This is a predictable, dose-related, and usually reversible complication of treatment with such agents and will not be discussed further. We will instead consider unpredictable instances of drug-associated neutropenia that tend not to be dose related and are generally ascribed to "idiosyncrasy" (Kaufman and Jurgelon 1996; Andrès et al. 2006; Andersohn et al. 2007; Andrès and Maloisel 2008). Evidence for a cause-and-effect relationship between drug exposure and neutropenia is in most cases based on epidemiologic grounds because of a lack of reliable laboratory methods for identifying the responsible drug.

Two distinctly different mechanisms appear to cause idiosyncratic drug-induced neutropenia (DINP). One is more or less dose related, appears not to have an immune etiology, and probably involves interference by the drug with critical cell functions such as mitosis or protein synthesis. Underlying poorly defined genetic or acquired abnormalities may predispose individuals to this complication but the underlying mechanism(s) are poorly understood. Drugs that appear to cause neutropenia by nonimmune mechanisms include the phenothiazine group of tranquilizers, antithyroid drugs such as propylthiouracil, anticonvulsants, and the antipsychotic drug clozapine. Of these, clozapine is remarkable in that about 2–3% of patients treated chronically develop neutropenia and a subset of them experience agranulocytosis, which may or may not be reversible. A reactive metabolite of clozapine has been shown to induce apoptosis of neutrophils at pharmacologic concentrations (Williams et al. 2000), but whether this mechanism accounts for clozapine-induced neutropenia is uncertain. Certain drug metabolites may cause neutropenia by affecting supporting tissues of the bone marrow (stromal cells) rather than hematopoietic cells themselves (Guest and Uetrecht 1999).

Drugs can also cause idiosyncratic neutropenia through immune mechanisms (Andersohn et al. 2007; Andrès and Maloisel 2008). Individual reports have described patients who developed severe neutropenia after a week or more of drug treatment and who possessed antibodies that reacted with neutrophils only when the implicated drug was present (Stroncek et al. 1994). The underlying mechanism is probably similar to that involved in drug-induced immune thrombocytopenia and immune hemolytic anemia but has not been as thoroughly studied. In patients who develop agranulocytosis as well as neutropenia, the antibodies presumably affect myeloid elements of the bone marrow as well as peripheral blood neutrophils, but this has not been directly demonstrated.

Certain agents, like propylthiouracil, may cause neutropenia by immune mechanisms in some individuals (Fibbe et al. 1986) and by nonimmune mechanisms in others. The target molecules recognized by drug-dependent, neutrophil-specific antibodies have not been identified, although it has been shown that quinine-dependent neutrophil antibodies react with several surface glycoproteins

ranging from 32 to 85 kDa in molecular mass (Stroncek et al. 1994). The paucity of available detail about drug-dependent, neutrophil-reactive antibodies and their target molecules is due at least in part to the technical difficulties inherent in working with granulocytes in the laboratory.

3.2 Implicated Drugs

Drugs generally thought to be triggers for neutropenia and agranulocytosis are listed in Table 3 but many others have been implicated in reviews and individual case reports. For additional information, the reader is referred to Kaufman et al. (1996), Andersohn et al. (2007) and Andrès and Naloisel (2008).

3.3 Clinical Findings

As with drug-induced hemolytic anemia and thrombocytopenia, affected patients will usually have taken the responsible drug for at least 1 or 2 weeks before presenting with symptoms such as sore throat, pneumonia, and sometimes overwhelming sepsis. Profound neutropenia is found in the blood, but platelet levels and hematocrit are usually normal. Identifiable neutrophil precursors may be completely absent from the marrow or may be "arrested" in development, whereas cells of the megakaryocyte and erythroid lineages are normal.

3.4 Diagnosis

In the absence of a convenient and sensitive way to document that a particular drug is actually the cause of neutropenia, a high index of suspicion is particularly important. Drug-dependent antibodies can sometimes be identified using methods comparable to those described above for platelet-dependent antibodies. Whether the paucity of

Table 3 Drugs implicated in neutropenia/agranulocytosis[a]

Class of drug	Examples
Analgesic/ antiinflammatory	Diclofenac, **dipyrone**, ibuprofen, indomethacin, **phenylbutazone** sulfapyridine, **sulfasalazine**
Antibiotic	Ampicillin, cefotaxime, ceftazidine, ciprofloxacin, methicillin, sulfathiazole, **sulfamethoxazole**
Anticonvulsant	Carbamazepine, felbamate, mesantoin, phenytoin, valproic acid
Antihistamine	Cimetidine, ranitidine
Antimalarial	Amodiaquin, chloroquine, quinine
Antipsychotic/ tranquilizer	Chlorpromazine, **clozapine**, mianserin
Antithyroid	**Carbimazole. propylthiouracil**, thiocyanate
Other	**Allopurinol**, penicillamine, **procainamide**, **gold salts**, **ticlopidine**

[a]Drugs associated with higher risk are in bold. For more extensive listings see Kaufman et al. (1996), Andersohn et al. (2007) and Andrès et al. 2008

reports describing such antibodies is due to the fact that they are rare or because few laboratories are capable of carrying out the required diagnostic tests is uncertain.

3.5 Treatment

Patients being treated with drugs that have a relatively high risk for agranulocytosis should be advised to contact their physician if unexplained symptoms suggestive of infection develop, especially sore throat or other upper respiratory symptoms. In patients with possible drug-induced neutropenia, each of the potentially responsible drugs should be discontinued and replaced by pharmacologic equivalents when necessary. Supportive care, including appropriate antibiotics when necessary, should be provided. The effectiveness of prophylactic antibiotics has not been fully established. Granulocyte colony-stimulating factor (G-CSF) can be helpful in some cases (Bhatt and Saleem 2004; Ibáñez et al. 2008). In patients with agranulocytosis, mortality rates formerly ranged from 10 to 25% but are now 2 to 10% with optimal supportive care (Andrès et al. 2002; Maloisel et al. 2004). Patients who have detectable neutrophil precursors in their bone marrow are more likely to recover than those in whom precursors are absent. In general, it is impractical to monitor neutrophil levels routinely in patients taking drugs that carry a relatively high risk of neutropenia. However, regular blood counts are recommended for patients being treated with clozapine.

4 Drug-Induced Aplastic Anemia

4.1 Pathogenesis

Drug-induced failure of all myeloid elements of the bone marrow, leading to "aplastic anemia", affects about 2–5 per million individuals and is more common in Asia than in Western populations (Montané et al. 2008; Young and Kaufman 2008). As with drug-induced neutropenia, in aplastic anemia both nonimmune and immune mechanisms appear to be operative. Agents that may act by affecting critical cell functions through nonimmune processes include benzene, to which industrial workers were formerly exposed, and chloramphenicol, an antibiotic shown more than 50 years ago to be associated with a relatively high incidence of the disease. Whether specific underlying, genetically determined or acquired factors predispose selected individuals treated with these drugs to develop aplastic anemia is uncertain. It is likely that immune mechanisms are responsible for most cases of drug-associated marrow aplasia. Some drugs that carry a relatively high risk for aplastic anemia are metabolized to reactive intermediates which, in theory, could produce immunogenic adducts capable of triggering an immune response. However, there is virtually no experimental evidence to document a cause-and-effect relationship between drug exposure

and marrow failure through an immune mechanism. It is now thought that autoimmunity accounts for a high percentage of cases of "idiopathic" aplastic anemia, which often respond to immunosuppressive therapy (Young et al. 2008). Patients with drug-induced aplastic anemia appear to be as likely to respond to immunosuppressive therapy as those whose disease is 'idiopathic", suggesting that the drug-induced form of the disease also has an underlying immune etiology. However, there is little direct evidence to support such a connection.

4.2 Implicated Drugs

Drugs implicated on epidemiologic grounds as likely causes of aplastic anemia are listed in Table 4. Many others have been described as possible triggers in individual case reports.

4.3 Clinical Presentation

Patients developing aplastic anemia often present with generalized weakness secondary to anemia, with infectious complications related to neutropenia or with bleeding secondary to thrombocytopenia. In general, patients will have had exposure to the medication suspected of triggering their condition for months prior to the onset of symptoms.

4.4 Diagnosis

No laboratory tools are presently available to identify the drug responsible for triggering aplastic anemia in an individual case. Accordingly, the diagnosis is based on the finding of pancytopenia in the peripheral blood and deficiency of myeloid and erythroid precursor cells in the bone marrow.

Table 4 Drugs implicated in aplastic anemia[a]

Class of drug	Examples
Analgesic/ antiinflammatory	**Dipyrone**, **felbamate**, diclofenac, **indomethacin**, naproxen, **phenylbutazone**, **piroxicam**, sulfasalazine
Antibiotic	Cephalosporins, chloramphenicol, methicillin, sulfonamides
Anticonvulsant	**Carbamazepine**, phenytoin
Antihistamine	Cimetidine, chlorpheniramine, ranitidine
Antimalarial	Chloroquine, quinacrine,
Antipsychotic/ tranquilizer	Chlorpromazine, prochloperazine
Antithyroid	Carbimazole, methimazole, propylthiouracil
Other	**Allopurinol**, chlopropamide, **gold salts**, **penicillamine**, tolbutamide

[a]Drugs associated with higher risk are shown in bold. For more extensive listings see Montané et al. (2008) and Young and Kaufman 2008.

4.5 Treatment

All medications suspected to be possible triggers for the disease should be discontinued. Supportive measures, especially blood transfusions, are often necessary for treatment of anemia and bleeding complications. Drug-induced aplastic anemia is treated like the idiosyncratic form of the disease, and responds to therapy at about the same rate. Overall, immunosuppressive therapy produces significant improvement in more than half of all patients; younger individuals have a considerably better prognosis than those who are older than 45 years (Young et al. 2008).

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Molecular Mechanisms of Adverse Drug Reactions in Cardiac Tissue

Reza Tabrizchi

Contents

Abstract The myocardium is the target of toxicity for a number of drugs. Based on pharmacological evidence, cellular targets for drugs that produce adverse reactions can be categorized into a number of sites that include the cell membrane-bound receptors, the second messenger system, ionic channels, ionic pumps, and intracellular organelles. Additionally, interference with the neuronal input to the heart can also present a global site where adverse drug effects can manifest themselves. Simply, a drug can interfere with the normal cardiac action by modifying an ion channel function at the plasma membrane level leading to abnormal repolarization and/or depolarization of the heart cells thus precipitating a disruption in the rhythm and causing dysfunction in contractions and/or relaxations of myocytes. It is now recognized that toxic actions of drugs against the myocardium are not exclusive to the antitumor or the so-called cardiac drugs, and many other drugs with diverse chemical structures, such as antimicrobial, antimalarial, antihistamines, psychiatric, and gastrointestinal medications, seem to be capable of severely compromising myocardium function. At present, great emphasis in terms of drug safety is being placed on the interaction of many classes of drugs with the hERG potassium channel in cardiac tissue. The interest in the latter channel stems from the simplified view that drugs that block the hERG potassium channel cause prolongation of the QT

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interval, and this can cause life-threatening cardiac arrhythmias. Based on the evidence in the current literature, this concept does not seem to always hold true.

Keywords Cardiac · Ion channels · Depolarization · Repolarization · Arrhythmias

1 Introduction

In the treatment of a variety of diseases and disorders, the myocardium has frequently become a major site of toxicity for a host of drugs. In a broad sense, at the myocyte level, the sites of drug action that can lead to an adverse reaction can be categorized into a number of specific locales. For example, cell membrane-bound receptors, second messenger systems and associated enzymes, ionic channels (i.e., Na, K, Ca), ion exchangers, ionic pumps, intracellular organelles (i.e., mitochondria, sarcoplasmic reticulum, myofilaments), and DNA/mRNA molecules (Fig. 1) are sites on which drugs can act to produce an adverse cardiac event. In addition, interference at the level of neuronal input to the heart can present yet another site where adverse drug effects can manifest themselves. A relatively simple scenario is that drugs can interfere with cardiac function via the modification of the activities of an ionic channel, either at the plasma membrane or the intracellular level, causing abnormal depolarization and/or repolarization in the pacemaker cells, the conduction fibers and/or the contractile cells, and hence precipitate a disruption in the rhythm and causing dysfunction in the contraction and/or relaxation of myocytes (Fig. 2). Needless to say, a persistent and significant disruption in the process of

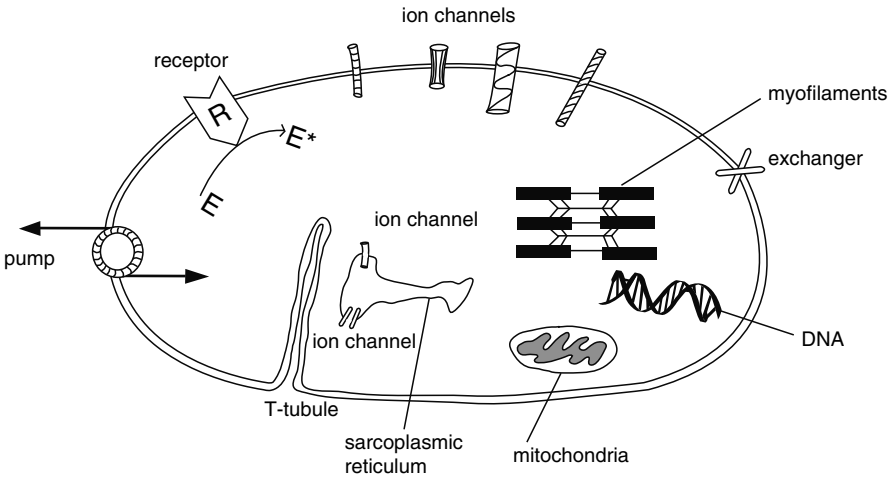

Fig. 1 A schematic of sites (i.e., channels, pumps, etc.) in a myocyte on which drugs can interact to produce adverse cardiac effects

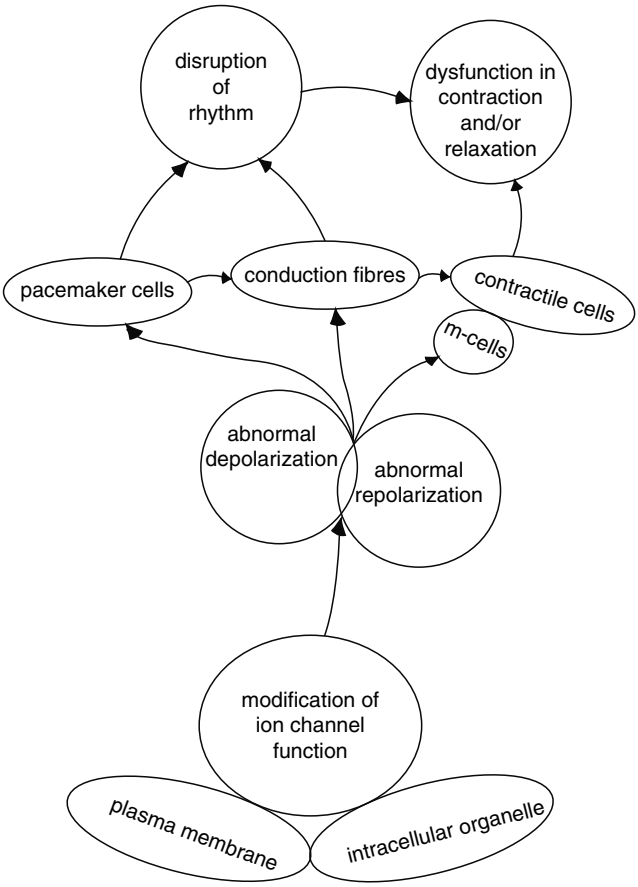

Fig. 2 Broad generalized schematic of modes of disruption in the function of the heart

contraction/relaxation will eventually lead to inadequate perfusion of blood to vital organs (i.e., brain, heart, lungs, kidney, liver) ultimately leading to death.

It is now recognized that toxic actions of drugs to the myocardium are not exclusive to antitumour and/or the so-called cardiac drugs, and that many other drugs with diverse chemical structures, such as antimicrobials, antimalarials, antihistamines, psychiatric, and gastrointestinal medications, can profoundly disrupt myocardium function, which at times can be fatal (Tamargo 2000; Haverkamp et al. 2000; Vandenberg et al. 2001).

2 Nature of the Disruption in Cardiac Function

The genesis of the disruption in the rhythm of the heart manifesting as arrhythmias can have its roots in the abnormal electrical events in the conducting fibers (i.e., Purkinje fibers), cardiac myocytes, and/or pacemaker cells. Drug-induced

disturbances in the electrical activity of the heart can have many different facets. For example, the cardioglycoside, digitalis, has been found to cause transient depolarization leading to extra-systole or trains of extra-systole (Ferrier et al. 1973). Moreover, digitalis not only causes transient depolarization in Purkinje fibers but can also block electrical conduction which may lead to re-entry cardiac arrhythmias manifesting as ventricular tachycardia with the possibility of evolving into ventricular fibrillation (Sounders et al. 1973).

Other major forms of abnormal behavior in cardiac tissue due to toxic effects of drugs are *early after depolarization* (EAD) and *delayed after depolarization* (DAD). EAD can result from oscillation in membrane potential that interferes with the repolarization phase of the cardiac action potential. DAD can result from oscillations in the membrane potential after the completion of repolarization. Under circumstances where EADs and DADs are large enough to produce sufficient depolarization in the membrane potential to trigger contractility, the event can lead to extra-systole, which disrupts the regular activity of the heart. For example, in examining the mechanisms of EAD and DAD in the ferret ventricular muscle, Merban and colleagues reported that digitalis and the β-adrenoceptor agonist, isoprenaline, were capable of producing EADs while cesium could evoke DADs (Merban et al. 1986). Further, it was revealed that inhibition of Ca^{2+} release from the sarcoplasmic reticulum (SR) or chelation of intracellular Ca^{2+} suppresses DADs but not EADs. Moreover, the putative L-type Ca channel agonist, BAY-K 8644, potentiated EADs, which were blocked by the L-type Ca channel antagonist, nitrendipine (Merban et al. 1986). In investigating EAD in canine Purkinje fibers, January and colleagues reported that BAY-K 8644 could evoke EADs which were enhanced by low stimulation frequencies, lowering the concentration of extracellular potassium ions ($[K^+]_o$), the presence of tetraethylamoium (K channel blocker), or the application of depolarizing constant current (January et al. 1988). In contrast, EADs were abolished by the increases in frequency of stimulation, raising $[K^+]_o$, addition of tetrodotoxin (Na channel blocker), lidocaine (local anesthetic and Na channel blocker), verapamil (L-type Ca channel antagonist), nitrendipine, or the application of a depolarizing constant current (January et al. 1988). It would appear that, mechanistically, EADs show time and voltage dependence, and they require lengthening of the action potential duration during the plateau voltage range and recovery from inactivation and reactivation of an inward current (January et al. 1988).

Further, in examining the role of the adrenoceptors in the induction of EADs and DADs, it has been determined that stimulation of α-adrenoceptors in isolated canine myocytes with phenylephrine resulted in dose-dependent prolongation of action potential duration (APD) without the appearance of EADs or DADs (Priori and Corr 1990). In contrast, isoprenaline (β-adrenoceptor agonist) caused both DADs, single or multiple (2–5), and EADs with 2:1 or 3:1 patterns. Isoprenaline-induced DADs and EADs were reversed by ryanodine (intracellular Ca^{2+} release inhibitor), benzamil (Ca channel antagonist), or a low concentration of extracellular Na^+ (Priori and Corr 1990). The Na^+/Ca^{2+} exchanger has also been implicated in the development of EADs (Szabo et al. 1994) while Ca loading has been

suggested to be responsible for the development of both DADs and EADs (Szabo et al. 1995).

Cesium-induced EADs that occur during phase 3 of the action potential have been associated with long QT syndrome and ventricular arrhythmias in vivo (Levine et al. 1985). It has also been suggested that EADs correspond to distinct and prominent U-waves on the electrocardiogram (ECG), and that ventricular premature depolarization arising from U or TU complex can initiate polymorphic ventricular arrhythmias, which can terminate spontaneously or degenerate into ventricular fibrillation (el-Sherif et al. 1988). Essentially, EADs have been proposed to cause tachyarrhythmia. For instance, administration of cesium (168 mg kg^{-1}, i.v.) to dogs has been found to prolong the QT interval and cause EADs leading to monomorphic, polymorphic ventricular tachycardia, and/or ventricular fibrillation while the incidence of such events was reduced by the infusion of magnesium (1–2 mg kg^{-1} per min for 20–30 min) (Bailie et al. 1988). Elevation of the extracellular concentration of magnesium ($[Mg^{2+}]_o$) has been found to reversibly abolish EADs that are induced by quinidine (Class IA antiarrhythmic) (1–4 mM), cesium (5–12 mM), or 4-aminopyridine (K channel blocker; 1.5–5 mM) in canine Purkinje fibers (Kaseda et al. 1989). EADs caused by quinidine were also noted to be sensitive to rate, $[K^+]_o$, and $[Mg^{2+}]_o$. Slow rate and low $[K^+]_o$ helped induce EADs in the presence of quinidine while an elevation in $[Mg^{2+}]_o$ suppressed EADs (Davidenko et al. 1989). A number of antidepressants and neuroleptic drugs have also been noted to cause EADs in guinea-pig Purkinje fibers, but only at low $[K^+]_o$ (Studenik et al. 1999). While amitriptyline (tricyclic antidepressant), doxepin (tricyclic antidepressant), chlorpromazine (neuroleptic), and thioridazine (neuroleptic) did not elicit EADs at $[K^+]_o$ of 2.7 mM, they did so when $[K^+]_o$ was lowered to 1.35 mM. Also, the EADs induced by these antidepressants and neuroleptics were reversed by the presence of tetrodotoxin (Studenik et al. 1999). In essence, generation of EADs and DADs can be disruptive to the regular function of the heart. Thus drug-induced arrhythmias can have their roots in the abnormal behavior of the electrical events in the heart.

3 Sites of Drug Action

3.1 Plasma Membrane

3.1.1 Receptors

Cardiac function can be impaired by the action of drugs on specific receptors. To deal with every adverse reaction that is mediated via the effects of drugs acting on specific cardiac receptors is beyond the scope of this chapter. Among the most dominant membrane-bound receptors in the cardiac muscle that can contribute to adverse drug reactions are the β-adrenergic and cholinergic (muscarinic) receptors.

Either over-stimulation or excessive inhibition of these receptors can produce unwanted cardiac events.

For example, β-adrenoceptor antagonists (β-blockers), while very useful in the treatment of many cardiovascular disorders (e.g., angina pectoris, hypertension, cardiac arrhythmias, and heart failure), can produce significant negative chronotropic, inotropic, and dromotropic effects causing substantial disruption in cardiac function (Zeltser et al. 2004; Thackray et al. 2006; Dobre et al. 2007). In contrast, sinus tachycardia is a common side effect associated with the use of tricyclic antidepressant agents, an effect that can be attributed to the anticholinergic effects as well as the inhibition of noradrenaline uptake caused by this class of drugs (Thanacoody and Thomas 2005). Disruption in the rhythm of the heart can also occur "indirectly". A typical example is the effect of cardioglycosides on heart rate. These drugs can have direct effects via the autonomic nervous system as well as indirect effects (Gillis and Quest 1980). Digitalis has been found to produce bradycardia by acting on nodose ganglia and in the efferent vagal pathways as well as on carotid sinus receptors and the central vagal nucleus (Chai et al. 1967). Ten Eick and Hoffman (1969) also reported that cardiac glycosides produced negative chronotropic effects by direct actions on the sinoatrial node. The chronotropic effects of cardioglycosides are attributed to slowing of the sinus node impulse, hyperpolarization of cells in the atria, enhanced atrial conduction, slow atrioventricular nodal effective period, and prolongation of atrioventricular nodal effective refractory period (Rosen et al. 1975). In short, a kaleidoscope of drug-induced events mediated via the membrane-bound receptors located on cardiac tissue can, either "*directly*" or "*indirectly*", cause detrimental effects on heart function.

3.1.2 Ionic Pumps

The major ionic pump that has received much attention in the myocardium is the Na^+/K^+-ATPase pump, which is the site of action for cardiac glycosides (Langer 1972). This is a critical site in which cardioglycoside toxicity is initiated and can subsequently lead to severe diastolic and systolic dysfunction. Excessive inhibition of the Na^+/K^+-ATPase pump can eventually lead to disruption of intracellular K^+ and Ca^{2+} homeostasis by lowering intracellular potassium ($[K^+]_i$) and increasing the intracellular levels of free Ca^{2+} thus causing electrical disturbances in conduction fibers as well as in the contractile myocytes (Sounders et al. 1973; Rosen et al. 1973). A progressive lowering of $[K^+]_i$ can lead to an increase in the automaticity and subsequently cause inexcitability (i.e., from a period of ectopic arrhythmia to a period of block) (Langer 1972). Cardioglycosides can cause shortening of the action potential, and the combination of block and shortened action potential can set the stage for re-entry and subsequently precipitate fibrillation (Langer 1972; Sounders et al. 1973; Rosen et al. 1973). These events in an already dysfunctional myocardium can ultimately cause major disturbances of electrical events, more specifically causing EADs and DADs leading to life-threatening cardiac arrhythmias (Ferrier

et al. 1973; Merban et al. 1986; Sicouri and Antzelevitch 1993). Superimposed on such events is slowing of the heart by cardiac glycosides via direct and indirect vagomemetic actions, which will further cause disturbances in myocardial function (Rosen et al. 1975; Gillis and Quest 1980).

In cardiac myocytes, interaction of Na^+/K^+-ATPase and cardioglycosides leading to an increase in intracellular Ca^{2+} has been associated with Ca^{2+} influx near the sarcolemmal surface including the T-tubules (Langer 1972; Barry et al. 1985). The increase in intracellular Ca^{2+} by cardioglycosides has been associated with inhibition of the Na^+/K^+–ATPase pump linked closely to Na^+/Ca^{2+} exchangers (Dostanic et al. 2004) and also activation of protein kinases (Tian et al. 2006). Over the past decade, the Na^+/K^+-ATPase pump has been the subject of much investigation. This enzyme belongs to the P-type ATPase family of isozymes and is composed of two major polypeptides with two catalytic units, α and β (Pierre and Xie 2006). Each α and β subunit consists of multiple isoforms (α1-4 and β1,2) (McDonough et al. 2002; Lingrel et al. 2003). Three isoforms of α-subunits (α1-, α2-, α3) are expressed in cardiac myocytes (McDonough et al. 1996; Wang et al. 1996; Harada et al. 2006). It has been suggested that the α3-subunit is localized between cardiac myocytes and the heart conduction system (Zahler et al. 1996) while the α1-subunit is predominant in cardiac tissue and seems to be uniformly distributed (Despa and Bers 2007). However, it has been proposed that the α2-subunit is preferentially localized in the cardiac myocyte T-tubules (Despa and Bers 2007) and functionally coupled to the Na^+/Ca^{2+} exchanger (Swift et al. 2007). It should also be mentioned that there are functional and structural similarities between Na^+/K^+-ATPase and the nonheavy metal (P2) subtype of P-type ATPase, which includes H^+/K^+-ATPase, sarcoplasmic-endoplasmic reticulum ATPase (SERCA), and plasma membrane Ca^{2+} ATPase (PMCA) (Xie and Cai 2003; Pierre and Xie 2006). Recent evidence has linked the binding of cardioglycosides and the Na^+/K^+-ATPase to the activation of *c-src* (Xie and Cai 2003; Pierre and Xie 2006). Furthermore, a link between the activation of Na^+/K^+-ATPase and *c-src* has been extended to the transactivation of epithelial growth factor receptor leading to production of intracellular messengers such as diacylgylcerol, inositol 1,4,5-trisphosphate, reactive oxygen species, and disturbance in Ca^{2+} homeostasis in cardiac myocytes (Xie and Cai 2003; Pierre and Xie 2006). In addition, Na^+/K^+-ATPase has been suggested to act as a scaffold involved in both regulation and activation of various enzymes (Pierre and Xie 2006). These events may well account for the electrical abnormalities associated with actions of toxic levels of cardioglycosides in cardiac myocytes. Thus, therapeutic and adverse effects of cardioglycosides on the myocardium may well extend beyond just an increase in the intracellular concentration of Na^+ via inhibition of the Na^+/K^+-ATPase pump linked to the Na^+/Ca^{2+} exchanger and could involve the activation of second messenger cascades, disturbances in Ca^{2+} homeostasis, and activation of gene expression. It is prudent to suggest that the exact nature of the long- and short-term adverse effects of cardioglycosides remains to be fully elucidated, but the fact remains that ionic pumps are sites that drugs interact with and can cause cardiotoxic effects.

3.1.3 Ion Channels

The three main ion channels that have been at the centre of both therapeutic and toxic reactions in the myocardium are Na, Ca, and K channels, with the latter receiving an enormous amount of attention because the outward K^+ rectifier currents play a pivotal role in phase 3 of the action potential in cardiac tissue, and they are influenced by a variety of chemicals of differing structural features (Haverkamp et al. 2000; Roden 2004). The action potential of cardiac myocytes can be divided into five distinct phases: phase 0 being the inward fast Na^+ current, phase 1 being the transient K^+ outward currents, phase 2 being the slow inward Ca^{2+} and Na^+/Ca^{2+} exchange current, phase 3 being the outward K^+ rectifier currents, and finally phase 4 being the Na^+/K^+ exchanger resulting from activation of Na^+/K^+-ATPase (Fig. 3). Various currents that are involved in depolarization and repolarization are listed in Table 1.

3.1.4 Sodium Channels

The fast Na^+ inward current is obviously a critical component of the action potential in the heart. Since this current forms the basis of the initial phase of the action

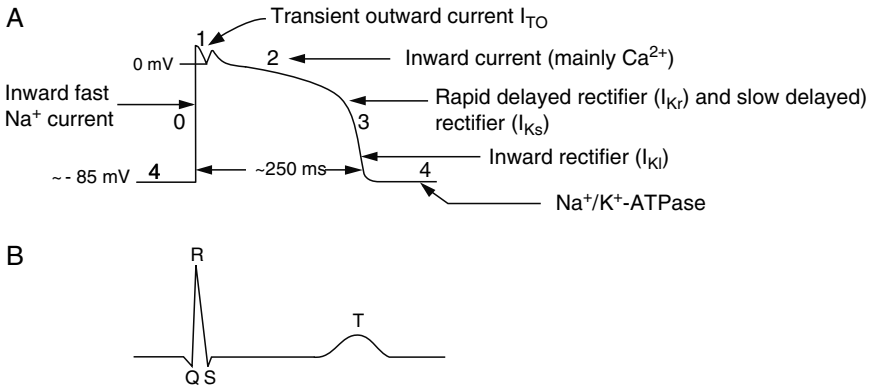

Fig. 3 Schematics of (A) typical cardiac (ventricular) action potential and (B) the corresponding electrocardiogram

Table 1 Some of the main ionic currents for the cardiac action potential consisting of depolarizing inward and repolarizing outward currents

Ionic current	Direction
I_{Na}	Inward
I_{Ca}	Inward
$I_{Na/Ca}$	Inward
I_{KI}	Inward
$I_{to,1,2}$	Outward
I_{Kr}	Outward
I_{Ks}	Outward

Fast sodium current (I_{Na}); slow calcium current (L-type channels; I_{Ca}); sodium/calcium exchange current ($I_{Na/Ca}$); inward potassium rectifier current (I_{KI}); transient currents (I_{to}); rapid delayed potassium rectifier current (I_{Ks}); slow potassium delayed rectifier current (I_{Kr})

potential in cardiac myocytes, its inhibition would virtually stop the heart from functioning. Therefore, chemicals that interfere with Na^+ influx can have a profound impact on the ensuing excitation-contraction coupling process in myocytes. Drugs such as quinidine and lidocaine are known to interfere with the fast Na^+ inward current but to also have additional effects (Colatsky 1982). In terms of its actions, quinidine is believed to block the fast Na^+ current and delay its reactivation (Chen et al. 1975; Chen and Gettes 1976; Lee et al. 1981). Quinidine-induced block shows voltage dependence, and the nature of the inhibition is frequency-dependent. A greater degree of block develops at a shorter cycle length while the block is compromised and diminishes at a long cycle length (>10 s), which has led to the suggestion that quinidine binds preferentially to an open state of the Na channel (Hondeghem and Katzung 1977). A number of tricyclic antidepressants have also been noted for their "quinidine-like" actions at high doses (Spiker 1978). They have been found to produce electrical disturbances in the heart in clinical studies, for example, significantly increasing the QRS duration (i.e., $\geq$ 100 ms) of the ECG, while in experimental studies, it has been revealed that this class of drugs can cause significant negative chronotropic and inotropic effects (Spiker 1978). Clearly, toxicity associated with inhibition of the fast Na^+ current interferes with both the electrical and mechanical events in cardiac tissue.

Calcium Channels

Ca^{2+} is a critical cation necessary for cardiac function in terms of automaticity/pacemaker activity, conduction of electrical signals, and excitation-contraction coupling of myocytes. Drugs and chemicals that influence Ca^{2+} flux in cardiac tissue also have a profound effect on the electrical and mechanical function of the heart. The slow Ca^{2+} current is mediated, in part, via the voltage-gated L-type Ca channels, one that can be influenced by Ca channel antagonists such as verapamil, D600, and diltiazem. Cardiac toxicity associated with the blockade of this channel can result in the disruption of rhythm and rate, as well as contraction and relaxation, of the heart.

For example, verapamil, the so-called "cardiac selective" Ca channel blocker, has been noted to cause the disappearance of the P-wave and impair atria-ventricular conduction in chloralose-anesthetized dogs (Garvey 1969). In canine isolated cardiac Purkinje fibers, verapamil reportedly suppressed spontaneous activity in these fibers and abolished the sustained rhythmic activity evoked by externally applied depolarizing pulses, essentially reducing the rate at which quiescent fibers could be driven (Cranefield et al. 1974). Also, in sodium pentobarbital-anesthetized dogs, intra-arterial administration of verapamil and D600 depressed the frequency of sinus node discharge and lengthened atrioventricular (AV) nodal conduction time, while prolonging AV nodal refractoriness, actions that are similar to those of inorganic Ca channel antagonists, $MnCl_2$, and $LaCl_3$ (Zipes and Fischer 1974). These compounds are capable of impairing AV nodal conduction leading to a 2–1 or Wenckebach supra-His AV block. It has been noted that the actions of organic (e.g., verapamil)

and inorganic ($MnCl_2$) Ca channel antagonists can be reversed by the administration of the β-adrenoceptor agonists, isoprenaline or adrenaline, in anesthetized dogs (Zipes and Fischer 1974).

In rabbit isolated cardiac tissue, verapamil has been reported to significantly reduce sinoatrial nodal action potentials with a modest decline in the maximum diastolic potential, which was of borderline significance (Wit and Cranefield 1974). In the same preparation, AV nodal action potentials were also significantly depressed while the AV nodal conduction time was prolonged, and no discernible effects were noted on the action potentials generated in the Bundle of His. Notwithstanding the latter action, these negative effects of verapamil in the rabbit isolated cardiac tissue could be reversed by the presence of adrenaline (Wit and Cranefield 1974). Conversely, β-blockers (e.g., propranolol) are able to enhance the pharmacological actions of Ca channel antagonists on the heart resulting in substantial depression of electrical activity, both in pacemaker cells (i.e., producing severe bradycardia) as well as in conduction fibers (i.e., leading to heart block due to inhibition of A-V conduction). This can produce extensive depression of contractility, which can ultimately result in death (Vick et al. 1983). However, it has also been found that these synergistic effects of Ca channel antagonists and β-blockers can be partially reversed by the administration of Ca^{2+} (Vick et al. 1983; Henry et al. 1985).

It seems that verapamil shows differential effects in attenuating electrical activity compared to its contractile effects on cardiac tissue. In the rabbit isolated cardiac tissue, verapamil, up to a concentration of 2 mg l^{-1}, has been found to preferentially impair the electrical events in the sinoatrial (SA) node and atrioventricular (AV) node and reduce conduction velocity while very modestly affecting contractility in the atrial muscle (Wit and Cranefield 1974; Okada and Konishi 1975).

In isolated and cross-circulated canine AV nodal preparations, high doses of verapamil have been reported to produce AV conduction block (Iijima and Taira 1976). In sodium pentobarbital-anesthetized dogs, verapamil was noted to be capable of impairing ventricular contractility (Smith et al. 1976). Organic Ca channel blockers (e.g., verapamil) have also been shown to impair contractility in the rat isolated perfused Langendorff preparation while this toxicity could be reversed by simultaneous perfusion with glucagon (Zaritsky et al. 1988). Verapamil-induced cardiac toxicity in sodium pentobarbital-anesthetized dogs has also been found to be reversed by the administration of the phosphodiesterase inhibitor, amrinone (Koury et al. 1996). While amrinone was noted to reverse depression of the cardiac index, it did not appear to significantly impact verapamil-induced bradycardia (Koury et al. 1996). Significant cardiac depression can be produced by co-administration of Ca channel blockers (verapamil and diltiazem) and β-blockers leading to profound bradycardia and depression of myocardial function, which if not dealt with appropriately could be fatal (DeWitt and Waksman 2004). In general, in an individual with Ca channel blocker toxicity, infusion of calcium glucagon, along with supportive measures, appears to be the best treatment (Ashraf et al. 1995; DeWitt and Waksman 2004).

In summary, impediment to Ca^{2+} entry through the slow Ca channels can interfere with the sinus node action potential, AV node action potential, and AV

node conduction time, as well as contractility. In addition, the toxicity associated with Ca channel antagonists that show cardiac effects can be significantly enhanced by the simultaneous presence of β-blockers and/or in cardiac tissue with compromised β-adrenoceptor function with the end event being reduced Ca^{2+} flux into the cardiac myocytes causing myocardial dysfunction. Essentially, the consequence of blocking the slow Ca channel overtly is negative chronotropic, dromotropic, and inotropic effects.

Potassium Channels

The relatively recent finding that many common drugs can cause potentially fatal arrhythmias has focused attention on trying to predict which drugs are most likely to cause this adverse reaction. In general, these drugs cause a prolongation of the QT interval of the ECG and are associated with a form of cardiac arrhythmia known as torsade de pointes (*TdP*) which can also be described as "polymorphic ventricular tachycardia" (Jackman et al. 1988; Roden 2004; Antzelevitch 2007). The term *TdP* was first used to describe a peculiar form of ventricular tachycardia in which each QRS is somewhat different from the preceding one (Dessertenne 1966), and this can cause short episodes of loss of consciousness (syncope) or sudden death (Jackman et al. 1988; Roden 2004; Antzelevitch 2007). While there is an association between a drug-induced increase in QT interval and the occurrence of *TdP*, there are many issues that preclude a direct linear relationship between the two events. The QT interval is believed to reflect the time required for the ventricular muscle to repolarize during a single cardiac cycle. However, at the forefront of the difficulties in attempting to directly associate the prolongation of QT interval to the occurrence of *TdP* is the ability to correctly measure the QT interval in an individual (Ahmad and Dorian 2007). For example, there are inherent and pathophysiological conditions (e.g., morphological and structural) that may cause modification in the standard of the measurements of a true QT interval from an ECG recording (Ahmad and Dorian 2007). Such a fundamental issue has undoubtedly cast a shadow on the assumption that a linear relationship exists between drug-induced prolongation of QT interval and the development of *TdP*. However, this is just one facet among many issues in this complex paradigm.

Nonetheless, even if an appropriate measurement is made that reflects a true measure of the QT interval over a minimum required number of beats, it has to be understood that such a measurement is the summation of repolarization for the entire ventricular muscle over the course of a cardiac cycle with the caveat that different cardiac cells repolarize at differing rates (Sicouri and Antzelevitch 1991a; Sicouri et al. 1994; Anyukhovsky et al. 1996). It has been recognized that ventricular muscle, not including the Purkinje fibers, are composed of at least three electrophysiologically distinct cell types: epicardial, mid-myocardial (M cells), and endocardial cells (Sicouri and Antzelevitch 1991a; Anyukhovsky et al. 1996; Yan et al. 1998). In the canine heart, M cells and transitional cells occupy 30–40% of the left ventricle, and it is estimated that such cells occupy 20–40% of the mass of

Table 2 The complete action potential duration (APD) and peak maximal voltage (PMV) change in various canine cardiac ventricular cells at a rate of 60 and 15 cycles per minute

Cell type	APD (ms)		PMV (mV)	
	60/min	15/min	60/min	15/min
Epicardial	~220	~260	~100	~100
Endocardial	~260	~320	~100	~100
Mid-myocardial	~280	~340	~100	~100
Purkinje-fibers	~360	~460	~100	~100

the total normal heart (Sicouri and Antzelevitch 1995). Excluding the Purkinje fiber cells, M cells have the longest action potential duration (APD) of the mentioned cardiac myocytes (Table 2), which appears to become even longer at a lower heart rate (Sicouri and Antzelevitch 1991a; Yan et al. 1998). In the guinea pig ventricle, differences in the APD have also been reported among different cardiac myocytes with the M cells having the longer APD (Sicouri et al. 1996). Furthermore, M cells ($240 \pm 13\ Vs^{-1}$) (mean ± SD) seem to have a faster rate of rise (i.e., V_{max}) of the action potential compared to either epicardial ($129 \pm 13\ Vs^{-1}$) or endocardial ($192 \pm 28\ Vs^{-1}$) cells (Sicouri et al. 1996).

While M cells appear to be similar to epicardial and endocardial cells histologically, they have different electrophysiological characteristics and appear to be a hybrid between ventricular myocytes and Purkinje fiber cells (Sicouri and Antzelevitch 1991a, 1995). Similar to Purkinje cells, M cells exhibit large V_{max} and steep APD-rate relationships, which are influenced by changes in $[K^+]_o$ (Sicouri and Antzelevitch 1995). M cells are not like Purkinje fibers in that they do not appear in bundles. In addition, they do not exhibit phase 4 depolarization even in low $[K^+]_o$ or even after exposure to a catecholamine (Sicouri and Antzelevitch 1991a, 1995).

In the human ventricle, Doruin and colleagues) have reported that the transition in the action potential between epicardial and M cells was quite sharp, this being indicative of poor coupling between these regions; this is in contrast to the more gradual transition between the electrical coupling of M cells and endocardial cells (Doruin et al. 1995). The poor coupling between M cells and epicardial cells could be due to their morphological arrangements because the orientation of the epicardial layer appears to be perpendicular to that of the subepicardial layer (i.e., M cell region) as noted in human and swine hearts (Doruin et al. 1995; Vetter et al. 2005). M cells of human ventricles also have a longer APD duration, which is rate dependent as compared to the epicardial or endocardial cells (Doruin et al. 1995). Values for APD at the level of 90% repolarization for epicardial, mid-myocardial (M cells), and endocardial cells of human ventricles at 60 cycles per minute are 351 ± 14, 439 ± 22, and 330 ± 16 ms, respectively (mean ± SEM). In addition, the M cell action potential exhibites a faster V_{max} compared with the epicardial or the endocardial cells. V_{max} recorded at 60 cycles per minute is 228 ± 11, 326 ± 16, and $234 \pm 28\ Vs^{-1}$ in epicardial, M cells, and endocardial cells, respectively. The M cells, unlike Purkinje fibers, do not exhibit phase 4 depolarization or automatic activity in low $[K^+]_o$ or on exposure to adrenaline (1.0 μM) (Doruin et al. 1995).

M cells, compared to epicardial and endocardial cell types, characteristically appear to have a larger late Na^+ current and a larger Na/Ca exchange current while possessing a smaller slowly activating delayed rectifier current (I_{Ks}) (Liu and Antzelevitch 1995). Certainly, regional disparity in the expression of different K channels has been reported in different myocardial cells (Brahmajothi et al. 1996, 1997). Furthermore, in canines, the slowly activating delayed rectifier current of the M cells was found to be considerably larger in the right compared to the left ventricle, with no discernible differences noted in the rapidly activating delayed rectifier current (I_{Kr}) between the two ventricles (Volders et al. 1999). Activation-recovery intervals have been noted to be longer in the M cell regions than in epicardial and endocardial cell layers in the canine myocardium leading to non-homogenous recovery causing dispersion of APD (Taccardi et al. 2005). At short cycle lengths, the effect of M cells is less prominent and does not disrupt excitation and recovery sequences. In contrast, at longer cycle lengths, the recovery is perturbed (Taccardi et al. 2005).

The pharmacological actions of drugs are quite different in M cells when compared with the epicardial and the endocardial cells. For instance, EADs and DADs were produced by the cardioglycoside, digitalis (0.1 μg ml^{-1}), and the L-type Ca channel agonist, BAY K-8644 (1.0 μM), in M cells, but not in the epicardial or the endocardial cells of canine myocardium (Sicouri and Antzelevitch 1991b). Similar findings were observed with quinidine (0.1–0.5 μg ml^{-1}) and higher concentrations of digitalis (1.0 μg ml^{-1}) in which EADs and DADs were produced in Purikinje fibers and M cells, but not in epicardial or endocardial myocytes (Sicouri and Antzelevitch 1993). In another study, exposure of M cells to *d,l*-sotalol (10 and 100 μM) caused a greater increase in the APD than in either the epicardial or endocardial cells of guinea-pig ventricles. Moreover, *d,l*-sotalol also induced EADs and DADs in M cells, but not in epicardial or endocardial cells (Sicouri et al. 1996).

The emphasis, in terms of drug-induced prolongation of QT interval due to a decrease in the ability of myocytes to repolarize, has been squarely placed on the notion that this is due to the inhibition of I_{Kr} (i.e., hERG potassium channel). However, an increase in the time constant for a myocyte to repolarize can also occur for reasons other than the inhibition of I_{Kr}. Other reasons for the prolongation of repolarization include an increase in the plateau of I_{Ca} (January and Riddle 1989), an increase in the plateau of I_{Na} (Lee 1992), and/or a decrease in I_{Ks} (Busch et al. 1994). The propensity of a drug to increase the QT interval and cause *TdP* may increase if it is a blocker of hERG potassium channel and also has the ability to increase I_{Ca}, and I_{Na} and/or inhibit I_{Ks}, simultenaously. Thus, not surprisingly, other pharmacological characteristic of chemicals will also determine whether they are proarrhythmic in addition to their ability to inhibit the hERG potassium channel.

Warmke and Ganetzky (1994) were the first to identify the gene for the *ether-à-go-go-related gene* (hERG). Subsequently, genetic studies have identified a number of genes that, if mutated, will cause congenital long QT syndrome, and members of a family with near normal QT intervals may have mutations in their genes that are associated with long QT syndrome. They are at risk if exposed to drugs that prolong QT interval (Curran et al. 1995; Trudeau et al. 1995). The latter gene was linked to

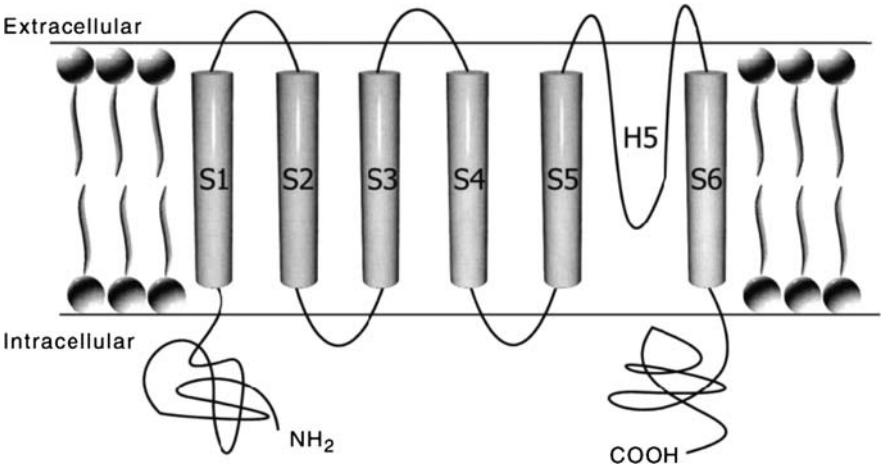

Fig. 4 Schematic representation of a single human *ether-à-go-go* related gene (hERG) potassium channel protein α-subunit. The channel has six transmembrane spanning domains where the S4 domain is the *voltage sensor* and the *pore* is located between S5 and S6 regions. Reproduced with permission from Wiley-Blackwell Publishing, Witchel and Hancox (2000)

encoding the I_{Kr} K channel (Sanguinetti et al. 1995; Trudeau et al. 1995). Needless to say, the interest has been to study the human *ether-à-go-go-related gene,* which is responsible for encoding a K channel that is prominently involved in the repolarization of cardiac ventricular cells. Thus, the rapidly activating delay rectifier current (I_{Kr}) in the heart is believed to be generated by the hERG potassium channel (Hancox et al. 1998), which has six transmembrane units (S1–S6) where the charged S4 region is the voltage sensor that responds to changes in the membrane potential, and between the S5 and S6 region is the looping pore domain (Witchel and Hancox 2000) (Fig. 4). Considerable effort has also been placed in delineating the different functional regions of the hERG potassium channel. As such, it has been determined that mutation in the N-terminal domain leads to changes in inactivation: mutation in S3/S4 affects activation, and mutation in the extracellular region of between S5 and S6, and S6, and the pore loop tend to affect inactivation (Witchel 2007). There are two isoforms of hERG (hERG1a and hERG1b), and it seems that heteromultimers and hERG1b have a more rapid deactivation time course than the homomultimer, hERG1a. However, most, if not all, of the pharmacological studies have been conducted using the isoform homomultimer, hERG1a (Witchel 2007).

Numerous studies have been undertaken to delineate the nature of drug binding to hERG potassium channels because (1) inhibition of hERG potassium channel is associated with prolongation and QT interval, and (2) a relationship has been discovered between prolongation of the QT interval and *TdP* (Table 3). Needless to say, this is a major drug safety issue because *TdP*, a form of polymorphic cardiac tachycardia, can degenerate into life-threatening ventricular fibrillation (Haverkamp et al. 2000; Vandenberg et al. 2001).

Table 3 Examples of drugs that have been reported to cause *torsade de pointes*

Drug name	Clinical class
Arsenic trioxide	Anticancer
Astemizole	Antihistamine
Bepridil	Antianginal
Chloroquine	Antimalarial
Chlorpromazine	Psychiatric/Antiemetic
Cisapride	Gastorintestinal stimulant
Clarithromycin	Antimicrobial
Disopyramide	Antiarrhythmic
Dofetilide	Antiarrhythmic
Erythromycin	Antimicrobial
Haloperidol	Psychiatric
Ibutilide	Antiarrhythmic
Ketoconazole	Antimicrobial
Pentamidine	Antimicrobial
Pimozide	Psychiatric
Procainamide	Antiarrhythmic
Quinidine	Antiarrhythmic
Sotalol	Antiarrhythmic
Terfenadine	Antihistamine
Terodiline	Antimuscarinic

Many factors appear to influence the binding of a drug molecule to the hERG potassium channel; these include time, frequency, voltage, and temperature, as well as ion concentration, which also seems to affect drug-induced blockade of the hERG channel. For instance, the block of hERG potassium channel expressed in Chinese Hamster Ovary (CHO-K1) cell lines induced by the prokinetic agent, cisapride, showed time-, voltage-, and frequency dependence with features consistent with the block occurring in the open state of the channel; specifically, an IC_{50} of 16.4 nM (95% CI: 11.0–24.4 nM and a n_H -0.8 ± 0.01 (mean $\pm$ SEM, $n = 8$) and 23.6 nM (95% CI: 20.7–27.0 nM), $n_H - 0.8 \pm 0.01$ (mean $\pm$ SEM, $n = 4$–7) at 20–22°C and 37°C, respectively (Walker et al. 1999). The potency of cisapride was also influenced by $[K^+]_o$, and was reduced by 26% when $[K^+]_o$ was increased from 1.0 to 10 mM (Walker et al. 1999). The tricyclic antidepressant, imipramine, reversibly inhibited hERG expressed in CHO cells (IC_{50} 3.4 $\pm$ 0.4 μM; n_H 1.17 $\pm$ 0.03; mean $\pm$ SEM, $n = 5$) at ambient temperature in both closed and open states of the channel, but the block showed weak voltage dependence (Teschemacher et al. 1999) similar to a block caused by the class III antiarrhythmic agent, azimilide (Busch et al. 1998).

Although there was an initial indication that drugs with high affinity for hERG potassium channels exhibited an open-state-dependent block mechanism (Spector et al. 1996), it was also recognized that a single site would not likely confer drug sensitivity in hERG potassium channels. Since then, a variety of different binding scenarios have been postulated to account for drug interaction with this K channel. For example, Zou and colleagues proposed that the antiarrhythmic, MK-499, a methanesulfonanilide, binds to the channel from the intracellular site within the cavity, between the selectivity filter and the activation gate (Zou et al. 1997). Most

likely it inhibits the channel by being trapped, as opposed to the so-called "foot in the door" type of inhibition (Mitcheson et al. 2000a). The latter evidence was obtained following replacement of Asp with Lys at position 540 in the S4–S5 region of the hERG potassium channel. This caused destabilization of the closed state and permitted the channel to reopen in response to membrane hyperpolarization (Sanguinetti and Xu 1999), thus substantially reducing the potency of MK-499, which could not get trapped within the inner cavity of the hERG potassium channel (Mitcheson et al. 2000a). MK-499 is nearly all charged at the physiological pH, and, in addition, it is a relatively large molecule (140 Å) compared to, for example, the K channel blocker, tetraethyl ammonium (~6.9 Å). MK-499 would thus likely require a large space within the pore of the channel as it is trapped within the inner cavity of the hERG potassium channel in order to produce an effective blockade (Mitcheson et al. 2000a). This implies such a space is available within the inner cavity of the hERG potassium channel.

An earlier observation revealed that mutation at the Phe amino acid residue located in the S6 domain decreased the block produced by the antiarrhythmic agents, dofetilide and quinidine (Lees-Miller et al. 2000). A subsequent investigation identified high affinity binding sites on the S6 domain located at amino acid residues Y652 and F656 for molecules such as terfenadine (antihistamine), cisapride (gastrointestinal prokinetic), and MK-499 (Mitcheson et al. 2000b). In other voltage-gated K channels (i.e., Kv1), a highly conserved proline-X-proline motif exists which has been purported to "kink" at the inner helices and thus reduce the space within the inner cavity of the channel (Del Camino et al. 2000). This precludes such molecules as MK-499 from being trapped within such a small space. It would seem that the hERG potassium channel does not have these proline residues in the S6 domain, and in the equivalent location (i.e., F656 and Y652), aromatic amino acids (i.e., Ile-Phe (656)-Glu and Met-Tyr (652)-Arg) occupy the space (Mitcheson et al. 2000b). This is in contrast to other voltage-gated K channels that have hydrophobic amino acid residues (i.e., Ile, Leu, or Val) in the equivalent positions within the helices, and consequently such an arrangement will not allow a large molecule such as MK-499 to enter the inner cavity of the channel and cause impediment to the movement of K^+ through the channel. It has been further proposed that the binding of a molecule such as MK-499 within the hERG potassium channel occurs via electrostatic interactions between π electrons and hydrogen atoms of the aromatic rings of Y652 and F656 and the drug molecule (Mitcheson et al. 2000b). However, mutagenesis studies revealed that the replacement of Y652 and F656 amino acid residues with other neutral aromatic amino acids, i.e., Tyr replaced by Trp, or Phe replaced by Tyr, respectively, did not appreciably affect the binding of the three structurally different drug molecules (MK-499, cisapride, and terfenadine) to the hERG potassium channel (Fernandez et al. 2004). In addition, the aromatic amino acid residues in position 656 are not paramount for the high affinity binding as long as the replacement amino acid is neutral. Nonetheless, the replacement of the aromatic amino acid residues at F656 with charged polar amino acid residues (e.g., Gly, Glu, or Arg), severely disrupted channel gating (i.e., closure), significantly reducing the effectiveness of MK-499, cisapride, and terfenadine as inhibitors of hERG potassium channel

because they cannot be trapped within the cavity of the channel (Fernandez et al. 2004). Moreover, mutations at Phe-656 with hydrophobic amino acids (e.g., Met, Leu, or Ile) only moderately reduced the affinity of cisapride, terfenadine, and MK-449. This has led to the proposal that the aromatic side chain at residue 652 is essential for high affinity block, resulting in cation-π interaction between Tyr-652 and the basic nitrogen of the drug molecule, while disruption of gating by the replacement of Ile-Phe-Gly at position 655, 656, and 657 with Pro-Val-Pro, respectively, reduces the potency of drug molecules such as MK-499 because it is no longer trapped with the inner cavity and will not produce effective inhibition of the hERG potassium channel (Fernandez et al. 2004).

There is evidence in the literature implying that inhibition of the hERG potassium channel by chemicals can occur via their interaction with sites other than Y652. Mergenthaler and colleagues reported that the Class IC antiarrhythmic agent, propafenone, produced an open channel block that most likely occurred at an intracellular site of the hERG channel in oocytes *Xenopus laevis* (Mergenthaler et al. 2001). Further indication that drugs may bind to different sites on the hERG potassium channel was presented in studies by Paul and colleagues, where in human embryonic kidney (HEK 293) expressed hERG, it was reported that a number of Class I antiarrhythmic agents (Table 4) produced a similar rapid activation-dependent, open channel block with a n_H of close to unity, indicative of one molecule binding to the channel (Paul et al. 2001, 2002). It seems that channel inactivation is not obligatory for hERG blockade for flecainide, quinidine, propafenone, and disopyramide. These agents were also capable of inhibiting the hERG potassium channel at concentrations well within clinically relevant levels. Thus, this would imply that the site of actions of these drugs could be different than that for methanesulfonanilides (Paul et al. 2001, 2002).

There are also indications that some hERG blockers such as imipramine (Teschemacher et al. 1999) and the selective serotonin uptake inhibitors (e.g., citalopram and fluoxetine) (Thomas et al. 2002; Witchel et al. 2002) exhibit a different profile of block that seemed to occur early during the depolarization pulse. It seems that, while mutations in position 540 in the S4–S5 region of hERG causes destabilization of the closed state permitting the channel to reopen in response to membrane hyperpolarization (Sanguinetti and Xu 1999), substitution of the amino acid residue with alanine in the S6 domain at S631 results in reduced inactivation (Zou et al. 1998; Hancox et al. 1998). Thus, Milnes and colleagues reported that mutations with alanine at amino acid residues S631A, Y652A, and F656A only partially reduced the inhibitory action of fluvoxamine, which has an IC_{50} of 3.8 μM

Table 4 IC_{50} (μM) and Hill coefficient (n_H) values for some Class I antiarrhythmic agents against hERG potassium channels expressed in the human embryonic kidney (HEK 293) cell line

	IC_{50}	n_H
Flecainide	3.91 ± 0.68	0.76 ± 0.09
Quinidine	0.41 ± 0.04	0.76 ± 0.05
Propafenone	0.44 ± 0.07	0.79 ± 0.10
Disopyramide	7.23 ± 0.72	0.89 ± 0.06
Lidocaine	262.9 ± 22.4	0.83 ± 0.07

(95% CI: 2.7–5.4) with a n_H of 0.9 (95% CI: 0.6–1.2) in wild-type. The characteristics of fluvoxamine-induced block were mixed and consistent with closed or extremely rapidly developing open channel block (Milnes et al. 2003). The actions of fluvoxamine appear distinct, and its inhibitory profile is not entirely dependent upon the presence of Y652 and F656 or inactivation of the channel by mutation of amino acid residue at S631. Yet another study that provided evidence in support of other binding sites for potent hERG blockers was carried out in the HEK 293 cell line expressing the hERG potassium channel at 37°C (Ridley et al. 2004). A comparison between amiodarone (Class III antiarrhythmic) and its noniodinated analogue, dronedarone, with IC_{50} and n_H in the wild-type of 70.0 nM (95% CI: 53.6–91.3), 59.1 nM (95% CI: 50.8–68.7), and 1.1 (95% CI: 0.8–1.4), 0.8 (95% CI: 0.7–0.9), respectively. The characteristics of the inhibition for agents were that, while the block developed rapidly on membrane depolarization, no preference was noted for either activated or inactivated states. Furthermore, mutagenic studies and replacement of aromatic amino acid residues with alanine in the S6 domain for F656 failed to influence the binding of either molecule to the hERG channel, while mutations at residue Y652 had little effect on dronedarone, but they did have some effect on the binding of amiodarone, thus suggesting that high affinity binding can occur without strong dependence on Y652 or F656 (Ridley et al. 2004). Interestingly, high $[K^+]_o$ (98 mM) reduced potency of hERG inhibition produced by both amiodarone and dronedarone. The cause of the negative impact of high $[K^+]_o$ on hERG blockers has been suggested to result from electrostatical repulsion against the drug molecules (Wang et al. 1997). Recent evidence seems to imply that hERG channel inactivation involves two synergistic processes that can be affected by mutation of N588K and S631A (McPate et al. 2008). It is evident that the potency of disopyramide as an inhibitor of hERG is only marginally affected by a single mutation while it is significantly affected with the double mutations (McPate et al. 2008).

Even though channel inactivation has been proposed to be an integral part of the interaction between the so-called "high affinity' blockers and the hERG potassium channel, there is evidence to indicate that inactivation per se is not a determinant of hERG potassium channel inhibition by a high affinity blocker such as cisapride (Chen et al. 2002). Recently, Perrin and colleagues presented evidence to support the notion that preferential binding of high affinity drugs (e.g., cisapride, dofetilide, terfenadine, and astemizole) to an inactivated state, while necessary, is not sufficient to yield high affinity binding to the hERG potassium channel (Perrin et al. 2008). However, one question that perhaps still remains unanswered is whether the binding of the drug at the receptive sites within the channel and the resulting allosteric changes that could occur act as a positive feedback loop for inactivation of the channel. This gives the impression that inactivation is necessary for binding of the high affinity blockers.

It is evident from the literature that there is variance in the IC_{50} values in cloned hERG channels expressed in native I_{Kr} mammalian myocytes and in clonally derived mammalian cell lines (Kirsch et al. 2004). Clearly, both temperature and pulse (duration and frequency) patterns can influence the inhibitory

profile of chemicals on hERG. Furthermore, it is worthwhile noting that hERG inhibitors bind most efficiently to the channel in activated/inactivated conformational states and dissociate at different rates from the resting state of the channel. The enormous variability that seems to shape and define an IC_{50} value for a compound that affects the hERG potassium channel means that, when a drug is found to be an inhibitor of this channel, experimental conditions have to be scrutinized carefully if a meaningful prediction is to be sought for an in situ effect. Recently, in a study by Lu and colleagues, it was revealed that not all drugs that were capable of inhibiting the hERG potassium channel were also, in parallel, capable of causing prolongation of APD and QT intervals (Lu et al. 2008). Evidence was presented on the cardiac action of 170 compounds, both on APD in isolated Purkinje fibers or isolated Langendroff-perfused hearts as well as against hERG current (in hERG-transfected HEK 293 cells). The data indicated that, of 170 compounds, 92 (54%) were found to inhibit hERG current, but 70 (41%) were found to be infective, while 8 (5%) were noted to be able to activate hERG. Moreover, of the 92 compounds that were found to inhibit hERG, only 51 (55.4%) prolonged the APD, while 15 (16.3%) shortened it, and 26 (28.3%) were without an effect. Interestingly, among the 70 compounds that did not inhibit hERG, 38 (54.3%) did not change the APD, while 18 (25.7%) still managed to prolong the APD, and 14 (20%) were noted to shorten the APD (Lu et al. 2008). Such data simply imply that a mere inhibition or lack of inhibition of hERG current by a drug cannot be readily taken as evidence for or against that compound having an impact on APD and QT intervals.

Therefore, translation of information obtained for a drug as an inhibitor of hERG to prolongation of the QT interval and ultimately causing *TdP* in humans is not an easy task (Pugelsy et al. 2008a, b). Also, discarding drugs that are found to be inhibitors of hERG purely based on electrophysiological evidence because of concerns over the development of *TdP* in humans can easily result in false positive information. It is pertinent to bear in mind that it is possible that additional and other pharmacological properties of a compound other than whether they cause prolongation of the QT interval may counter the development of *TdP*.

With regard to drug-induced cardiac arrhythmias, a number of *markers* are thought to provide the impetus for the development of *TdP* and ventricular fibrillation. Among these are *instability,* where the APD of the consecutive action potential becomes unstable, *reverse use dependence,* which could be the basis of instability, *triangulation*, i.e., slowing of the repolarization, and *dispersion* or repolarization from nonuniform triangulation and instability (Hondeghem 2008). There are also patient risk factors that could increase the propensity of a drug to induce long QT syndrome causing *TdP* (Table 5).

In pursuit of a suitable means to evaluate compounds that could possibly cause *TdP*, Hondegham and colleagues employed a bioassay as a means of assessing the proarrhythmic effects of a host of drugs in the isolated female rabbit hearts using the Langendroff perfusion method (Hondegham et al. 2003). Monophasic action potentials (MAP) measurements were used to determine intravascular conduction, APD,

Table 5 Some examples of risk factors that could increase the propensity for drug-induced long QT syndrome

Risk factors
Bradycardia
Dietary deficiencies
Digitalis therapy
Dilated cardiomyopathy
Electrolyte abnormalities (e.g., hypokalemia)
Gender (females)
Intracranial disease
Mitral valve prolapse
Myocardial infarction
Severe hypomagnesemia
The use of other drugs that prolong QT interval

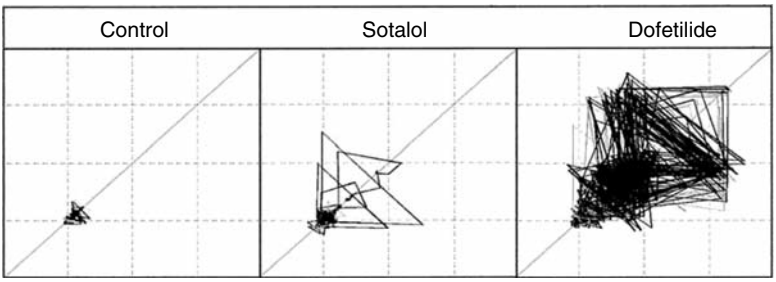

Fig. 5 *Poincaré plots.* Control and drug-induced chaotic behavior determined by plotting action potential duration (APD_{60}) for each action potential against the APD_{60} of the preceding action potential for sotalol and doftetilide. Each figure box represents an 800×800 ms square while each small box represents 200×200 ms. Reproduced with permission from Wiley-Blackwell Publishing, Hondeghem et al. (2003)

triangulation (i.e., ratio of APD_{30} to APD_{90}), reverse use-dependence, instability, and the presence of chaotic behavior such as EADs, *TdP,* and ventricular fibrillation in the preparation. The data revealed that while sotalol, which has been noted to clinically cause significant cardiac arrhythmias (Waldo et al. 1996), produced modest Class III effects in increasing APD, it also caused marked use-dependence, triangulation, and EADs. In contrast, pimozide caused marked prolongation of APD but little triangulation; Amiodarone also caused marked prolongation of APD, but it is not noted for overtly causing *TdP*. In essence, this study revealed that there appears to be a weak correlation between prolongation of APD duration and proarrhythmias (Hondeghem et al. 2003). Also of interest was an assessment of chaotic behavior using *Poincaré plots.* Drug-induced chaotic behavior was determined by plotting APD_{60} for each action potential against the APD_{60} of the preceding action potential. This revealed that both sotalol and dofetilide, both known for causing *TdP* (Haverkamp et al. 2000), produced marked chaotic behavior (Fig. 5). The blind bioassay revealed a number of compounds, namely droperidol (dopamine receptor antagonist), sotalol (Class

III antiarrhythmic and β-blocker), bepridil (Ca channel blocker), lidoflazine (Ca channel blocker), ketanserin (serotonin, α-adrenergic and dopamine receptor antagonist), sertindole (dopamine, serotonin, α-adrenergic, and histamine receptor antagonist), terfenadine (histamine receptor antagonist), haloperidol (dopamine receptor antagonist), astemizole (histamine and muscarinic antagonist), cisapride (serotonine receptor agonist), ziprasidone (dopamine, serotonin, α-adrenergic, and histamine receptor antagonist), dofetilide (Class III antiarrhythmic), quinidine (Class IA antiarrhythmic), and ibutilide (Class III antiarrhythmic), as having proarrhythmic properties similar to those described for the Class III antiarrhythmics (Hondeghem et al. 2003).

Another example that merely being a potent hERG blocker does not necessarily translate into the compound having proarrhythymic features comes from an investigation comparing the effects of two antimuscarinic and antispasmodic agents: tolterodine and terodiline. Both compounds are relatively potent hERG blockers with an IC_{50} (nM) of 9.6 ± 0.6 (mean ± SEM, n=4-5) 375 ± 4 and n_H of 1.09 ± 0.07, 1.1 ± 0.01 (n=5), respectively (Martin et al. 2006). While tolterodine is considered safe, terodiline has been withdrawn from the market because of adverse cardiac events associated with its use (Thomas et al. 1995). Evaluation of triangulation (APD_{50}/APD_{90}) produced by terodiline was 0.94 and 0.59 at 1.0 and 10 μM, and for tolterodine they were 0.99 and 0.97 at 7 and 70 nM, respectively; the implication being that the benign effects of tolterodine could, in part, be due to lack of triangulation of action potential which was prominent with terodiline (Martin et al. 2006).

One other interesting example in the literature involves an investigation comparing the effects of a number of quinolone antibiotics (ciprofloxacin, ofloxacin, moxifloxacin, and levofloxacin) on cardiac electrical events in isolated Langandroff-perfused rabbit hearts (Milberg et al. 2007a). It was apparent that all the quinolones tested produced concentration-dependent prolongation of the QT interval and MAP. Reverse use-dependence was quite marked with ofloxacin and moxifloxacin. It was also evident that hearts that developed *TdP* in low $[K^+]_o$ showed significantly greater dispersion of repolarization (Fig. 6). Moreover, triangulation (MAP_{90}/MAP_{50}) was noted for all quinolones at high concentrations. It was apparent that MAP_{90} was markedly lengthened whereas MAP_{50} was modestly prolonged causing a significant increase in the MAP_{90}/MAP_{50} ratio. More triangulation of the action potential resulted in a significant occurrence of *TdP* (Fig. 7). Furthermore, *TdP* developed in low $[K^+]_o$ solution which was accompanied by more marked triangulation of the action potential. EADs and *TdP* were demonstrated in the presence of ciprofloxacin, ofloxacin, levofloxacin, and was greater for moxifloxacin. These findings seem to support the view that an increase in dispersion of repolarization with an increase in triangulation of the action potential is a predictor for the occurrence of *TdP*.

In view of the fact that different cardiac cells seem to have varying rates of repolarization, it is not surprising that significant dispersion and triangulation may occur to varying degrees in the different segments of the heart, which could then lead to cardiac arrhythmias.

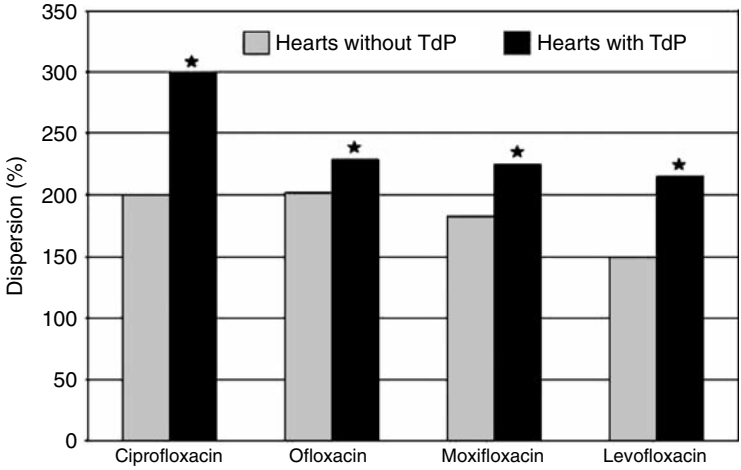

Fig. 6 More increase in dispersion of repolarization in hearts with later *torsade de pointes* (*TdP*) as compared with hearts without developing *TdP* $*P < 0.05$ as compared to hearts without *TdP*). Reproduced with permission from Wiley-Blackwell Publishing, Milberg et al. (2007a)

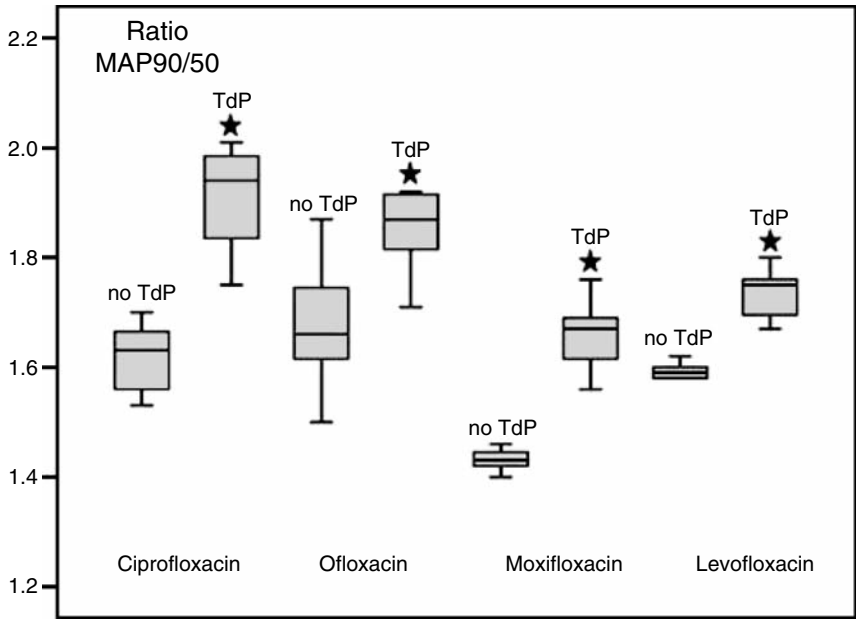

Fig. 7 More triangulation of action potential configuration in hearts with later *torsade de pointes* (*TdP*) as compared with hearts without developing *TdP* $*P < 0.05$ as compared to hearts without *TdP*). Reproduced with permission from Wiley-Blackwell Publishing, Milberg et al. (2007a)

3.2 *Intracellular Sites and Organelles*

Anthracyclines (e.g., daunorubicin, doxorubicin) are notably used for cancer chemotherapy, and they produce well-described cardiac toxicity, but the mechanism for their action is not completely understood (Olson and Mushlin 1990). Several mechanisms have been proposed to account for cardiac injuries produced by anthracyclines, which include the generation of reactive oxygen species (Bachur et al. 1979), lipid peroxidation (Goodman and Hochstein 1977; Myers et al. 1977), impairment of mitochondrial function (Ferrero et al. 1976; Seraydarian et al. 1977), and modifications of gene transcription (Kim et al. 2003) (Fig. 8). However, the main shortcoming of many observations describing the toxic cardiac actions of anthracyclines is the lack of appropriate correlation between the concentrations employed to produce toxic effects and the cardiotoxic dose in vivo. Notwithstanding the latter, it is believed that certain anthracyclines may accumulate in cardiac tissue by an active process (Kang and Weiss 2003a, b). Such an action could result in high concentration of the drug becoming localized in cardiac myocytes.

It is evident that anthracyclines cause congestive heart failure, which appears to be dose-dependent (Bristow et al. 1978, 1981). Severe cardiotoxicity has been noted in >20% of patients treated with anthracyclines (Bristow et al. 1978; Singal and Iliskovic 1998). Furthermore, it would seem that toxicity associated with

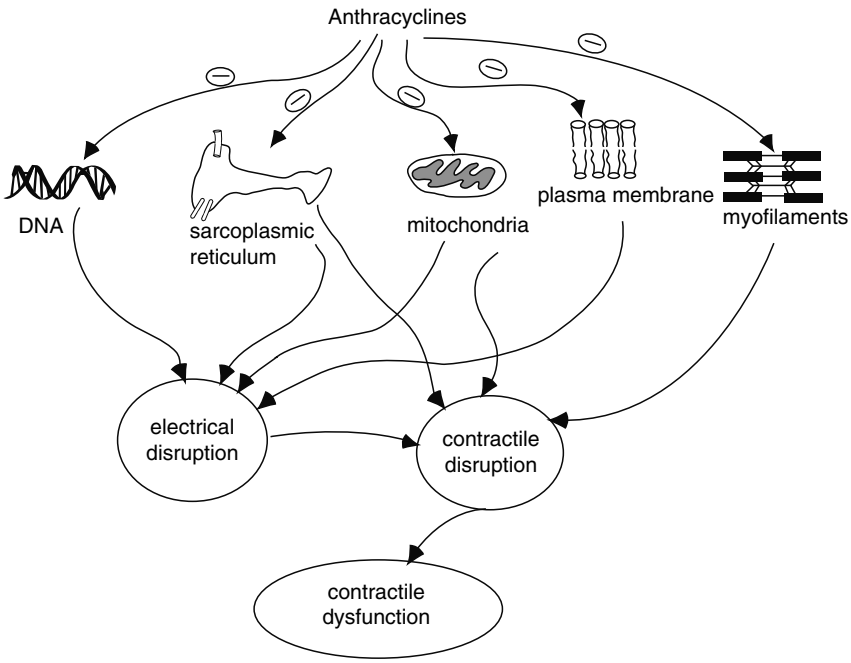

Fig. 8 Various cellular sites in the cardiac myocyte that anthracyclines can act upon to cause cardiac toxicity

anthracyclines is, at least in part, due to the formation of toxic metabolites (Zucchi and Danesi 2003). A report by Sarubbi and colleagues indicated that short periods of treatment of patients with doxorubicin can result in a significant increase in ventricular recovery time dispersion indexes, which in turn, can lead to an enhanced susceptibility to sustained ventricular tachycardia (Sarubbi et al 1997). Furthermore, Milberg and colleagues have suggested that the anthracycline, doxorubicin, reduces repolarization reserve and may facilitate *TdP* by drugs that block hERG (Milberg et al. 2007b).

3.2.1 Sarcoplasmic Reticulum

The acute in vitro anthracycline-mediated cardiac effects appear to be biphasic with an initial early effect that is characterized by an increase in contractile function, and a secondary delayed effect that seems to decrease cardiac function (Hagane et al. 1988). Chronic treatment of rats with doxorubicin (2 mg kg^{-1} i.v. per week for 4 weeks) revealed that tension of permeabilized cardiac trabeculae was significantly reduced compared to saline-treated animals (Bottone et al. 1998). Moreover, while cardiac tissue from doxorubicin-treated animals had a lower Ca^{2+} sensitivity in permeabilized tissue, the amplitude of caffeine-induced contractions was similar in drug- versus saline-treated animals (Bottone et al. 1998). Based on the latter study, it would seem that treatment with doxorubicin may impair actin–myosin interaction but does not seem to significantly attenuate the functionality of the SR. Sawyer and colleagues (2002) have presented data to suggest that, in adult rats, ventricular myocyte exposed to doxorubicin (0.1–0.5 μM) showed concentration-dependent increases in myofilament disarray. Therefore, it seems that detrimental effects of doxorubicin, at least in part, could be due to disturbance of the contractile elements in the cardiac tissue.

Boucek and colleagues (1993) examined the effect of doxorubicin on the SR function in isolated intact and permeabilized rabbit cardiac tissue. In the latter study, it was noted that doxorubicin (10–120 μM) was able to cause contractions in a similar manner to caffeine by releasing Ca^{2+} from SR and seemed to have no impact on the function of the myofilaments. Evidence has also been presented to indicate that anthracyclines (daunorubicin; 10–300 μM) can perturb Ca^{2+} handling by the SR in a free radical-independent manner in rabbit cardiac tissue; however, the quinone moiety of the molecule appears to be a prerequisite for such an action (Shadle et al. 2000). It is possible that alterations in Ca^{2+} release from the SR by anthracyclines could contribute to the inotropic and lusitropic dysfunction observed in cardiac tissue with this class of drugs.

3.2.2 Mitochondria

Chronic treatment of rats with doxorubicin (2 mg kg^{-1} per week s.c. for 13 weeks) has been reported to cause Ca^{2+} cycling and disruption of mitochondrial Ca^{2+}

homeostasis, leading to the suggestion that this effect may be important in cardiomyopathy induced by anthracyclines (Solem et al. 1994). In mice, treatment with doxorubicin caused bradycardia, prolongation of ventricular repolarization, and mitochondrial swelling as well as dissolution and disruption of mitochondrial cristae. The damage to the mitochondria was found to be specific to the heart and not found to occur in the liver (Papadopoulou et al. 1999). Anthracyclines, in a dose- and time-dependent manner, have been found to impair mitochondrial creatine kinase activity (Tokarska-Schlattner et al. 2002). Doxorubicin has also been reported to induce oxidative stress and mitochondrial-mediated apoptosis (Childs et al. 2002), while electron microscopy data has revealed that treatment with doxorubicin causes matrix swelling and cristae disorganization in mitochondria (Ogihara et al. 2002). It seems that injections of rats with doxorubicin (2 mg kg^{-1} per week, i.p.) damaged cardiac mitochondrial DNA (Serrano et al. 1999). While both reactive oxygen species and reactive nitrogen products have been implicated in acute doxorubicin-induced cardiac mitochondrial injury (Chaiswing et al. 2005), oxidative damage seems to precede nitrative damage in cardiac tissue (Chaiswing et al. 2004). Needless to say, it would seem that one specific site of toxic action by anthracyclines is cardiac mitochondria (Lebrecht and Walker 2007).

3.2.3 DNA/mRNA

In broad terms, an effect on cardiac DNA/mRNA molecules may also account for the negative impact of anthracyclines on the heart. Papoian and Lewis (1992) reported that anthracyclines (doxorubicin, daunorubicin, and epirubicin) decreased α-actin mRNA. Furthermore, an ultrastructural examination of the cardiac tissue showed loss of myofilaments (Papoian and Lewis 1992). Anthracyclines have also been reported to cause DNA-damage by producing superoxide resulting in production of peroxide and hydroxyl radicals in cardiac tissue (Shadle et al. 2000). However, in cardiac tissue, damage to DNA caused by anthracyclines has been dissociated from damage caused by these agents to the mitochondia. This has led to the suggestion that damage caused by doxorubicin to DNA is proximal to damage to mitochondia in cardiac tissue (L'Ecuyer et al. 2006). Nonetheless, significant damage to cardiac DNA can ultimately compromise myocardial function.

4 Concluding Remarks

It is apparent that many different classes of drugs can produce toxic effects on the myocardium. Such agents do not necessarily include those used to treat cardiovascular disorders and can include other therapeutic drugs that are used to treat pathophysiological conditions other than cardiovascular disease. It is also apparent that the hERG potassium channel is a ubiquitous site of action for many drugs and, inappropriately at times, assessment of the cardiotoxicity of drugs is being

intimately linked to the inhibition of hERG. The fact that many drugs seem to bind to this particular channel seems to be related, in part, to the nature of the structure of this channel. In addition, it seems that molecules can get trapped within the channel, an event that may indirectly result in the amplification of the pharmacological effect of the chemical. Nonetheless, for a drug that is found to be an inhibitor of the hERG potassium channel, it is pivotal that other pharmacological properties of the molecule be taken into account in an assessment of safety for clinical use. Importantly, not all molecules that are effective inhibitors of hERG will result in fatal adverse cardiovascular events.

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Mechanisms of Drug-Induced Nephrotoxicity

Thomas D. Nolin and Jonathan Himmelfarb

Contents

Abstract Drug-induced nephrotoxicity is a common complication of several medications and diagnostic agents. It is seen in both inpatient and outpatient settings with variable presentations ranging from mild, reversible injury to advanced kidney disease. Manifestations of drug-induced nephrotoxicity include acid–base abnormalities, electrolyte imbalances, urine sediment abnormalities, proteinuria, pyuria, hematuria, and, most commonly, a decline in the glomerular filtration rate. The mechanisms of drug-induced nephrotoxicity may differ between various drugs or drug classes, and they are generally categorized based on the histological component of the kidney that is affected. Aminoglycoside antibiotics, radiocontrast media, conventional nonselective nonsteroidal anti-inflammatory drugs, and selective cyclooxygenase-2 inhibitors, amphotericin B, and angiotensin-converting enzyme inhibitors have been frequently implicated. This chapter reviews the clinical presentation and basic mechanisms of drug-induced nephrotoxicity.

Keywords Nephrotoxicity · Kidney disease · Acute kidney injury

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Abbreviations

ACEI	Angiotensin-converting enzyme inhibitor
AIN	Allergic interstitial nephritis
AKI	Acute kidney injury
ANCA	Antineutrophil cytoplasmic antibody
ARB	Angiotensin II receptor blocker
ATN	Acute tubular necrosis
BUN	Blood urea nitrogen
CKD	Chronic kidney disease
COX	Cyclooxygenase
ESRD	End-stage renal disease
FSGS	Focal segmental glomerulosclerosis
GFR	Glomerular filtration rate
HIV	Human immunodeficiency virus
NSAID	Nonsteroidal anti-inflammatory drug
PGE_2	Prostaglandin E_2

1 Mechanisms of Drug-Induced Nephrotoxicity

Drug-induced nephrotoxicity is a relatively common complication of several diagnostic and therapeutic agents. Up to 20% of hospital admissions due to acute kidney injury (AKI) have been attributed specifically to drug-induced nephrotoxicity occurring in the community (Elasy and Anderson 1996). In the acute care hospital setting, drug-induced nephrotoxicity has been implicated in 8–60% of all cases of in-hospital AKI, and as such, is a recognized source of significant morbidity and mortality (Schetz et al. 2005). In-hospital drug use may contribute to 35% of all cases of acute tubular necrosis, most cases of allergic interstitial nephritis, as well as nephrotoxicity due to alterations in renal hemodynamics and postrenal obstruction (Thadhani et al. 1996). The incidence of antibiotic-induced nephrotoxicity alone may be as high as 36% (Choudhury and Ahmed 2006). Aminoglycoside antibiotics, radiocontrast media, conventional nonselective nonsteroidal anti-inflammatory drugs (NSAIDs), and selective cyclooxygenase-2 (COX-2) inhibitors, amphotericin B, and angiotensin-converting enzyme inhibitors (ACEIs) have also been frequently implicated (Nash et al. 2002; Markowitz and Perazella 2005; Perazella 2005). The mechanisms of drug-induced nephrotoxicity may differ between various drugs or drug classes, and, as depicted in Table 1, they are generally categorized based on the histological component of the kidney that is affected (Perazella 2005). This chapter reviews the clinical presentation and basic mechanisms of drug-induced nephrotoxicity.

Table 1 Types of drug-induced nephrotoxicity and associated drugs

Types of toxicity	Drugs
Hemodynamically-mediated kidney injury	Angiotensin-converting enzyme inhibitors
	Angiotensin II receptor blockers
	Nonsteroidal anti-inflammatory drugs
	Cyclosporine, tacrolimus
	OKT3
Tubular epithelial cell damage	
Acute tubular necrosis	Aminoglycoside antibiotics
	Radiographic contrast media
	Cisplatin, carboplatin
	Amphotericin B
	Cyclosporine, tacrolimus
	Adefovir, cidofovir, tenofovir
	Pentamidine
	Foscarnet
	Zoledronate
Osmotic nephrosis	Mannitol
	Dextran
	Intravenous immunoglobulin
Tubulointerstitial disease	
Acute allergic interstitial nephritis	Penicillins
	Ciprofloxacin
	Nonsteroidal anti-inflammatory drugs, cyclooxygenase-2 inhibitors
	Proton pump inhibitors
	Loop diuretics
Chronic interstitial nephritis	Cyclosporine
	Lithium
	Aristolochic acid
Nephrocalcinosis	Oral sodium phosphate solution
Papillary necrosis	NSAIDs, combined phenacetin, aspirin, and caffeine analgesics
Glomerular disease	Gold
	Lithium
	Nonsteroidal anti-inflammatory drugs, cyclooxygenase-2 inhibitors
	Pamidronate
Renal vasculitis and thrombosis	Hydralazine
	Propylthiouracil
	Allopurinol
	Penicillamine
	Gemcitabine
	Mitomycin C
	Methamphetamines
	Cyclosporine, tacrolimus
Obstructive nephropathy	
Intratubular obstruction	Acyclovir
	Sulfonamides
	Indinavir
	Foscarnet
	Methotrexate

(*continued*)

Table 1 (continued)

Types of toxicity	Drugs
Nephrolithiasis	Sulfonamides
	Triamterene
	Indinavir
	Thrombolytic agents

1.1 Hemodynamically-Mediated Kidney Injury

The kidneys constitute only 0.4% of body weight, but receive approximately 25% of resting cardiac output (Choudhury and Ahmed 2006). This enhances the kidney's exposure to circulating drugs. Within each nephron, blood flow and pressure are regulated by glomerular afferent and efferent arterioles to maintain intraglomerular capillary hydrostatic pressure, glomerular filtration, and urine output. Afferent and efferent arteriolar vasoconstriction are primarily mediated by angiotensin II, whereas afferent vasodilation is primarily mediated by prostaglandins. This specialized blood flow is precisely regulated by arachidonic acid metabolites, natriuretic factors, nitric oxide, the sympathetic nervous system, the renin-angiotensin system, and the macula densa response to distal tubular solute delivery. Hemodynamically-mediated kidney injury results from a decrease in intraglomerular pressure. Mechanisms commonly include a decrease in renal blood flow, vasoconstriction of glomerular afferent arterioles, or vasodilation of glomerular efferent arterioles.

The kidney normally attempts to maintain glomerular filtration rate (GFR) by dilating the afferent arteriole and constricting the efferent arteriole in response to a decrease in renal blood flow. During states of reduced blood flow, the juxtaglomerular apparatus increases renin secretion. Plasma renin converts angiotensinogen to angiotensin I, and ultimately angiotensin II (AII) by angiotensin-converting enzyme. AII constricts the afferent and efferent arterioles resulting in a net increase in intraglomerular pressure. Additionally, renal prostaglandins, prostaglandin E_2 (PGE_2) in particular, are released and induce a net dilation of the afferent arteriole, thereby improving blood flow into the glomerulus. Together these processes maintain GFR and urine output.

1.1.1 Angiotensin-Converting Enzyme Inhibitors and Angiotensin II Receptor Blocking Agents

ACEI-induced AKI has accounted for 9% of all cases of AKI requiring hospitalization (Wynckel et al. 1998), but reductions in GFR have been reported primarily in patients with severe atherosclerotic renal artery stenosis (Epstein 2004). The rise is often minimal in renovascular disease if only one renal artery is stenotic, but is more apparent in patients with a single kidney with renovascular disease, congestive heart failure, volume depletion, or bilateral renal small vessel disease. Up to

one-third of patients with bilateral renal artery stenosis demonstrate a rise in serum creatinine >30% after starting ACEI therapy (Wynckel et al. 1998).

Kidney injury mediated by ACEIs or angiotensin II receptor blockers (ARBs) is the result of a decrease in glomerular capillary hydrostatic pressure sufficient to reduce glomerular ultrafiltration (Perazella 2005). When ACEI therapy is initiated, the synthesis of angiotensin II is decreased, thereby preferentially dilating the efferent arteriole. This reduces outflow resistance from the glomerulus and decreases hydrostatic pressure in the glomerular capillaries, which alters Starling's forces across the glomerular capillaries to decrease intraglomerular pressure, GFR, and then often leads to nephrotoxicity, particularly in the setting of reduced renal blood flow or effective arterial blood volume, for example "prerenal" settings in which glomerular afferent arteriolar blood flow is reduced and the efferent arteriole is vasoconstricted to maintain sufficient glomerular capillary hydrostatic pressure for ultrafiltration. Patients at greatest risk are those dependent on angiotensin II to maintain blood pressure and renal efferent arteriolar constriction. These include patients with hemodynamically significant renal artery stenosis, particularly bilateral stenosis, and those with decreased effective arterial blood volume, especially those with congestive heart failure, volume depletion from excess diuresis or gastrointestinal fluid loss, hepatic cirrhosis with ascites, and the nephrotic syndrome (Perazella 2003, 2005).

1.1.2 Nonsteroidal Anti-Inflammatory Drugs and Selective Cyclooxygenase-2 Inhibitors

Fifty million US citizens report NSAID use, and it has been estimated that 500,000–2.5 million people will develop NSAID nephrotoxicity in the US annually (Whelton 1999). Kidney injury can occur within days of initiating therapy, particularly with a short-acting NSAID such as ibuprofen (Whelton 1999). Patients typically present with complaints of diminished urine output, weight gain, and/or edema. Urine volume and sodium concentration are usually low, and blood urea nitrogen (BUN), serum creatinine, and potassium are typically elevated. The urine sediment is usually unchanged from baseline, but may show granular casts. Hemodynamically-mediated kidney injury associated with COX-2 inhibitors presents similarly (Perazella 2005).

NSAIDs inhibit cyclooxygenase-catalyzed prostaglandin production and impair renal function by decreasing synthesis of vasodilatory prostaglandins from arachidonic acid (Whelton 1999). Renal prostaglandins are synthesized in the renal cortex and medulla by vascular endothelial and glomerular mesangial cells. Their effects are primarily local and result in renal vasodilation (particularly prostacyclin and PGE_2). They have limited activity in states of normal renal blood flow, but, in states of decreased renal blood flow, their synthesis is increased and they protect against renal ischemia and hypoxia by antagonizing renal vasoconstriction due to angiotensin II, norepinephrine, endothelin, and vasopressin. Administration of NSAIDs in the setting of renal ischemia and compensatory increased prostaglandin activity

may thus alter the balance of activity between renal vasoconstrictors and vasodilators. This leaves the activity of renal vasoconstrictors unopposed and promotes renal ischemia with loss of glomerular filtration (Perazella 2005; Choudhury and Ahmed 2006).

People at greatest risk for nephrotoxicity have chronic kidney disease (CKD), hepatic disease with ascites, decompensated congestive heart failure, intravascular volume depletion, or systemic lupus erythematosus (Perazella 2005). Additional risk factors include atherosclerotic cardiovascular disease and concurrent diuretic therapy. The elderly are also at higher risk due to interaction of prevalent medical problems, multiple drug therapies, and reduced renal hemodynamics. NSAID use in patients older than 65 years may increase the risk of AKI by up to 58% (Griffin et al. 2000). Combined NSAID or COX-2 inhibitor and ACEI or ARB therapy is also a concern and should be avoided in high risk patients.

1.1.3 Calcineurin Inhibitors

The calcineurin inhibitors, cyclosporine and tacrolimus, have dramatically enhanced the success of solid organ transplantation. Nephrotoxicity, however, remains a major dose-limiting adverse effect of both drugs (Ojo et al. 2003). Although delayed chronic interstitial nephritis has been reported, acute hemodynamically-mediated kidney injury is the primary mechanism of nephrotoxicity (Schetz et al. 2005; Perazella 2005). Acute kidney injury may occur within days of initiating therapy. Hypertension, hyperkalemia, sodium retention, and hypomagnesemia may occur. Typically, no urine sediment abnormalities are seen. Renal biopsy usually reveals thickening of arterioles, mild focal glomerular sclerosis, proximal tubular epithelial cell vacuolization and atrophy, and interstitial fibrosis. Biopsy is useful to distinguish acute cyclosporine nephrotoxicity from renal allograft rejection, the latter being evidenced by cellular infiltration (de Mattos et al. 2000).

A dose-related hemodynamic mechanism is likely during the initial months of therapy because renal function improves rapidly following dose reduction. Reversible vasoconstriction and injury to glomerular afferent arterioles occurs, possibly due to increased activity of thromboxane A_2, endothelin, and the sympathetic nervous system, or diminished activity of nitric oxide or prostacyclin (Burdmann et al. 2003; Liptak and Ivanyi 2006). Vasoconstriction due to increased renin-angiotensin system activity may also contribute. In contrast, renal arteriolar hyalinization and chronic renal ischemia as well as increased extracellular matrix synthesis appear to be the primary mechanisms that contribute to cyclosporine-induced chronic kidney disease (Liptak and Ivanyi 2006; Burdmann et al. 2003). Risk factors include increased age and higher initial cyclosporine dose and/or serum concentrations, as well as renal graft rejection, hypotension, infection, and concomitant therapy with nephrotoxic drugs such as aminoglycosides, amphotericin B, acyclovir, NSAIDs, and radiocontrast agents (de Mattos et al. 2000).

1.2 Tubular Epithelial Cell Damage

1.2.1 Acute Tubular Necrosis

Renal tubular epithelial cell damage may be caused by either direct toxic or ischemic effects of drugs. Damage is most often localized in the proximal and distal tubular epithelia, and, when observed as cellular degeneration and sloughing from proximal and distal tubular basement membranes, is termed acute tubular necrosis (ATN) (Silva 2004). Swelling and vacuolization of proximal tubular cells may also be noted in those with osmotic nephrosis (Perazella 2005). ATN is the most common presentation of drug-induced nephrotoxicity in the inpatient setting. The primary agents implicated in renal tubular epithelial cell damage are aminoglycosides, radiocontrast media, cisplatin, amphotericin B, foscarnet, and osmotically active agents such as immunoglobulins, dextrans, and mannitol (Choudhury and Ahmed 2006; Perazella 2005).

Aminoglycosides

ATN has been reported in 5–15% of patients receiving aminoglycoside therapy (Streetman et al. 2001; Slaughter and Cappelletty 1998). A gradual progressive rise in the serum creatinine concentration and decrease in creatinine clearance after 6–10 days of therapy are the initial clinical manifestations of toxicity. Patients typically present with nonoliguria, maintaining urine volumes greater than 500 mL/day. Severe kidney injury does not usually develop if aminoglycoside therapy is stopped immediately once toxicity becomes apparent. Aminoglycoside-associated nephropathy must be evaluated carefully since not all acute kidney injury during a course of therapy is due to the aminoglycoside. Dehydration, sepsis, ischemia, and other nephrotoxic drugs frequently contribute and complicate the identification of the causative agent or condition.

The reduction of GFR in patients receiving aminoglycosides is predominantly the result of proximal tubular epithelial cell damage leading to obstruction of the tubular lumen and backleakage of the glomerular filtrate across the damaged tubular epithelium (Mingeot-Leclercq and Tulkens 1999). Toxicity may be related to cationic charge, which facilitates binding of filtered aminoglycosides to renal tubular epithelial cell luminal membranes, followed by intracellular transport and concentration in lysosomes (Choudhury and Ahmed 2006; Nagai and Takano 2004; Mingeot-Leclercq and Tulkens 1999). Cellular dysfunction and death may result from release of lysosomal enzymes into the cytosol, generation of reactive oxygen species, altered cellular metabolism, and alterations in cell membrane fluidity, leading to reduced activity of membrane-bound enzymes, including Na^+-K^+-ATPase, dipeptidyl peptidase IV, and neutral aminopeptidase. Multiple risk factors for aminoglycoside nephrotoxicity have been identified. These relate to the aggressiveness of aminoglycoside dosing, synergistic toxicity as the result of combination

drug therapy, and preexisting clinical conditions of the patient (Mingeot-Leclercq and Tulkens 1999).

Radiocontrast Media

ATN associated with the administration of radiographic contrast agents is the third leading cause of hospital-acquired acute kidney injury (Waybill and Waybill 2001; Barrett and Parfrey 2006). The incidence rises from $<2\%$ in patients with low risk to 40–50% in high-risk patients such as those with CKD or diabetes mellitus (Waybill and Waybill 2001; Rudnick et al. 2006; Maeder et al. 2004). The risk of contrast-induced nephrotoxicity increases as the number of risk factors increases, and diabetic patients with CKD have the greatest risk (Rudnick et al. 2006; Murphy et al. 2000).

Contrast nephrotoxicity presents most commonly as nonoliguric, transient tubular enzymuria. However, irreversible oliguric (urine volume <500 mL/day) kidney injury requiring dialysis has been reported in high risk patients including diabetics with pre-existing kidney disease. Kidney injury typically manifests within the first 12–24 h after the contrast study. The serum creatinine concentration usually peaks between 2 and 5 days after exposure, with recovery after 4–10 days. Urinalysis typically reveals only hyaline and granular casts, but may also be completely bland (Murphy et al. 2000). The urine sodium concentration and fractional excretion of sodium are frequently low.

The mechanism of contrast-induced nephrotoxicity appears to be direct tubular toxicity and/or renal ischemia (Rudnick et al. 2006; Maeder et al. 2004). Direct tubular toxicity is suggested by the frequent presence of renal tubular enzymuria and biopsy findings of proximal tubular epithelial cell vacuolization and acute tubular necrosis. In contrast to these findings, the frequent finding of a low urine sodium concentration and low fractional excretion of sodium suggests that renal tubular function is preserved. Renal ischemia may result from systemic hypotension associated with contrast injection, as well as renal vasoconstriction mediated by an imbalance of humoral agents, including prostaglandins, adenosine, atrial natriuretic peptide, nitric oxide, and endothelin (Murphy et al. 2000; Rudnick et al. 2006). Renal ischemia may also result from dehydration due to osmotic diuresis accompanying use of hyperosmolar agents (900–1,780 mOsm kg^{-1}) and increased blood viscosity due to red blood cell crenation and aggregation (Rudnick et al. 2006). Pre-existing kidney disease, particularly in diabetic patients, is the major risk factor (Rudnick et al. 2006; Murphy et al. 2000). Conditions associated with decreased renal blood flow, including congestive heart failure and dehydration, also confer risk.

Platin-Containing Compounds

Platin-containing compounds are important chemotherapeutic agents that frequently cause ATN (Kintzel 2001; Hartmann and Lipp 2003; Taguchi et al. 2005). The

incidence of cisplatin nephrotoxicity is 6–13%, down from the much higher rate of >50% observed in the 1980s. This reduction in toxicity is due primarily to limiting the total dose and reducing the rate of administration. Carboplatin is associated with a much lower incidence of nephrotoxicity than cisplatin and is generally preferred in high risk patients (Hartmann and Lipp 2003).

Peak serum creatinine concentrations occur approximately 10–12 days after initiation of therapy, with recovery by 21 days. However, kidney damage is dose-related and cumulative with subsequent cycles of therapy, so the serum creatinine concentration may continue to rise. Irreversible kidney injury may result. Renal magnesium wasting is common and can be accompanied by hypocalcemia and hypokalemia. Hypomagnesemia may be severe, causing seizures, neuromuscular irritability, or personality changes, and persist long after chemotherapy has ended. Hypomagnesemia results primarily from increased urinary losses due to renal tubular damage as well as saline hydration and diuretic therapy to prevent toxicity. Anorexia and diarrhea also contribute, due to decreased intake and increased loss of magnesium, respectively.

Proximal tubular damage appears acutely after administration of platin-containing compounds, as the result of impairment of cell energy production, possibly by binding to proximal tubular cellular proteins and sulfhydryl groups with disruption of cell enzyme activity and uncoupling of oxidative phosphorylation (Taguchi et al. 2005). The initial proximal tubular damage is followed by a progressive loss of glomerular filtration and impaired distal tubular function (Kintzel 2001). Renal biopsies generally show sparing of glomeruli with necrosis of proximal and distal tubules and collecting ducts. Risk factors include increased age, dehydration, renal irradiation, concurrent use of aminoglycoside antibiotics, and alcohol abuse (Kintzel 2001).

Amphotericin B

Amphotericin B-induced ATN may be seen with cumulative doses as low as 300–400 mg, and reaches an incidence of 80% when cumulative doses approach 4 g (Fanos and Cataldi 2000; Costa and Nucci 2001). Toxicity usually manifests after administration of 2–3 g as renal tubular potassium, sodium, and magnesium wasting, impaired urine concentrating ability, and distal renal tubular acidosis due to a leak of hydrogen ions back out of the tubular lumen (Costa and Nucci 2001).

The mechanisms of kidney injury include direct tubular epithelial cell toxicity with increased tubular permeability and necrosis, as well as arterial vasoconstriction and ischemic injury (Fanos and Cataldi 2000). Overall, the combined effects of increased cell energy and oxygen requirements due to greater cell membrane permeability, and reduced cellular oxygen delivery due to renal vasoconstriction, results in renal medullary tubular epithelial cell necrosis and kidney injury. Risk factors include CKD, higher average daily doses, volume depletion, and concomitant administration of diuretics and other nephrotoxins (e.g., cyclosporine) (Deray 2002; Costa and Nucci 2001). Rapid infusions of amphotericin B have the potential

to increase toxicity. A recent comparison of 24-h continuous infusions with conventional 4-h infusions revealed a significant reduction of toxicity, attributed to decreased "pretubular" effects (e.g., effects on renal blood flow and GFR) (Eriksson et al. 2001).

1.2.2 Osmotic Nephrosis

Several drugs, including mannitol, low-molecular-weight dextran, and radiographic contrast media, or drug vehicles such as sucrose and propylene glycol, have been associated with vacuolization, swelling, and ultimately necrosis of proximal tubular epithelial cells with a decline in renal function (Perazella 2005). The decline in renal function may be due to the hypertonic and osmotically active nature of these agents. Intravenous immunoglobulin solutions contain hyperosmolar sucrose and may cause osmotic nephrosis and acute kidney injury, which is usually reversible shortly after discontinuing therapy (Orbach et al. 2004). Toxicity may be prevented by diluting the solution and reducing the rate of infusion. Hydroxyethylstarch, used as a plasma volume expander, has also been implicated in the development of osmotic nephrosis (Perazella 2005). Mannitol may rarely cause oligo-anuric kidney injury with proximal tubular cell vacuolization on biopsy (Choudhury and Ahmed 2006). It can also cause direct renal vasoconstriction or induce an osmotic diuresis with increased solute delivery to the macula densa and subsequent tubuloglomerular feedback, leading to vasoconstriction of the glomerular afferent arteriole and decreased renal blood flow. Risk factors for mannitol toxicity include excessive doses, pre-existing kidney disease, and concomitant diuretic or cyclosporine therapy.

1.3 Tubulointerstitial Disease

1.3.1 Acute Allergic Interstitial Nephritis

Tubulointerstitial diseases involve the renal tubules and the surrounding interstitial tissue (Silva 2004). Acute allergic interstitial nephritis (AIN) is the underlying cause for up to 3% of all cases of AKI (Rossert 2001). Unlike ATN, which is typically dose-dependent toxicity, AIN is a nondose-dependent (within the therapeutic range) idiosyncratic reaction (Perazella 2005). AIN is characterized as a diffuse or focal interstitial infiltrate of lymphocytes, plasma cells, eosinophils, and occasional polymorphonuclear neutrophils (Rossert 2001; Silva 2004). Granulomas and tubular epithelial cell necrosis are relatively common with drug-induced AIN. The pathogenesis is an allergic hypersensitivity response. Occasionally a humoral antibody-mediated mechanism is implicated by the presence of circulating antibody to a drug hapten–tubular basement membrane complex, low serum complement levels, and deposition of IgG and complement in the tubular basement membrane. More commonly, a cell-mediated immune mechanism is suggested by the absence

of these findings and the presence of a predominantly T-lymphocyte infiltrate (Silva 2004; Markowitz and Perazella 2005). Numerous drugs, including several antimicrobials, analgesics, diuretics, and gastrointestinal agents, have been associated with AIN (Table 1).

β-Lactams

Although methicillin-induced allergic interstitial nephritis is the prototype for AIN, it is now recognized that AIN is associated with virtually all β-lactam antibiotics (Rossert 2001). Clinical signs present approximately 14 days after initiation of therapy, but may occur sooner if the patient has been previously sensitized (Markowitz and Perazella 2005). Signs of AIN include fever, maculopapular rash, and eosinophilia, associated with renal findings of pyuria and hematuria, low-level proteinuria, and oliguria. Systemic hypersensitivity findings of fever, rash, eosinophilia, and eosinophiluria suggest the diagnosis, but this group of findings is not consistently reliable since one or more are frequently absent. Anemia, leukocytosis, and elevated IgE concentrations may be present. Tubular dysfunction may be manifested by acidosis, hyperkalemia, salt wasting, and concentrating defects (Rossert 2001; Silva 2004; Markowitz and Perazella 2005). β-lactam-induced AIN is likely an immune T cell-mediated reaction, since the interstitial infiltrate is predominantly comprised of lymphocytes, monocytes, and eosinophils.

NSAIDs

NSAID-induced AIN has a different clinical presentation than that seen with most other drugs (Rossert 2001; Perazella 2005). Patients are typically over age 50 (likely reflecting NSAID use for degenerative joint disease), the onset is delayed a mean of 6 months from initiation of therapy compared to 2 weeks with β-lactams (Perazella 2005). Also, there is a much lower incidence of fever, rash, and eosinophilia, and milder interstitial infiltration and eosinophilic infiltration are seen, which may be related to the anti-inflammatory properties of NSAIDs (Perazella 2005).

1.3.2 Chronic Interstitial Nephritis

Lithium

Lithium and only a few other drugs have been reported to cause chronic interstitial nephritis, which is usually a progressive and irreversible lesion (Choudhury and Ahmed 2006; Silva 2004). Several renal tubular lesions have been associated with lithium therapy; an impaired ability to concentrate urine (nephrogenic diabetes insipidus) has been seen in up to 87% of patients (Markowitz et al. 2000). Acute

tubular necrosis and chronic tubulointerstitial nephritis are less frequently noted, and incomplete distal renal tubular acidosis is observed in up to 50% of patients (Braden et al. 2005). Historically, the most important question regarding lithium use was whether long-term therapy, with lithium concentrations maintained in the therapeutic range, caused chronic tubulointerstitial nephritis with kidney disease. It is now known that long-term lithium therapy is associated with nephrotoxicity in the absence of episodes of acute intoxication, and that the duration of therapy and the cumulative dose are the major determinants of toxicity (Presne et al. 2003). Polydipsia and polyuria are observed in 40 and 20%, respectively, of patients with nephrogenic diabetes insipidus (Braden et al. 2005). Patients adapt well to their urinary-concentrating defect and these concerns are usually minimal. Impaired ability to concentrate urine is due to a dose-related decrease in collecting duct response to antidiuretic hormone. This results from impaired formation of cellular cyclic adenosine monophosphate in response to antidiuretic hormone. Lithium-induced AKI occurs predominantly during episodes of acute lithium intoxication. Urinalysis may show moderate proteinuria, a few red and white blood cells, and granular casts. Renal function usually returns to baseline values after lithium concentrations are reduced to the therapeutic range. Nephrotoxicity may develop insidiously and only be recognized by rising BUN or creatinine concentrations or the onset of hypertension.

The pathogenesis includes dehydration secondary to nephrogenic diabetes insipidus, as well as direct proximal and distal tubular cell toxicity resulting in ATN. Chronic tubulointerstitial nephritis attributed to lithium is evidenced most commonly by biopsy findings of interstitial fibrosis, tubular atrophy, and glomerular sclerosis (Presne et al. 2003; Silva 2004). The pathogenesis may involve cumulative direct lithium toxicity since duration of therapy has correlated with the decline in the GFR. The major risk factor for AKI is an elevated lithium concentration, particularly in association with dehydration.

Cyclosporine

Delayed chronic interstitial nephritis has been reported after 6–12 months of therapy and can result in irreversible kidney disease. Toxicity usually manifests as a slowly rising serum creatinine concentration and decreased creatinine clearance that may not reflect the severity of histopathologic changes. Typical biopsy findings include arteriolar hyalinosis, glomerular sclerosis, and a striped pattern of tubulointerstitial fibrosis (de Mattos et al. 2000; Burdmann et al. 2003; Liptak and Ivanyi 2006; Braden et al. 2005). The pathogenesis appears to involve sustained renal arteriolar endothelial cell injury which ultimately results in chronic renal ischemia because of increased release of endothelin-1, decreased production of nitric acid, and increased expression of transforming growth factor-β (Olyaei et al. 2001). Nephrotoxicity has been dose dependent in some, but not all analyses, and occurs even during low-dose therapy (Burdmann et al. 2003).

Aristolochic Acid

Aristolochic acid is the major alkaloid of the botanical species *Aristolochia*, a common ingredient of various Chinese herbal remedies that has been associated with the development of chronic interstitial injury (Cosyns 2003). Aristolochic acid-induced nephrotoxicity, also known as "Chinese herb nephropathy", typically present with mild to moderate hypertension, mild proteinuria, glucosuria, and moderately elevated serum creatinine concentrations (Cosyns 2003). Anemia and shrunken kidneys are also common on initial presentation. The main pathologic lesions observed in the kidneys are interstitial fibrosis with atrophy and destruction of tubules throughout the renal cortex. Glomeruli are generally not affected. Perhaps the most remarkable feature of Chinese herb nephropathy is the rate at which it progresses. In most individuals, end-stage renal disease (ESRD) requiring dialysis or transplantation develops within 6–24 months of exposure (Reginster et al. 1997). Although the precise mechanism of aristolochic acid–induced nephropathy is yet to be characterized, recent data indicate direct DNA damage may be the cause. The major components of aristolochic acid are metabolized to mutagenic compounds called aristolactam I and aristolactam II, respectively, which have been demonstrated to form DNA adducts in humans (Cosyns 2003). Direct cellular toxicity is not a likely mechanism because the onset is delayed and progression of kidney disease continues after exposure (Reginster et al. 1997).

1.3.3 Nephrocalcinosis

Oral Sodium Phosphate Solution

Nephrocalcinosis is a clinical-pathologic condition characterized by extensive tubulointerstitial precipitation and deposition of calcium phosphate leading to marked tubular calcification (Markowitz et al. 2005; Gonlusen et al. 2006). Typical risk factors for developing nephrocalcinosis include clinical conditions associated with hypercalcemia, including hyperparathyroidism, increased bone turnover, hypercalcemia of malignancy, and increased intake of calcium or vitamin D. Recently, however, several documented cases of nephrocalcinosis in patients without hypercalcemia have pointed to oral sodium phosphate solution (OSPS) used for bowel cleansing as the causative agent (Markowitz et al. 2005; Gonlusen et al. 2006). The term "acute phosphate nephropathy" has been coined specifically to describe OSPS-induced nephrocalcinosis, since its pathogenesis is the result of increased phosphate intake rather than hypercalcemia (Markowitz et al. 2005). The incidence of acute phosphate nephropathy is unknown but rare. Patients usually present with acute kidney injury several days to months after exposure to OSPS. Patients in one cohort of 21 cases of acute phosphate nephropathy presented with AKI and a mean serum creatinine of 3.9 mg dL^{-1} at a median of 1 month after colonoscopy (Markowitz et al. 2005). Low grade proteinuria (< 1.0 g/day), normocalcemia, and a bland urinary sediment is usually observed. Extensive deposition of

calcium phosphate in the distal tubules and collecting ducts without glomerular or vascular injury is the hallmark of OSPS-induced nephrocalcinosis (Markowitz et al. 2005; Gonlusen et al. 2006). Risk factors include bowel conditions associated with prolonged intestinal transit, and high sodium phosphate dosage, along with concomitant volume depletion and diuretic, NSAID, ACEI, or ARB therapy. Advanced age may also be a risk factor since most reported cases are in the elderly. OSPS should be avoided in patients with CKD.

1.3.4 Papillary Necrosis

Analgesics

Papillary necrosis is a form of chronic tubulointerstitial nephritis characterized by necrosis of the renal papillae (Silva 2004). Analgesic use is the most common cause of papillary necrosis, accounting for a third of all cases (Brix 2002). Although chronic excessive consumption of combination analgesics containing phenacetin was believed to be the major cause, contemporary analgesics, particularly aspirin, acetaminophen, and NSAIDs have also been associated with the development of analgesic nephropathy. Analgesic nephropathy has been reported as the primary cause of ESRD by as many as 9% of dialysis patients (De Broe and Elseviers 1998). It evolves insidiously over years, is difficult to recognize, and may be underdiagnosed as a cause of ESRD. Imaging often only demonstrates chronic pyelonephritis and small kidneys with thin renal cortices and blunted calyces. Hypertension and atherosclerotic cardiovascular disease are common. Early renal manifestations include impaired maximal urinary concentration, sterile pyuria, microscopic hematuria, and low levels of proteinuria. Urinary tract infection is common. Renal biopsy reveals nonspecific chronic interstitial inflammation and scarring (Silva 2004).

Mechanisms of analgesic nephropathy remain unclear. The renal lesion begins in the papillary tip as a result of accumulated toxic metabolites, decreased blood flow, and impaired cellular energy production. The metabolism of phenacetin to acetaminophen, which is then oxidized to toxic free radicals that are concentrated in the papilla, appears to be the initiating factor that causes toxicity by mechanisms analogous to acetaminophen hepatotoxicity. Toxicity is prevented by availability of reduced glutathione. However, salicylates deplete renal glutathione and thereby facilitate phenacetin and acetaminophen toxicity (Silva 2004; Braden et al. 2005).

1.4 Glomerular Disease

Proteinuria with or without a decline in the GFR is a hallmark sign of glomerular injury. Several different glomerular lesions may occur, mostly by immune mechanisms rather than direct cellular toxicity. Although drug-induced glomerular disease is uncommon, a variety of agents have been implicated (Izzedine et al. 2006).

Minimal change glomerular injury with nephrotic range proteinuria (i.e., > 3.5 g/day) due to drugs is frequently accompanied by interstitial nephritis and is most common during NSAID therapy (Whelton 1999; Izzedine et al. 2006). Ampicillin, rifampin, phenytoin, and lithium have also been implicated. The pathogenesis is unknown, but nephrotic-range proteinuria due to NSAID therapy is frequently associated with a T-lymphocytic interstitial infiltrate, suggesting disordered cell-mediated immunity. Proteinuria usually resolves rapidly after discontinuation of the offending drug, and a 3–4 week course of corticosteroids may help resolve the lesion (Rossert 2001).

Focal segmental glomerulosclerosis (FSGS) is characterized by patchy areas of glomerular sclerosis with interstitial inflammation and fibrosis. FSGS has been described in the setting of chronic heroin abuse (heroin nephropathy) (D'agati 2003; Jaffe and Kimmel 2006). The pathogenesis is unknown but may include direct toxicity by heroin or adulterants and injury from bacterial or viral infections accompanying intravenous drug use. FSGS is also the predominant renal lesion in AIDS patients and may result from human immunodeficiency virus (HIV) infection or heroin abuse. Glomerulosclerosis due to HIV infection may be distinguished from heroin nephropathy by tubuloreticular structures in endothelial cells on electron microscopy and the more rapid course and poorer prognosis. The bisphosphonate, pamidronate, commonly used to treat malignancy-associated hypercalcemia, has also been associated with the development of collapsing FSGS. Patients receiving either high doses or prolonged therapy are at highest risk (Markowitz et al. 2001; Albaqumi et al. 2006).

Membranous nephropathy is characterized by immune complex formation along glomerular capillary loops and, although rarely seen, has classically been associated with gold therapy, penicillamine, and NSAID use (Perazella 2005; Izzedine et al. 2006). The pathogenesis may involve damage to proximal tubule epithelium with antigen release, antibody formation, and glomerular immune complex deposition.

1.5 Renal Vasculitis and Thombosis

Numerous drugs have been associated with the development of vasculitis (ten Holder et al. 2002; Cuellar 2002). For example: propylthiouracil is associated with cutaneous, renal, and pulmonary vascultis; allopurinol is associated with cutaneous, renal, and hepatic vasculitis; hydralazine is associated with cutaneous, renal, and pulmonary vascultis; and isotretinoin is associated with cutaneous, renal, pulmonary, and gastrointestinal vascultis (Cuellar 2002; ten Holder et al. 2002). Systemic polyarteritis nodosa, a vasculitis with involvement of small- and medium-sized renal arteries, has been described following minocycline use (Cuellar 2002). Patients may present with hematuria, proteinuria, reduced renal function, and hypertension. Hydralazine, propylthiouracil, allopurinol, and penicillamine have been implicated in the development of antineutrophil cytoplasmic antibody (ANCA)-positive vasculitis (Choi et al. 2000). Patients exposed to these drugs

who subsequently develop ANCA-positive vasculitis appear to exhibit high titers of antimyeloperoxidase antibodies. Treatment of vasculitis typically consists of withdrawing the offending drug and a tapering course of prednisone, which usually leads to resolution of symptoms within weeks to months (ten Holder et al. 2002).

Numerous medications, including mitomycin C, oral contraceptive agents, cyclosporine, tacrolimus, muromonab-CD3, antineoplastic agents, interferon, ticlopidine, clopidogrel, and quinine can cause a thrombotic microangiopathy (hemolytic uremic syndrome or thrombotic thrombocytopenic purpura) manifested by endothelial proliferation and thrombus formation in the renal and central nervous system vasculature (Pisoni et al. 2001; Dlott et al. 2004). The association with mitomycin C is notable since the pathogenesis appears to be a direct, dose-related toxic effect, rarely occurring in patients who receive doses <30 mg m^{-2}. Nephrotoxicity has occurred following chemotherapy with mitomycin C alone or with 5-fluorouracil, cisplatin, bleomycin, a vinca alkaloid, and tamoxifen. Microangiopathic hemolytic anemia and thrombocytopenia are usually present. Systemic endothelial damage with multisystem organ failure has occurred. Kidney injury can be severe and irreversible, although corticosteroids, antiplatelet agents, vincristine, plasma exchange, plasmapheresis, and high-dose intravenous IgG have each induced clinical improvement. Gemcitabine is a pyrimidine analog used for the treatment of various solid tumors which was recently associated with the development of hemolytic uremic syndrome, with an estimated incidence of 0.015% (Kintzel 2001; Perazella 2005).

1.6 Obstructive Nephropathy

1.6.1 Intratubular Obstruction

Obstructive nephropathy is the result of mechanical obstruction to urine flow following glomerular filtration, and is most commonly due to intratubular obstruction or post-renal obstruction secondary to nephrolithiasis or prostatic hypertrophy (Perazella 2005; Daudon and Jungers 2004). Drug-induced renal tubular obstruction can be caused by intratubular precipitation of tissue degradation products or drugs and/or their metabolites. Acute uric acid nephropathy following tumor tissue degradation as the result of chemotherapy (i.e., tumor lysis syndrome) is the most common cause of this type of kidney injury. Acute oliguric or anuric kidney injury develops rapidly. The diagnosis is supported by a urine uric acid:creatinine ratio greater than 1.

Drug-induced rhabdomyolysis can lead to intratubular precipitation of myoglobin, and if severe, acute kidney injury. The most common cause of drug-induced rhabdomyolysis is direct myotoxicity from HMG-CoA reductase inhibitors, including lovastatin and simvastatin (Vanholder et al. 2000). The risk of rhabdomyolysis is increased when this class of drugs is administered concurrently with gemfibrozil, niacin, or inhibitors of the cytochrome P450 3A4 metabolic pathway

(e.g., erythromycin or ketoconazole). Rhabdomyolysis may also result from pressure necrosis as the result of stupor or coma following ingestion of CNS depressants (e.g., alcohol or narcotics), or extreme neuromuscular agitation associated with abuse of central nervous system stimulants (e.g., amphetamines, cocaine, ecstasy, or phencyclidine) (Vanholder et al. 2000).

Intratubular precipitation of drugs or their metabolites can also cause acute kidney injury (Perazella 2005; Perazella 1999). Urine pH decreases to approximately 4.5 during maximal stimulation of renal tubular hydrogen ion secretion. Certain solutes can precipitate and obstruct the tubular lumen at this acid pH, particularly when urine is concentrated, e.g., in patients with volume depletion. For example, acyclovir is relatively insoluble at physiological urine pH and has been associated with intratubular precipitation in dehydrated oliguric patients (Perazella 2005; Daudon and Jungers 2004; Izzedine et al. 2005). Sulfadiazine, when used at high doses, and methotrexate may also precipitate in acidic urine and can cause oligo-anuric kidney injury. Intravenous and high-dose oral acyclovir therapy for acute herpes zoster has also been associated with intratubular precipitation in dehydrated oliguric patients. Massive administration of ascorbic acid can also result in obstruction of renal tubules with calcium oxalate crystals. Oxalate, a poorly soluble ascorbic acid metabolite, can also precipitate and worsen renal function when ascorbic acid is administered to patients with acute kidney injury or the congenital nephrotic syndrome. Low-molecular-weight dextran therapy for volume expansion and rheologic effects has also caused kidney injury, possibly by intratubular precipitation of filtered dextran. Triamterene may also precipitate in renal tubules and cause kidney injury (Perazella 1999). Foscarnet complexation with ionized calcium may result in precipitation of calcium-foscarnet salt crystals in renal glomeruli, causing primarily a crystalline glomerulonephritis. The salt crystals may then secondarily precipitate in the renal tubules causing tubular necrosis (Maurice-Estepa et al. 1998). The protease inhibitor indinavir has been associated with crystalluria, crystal nephropathy, dysuria, urinary frequency, back and flank pain, or nephrolithiasis in approximately 8% of treated patients (Perazella 2005).

1.6.2 Nephrolithiasis

Nephrolithiasis does not present as classic nephrotoxicity because GFR is usually not decreased. Drug-induced nephrolithiasis can be the result of abnormal crystal precipitation in the renal collecting system, potentially causing pain, hematuria, infection, or, occasionally, urinary tract obstruction with kidney injury. The overall prevalence of drug-induced nephrolithiasis is estimated to be 1% (Daudon and Jungers 2004).

Renal stone formation, possibly also accompanied by intratubular precipitation of crystalline material, has been a rare complication of drug therapy. Until the AIDS era, triamterene had been the drug most frequently associated with renal stone formation, with a prevalence of 0.4% (Daudon and Jungers 2004). However, it has been unclear whether triamterene or its metabolites actually initiated stone formation, or are

passively absorbed onto the organic matrix of pre-existing calculi. Sulfadiazine is a poorly soluble sulfonamide that has caused symptomatic acetylsulfadiazine crystalluria with stone formation and flank or back pain, hematuria, or kidney injury in up to 29% of patients treated with the drug (Perazella 2003). A high urine volume and urinary alkalinization to pH > 7.15 may be protective. AKI due to intratubular precipitation of indinavir and collecting system obstruction from nephrolithiasis have occurred (Daudon and Jungers 2004). Numerous other drugs have been implicated in the development of nephrolithiasis, including the antiviral drugs nelfinivir and foscarnet, the antibacterial agents ciprofloxacin, amoxicillin, and nitrofurantoin, and various products containing ephedrine, norephedrine, and pseudoephedrine (Daudon and Jungers 2004).

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Receptor- and Reactive Intermediate-Mediated Mechanisms of Teratogenesis

Peter G. Wells, Crystal J.J. Lee, Gordon P. McCallum, Julia Perstin, and Patricia A. Harper

Contents

Abstract Drugs and environmental chemicals can adversely alter the development of the fetus at critical periods during pregnancy, resulting in death, or in structural and functional birth defects in the surviving offspring. This process of teratogenesis may not be evident until a decade or more after birth. Postnatal functional abnormalities include deficits in brain function, a variety of metabolic diseases, and cancer. Due to the high degree of fetal cellular division and differentiation, and to differences from the adult in many biochemical pathways, the fetus is highly susceptible to teratogens, typically at low exposure levels that do not harm the mother. Insights into the mechanisms of teratogenesis come primarily from animal models and

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in vitro systems, and involve either receptor-mediated or reactive intermediate-mediated processes. Receptor-mediated mechanisms involving the reversible binding of xenobiotic substrates to a specific receptor are exemplified herein by the interaction of the environmental chemical 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or "dioxin") with the cytosolic aryl hydrocarbon receptor (AHR), which translocates to the nucleus and, in association with other proteins, binds to AH-responsive elements (AHREs) in numerous genes, initiating changes in gene transcription that can perturb development. Alternatively, many xenobiotics are bioactivated by fetal enzymes like the cytochromes P450 (CYPs) and prostaglandin H synthases (PHSs) to highly unstable electrophilic or free radical reactive intermediates. Electrophilic reactive intermediates can covalently (irreversibly) bind to and alter the function of essential cellular macromolecules (proteins, DNA), causing developmental anomalies. Free radical reactive intermediates can enhance the formation of reactive oxygen species (ROS), resulting in oxidative damage to cellular macromolecules and/or altered signal transduction. The teratogenicity of reactive intermediates is determined to a large extent by the balance among embryonic and fetal pathways of xenobiotic bioactivation, detoxification of the xenobiotic reactive intermediate, detoxification of ROS, and repair of oxidative macromolecular damage.

Keywords Aryl hydrocarbon receptor · Dioxin · Reactive intermediates · Oxidative stress · Teratogenesis

Abbreviations

AED	Antiepileptic drug
AHR	Aryl hydrocarbon receptor
AHRE	Aryl hydrocarbon responsive element
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATM	Ataxia telangiectasia mutated
ATRA	All-trans retinoic acid
B[a]P	Benzo[a]pyrene
CAR	Constitutive androstane receptor
CYP	Cytochrome P450
G6PD	Glucose-6-phosphate dehydrogenase
CSB	Cockayne Syndrome B
GSH	Glutathione
GST	Glutathione S-transferase
LPO	Lipoxygenase
NF-kB	Nuclear factor *kappa* B
OGG1	Oxoguanine glycosylase 1
PHS	Prostaglandin H synthase
PXR	Pregnane X receptor
RNS	Reactive nitrogen species

ROS Reactive oxygen species
RAR Retinoic acid receptor
SOD Superoxide dismutase
TCDD 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin ("dioxin")
UGT UDP-glucuronosyltransferase

1 Introduction

1.1 Embryonic and Fetal Development and Windows of Susceptibility

Teratogenesis refers to irreversible birth defects occurring in the developing embryo or fetus that persist after birth. Structural birth defects caused by drugs and environmental chemicals, collectively termed xenobiotics, are exemplified by the shortened or absent arms and or legs (phocomelia) that result from in utero exposure to the sedative drug thalidomide, and cleft lip/palate caused by the antiepileptic (AED) phenytoin. At least as important are functional birth defects, including cognitive and motor deficits in brain function, biochemical imprinting of permanently altered enzymatic activities, postnatal carcinogenesis, and an expanding range of metabolic disorders including obesity, diabetes, and cardiovascular disease (Wells et al. 2009). The role of xenobiotics in such functional birth defects is less well appreciated and constitutes a rapidly growing area of research. Some well-known examples include cognitive deficits and behavioral disorders in children who were exposed in utero to the AEDs, valproic acid and phenytoin, and to alcohol. The most severe cases of structural or functional birth defects can result in in utero or neonatal death. The range of potential adverse developmental consequences of in utero xenobiotic exposure is shown in Fig. 1. Although over 1,000 chemicals are teratogenic in animal models, only about 60 have been shown to adversely affect human development (Table 1). Such adverse developmental consequences are most often exhibited at lower xenobiotic doses or maternal plasma concentrations that have no adverse effects on the mother. This differential susceptibility is due in part to the extensive degree of cell division and differentiation that are unique to the embryo and fetus, along with the correspondingly unique sensitivity of developing structures and functions to teratological alterations. A broader review of the history of teratogenesis, principles of relevant developmental biology, and a comprehensive discussion of chemical teratogens is presented elsewhere (Rogers and Kavlock 2008).

Susceptibility to teratological effects is different from adverse drug effects in the adult, in that the risk changes during development from the fertilization of the egg and implantation of the blastocyst, and through the embryonic and fetal periods to birth (Fig. 2). This changing pattern of risk for a given developing structure or function is often referred to as a "window" of susceptibility, the gestational timing of which varies with both the target tissue and the xenobiotic.

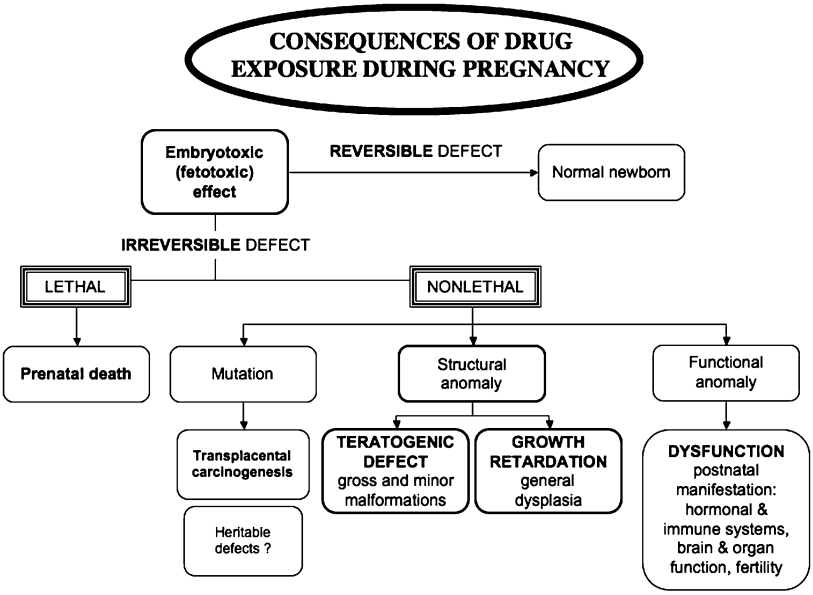

Fig. 1 Consequences of drug exposure during pregnancy (modified from Neubert et al. 1980)

In humans, xenobiotic toxicity in the first 2 weeks following fertilization results either in the death of the organism or no effect, since the cells have not begun to differentiate and there is a high degree of redundant backup. Exposure to toxic insult during the embryonic period, when organs are being formed (organogenesis), typically results in structural birth defects, while exposure during the later fetal period results in functional birth defects. However, there are exceptions to these generalizations, including xenobiotics that are poorly eliminated and accumulate in the embryo and fetus, and xenobiotics that cause permanent damage to cellular macromolecules like DNA, which can persist throughout development.

A final remarkable feature of teratogenesis is the dramatic species- and strain-dependent variability in susceptibility for some xenobiotics. For example, some strains of mice are sensitive to phenytoin teratogenicity, while other strains are resistant. In the case of thalidomide, primates (including humans) and rabbits are sensitive, while all rodent (mice and rats) strains tested are resistant. These differences must be considered in the design and interpretation of data from animal studies when directed towards an evaluation of human risk.

1.2 Mechanisms Involving Receptors and Reactive Intermediates

Xenobiotics typically adversely affect development in one of two general ways (Fig. 3). The first mechanism involves the reversible binding of a parent xenobiotic

Table 1 Human teratogens

Aminoglycosides
Androgenic hormones
Angiotensin converting enzyme inhibitors:
Captopril, enalapril
Angiotensin receptor antagonists:
Sartans
Anticonvulsants:
Carbamazepine, phenytoin,
Trimethadione, valproic acid
Busulfan
Carbon monoxide
Chlorambucil
Cocaine
Coumarins
Cyclophosphamide
Cytarabine
Diethylstilbestrol
Danazol
Egotamine
Ethanol
Ethylene oxide
Fluconazole
Folate antagonists:
Aminopterin, methotrexate
Iodides
Lead
Lithium
Mercury, organic
Methimazole
Methylene blue
Misoprostal
Penicillamine
Polychlorinated biphenyls
Quinine (high dose)
Retinoids:
Isotretinoin, etretinate, acitretin
Tetracyclines
Thalidomide
Tobacco smoke
Toluene
Vitamin A (high dose)

Extracted from Rogers and Kavlock (2008)

or stable metabolite to a specific receptor, such as the aryl hydrocarbon receptor (AHR) or the retinoic acid receptor (RAR), that activates a chain of events leading to altered gene transcription. The second mechanism involves the conversion or bioactivation of a relatively nontoxic parent xenobiotic to either an electrophilic or free radical reactive intermediate that respectively bind permanently (covalently) to cellular macromolecules like proteins and DNA, or react with molecular oxygen to form reactive oxygen species (ROS), which in turn can alter signal transduction or

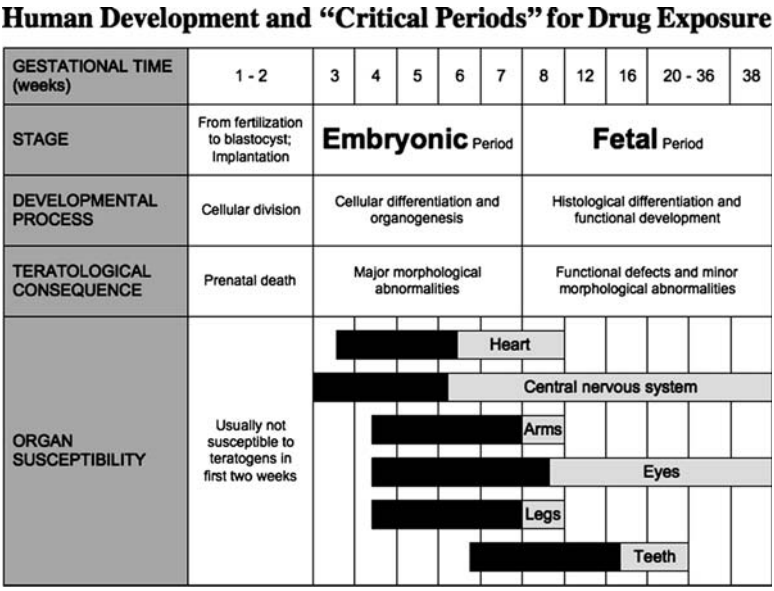

Fig. 2 Human development and critical periods for drug exposure (modified from Wells 2007)

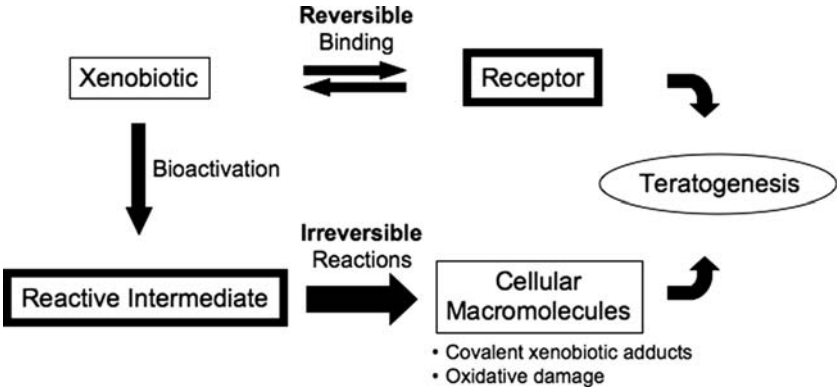

Fig. 3 Receptor versus reactive intermediate-mediated mechanisms of teratogenesis (modified from Wells and Winn 1996)

oxidatively damage cellular macromolecules such as lipids, protein, and DNA. Receptor-mediated mechanisms tend to result in predictable risks, since they require exposure to a toxic plasma concentration, if not dose, of the xenobiotic. In contrast, the risk due to reactive intermediate-mediated mechanisms can be unpredictable, since the possibility of a teratological outcome will depend to a large degree on the individual's balance among pathways that form the reactive intermediate, vs pathways that detoxify the xenobiotic reactive intermediate and/or

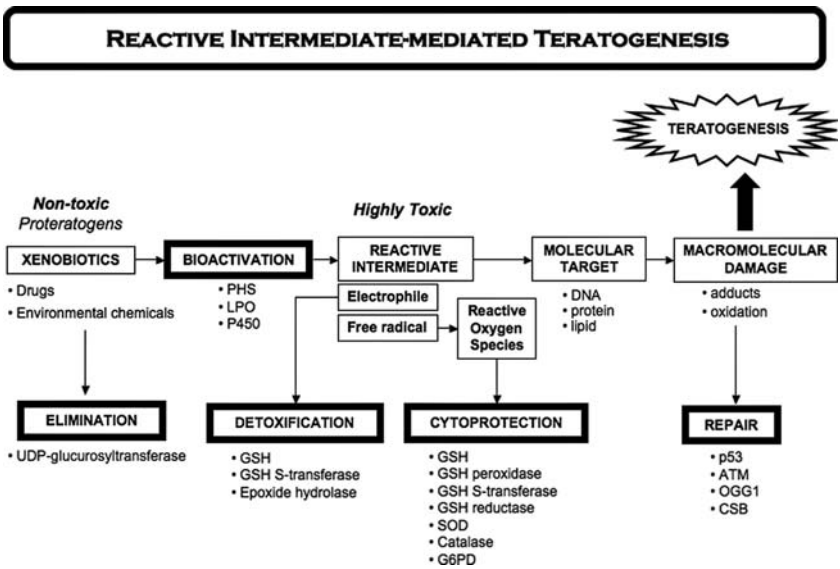

Fig. 4 Enzymatic pathways involved in reactive intermediate-mediated teratogenesis (modified from Wells and Winn 1996)

ROS, or repair the macromolecular damage (Fig. 4). If there is an imbalance favoring accumulation of the reactive intermediate and/or macromolecular damage, that individual can experience developmental toxicity at a therapeutic dose or concentration of a drug, or a normally safe level of exposure to an environmental chemical. Particularly for reactive intermediate-mediated mechanisms, most of the data are derived from animal studies, and very little has been confirmed in humans.

The ability of a xenobiotic to bind reversibly to a receptor does not preclude its being bioactivated to a reactive intermediate, or vice versa (Fig. 5). Similarly, for some birth defects, both mechanisms potentially could contribute to the same teratological outcome, and different mechanisms may predominate in different strains and species at different gestational times of pregnancy, or in different embryonic-fetal target tissues or cell types. These possibilities often confound a precise elucidation of the mechanism of teratogenesis for a given xenobiotic.

1.3 Drug Metabolism in the Mother Versus the Embryo and Fetus

Compared to the mother, the developing embryo and, to a somewhat lesser extent, the fetus have relatively low levels of most xenobiotic-metabolizing enzymes, including most cytochromes P450 (CYPs) that catalyze phase I reactions, phase II enzymes like the UDP-glucuronosyltransferases (UGTs) and, perhaps most

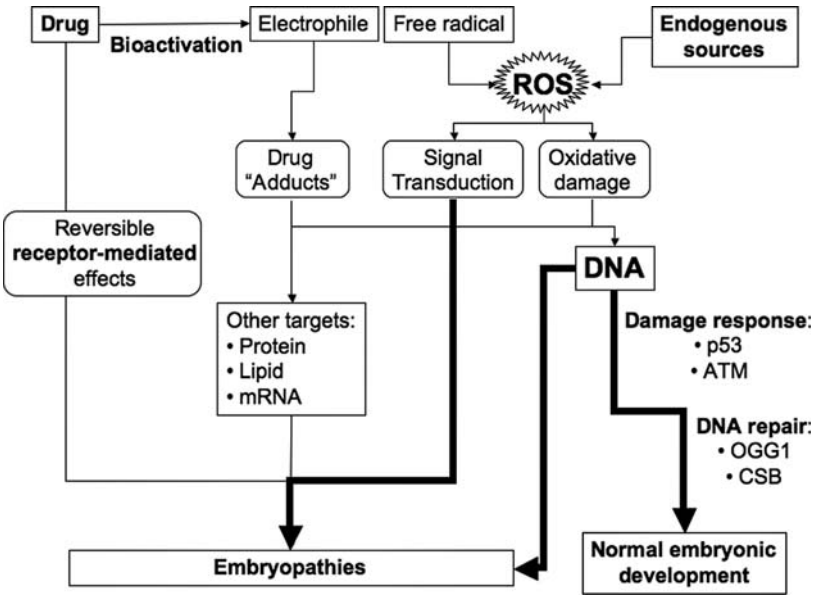

Fig. 5 Competing mechanisms potentially leading to teratogenesis. Changes in teratological outcomes resulting from modifying the pathways involved in DNA damage response and repair help distinguish the role of oxidative DNA damage from oxidative damage to other cellular macromolecules (protein, RNA, lipids, carbohydrates), as well as from ROS effects via signal transduction. Similarly, changes in teratological outcomes due to modifications in antioxidants and antioxidative enzymes help distinguish the role of ROS from mechanisms involving electrophilic xenobiotic reactive intermediates, and reversible, receptor-mediated interactions (from Wells et al. 2009)

importantly, critical detoxifying enzymes such as glutathione S-transferases (GSTs) and epoxide hydrolases (Fig. 4) (Hines 2008; Wells et al. 2009). The embryo and fetus also have very low levels of enzymes that catalyze the detoxification of ROS. Their low activity of most detoxifying enzymes leaves the embryo and fetus more susceptible than adults to reactive intermediate-mediated toxicities.

Maternal xenobiotic metabolism plays an important role in determining how much xenobiotic reaches the embryo and fetus, and hence can modulate developmental toxicity due to either receptor- or reactive intermediate-mediated mechanisms. For example, pregnant Gunn rats with low UGT activity exhibit decreased glucuronidation of benzo[a]pyrene metabolites and a three-fold increase in fetal resorptions (in utero death) (Wells et al. 2004).

In the case of reactive intermediate-mediated teratogenesis, the reactive intermediates and ROS are typically too unstable to be formed maternally and cross the placenta. Accordingly, maternal pathways do not contribute directly to this mechanism, whereas the activities of embryonic and fetal enzymes catalyzing xenobiotic bioactivation and the detoxification of xenobiotic reactive intermediates and ROS play a key role in determining the adverse developmental consequences of xenobiotic exposure (see Sect. 3 on Reactive intermediate-mediated mechanisms).

2 Receptor-Mediated Mechanisms

2.1 Introduction

The aryl hydrocarbon receptor (AHR) and retinoic acid receptor (RAR) are ligand-activated transcription factors that regulate gene transcription by binding to specific DNA sequences known as enhancers. Binding by endogenous or xenobiotic ligands to these receptors initiates a cascade of events resulting in changes in target gene transcription.

The RAR is a member of the nuclear receptor superfamily, which includes steroid hormone receptors, such as the estrogen and glucocorticoid receptors, as well as xenobiotic-activated receptors, such as the pregnane X receptor (PXR) or constitutive androstane receptor (CAR). The most well-known xenobiotic receptor is the AHR. Initially, the AHR was thought to be a member of the nuclear receptor superfamily because its mechanism of action is similar to that of the glucocorticoid receptor. However, extensive characterization at the molecular level has revealed that the AHR does not have the "zinc finger" DNA-binding domain characteristic of the receptors for steroid hormones and does not have any significant sequence similarity with the steroid hormone receptors. Rather, the ligand-binding subunit of the AHR is a member of the basic-helix-loop helix family of DNA-binding proteins.

Nuclear receptors play an essential role in embryonic morphogenesis and organogenesis and disruption of their function can have a catastrophic impact on the developing organism. Fetuses with targeted inactivation of RARs die in utero or at birth from severe developmental defects (Mark et al. 2006). Though not as severe, targeted inactivation of the AHR also has an adverse impact on fetal development with effects that are observed throughout the life of the animal (Gu et al. 2000).

Many xenobiotics, such as the environmental contaminant benzo[a]pyrene (B[a]P) found in cigarette smoke, or a therapeutic drug such as acitretin, a retinoic acid derivative used to treat acne, are agonists for nuclear receptors and can initiate receptor-mediated gene transcription. Gene expression during development is finely controlled, and exposure to a xenobiotic has the potential to disrupt the normal pattern of gene expression. Whether dysregulation of gene expression is deleterious depends on which genes are perturbed and when in the course of development they are perturbed.

2.2 Aryl Hydrocarbon Receptor (AHR)

The aryl hydrocarbon receptor (AHR), a soluble intracellular protein found in most if not all vertebrate cells and tissues, is the prototypical xenobiotic sensing receptor. The AHR is an essential component of an organism's adaptive response to xenobiotic exposure. Phylogenetic evidence indicates that the AHR may have evolved as a mechanism by which animals adapt to potential toxicants in plant food sources, but

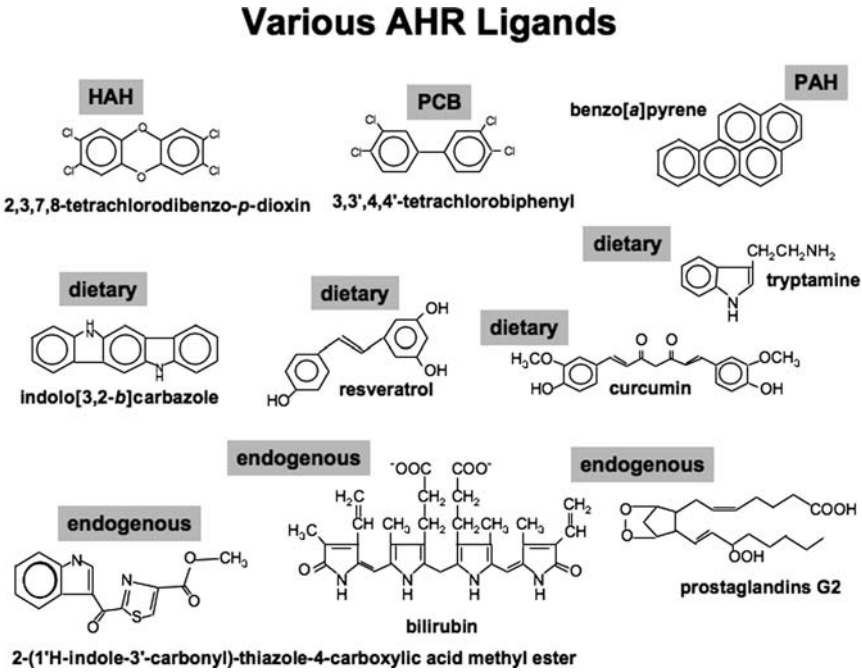

Fig. 6 Examples of ligands for the aryl hydrocarbon receptor (AHR)

it is now clear that the AHR is an important signaling pathway and plays key roles in mammalian development (Hahn 2002).

The AHR has a promiscuous ligand-binding site and is activated by a range of structurally diverse chemicals (Denison and Nagy 2003), many of which are found as environmental contaminants. The most potent ligand for the AHR is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, also known as TCDD or dioxin (Fig. 6). TCDD is used in experimental models because of its high potency and resistance to metabolism resulting in few if any effects not directly AHR-mediated. Whether or not a physiologic ligand exists for the AHR remains a matter of debate; however, there is convincing evidence that receptor activation by an endogenous ligand is essential for normal development (Nguyen and Bradfield 2008).

2.2.1 Outline of the AHR Regulatory Mechanism

Figure 7 shows the general AHR mechanism (Kawajiri and Fujii-Kuriyama 2007). Unliganded AHR is located in the cytoplasm complexed with various chaperone proteins. Ligand binding transforms the AHR, resulting in nuclear translocation, shedding of chaperone proteins, and dimerization with the aryl hydrocarbon receptor nuclear translocator (ARNT). As a transcription factor, the ligand-AHR-ARNT complex binds specific DNA sequences known as AH responsive elements (AHREs) typically located in the 5'-flanking region of target genes. This provides

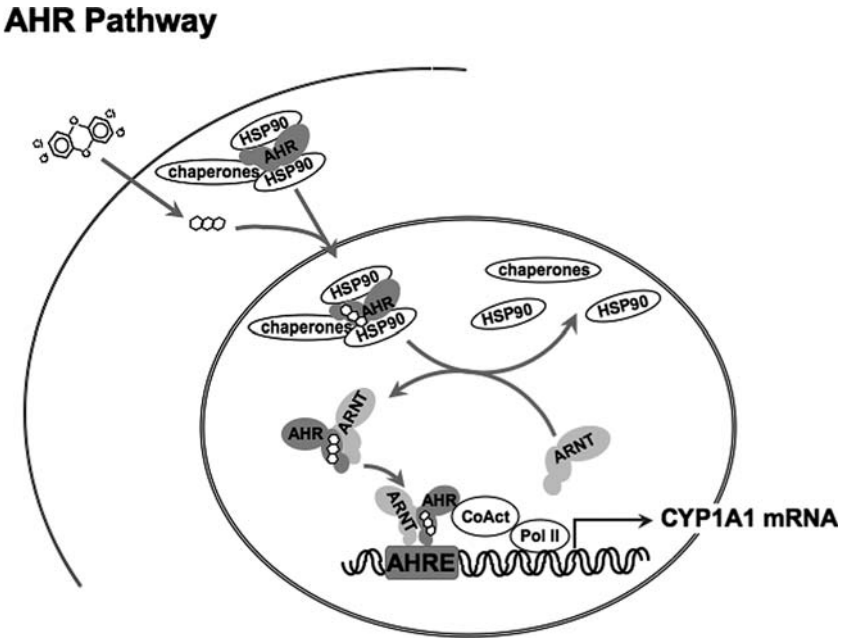

Fig. 7 The prototypical aryl hydrocarbon receptor (AHR) signaling pathway for the induction of AHR target gene expression. In the unliganded form, the AHR resides in the cytoplasm complexed with several chaperone proteins including HSP90 (heat shock protein 90). Ligand binding induces a conformational change in the AHR, revealing a nuclear localization signal that targets the AHR for nuclear translocation. In the nucleus, the AHR dimerizes with ARNT and the ligand-AHR-ARNT complex interacts with the regulatory regions of target genes at AHREs. AHR-DNA-binding recruits various coactivators to initiate the transcription of these target genes, the most characterized being those that encode xenobiotic metabolizing enzymes such as CYP1

a platform for recruiting multiple co-activator proteins that enhance (or, in some cases, inhibit) gene transcription. In either event, the outcome is a change in gene transcription resulting in a change in biological response.

2.2.2 Evidence That the AHR Plays a Role in Toxicity

Initial evidence that the AHR played a role in the toxicity response came from pharmacological and pharmacogenetic studies. Studies of quantitative structure–activity relationships clearly demonstrate that the binding affinity of a xenobiotic to the receptor correlates with its potency as an inducer of drug-metabolizing enzymes such as the CYPs, and hence as an inducer of toxic metabolites. Within a particular series of halogenated aromatic hydrocarbons, e.g. polychlorinated dioxins, the CYPlAl induction potency is correlated with the affinity with which the congeners bind to the AH receptor (Safe 1986). In mice, the AHR is encoded by four alleles. Greater susceptibility to a wide variety of toxic effects of TCDD in mice segregates with the Ahr^{b1} allele that encodes a 'high affinity' form of AHR as compared with

mice that express the *Ahr*^{d} allele. The *Ahr*^{d} allele possess a mutation in the ligand binding domain resulting in a much lower affinity for TCDD and hence less AHR activation at any given concentration (Ema et al. 1994).

Our understanding of the role of the AHR in toxicity has been more clearly defined as sophisticated techniques to dissect proteins at the molecular level have been developed. The most blunt approach is to create mice that express an inactivating mutation of the AHR. Typically, this involves the creation of knockout mice where the *Ahr* gene is deleted from mouse genome (Lahvis and Bradfield 1998). Not only are AHR-knockout mice insensitive to most if not all aspects of TCDD toxicity including teratogenesis, they also display several developmental abnormalities consistent with a role of the AHR in development. The mechanism of AHR-mediated toxicity can be more clearly defined if one more finely dissects the protein, allowing creation of mice expressing specific truncations of the AHR (Table 2). Inactivating mutations targeted to specific functions of the receptor reveal the importance of receptor activation along several steps of the pathway.

2.2.3 Consequences Arising from Actions Mediated by the AH Receptor

Consequences of AHR activation may be divided into two distinct pathways: (1) a *developmental pathway* where the role of AHR in normal development has been inferred from the phenotypes arising from several *Ahr*-deficient models (Table 3); and (2) an *adaptive pathway* resulting in altered xenobiotic metabolism and elimination. For the most part, the adaptive pathway is protective of the organism,

Table 2 Phenotypes arising from aryl hydrocarbon receptor (AHR)-deficient models

Gene	Mutation	Lost function	Outcome	References
Ahr$^{-/-}$	Knockout	AHR protein expression	No teratogenesis Aberrant development	Lahvis and Bradfield (1998)
Ahr^{d}	Natural polymorph	Ligand-binding to AHR	Decreased incidence	Couture et al. (1990)
Ahr^{nls}	Deleted NLS signal	Nuclear translocation Aberrant development	No teratogenesis Aberrant development	Bunger et al. (2003)
Arnt^{fxneo}	Conditional ARNT deletion	Hypomorphic ARNT expression AHR-null phenocopy	No toxicity Aberrant development	Walisser et al. 2004)
Ahr^{dbd}	Deleted DNA-binding domain	AHRE-binding AHR-null phenocopy	No teratogenesis Aberrant development	Bunger et al. (2008)
Ara9^{fxneo}	Conditional ARA9 deletion	Hypomorphic ARA9 expression AHR-null phenocopy	Teratogenesis not tested Aberrant development	Lin et al. (2008)

Table 3 Role of the AHR in development from *Ahr* gene deletion studies

Species	Target tissue	References
C. elegans	Neuronal fate	Qin and Powell-Coffman (2004)
D. melanogaster	Antenna:tarsus Specialization Color vision Dendrite arborization	Duncan et al. (1998) Wernet et al. (2006) Kim et al. (2006)
M. musculus	Various tissues	Fernandez-Salguero et al. (1995) Schmidt et al. (1996) Mimura et al. (1997) Hernandez-Ochoa et al. (2009)

providing a robust mechanism for the detoxification and elimination of harmful chemicals (Nebert et al. 2004).

Perturbation of AHR activity in either pathway can have a deleterious impact. During development, exposure to xenobiotics that are ligands for the AHR will activate the AHR and disrupt the normal pattern of AHR-regulated gene expression resulting in teratogenesis. Alternatively, as a vital component of the adaptive pathway, induction of xenobiotic metabolizing enzymes often increases the production of reactive intermediates beyond the detoxification capacity of conjugating and exporting enzymes. In some instances, the reactive intermediate is necessary for the desired pharmacological effect of a drug, but not usually, and such reactive intermediates may accumulate and cause macromolecular damage.

2.2.4 The AHR Regulates Pathways of Toxicity Involving Metabolism of the Ligand

The classical and best-characterized AHR function is regulation of CYP1A1, a cytochrome P450 enzyme that is highly inducible in virtually all tissues of most mammalian species. CYP1A1 is toxicologically important because it converts environmental polycyclic aromatic hydrocarbons into metabolites that are highly mutagenic or carcinogenic, as well as teratogenic. Many chemicals that are substrates for CYP1A1 (e.g., B[a]P) are also ligands for the AHR; these agents stimulate their own metabolism by inducing CYP1A1 as illustrated in Fig. 8. It is often these metabolites, as distinct from AHR activation, that are ultimately teratogenic via pathways outlined in the following section on reactive intermediate-mediated mechanisms.

The AHR regulates three main additional CYP enzymes and new ones are still being discovered (Jonsson et al. 2007): CYP1A2 activates procarcinogenic aromatic amines and heterocyclic amines; CYP1B1 bioactivates structurally diverse procarcinogens including endogenous estrogens; and CYP2S1 metabolizes retinoids, important in cell differentiation. In addition to regulating "phase I" enzymes (CYPs) the AHR also regulates multiple phase II conjugating enzymes such as the UDP-glucuronosyltransferases UGT1A1 and UGT1A6, aldehyde dehydrogenase-3, and some forms of glutathione *S*-transferase. Most recently, aldehyde oxidases were shown to

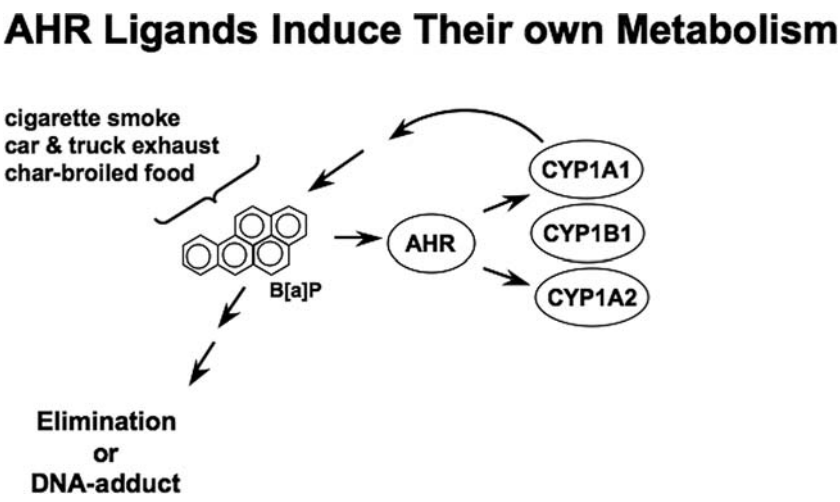

Fig. 8 Ligands of the aryl hydrocarbon receptor (AHR), exemplified by the environmental chemical benzo[a]pyrene (B[a]P), induce their own metabolism by cytochromes P450 (CYPs), resulting in their enhanced elimination or bioactivation to potentially toxic reactive intermediates

be induced by TCDD via an AHR-dependent pathway; these enzymes are important in the metabolism of retinoids, heterocyclic carcinogens, and antineoplastic agents.

2.2.5 The AHR Regulates Pathways of Toxicity Not Involving Metabolism of the Ligand

Enzyme induction mediated by the AHR has both metabolic and toxicological consequences as outlined above and depicted in Fig. 8. However, a major complexity in examining AHR-mediated toxicity is that induction of phase I and phase II enzymes regulated by the AHR does not account for the toxicity of xenobiotic substrates that do not form reactive metabolites, such as TCDD. It is the parent compound, working through the AHR, that is toxic.

Studies of changes in global gene expression by microarray analysis of mRNA expression indicate that the expression of many hundreds of genes respond both positively and negatively to AHR signaling (Boutros et al. 2008). Furthermore, detailed analyses indicate a high degree of cross-talk between the AHR pathway and other signaling pathways (Frericks et al. 2008). There are many candidate pathways (Puga et al. 2009) and proposed crosstalk mechanisms include competition for co-factors, direct protein interactions, and competition for DNA binding sites (Table 4). While AHR activation is the essential first step in the pathogenesis of the teratogenic response, the developmental pathways that are disrupted by TCDD have yet to be identified with certainty.

The induction of CYP1A1, the prototypical target gene transcriptionally regulated by the AHR, together with other CYP1 enzymes accompanies toxicity in

Table 4 AHR crosstalk with other signaling pathways

Pathway	Interaction	References
WNT	AHR → WNT	Mathew et al. (2009)
	WNT → AHR	Chesire et al. (2004)
ER	AHR → ER	Khan et al. (2006)
	ARNT → ER	Ruegg et al. (2008)
	ER → AHR	Matthews and Gustafsson (2006)
EGFR	AHR → EGFR	Choi et al. (2006); Patel et al. (2006)
TGFβ	AHR → TGFβ	Chang et al. (2007)
	TGFβ → AHR	Wolff et al. (2001)
NRF2	AHR → NRF2	Miao et al. (2005)
	NRF2 → AHR	Shin et al. (2007)
HIF1α	AHR → Hif1α	Chan et al. (1999); Zhang and Walker (2007)
	Hif1α → AHR	Khan et al. (2007)
RAR	AHR → RAR	Murphy et al. (2007)
TR	AHR → TR	Nishimura et al. (2005)

animals exposed to TCDD. Such an increase in CYP1 enzymes can result in increased levels of reactive oxygen-mediated oxidative stress from metabolism of endogenous substrates or xenobiotics (Reichard et al. 2005). In this regard, TCDD is extremely potent since it is not extensively metabolized by CYP1 resulting in persistent AHR-activation and chronic oxidative stress (Shertzer et al. 1998). Whether or not AHR-mediated toxicity is the result of reactive oxygen-mediated oxidative stress has not been unequivocally determined. *Ahr*-null mice, which are resistant to the toxic effects of TCDD, do not exhibit induction of CYP1 enzymes when treated with TCDD (Fernandez-Salguero et al. 1995; Lin et al. 2001; Tijet et al. 2006), whereas in *Ah*r+/+ mice, sustained AHR-activation by TCDD induces hypertension and cardiac hypertrophy accompanied by increased production of reactive oxygen species (Kopf et al. 2008). Further, recent experiments in which *Cyp1a1* or *Cyp1a2* have been deleted in mice show that these mice are highly resistant to TCDD-induced thymic atrophy, hepatic toxicity, wasting, and lethality (Uno et al. 2004). The *Cyp1a1*-null and *Cyp1a2*-null experiments in mice indicate that these P450s make a significant contribution to dioxin toxicity in rodents. However, their upregulation alone is not sufficient to cause the full spectrum of toxicity. In utero exposure to TCDD together with antioxidants (vitamin E succinate or ellagic acid) significantly reduced some fetotoxic effects such as fetal growth retardation or fetal death, but had no impact on other teratogenic endpoints such as cleft palate and hydronephrosis of the kidney (Hassoun et al. 1997). An extensive structure:activity study shows clearly that more than half the chemical agents that induce CYP1A1 (including several therapeutic agents) do not produce TCDD-like toxicity in rodents or in humans (Hu et al. 2007). Therefore, upregulation of CYP1A1 or CYP1A2 and oxidative stress influences some forms of dioxin toxicity, but are not sufficient to cause TCDD-like toxic responses. Some genes under AHR control, in addition to CYP1A1 or CYP1A2, must be dysregulated in order to provoke major TCDD toxicities.

2.3 AHR-Mediated Teratogenesis

In as much as the AHR is important for normal development, proper regulation of the AHR itself is vital for its ability to regulate such development (Harper et al. 2006). Activating the AHR above physiological levels by means of exposure to a potent agonist affects the developing embryo of several species. These teratogenic events occur through exposure to exogenous ligands, such as TCDD, which act via the AHR to produce teratogenesis. Thus, TCDD-induced teratogenesis requires the AHR, and the first step in the mechanism of such teratogenesis is ligand activation of the AHR. In developing mice, prenatal exposure to the most potent AHR ligand, TCDD, results in teratogenesis at doses below those that cause maternal or embryo/fetal toxicity (Birnbaum and Tuomisto 2000). Such teratogenic outcomes include thymic involution, cleft palate, hydronephrosis (Couture et al. 1990), aberrant cardiac development (Thackaberry et al. 2005), and inhibition of ventral, dorsolateral, and anterior prostatic bud development (Vezina et al. 2009). Thymic involution and a kidney phenotype is also observed in mice harboring a mutant AHR that is active in the absence of ligand, further demonstrating the importance of proper AHR regulation in development (Brunnberg et al. 2006). Evidence suggests that TCDD may also be teratogenic in humans where tooth development appears to be adversely affected (Alaluusua and Lukinmaa 2006). The ability of TCDD to disrupt normal development and act as a multi-organ teratogen is a universal phenomenon occurring in several mammalian and nonmammalian species (Table 5).

Most if not all of the toxic effects produced by TCDD are mediated by the AHR. The same holds true for the teratogenic effects of TCDD, in that the AHR is critical for the occurrence of TCDD-initiated teratogenesis. In mice, evidence for this conclusion first came from studies using the AHR nonresponsive mouse strain, DBA/2. This strain of mice expresses an AHR encoded by the Ahr^{d} allele that binds TCDD with low affinity, whereas the AHR responsive mouse strain, C57BL/6, expresses a high-affinity receptor encoded by the Ahr^{b1} allele (Okey et al. 1989; Poland et al. 1994). When compared to C57BL/6J fetuses, DBA/2J fetuses exposed to TCDD develop cleft palate and hydronephrosis to a much lower extent at a given dose. Studies show that TCDD must be given at doses at least 2–3 times higher in DBA/2J mice than in C57BL/6J mice to elicit the same effects (Hassoun and Stohs 1996). A similar effect of AHR genotype was observed for TCDD-initiated reduction of fetal thymic weight. Thus, the affinity of the AHR for TCDD plays a significant role in mediating the teratogenic effects of TCDD.

Table 5 AHR-mediated teratogenesis (from exposure to TCDD)

Species	Target tissue	References
Rodent (mouse, rat, guinea pig, hamster)	Palate, heart, kidney, thymus	Couture et al. (1990); Kransler et al. (2007)
Fish	Heart, vascular tissue, craniofacial	Hahn (2001)
Chicken	Heart, brain	Ivnitski-Steele et al. (2004)
Human	Skin, tooth	Alaluusua and Lukinmaa (2006)

The AHR is not merely important in mediating TCDD-initiated teratogenesis, but necessary. Studies using $Ahr^{-/-}$ mice unequivocally demonstrate this requirement for the AHR. For example, fetuses from $Ahr^{-/-}$ dams exposed to a high dose of TCDD, display minimal incidences of cleft palate and hydronephrosis when compared to $Ahr^{+/+}$ fetuses (Lin et al. 2001; Mimura et al. 1997; Peters et al. 1999). Therefore, AHR nonresponsive mouse strains and AHR-deficient mice do not display the characteristic developmental abnormalities caused by TCDD exposure to the same extent as observed in AHR-responsive strains, indicating the requirement of TCDD activation of the AHR in teratogenesis.

The role of the AHR as a transcription factor has mechanistic significance in teratogenesis. Mice expressing an AHR protein deficient in the nuclear localization signal ($Ahr^{nls/nls}$) were resistant to TCDD-initiated teratogenesis (Bunger et al. 2003). Thus, the mechanistic role of the AHR in mediating both normal development and teratogenesis shares the same steps as the mechanism leading to AHR-mediated transactivation of gene expression. In TCDD-initiated teratogenesis, the AHR is thought to dysregulate the expression of developmentally important genes so that they are inappropriately expressed or downregulated at an inappropriate time. In models of TCDD-initiated teratogenicity of the mouse heart (Thackaberry et al. 2005) and zebrafish heart (Handley-Goldstone et al. 2005), many genes respond transcriptionally to exposure to TCDD. For example, in the mouse, these genes include those involved in regulating the cell cycle, cell growth, and embryonic development (including genes with known roles in cardiovascular development), as well as numerous genes encoding transcription factors, transporters and ion channels. Furthermore, studies have shown that the AHR directly modulates the expression of developmentally important genes at the transcriptional level, such as transforming growth factor-alpha (Choi et al. 1991; Davis et al. 2003; Gaido et al. 1992), epiregulin (Patel et al. 2006), amphiregulin (Choi et al. 2006), and prostaglandin-endoperoxide synthase 2, also known as prostaglandin H synthase (PHS) (Kraemer et al. 1996). Together, the literature demonstrates that TCDD-initiated teratogenicity is mediated via the AHR and suggests that the mechanism of action largely involves the transcriptional activity of the AHR.

2.4 Nuclear Receptor Superfamily

Historically, the AHR is the prototypical xenobiotic-activated transcription factor, but certain members of the nuclear receptor superfamily may also be classified as xenobiotic receptors. The nuclear receptor superfamily is a family of structurally similar ligand-activated transcription factors, which play important roles in many physiological and pathological processes (Lanz et al. 2006). Thus far, 48 individual nuclear receptor genes (not including the AHR) have been identified in the human genome with the potential for more to be discovered (Robinson-Rechavi et al. 2001). This large family of proteins includes not only the familiar hormone receptors such as the estrogen receptor, glucocorticoid receptor, mineralocorticoid

receptor, progesterone receptor, androgen receptor, thyroid hormone receptor, vitamin D receptor, and retinoic acid receptor, but also less well-known receptors such as peroxisome proliferator activated receptor, oxysterol receptor, farnesoid receptor, pregnane x receptor, and constitutive androstane receptor (Pascussi et al. 2008).

The mechanism of transcriptional regulation by these nuclear receptors is similar to that of the AHR. Typically, binding of ligand to the receptor promotes dimerization and binding to DNA at regulatory sites in the genome (enhancers), followed by recruitment of chromatin remodeling agents and transcriptional co-factors that regulate mRNA synthesis at the promoters of target genes. Analogous to the AHR, the biological impact of receptor activation is through changes, both positive and negative, in expression of a multitude of genes (Kininis and Kraus 2008).

Two important members of the nuclear receptor superfamily are CAR and PXR. These receptors were initially identified as xenobiotic receptors because ligands included xenobiotics such as pesticides (dichlorodiphenyltrichloroethane, DDT) and therapeutic drugs (phenobarbital and rifampin). CAR and PXR are now known to play an integral role in lipid homeostasis (Kakizaki et al. 2008; Kretschmer and Baldwin 2005; Moreau et al. 2008). Analogous to the AHR, CAR and PXR regulate numerous genes involved in drug metabolism and transport, and it is this activity that is often the basis for receptor-mediated toxicity. CAR and PXR regulate the expression of cytochromes CYP3A and CYP2B, which are involved in the metabolism of steroid hormones. Thus, the CAR agonists, phenobarbital or phenytoin, taken in pregnancy may effectively reduce the levels of circulating hormone resulting in teratogenic endocrine disruption in the fetus (Kretschmer and Baldwin 2005).

Teratogenesis may also occur when a xenobiotic hijacks the normal physiological function of a nuclear receptor during development. The retinoic acid receptors (RARα, RARβ, and RARγ) play a fundamental role in embryonic morphogenesis and organogenesis as well as cell growth arrest, differentiation, and apoptosis in adult life (Mark et al. 2006). All-trans retinoic acid (ATRA) is a potent ligand for the RARs and may be used clinically to treat acne or psoriasis. However, ATRA at pharmacological levels is teratogenic during early organogenesis, leading to craniofacial, cardiac, thymic, and central nervous system structures defects similar to those seen in animal studies (Collins and Mao 1999). In this instance, toxicity arises from the dysregulation of RAR activity resulting in the inappropriate expression of RAR target genes during critical developmental windows.

Recently, the inappropriate activation of nuclear receptors has gained much attention in the context of endocrine disruptors. Environmental contaminants such as bisphenol A (BPA), primarily used to make plastics or phthalate esters added to plastics to increase flexibility, are ligands for the estrogen receptor (Blair et al. 2000; Matthews et al. 2001). There is concern and controversy that current exposure levels could have a detrimental effect on in utero development (NTP 2008; vom Saal et al. 2007).

3 Reactive Intermediate-Mediated Mechanisms

3.1 Introduction

Many xenobiotics can be enzymatically bioactivated by enzymes like the cytochromes P450 (CYPs) and prostaglandin H synthases (PHSs) to either electrophilic reactive intermediates that covalently bind to cellular macromolecules, or free radical reactive intermediates that react with molecular oxygen to produce ROS, which can alter signal transduction and/or oxidatively damage cellular macromolecules (lipids, proteins, DNA, RNA, etc.; Figs. 4 and 5). Although different enzymes may be involved in both the formation and detoxification of electrophilic reactive intermediates and ROS, the risk of adverse developmental outcomes is determined in both cases by the balance among embryonic-fetal pathways for reactive intermediate formation (bioactivation) versus pathways for the detoxification of electrophilic reactive intermediates and ROS, and repair of macromolecular damage.

3.2 Electrophilic Reactive Intermediates

Bioactivation to an electrophilic reactive intermediate, catalyzed primarily by CYPs (Fig. 9), and in some cases by other enzymes such as the PHSs, has been implicated in the mechanism of teratogenicity for a number of xenobiotics, including phenytoin, benzo[a]pyrene, and the anticancer drug cyclophosphamide (Juchau 1981, 1989; Juchau et al. 1998; Wells et al. 1997, 2009; Winn and Wells 1995). Reactive intermediates like epoxides contain an electron-deficient (electrophilic), a relatively positively charged center that reacts with electron-rich (nucleophilic) groups such as sulfhydryl groups on proteins or similar nucleophilic centers on nucleic acids, forming a covalent (irreversible) bond, and a permanent xenobiotic-protein or xenobiotic-DNA adduct. Although much covalent binding is without affect, a small proportion of the xenobiotic may form an adduct at a critical region of a developmentally important protein or gene, resulting in altered development or death of the embryo-fetus. In adults, the higher activities of detoxifying enzymes usually balance the formation of the reactive intermediate with no adverse consequences. In contrast, the embryo-fetus has relatively low levels of detoxifying enzymes like epoxide hydrolases and glutathione (GSH) S-transferases (GSTs), which could contribute to the typically increased susceptibility in the embryo-fetus compared with the mother. The embryonic activity of most CYPs, as distinct from their mRNAs, is low to negligible (Hines 2008), and their role in xenobiotic bioactivation remains unclear, although CYP1A1/2 have been shown to exhibit embryopathically relevant bioactivation in embryo culture for xenobiotics such as benzo[a]pyrene and 2-acetylaminofluorene (Juchau et al. 1992). In humans, CYP3A5 and CYP2C19 are expressed throughout pregnancy (Hines 2008), and therefore might play a role in teratogen bioactivation. Human CYP-dependent

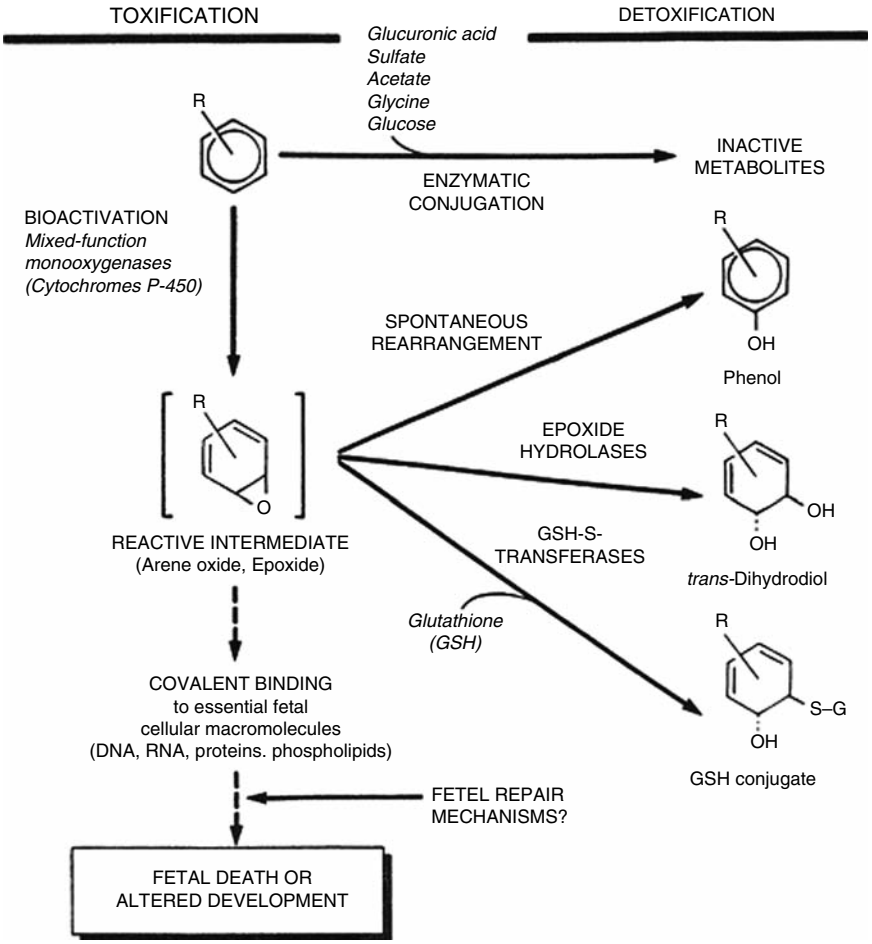

Fig. 9 Formation and detoxification on an electrophilic reactive intermediate (modified from Wells 2007)

bioactivation has been implicated but not proven in the fetal hydantoin syndrome caused by in utero exposure to phenytoin (Wells and Winn 2009). The activities of some CYPs increase during the later fetal period and could contribute to functional teratogenesis. In contrast, there are some developmentally-expressed CYPs, such as CYP 1B1/2 in rodents and CYP3A7 in humans, with high activity in the embryo-fetus that decline rapidly at birth (Hines 2008; Juchau et al. 1992). Such embryonic-fetal CYPs could play an important role in the bioactivation of teratogens, such as benzo[a]pyrene and aflatoxin B1 in the case of CYP3A7 (Wells and Winn 2009). Other enzymes with high embryonic-fetal activity, such as PHSs, can bioactivate xenobiotics, such as benzo[a]pyrene, to electrophilic reactive intermediates (e.g., epoxides) either directly or via the formation of epoxidizing peroxyl radicals (Wells et al. 1997; Wells and Winn 2009), which could contribute to teratogenesis.

3.3 *Free Radical Reactive Intermediates*

3.3.1 Bioactivation to Free Radical Intermediates

Bioactivation to a free radical intermediate has been implicated in the teratological mechanism for a number of xenobiotics, including phenytoin and structurally-related AEDs, benzo[a]pyrene, thalidomide, methamphetamine, valproic acid, and cyclophosphamide (Fantel 1996; Wells et al. 2009; Wells and Winn 1996). Unlike in the case of most CYPs, the embryo-fetus has relatively high activities of PHSs and lipoxygenases (LPOs), which via intrinsic or associated hydroperoxidase activity can oxidize xenobiotics to free radical intermediates (Fig. 10) (Wells et al. 2009). These xenobiotic free radical intermediates can in some cases react with double bonds in cellular macromolecules to form covalent adducts, or more often react directly or indirectly with molecular oxygen to initiate the formation of potentially teratogenic reactive oxygen species (ROS).

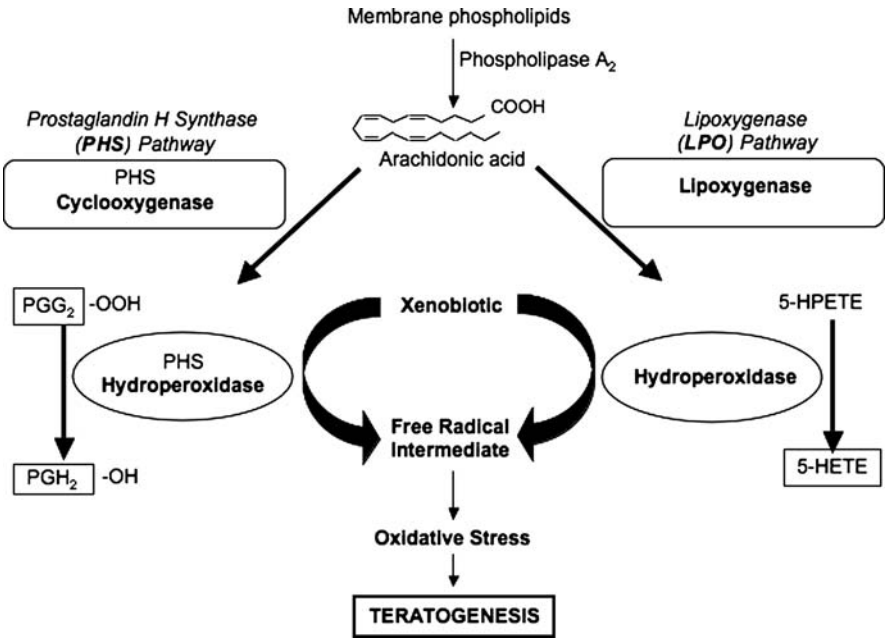

Fig. 10 Bioactivation of xenobiotics via the prostaglandin H synthase (PHS) and lipoxygenase (LPO) pathways-postulated role in teratogenesis. The hydroperoxidase component of embryonic and fetal PHSs, and hydroperoxidases associated with LPOs, can oxidize xenobiotics to free radical intermediates that initiate the formation of reactive oxygen species causing oxidative stress (modified from Yu and Wells 1995)

3.3.2 Oxidative Stress and Reactive Oxygen Species (ROS)

Oxidative stress occurs when the production of cellular oxidants, or ROS, exceeds the antioxidative capacity of the cell (Halliwell 2007; Halliwell and Gutteridge 1999, 2007; Sies 1985). Following a variety of exogenous exposures, ROS can be generated in the embryo-fetus via several mechanisms, including the enzymatic bioactivation of xenobiotics, redox cycling of catechol molecules, reperfusion following cardiosuppression, and exposure to ionizing radiation (Fig. 11) (Halliwell and Gutteridge 1999, 2007; Wells et al. 2009). Superoxide generated via one of these mechanisms is converted by superoxide dismutase (SOD) to hydrogen peroxide, which can react with superoxide and iron to produce ultra-reactive hydroxyl radicals. ROS can affect development by altering signal transduction and/or causing oxidative damage to cellular macromolecules like lipids, protein, and DNA (Wells et al. 2009).

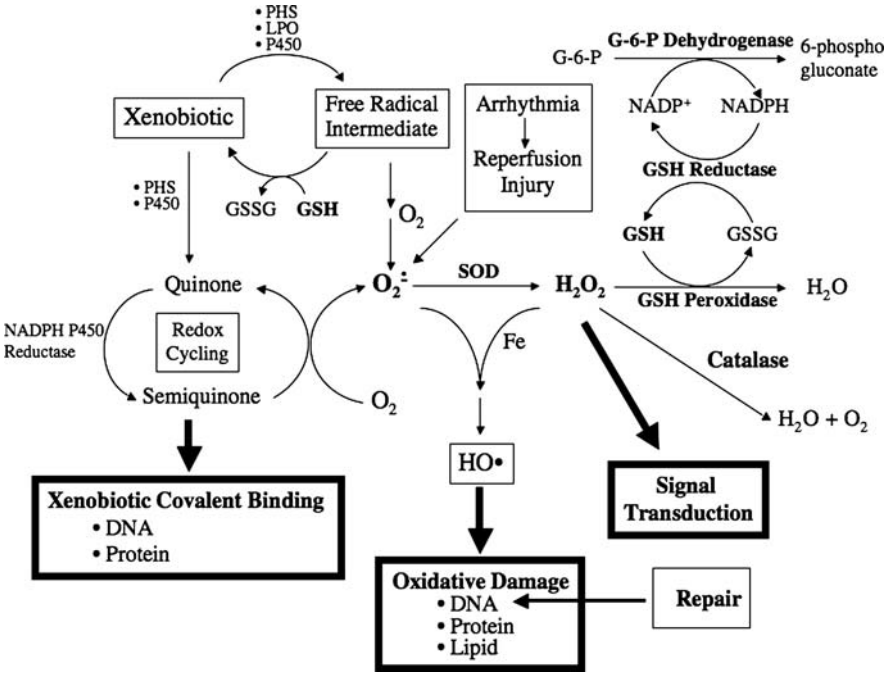

Fig. 11 Biochemical pathways for the formation, detoxification, and cellular effects of xenobiotic free radical intermediates and reactive oxygen species (ROS). *Fe* iron, *G-6-P* glucose-6-phosphate, *GSH* glutathione, *GSSG* glutathione disulfide, H_2O_2 hydrogen peroxide, $HO^{\cdot}$ hydroxyl radical, *LPO* lipoxygenase, $NADP^+$ nicotinamide adenine dinucleotide phosphate, $O_2^{\bullet-}$ superoxide, *P450* cytochromes P450, *PHS* prostaglandin H synthase, *SOD* superoxide dismutase. (Modified from Wells et al. 1997)

3.3.3 Reactive Nitrogen Species (RNS)

Although little is known about the teratological relevance of reactive nitrogen species (RNS), they might be expected to alter development indirectly by reacting with ROS, or directly by altering signal transduction and/or causing macromolecular damage (Fig. 12). RNS appear to contribute at least in part to the mechanism of phenytoin teratogenicity in mice (Wells et al. 2009).

3.3.4 Oxidative Damage to Cellular Macromolecules

ROS can initiate embryonic-fetal lipid peroxidation and oxidatively damage proteins and DNA, all or any one of which could adversely affect embryonic-fetal

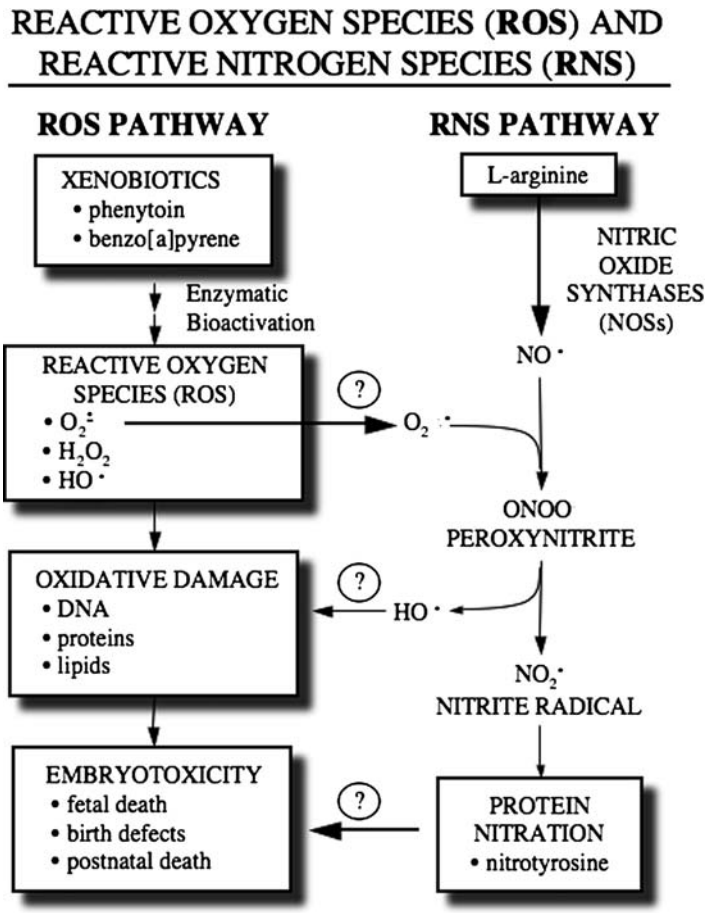

Fig. 12 Potential interactions between the pathways for reactive oxygen species (ROS) and reactive nitrogen species (RNS) (from Kasapinovic et al. 2004)

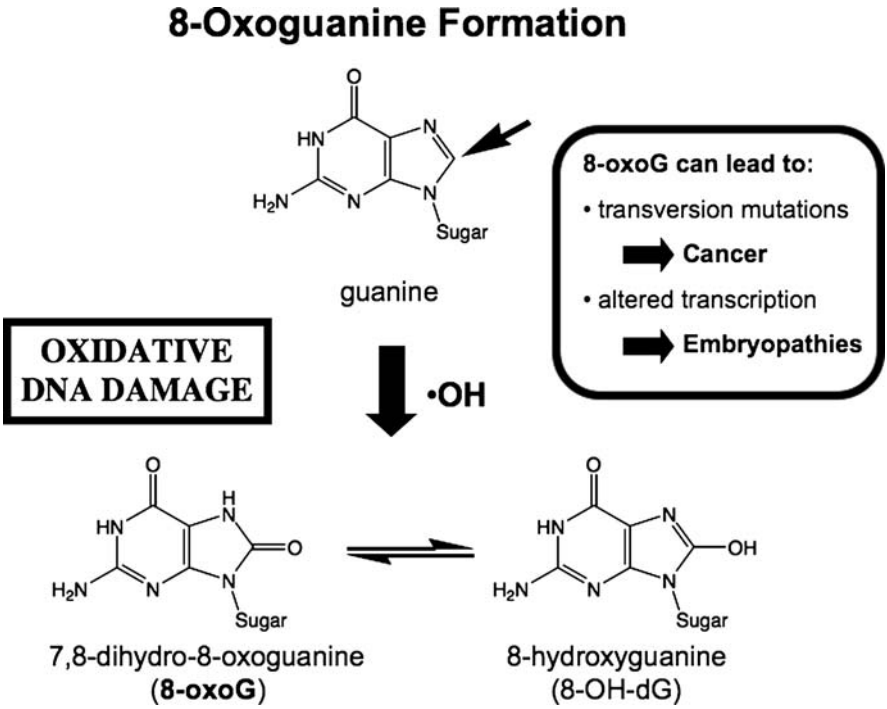

Fig. 13 Reaction of hydroxyl radicals (HO•) with guanine residues of DNA to form the molecular lesion 7-8,dihydro-8-oxoguanine (8-oxoguanine). If not repaired, this oxidative damage can cause mutations and/or altered gene transcription, which may lead to cancer and/or embryopathies (from Wells et al. 2009)

development (Fig. 11) (Wells et al. 1997, 2009). In the case of DNA, one of the most prevalent of about 20 different oxidative lesions formed by ROS is 8-oxoguanine (Fig. 13). This macromolecular lesion is known to be mutagenic, and recent evidence from DNA repair knockout mice (see Sect. 3.3.6 below) suggests that it also is teratogenic.

3.3.5 Antioxidants and Antioxidative Enzymes

In embryonic and fetal target tissues, antioxidative enzymes (Fig. 11) and antioxidants such as vitamin E in the membranes and vitamin C in the cytosol provide important cytoprotection against potentially embryopathic ROS. A reduction in the activity of any one of these antioxidative enzymes, either by dietary methods or by genetic manipulation, enhances susceptibility to ROS-initiating teratogens (Wells et al. 2009). This is despite the fact that the embryonic-fetal activity of most such enzymes, with the exception of glucose-6-phosphate dehydrogenase

(G6PD), are less than 5% of maternal levels. Conversely, protein therapy with a stabilized form of catalase will protect against the embryopathic effects of benzo [a]pyrene and phenytoin.

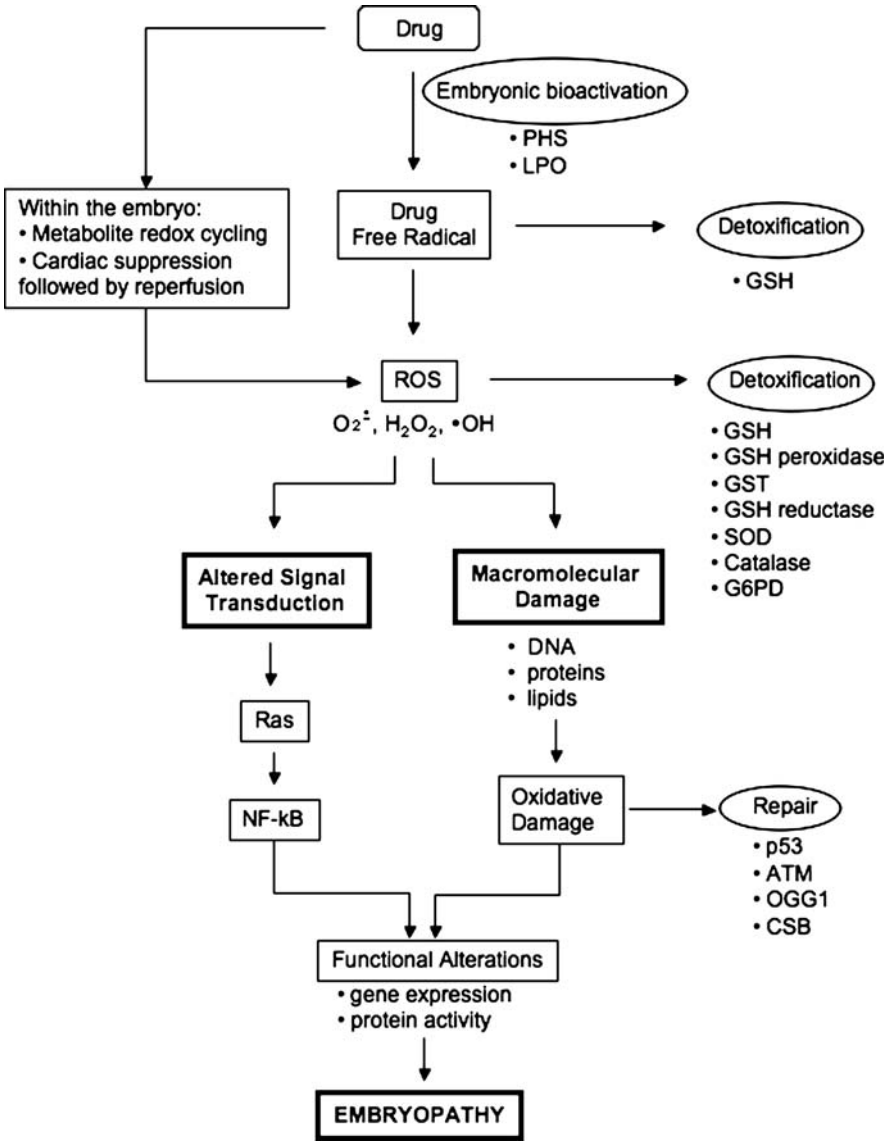

Fig. 14 Potential contribution of Ras and nuclear factor-kappa B (NF-kB) proteins in signal transduction pathways initiated by drug-enhanced formation of reactive oxygen species (ROS) (see Figs. 2 and 6 for remaining abbreviations) (modified from Kennedy et al. 2004)

3.3.6 Detection and Repair of Oxidative DNA Damage

The embryopathic role of oxidative DNA damage (Figs. 4 and 11) is exhibited in knockout mice lacking key proteins involved in the detection/response of DNA damage (p53, ataxia telangiectasia mutated [ATM]), and repair of oxidative DNA damage in particular (oxoguanine glycosylase 1 [OGG1], Cockayne syndrome B [CSB]) (Fig. 5), which are more sensitive to the embryopathic effects of benzo[a] pyrene, phenytoin, methamphetamine, and ionizing radiation (Wells et al. 2009). Conversely, these studies suggest that interindividual variability in the activities of such DNA damage response/repair pathways likely constitutes an important component of teratological risk.

3.3.7 ROS/RNS-Mediated Signal Transduction

Apart from causing oxidative macromolecular damage, ROS and RNS can alter signal transduction, with teratological consequences. This rapidly growing field of investigation is reviewed elsewhere (Hansen 2006; Hitchler and Domann 2007; Kovacic and Pozos 2006; Wells et al. 2009), and is only briefly exemplified here. Ras and nuclear factor kappa B (NF-kB) are transduction proteins commonly involved in ROS-mediated signaling pathways, and embryonic Ras activation and NF-kB expression are both increased by phenytoin (Wells et al. 2009). Pretreatment with a farnesyltransferase inhibitor, which blocks Ras activation, or inhibition of NF-kB expression with antisense oligonucleotides, both block the embryopathic effects of phenytoin, suggesting that ROS-mediated signal transduction, in addition to oxidative macromolecular damage, play an important role in the teratological mechanism (Fig. 14). Similarly for thalidomide, in addition to ROS-initiated macromolecular damage (Parman et al. 1999), ROS-mediated alterations in signaling involving GSH and the transduction proteins, NF-kB, Wnt, and Akt, have been implicated in the mechanism of teratogenesis (Hansen 2006; Knobloch and Rüther 2008).

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Part II
Mediators/Biochemical Pathways

Role of Reactive Metabolites in Drug-Induced Hepatotoxicity

A. Srivastava, J.L. Maggs, D.J. Antoine, D.P. Williams, D.A. Smith, and B.K. Park

Contents

Abstract Drugs are generally converted to biologically inactive forms and eliminated from the body, principally by hepatic metabolism. However, certain drugs undergo biotransformation to metabolites that can interfere with cellular functions through their intrinsic chemical reactivity towards glutathione, leading to thiol depletion, and functionally critical macromolecules, resulting in reversible modification, irreversible adduct formation, and irreversible loss of activity. There is now a great deal of evidence which shows that reactive metabolites are formed from drugs known to cause hepatotoxicity, such as acetaminophen, tamoxifen, isoniazid,

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and amodiaquine. The main theme of this article is to review the evidence for chemically reactive metabolites being initiating factors for the multiple downstream biological events culminating in toxicity. The major objectives are to understand those idiosyncratic hepatotoxicities thought to be caused by chemically reactive metabolites and to define the role of toxic metabolites.

Keywords Metabolic activation · Reactive metabolites · Hepatotoxicity

Abbreviations

ADRs	Adverse drug reactions
DILI	Drug-induced liver injury
APAP	Acetaminophen
NAPQI	N-acetyl-p-benzoquinoneimine
GSH	Glutathione
ARE	Antioxidant response element
CCl_4	Carbon tetrachloride
NVP	Nevirapine
INH	Isoniazid
AQ	Amodiaquine
AQQI	Amodiaquine quinoneimine
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ULN	Upper limit of normal

1 Introduction

Adverse drug reactions (ADRs) are a major cause of patient morbidity and a significant cause of patient mortality (Lazarou et al. 1998; Pirmohamed et al. 1998). There are many types of ADRs, affecting every organ in the body (Table 1). However, drug-induced liver injury (DILI) is the most frequent reason for the withdrawal of an approved drug from the market (Temple and Himmel 2002), and it accounts for approximately 50% of all acute liver failure cases (Kaplowitz 2001; Lee 2003; Ostapowicz 2002) and 15% of all liver transplantations in the United States (Russo et al. 2004).

Most drug-induced hepatotoxicities are unpredictable and poorly understood. The liver is the principal site of drug metabolism; therefore, it is often the target of toxicity. Typically they are idiosyncratic in nature. The manifestations range from mild, asymptomatic changes in serum transaminases, which are relatively common,

Table 1 Classification of adverse drug reactions on the basis of pathological effect (Liebler and Guengerich 2005)

Type	Definition	Examples
On-target or mechanism-related toxicity	Toxicity that occurs because of modulation of the primary pharmacological target (for example, receptor or enzyme), whether in the same cell/tissue or not	Statins
Off-target pharmacology	Adverse effects resulting from interaction of a drug with targets other than intended therapeutic targets	Terfenadine & hERG channel effects
Hypersensitivity & immunological reactions	Hypersensitivity refers to undesirable (damaging, discomfort-producing and sometimes fatal) reactions produced by the normal immune system	Penicillins
Bioactivation to reactive intermediates	The biological transformation of a drug to toxic metabolites, which often results in organ-and tissue-specific toxicity	Acetaminophen
Idiosyncratic toxicities	Rare toxicities that are thought to represent unique susceptibility of affected individuals	Nevirapine Isoniazid

to fulminant hepatic failure, which although rare, is potentially life threatening and may necessitate a liver transplant (Park et al. 1998).

The complete mechanisms of DILI remain largely unknown but appear to involve two pathways: direct hepatotoxicity and adverse immune reactions. In many instances, liver injury is thought to be initiated by the bioactivation of the drug to chemically reactive metabolites, which have the potential to modify the function of various critical cellular macromolecules and are therefore able to cause direct damage.

1.1 Drug Metabolism and Reactive Metabolites

The biotransformation of lipophilic compounds into water-soluble derivatives that are more readily excreted is the physiological role of drug metabolism. The principal site of drug metabolism is the liver. The liver is exposed to xenobiotics immediately after their absorption from the gastro-intestinal tract and has a high capacity for both phase I and phase II biotransformations. Usually, this conversion of the xenobiotic from a lipid to water soluble form results in loss of pharmacological/biological activity. Such metabolic reactions are, therefore, regarded as true "detoxication reactions", but certain xenobiotics additionally undergo biotransformation to toxic/reactive metabolites that can interfere with cellular functions and may have intrinsic chemical reactivity towards certain types of cellular macromolecules. Such biotransformations are therefore termed "intoxication" reactions. These toxic/reactive metabolites have the ability to interact with cellular proteins, lipids and nucleic acids, leading to protein dysfunction, lipid peroxidation, DNA

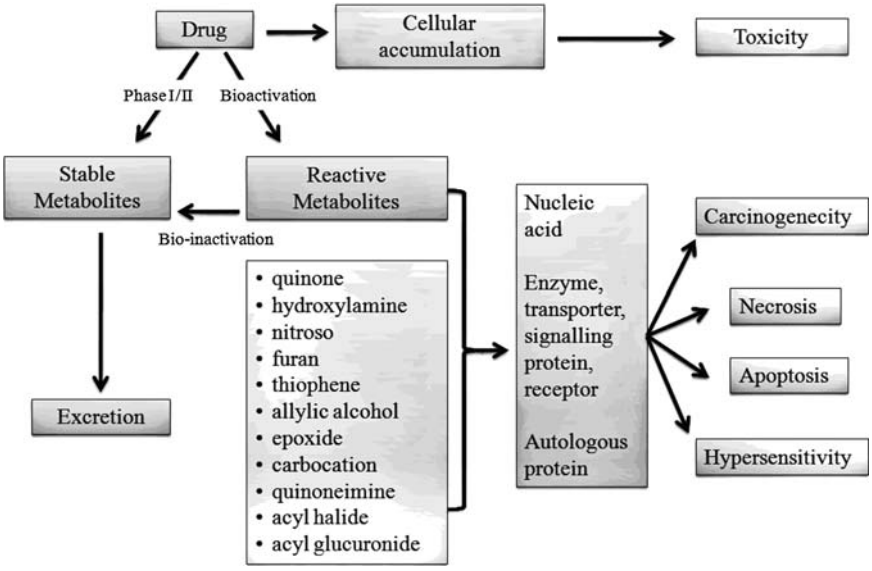


Fig. 1 Relationship between drug metabolism and toxicity. Toxicity may accrue through accumulation of parent drug or, via metabolic activation, through formation of a chemically reactive metabolite, which, if not detoxified, can effect covalent modification of biological macromolecules. The identity of the target macromolecule and the functional consequence of its modification will dictate the resulting toxicological response

damage, and oxidative stress. Additionally, the metabolites may induce disruption of ionic gradients and intracellular calcium stores, resulting in mitochondrial dysfunction and loss of energy production. This impairment of cellular function can result in cell death and possible liver failure (Fig. 1).

The propensity of a molecule to form either toxic and/or chemically reactive metabolites is simply a function of its chemistry. Such metabolites are typically short-lived, with half-lives of generally less than 1 s, and are not usually detectable in plasma. Toxicophores are functional groups present in drugs which can be transformed into reactive species by normal biotransformations. The concept that small organic molecules can undergo bioactivation to electrophiles and free radicals, and elicit toxicity by covalent modification of cellular macromolecules, has its basis in chemical carcinogenicity and the pioneering work of the Millers (Miller and Miller 1947, 1952). They studied the hepatotoxic effects of p-dimethylaminoazobenzene in the rat and found that aminoazo dyes become tightly bound to the protein constituents of liver tissue. The application of such concepts to human drug-induced hepatotoxicity was established through the studies of Brodie, Gillette, and Mitchell (Brodie et al. 1971; Gillette et al. 1974) on the covalent binding to hepatic proteins of toxic (over) doses of the widely used analgesic acetaminophen.

Reactive metabolites may be broadly classified as either electrophiles or free radicals (Williams et al. 2002). In the vast majority of cases, the ultimate reactive

species is electrophilic in nature (Parkinson 1996), for example, epoxides and quinoids. Electrophiles are reactive because they are electron deficient and have either a high positive charge density (hard electrophiles) or a lower positive charge density (soft electrophiles) at the electrophilic center. Reactive metabolites that possess unpaired electrons are free radicals. Free radicals usually abstract a hydrogen atom from other molecules rather than becoming covalently bound; however, they can also add to double bonds. Free radical reactions can be self propagating: abstraction of a hydrogen atom from a lipid can initiate a chain reaction leading to lipid peroxidation, oxidative stress, or modification of other types of biological molecules by free radicals (Uetrecht 1995).

Formation of chemically reactive metabolites is mainly catalyzed by cytochromes P450 (Fig. 1). Quantitatively, the P450 isoforms in the endoplasmic reticulum are the most important group of enzymes involved in this process. Products of phase-II metabolism can also lead to toxicity. Additionally, non-cytochrome P450 oxidative enzymes, such as myeloperoxidase and prostaglandin H synthetase, have been implicated in the bioactivation of drugs and other chemicals, the metabolites of which are thought to be responsible for observed toxicity, e.g., clozapine and agranulocytosis, benzene and aplastic anemia (Fischer et al. 1991; Mason and Fischer 1992; Ross et al. 1996; Smith et al. 1989). Cytochrome P450 isoforms are present in different proportions in many organs, though most abundantly in the liver, and thus bioactivate the chemicals to cause organ-specific toxicity (Kao and Carver 1990; Pelkonen and Raunio 1997; Uetrecht 1992).

1.2 Relationship Between Metabolic Activation and Hepatotoxicity

The relationship between bioactivation and the occurrence of hepatic injury is not simple. For example, many chemicals undergo bioactivation in the liver but are not hepatotoxic. The best example is the absence of hepatotoxicity with therapeutic doses of acetaminophen. Tight coupling of bioactivation with bioinactivation may be one reason for this. Many enzymic and nonenzymic pathways of bioinactivation are present in the liver, which is perhaps the best equipped of all the organs in the body to deal with chemically reactive toxins. Typical examples of bioinactivation pathways include glutathione conjugation of quinones by glutathione *S*-transferases and hydration of arene oxides to dihydrodiols by epoxide hydrolases. The efficiency of a bioinactivation process is dependent on several factors including the inherent chemical reactivity of the substrate, substrate-selectivity of the enzymes, which is usually very broad, tissue expression of the enzymes, and the rapid upregulation of enzyme(s) and cofactors mediated by cellular sensors of chemical stress. It is only when a reactive metabolite can bypass or saturate these defense systems of bioinactivation and thereby damage proteins and nucleic acids through covalent binding that it exerts significant toxic effects.

Table 2 Examples of hard and soft electrophiles and hard and soft nucleophiles

Type	Electrophiles	Nucleophiles
Hard	Alkyl carbonium ions	Oxygen atoms of purine/pyrimidine bases in DNA
	Benzylic carbonium ions	Endocyclic nitrogens of purine bases in DNA
	Iminium ions	Oxygen atoms of protein serine and threonine residues
	Aldehydes	
Soft	Epoxides	Protein thiol groups
	Enones	Sulfhydryl groups of glutatione
	Quinone imines	Primary/secondary amino group of protein lysine and histidine residues
	Quinone methides	
	Michael acceptors	

Moreover, covalent binding of a drug does not necessarily lead to hepatotoxicity. A regioisomer of acetaminophen, 3-hydroxyacetanilide, becomes covalently bound to hepatic proteins in rodents without inducing hepatotoxicity (Qiu et al. 2001; Tirmenstein and Nelson 1989). It is therefore necessary to identify the subset of targets, i.e., covalently modified macromolecules, that are critical to the toxicological process. Unfortunately, although some liver proteins have been identified which are modified covalently by several xenobiotics (Hanzlik et al. 2007), there are currently no techniques to predict the target macromolecule(s) for a particular chemically reactive metabolite let alone the biological consequences of a particular modification. However, from a simple chemical perspective, it is known that hard electrophiles generally react with hard nucleophiles, such as functional groups in DNA and lysine residues in proteins, whereas soft electrophiles react with soft nucleophiles, which include cysteine residues in proteins and in glutathione (Table 2), which has a concentration of approximately 10 mM in the liver.

Furthermore, noncovalent drug–protein interactions also play a role because covalent binding of hepatotoxins is not indiscriminate with respect to cellular proteins (Koen et al. 2007; Qiu et al. 1998). Even within a single liver protein there can be selective modification of an amino acid side-chain found repeatedly in the primary structure (Koen et al. 2006; Nerland et al. 2003). Thus, the microenvironment (pKa, hydrophobicity, etc.) of the amino acid in the tertiary structure appears to be the crucial determinant of selective binding, and therefore the impact of covalent binding on protein function. The extent of binding and the biochemical role of the protein will, in turn, determine the toxicological insult of drug bioactivation. The resulting pathological consequences will be a balance between the rates of protein damage and the rates of protein replacement and cellular repair.

It is therefore not surprising that irreversible chemical modification of a protein, when it has a profound effect on function, is a potential mechanism of drug-induced hepatotoxicity. However, it is also important to note that a number of drugs (e.g., penicillins, clopidogrel, and omeprazole) rely on covalent binding to proteins for their efficacy, and thus prevention of adverse covalent binding through chemical modification of the compound may also inadvertently lead to loss of efficacy. Similarly, certain endogenous electrophiles, such as cyclopentenone prostaglandins, are Michael acceptors, which can react with specific cysteine residues in transcription factors to elicit their physiological effects in cell signaling (Kawamoto et al. 2000).

The considerable task, therefore, facing the molecular toxicologist and drug metabolist is to differentiate between those protein modifications that are critical for a particular type of drug toxicity (and drug efficacy) and functionally inconsequential "background" covalent binding.

1.3 Relationship Between Dose of Drug and Incidence of Hepatotoxicity

Although liver toxicity does not show a classical dose response for any one drug, many of the drugs that are associated with high incidence of hepatotoxicity are given at high doses, a factor that drives the relatively high amounts of reactive metabolite formed (Smith and Obach 2005; Uetrecht 1999). Moreover, in a recent study of 233 most commonly prescribed oral medications that are associated with significantly higher incidences of DILI, it has been reported that the drugs that are prescribed at higher dose have a higher incidence of hepatotoxicity: out of 598 cases reported, 77% occurred in patients taking drugs at daily doses of at least 50 mg (higher doses), 14% at doses of 11-49 mg (intermediate doses), and only 9% for drugs at doses less than 10 mg (lower doses) (Lammert et al. 2008).

Table 3 lists the dose and incidence of the hepatotoxicity for various drugs, some of which have been withdrawn as a result of these findings. Some of the drugs show effects on liver function earlier than the actual onset of liver toxicity, with serum aminotransferase levels raised by threefold the upper limit of normal (ULN). The incidence of this is much higher and can show a classical dose response: for instance, tolcapone, a catechol-O-methyltransferase inhibitor used as an adjunct to levodopa in Parkinson's disease, produces threefold the ULN in 1–3% of patients receiving 100 mg TID and 3.7% of patients receiving 200 mg TID (Olanow and Watkins 2007). Tolcapone, an *o*-nitrocatechol, is metabolized to reactive intermediates – o-quinone or quinoneimine species – by human liver microsomes (Smith et al. 2003).

In the next section, we discuss a selection of hepatotoxic drugs and nondrug xenobiotics, and summarize the evidence for reactive metabolite formation and the current hypotheses of how this might lead to liver injury and defensive cellular responses.

2 Model Hepatotoxins: Role of Reactive Metabolite Formation

A number of simple chemical compounds such as acetaminophen, bromobenzene, furosemide, methapyrilene (Graham et al. 2008), and thiobenzamide (Ikehata et al. 2008) produce hepatotoxicity, with damage to extrahepatic tissues

Table 3 Incidance of hepatotoxicity associated with various drugs ([a]drugs discussed in this review, [b]cases with serum aminotransferase levels raised 3x ULN, but acute liver failure cases reported are 1:3)

Drugs	Dose (mg/day)	Incidence per treated patient	Metabolic activation
Acetaminophen[a]	>4,000	1:1[b] (Ostapowicz et al. 2002)	Yes (Mitchell et al. 1973; Raucy et al. 1989)
Acetaminophen[a]	<4,000	1:250 (Dart and Bailey 2007)	Yes (Mitchell et al. 1973; Raucy et al. 1989)
Nevirapine[a]	200–400	1:30 (de Maat et al. 2002)	Proposed (Uetrecht 2006)
Isoniazid[a]	300	1:250 (Derby et al. 1993)	Yes (Nelson et al. 1976; Sarich et al. 1995)
Troglitazone	400	1:3,000 (Graham et al. 2003)	Yes (He et al. 2004)
Bromfenac	100–200	1:15,000 (Goldkind and Laine 2006)	–
Amodiaquine[a]	200–400	1:15,650 (Phillips-Howard and West 1990)	Yes (Harrison et al. 1992; Jewell et al. 1995)
Clozapine	300	1:16,000 (Macfarlane et al. 1997)	Yes (Pirmohamed et al. 1995)
Trovafloxacin	200	1:18,000 (Ball et al. 1999)	Proposed (Sun et al. 2008)
Zileuton	2,400	1:30,000 (Watkins et al. 2007)	Yes (Joshi et al. 2004)
Tolcapone	300–600	1:30,000 (Olanow and Watkins 2007)	Yes (Smith et al. 2003)
Nimesulide	200	1:10,00000 (Boelsterli 2002)	Proposed (Boelsterli et al. 2006)

in certain cases (bromobenzene is also pneumotoxic and nephrotoxic; Dahl et al. 1990), in one or more rodent species after a single dose or a short-term regimen. The reproducibility of these injuries permits detailed mechanistic investigations that are impractical or unachievable in the case of idiosyncratic reactions; however, they may provide a source of crucial insights into the mechanisms of such reactions.

It is generally accepted that the toxicities of these model hepatotoxins are associated with the generation of chemically reactive metabolites, which can be detected and estimated indirectly through the irreversible binding of uncharacterized radiolabeled material to hepatic protein (Evans et al. 2004) and/or the formation of stable metabolites such as glutathione conjugates (Masubuchi et al. 2007). Until recently, most assays of reactive metabolite formation in vivo and in vitro depended on measuring irreversible binding of radioactivity using relatively laborious methods of exhaustive solvent extraction (Evans et al. 2004; Masubuchi et al. 2007; Takakusa et al. 2008). The possibility of an alternative, semi-quantitative assay of reactive metabolites in microsomal incubations based upon a novel glutathione analog and LC-MS/MS has been established by Soglia et al. (2006). Advanced mass spectrometry techniques now enable not only high-throughput screening of glutathione-trapped reactive metabolites but also structural characterization of adducts, which provides fundamental insights into the identities of the reactive metabolites (Ma et al. 2008; Wen et al. 2008). A complementary method exists for cyanide trapping of iminium ion intermediates

generated in microsomal incubations (Argoti et al. 2005). As an alternative technique, a peptide-based in vitro method for the detection of reactive metabolites, in which covalently bound adducts are detected by mass spectrometry, has been developed (Mitchell et al. 2008). A recent study on the mechanism-based inactivation of cytochrome P450s by 17-α-ethynylestradiol illustrated how the characterization of GSH adducts of reactive metabolites can complement parallel analyses of modified proteins (Kent et al. 2006). However, the structure of a GSH adduct of a reactive species is not invariably analogous to that of the protein adduct (Koenigs et al. 1999).

The metabolic activation of hepatotoxins and the covalent modification of cellular, sub-cellular, and blood plasma proteins by reactive metabolites, in general, has received considerable attention (Caldwell and Yan 2006; Kalgutkar and Soglia 2005; Park et al. 2005). Nevertheless, the structure of the metabolite-protein adduct has been determined in only a few cases (Baer et al. 2007; Bambal and Hanzlik 1995; Damsten et al. 2007; Sleno et al. 2007; Yukinaga et al. 2007; Zhang et al. 2003), and identification of the modified amino acid residue in vivo remains a major analytical challenge (Koen et al. 2006). Greater progress has been made in identification of the cellular proteins that are modified in vivo (Druckova et al. 2007; Ikehata et al. 2008; Koen et al. 2007; Qiu et al. 1998; Shipkova et al. 2004). Potential targets within individual organelles can now be identified by using model electrophiles in cell fractions (Shin et al. 2007; Wong and Liebler 2008). Adducted amino acid residues of hepatic proteins are likely to be more easily identified in vivo if an intrinsically reactive compound is administered (Nerland et al. 2003). However, the usefulness of this approach for identifying potential binding sites of reactive metabolites has not been established. Nevertheless, it has the potential to confirm the association of selective modification of liver proteins with loss of critical enzyme activities (Campian et al. 2002) suggested by various studies on APAP (Park et al. 1998). The extent to which a particular loss of activity in vivo is due to adduction by a reactive metabolite or drug-induced oxidative modifications of amino acid residues is a complex analytical problem (Andringa et al. 2008). Despite these difficulties, the expectation is that a comprehensive database of cellular proteins modified covalently by reactive metabolites in vivo should ultimately facilitate elucidation of the mechanisms of associated toxicities (Hanzlik et al. 2007). It is already apparent that, although each bioactivated xenobiotic may modify a unique set of hepatic proteins, there is a partial commonality with the proteins modified by other compounds (Koen et al. 2007). A similar selectivity is seen with model electrophiles in vitro (Shin et al. 2007). The proteins modified perform a great variety of biological functions, with corresponding potential for disruption of those functions. The relationship of the adduction of these proteins to the toxicity of reactive metabolites has been discussed in terms of inhibition of enzymes critical to maintenance of cellular energy and homeostasis, the unfolded protein response, and interference with kinase-based signaling pathways (Ikehata et al. 2008). The targeting of components of signaling pathways and metabolic networks has been proposed, alongside the need to understand mechanisms of damage at a systems level (Liebler 2008).

2.1 *Acetaminophen*

Acetaminophen (APAP) is a major cause of drug-related morbidity and mortality in humans, producing massive hepatic necrosis after a single toxic dose. APAP is thought to be safe at therapeutic doses, but some studies have claimed that it may be hepatotoxic at doses below 4 g per day (Dart and Bailey 2007; Watkins et al. 2006) (Fig. 2).

At therapeutic doses, APAP is safely cleared by glucuronylation and sulphation to metabolites that are rapidly excreted in urine. However, a proportion of the drug undergoes bioactivation to N-acetyl-p-benzoquinoneimine (NAPQI) by CYP2E1, CYP1A2, and CYP3A4 (Raucy et al. 1989; Thummel et al. 1993). NAPQI is rapidly quenched by a spontaneous and enzymatic reaction (Coles et al. 1988) with hepatic glutathione after a therapeutic dose of APAP (Fig. 3). However, after a toxic (over) dose, glutathione depletion occurs, which is an obligatory step for covalent binding and toxicity (Davis et al. 1974). The standard treatment for APAP intoxication is N-acetylcysteine, which replaces hepatic glutathione and prevents toxicity. N-acetylcysteine is most beneficial if given within 16 h of the overdose. The early signs of cellular disruption in isolated hepatocytes can be reversed by a disulphide reductant, dithiothreitol (Albano et al. 1985; Rafeiro et al. 1994).

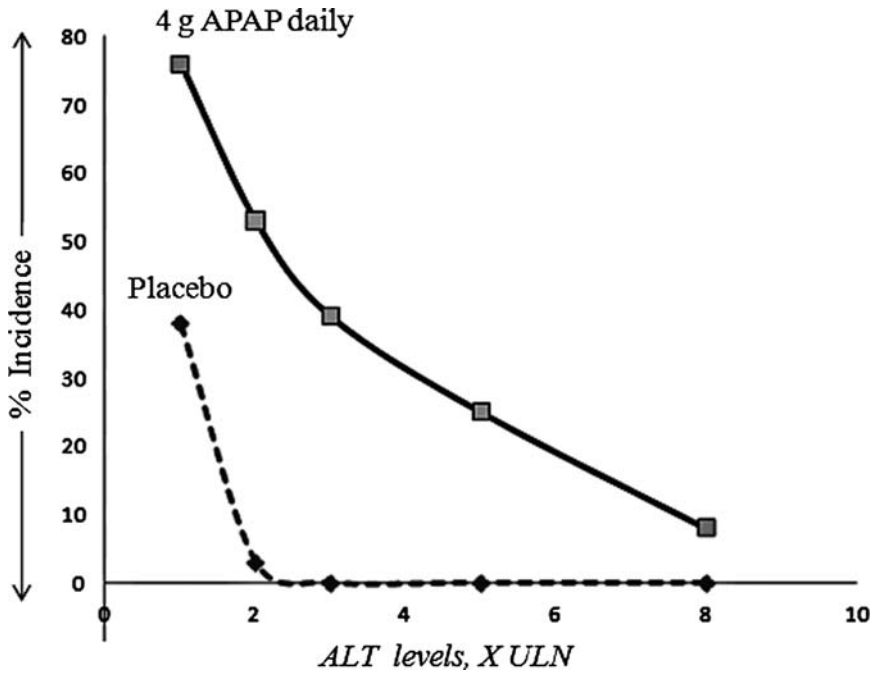


Fig. 2 Incidence of peak alanine aminotransferase elevations by multiples of upper limit of normal for therapeutic dose (4 g/day) of APAP and placebo, in 145 healthy adults (Watkins et al. 2006)

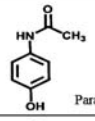
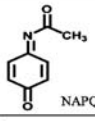
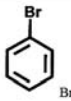
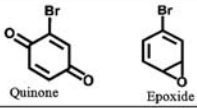
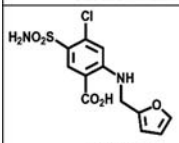
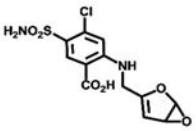
Parent Compound	Reactive Metabolite	GSH Depletion	Covalent Binding
 Paracetamol	 NAPQI	Required	Yes
 Bromobenzene	 Quinone Epoxide	Required	Yes
CCl_4 Carbon Tetrachloride	$CCl_3^{\bullet}$ Trichloromethyl radical	May occur	Yes
 Furosemide	 Furosemide epoxide	May occur	Yes

Fig. 3 Bioactivation of model hepatotoxins. The structures of the toxic metabolites that are formed from oxidative metabolism of acetaminophen, bromobenzene, carbon tetrachloride, and furosemide are given alongside the requirement for GSH depletion and covalent binding

The massive biochemical stress produced by an APAP overdose leads to an immediate adaptive defense response in the hepatocyte. This involves various mechanisms, including the nuclear translocation of redox-sensitive transcription factors such as Nrf-2, which "sense" chemical danger and orchestrate cell defense. Thus, with respect to APAP, Nrf-2-activated genes of immediate significance are those coding for γ-glutamylcysteine synthetase (γ-GCS), GSTs, glucuronyltransferases, and heme oxygenase. Importantly, it has been observed that nuclear translocation occurs at nontoxic doses of APAP and at time-points before overt toxicity is observed (Goldring et al. 2004). However, with increasing doses of APAP, there is progressive dislocation of nuclear translocation, transcription, translation, and protein activity (Kitteringham et al. 2000), as the rate of drug bioactivation overwhelms cell defense through the destruction of critical proteins.

The cytoplasmic protein Keap1 directly binds to Nrf2 and represses transcription by promoting proteasome-dependent degradation of the protein. It has also been demonstrated that Keap1 is an adaptor molecule for the ubiquitin ligase complex and directs the rapid degradation of Nrf2. When cells are exposed to electrophiles, Nrf2 is liberated from the Keap1-mediated degradation process and accumulates in the nucleus to activate antioxidant response element (ARE)-mediated gene transcription (Okawa et al. 2006). Somatic disruption of the Keap1 gene does not interfere with the development of the morphological and physiological integrity of the liver. However, specific knockout of the Keap1 gene in mouse hepatocytes confers a strong resistance to drug-induced toxicity (Okawa et al. 2006). This indicates that the constitutive activation of Nrf2, and concomitantly activation of its target genes, is advantageous for mice to overcome xenobiotic toxicity.

2.2 Bromobenzene

Bromobenzene is an industrial solvent and environmental pollutant, which causes centrilobular hepatic necrosis in rats and mice (Casini et al. 1985; Jollow et al. 1974) and is toxic to isolated rodent and human hepatocytes (Grewal et al. 1996; Mennes et al. 1994). The hepatic damage is generally believed to be mediated through P450-generated reactive intermediates (Fisher et al. 1993), especially bromobenzene 3,4-epoxide, although alkylation of protein sulfur nucleophiles in vivo by quinone metabolites is 10–15 times more extensive than alkylation by epoxides (Slaughter and Hanzlik 1991) (Fig. 3). The 3, 4-epoxide can be hydrolysed by microsomal epoxide hydrolase to form the dihydrodiol (den Besten et al. 1994) or detoxified by conjugation to GSH (Jollow et al. 1974). The epoxide intermediate may also undergo nonenzymatic isomerisation to 4-bromophenol (Selander et al. 1975), which can be further metabolized to yield catechols and quinones, some of which have been associated with hepatotoxicity via redox cycling and oxidative stress (Mizutani and Miyamoto 1999). The postulated mechanisms of bromobenzene-induced hepatotoxicity include GSH depletion, lipid peroxidation, covalent modification of critical cellular proteins, and mitochondrial dysfunction (Casini et al. 1985; Fisher et al. 1993; Koen et al. 2007; Maellaro et al. 1990). Modification of rat hepatic proteins by bromobenzene's reactive metabolites has been analyzed in some detail and undoubtedly appears to be a highly selective process at both the molecular (Koen et al. 2006) and cellular levels (Koen et al. 2007), although there is partial commonality with the proteins modified by other bioactivated hepatotoxins such as APAP (Koen et al. 2007). Nevertheless, no specific mechanistic linkage with hepatotoxicity has been elucidated to date. Some early studies obtained a dissociation of bromobenzene-induced liver necrosis from covalent binding in mice administered an antioxidant that largely blocked the necrosis (Casini et al. 1984, 1985). Major interindividual differences between the susceptibility of isolated primate hepatocytes to bromobenzene were observed by Mennes et al. (1994), which could not be attributed to rates of cytochrome P450-mediated bioactivation. This suggested that the combined activities of metabolic deactivating reactions might be the major determinant of cell damage.

2.3 Carbon Tetrachloride

Carbon tetrachloride (CCl_4) was a widely used industrial solvent and cleaner. It produces liver injury in rats and mice that has served as a useful experimental model for certain hepatotoxic effects (Renner 1985; Slater 1981). A single dose of CCl_4 when administered to a rat produces centrilobular necrosis and fatty degeneration of the liver, and chronic low dose exposure is associated with liver cirrhosis (Slater et al. 1985). These hepatotoxic effects of CCl_4 are mediated through its metabolic activation in the liver by CYP2E1 to reactive intermediates, including the

trichloromethyl free radical (Fig. 3). The radicals formed are capable of covalently binding to lipids and proteins, with lipids being the favored option. The availability of oxygen determines the nature of the pathological outcome, with low oxygen levels favoring covalent binding to cellular protein as well as to CYP450s (Recknagel et al. 1989). Hepatotoxic mechanisms are thought to be through lipid peroxidation and disruption of cellular homeostasis, which can lead to disruption of the cytoskeleton, cell signaling, and gene expression pathways (Clawson 1989; Recknagel et al. 1989).

2.4 Furosemide

Furosemide is a highly potent loop diuretic, which causes massive hepatic necrosis in mice (Mitchell et al. 1974; Williams et al. 2007; Wong et al. 2000). It has been postulated that the hepatocellular damage caused by furosemide is a result of cytochrome P450-mediated bioactivation of the furan ring to an epoxide intermediate (Mitchell et al. 1974, 1976a), which binds covalently with hepatic macromolecules both in vivo and in vitro (Mitchell et al. 1976a) (Fig. 3). The proteins modified by the reactive metabolite have not been identified. Recently, we have found that furosemide is metabolized in mice to a γ-ketocarboxylic acid, a product of furan-ring opening, which further indicates the formation of a reactive epoxide intermediate (Williams et al. 2007). Detoxification of furosemide's epoxide is thought to occur via hydration to a dihydrodiol and conjugation with GSH (Williams et al. 2007). Depletion of GSH has been observed in mouse (Grewal et al. 1996) and rat (Williams et al. 2007) hepatocytes. However, furosemide has no effect on hepatic GSH levels in vivo (Mitchell et al. 1974; Wong et al. 2000).

3 Hepatocarcinogenesis: Role of Reactive Metabolite Formation

The relationship between bioactivation, bioinactivation, and DNA adduct formation has been well established for a number of hepatocarcinogens. Aflatoxin, which is a hepatocarcinogen and a hepatotoxin, is converted into an epoxide, which is more readily detoxified by GST enzymes than by epoxide hydrolase. The balance of these reactions explains the greater DNA damage in humans compared with rodents because human forms of GST are less able to catalyze the conjugation of aflatoxin than their rodent counterparts (Guengerich et al. 1996; Wilson et al. 1997). Transgenic knockout mice have been used to establish the role of bioactivation by P450 (Gonzalez and Kimura 1999) and bioinactivation by GSTs (Henderson et al. 1998) for a number of carcinogenic polyaromatic hydrocarbons.

3.1 *Tamoxifen*

Tamoxifen is a nonsteroidal antiestrogen used for the treatment of breast cancer (Benson and Pitsinis 2003; Clemons et al. 2002; Fisher et al. 1998; Furr and Jordan 1984; U.S. Food and Drug Administration 2005b). It has a good tolerability profile and moreover, unlike many other endocrine therapies, it is efficacious in both pre- and postmenopausal women (Clemons et al. 2002). However, an increased risk of developing uterine sarcomas and endometrial carcinomas has been observed in patients treated with tamoxifen (Lavie et al. 2008). Although this drug has been reported to be a potent genotoxic hepatocarcinogen in rats (Williams et al. 1993), it does not cause hepatic tumors in women after either prophylaxis or treatment. A consideration of the relative rates of bioactivation and bioinactivation provides a metabolic rationale for the safety of the drug in women.

Tamoxifen is mainly metabolized to *N*-oxide, *N*-desmethyl, α-hydroxy, and 4-hydroxy metabolites (Carter et al. 2001; De Vos et al. 2001; Li et al. 2001; MacCallum et al. 2000). Multiple CYPs including CYP 1A1, 2D6, 3A4, 2B6, and 3A5 are involved in the metabolism of tamoxifen to reactive catechol products that have the potential to form protein and DNA adducts (Davis et al. 1998; Dehal and Kupfer 1999; Fan and Bolton 2001; Notley et al. 2002). The major route of bioactivation of tamoxifen to a genotoxic metabolite is thought to be by sequential α-hydroxylation and sulphonation to a sulfate ester that collapses to a reactive carbocation and forms DNA adducts (Martin et al. 1995) (Fig. 4). Importantly, it was observed that the corresponding glucuronide of α-hydroxytamoxifen is chemically very stable, and thus this biotransformation represents bioinactivation. There is no glutathione conjugate formed because the carbocation is a hard electrophile. A comparison of the relative rates of hydroxylation, sulphonation, and glucuronylation was performed in vitro between human and rodent enzymes. Rats had a greater propensity for sulphonation (bioactivation), whereas human liver had a much greater ability to effect glucuronylation (bioinactivation) (Boocock et al. 1999, 2000). An overall analysis of risk based on dose and the relative rates of metabolism suggested a 150,000-fold safety factor for the development of liver cancer from tamoxifen in humans when compared with rats.

4 Idiosyncratic Hepatotoxicity: Role of Reactive Metabolite Formation

Idiosyncratic reactions are extremely host-dependent and uncommon reactions that cannot be either predicted or rationalized from the known pharmacology of the drug. The exact mechanism behind these idiosyncratic reactions is still unclear; however, it is believed that many idiosyncratic reactions are initiated by reactive drug metabolites, e.g., nevirapine, isoniazid, and amodiaquine, which bind covalently to macromolecules and either cause direct cell damage or trigger an immune response.

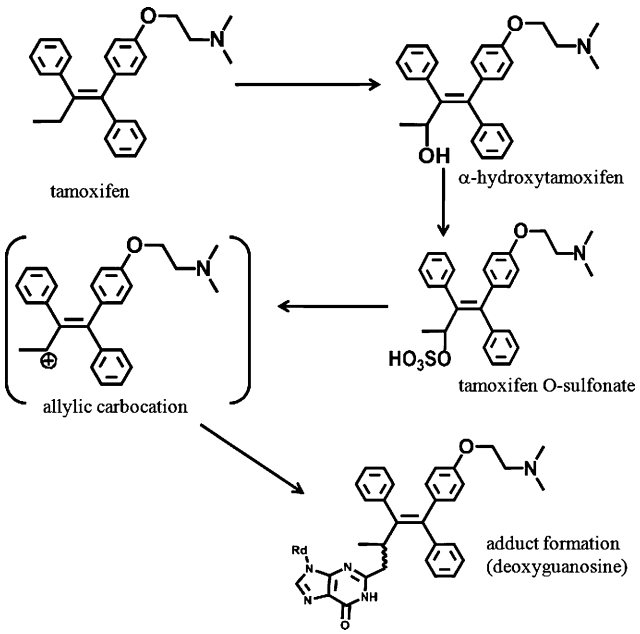


Fig. 4 Metabolic activation of tamoxifen

4.1 Nevirapine

Nevirapine (NVP), a nonnucleoside reverse transcriptase inhibitor, is widely used for the treatment of human immunodeficiency virus (HIV) infections. It is the main option for the first-line treatment of HIV-1, together with two nucleoside reverse transcriptase inhibitors, in countries with limited resources. NVP is associated with two serious clinically restrictive side effects: skin reactions and hepatotoxicity. Severe, life threatening, and in some cases fatal hepatotoxicity, including fulminant and cholestatic hepatitis, hepatic necrosis, and hepatic failure, has been reported in HIV-infected patients taking NVP (DHHS Panel on Antiretroviral Guidelines for Adults and Adolescents 2008). For this reason, NVP is given a black box warning for hepatotoxicity, and concern has been raised over NVP-based treatment.

NVP-induced hepatotoxicity is idiosyncratic in nature and no clear mechanistic understanding of human toxicity exists. Thus, it is impossible to predict which patients will suffer from hepatic damage. For instance, among persons taking NVP, the incidence of an asymptomatic increase of liver enzyme levels in plasma is approximately 5–15% (de Maat et al. 2003; Martin-Carbonero et al. 2003; Martinez et al. 2001; Prakash et al. 2001), and the occurrence of clinically symptomatic hepatotoxicity of NVP is approximately 4% (Boehringer-Ingelheim International 2005; DHHS Panel on Antiretroviral Guidelines for Adults and Adolescents 2008; Martinez et al. 2001; U.S. Food and Drug Administration 2005a). However, patients having concomitant infection with hepatitis viruses and a higher CD4

cell count prior to starting NVP ($\geq$250 cells/mm^3 in females and 400 cells/mm^3 in males) are at a higher risk of developing hepatotoxicity (Boehringer-Ingelheim International 2005; de Maat et al. 2003, 2005; Piliero and Purdy 2001). Multivariate analysis in a recent study showed that the independent risk factors for severe hepatotoxicity were a body mass index (BMI) lower than 18.5, female sex, serum albumin level of less than 35 gl^{-1}, mean corpuscular volume above 85 fl, plasma HIV-1 RNA load of lower than 20,000 copies/ml, aspartate aminotransferase level of less than 75 IU l^{-1}, and lactate dehydrogenase level of less than 164 IU l^{-1}. It is unclear why low baseline enzyme levels should act as predictive factors for hepatotoxicity (Sanne et al. 2005).

The pattern of NVP-induced liver injury varies, not only in terms of severity (Buyse et al. 2006; Centers for Disease Control and Prevention. 2001; Maniar et al. 2006) but also in time to onset (Clarke et al. 2000; de Maat et al. 2003). For instance, some reactions occur early while others occur late after prolonged exposure and, indeed, are not always accompanied by hypersensitivity manifestations, such as fever and eosinophilia. It is possible that the late-occurring reactions may be nonimmune in nature, while those occurring early (e.g., within the first 3 months) may have an immune pathogenesis. This is consistent with our recent observation of NVP-reactive T cells in a patient with early-onset hepatitis in the absence of any cutaneous manifestations (Drummond et al. 2006). The patient's T cells proliferated in vitro on exposure to NVP but not its stable metabolites.

Studies with human liver microsomes suggest that oxidative metabolism of NVP is mediated primarily by cytochrome P450 (CYP) isozymes CYP3A4 and CYP2B6. Among the phase-1 metabolites, 2-hydroxy NVP, 3-hydroxy NVP, 8-hydroxy NVP, and 12-hydroxy NVP are the major metabolites (Riska et al. 1999), mainly formed by CYP3A4, CYP2B6, CYP3A4, and CYP2D6, respectively (Erickson et al. 1999).

The enzymes involved in the metabolism of NVP show considerable variation in expression and activity. CYP3A4 is the most variable enzymatic complex and displays a wide range of inter-individual polymorphic expression (Dai et al. 2001; Wojnowski 2004). In one study, association between the frequent CYP2B6 variant (516G$\rightarrow$T) and NVP pharmacokinetics has been observed. Patients homozygous for the variant allele (516G$\rightarrow$T/T) presented 1.7-fold higher NVP plasma levels than those homozygous for the common allele (516G$\rightarrow$G/G) (Rotger et al. 2005). The clinical implication of this observation remains unclear; however, high NVP plasma levels have been associated with an increased risk of liver toxicity (De Requena et al. 2005; Nunez et al. 2003). Although this variable expression may result in hepatotoxicity in patients homozygous for the variant allele, the relationship between NVP plasma concentrations and the risk of skin rash and hepatic injury appears to be unclear because contradictory reports also exist (Almond et al. 2004). The association of these adverse effects with a higher starting dose, low BMI, and increased age (all of which are associated with higher plasma concentrations) would suggest that there is a relationship with NVP pharmacokinetics (Pirmohamed et al. 2007).

In another study, MDR1 3435C$\rightarrow$T was significantly associated with decreased risk of hepatotoxicity (Haas et al. 2006). This gene is thought to be associated with

altered expression of the P-glycoprotein. It is possible that altered P-glycoprotein activity in the intestine associated with MDR1 variants alters disposition of NVP and/or its metabolites, which in turn affects intracellular concentrations of NVP and/or its metabolites and ultimately toxicity in the liver (Saitoh and Spector 2008).

Although the role of an immune-mediated mechanism in NVP-induced skin rash and hepatotoxicity has been strongly advocated (Pirmohamed et al. 2007; Popovic et al. 2006; Shenton et al. 2007), it is not yet clear whether immune induction in patients is due to a (reactive) metabolite or NVP itself. Highly circumstantial evidence for reactive metabolite involvement comes from a case reported by Claes et al. (2004), where a patient suffering from NVP-induced toxic epidermal necrolysis and toxic hepatitis was successfully treated with intravenous human immunoglobulins and high doses of N-acetylcysteine (300 mg kg^{-1} per day in a continuous infusion until recovery). As a precursor of glutathione, N-acetylcysteine may have helped to restore levels of glutathione, thus enhancing detoxification of toxic metabolites (De Rosa et al. 2000), and resulted in exceptionally fast clinical recovery in this case.

Hypothetically, there may be several pathways for NVP bioactivation. For example, the cyclopropylamine group has the potential to become bioactivated to an aminium cation radical (Shaffer et al. 2001). 12-Hydroxy NVP, which is the major phase-1 metabolite in human liver microsomes, is a substrate for sulphotransferase, and it has been suggested that the sulfate ester dissociates to form a reactive quinonemethide intermediate (Uetrecht 2006; Chen et al. 2008). However, in the case of the hepatotoxicity associated with NVP in humans, Chen et al. (2008) have proposed that tissue injury is due to quinonemethide produced by P450. NVP also has the potential to form an epoxide intermediate in either of the pyridine rings (Fig. 5). Srivastava et al. (2009) have found a pyridino-substituted mercapturate of NVP in human urine that could be derived from a glutathione conjugate of an epoxide. A reactive intermediate (aminium cation/epoxide/quinonemethide) might deplete hepatic glutathione (GSH) in certain patients because the synthesis of GSH is often reduced in HIV infection (Otis et al. 2008; Stehbens 2004). Depletion of GSH, if it is uncompensated, may lead to oxidative stress and covalent binding of NVP to critical hepatic proteins; as a consequence, triggering apoptosis and necrosis of liver cells. Adduct formation with proteins under these circumstances might also lead to the initiation of the immune response, and possibly explain the hypersensitivity observed with NVP.

A fully robust animal model of NVP-induced hepatotoxicity has yet to be developed (Walubo et al. 2006), but Uetrecht and colleagues have characterized extensively a dose-dependent, NVP-induced, skin rash in female Brown Norway rats that resembles the idiosyncratic cutaneous reaction seen in humans and appears to be immune-mediated (Popovic et al. 2006; Shenton et al. 2007). An association between 12-hydroxy NVP metabolism and skin toxicity has been shown in a recent study (Chen et al. 2008), when it was found that 12-OH-NVP caused a rash at a lower dose than required for NVP. The authors proposed that the rash produced in rats may be due to quinonemethide formed in the skin via sulfation of 12-hydroxy NVP metabolite followed by loss of inorganic sulfate.

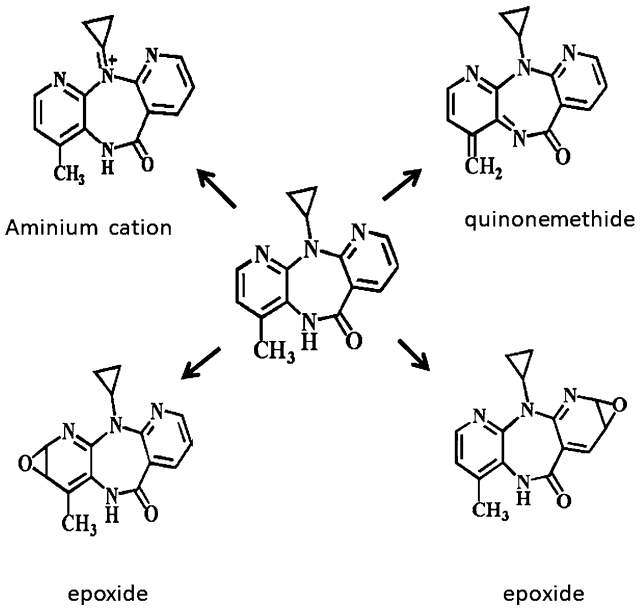


Fig. 5 Possible bioactivation pathways for nevirapine

4.2 Isoniazid

Isoniazid (INH) is still the most widely used drug in the treatment of tuberculosis (TB) and has high activity against *Mycobacterium tuberculosis*, although resistant strains have emerged. INH is used in combination with drugs such as rifampicin and pyrazinamide to reduce the chance of inducing resistant strains of the mycobacterium.

INH causes two major adverse reactions: hepatitis and peripheral neuropathy. The incidence and severity of the adverse drug reactions are related to dose and duration of therapy. Toxicity may be delayed by several weeks. A minor asymptomatic increase in liver aminotransferases (less than threefold) is seen in 10–20% of patients within the first 2 months of therapy, whereas fatal hepatitis is seen in less than 1% of patients. Mortality is greater than 10% in patients with jaundice (Mitchell et al. 1976b; Zimmerman 1990). INH typically produces diffuse massive necrosis or chronic hepatitis. Clinical features resemble acute viral-induced hepatitis. Anorexia, fatigue, nausea, and vomiting are the usual prodromal features, but jaundice and dark urine may be the first evidence of injury (Ishak and Zimmerman 1995). Combination therapy is a risk factor for hepatitis, although formal studies evaluating the mechanisms of this have not been undertaken. Interestingly, of the three anti-TB compounds, it has been suggested that pyrazinamide is the most hepatotoxic, with a rate of hepatitis three and five times higher than that of rifampicin and INH, respectively (Durand et al. 1995; Ormerod et al. 1996; Schaberg et al. 1996).

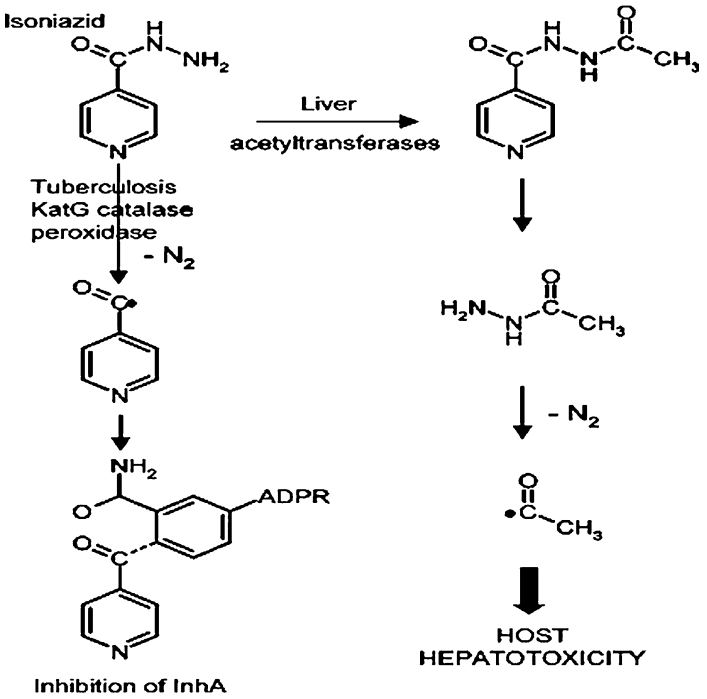


Fig. 6 Metabolic activation of isoniazid. Reactive metabolites are responsible for the pharmacology and toxicology of isoniazid. In *Mycobacterium tuberculosis*, generation of the isonicotinoyl radical leads to the formation of an adduct with NADH, which in turn inhibits an enoyl-acyl carrier protein reductase (InhA) (Rawat et al. 2003; Rozwarski et al. 1998)

Studies in the rat (Nelson et al. 1976) and rabbit (Sarich et al. 1995, 1996), along with in vitro studies, indicate that INH undergoes acetylation to give N-acetylisoniazid. This is hydrolyzed to acetylhydrazine (Lauterburg et al. 1985a, b; Preziosi 2007; Ryan et al. 1985; Sarich et al. 1996), which undergoes bioactivation by P450 to give the acetyl radical (Fig. 6); a reactive species identified by trapping as a glutathione conjugate (Nelson et al. 1976). Precisely how such a reactive intermediate induces hepatocyte damage remains to be elucidated, as do the reasons for the increased incidence of hepatotoxicity when combination therapy is used. However, it has been found that rifampicin exacerbates INH-induced toxicity in human though not in rat hepatocytes (Shen et al. 2008). In another study, GST-T1 homozygous null polymorphism was found to be a risk factor for antituberculosis drug-induced hepatotoxicity in humans (Leiro et al. 2008). Target proteins have not been identified for the reactive metabolite formed from INH. To date, there is no convincing clinical or laboratory evidence to suggest an immunological mechanism.

Interestingly, bioactivation plays a role in the pharmacology of INH, with elimination of nitrogen being the driving force for the formation of an isonicotinoyl radical intermediate (Fig. 6). INH can thus be considered a prodrug, which is

activated by the mycobacterial catalase-peroxidase enzyme KatG. The product of bioactivation forms a covalent adduct with NADH, which is an inhibitor of InhA, an enoyl-acyl carrier protein reductase that is involved in the biosynthesis of mycolic acids present in the mycobacterium cell wall (Rawat et al. 2003; Rozwarski et al. 1998).

4.3 Amodiaquine

Amodiaquine (AQ), a 4-aminoquinoline antimalarial which is effective against many chloroquine-resistant strains of *Plasmodium falciparum*, produces idiosyncratic toxicity in man. Clinical use of AQ has been severely restricted because of life-threatening agranulocytosis and hepatotoxicity seen in about 1 in 2,000 patients during prophylactic administration (Hatton et al. 1986; Larrey et al. 1986; Neftel et al. 1986). The metabolism of AQ has been clearly implicated and there is potential for designing out the toxicity.

AQ, like APAP, undergoes extensive bioactivation to an electrophilic quinoneimine metabolite (AQQI) (Fig. 7), which has been detected in vivo in rats (Harrison et al. 1992) and in vitro (Jewell et al. 1995). However, unlike APAP, AQ can undergo autoxidation in solution. Subsequent oxidative stress or conjugation to

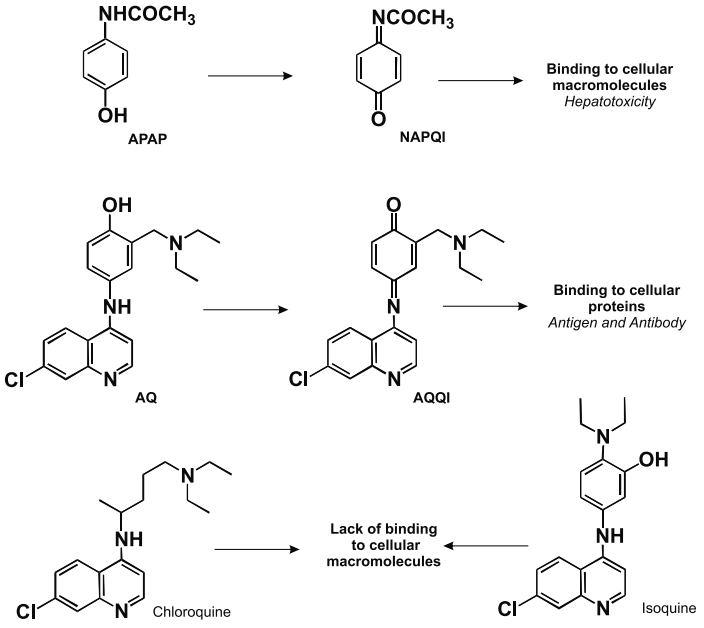


Fig. 7 Metabolic activation of acetaminophen (APAP) and amodiaquine (AQ) to their respective quinoneimines. The structures of chloroquine and isoquine demonstrate their relative lack of chemical reactivity

cysteinyl sulfhydryl groups on proteins is likely to be involved in the induction of toxicity by either cytotoxic or immunological mechanisms (Clarke et al. 1990; Maggs et al. 1988). IgG antibodies, which recognize the 5′-cysteinyl group, have been detected in patients with adverse reactions to AQ (Clarke et al. 1990). However, the factors determining individual susceptibility are unknown.

Modification of AQ has been carried out in an attempt to design a safe replacement for AQ. Interchange of the 3′ hydroxyl and the 4′ Mannich side-chains provided a new series of analogs that cannot form toxic quinoneimine metabolites via cytochrome P450-mediated metabolism (Fig. 7). The prototype, isoquine, emerged as an AQ regioisomer that cannot form toxic metabolites by simple oxidation, and which is potent against chloroquine-resistant parasites in vitro. In sharp contrast to AQ, isoquine (and phase I metabolites) undergoes clearance by glucuronidation instead of GSH conjugation (O'Neill et al. 2003). Isoquine has the potential to be a cost-effective and safer alternative to AQ. Knowledge of AQ's mechanism of toxicity was essential for this development.

5 Conclusion

Clearly, drug metabolism can play an important initiating step in the development of hepatotoxicity, and chemically reactive metabolites have been correlated with the hepatotoxicity of most of the drugs. For this reason, it appears that, if a pharmacophore replaces the potential toxicophore in a new chemical entity, toxicity can be prevented. Additionally, the propensity of a new chemical entity to undergo bioactivation needs to be determined at an early stage of the drug developmental process. High throughput screens have been developed to identify reactive metabolites early in lead optimization based inter alia on covalent binding potential and glutathione adduct formation (Caldwell and Yan 2006; Kalgutkar and Soglia 2005). A retrospective analysis of covalent binding of drugs associated with idiosyncratic drug toxicity (Takakusa et al. 2008) concluded that such studies could be informative in terms of risk assessment. However, it is clear that such studies, when conducted in the preclinical phase of drug development, cannot predict the risk of drug toxicity. Moreover, the use of transgenic animals has provided enhanced definition of the role of P450 enzymes in chemical-induced toxicity. However, these studies, at best, predict the potential of a compound to undergo bioactivation, with the ultimate effect on the liver dependent upon a range of factors such as dose, fractional clearance, rate of metabolic turnover, systemic exposure, activation of cellular signaling pathways, and host-specific factors. In particular, the administration of higher mass doses of drug has been associated with a greater incidence of ADRs (Lammert et al. 2008). Therefore, the development of improved physiological test systems based on information gained from studies with model hepatotoxins such as APAP are required that encompass both chemical and biological factors associated with hepatotoxicity to try and screen for rare but often fatal idiosyncratic hepatotoxicities earlier on in drug development. Finally, the methodology used to

identify protein modification through the irreversible association of radiolabeled compound to general microsomal protein has not changed since the 1960s. Further utilization of shotgun proteomic mass spectrometric techniques combined with LC-MS-MS now allow the identification of adducted amino acids within peptides (Koen et al. 2006). Identification of critical residues within proteins that become adducted, leading to loss of protein function, enhances the biological information that can be obtained from these outdated irreversible binding assays.

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The Role of Cytokines in the Mechanism of Adverse Drug Reactions

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Contents

Abstract Cytokines are thought to play a role in acute and/or immune-mediated adverse drug reactions (ADRs) due to their ability to regulate the innate and adaptive immune systems. This role is highly complex owing to the pluripotent

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nature of cytokines, which enables the same cytokine to play multiple roles depending on target organ(s) involved. As a result, the discussion of cytokine involvement in ADRs is organized according to target organ(s); specifically, ADRs targeting skin and liver, as well as ADRs targeting multiple organs, such as drug-induced autoimmunity and infusion-related reactions. In addition to discussing the mechanism(s) by which cytokines contribute to the initiation, propagation, and resolution of ADRs, we also discuss the usefulness and limitations of current methodologies available to conduct such mechanistic studies. While animal models appear to hold the most promise for uncovering additional mechanisms, this field is plagued by a lack of good animal models and, as a result, the mechanism of cytokine involvement in ADRs is often studied using less informative in vitro studies. The recent formation of the *Drug-Induced Liver Injury Network*, whose goal is collect thousands of samples from drug-induced liver injury patients, has enormous potential to advance knowledge in this field, by enabling large-scale cytokine polymorphism studies. In conclusion, we discuss how further advances in this field could be of significant benefit to patients in terms of preventing, predicting, and treating ADRs.

Keywords Cytokines · Immune-mediated adverse drug reactions · Skin · Drug-induced autoimmunity · Drug-induced liver injury

1 Introduction

Cytokines are thought to play a role in adverse drug reactions (ADRs) due to their ability to regulate both innate and adaptive immune responses. The role of cytokines in ADRs can vary dramatically because cytokines exert multiple effects depending on the type of target cell and are believed to be involved in a variety of ADRs affecting both multiple (drug-induced hypersensitivity and autoimmunity) and single (liver and skin) organs.

2 What Are Cytokines?

Cytokines are a group of soluble, low molecular weight messenger proteins secreted by virtually all the cells in the body, but particularly by cells of the immune system. As such, the main function of cytokines is to regulate immune and inflammatory responses. Cytokines control both the intensity and type of immune response mounted by stimulating/suppressing the different cells involved in an immune response. Although each cytokine exerts a specific effect on the target cell, they rarely act alone. Usually, the target cell is exposed to many cytokines that have synergistic, antagonistic, or redundant effects. Cytokines exert these effects by binding to a specific receptor on target cells, which induces or inhibits gene

expression. The binding of cytokines to the target receptor occurs with high affinity and, as a result, the actions of cytokines are highly potent. For this reason, cytokine release is very tightly regulated.

Table 1 lists the main cytokines implicated in ADRs and provides a brief outline of their functions. While this list identifies the major functions of each cytokine, it is by no means complete. There is an excellent resource (Paul 2003) to obtain additional information on cytokines and their functions.

3 Cytokines in Skin ADRs

Current evidence suggests that cytokines play an important role in many skin ADRs (Naisbitt et al. 2007; Roychowdhury and Svensson 2005), ranging from mild rashes to life-threatening reactions such as Steven's Johnson Syndrome (SJS) or toxic epidermal necrolysis (TEN). Skin reactions can be broadly classified into two major categories based on time-to-onset: immediate (within 24 h) and delayed reactions (greater than 48 h). Because the type of immune response and the cytokines involved in immediate versus delayed skin reactions varies, each category will be discussed separately.

3.1 Immediate Skin ADRs

Immediate skin reactions occurring within 24 h are mainly IgE-mediated, and are often described as 'true allergic' or hypersensitive immune responses (Guglielmi et al. 2006a). The drugs most commonly associated with this type of reaction are the β-lactam antibiotics. Clinical symptoms range from mild urticaria and erythema and can be associated with anaphylaxis (Guglielmi et al. 2006a).

IgE-mediated skin reactions arise as a consequence of the haptenization (formation of drug–protein conjugates) of cell and serum proteins by β-lactams. Subsequent recognition of the hapten by IgE antibodies located on mast cells and basophils leads to the rapid release of histamine and other inflammatory mediators involved in the allergic reaction (Antunez et al. 2006). T cell helper (Th2) cytokines are thought to be involved in immediate skin reactions due to their ability to modulate antibody responses (Guglielmi et al. 2006b; Hershey et al. 1997; Mayorga et al. 2006). They include IL-4 and IL-13, which promote B cell proliferation, differentiation, and isotype class-switching to IgE antibodies (Paul 2003). In contrast, IFN-γ, a Th1 cytokine, negatively regulates antibody-mediated reactions by inhibiting IgE production (Gao et al. 1999; Nelms et al. 1999).

To investigate the role of these cytokines in immediate skin reactions in humans, single nucleotide polymorphism (SNP) studies were conducted within small subsets of patients known to have allergies to β-lactams (Guglielmi et al. 2006a; Qiao et al. 2007; Yang et al. 2005). SNPs within the IL-4, IL-13, IFN-γ, and their respective

Table 1 Major functions of cytokines involved in ADRs

Cytokine	Secreted by	Function
Interleukin (IL)-1	• Monocytes • Activated macrophages • B cells • Dendritics	• Inflammatory cytokine • Recruits macrophages and neutrophils to site of infection • Activates vascular endothelium • Induces acute-phase protein production by hepatocytes
IL-2	• T cells	• T cell growth factor • Activates NKT and NK cells
IL-4	• Th2 cells • Mast cells • NKT and NK cells	• Activates B cells, induces antibody class-switching, and upregulates MHCII expression • Activates macrophages by inducing MHCII expression and increasing phagocytic activity
IL-6	• Monocytes • Activated macrophages • CD4 T cells	• Activates T cells • Increases antibody production by B cells • Induces acute-phase protein production by hepatocytes
IL-10	• Activated macrophages • Th2 cells • Sinusoidal endothelial cells • Hepatocytes	• Inhibits macrophage activation • Inhibits Th1 cells
IL-12	• Activated macrophages • B cells	• Activates NK cells • Induces differentiation of CD4 T cells into Th1 cells
IL-13	• Th2 cells • Kupffer cells • NKT cells	• Inhibits macrophage activation • Activates B cells, induces antibody class-switching • Upregulates MHCII expression
IL-18	• Keratinocytes • Macrophages	• Activates IFN-γ production by T cells and NK cells
IL-21	• CD4 T cells	• Activates antigen-specific CD8 T cells and NKT cells • Increases the production of IgG by mature B cells
Interferon (IFN)-γ	• Th1 cells • NKT and NK cells	• Inhibits proliferation of Th2 cells • Activates macrophages • Activates NK cells • Inhibits viral replication and promotes MHCI expression in virus-infected cells
Macrophage inflammatory protein (MIP-2)	• Monocytes • Activated macrophages • Endothelial cells	• Chemotactic factor that recruits neutrophils and basophils to site of inflammation
Macrophage migration inhibitory factor (MIF)	• Macrophages • T cells	• Inflammatory cytokine • Activates macrophages and T cells • Involved in IgE synthesis, insulin release, cell growth, and apoptosis

(*continued*)

Table 1 (continued)

Cytokine	Secreted by	Function
Monocyte chemoattractant Protein (MCP-1)	• Monocytes • Endothelial cells	• Chemoattractant protein that recruits monocytes, T lymphocytes, eosinophils, and basophils to the site of inflammation
Transforming growth factor (TGF)-β	• CD4 T cells • Monocytes • Hepatic stellate cells	• Inhibits macrophage and T cell activation • Induces antibody class-switching to IgA • Activates neutrophils • Active in wound healing process
Tumor necrosis factor (TNF)-α	• Monocytes • Activated macrophages • CD4 and CD8 T cells	• Inflammatory cytokine • Activates vascular endothelium • Activates macrophages and stimulates nitric oxide (NO) production • Induces fever and septic shock

receptors' (R) were analyzed based on their known association with other types of IgE-mediated allergies (Gao et al. 1999; Guglielmi et al. 2006b; Hershey et al. 1997; Mayorga et al. 2006; Naisbitt et al. 2007). In addition, SNPs within the IL-21 and IL-10 promoter regions were analyzed due to their ability to regulate antibody responses. Specifically, IL-21 has been shown to enhance humoral immune responses by promoting IL-4-driven IgE production (Ozaki et al. 2002; Wood et al. 2004), while IL-10 is known to downregulate humoral immune responses by opposing the actions of IL-4 (Reuss et al. 2002; Turner et al. 1997). The results obtained from two independent studies were somewhat conflicting. The first group found an association between IL-4Rα Gln576 and β-lactam-specific IgE allergy within a Chinese population (Qiao et al. 2005). This study also reported decreased frequency of an IL-10 allele (−1082) that is associated with decreased IL-10 levels in patients with β-lactam-specific IgE antibodies (Qiao et al. 2007). No associations between IL-13 polymorphisms and β-lactam allergies were observed in this study (Yang et al. 2005). In contrast, the second group did not observe an association between IL-4 or IL-4Rα polymorphisms and immediate β-lactam allergic reactions within a French population (Guglielmi et al. 2006a). However, when the analysis was limited to atopic patients (defined by one or more positive skin prick tests to irrelevant antigens), an association between IL4Rα Ile75 and β-lactam allergy was found in atopic women. They also found an association between immediate β-lactam allergies and two linked SNPs within the IL-10 promoter region, -819C and -592C, in atopic women. No associations were observed in atopic males, nor were there any associations between IL-21 polymorphisms and β-lactam allergies (Guglielmi et al. 2006a). The ability of the first group (Qiao et al. 2005, 2007) to associate SNPs within the IL-4Rα and IL-10 promoter region with immediate β-lactam allergies without discriminating between atopic and nonatopic patients might be due to the fact that the Chinese population is likely more genetically homogenous than the French population (Guglielmi et al. 2006a). Despite the differences, these studies suggest that polymorphisms in IL-4 and IL-10 can influence susceptibility to immediate skin reactions to β-lactams.

3.2 *Delayed Skin ADRs*

Delayed skin ADRs can occur anywhere from 48 h to several weeks after initiating drug therapy (reviewed in Roychowdhury and Svensson 2005). Numerous drugs from many pharmaceutical classes have been associated with delayed skin reactions. The drugs most commonly associated with these reactions are antimicrobials (e.g., sulfonamides), nonsteroidal antiinflammatory (NSAID) agents (e.g., diclofenac), and anticonvulsants (e.g., carbamazepine). The clinical manifestations of delayed skin reactions are highly variable, ranging from mild maculopapular reactions to severe and life-threatening SJS and TEN. Although delayed skin reactions are often sub-classified based on these clinical manifestations (reviewed in Khan et al. 2006), the difficulties associated with sub-classifying such clinically diverse reactions often result in conflicting reports between laboratories.

3.2.1 Maculopapular Exanthema

The most common type of delayed skin reaction appears as wide spread erythematosus macules (flat, discolored areas) and/or papules (small red bumps) on the trunk within 1–2 weeks of drug initiation (reviewed in Khan et al. 2006). These types of reactions are generally mild and disappear shortly after discontinuing the drug. Histologically, necrotic keratinocytes are surrounded by cellular infiltrates consisting of T cells and eosinophils. IL-5 is thought to be a major cytokine involved in this type of skin ADR due to its ability to recruit eosinophils to the site of inflammation (Pichler et al. 1997).

3.2.2 Fixed Drug Eruptions (FDE)/Exanthema Pustulosis

This type of skin reaction consists of pustules (small, pus-filled lesions) limited to specific areas of the body that appear within 1–2 days of initiating drug treatment. These reactions are considered intermediate in severity and resolve quickly upon discontinuation of therapy (reviewed in Khan et al. 2006). Mononuclear cell infiltrates containing lymphocytes, neutrophils, and eosinophils are found at the dermal-epidermis junction. Isolated T cells secreting IL-8 and IFN-γ suggest a role for these cytokines in FDE (Britschgi et al. 2001; Roujeau 2006). IL-8 is likely important for recruiting neutrophils to the site of inflammation, while IFN-γ could enhance T cell cytotoxicity by upregulating MHCII expression on keratinocytes (Friedmann et al. 1994). In addition, after drug rechallenge, memory CD8 T cells secreting high levels of IFN-γ were detected in blisters prior to the onset of keratinocyte apoptosis, suggesting a direct role for IFN-γ in the initiation of FDE (Roujeau 2006).

3.2.3 SJS/TEN

SJS/TEN are considered the same disease with different degrees of severity. While rare, these reactions are life-threatening diseases with mortality rates as high as 5% for SJS and 30% for TEN (Roujeau and Stern 1994). Clinically, these reactions appear as widespread blistering on the skin and mucus membranes accompanied by skin detachment (Roujeau 2006). Histological findings reveal keratinocyte necrosis in the epidermis along with inflammatory cell infiltrates (Villada et al. 1992). Although cytotoxic CD8 drug-specific T cells found within the blisters mediate keratinocyte killing via a perforin/granzyme pathway (Nassif et al. 2004a), the blister fluid contains high levels of IFN-γ and TNF-α (Nassif et al. 2004b). TNF-α likely contributes to skin lesions by inducing keratinocyte apoptosis (Groves et al. 1995; Paquet et al. 1994). In addition, TNF-α and IFN-γ have both been shown to enhance keratinocyte cytotoxicity by upregulating MHCII expression (Friedmann et al. 1994).

3.2.4 Drug Hypersensitivity Syndrome (DHS)

The term DHS refers to skin rash with systemic symptoms, often involving liver toxicity, fever, and eosinophilia. This type of reaction usually appears 3–4 weeks after initiating drug therapy (Roujeau 2006). Most of the research on DHS has been conducted by studying drug-specific T cells isolated from the peripheral blood of patients both during and after resolution of the reaction. This in vitro approach has failed to identify a predominant Th1 or Th2 cytokine profile for drug hypersensitivities (Mayorga et al. 2006), as IFN-γ-, IL-4-, or both IFN-γ- and IL-4-secreting T cells have been isolated from DHS patients (Naisbitt et al. 2003). One possibility is that both humoral and cell-mediated immune responses could be involved in this syndrome. Alternatively, the results could be confounded by clinical heterogeneity or by the fact that T cells are isolated from patients at different phases of disease progression (Mayorga et al. 2006). Another research approach has been to monitor serum cytokine levels throughout the course of disease (Choquet-Kastylevsky et al. 1998). This has led to the discovery that IL-5 levels are only elevated in patients with eosinophilia, suggesting that this cytokine is a key recruiter of eosinophils in DHS.

3.2.5 Role of Cytokines in Delayed Skin ADRs

With the exception of DHS, the predominant cytokines at the site of tissue damage are characteristic of each sub-category of delayed skin reactions suggesting that cytokines might play an important role in determining the clinical manifestations of skin reactions. Cytokines might also play a role in controlling the severity of delayed skin ADRs. When comparing mild versus severe delayed skin rashes, the severity of the reactions positively correlated with IFN-γ and TNF-α levels (Posadas et al. 2002). There is also evidence to suggest that cytokines might play a

regulatory role in delayed skin reactions by limiting the extent of injury. Specifically, the presence of IL-10 in FDE blisters is thought to limit the extent of injury associated with this type of skin ADR (Roujeau 2006). Although these results suggest that cytokines are involved in delayed skin reactions, it is clear that additional research is necessary. Attempts to study the role of cytokines in skin ADRs using animal models will be discussed next.

3.3 Animal Models of Delayed Skin Reactions

3.3.1 Contact Hypersensitivity Syndrome (CHS) Model

To date, most of the animal research on delayed skin reactions has been conducted using the CHS model, which involves a classic T cell-mediated immune response directed against topically applied skin sensitizing agents. The major difference between the CHS model and delayed skin reactions in humans is that the skin sensitizing agents are applied topically while the drugs associated with skin ADRs in humans are mostly administered systemically. Despite this important difference, the CHS model has been widely used as a model of delayed skin ADRs because the two reactions are thought to involve similar mechanisms.

Evidence suggests that cytokines play a role in many aspects of CHS including the initiation, propagation, and regulation of the immune response (reviewed in Khan et al. 2006). During the sensitization phase, sensitizing agents (e.g., dinitrochlorobenzene) chemically bind to keratinocytes resulting in cell stress and the release of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-18 (Cumberbatch et al. 2001). These cytokines are important for the activation and mobilization of Langerhan's cells, which then migrate to the draining lymph nodes to activate antigen-specific T cells. During the effector phase, activated T cells travel back to the skin where they secrete high levels of IFN-γ and TNF-α (Piguet et al. 1991; Xu et al. 1996). As noted previously, both these cytokines can contribute to keratinocyte cytotoxicity by upregulating MHCII expression (Friedmann et al. 1994). TNF-α can also induce keratinocyte apoptosis by interacting with the TNF death receptor (Groves et al. 1995; Paquet et al. 1994). Neutralization of either of these cytokines limits the extent of tissue injury (Fong and Mosmann 1989; Piguet et al. 1991). In addition, Th2 cytokines are thought to play a regulatory role in CHS (Xu et al. 1996). The depletion of IL-4- and IL-10-secreting CD4 cells prior to drug sensitization increases the extent of CHS-mediated tissue injury (Xu et al. 1996). Together, these results suggest that cytokines play an important role in immune responses directed against a foreign chemical in the skin.

3.3.2 Nevirapine-Induced Skin Rash in Brown Norway Rats

The main obstacle to studying the mechanism of delayed skin ADRs is a lack of animal models. With the exception of nevirapine (Shenton et al. 2005), the vast

majority of drugs associated with these reactions in humans do not produce immune-mediated skin rash in animals. Consequently, this model could be important to the study of cytokines in skin ADRs. Although not much work has been done on this aspect of the model to date, high levels of IFN-γ are detected in the serum upon the first day of nevirapine rechallenge (Popovic et al. 2006). Additional studies examining cytokine expression at the site of tissue injury could provide valuable insight into the role of cytokines in initiating and regulating delayed skin reactions.

3.4 Human Studies of Delayed Skin ADRs

Studying the role of cytokines in delayed skin reactions in humans is complicated by the low incidence of these reactions, which often makes it difficult to recruit sufficient patients to conduct clinical studies. Consequently, research is most often conducted by analyzing drug-specific T cells cloned from patients long after the disease has resolved, as indicated earlier. The use of these cloned T cells is not ideal for several reasons. Namely, the T cells are collected from the peripheral blood and not the site of tissue injury; hence, the cytokines secreted by these cells cannot be directly linked to the reaction in the skin. In addition, the T cell clones are often cloned for several generations prior to activation, which could confound the data as the method by which T cells are activated in vitro might differ significantly from what occurs in vivo (Mayorga et al. 2006). Cloning peripheral T cells after disease resolution could also skew the data to suggest an inflated role for CD4 cells in delayed skin ADRs, as more CD4 cells might persist as memory cells than CD8 cells (Seder and Ahmed 2003). For these reasons, it is preferable to conduct research in patients whose disease has not yet resolved (Mayorga et al. 2006). Cytokine production could be analyzed directly at the site of injury by taking small tissue samples over a time course. By doing so, invaluable information regarding the role of cytokines in disease progression could be obtained.

4 Cytokines in Drug-Induced Autoimmunity

Cytokines have also been implicated in the mechanism of drug-induced autoimmune syndromes, which result from immune responses directed against autoantigens as a consequence of drug-treatment. The symptoms of drug-induced autoimmunity (DIAI) most often resemble systemic autoimmune diseases, such as idiopathic systemic lupus erythematosus (SLE), due to the presence of autoantibodies, dermatitis, glomerulonephritis, and myalgia. In contrast, other types of DIAI, such as autoimmune hemolytic anemia or autoimmune hepatitis, are more organ-specific. In the past, the systemic type of DIAI was not considered a serious, life-threatening ADR, as the symptoms were generally mild and reversible upon discontinuation of drug (reviewed in Olsen 2004). The drugs most often associated

with DIAI at that time were procainamide, hydralazine, α-methyldopa, and D-penicillamine. The discovery of new classes of drugs (TNF-α blockers, recombinant interferon-α, and minocycline) that induce severe DIAI with life-threatening symptoms, such as vasculitis and pulmonary failure, has led to renewed interest in the mechanistic study of DIAI (Geddes 2007; Olsen 2004). As most of the research to date has focused on the systemic type of DIAI, the role of cytokines will be discussed in this context.

4.1 Immune Modulator-Induced DIAI

It has recently been discovered that immune modulating biologic agents can induce DIAI in a small percentage of patients (Pichler 2006; Weber 2004). Currently, there are no animal models to study the mechanism of these diseases, and, as a result, mechanistic insight into the role of cytokines has been derived from animal models of idiopathic SLE.

4.1.1 TNF-α Blockers

While TNF-α blockers such as etanercept and infliximab are highly effective treatments for rheumatoid arthritis, these drugs have also been associated with a significant number of DIAI cases, a few of which have been life-threatening (Kollias and Kontoyiannis 2002; Olsen 2004). TNF-α blockers are thought to induce DIAI by shifting the Th1/Th2 balance from the Th1-mediated response characteristic of rheumatoid arthritis to a Th2 response (Jarrett et al. 2003). In support of this hypothesis, TNF-α blocking antibodies exacerbated autoimmunity in an animal model of idiopathic SLE (Segal et al. 2001). Similarly, recombinant TNF-α treatment was found to protect NZB/W and NOD mice from idiopathic SLE (Jacob et al. 1991; Satoh et al. 1989).

4.1.2 Recombinant IFN-α

Recombinant IFN-α is a relatively new therapy designed to treat certain types of malignancies and is also useful in the management of hepatitis C. DIAI is a rare side effect of treatment, characterized by ANA and vasculitis with occasional renal involvement. Recombinant IFN-α is thought to induce DIAI in susceptible patients by directly stimulating the immune system (Olsen 2004; Pichler 2006). This stimulation could involve IFN-α-induced upregulation of MHCI and MHCII expression on antigen presenting cells (Rifkin et al. 2005). IFN-α might also induce DIAI by extending the life of B cells through its ability to increase BcL-2 oncogene expression (Chaouchi et al. 1994).

4.2 Animal Models of DIAI

Unlike most ADRs that are mediated by the adaptive immune system, there are some very good animal models of systemic DIAI involving autoantibody production. While these models have revealed that there are multiple mechanisms by which drugs initiate autoimmunity, it is clear that Th2 cytokines, such as IL-4 and IL-6, play an important role in all types of systemic DIAI (Kroemer et al. 1996; Qasim et al. 1997; Yung et al. 1995). These cytokines mediate the humoral aspects of DIAI, including autoantibody production and B cell proliferation and differentiation. Neutralizing either IL-4 (Ochel et al. 1991) or IL-6 (Finck et al. 1994) decreases the severity of DIAI in animal models. In contrast, treatments that enhance Th2 immunity by inhibiting Th1 cytokines such as TNF-α increase the severity of DIAI (Ishida et al. 1994).

4.2.1 Procainamide- and Hydralazine-Induced Autoimmunity in Mice

Due to their inhibitory effects on DNA methylation, procainamide and hydralazine can induce autoreactive T cells by upregulating the expression of lymphocyte function-associated antigen-1 (LFA-1), which causes the cells to proliferate in response to normally subthreshold stimuli, including self (Yung et al. 1995). As a result, the adoptive transfer of T cells modified by in vitro treatment with procainamide or hydralazine induces an autoimmune disorder characterized by antinuclear antibodies (ANAs) and immune complex glomerulonephritis in naïve mice (Quddus et al. 1993).

These autoreactive T cells secrete the Th2 cytokines, IL-4 and IL-6 (Quddus et al. 1993). Interestingly, the autoimmune disease was more severe when autoreactive cloned Th2 T cells were transferred to AZR mice than when autoreactive polyclonal T cells were transferred to DBA mice (Quddus et al. 1993; Yung et al. 1995). While genetic background could play a role in determining disease susceptibility, it is also possible that the presence of Th1 IFN-γ-secreting T cells within the polyclonal population transferred to DBA mice could dampen the Th2-mediated autoimmunity (Quddus et al. 1993).

4.2.2 D-Penicillamine (D-Pen)-Induced Autoimmunity in the Brown Norway (BN) Rat

One of the most important characteristics of this model is that oral dosing with D-pen induces autoimmune disease, thereby mimicking what occurs clinically. The clinical manifestations are also similar to those observed in humans; specifically, both diseases are characterized by the production of ANAs, immune-complex glomerulonephritis, dermatitis, and arthralgia (Donker et al. 1984; Enzenauer et al. 1990). Another unique feature of this model is that the disease is idiosyncratic

in both humans and rats. While a high percentage of patients taking drugs associated with DIAI develop ANA, only a fraction of those patients develop autoimmune disease (Mitchell et al. 1990). Similarly, despite the fact that IgE levels rise in 100% of BN rats treated with D-pen, only 60–80% of BN rats develop autoimmune disease (Tournade et al. 1990).

Susceptibility to D-pen-induced autoimmunity is influenced by a genetic propensity towards Th2 immune responses. The BN rat used in this model is highly susceptible to Th2-mediated diseases, such as DIAI, and is resistant to Th1-mediated diseases, such as experimental autoimmune encephalomyelitis (EAE) (Fournie et al. 2001; Happ et al. 1988). The opposite is true of the Lewis rat, which is resistant to DIAI but highly susceptible to EAE. Mechanistic evidence to support the role of cytokines in the susceptibility to DIAI has been found in the related model of mercuric chloride ($HgCl_2$)-induced autoimmunity in the BN rat. In vitro incubation of BN splenocytes with $HgCl_2$ results in IL-4 production, while incubation of Lewis splenocytes with $HgCl_2$ has no effect on IL-4 production (Delespesse et al. 1991). In vivo evidence is derived from the F1 offspring of BN and Lewis rats, which are susceptible to both DIAI and EAE (Saoudi et al. 1993). In this F1 generation, pretreatment with $HgCl_2$ provides resistance to EAE and skews cytokine production towards a Th2 response. These findings suggest that genetic control of the Th1/Th2 balance might be an important factor in determining the susceptibility of patients to DIAI.

The ability to induce immune tolerance to D-pen by pretreating BN rats with low dose D-pen prior to high dose D-pen has yielded additional evidence that cytokines might play a role in determining susceptibility to DIAI (Masson and Uetrecht 2004). Specifically, low dose tolerance to D-pen is associated with increased expression of the regulatory cytokines, IL-10 and TGF-β, in splenic $CD4^+$ and $CD8^+$ T cells (Donker et al. 1984; Masson and Uetrecht 2004). Both of these cytokines have been shown to suppress disease in other models of autoimmunity (Blenman et al. 2006; Rubtsov and Rudensky 2007). Th1 cytokines also appear to be involved in the mechanism of immune tolerance to D-pen, as low dose pretreatment increased IFN-γ expression in $CD8^+$ T cells (Masson and Uetrecht 2004). These observations indicate that regulatory and Th1 cytokines might be at least partially responsible for the resistance to D-pen-induced autoimmunity.

4.2.3 Disruption of Central Tolerance by a Reactive Metabolite of Procainamide Induces Autoimmunity in Mice

In this model, the injection of a reactive metabolite of procainamide into the thymus of young mice disrupts central tolerance and leads to the production of ANAs (Kretz-Rommel et al. 1997). To date, the role of cytokines has not been investigated in this model.

5 Cytokines in Infusion-Related Toxicities

The adverse effects associated with infusion-related toxicities are induced by the massive release of inflammatory cytokines following intravenous administration of certain drugs. The drugs most commonly associated with infusion-related toxicities include amphotericin B and rituximab, an anti-CD20 antibody (Rogers et al. 2000). The syndrome is typically characterized by mild symptoms, such as fever, chills, nausea, vomiting, and headache. In rare cases, these reactions can be severe, or even fatal, due to respiratory failure and/or hypotension (Gutierrez et al. 2006). The onset of the reaction ($\approx$ 3 h) coincides with the peak release of IL-1β and TNF-α (Gutierrez et al. 2006), and the severity of the reaction positively correlates with cytokine levels (Bienvenu et al. 2001). The clinical symptoms are mediated by the inflammatory actions of IL-1β and TNF-α, which can induce fever and chills by directly stimulating the hypothalamus (Kuby 1997), and can cause hypotension by enhancing nitric oxide production (Cattell et al. 1993; Liew et al. 1990). TNF-α can also perpetuate the inflammatory response by stimulating leukotriene production (Cattell et al. 1993). These observations have led to the hypothesis that TNF-α blockers might be an effective therapy for severe cases of infusion-related toxicities.

Cytokine release is thought to result from macrophage activation following drug in fusion (Pai et al. 2005). Amphotericin B induces IL-1β, TNF-α, and IL-8 release by interacting with Toll-like receptor 2 (TLR2) on macrophages (Rogers et al. 1998, 2000; Sau et al. 2003). In the case of rituximab, it has been speculated that the anti-CD20 antibody induces cytokine release by interacting with inhibitory Fcγ receptors on macrophages (Clynes et al. 2000).

6 Cytokines in Drug-Induced Liver Injury (DILI)

The role of cytokines in determining susceptibility to ADRs has been most fully explored in the liver. The liver produces both hepatoprotoxicant and hepatoprotective cytokines in response to injury, and it is thought that the balance between these cytokines affects an individual's predisposition to DILI. The low incidence of DILI has led to the hypothesis that a cytokine imbalance that promotes a protoxicant immune response may increase the risk of DILI. While the incidence is low (0.01–1%), for most drugs that cause liver injury, DILI is actually the most common cause of acute liver failure in the United States due to the relatively high number of drugs associated with hepatotoxicity (Bissell et al. 2001). The injury can be cholestatic, hepatic, or mixed, and typically resolves after stopping the drug (Liu and Kaplowitz 2002). For the most part, these reactions are idiosyncratic in that they are unpredictable and are highly host-dependent. In addition, both allergic and nonallergic processes have been implicated in the mechanism of DILI (Gunawan and Kaplowitz 2007).

Evidence, such as antidrug antibodies, drug-reactive T cells, and faster time to onset upon rechallenge, suggests that at least some types of DILI (e.g., halothane, tienilic acid, dihydralazine, and diclofenac) are immune-mediated (Liu and Kaplowitz 2002). This type of DILI is often referred to as "allergic hepatitis" due to the ability of the drug to initiate an adaptive immune response by acting as a hapten. In allergic hepatitis, it is generally believed that a small degree of liver injury is necessary to initiate a drug-specific immune response in the liver (Pessayre 1995). The initial liver injury is thought to be induced, at least in part, by drug–protein adducts, which are formed following the covalent modification of endogenous proteins by metabolically activated drugs (Gunawan and Kaplowitz 2007). The subsequent release of these drug–protein adducts from dying hepatocytes (Bourdi et al. 2001) is thought to initiate drug-specific allergic hepatitis by activating the adaptive immune system. Cytokines could play an important role in allergic hepatitis by promoting either a tolerogenic or pathogenic adaptive immune response to drug–protein adducts (Bourdi et al. 2002a).

In contrast, some types of DILI (e.g., APAP-induced liver injury, AILI) do not appear to involve an allergic mechanism. In this case, liver injury may be potentiated by a down-stream inflammatory response in which cytokines modulate the extent of inflammation (Bourdi et al. 2002a, 2007; James et al. 2003, 2005b; Yee et al. 2007).

6.1 Animal Models of DILI

It is difficult to study the mechanism(s) of DILI due to a lack of animal models. While there is evidence to suggest that many types of DILI involve the adaptive immune system (Liu and Kaplowitz 2002), there are currently no working animal models of allergic hepatitis, presumably due to the induction of immunological tolerance against drug–protein adducts (Bourdi et al. 2002a). There are, however, a few animal models of DILI where drug–protein adducts induce liver injury by nonallergic mechanisms. Evidence suggests that cytokines have a role in these models of DILI.

6.1.1 Halothane-Induced Liver Injury

Acute exposure to the inhalational anesthetic halothane induces relatively mild and transient liver injury in both guinea pigs and mice (Bourdi et al. 2001; You et al. 2006). This liver injury is induced by trifluoroacetylated (TFA)-protein adducts, which are formed following the bioactivation of halothane to trifluoroacetyl chloride (Bourdi et al. 2001). Despite the appearance of anti-TFA antibodies in guinea pigs, it has not been possible to generate a model of allergic hepatitis in animals (Chen and Gandolfi 1997). Although cytokines (IL-1β, TNF-α, IL-8, and IL-6)

have been detected in the livers of halothane-treated mice, the role of these cytokines has not yet been determined (You et al. 2006).

6.1.2 Lipopolysaccharide (LPS)-Induced DILI

This model is based on the hypothesis that transient inflammatory episodes, induced by LPS released from gut bacteria, might increase the risk of DILI. By administering sub-toxic doses of LPS to rodents just prior to or after drug treatment, animals develop liver injury that is greater than that induced by LPS or drug alone (Ganey et al. 2004). To date, this has been demonstrated for ranitidine, trovafloxacin, diclofenac, and chlorpromazine (Shaw et al. 2007). Although several cytokines, including TNF-α, IL-1β, IL-10, IL-6, and MIP-2 (Shaw et al. 2007; Tukov et al. 2007), have been detected in the livers of treated rodents, TNF-α is the only cytokine studied in detail to date. Neutralizing experiments demonstrated that TNF-α plays a pathogenic role in trovafloxacin- and ranitidine-induced liver injury by promoting fibrin deposition, and activation of coagulation factors (Shaw et al. 2007; Tukov et al. 2007).

6.1.3 Acetaminophen-Induced Liver Injury

The vast majority of the research on the role of cytokines in DILI has been conducted using the mouse model of APAP-induced liver injury (AILI). In overdose situations, bioactivated APAP induces predictable and dose-dependent liver injury in both humans and rodents through the formation of reactive metabolites and APAP-protein adducts within hepatocytes. Although AILI involves a nonallergic mechanism and is not idiosyncratic, current evidence from AILI studies in mice suggests that the balance of hepatoprotoxicant versus hepatoprotective cytokines might be a factor in determining a person's inherent susceptibility to liver injury caused by allergic and nonallergic mechanisms of DILI.

6.2 Hepatoprotoxicant Cytokines

Hepatoprotoxicant cytokines increase susceptibility to liver injury by enhancing the inflammatory response, interfering with liver regeneration, and in some cases, by direct hepatotoxicity (Diehl 2000; Lacour et al. 2005). In addition to their role in AILI, these cytokines play a pathogenic role in viral hepatitis, carbon tetrachloride (CCl_4)-induced liver injury, concanavalin A-induced liver injury, ischemia/reperfusion (I/R) injury, and partial hepatectomy (Diehl 2000; Lacour et al. 2005).

6.2.1 IFN-γ

IFN-γ induces Th1 immune responses by activating macrophages and NK cells, and by upregulating MHCI and MHCII expression on antigen-presenting cells (Carnaud et al. 1999; Friedmann et al. 1994; Kleinschmidt and Schultz 1982). In the liver, IFN-γ induces injury by upregulating the production of other pro-inflammatory factors, recruiting inflammatory cells to the liver, and suppressing liver regeneration (Gao 2005). The role of IFN-γ in AILI was first investigated following the observation that patients on interferon therapy exhibited significant rises in liver transaminases in response to APAP treatment. This suggested that IFNs might increase susceptibility to liver injury (Kellokumpu-Lehtinen et al. 1989). Initial experiments in wild-type mice revealed that hepatic IFN-γ mRNA levels were significantly elevated following a hepatotoxic dose of APAP (Ishida et al. 2002). Subsequent experiments using anti-IFN-γ neutralizing antibodies or IFN-$\gamma^{-/-}$ mice demonstrated that IFN-γ plays a protoxicant role in AILI (Ishida et al. 2002). While the exact mechanism by which IFN-γ enhances AILI is not known, these results suggest that IFN-γ might promote liver injury by recruiting inflammatory cells to the liver and by upregulating the expression of other pro-inflammatory cytokines, such as IL-1 and TNF-α (Ishida et al. 2002). IFN-γ has also been shown to play a protoxicant role in knockout mice, including chemokine (C–C motif) receptor 2 (CCR2$^{-/-}$), MIF$^{-/-}$, IL-13$^{-/-}$, and IL4$^{-/-}$IL10$^{-/-}$ mice (Bourdi et al. 2002b, 2007; Hogaboam et al. 2000; Yee et al. 2007). Within these knockout mice, high IFN-γ levels correlated with increased susceptibility to AILI, and in the case of IL-13$^{-/-}$ and CCR2$^{-/-}$ mice, IFN-γ neutralizing antibodies partially inhibited liver injury (Hogaboam et al. 2000; Yee et al. 2007).

6.2.2 MIF

MIF exerts pro-inflammatory cytokine effects in response to liver injury (Kobayashi et al. 1999). This activity is associated with its ability to promote the expression of other inflammatory mediators, such as TNF-α, IL-1β, IL-8, and IFN-γ (Baugh and Bucala 2002; Lue et al. 2002). MIF can also contribute to inflammatory responses by countering the anti-inflammatory effects of glucocorticoids (Baugh and Bucala 2002; Lue et al. 2002). The role of MIF in DILI was investigated following the observation that MIF serum levels were elevated in both halothane- and APAP-treated animals (Bourdi et al. 2002b). The hepatoprotoxicant effects of MIF were confirmed using MIF$^{-/-}$ mice, which exhibited decreased liver injury and increased rate of survival in response to APAP treatment as compared to wild-type control mice (Bourdi et al. 2002b). While the mechanism by which MIF contributes to AILI is unknown, initial evidence suggests that MIF might potentiate liver injury by enhancing IFN-γ secretion or decreasing heat shock protein expression (Bourdi et al. 2002b).

6.2.3 IL-1

IL-1 is a pro-inflammatory cytokine released from sinusoidal endothelial cells and activated Kupffer cells in response to liver injury (Blazka et al. 1995). As part of the acute phase response, IL-1 exacerbates inflammation by inducing the release of other pro-inflammatory mediators, such as IL-6 and IL-8 (Kaplowitz and Tsukamoto 1996; Liu et al. 1999). Additionally, IL-1 can recruit inflammatory cells to the liver by upregulating the expression of adhesion molecules (Lukacs et al. 1994, 1995). The observation that IL-1-neutralizing antibodies partially prevented liver injury in response to APAP treatment indicates that IL-1 might play a pro-inflammatory role in AILI (Blazka et al. 1995).

6.2.4 TNF-α

The role of TNF-α in liver injury has been extensively investigated because TNF-α release is one of the earliest events to occur in hepatic inflammation. In addition, polymorphisms within the promoter region of the TNF-α gene have been linked to increased severity of inflammatory reactions in humans (Luster et al. 2000). Despite several investigations, the role of TNF-α remains controversial. TNF-α acts as a protective or a protoxicant factor depending on the circumstances. As a protective factor, TNF-α promotes tissue repair and liver regeneration by stimulating hepatocyte proliferation (Beyer and Stanley 1990; Diehl and Rai 1996). At other times, TNF-α can enhance inflammatory responses by inducing the release of other pro-inflammatory cytokines such as IL-1, IL-8, and IFN-γ (Diehl 2000; Kaplowitz and Tsukamoto 1996; Lacour et al. 2005; Tracey and Cerami 1993). TNF-α can also be directly cytotoxic to hepatocytes by activating type 1 TNF receptors (TNFR1) containing a Fas-like "death domain", although signaling through this receptor only triggers death in hepatocytes made vulnerable by protein synthesis inhibition (Diehl 2000).

Because of the dual nature of TNF-α, experiments investigating its role in liver injury have often yielded conflicting results. For example, TNF-α has been shown to play both a protoxicant and a protective role in CCl_4-induced liver injury. While one study using TNF-$\alpha^{-/-}$ and TNFR1$^{-/-}$ mice reported that TNF-α increases susceptibility to CCl_4-induced liver injury (Morio et al. 2001), another study, also using TNFR1$^{-/-}$ mice, demonstrated that TNF-α plays a protective role in CCl_4-induced liver injury by promoting liver regeneration (Yamada and Fausto 1998). Experiments investigating the role of TNF-α in AILI have also yielded conflicting results. An early study demonstrated that anti-TNF-α neutralizing antibodies partially inhibited liver injury in B6C3F1 mice treated with 500 mg kg^{-1} APAP (Blazka et al. 1995). In contrast, subsequent studies reported that TNF-α is not involved in AILI because liver injury was not affected by the absence of TNF-α in either TNF-$\alpha^{-/-}$ B6 mice treated with 400 mg kg^{-1} APAP (Boess et al. 1998) or CBA/J wild-type mice treated with anti-TNF-α-neutralizing antibodies prior to 300 mg kg^{-1} APAP (Simpson et al. 2000). Further attempts to clarify the role of TNF-α in AILI were conducted in

TNFR1$^{-/-}$ mice, TNF-α was found to play a protective role in AILI, as the extent of liver injury was significantly greater in TNFR1$^{-/-}$ B6 mice than in wild-type mice following treatment with 300 mg kg^{-1} APAP (Gardner et al. 2003). The researchers also found evidence of suppressed hepatocyte proliferation in TNFR1$^{-/-}$ B6 mice, suggesting that TNF-α might be involved in liver regeneration. In a second study of AILI, also using TNFR1$^{-/-}$ B6 mice treated with 300 mg kg^{-1} APAP, TNF-α was shown to play a role in liver regeneration, without apparently being involved in liver injury (James et al. 2005a). In contrast, other researchers found a protoxicant role for TNF-α in AILI using TNFR1$^{-/-}$ Balb/c mice (Ishida et al. 2004). These mice exhibited decreased liver injury, inflammatory cell infiltrates, and pro-inflammatory cytokines as compared to wild type mice in response to treatment with 600 mg kg^{-1} APAP. Additional research on the role of TNF-α in AILI has been conducted in other cytokine knockout models; however, the results obtained from these studies have also been conflicting. While anti-TNF-α neutralizing antibodies decreased the extent of liver injury in CCR2$^{-/-}$ mice (Hogaboam et al. 2000), they had no effect on AILI in IL-13$^{-/-}$ mice (Yee et al. 2007). Taken together, these data indicate that the role of TNF-α in AILI is very complex and may depend on factors such as dose of APAP, timing of TNF-α release, and genetic background.

6.3 *Hepatoprotective Cytokines*

Cytokines have also been shown to play protective roles in several liver injury models, including viral hepatitis, carbon tetrachloride (CCl_4)-induced liver injury, concanavalin A-induced liver injury, I/R injury, partial hepatectomy, and AILI, where they exert their hepatoprotective effects by downregulating inflammation and promoting liver regeneration (Diehl 2000; Gao 2005; James et al. 2005b; Lacour et al. 2005; Liu and Kaplowitz 2006).

6.3.1 IL-10

The antiinflammatory properties of IL-10 have been reported in many models of acute liver injury, including concanavalin A-induced liver injury, CCl_4-induced liver injury, partial hepatectomy, and alcoholic liver disease (reviewed in Louis et al. 2003). Activated Kupffer cells are the main source of IL-10 in the liver (Ju et al. 2002), but it can also be produced by sinusoidal endothelial cells, hepatocytes, and T cells (Le Moine et al. 1999). IL-10 exerts its protective effects in the liver by downregulating the production of protoxicant cytokines, including TNF-α, IL-1β, IL-8, and IFN-γ (Bogdan et al. 1991; Moore et al. 2001; Stordeur and Goldman 1998), and by promoting liver regeneration (Huang et al. 2006; Louis et al. 1998). It also functions as a regulatory cytokine by limiting T cell expansion in the liver through its inhibitory effects on antigen presentation and costimulatory activity (Knolle et al. 1998). Experiments of AILI in IL-10$^{-/-}$ B6 mice revealed that the

absence of IL-10 leads to increased liver injury, decreased survival, and increased levels of pro-inflammatory mediators, including IL-1, TNF-α, and inducible nitric oxide synthase (iNOS) (Bourdi et al. 2002a). Subsequent experiments in IL-$10^{-/-}$iNOS$^{-/-}$ mice indicated that a major way IL-10 exerts its hepatoprotective effects in AILI is by downregulating iNOS (Bourdi et al. 2002a). Similar observations have been noted in the clinic, where higher IL-10 levels have been associated with increased survival in patients with acute liver failure (Yamano et al. 2000).

6.3.2 IL-13

IL-13 can function as either a protoxicant or a protective cytokine in the liver depending on the disease model; for example, in *Schistosomiasis manisomi* infection-induced liver fibrosis, IL-13 plays a protoxicant role (Chiaramonte et al. 2001), while in ischemia/reperfusion-induced liver injury, it is hepatoprotective (Kato et al. 2003). A potential role for IL-13 in AILI was first suggested by the observation that IL-13 levels increase in response to AILI in wild-type B6 mice (Bourdi et al. 2002a). Experiments using anti-IL-13 neutralizing antibodies and IL-$13^{-/-}$ B6 mice demonstrated that the absence of IL-13 increases susceptibility to AILI (Yee et al. 2007). While the exact mechanism by which IL-13 protects against AILI is unknown, the findings suggest that IL-13 protects the liver, at least in part, by downregulating the effects of protoxicant factors such as IFN-γ, nitric oxide, and neutrophils (Yee et al. 2007).

6.3.3 IL-6

IL-6 is a multifunctional cytokine involved in a variety of cellular functions in the hematopoietic, immune, neuronal, and hepatic systems (reviewed in Gao 2005). Within the liver, IL-6 is not only a key component of the acute phase response (Gao 2005), but it also plays a protective role in models of liver injury, including ischemia/reperfusion (Selzner et al. 1999), partial hepatectomy (Cressman et al. 1996), and CCl_4-induced liver injury (Weber et al. 2003). In addition to protecting the liver from necrosis by sustaining the expression of antiapoptotic factors (Kovalovich et al. 2001), IL-6 also promotes liver regeneration by stimulating hepatocyte proliferation (Cressman et al. 1996; Taub 2003). Elevated serum levels of IL-6 have been observed in patients with liver injury due to APAP overdose (James et al. 2005b), acute hepatitis (Sun et al. 1992), and alcoholic liver disease (Hill et al. 1992). Similarly, elevated levels of IL-6 and IL-6 family members, IL-11, leukemia inhibitory factor (LIF), and oncostatin M (OSM) have been detected in wild-type B6 mice following a hepatotoxic dose of APAP (Masubuchi et al. 2003). Two reports investigated the role of IL-6 in AILI using IL-$6^{-/-}$ B6 mice. The first study reported a protective role for IL-6 in AILI by demonstrating that IL-$6^{-/-}$ B6 mice were more susceptible to liver injury than control B6 mice 24 h after treatment with either 200 or 300 mg kg^{-1} APAP (Masubuchi et al. 2003).

Decreased expression of several heat shock proteins (HSP25, HSP32, HSP40, and inducible HSP70) in IL-$6^{-/-}$ B6 mice suggested that IL-6 might exert its protective effects by upregulating the expression of these HSPs, which have been shown to play a protective role in AILI (Chiu et al. 2002; Salminen et al. 1997). The second study failed to detect a difference in liver injury between IL-$6^{-/-}$ B6 and control B6 mice 24 h after treatment with 300 mg kg^{-1} APAP (James et al. 2003); however, by 48 h, decreased hepatocyte proliferation and increased liver injury in IL-$6^{-/-}$ B6 mice relative to control mice suggested that IL-6 might exert its protective effects in AILI by promoting liver regeneration. Although the results from these two studies are not entirely consistent, both clearly demonstrate a protective role for IL-6 in AILI.

6.3.4 MIP-2 (Murine Homologue of IL-8)

MIP-2 is a chemokine released from hepatocytes in response to liver injury (Bautista 1997; Lentsch et al. 1998) whose primary role is to recruit neutrophils and other inflammatory cells to inflamed tissues. The role of exogenous MIP-2 in AILI was studied by using an adenovirus vector to deliver recombinant MIP-2 to wild-type mice prior to APAP treatment (Hogaboam et al. 1999). Decreased liver injury and increased survival in mice treated with the MIP-2 vector indicated that exogenous MIP-2 protects against AILI. Endogenous MIP-2 was also shown to play a protective role in AILI as the extent of liver injury was greater in MIP-2 receptor knockout mice (CXC chemokine receptor 2, CXCR$2^{-/-}$), compared to their wild-type counterparts following APAP treatment. Although the mechanism by which MIP-2 protects against AILI is unknown, the observation that hepatocyte proliferation was increased in mice treated with the MIP-2 vector suggests that MIP-2 might be involved in liver regeneration (Hogaboam et al. 1999). This hypothesis is supported by the observation that MIP-2 stimulates hepatocyte proliferation through a STAT-3-dependent mechanism (Ren et al. 2003). Although MIP-2 has clearly been shown to play a protective role in AILI in mice, elevated have been associated with decreased survival in APAP overdose patients (Williams et al. 2003), indicating that additional work is needed to clarify whether the results obtained from mouse studies can be applied to humans.

6.3.5 MCP-1

MCP-1 is a chemokine that signals through CCR2 to recruit monocytes and macrophages to inflamed tissues during an inflammatory response (Dambach et al. 2002). Activated macrophages, stellate cells, and endothelial cells all produce MCP-1 in response to liver injury (Dambach et al. 2002). Depending on the model, MCP-1 can have either pro-inflammatory or anti-inflammatory effects in the liver. In endotoxin-induced liver injury, MCP-1 plays a protective role by downregulating

the inflammatory response (Zisman et al. 1997); while in CCl_4-induced liver injury, MCP-1 plays a protoxicant role by stimulating the production of pro-inflammatory mediators (Zamara et al. 2007). Although the role of MCP-1 in liver injury in humans is not known, elevated levels of MCP-1 are observed in patients with acute liver failure due to APAP and alcoholic hepatitis (Devalaraja et al. 1999; James et al. 2005b; Maltby et al. 1996). The role of MCP-1 in AILI was first examined using MCP-1 receptor knockout, $CCR2^{-/-}$, mice on a B6129sv/J background. This study reported that MCP-1 plays a protective role in AILI by demonstrating that $CCR2^{-/-}$ B6129sv/J mice were dramatically more susceptible to liver injury in response to 300 mg kg^{-1} APAP than control mice (Hogaboam et al. 2000). The observation that IFN-γ and TNF-α levels were significantly elevated in $CCR2^{-/-}$ mice suggests that MCP-1 might protect against AILI by downregulating pro-inflammatory mediators. A subsequent report did not find a role for MCP-1 in AILI using both MCP-$1^{-/-}$ B6 mice and $CCR2^{-/-}$ 129 Sv/ICR mice in their studies (Dambach et al. 2002). In this study, the liver injury observed in response to treatment with 200 or 300 mg kg^{-1} APAP did not differ significantly between $CCR2^{-/-}$ 129 Sv/ICR mice and their wild-type controls. These results were confirmed in MCP-$1^{-/-}$ B6 mice, where again, the liver injury induced by APAP treatment did not differ significantly from controls. Although MCP-1 did not protect against necrosis in this study, fewer infiltrating macrophages, which are known to be involved in liver regeneration by initiating wound repair, were observed in $CCR2^{-/-}$ mice following APAP treatment (DiPietro 1995). Therefore, even though MCP-1 did not appear to play a protective role in AILI in this study, it might be involved in liver regeneration. These findings also illustrate the important influence of mouse strain background on susceptibility to liver injury.

6.3.6 IL-4

IL-4 is another cytokine that can have a hepatoprotoxicant or a hepatoprotective role in the liver depending on the disease model. IL-4 plays a protoxicant role in concanavalin A-induced liver injury by upregulating eotaxin and IL-5 expression, which in turn promotes liver injury by recruiting inflammatory neutrophils and eosinophils to the liver (Jaruga et al. 2003). In contrast, IL-4 plays a protective role in ischemia/reperfusion-induced liver injury by suppressing the expression of pro-inflammatory mediators and preventing the infiltration of neutrophils (Kato et al. 2000). IL-4 has also been shown to play a protective role in Schistosomiasis infection by tightly regulating the production of reactive oxygen and nitrogen intermediates in the liver (La Flamme et al. 2001). The role of IL-4 in AILI was studied following the observation that IL-4 levels were elevated in wild-type mice in response to a hepatotoxic dose of APAP (Bourdi et al. 2002a). Although preliminary studies using IL-$4^{-/-}$ B6 mice indicated a protective role for IL-4 in AILI (Bourdi et al. 2007), additional mechanistic studies are required and are ongoing.

6.3.7 IL-4 and IL-10 Synergism

While both IL-4$^{-/-}$ and IL-10$^{-/-}$ mice are more susceptible to AILI than their wild-type counterparts (Bourdi et al. 2002a, 2007), it appears that the simultaneous dysregulation of these cytokines in IL-4$^{-/-}$IL-10$^{-/-}$ double knockout mice increases susceptibility to AILI in a synergistic manner. Consequently, significant liver injury and mortality is observed in IL-4$^{-/-}$IL-10$^{-/-}$ mice following treatment with a low dose of APAP, which is nonhepatotoxic in IL-4$^{-/-}$ and mildly hepatotoxic in IL-10$^{-/-}$ mice (Bourdi et al. 2007). It appears that the simultaneous dysregulation of IL-4 and IL-10 increases susceptibility to AILI via an IL-6-dependent mechanism, because AILI was significantly decreased in IL-4$^{-/-}$IL-10$^{-/-}$IL-6$^{-/-}$ triple knockout mice compared to IL-4$^{-/-}$IL-10$^{-/-}$ mice (Bourdi et al. 2007). Although IL-6 is normally a protective cytokine in AILI (James et al. 2003; Masubuchi et al. 2003), the levels of IL-6 produced in IL-4$^{-/-}$IL-10$^{-/-}$ mice in response to APAP treatment were highly elevated compared to wild-type mice treated with the same dose of APAP, suggesting that the role of IL-6 in AILI might be highly complex, and that its effects in AILI might depend on expression levels and timing of release (Bourdi et al. 2007).

6.4 Human Studies of DILI

6.4.1 Acetaminophen

Although AILI is not idiosyncratic in nature, overdoses (greater than 15 mg) can result in highly variable clinical manifestations, ranging from hepatic necrosis and coagulopathy to multi-organ failure requiring immediate liver transplant (Bernal et al. 1998). Polymorphisms within the TNF-α gene were investigated to determine whether they might influence the outcome of APAP overdose (Bernal et al. 1998). This gene was chosen, in part, because polymorphisms within the TNF-α promoter region have been shown to alter TNF-α production, susceptibility to inflammatory responses (Luster et al. 2000), and clinical outcome in sepsis patients (Leeder and Kearns 1997). Despite the significant number of polymorphisms in the TNF-α gene, there were no associations between the pathogenesis of AILI and specific TNF-α polymorphisms (Bernal et al. 1998). Even though the results of this study were negative, additional polymorphism studies would be useful to determine the role of other cytokines in AILI.

Another related approach to studying the role of cytokines in AILI in humans has been to correlate the severity of AILI with serum cytokine levels. To do so, serum levels of IL-6, IL-8, IL-10, MCP-1, and MIP-2 were compared between AILI patients with low, moderate, or severe liver injury (James et al. 2005b). Of these cytokines, MCP-1 was the only cytokine that correlated with the severity of liver injury (James et al. 2005b). Interestingly, MCP-1 also correlates with the severity of liver injury in a variety of other liver diseases, including acute viral

hepatitis and alcoholic hepatitis (Devalaraja et al. 1999; Leifeld et al. 2003; Maltby et al. 1996). While these types of studies are useful in that they are able to identify correlations between cytokine levels and liver injury, they do not provide any information regarding the role of cytokines in AILI. As a result, correlative studies in humans have limited usefulness in terms of uncovering mechanisms involved in ADRs.

6.4.2 Other Drugs

To date, only two studies have attempted to identify polymorphisms in cytokine genes that influence susceptibility to idiosyncratic DILI. The first study searched for associations between polymorphisms in the IL-4 and IL-10 genes and diclofenac-induced liver injury (Aithal et al. 2004), which is thought to be mediated at least in part by an adaptive immune response against diclofenac-protein adducts (Pumford et al. 1993). The IL-10 gene was examined as it contains a polymorphism in the promoter region (−627A) that is present in a high percentage of the population and is associated with a decreased IL-10 response (Grove et al. 2000; Lazarus et al. 1997; Lim et al. 1998; Turner et al. 1997). The IL-4 gene was also examined, as it contains a polymorphism (−590 T) associated with elevated levels of IL-4 and IgE (Rosenwasser et al. 1995) and increased incidence of asthma and atopic dermatitis (Noguchi et al. 1998). While the frequency of the IL-10 −627A allele was significantly increased in patients with diclofenac-induced liver injury, no association was found between the IL-4 −590 T allele and liver injury (Aithal et al. 2004). However, when the IL-10 −627A and the IL-4 −590 T alleles were considered together as a haplotype, the frequency of this haplotype was significantly greater in patients with diclofenac-induced liver injury than in controls (Aithal et al. 2004). As the IL-10 −627A allele is associated with a decreased IL-10 response, these results are consistent with the protective role of IL-10 in AILI in mice (Bourdi et al. 2002a). In contrast, the increased frequency of the IL-4 −590 T allele in patients with diclofenac-induced liver injury, which indicates a protoxicant role for IL-4 in idiosyncratic DILI, is not consistent with the protective role of IL-4 in AILI observed in mice (Bourdi et al. 2007). Consequently, this study reveals that not all data extracted from animal models of AILI may be applicable to idiosyncratic DILI in humans.

The second study was conducted in patients with tacrine-induced liver injury which, unlike diclofenac-induced liver injury, does not appear to involve the adaptive immune system (Carr et al. 2007). Tacrine also produces transient and mild liver injury in rodents (Ma et al. 2003; Stachlewitz et al. 1997), and, as a result, preliminary experiments were able to identify genes associated with tacrine-induced liver injury in rats using gene chip analysis (Carr et al. 2007). These experiments revealed a significant increase in acute phase proteins in response to tacrine-induced liver injury (Carr et al. 2007). As IL-6 is a key regulator of the acute phase response, an attempt was made to correlate polymorphisms in the IL-6 gene with increased susceptibility to tacrine-induced liver

injury in humans. Frequencies of the IL-6 −597A and IL-6 variable nucleotide random repeat (VNTR) D allele were significantly greater in patients who experienced elevations in serum alanine transaminases (ALT) at least $> 2\times$ upper limit of normal (ULN) in response to tacrine treatment. When these polymorphisms were considered together as a haplotype, the IL-6 −597A and VNTR D haplotype was not only associated with increased susceptibility to liver injury, but was also completely absent in patients in whom tacrine failed to induce liver injury. The association of the IL-6 −597A and VNTR D haplotype with a decreased IL-6 response (Terry et al. 2000) suggests that IL-6 plays a protective role in DILI, which is consistent with the observation that IL-6 is protective in AILI in mice (James et al. 2003; Masubuchi et al. 2003).

6.4.3 Ex Vivo Cytokine Production

Another approach to studying the role of cytokines in DILI has been to correlate ex vivo cytokine production with liver injury. To do so, intracellular levels of IFN-γ were measured in CD8 T cells following ex vivo stimulation with drug, or with hepatocyte lysates containing drug–protein adducts, which were generated by incubating control hepatocytes with drugs (Murata et al. 2003). High IFN-γ levels were only observed in patients with moderate to severe liver injury, suggesting that IFN-γ levels in CD8 T cells rise in response to liver injury. Despite this association, it was not possible to determine whether IFN-γ plays a direct role in the pathology of DILI because IFN-γ could not be detected in liver biopsies of DILI patients by immunohistochemical analysis.

7 Increased Susceptibility to ADRs During Concomitant Viral Infections

Viral infections, such as HIV or hepatitis C, have been identified as risk factors of idiosyncratic ADRs associated with numerous drugs, including nevirapine, isoniazid, and carbamazepine (Ackerman and Levy 1987; Sullivan and Shear 2001; Ungo et al. 1998). The mechanism by which this occurs is unknown; however, it has been hypothesized that viral infections could enhance the antigen presentation and costimulatory signals required to initiate an inappropriate immune response to drugs (Uetrecht 1999). This hypothesis is derived from the "danger" model (Matzinger 1994), which states that the dominant immune response is tolerance and that the immune system does not respond to a foreign antigen, such as a drug, unless the antigen is associated with a "danger signal". While the exact nature of what constitutes a "danger signal" is not known, it is possible that cytokines, released in response to viral infections, could provide such a signal. Although there is currently no evidence to support

this hypothesis in humans, there are some preliminary results from animal studies to suggest that cytokines might act as 'danger signals' during concomitant viral infections. Specifically, a single dose of polyinosinic:polycytidylic acid (poly I:C), a synthetic double-stranded RNA that upregulates IFN-α production (Sobel et al. 1994), increased both the severity and incidence of D-pen-induced DIAI in BN rats (Sayeh and Uetrecht 2001). In addition, a single dose of poly I:C was also sufficient to break specific immune tolerance to D-pen (Masson and Uetrecht 2004). Although further investigation is required to demonstrate that IFN-α is essential to the immune modulating effects of poly I:C, it seems likely that cytokines are potential "danger signals" associated with concomitant viral infections.

8 Th17 Cells and IL-17

The recent discovery of the role of Th17 cells in many autoimmune disorders (reviewed in Wang and Liu 2008) has led to speculation that this novel T cell subset might also play a role in DIAI due to the many similarities between DIAI and idiopathic autoimmunity. Th17 cells are defined by the secretion of proinflammatory cytokines IL-17, IL-21, and IL-22 (Wang and Liu 2008). It is thought that these cytokines contribute to autoimmune pathogenesis through their ability to chemoattract tissue-damaging inflammatory cells (Wang and Liu 2008). Specifically, there is significant evidence to suggest that IL-17, in particular, is a major pro-inflammatory mediator in Th17-induced autoimmunity. Mechanistically, it induces the release of other pro-inflammatory mediators, including TNF-α, IL-1β, granulocyte colony stimulating factor (G-CSF), IL-8, and MCP-1 (Benghiat et al. 2009), which in turn leads to tissue injury via neutrophil and macrophage activation (Benghiat et al. 2009). Increased IL-17 levels have been observed in multiple sclerosis (MS) (Lock et al. 2002), RA (Aarvak et al. 1999), inflammatory bowel disease (IBD) (Fujino et al. 2003), and psoriasis patients (Teunissen et al. 1998). In addition, IL-17 deficiency reduces collagen-induced arthritis (Lubberts et al. 2004) and EAE symptoms in mice (Cua et al. 2003). Likewise, evidence suggests that IL-22 plays a role in Th17-mediated autoimmune pathogenesis (reviewed in Ouyang et al. 2008) as IL-22 expression is upregulated in psoriasis (Wolk et al. 2006) and Crohn's disease patients (CD) (Andoh et al. 2005), and treatment with anti-IL-22 antibodies ameliorates disease symptoms in a mouse model of psoriasis (Ma et al. 2008). IL-21 also contributes to Th17-mediated autoimmunity by supporting further proliferation of Th17 cells in an autocrine fashion (reviewed in Ouyang et al. 2008). Anti-IL-21 antibody neutralization has been shown to ameliorate SLE symptoms in a mouse model (Herber et al. 2007).

Th17 cytokines might also play an important role in liver injury (Zenewicz et al. 2007). In contrast to the pro-inflammatory role observed in autoimmune disease, IL-22 exerted protective effects in the concanavalin A model, and IL-17 deficiency surprisingly had no effect on liver injury (Zenewicz et al. 2007). Besides

highlighting the multiplicity of cytokine effects, these studies suggest that it is of mechanistic importance to investigate the possible role of Th17 cytokines in DIAI and DILI.

9 Why Study Cytokines in ADRs

Further mechanistic insight into the role of cytokines in ADRs would be of clinical benefit for the following reasons:

9.1 To Assess the Potential of New Drugs to Cause ADRs

The discovery of an animal model or an in vitro assay that accurately detects the potential of new chemicals to cause toxicities would be highly valued by the pharmaceutical industry. Towards this goal, an in vitro assay was designed to assess the potential of drugs to induce skin ADRs by detecting IL-1β, TNF-α, and IL-18 secretion by keratinocytes following incubation with various known irritants and contact sensitizers (Vandebriel et al. 2005). While this approach was overly simplistic, further mechanistic insight into antigen processing and presentation might lead to the development of more sophisticated in vitro assays with increased sensitivity.

9.2 To Predict Susceptible Individuals

It would be extremely beneficial to predict an individual's susceptibility to drug-induced ADRs prior to the initiation of drug therapy. By doing so, many severe reactions could be avoided. One approach is to identify cytokine gene SNPs with strong associations to severe drug toxicities. An example highlighting the potential of this approach is the discovery of a link between a polymorphism in the TNF-α promoter with severe skin reactions to carbamazepine (Pirmohamed et al. 2001). This approach might allow highly useful drugs to still be used in patients who have been determined to be resistant to the ADR associated with the specific drug.

9.3 To Develop Interventional Therapies

There are not many effective therapies to treat severe drug toxicities such as SJS/TEN or acute hepatitis. Further mechanistic understanding of the role of cytokines could lead to the development of effective treatments (e.g., neutralizing antibodies, soluble blocking receptors, and recombinant cytokines) for severe drug toxicities.

10 Conclusions

Major progress has been made in our understanding of the role of cytokines in ADRs; however, there is still much work to be done. Further mechanistic insight could help identify individuals at risk of developing an ADR, or more importantly, assess the potential of new drugs to cause ADRs long before the drug enters the market. As discussed in this chapter, it is likely that animal models represent the best course of action to study the role of cytokines in drug toxicities. The recent discovery of the model of nevirapine-induced skin rash in BN rats appears to be a highly promising tool to study the role of cytokines in initiating and regulating specific immune responses to drugs in the skin. In addition, it appears that the model of D-pen-induced DIAI could be used to study the potential of cytokines to increase susceptibility to ADRs by acting as 'danger signals'. Further research must also be conducted to determine the role of cytokines in promoting drug-specific immune tolerance. Although a lack of animal models impedes mechanistic of DILI studies, the APAP model has identified several cytokines that might affect susceptibility to DILI. These findings in animal models must be confirmed in humans. Due to the difficulties in obtaining sufficient patient numbers and tissue samples for other types of clinical studies, large-scale polymorphism studies might be the best approach for conducting these studies. The *Drug-Induced Liver Injury Network* has been established to conduct research into the causes of DILI and is currently collecting biosamples and information on the history, causes and outcomes of DILI in the United States to make these types of studies possible, see http://dilin.dcri.duke.edu/.

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The Keap1-Nrf2 Cellular Defense Pathway: Mechanisms of Regulation and Role in Protection Against Drug-Induced Toxicity

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Contents

Abstract Adverse drug reactions pose a significant public health problem. In some cases, the process of drug metabolism can contribute to the onset of toxicity through the bioactivation of a parent molecule to a chemically reactive intermediate. In order to maintain a favorable balance between bioactivation and detoxification, mammalian cells have evolved an inducible cell defense system known as the antioxidant response pathway. The activity of this cytoprotective pathway is largely regulated by the transcription factor Nrf2, which governs the expression of many phase II detoxification and antioxidant enzymes. In turn, the activity of Nrf2 is regulated by the cysteine-rich cytosolic inhibitor Keap1, which acts as a "sensor" for chemical/oxidative stress. This article summarizes our current

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understanding of the molecular mechanisms that regulate the function of the Keap1-Nrf2 pathway and highlights the importance of Nrf2 in the protection against drug-induced toxicity.

Keywords Nrf2 · Keap1 · Antioxidant response element · Cell defense

1 Introduction

Adverse drug reactions constitute a major cause of patient morbidity and mortality (Park et al. 2005), and they have been responsible for the withdrawal of 4% of all drugs licensed in the United Kingdom between 1974 and 1994 (Jefferys et al. 1998). Therefore, adverse drug reactions pose a significant public health problem. The process of drug metabolism can, in some cases, contribute to the onset of toxicity through the generation of chemically reactive intermediates that can promote oxidative stress and/or inhibit the function of critical cellular macromolecules through the process of covalent modification (for a review, see Park 1986). The propensity of a xenobiotic to form a reactive intermediate is a function of its chemistry, with structural "alerts" now well defined; examples include epoxides, quinones, hydroxylamines, and furans (Park et al. 2005). The ability of an organism to withstand the potential toxic effect(s) of a given molecule is often determined by the balance between bioactivation and detoxification. In order to maintain a favorable balance between bioactivation and detoxification, mammalian cells have evolved a multi-faceted, highly regulated cell defense system, termed the antioxidant response pathway, which affords protection against the deleterious effects of endogenous and exogenous reactive species via the transcriptional upregulation of an array of detoxification and antioxidant enzymes (Primiano et al. 1997). The three regulatory components of the antioxidant response pathway are (1) the antioxidant response element (ARE), a DNA motif found within the promoter regions of numerous cytoprotective genes, (2) nuclear factor erythroid 2-related factor 2 (Nrf2), the redox-sensitive transcription factor that binds to the ARE, and (3) Kelch-like ECH-associated protein 1 (Keap1), the cytosolic repressor of Nrf2. An appreciation of the molecular mechanisms that underlie the adaptive response to cellular stress is vital to gain insights into the signaling events that determine the progression and outcome of adverse drug reactions. Therefore, the aims of this article are to provide a concise review of the mechanisms that underlie the activity of the antioxidant response pathway, and to highlight the important role of Nrf2 in protecting against drug-induced toxicity. In addition, this article will consider the future direction of research in this field, emphasizing some of the technical challenges that must be overcome to enable a better understanding of this important cytoprotective pathway.

2 Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) and the Antioxidant Response Element (ARE)

Nrf2 was first isolated during a screen for nuclear factor erythroid 2 (NF-E2)-regulating proteins in a complementary DNA (cDNA) expression library derived from hemin-induced erythroleukemia cells (Moi et al. 1994). Unlike NF-E2, which regulates globin gene expression in developing erythroid cells (Igarashi et al. 1994), Nrf2 is expressed in many tissues (Moi et al. 1994), particularly those associated with detoxification (liver and kidney) and those that are exposed to the external environment (skin, lung, and gastrointestinal tract) (Motohashi et al. 2002). As with other members of the CNC family of transcription factors (Itoh et al. 1995), so named because of structural similarities with the *Drosophila* protein cap 'n' collar (CNC), Nrf2 contains a C-terminal basic leucine zipper (bZip) structure that facilitates dimerization with other bZip proteins and DNA binding (Moi et al. 1994).

Through reporter transgene (Venugopal and Jaiswal 1996) and electrophoretic mobility shift assay (Nguyen et al. 2000) experiments, Nrf2 was shown to bind to the ARE and upregulate the expression of target genes. The ARE, a *cis*-acting DNA enhancer motif with a consensus sequence defined as 5′-gagTcACaGTgAGtCgg-CAaaatt-3' (where essential nucleotides are in capitals and the core is in bold) (Nioi et al. 2003), was originally identified within a 41-bp section from the 5'-flanking region of the rat glutathione S-transferase A2 gene that was responsive to the phenolic antioxidant β-naphthoflavone (Rushmore and Pickett 1990). Nrf2 only binds with high affinity to the ARE as a heterodimer with the bZip domain-containing small Maf proteins MafF, MafG, or MafK (Itoh et al. 1997). However, small Maf proteins lack transactivation domains, and thus the ability of the Nrf2-Maf heterodimer to promote transcription is reliant on the transactivation faculty of Nrf2 (Motohashi et al. 2002). Indeed, overexpression of small Maf proteins represses Nrf2-mediated transactivation of cell defense genes (Dhakshinamoorthy and Jaiswal 2000; Nguyen et al. 2000; Dhakshinamoorthy and Jaiswal 2002) through binding of small Maf homodimers, which lack intrinsic transcriptional activity, to the ARE (Dhakshinamoorthy and Jaiswal 2000).

Structural comparison of the chicken homolog of Nrf2 (erythroid cell-derived protein with CNC homology, ECH) (Itoh et al. 1995) with the human and mouse proteins enabled the identification of six highly conserved regions, termed Nrf2-ECH homology (Neh) domains (Itoh et al. 1997) (Fig. 1 and Table 1). The Neh1 domain contains the conserved CNC and bZip motifs (Itoh et al. 1999), and is therefore important for DNA binding and dimerization with small Maf proteins. The Neh4 and Neh5 domains of Nrf2 are involved in the recruitment of transcriptional coactivators (Lin et al. 2006), particularly cyclic adenosine monophosphate (cAMP) responsive element binding protein (CREB)-binding protein (CBP) (Katoh et al. 2001; Zhu and Fahl 2001), and are therefore important in driving the transcription of target genes following the binding of Nrf2 to the ARE. Through the upregulated expression of numerous cytoprotective genes (see Table 2 for

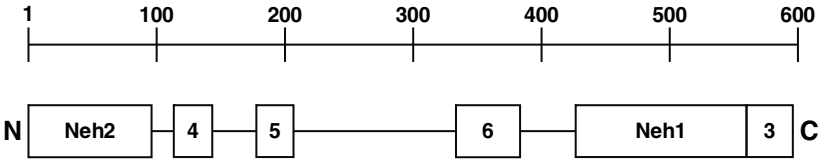


Fig. 1 Nrf2 functional domains. Schematic overview of the six Nrf2-ECH homology (Neh) functional domains in the mouse Nrf2 protein, with each domain labeled. The *scale* at the top of the panel indicates 100 amino acid sections of the protein (597 amino acids in total). See Table 1 for functional characteristics of each domain

Table 1 Nrf2 functional domains

Domain	Location (in mouse protein)	Function(s) and features	Reference(s)
Neh2	1–96	Contains ^{29}DLG^{31} and ^{79}ETGE^{82} motifs (points of interaction with Keap1)	Itoh et al. (1999) Katoh et al. (2005)
		Contains lysine-rich region (target for ubiquitination)	McMahon et al. (2004) McMahon et al. (2006)
		Contains DIDLID element (regulation of Nrf2 turnover under homeostatic conditions)	Tong et al. (2006a)
Neh4	111–141	Transactivation Interaction with co-activator CBP	Katoh et al. (2001)
Neh5	172–201	Transactivation	Katoh et al. (2001)
		Interaction with co-activator CBP	Li et al. (2006)
		Contains nuclear export signal	Zhang et al. (2007b)
Neh6	330–380	Regulation of Nrf2 turnover under stressed conditions	McMahon et al. (2004)
Neh1	427–560	Contains CNC and bZip regions	Bloom et al. (2002)
		ARE binding	Itoh et al. (1999)
		Dimerization with other bZip proteins (small Mafs)	Jain et al. (2005)
		Contains nuclear localization and export signals	
Neh3	561–597	Transactivation Interaction with putative co-activator proteins	Nioi et al. (2005)

examples), activation of Nrf2 promotes cell survival through the detoxification and/or elimination of chemical/oxidative stressors (Fig. 2).

Although Nrf2 is by far the most potent transcriptional activator of the ARE amongst members of the CNC-bZip family (Kobayashi et al. 1999; Papaiahgari et al. 2006), Nrf1 (Chan et al. 1993) also appears to play a limited role in protecting against the deleterious effects of chemical/oxidative stress, by controlling the expression of certain ARE-regulated genes (Venugopal and Jaiswal 1996; Kwong et al. 1999; Myhrstad et al. 2001; Xu et al. 2005; Hernandez-Montes et al. 2006). Furthermore, Nrf1 is important for embryonic development, as murine *Nrf1*$^{-/-}$ embryos die within 17–18 days of gestation (Chan et al. 1998). The lethal

Table 2 Nrf2-regulated cell defense genes and the major functions of the proteins they encode

Gene	Protein function	Reference(s)
Aldo-keto reductases (AKR)	Reduce aldehydes and ketones to yield primary and secondary alcohols	Lou et al. (2006) Nishinaka and Yabe-Nishimura (2005)
Glutamate cysteine ligase, catalytic subunit (GCLC)	Catalyzes the conjugation of cysteine with L-glutamate, to form γ-glutamylcysteine	Chan and Kwong (2000) Jeyapaul and Jaiswal (2000) Sekhar et al. (2000) Wild et al. (1999)
Glutamate cysteine ligase, regulatory subunit (GCLM)	Lowers the K_m of GCLC for glutamate and raises the K_i for glutathione (GSH)	Moinova and Mulcahy (1999) Wild et al. (1999) Chan and Kwong (2000)
Glutathione peroxidases (GPX)	Catalyze the reduction of H_2O_2, organic hydroperoxide, and lipid peroxides, using GSH as a substrate	Banning et al. (2005); Singh et al. (2006b)
Glutathione reductase (GSR)	Catalyzes the reduction of oxidized glutathione (GSSG) to GSH	Thimmulappa et al. (2002)
Glutathione synthetase (GS)	Catalyzes the conjugation of glycine with γ-glutamylcysteine	Lee et al. (2005)
Glutathione S-transferases (GST)	Reduces pK_a of GSH, catalyzing its conjugation to electrophiles	Chanas et al. (2002) Hayes et al. (2000) McMahon et al. (2001)
Heme-oxygenase 1 (HO-1)	Catabolizes heme to yield biliverdin, carbon monoxide and free iron	Alam et al. (1999); Ishii et al. (2000)
Microsomal epoxide hydrolase (MEH)	Hydrates simple epoxides and arene oxides to more polar vicinal diols and *trans*-dihydrodiols	Ramos-Gomez et al. (2001); Slitt et al. (2006); Thimmulappa et al. (2002)
NAD(P)H:quinone oxidoreductases (NQO)	Catalyzes two-electron reduction and detoxification of quinones	Venugopal and Jaiswal (1996) Wang and Jaiswal (2006)
Peroxiredoxin 1 (PRX1)	Reduces H_2O_2, peroxynitrite and other organic hydroperoxides	Kim et al. (2007)
Superoxide dismutases (SOD)	Catalyzes the dismutation of superoxide radicals to O_2 and H_2O_2	Park and Rho (2002)
Thioredoxins (TRX)	Catalyzes the reversible reduction of disulfides to sulfhydryls	Kim et al. (2001) Kim et al. (2003)
Thioredoxin reductases (TRX-R)	Reactivates thioredoxin by catalyzing the reduction of its active site disulfide	Sakurai et al. (2005)
UDP-Glucuronosyltransferases (UGT)	Catalyzes conjugation of uridine diphosphate glucuronic acid (UDPGA) to lipophilic substrates	Shelby and Klaassen (2006) Yueh and Tukey (2007)

consequence of Nrf1 knockout is thought to be, at least in part, caused by the reduced survival of hepatocytes during the developmental process (Chen et al. 2003). Notably, despite Nrf2 being dispensable during embryonic development (Chan et al. 1996), $Nrf1^{-/-}::Nrf2^{-/-}$ embryos die earlier than $Nrf1^{-/-}$ counterparts (between days 9–10 of gestation), due to increased levels of oxidative stress (Leung

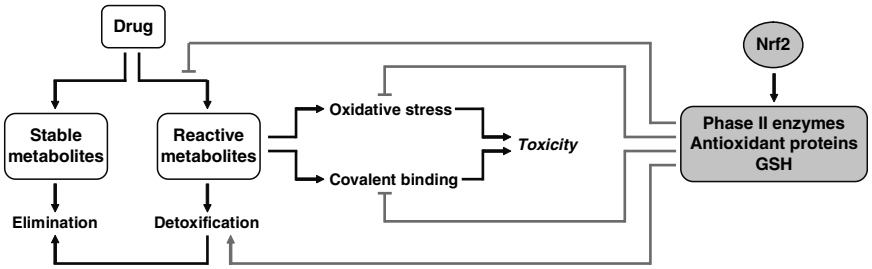


Fig. 2 The protective effects of Nrf2 on drug-induced toxicity. The metabolic biotransformation of drugs can, in some instances, lead to the formation of chemically reactive intermediates including quinones, epoxides, and thiophenes. Unless detoxified, these intermediates may cause toxicity, often via the generation of oxidative stress and/or through the process of covalent binding to critical macromolecules, such as DNA and proteins. Mammalian cells have evolved means of "sensing" and responding to chemical stress through the upregulated expression of detoxification and antioxidant enzymes. This process is largely mediated by the transcription factor Nrf2, which serves to protect against the potential deleterious effects of chemically reactive intermediates, and thus promotes cell survival

et al. 2003). This evidence suggests that both Nrf1 and Nrf2 have important cooperative roles in ensuring embryonic survival, further emphasizing the importance of ARE-transactivating factors in the process of cytoprotection.

At the time of writing, our understanding of the role of Nrf3 in the protection against cellular stress is less advanced than that of Nrf1 and Nrf2. Although it is known that Nrf3 can dimerize with MafK, bind to the ARE, and activate transcription (Kobayashi et al. 1999), Nrf3 does not appear to be expressed in the liver, the major site of xenobiotic metabolism and detoxification within the body (Derjuga et al. 2004; Kobayashi et al. 2004). Furthermore, loss of Nrf3 does not appear to have any phenotypic consequences, at least in the absence of cellular stress (Derjuga et al. 2004; Kobayashi et al. 2004). Therefore, the physiological function of Nrf3 in driving the cellular defense response is still unclear. However, evidence suggests that Nrf3 (Sankaranarayanan and Jaiswal 2004), in addition to other members of the CNC-bZip family, including the p65 isoform of Nrf1 (Wang et al. 2007), bric-a-brac/tram-track/broad complex (BTB) and CNC homolog 1 (Bach1) (Sun et al. 2002; Dhakshinamoorthy et al. 2005; Reichard et al. 2007), and Bach2 (Muto et al. 2002), may act as negative regulators of ARE gene expression, in part by competing with Nrf2 for binding to the ARE. Future investigations should enable a better understanding of the functional significance of this apparent interplay between members of the CNC-bZip family.

3 Kelch-Like ECH-Associated Protein 1 (Keap1)

In the absence of cellular stress, Nrf2 is tethered within the cytosol by an inhibitory partner, which binds to Nrf2 via the Neh2 domain of the transcription factor (Itoh et al. 1999). Due to similarities with sequence motifs found in the *Drosophila*

cytoskeleton-binding protein Kelch (Xue and Cooley 1993), the repressor of Nrf2 was named Kelch-like ECH-associated protein 1 (Keap1; Fig. 3). The features of the three major functional domains of Keap1 are summarized in Table 3. Keap1 resides within the cytosol of mammalian cells, where it interacts with the actin cytoskeleton (Kang et al. 2004) and, in the absence of chemical/oxidative stress, antagonizes the activity of Nrf2 (Itoh et al. 1999; Dhakshinamoorthy and Jaiswal 2001). Overexpression of Keap1 reduces Nrf2-mediated transactivation of ARE-regulated genes (Itoh et al. 1999; Dhakshinamoorthy and Jaiswal 2001; Wakabayashi et al. 2004). Under conditions of chemical/oxidative stress, Nrf2 evades Keap1-mediated repression, accumulates within the nucleus via a nuclear localisation signal located within the Neh1 domain of the transcription factor (Jain et al. 2005), and transactivates ARE target genes (Itoh et al. 1999; Dhakshinamoorthy and Jaiswal 2001).

Unlike Nrf2, the cellular localization and functional activity of Nrf1 does not appear to be regulated by Keap1 (Papaiahgari et al. 2006; Wang and Chan 2006).

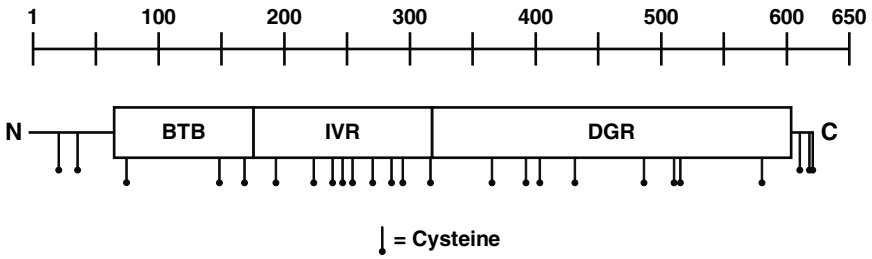


Fig. 3 Keap1 functional domains. Schematic overview of the three major functional domains in the mouse Keap1 protein, with each domain labeled. BTB, bric-a-brac/tram-track/broad complex; IVR, intervening region; DGR, double glycine repeat. The *scale* at the top of the panel indicates 50 amino acid sections of the protein. The position of each cysteine residue in the protein is indicated. See Table 3 for functional characteristics of each domain

Table 3 Keap1 functional domains

Domain	Location (in mouse protein)	Function(s) and features	Reference(s)
BTB	67–178	Bric-a-brac/tram-track/broad complex Homodimerisation	Zipper and Mulcahy (2002) Furukawa and Xiong (2005)
		Interaction with Cul3	Cullinan et al. (2004)
IVR	179–321	Intervening region Cysteine-rich (6.3% of amino acids)	
DGR	322–608	Double-glycine (kelch) repeat Interaction with Nrf2	Dhakshinamoorthy and Jaiswal (2001); Itoh et al. (1999)
		Interaction with actin cytoskeleton	Kang et al. (2004) Li et al. (2004) McMahon et al. (2006) Tong et al. (2006a)

Notably, Nrf1 is a much larger protein than Nrf2 (741 versus 597 amino acids in the mouse proteins), and the majority of the additional residues found in Nrf1 are located at the N-terminal end of the protein (Chan et al. 1993). Some of these residues appear to form a transmembrane domain, which tethers Nrf1 to the endoplasmic reticulum (Papaiahgari et al. 2006; Wang and Chan 2006). In light of the observation that the transcriptionally active form of Nrf1 is slightly smaller in molecular weight than the inactive, membrane-bound form, it has been postulated that Nrf1 undergoes proteolytic cleavage under conditions of endoplasmic reticulum stress, enabling its accumulation within the nucleus (Wang and Chan 2006). Therefore, the activities of Nrf1 and Nrf2 are regulated by distinct mechanisms.

4 Regulatory Mechanisms in the Keap1-Nrf2 Pathway

Although the physical restriction of Nrf2 is an important aspect of its repression by Keap1, recent investigations have provided evidence to support the concept that the activation of Nrf2 is governed by a mechanism that is more complex than through its simple release from Keap1.

4.1 Keap1-Directed Ubiquitination of Nrf2

In the absence of cellular stress, Nrf2 exhibits a relatively short half-life of 10–30 min (Alam et al. 2003; Itoh et al. 2003; McMahon et al. 2003; Stewart et al. 2003; Zhang and Hannink 2003; Furukawa and Xiong 2005; He et al. 2006). Importantly, proteasome inhibition causes the stabilization and nuclear accumulation of Nrf2, which in turn leads to an increase in ARE-driven gene transactivation (Sekhar et al. 2000; Alam et al. 2003; Itoh et al. 2003; McMahon et al. 2003; Nguyen et al. 2003; Stewart et al. 2003; Chen and Regan 2005; Furukawa and Xiong 2005; Usami et al. 2005; Yamamoto et al. 2007). Furthermore, ubiquitinated Nrf2 has been detected under these conditions (Nguyen et al. 2003; Stewart et al. 2003; Zhang and Hannink 2003; Cullinan et al. 2004; Kobayashi et al. 2004). This evidence suggests that Nrf2 is rapidly degraded by the ubiquitin-proteasome pathway, thus accounting for its relatively short half-life and its very low levels in unstressed cells/tissues.

Recently, it has been demonstrated that, similar to other BTB family proteins (Pintard et al. 2004), Keap1 functions as a substrate adaptor for a Cullin-dependent E3 ubiquitin ligase complex (Cullinan et al. 2004; Kobayashi et al. 2004; Zhang et al. 2004; Furukawa and Xiong 2005). Cullin proteins (in this case Cul3) act as molecular bridges, bringing together a substrate adaptor protein and substrate (in this case Keap1 and Nrf2, respectively) and the ring-box protein Roc1/Rbx1, which recruits a ubiquitin-charged E2 protein (Pickart 2001). Indeed, immunoprecipitation

of Keap1 from established cell lines reveals association with Cul3 (Cullinan et al. 2004; Kobayashi et al. 2004; Zhang et al. 2004, 2005 Furukawa and Xiong 2005) and Roc1/Rbx1 (Zhang et al. 2004, 2005; Furukawa and Xiong 2005), and this association appears to occur via the BTB domain of Keap1 (Cullinan et al. 2004; Furukawa and Xiong 2005). Inhibition of Cul3 function, through expression of a dominant negative Cul3 mutant or targeted depletion by RNA interference (RNAi), results in a decrease in Nrf2 turnover, a concomitant increase in the basal levels of Nrf2 (Cullinan et al. 2004; Zhang et al. 2004; Furukawa and Xiong 2005), and induction of an ARE-driven reporter transgene (Cullinan et al. 2004). Cul3 associates with Nrf2, and promotes its ubiquitination (Cullinan et al. 2004; Zhang et al. 2004) in a Keap1-dependent manner (Cullinan et al. 2004). Compound mutation of the seven lysine residues located between the conserved ^{29}DLG^{31} and ^{79}ETGE^{82} motifs in the Neh2 domain of Nrf2 effectively abrogates Keap1-directed ubiquitination of the transcription factor, and increases its steady-state half-life threefold (Zhang et al. 2004). Reversion of individual mutant residues back to lysine facilitates Nrf2 ubiquitination (Zhang et al. 2004), indicating that the targeting of this subset of lysines within the Neh2 domain is critical for Keap1-mediated repression of Nrf2.

The ubiquitination and degradation of Keap1, via a proteasome-independent pathway, may contribute to the diminished repression of Nrf2 under certain conditions of chemical/oxidative stress (Hong et al. 2005b; Zhang et al. 2005). Indeed, the formation of high molecular weight forms of Keap1 has been observed following exposure of cells to *tert*-butylhydroquinone (tBHQ) (Zhang and Hannink 2003) and ebselen (Sakurai et al. 2006), and this phenomenon is prevented through mutation of Cys-151 (Zhang and Hannink 2003; Sakurai et al. 2006), implying that this residue plays an important role in "sensing" molecules that promote the formation of high molecular weight Keap1 complexes. Molecular deletion of the intervening region (IVR) attenuates the ubiquitination of Keap1 following exposure to tBHQ (Zhang et al. 2005), and tandem mass spectrometric analysis has provided evidence for the ubiquitination of Lys-298 within the IVR domain of Keap1 (Hong et al. 2005b). However, it appears that not all Nrf2-activating molecules induce the formation of high molecular weight Keap1 complexes (Hong et al. 2005a; Zhang et al. 2005; Sakurai et al. 2006). Hence, the general importance of Keap1 ubiquitination in the response to chemical/oxidative stress is yet to be fully determined.

4.2 Phosphorylation of Nrf2

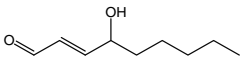
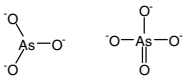
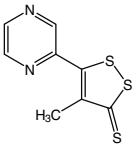
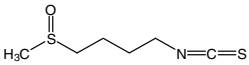
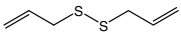
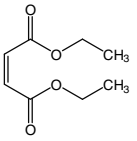
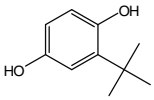
Although a consensus has yet to be reached on the general importance of phosphorylation as a regulatory mechanism controlling Nrf2 function, evidence suggests that the induction of Nrf2-dependent cell defense by some molecules may involve the stimulation of certain protein kinases. The majority of studies that have implicated phosphorylation as a regulatory influence on Nrf2 function have done so through the use of pharmacological inhibitors of specific protein kinases, which attenuate Nrf2 induction by known activating molecules (Nguyen et al. 2000; Johnson et al. 2002;

Bloom and Jaiswal 2003; Numazawa et al. 2003; Liby et al. 2005; Lee-Hilz et al. 2006). It is important to consider that inhibition of a protein kinase pathway will undoubtedly have significant effects on multiple cell signaling processes, which themselves may impact upon the integrity of the Nrf2 system. The specificity of some of the small-molecule inhibitors used to identify certain protein kinases as regulators of Nrf2 activity has also been questioned (Davies et al. 2000; Bain et al. 2003, 2007). Nevertheless, independent studies have demonstrated direct phosphorylation of Nrf2 by protein kinase C (Nguyen et al. 2000; Huang et al. 2002; Bloom and Jaiswal 2003), extracellular signal-regulated kinase 1 (Papaiahgari et al. 2006) and protein kinase R-like endoplasmic reticulum kinase (Cullinan et al. 2003). Furthermore, several recent reports have described the phosphorylation of Nrf2, at Tyr-568, by the tyrosine kinase Fyn, an event that is required for the nuclear export of the transcription factor (Jain and Jaiswal 2006, 2007; Kannan and Jaiswal 2006; Salazar et al. 2006). Chemical inhibition or RNAi depletion of Fyn, or its upstream regulator glycogen synthase kinase 3β, appears to attenuate nuclear export of Nrf2 and augment ARE-driven gene transactivation (Jain and Jaiswal 2006, 2007; Kannan and Jaiswal 2006; Salazar et al. 2006). Hence, phosphorylation may be an important signaling event in both the activation and deactivation of Nrf2, through promotion of both nuclear accumulation and export, respectively. However, it is not yet known whether specific inducers stimulate specific kinase pathways, perhaps in a cell- or species-dependent manner, or whether the simultaneous induction of multiple pathways is characteristic of all Nrf2-activating molecules.

4.3 Keap1 as a Redox Sensor

In addition to functioning as a physical repressor of Nrf2, by restraining its access to the nucleus, there is evidence that Keap1 acts as a "sensor" of chemical/oxidative stress, through its many cysteine residues. The human and mouse Keap1 proteins contain 27 and 25 cysteines respectively, representing 4.3 and 4.0% of the 624 total amino acids. This compares to the average cysteine frequency of 2.3% across all human and mouse proteins (Miseta and Csutora, 2000). Many of the cysteine residues in Keap1 have low predicted pK_a values, and thus high relative reactivities, as they are flanked by one or more basic amino acid (arginine, lysine, histidine), which stabilise cysteine in the more nucleophilic thiolate form (Snyder et al., 1981). Although the array of cell defence-inducing molecules is structurally diverse (Table 4), many are electrophilic (Prestera et al., 1993; Talalay et al., 1988), and almost all share a common capacity for modification of nucleophilic sulfhydryl groups via alkylation, oxidation or reduction (Dinkova-Kostova et al., 2001). Furthermore, the potency of benzylidene-alkanone and -cycloalkanone Michael acceptors (Dinkova-Kostova et al., 2001) and heavy metals (Prestera et al., 1993) as inducers of cytoprotective enzymes is related to their reactivity towards sulfhydryl groups. This evidence led to the proposal that Keap1 was the long-sought "sensor" for chemical/oxidative stress.

Table 4 Common classes of Nrf2-activating molecules

Category	Example	Reference(s)
Alkenes	4-Hydroxynonenal 	Chen et al. (2005) Ishii et al. (2004) Zhang et al. (2006) Zhang et al. (2007a)
Arsenicals	Arsenite / arsenate 	Aono et al. (2003) Gong et al. (2002) He et al. (2006) Pi et al. (2003)
Dithiolethiones	Oltipraz 	Pietsch et al. (2003) Ramos-Gomez et al. (2001)
Enones	Acrolein 	Kwak et al.(2003) Tirumalai et al. (2002)
Isothiocyanates	Sulforaphane 	Fahey et al. (2002) Jakubikova et al. (2006) Shinkai et al.(2006) Thimmulappa et al.(2002)
Mercaptans / disulfides	Diallyl disulfide 	Chen et al. (2004) Fisher et al. (2007)
Michael acceptors	Diethylmaleate 	Itoh et al. (1999)
Diphenols / quinones	*tert*-Butylhydroquinone 	Lee et al. (2001) Li et al. (2005)
Reactive oxygen / nitrogen species	Nitric oxide 	Buckley et al.(2003) Dhakshinamoorthy et al.(2004) Liu et al. (2007)

The extensive use of site-directed mutagenesis has served to highlight the critical roles of certain cysteine residues, particularly Cys-151, -273 and -288, in the function of Keap1 (Kobayashi et al., 2006; Levonen et al., 2004; Wakabayashi et al., 2004; Yamamoto et al., 2008; Zhang and Hannink, 2003). Cys-151, which resides within the BTB domain of Keap1, does not appear to be integral to the function of Keap1 in the absence of chemical/oxidative stress, but is important for the loss of Nrf2 repression and ubiquitination observed under these conditions (Yamamoto et al., 2008; Zhang and Hannink, 2003; Zhang et al., 2004). Therefore, the ability of the Keap1-Nrf2 pathway to "sense" and respond to conditions of chemical/oxidative stress is partly dependent on Cys-151 of Keap1. In contrast, Cys-273 and -288, both of which are located within the IVR domain of Keap1, are essential for the repressive activity of the protein under basal conditions (Kobayashi et al., 2006; Levonen et al., 2004; Wakabayashi et al., 2004; Yamamoto et al., 2008; Zhang and Hannink, 2003). Although mutation of Cys-273 and/or -288 to serine or alanine does not affect the association between Keap1 and Cul3 (Kobayashi et al., 2004), it does render Keap1 unable to direct ubiquitination of Nrf2, inhibit the nuclear accumulation of the transcription factor, and repress transactivation of an ARE-driven reporter transgene (Kobayashi et al., 2006; Levonen et al., 2004; Wakabayashi et al., 2004; Zhang and Hannink, 2003). Furthermore, the responsiveness of Nrf2 to known activating molecules is diminished or abolished in cells expressing Keap1 Cys-273/288 mutants (Levonen et al., 2004; Zhang and Hannink, 2003). Notably, the mutation of other cysteines within the IVR, N-terminal and C-terminal domains has essentially no effect on Keap1 function (Wakabayashi et al., 2004; Zhang and Hannink, 2003). Therefore, in light of the evidence discussed, the structural integrities of Cys-151, -273 and -288 are important for the function of Keap1, and these residues represent plausible targets for electrophilic inducers of Nrf2.

Compelling evidence for the direct chemical modification of Keap1 has been provided through the use of biotinylated analogues of Nrf2-activating molecules; exposure of cells to low micromolar concentrations of a biotinylated form of 15-deoxy-$\triangle^{12,14}$-prostaglandin J_2 (15d-PGJ_2), an endogenous cyclopentenone molecule with two electrophilic α,β-unsaturated carbonyl moieties, leads to the formation of adducts with Keap1 and an associated activation of Nrf2 (Itoh et al., 2004; Levonen et al., 2004). We have further characterized the modification of Keap1 by 15d-PGJ_2, employing mass spectrometry to identify specific target cysteine residues in a cellular model (Copple et al., 2008). Mass spectrometry has also been used to demonstrate residue-selective modification of Keap1 by N-acetyl-*p*-benzoquinoneimine, the hepatotoxic metabolite of acetaminophen (Copple et al., 2008), the thiol-reactive steroid dexamethasone 21-mesylate (Copple et al., 2008; Dinkova-Kostova et al., 2002), 1-chloro-2,4-dinitrobenzene (Copple et al., 2008; Liu et al., 2005), non-biotinylated (Copple et al., 2008) and biotinylated (Eggler et al., 2005; Hong et al., 2005b; Rachakonda et al., 2008) iodoacetamide, the isothiocyanate molecule sulforaphane (Hong et al., 2005a) and the natural chemopreventative compounds xanthohumol, isoliquiritigenin and 10-shogaol (Luo et al., 2007). These extensive investigations have demonstrated that no single cysteine in

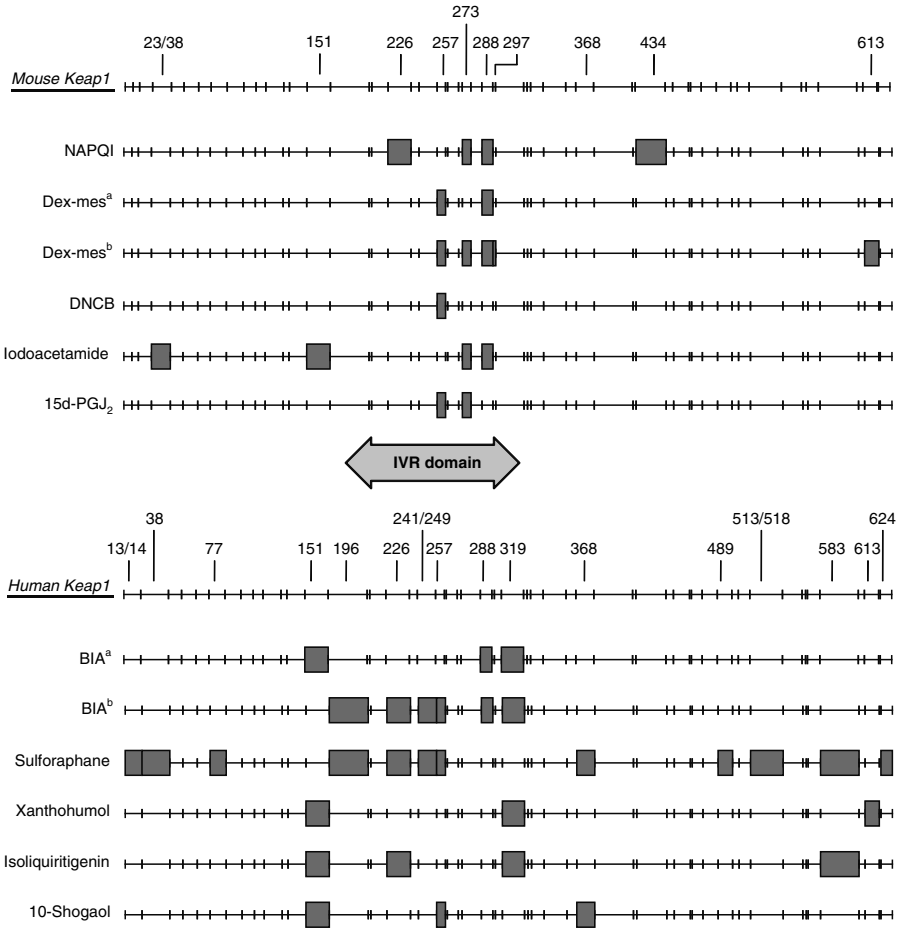


Fig. 4 Summary of Keap1 cysteine residues preferentially modified *in vitro* by Nrf2-activating molecules, as determined by tandem mass spectrometry. The *horizontal lines* represent the full-length mouse (*top*) and human (*bottom*) Keap1 proteins (amino acids 1-624). The *vertical lines* represent the boundaries between sequential tryptic peptides. *Filled boxes* represent cysteine-containing peptides found to be adducted by the indicated molecule at the lowest concentration (in cells) or molar ratio (molecule:Keap1, *in vitro*) at which there was reliable evidence for modification. The specific cysteines modified are noted. *NAPQI*, N-acetyl-*p*-benzoquinoneimine, 100 μM in HEK293T cells (Copple et al., 2008); *Dex-mes*, dexamethasone 21-mesylate, [a]30 μM in HEK293T cells (Copple et al., 2008), [b]molar ratio 33:1 *in vitro* (Dinkova-Kostova et al., 2002); *DNCB*, 1-chloro-2,4-dinitrobenzene, 100 μM in HEK293T cells (Copple et al., 2008); iodoacetamide, 30 μM in HEK293T cells (Copple et al., 2008); *15d-PGJ*$_2$, 15-deoxy-$\triangle^{12,14}$-prostaglandin J$_2$, 10 μM in HEK293T cells (Copple et al., 2008); *BIA*, biotinylated iodoacetamide, [a]molar ratio 1:1 *in vitro* (Eggler et al., 2005), [b]molar ratio 5:1 *in vitro* (Hong et al., 2005b); sulforaphane, molar ratio 0.1:1 *in vitro* (Hong et al., 2005a); xanthohumol, molar ratio 5:1 *in vitro* (Luo et al., 2007);

Keap1 appears to react preferentially with all of the Nrf2-activating molecules tested (Fig. 4). This may be a function of the inherent reactivity of a given electrophile toward a specific cysteine residue, or may simply reflect slight differences in experimental approaches, or both.

Aside from our recent work and that of Liebler and colleagues (Copple et al., 2008; Hong et al., 2005b; Rachakonda et al., 2008), the mass spectrometry-based studies highlighted above have been conducted *in vitro* with purified, recombinant Keap1 proteins in which all cysteines are free for modification by electrophiles, due to prior exposure to reducing agents such as dithiothreitol or tris(carboxyethyl) phosphine. However, it is important to note that, although the crystal structures of the DGR domain of human and mouse Keap1 indicate that none of the eight cysteines located within this region participate in disulfide bonds, at least in the absence of chemical/oxidative stress (Li et al., 2004; Lo et al., 2006; Padmanabhan et al., 2006), the structures of the remaining Keap1 domains have yet to be elucidated. Therefore, without an appreciation of the native redox states of all of the cysteine residues in Keap1, it is not possible to state how representative these *in vitro* models are of the situation in cells. In addition, the relative reactivities of Keap1 cysteines in the isolated recombinant protein may differ significantly from the situation in cells, due to protein folding, posttranslational modification(s), and/or the interaction with protein partners. These factors may cause some potential binding sites that are free for adduction *in vitro* to be obscured on the protein in cells. It should also be considered that in order to modify Keap1 within a cell, an electrophile must bypass various intracellular antioxidants and reductants, such as GSH, as well as other cellular proteins. These cellular obstacles may hinder the modification of some cysteines in Keap1 that would otherwise be free for modification *in vitro*. For these reasons, it is important that residue-specific modifications observed *in vitro* are validated in cell-based models and, where bioanalytical techniques permit, *in vivo*.

It is possible that modification of any single cysteine residue in Keap1 may be sufficient to trigger the activation of Nrf2. Such a nonspecific triggering mechanism may underlie the chemical versatility of the Keap1-Nrf2 pathway, in terms of its capacity to "sense" and respond to a variety of structurally distinct molecules. More specifically, it is possible that the modification of a single cysteine residue/group of residues within a critical domain of Keap1 provides the molecular trigger for Nrf2 activation. Indeed, it is clear that each of the Nrf2-activating molecules tested to date preferentially modify one or more cysteine residues within the IVR domain of Keap1 (Fig. 4). Consistent with this, it is notable that 1-biotinamido-4-(4'-[maleimidoethyl-cyclohexane]carboxamido)-butane, which does not activate Nrf2, modifies human Keap1 *in vitro*, but at cysteine residues outside of the IVR domain (Hong et al., 2005b). Taken together, these findings imply that a number of alternative target sets are present amongst the reactive cysteines of Keap1, but particularly within the IVR domain of the protein. Further investigations, particularly within a cellular context, are required to elucidate the importance of modification within the IVR domain in the activation of Nrf2.

5 The "Hinge and Latch" Model of Keap1-Nrf2 Interaction

Evidence suggests that Keap1 exists as a homodimer in mammalian cells (McMahon et al. 2006) and binds to a single molecule of Nrf2 in this form (Zipper and Mulcahy 2002; Wakabayashi et al. 2004; Lo et al. 2006; Tong et al. 2006a). The BTB domain of Keap1 is essential for homodimerization, which in turn is required for repression of Nrf2 (Zipper and Mulcahy 2002). Recently, Yamamoto and colleagues have proposed a "hinge and latch" interaction model (for a review, see Tong et al. 2006b), based on evidence that two distinct sites within the Neh2 domain of Nrf2, the conserved ^{29}DLG^{31} and ^{79}ETGE^{82} motifs, bind to a single overlapping site, comprising conserved arginine, serine, and asparagine residues, in the double glycine repeat (DGR) domain of Keap1 (Lo et al. 2006; McMahon et al. 2006; Padmanabhan et al. 2006; Tong et al. 2006a). Based on the observation that the ^{29}DLG^{31} and ^{79}ETGE^{82} motifs have different affinities for the DGR domain of Keap1 (Tong et al. 2006a, 2007), the "hinge and latch" model suggests that binding via the high-affinity ^{79}ETGE^{82} motif provides the "hinge", through which Nrf2 can move in space relatively freely. Concomitant binding via the lower affinity ^{79}ETGE^{82} motif provides the "latch", which tightly restricts Nrf2 to enable optimal positioning of target lysines for conjugation with ubiquitin (McMahon et al. 2006; Tong et al. 2006a). Consistent with this, deletion of the ^{79}ETGE^{82} motif attenuates the interaction between Nrf2 and Keap1 (Kobayashi et al. 2002, 2004; Furukawa and Xiong 2005), resulting in the stabilization of Nrf2 (Kobayashi et al. 2004; Furukawa and Xiong 2005). In contrast, deletion of the ^{29}DLG^{31} motif, or mutation of residues within it, has no effect on the association of Nrf2 and Keap1, but renders the latter unable to direct Nrf2 for degradation (McMahon et al. 2004, 2006), also causing an increase in the stability of the transcription factor.

Although chemical inducers are capable of promoting the stabilization and nuclear accumulation of Nrf2, evidence suggests that they do not evoke its complete dissociation from, nor impair its ability to associate with, Keap1 (Zhang and Hannink 2003; Zhang et al. 2004; Eggler et al. 2005; Kobayashi et al. 2006). In fact, such Nrf2-activating molecules may increase the association of the transcription factor with Keap1 (Hong et al. 2005b; He et al. 2006; Kobayashi et al. 2006), most probably due to diminished degradation of Keap1-bound Nrf2. Notably, when de novo protein synthesis is inhibited by cyclohexamide, Nrf2 does not accumulate within the nuclei of cells exposed to the inducers diethylmaleate (Itoh et al. 2003) or tBHQ (Kobayashi et al. 2006). This implies that the cytoprotective response is driven by newly synthesized Nrf2 protein, rather than Nrf2 molecules released from Keap1 repression. In the "hinge and latch" model, the ubiquitination of Nrf2 is attenuated under conditions of chemical/oxidative stress (Zhang et al. 2004; He et al. 2006; Kobayashi et al. 2006), and this is thought to be the result of disruption of the Nrf2-Keap1-Cul3 complex. This destabilization is postulated to occur through loss of ^{29}DLG^{31} motif binding, via a local conformational change in the cysteine-rich IVR domain of Keap1, which leads to the improper spatial positioning of target lysines in the Neh2 domain of Nrf2 (McMahon et al. 2006). Although conclusive evidence for

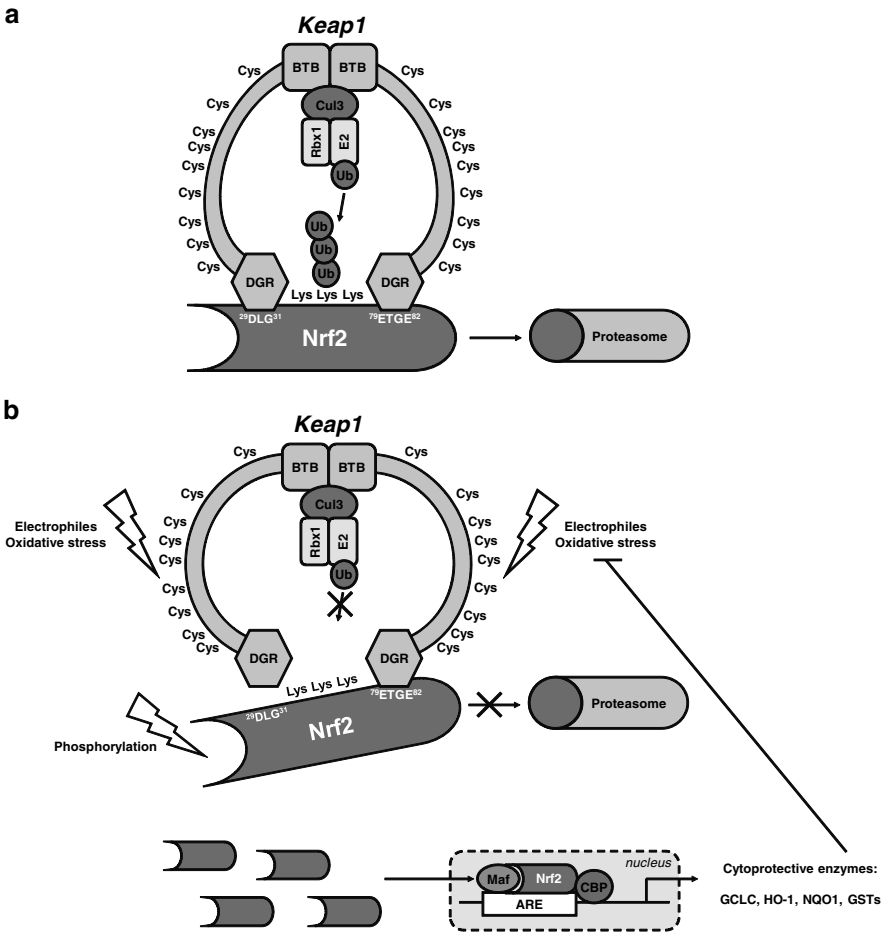


Fig. 5 Overview of the current "hinge and latch" model of Nrf2 regulation. (**a**) In the absence of cellular stress, the Keap1 homodimer binds both the ^{29}DLG^{31} and ^{79}ETGE^{82} motifs of a single Nrf2 molecule, tightly positioning the transcription factor to enable the efficient transfer of ubiquitin, and thus directing Nrf2 for proteasomal degradation. (**b**) Under conditions of chemical/oxidative stress, binding through the low-affinity ^{29}DLG^{31} "latch" is perturbed, probably via a conformational change in Keap1 brought about through modification of one or more cysteine residues, whilst binding through the high-affinity ^{79}ETGE^{82} "hinge" is maintained. Although Nrf2 still associates with Keap1, the transcription factor is no longer held in the correct position to facilitate ubiquitin transfer, and thus Nrf2 is not directed for proteasomal degradation. As a result, Keap1 becomes saturated by Nrf2, and any newly synthesized Nrf2 is able to accumulate within the nucleus and transactivate cytoprotective genes. Adapted from Tong et al. (2006b)

this concept has yet to be presented, recent reports have demonstrated that Nrf2-activating molecules do cause changes in the structural conformation of Keap1 (Dinkova-Kostova et al. 2005; Gao et al. 2007; Rachakonda et al. 2008) and antagonize the interaction between Keap1 and Cul3 (Gao et al. 2007; Rachakonda

et al. 2008). Following the destabilization of the Nrf2-Keap1-Cul3 complex, Nrf2 appears to remain associated with Keap1 via the high-affinity ^{79}ETGE^{82} motif, but is not directed for proteasomal degradation. This leads to the saturation of Keap1, such that any newly synthesized Nrf2 can evade Keap1 and accumulate within the nucleus, leading to the transactivation of ARE target genes (Tong et al. 2006b). An overview of the "hinge and latch" model of Nrf2 regulation is presented in Fig. 5.

6 Role of the Keap1-Nrf2 Pathway in Protecting Against Drug-Induced Toxicity

Since the discovery of Nrf2 in 1994 (Moi et al. 1994), several independent laboratories have generated transgenic mice in which specific components of the Keap1-Nrf2 pathway are not expressed, including *MafF*$^{-/-}$ (Onodera et al. 1999), *MafG*$^{-/-}$, and *MafK*$^{-/-}$ mice (Shavit et al. 1998). To date, the most widely used of these knockout animals are *Nrf2*$^{-/-}$ mice (Chan et al. 1996; Itoh et al. 1997; Ma et al. 2004). *Nrf2*$^{-/-}$ mice exhibit no significant early developmental phenotype (Chan et al. 1996), but aged mice develop vacuolar leukoencephalopathy (Hubbs et al. 2007) and lupus-like autoimmune symptoms (Yoh et al. 2001; Ma et al. 2006). Two notable characteristics demonstrate the severely compromised defense systems in *Nrf2*$^{-/-}$ mice: (1) lower basal and/or inducible expression of detoxification/antioxidant genes in a variety of tissues, including bladder (Iida et al. 2004), brain (Lee et al. 2003; Shih et al. 2005; Kraft et al. 2006), gastrointestinal tract (Itoh et al. 1997; McMahon et al. 2001; Ramos-Gomez et al. 2001; Khor et al. 2006), liver (Itoh et al. 1997; Chan and Kwong 2000a; Kwak et al. 2001; Ramos-Gomez et al. 2001; Chanas et al. 2002; Iida et al. 2004), lung (Chan and Kan 1999; Cho et al. 2002; Ishii et al. 2005; Rangasamy et al. 2005), and skin (Xu et al. 2006), and (2) enhanced susceptibility to the toxicities associated with various xenobiotics and environmental stresses (see Table 5 and Copple et al. 2008 for examples).

When considering the potential for extrapolation to a human context, the animal disease model which perhaps best indicates the importance of the Keap1-Nrf2 pathway for defining the threshold and response to cellular stress is that of acetaminophen-induced hepatotoxicity. Research in this laboratory has demonstrated that acetaminophen activates Nrf2-dependent cell defense in mouse liver, following the administration of both nontoxic and toxic doses *in vivo* (Goldring et al. 2004; Randle et al. 2008). This Nrf2-driven adaptive response, in addition to the protection afforded by the basal expression of Nrf2-regulated genes, may be an important determinant of the threshold for hepatotoxicity, as *Nrf2*$^{-/-}$ animals are more vulnerable to acetaminophen-induced hepatotoxicity (Chan et al. 2001; Enomoto et al. 2001). In contrast, hepatocyte-specific *Keap1*$^{-/-}$ mice, in which Nrf2-dependent cytoprotection is enhanced (Okawa et al. 2006), are highly resistant to doses of acetaminophen that are hepatotoxic and lethal in wild-type mice (Okawa et al. 2006). Furthermore, it has been demonstrated that known activators of Nrf2-dependent

Table 5 Examples of the enhanced sensitivities to xenobiotic-induced toxicity of transgenic Nrf2 knockout (*Nrf2*$^{-/-}$) mice compared to wild-type (*Nrf2*$^{+/+}$) counterparts

Target organ	Stressor	Toxicities observed	Reference(s)
Liver	Acetaminophen	Increased mortality, increased serum transaminase levels, greater depletion of GSH, increased tissue lesions, increased centrilobular hepatic necrosis	Chan et al. (2001); Enomoto et al. (2001)
Liver	Pentachlorophenol	Increased lipid peroxidation, increased DNA adducts, increased serum transaminases	Umemura et al. (2006)
Lung	Butylated hydroxytoluene	Acute respiratory distress, increased mortality, thickening of alveolar septa, destruction of alveolar architecture, extensive damage to alveolar capillaries, increased lung weight	Chan et al. (1999)
Lung	Cigarette smoke	Increased emphysema, increased tissue inflammation, increased vascular permeability, increased neutrophil invasion, reduced phagocytosis of neurophils by macrophages, increased apoptosis	Iizuka et al. (2005); Rangasamy et al. (2004)
Brain	Kainate	Increased mortality, increased seizure intensity and duration, increased hippocampal neuron damage, increased microglial infiltration	Kraft et al. (2006)
Colon	Dextran sulfate sodium	Increased expression of pro-inflammatory mediators (TNFα, iNOS, COX-2, cytokines), rectal bleeding, increased colitis severity, increased number of colonic aberrant crypt foci, increased lipid peroxidation	Khor et al. (2006); Osburn et al. (2007)
Stomach	Benzo[a]pyrene	Increased DNA adducts, increase in forestomach tumors	Ramos-Gomez et al. (2003); Ramos-Gomez et al. (2001)
Ovary	4-Vinylcyclohexene diepoxide	Greater loss of ovarian follicles, increased follicular apoptosis, accelerated reproductive failure	Hu et al. (2006)

cell defense can afford protection against acetaminophen-induced hepatotoxicity (Ansher et al. 1983; Hu et al. 1996; Sener et al. 2006a, b; Abdel-Zaher et al. 2008). The toxicities associated with acetaminophen (Bourdi et al. 2002; Ishida et al. 2002; Masubuchi et al. 2003; Liu et al. 2004) and some of the other xenobiotics to which *Nrf2*$^{-/-}$ mice are more vulnerable (see Copple et al. 2008) have an underlying immunological component. Therefore, it is possible that the disrupted immune systems of *Nrf2*$^{-/-}$ animals (Yoh et al. 2001; Vargas et al. 2006; Hubbs et al. 2007) may serve to potentiate the organ-directed toxicities associated with certain

chemicals. In conclusion, the generation of *Nrf2*$^{-/-}$ mice has facilitated investigations into the role of the Keap1-Nrf2 pathway in determining the threshold for, and response to, toxic insult.

Attempts to investigate the role of Keap1 in regulating Nrf2-mediated cell defense *in vivo* were initially hindered due to the retarded growth and death of *Keap1*$^{-/-}$ mice within 21 days of birth, due in part to malnutrition resulting from hyperkeratotic lesions in the eosophagus and forestomach, which obstruct the upper digestive tract (Wakabayashi et al. 2003). Co-knockout of *Nrf2* (*Keap1*$^{-/-}$*::Nrf2*$^{-/-}$) rescued this phenotype, indicating that Nrf2 is the central downstream target of Keap1 *in vivo* (Wakabayashi et al. 2003). Recently, however, hepatocyte-specific knockout of *Keap1* has been achieved using the Cre-loxP system, which facilitates tissue-specific gene knockout (Okawa et al. 2006). Briefly, an *Alb-Cre* mouse, expressing a Cre recombinase transgene under the control of the liver-specific albumin promoter, is crossed with a *Keap1-loxP* mouse, which transgenically expresses a *Keap1* gene in which exons 4–6 are flanked by loxP sites. In the double-transgenic *Alb-Cre::Keap1-loxP* mouse, Cre catalyzes recombination between target loxP sites, resulting in excision of the flanked segment (exons 4–6) within *Keap1* and thus translation of a truncated form of the protein, lacking the DGR domain that interacts with Nrf2 (Nagy 2000; Okawa et al. 2006). Without the growth retardation and malnutrition observed in *Keap1*$^{-/-}$ animals, hepatocyte-specific knockout of *Keap1* results in an increase in basal expression of numerous ARE-driven genes in the liver, including *Nqo1*, *Gclc*, *Gpx*, and carbonyl reductase (Okawa et al. 2006). Moreover, *Alb-Cre::Keap1-loxP* mice are highly resistant to doses of paracetamol that are hepatotoxic and lethal in wild-type mice (Okawa et al. 2006), further demonstrating that Keap1 regulates cell defense by repressing Nrf2 *in vivo*.

Recently, the lethality of the nontissue-specific *Keap1* knockout has been exploited to enable the development of an *in vivo* complementation rescue model, whereby the transgenic expression of Keap1 restores *Keap1*$^{-/-}$ mice to a viable state and enables development/growth that is analogous to wild-type mice (Yamamoto et al. 2008). Importantly, the rescue of *Keap1*$^{-/-}$ mice from lethality is abrogated when forms of Keap1 bearing mutations (specifically, BTB domain deletion or alanine substitution of Cys-273 and/or Cys-288) are transgenically expressed (Yamamoto et al. 2008). These observations provide strong evidence for the important roles of Cys-273 and Cys-288 in the function of Keap1 *in vivo*, consistent with the results of site-directed mutagenesis experiments in established cell lines (Zhang and Hannink 2003; Levonen et al. 2004; Wakabayashi et al. 2004; Kobayashi et al. 2006). Furthermore, the fact that the Keap1-Nrf2 pathway is rendered unable to respond to the electrophile tBHQ when Keap1 bearing a Cys-151-Ser substitution is transgenically expressed against a *Keap1*$^{-/-}$ background (Yamamoto et al. 2008) demonstrates the importance of Cys-151 in allowing Keap1 to "sense" chemical/oxidative stress *in vivo*, and also corroborates with evidence from cell-based molecular genetic studies (Zhang and Hannink 2003; Zhang et al. 2004).

7 Polymorphisms in the Keap1-Nrf2 Pathway

In light of the important role of the Keap1-Nrf2 pathway in regulating cellular defense, genetic variation in this pathway may have important consequences for human health. Research in our laboratory has identified several novel, albeit synonymous, polymorphisms in the genes encoding *Nrf2* and *Keap1*, through the screening of a cohort of healthy human volunteers (Wang et al. 2006). Single nucleotide polymorphisms have been identified within the promoter region of the human (Yamamoto et al. 2004; Arisawa et al. 2007; Marzec et al. 2007) and mouse (Cho et al. 2002) *Nrf2* genes, and these mutations are associated with an increase in susceptibility to certain diseases (Cho et al. 2002; Arisawa et al. 2007, 2008; Marzec et al. 2007). Furthermore, two nonsynonymous mutations in *Nrf2* have recently been identified in Japanese type II diabetes patients (Fukushima-Uesaka et al. 2007). Although a recent study has demonstrated that partial loss-of-function of Keap1 in male *Drosophila* is associated with an increase in resistance to oxidative stress and an extension of median lifespan of up to 10% (Sykiotis and Bohmann 2008), more comprehensive loss of Keap1 function has been associated with the process of carcinogenesis. Indeed, somatic loss-of-function mutations in *Keap1* have been identified in lung and breast carcinoma cell lines and in lung cancer patients (Padmanabhan et al. 2006; Singh et al. 2006a; Nioi and Nguyen 2007; Ohta et al. 2008). It will be important to determine whether there is variability in the competence of the Keap1-Nrf2 pathway amongst the general population, and whether such variability influences an individual's susceptibility to an adverse drug reaction.

8 Future Directions

In recent years, there have been great advances in our understanding of the chemical, biochemical, and molecular means by which the Keap1-Nrf2 pathway is regulated. Future research should consider the relative importance of different posttranslational modifications (direct adduction, oxidation, phosphorylation) in triggering Nrf2 activation, and the precise means by which these modifications are translated into biological effect. For instance, although there is strong evidence to suggest that Nrf2-activating molecules directly modify cysteine residues in Keap1 (Dinkova-Kostova et al. 2002; Eggler et al. 2005; Hong et al. 2005a, b; Luo et al. 2007), it has yet to be demonstrated unequivocally that modification of Keap1 triggers the activation of Nrf2 in cells or *in vivo*. The major bioanalytical constraint that hampers such investigations of protein modification and function in parallel is that modifications are often substoichiometric in nature, with perhaps only 1–2% of the total amount of a given protein modified under physiological conditions. In a recent review of this subject, Liebler (2008) likened this problem to "looking for dozens of needles in thousand of haystacks". In the study of the Keap1-Nrf2 pathway, this problem is further compounded by the fact that Keap1 appears to be expressed at a level of 10 parts per million or less in cells (McMahon et al. 2006). These issues

make it particularly difficult to detect modifications of endogenous Keap1, and even more difficult to identify the site(s) of modification, from a cell/tissue lysate containing thousands of proteins with different levels of abundance. Biochemical enrichment strategies and/or advances in mass spectrometry, such as multiple reaction monitoring (Unwin et al. 2005), may facilitate the reliable and sensitive analysis of low-abundance posttranslational modifications of Keap1 in the near future.

In addition to correlating the activation of Nrf2 with the occurrence of a particular modification per se, it will also be important to understand how the extent of modification influences the biological response. It is possible that Keap1 functions as a redox rheostat, in that the modification of a single reactive cysteine is sufficient to trigger the activation of Nrf2, with additional modifications of other cysteine residues augmenting this response. In this regard, it is vital to obtain quantitative measurements of the ratio of modified versus unmodified residues in Keap1, and to relate this to biological outcome. It is also important to consider that signaling events other than the direct modification of Keap1 may contribute to the activation of the Keap1-Nrf2 pathway by some inducers. Recently, much attention has focused on the importance of reversible oxidation of thiols to sulfenic acid as a signaling mechanism within cells (for a review, see Biswas et al. 2006). In fact, there is evidence to suggest that pro-oxidants can activate Nrf2 (Gong and Cederbaum 2006; Lee-Hilz et al. 2006; Purdom-Dickinson et al. 2007). Therefore, it is possible that Nrf2 may be activated by either direct adduction or oxidation of cysteines in Keap1. However, testing such a hypothesis is difficult, as almost all the molecules that have been demonstrated to activate Nrf2 have the ability to directly modify Keap1 and alter the cellular redox balance by depleting levels of GSH. The challenge therefore lies in determining which of these modifications represents the underlying trigger for Nrf2 activation by a given molecule.

By furthering our understanding of the regulatory mechanisms that govern the activity of the Keap1-Nrf2 pathway, we may be better placed to explore the potential benefits of modulating the activity of Nrf2 as a therapeutic intervention (for recent reviews on this prospect, see Lee and Surh 2005; van Muiswinkel and Kuiperij 2005). Recent associations between Keap1 loss-of-function mutations (i.e. overactivation of Nrf2) and carcinogenesis (Padmanabhan et al. 2006; Singh et al. 2006a; Nioi and Nguyen 2007; Ohta et al. 2008) suggest that the comprehensive induction of Nrf2-dependent cell defense over a prolonged period of time may be less beneficial as a therapeutic strategy than originally envisaged. As such, a more careful approach, informed by a detailed understanding of the chemistry, biochemistry, and molecular biology of the Keap1-Nrf2 pathway, may be required in order to maximize the benefit: harm ratio of such an intervention. Nevertheless, pharmacological manipulation of Nrf2 by the isothiocyanate sulforaphane, a derivative of glucoraphanin, which is present at high concentrations in broccoli, Brussel sprouts, and cabbage (Zhang et al. 1992), is currently being trialed as a means of preventing breast cancer (Dinkova-Kostova et al. 2006, 2007; Shapiro et al. 2006; Cornblatt et al. 2007). An alternative strategy can also be envisaged whereby the enhanced cellular protection that appears to be afforded by the upregulation of Nrf2 activity in certain cancers (Padmanabhan et al. 2006; Singh et al. 2006a; Nioi and Nguyen 2007; Ohta et al.

2008) might be inhibited in order to improve the efficacy of conventional chemotherapeutics. It is also possible that a better understanding of the role of the Keap1-Nrf2 pathway in "sensing" cellular stress may enable the activation of Nrf2 to be used as an indicator of toxicity during the drug development process, with a view to filtering out candidate molecules that may damage cells upon exposure *in vivo*.

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Signal Transduction Pathways Involved in Drug-Induced Liver Injury

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Abstract Hepatocyte death following drug intake is the critical event in the clinical manifestation of drug-induced liver injury (DILI). Traditionally, hepatocyte death caused by drugs had been attributed to overwhelming oxidative stress and mitochondria dysfunction caused by reactive metabolites formed during drug metabolism. However, recent studies have also shown that signal transduction pathways activated/inhibited during oxidative stress play a key role in DILI. In acetaminophen (APAP)-induced liver injury, hepatocyte death requires the sustained activation of c-Jun kinase (JNK), a kinase important in mediating apoptotic and necrotic death. Inhibition of JNK using chemical inhibitors or knocking down JNK can prevent hepatocyte death even in the presence of extensive glutathione (GSH) depletion, covalent binding, and oxidative stress. Once activated, JNK translocates to mitochondria, to induce mitochondria permeability transition and trigger hepatocyte death. Mitochondria are central targets where prodeath kinases such as JNK, prosurvival death proteins such as bcl-xl, and oxidative damage converge to determine hepatocyte survival. The importance of mitochondria in DILI is also observed in the Mn-SOD heterozygous (+/−) model, where mice with less mitochondrial Mn-SOD are sensitized to liver injury caused by certain drugs. An extensive body of research is accumulating suggesting a central role of mitochondria in DILI. Drugs can also cause redox changes that inhibit important prosurvival pathways such as NF-κB. The inhibition of NF-κB by subtoxic doses of APAP sensitizes hepatocyte to the cytotoxic actions of tumor necrosis factor (TNF). Many drugs will induce liver injury if simultaneously treated with LPS, which promotes inflammation and cytokine release. Drugs may be sensitizing hepatocytes to the cytotoxic effects of cytokines such as TNF, or vice versa. Overall many signaling pathways are important in regulating DILI, and represent potential therapeutic targets to reduce liver injury caused by drugs.

Keywords Acetaminophen · Oxidative stress · Redox · JNK · Mitochondria

Abbreviations

APAP	Acetaminophen
AMAP	*N*-acetyl-*m*-aminophenol
ASK-1	Apoptosis signaling-regulating kinase 1
CYP	Cytochrome P450
DILI	Drug-induced liver injury
DNASE1	Deoxyribonuclease 1
GSH	Glutathione
GSK-3β	Glycogen synthase kinase-3β
JNK	cJun N-terminal protein kinase
LPS	Lipopolysaccharide
MPT	Mitochondria permeability transition

NAC	*N*-acetylcysteine
NAPQI	*N*-acetyl-*p*-benzo-quinoneimine
NO	Nitric oxide
Nrf-2	NF E2-related factor 2
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TNF	Tumor necrosis factor

1 Introduction

Liver, as the major site of drug metabolism, is also the major site of drug injury. Drug-induced liver injury (DILI) continues to be a problem for many commonly used drugs, and represents a major challenge in designing potential therapies (Kaplowitz 2005; Thames 2004). Acetaminophen (APAP; Tylenol – an analgesic), valproic acid (an anticonvulsant drug), isoniazid (tuberculosis medication), and zafirlukast (a drug used for asthma) are examples of commonly used drugs that are associated with liver injury, requiring warning labels. Troglitazone (an antidiabetic drug) and bromfenac (an analgesic) were promising drugs that were recently withdrawn due to unacceptable liver injury. The development of strategies to reduce DILI not only has important implications in liver injury, but may also increase the availability of many drugs involved in treatment of a wide range of diseases.

Hepatocyte injury and death is the critical initiating event leading to the clinical manifestations of DILI. Hepatocyte injury can be triggered directly by some drugs (parent compound), but in most cases involves formation of reactive metabolites generated during metabolism in hepatocytes (Kaplowitz 2005; Uetrecht 2008). Since most drugs are lipophillic, drug clearance involves biotransformation of the drug by cytochrome P450 (CYP) or other phase I enzymes to reactive metabolites for conjugation with hydrophilic carriers (e.g., glucuronide, GSH) for excretion into urine or bile. However, as the name suggests, reactive metabolites are highly reactive molecules that not only covalently bind with hydrophilic carriers, but also covalently bind with proteins (Jollow et al. 1973; Kaplowitz 2005). Reactive metabolites can also directly, through redox cycling, or indirectly, through glutathione (GSH) depletion, can increase reactive oxygen species (ROS; superoxide ($O_2^{\cdot}-$), H_2O_2, hydroxyl radical ($HO^{\cdot}$)) generation in hepatocytes (Hanawa et al. 2008; Lores Arnaiz et al. 1995). Covalent binding and ROS production caused by reactive metabolites can modify proteins and other macromolecules to cause hepatocyte stress and injury. The stress and injury caused by reactive metabolites can activate and/or inhibit a wide range of signaling pathways. Whether a hepatocyte survives or undergoes cell death following injury by reactive metabolites is determined, in great part, by the balance of prodeath and prosurvival signaling pathways that become activated. If prodeath pathways (e.g., JNK, bax, etc.) are

primarily activated, these signaling pathways will initiate hepatocyte death following hepatocyte injury. Even in necrotic death, prodeath pathways such as JNK can trigger cell death to occur (Hanawa et al. 2008). Consequently, pharmacological inhibition of these prodeath pathways can often prevent cell death, even in the presence of extensive cell injury. On the other hand, the activation of prosurvival pathways (e.g., Akt, DNA repair enzymes) may inhibit cell death by directly inhibiting prodeath signaling, or indirectly by increasing cell repair or metabolism (Saberi et al. 2008). The modulation of these key prodeath and prosurvival signaling pathways in hepatocytes may decrease the extent of liver injury caused by drugs and, therefore, represent potential therapeutic targets for treatment of DILI.

In this chapter, the intrinsic signaling pathways in hepatocytes that are important in mediating cell death/survival in the context of drug hepatotoxicity will be discussed. Key prodeath or prosurvival signaling pathways activated by drugs in hepatocytes will be reviewed. We will focus on hepatocellular death and not consider cholestatic drug injury or immune-mediated hypersensitivity. However, before discussing signaling pathways involved in DILI, a brief overview of DILI will be presented.

2 Drug-Induced Liver Injury

DILI is a term that describes a condition in which medical intake of a drug(s) causes an individual to have abnormalities in liver tests, often manifested by an increase in serum ALT levels. DILI can cause severe liver injury, with the most catastrophic consequence being acute liver failure leading to death or requiring a liver transplant (Ulrich 2007). It has been estimated that around 60% of all cases of acute liver failure are caused by drugs (Kaplowitz 2005; Ostapowicz et al. 2002). The clinical cases of acute liver failure due to DILI are primarily due to overdose of APAP (~50% of acute liver failure due to drugs) and drugs that cause idiosyncratic liver injury (~10%).

2.1 Acetaminophen

APAP is the most common cause of drug-induced liver failure due to unintentional or deliberate overdose (Ostapowicz et al. 2002). It has been estimated that APAP causes 10,000 hospital visits and 500 deaths a year in the United States. If APAP-induced liver injury is caught early, it can be readily treated with *N*-acetylcysteine (NAC), a cysteine prodrug and precursor of GSH, generally without significant liver injury (Rumack et al. 1981). Unfortunately, many cases of APAP-induced hepatotoxicity are not diagnosed or seen early enough, leaving little available treatment to prevent liver injury. Consequently, the development of new therapeutic strategies to treat APAP-induced liver injury, particularly during later stages of liver injury, remains an important field of research.

2.2 *Idiosyncratic Drug Hepatotoxicity*

Idiosyncratic hepatotoxicity refers to the unpredictable clinical overt liver injury that occurs in a small subset of patients (~ between 1 in 1,000 to 1 in 10,000) taking therapeutic doses of certain drugs (Kaplowitz 2005; Watkins 2005). The underlying reasons of why a small subpopulation of patients develop liver injury following therapeutic levels of drugs, while the vast majority of patients do not develop liver toxicity, is complex. There is probably no single factor that causes idiosyncratic liver injury; rather, it is the result of a convergence of risk factors such as genetics, age, gender, diet, infections, etc. (Ulrich 2007). In addition, each drug that causes idiosyncratic drug injury has certain risk factors unique to that drug. For example, young age and viral infections are believed to be the risk factors associated with aspirin intake triggering Reye's syndrome, a disease associated with microvesicular steatosis and mitochondrial abnormalities in the liver of patients (Iancu et al. 1977; Osterloh et al. 1989). Idiosyncratic drug hepatotoxicity is difficult to predict since it is not generally observed in animal models, and because most clinical trials generally use insufficient population sizes (~ low thousands). Consequently, only when a drug reaches the general population and is taken by a large number of patients does idiosyncratic DILI become unequivocally recognized. This was recently illustrated in the 2000 recall of troglitazone, a peroxisome proliferator-activated receptor gamma agonist used in the treatment of diabetes (Watkins 2005). During clinical trials of troglitazone, roughly 1 in 1,000 patients had elevated ALT levels and jaundice (overt injury). However, following its release in 1997, after some 2 million patients began using the drug, nearly 100 cases of acute liver failure were reported to the FDA (thus 1 in 20,000 patients suffered DILI) (Kaplowitz 2005). Thus, idiosyncratic DILI is the major reason for postmarketing withdrawals and warnings of drugs. In addition, idiosyncratic DILI remains a major problem in drug development, and understanding its mechanism may open up a wide range of new therapeutic options.

Idiosyncratic drug hepatotoxicity falls into two categories, *allergic* or *nonallergic*. Allergic idiosyncratic DILI is characterized by an immune attack on liver, while nonallergic idiosyncratic DILI is the result of biochemical effects of toxic metabolites causing hepatocyte injury and death. Allergic reactions involve the adaptive immune system, a short latency period, hypersensitivity, and, in many instances, production of auto-antibodies (Castell and Castell 2006; Seguin and Uetrecht 2003). Allergic idiosyncratic hepatotoxicity is not well understood, and most of our knowledge comes from our understanding of autoimmune diseases. Since allergic idiosyncratic liver injury mainly involves signaling in the adaptive immune system (extrinsic signaling), it will not be a focus of this chapter. On the other hand, nonallergic idiosyncratic drug hepatotoxicity is characterized by a lack of apparent hypersensitivity and a variable, sometimes long, latency period (Kaplowitz 2005). Although allergic reactions are not a central feature of this type of idiosyncratic drug hepatotoxicity, its participation cannot be completely ruled out. Nonallergic idiosyncratic drug hepatotoxicity generally involves hepatocyte

injury and death probably initiated by reactive metabolites, as previously discussed. Nonallergic idiosyncratic DILI will be the major focus of this chapter. It should be noted that, while liver injury caused by APAP is dose dependent, it also displays characteristics of nonallergic idiosyncratic hepatotoxicity. Although the majority of hospitalizations occur from overdosing on APAP, about 1 in 5 cases of APAP-induced liver injury appear to occur in people who have taken therapeutic doses of APAP (Kaplowitz 2005). This suggests that APAP may also cause idiosyncratic liver injury in humans at low rates. The idiosyncratic nature of APAP has been supported in animal models, which show there are clear differences in susceptibility to APAP-induced liver injury between strains, sexes, and due to diet (Dai et al. 2006; Ito et al. 2006).

3 APAP-Induced Liver Injury as a Model of DILI

Studying the intrinsic signaling pathways involved in DILI, as well as other steps involved in DILI, has been hampered by the availability of good animal models (Dixit and Boelsterli 2007; Kaplowitz 2005). All prospective drugs are screened in animals for potential adverse effects on liver and other organs, and those drugs found to cause liver injury in animals are rarely developed for human consumption. Consequently, most drugs that cause idiosyncratic liver injury in humans generally do not cause liver injury in animals, having already passed screening. Very few drugs that cause liver injury in humans can be studied in animals and, consequently, a mechanistic understanding of how drugs cause liver injury is lacking. The noted exception is APAP, which causes liver injury in animals in a dose-dependent manner. Accordingly, most of our knowledge about DILI has come from work with APAP at high doses that cause liver injury in animals (Kaplowitz 2005). In addition, since APAP-induced liver injury exhibits idiosyncratic features in animals, a number of recent metabolomics and genomic studies have focused on understanding the underlying mechanism(s) responsible for the idiosyncratic nature of APAP (Welch et al. 2005, 2006). Our examination of the role of signaling pathways involved in DILI will begin by examining the signaling pathways that are important in mediating APAP-induced liver injury.

3.1 Mode of Hepatocyte Death During APAP-Induced Liver Injury

Hepatocyte death following APAP intake is the critical event in the clinical manifestation of liver injury. Hepatotoxic doses of APAP are believed to mainly cause necrotic cell death in hepatocytes (Gujral et al. 2002), predominantly in the centrilobar regions of liver. Apoptotic death or programmed cell death has been

suggested to occur during APAP hepatotoxicity in some studies (Hu and Colletti 2008; Nakagawa et al. 2008), but the extent is controversial (Gujral et al. 2002; Kaplowitz 2005; Malhi et al. 2006). Although apoptosis can be readily identified in cultured cells, the detection of apoptosis in vivo is technically more difficult and lends itself more to subjective interpretations (Jaeschke et al. 2004; Malhi et al. 2006). Studies investigating caspase activation during APAP-induced liver injury have supported the notion that necrosis is the primary mode of hepatocyte death following APAP treatment. Apoptosis requires the activation of caspases, cysteine protease important in causing protein cleavage that initiates formation of apoptotic bodies, the hallmark morphologic characteristic of apoptosis (Malhi et al. 2006). In APAP hepatotoxicity, very little caspase cleavage is observed in vivo, and caspase inhibitors cannot prevent APAP-induced injury in liver (Gujral et al. 2002; Jaeschke et al. 2006). Apoptosis can generally be prevented by inhibiting caspase activation, through chemical inhibitors (e.g., z-VAD) or genetic modification, even in the presence of cellular injury in hepatocytes and other cells (Han et al. 2006a; Matsumaru et al. 2003). Necrosis rather than apoptosis being the primary mode of hepatocyte death following APAP is significant in that necrosis is associated with much greater inflammation than apoptosis. The rupture of the plasma membrane during necrosis releases proteases, cathepsins, and proinflammatory factors that activate the innate immune system. Consequently, APAP-induced liver injury is associated with activation of the innate immune system (e.g., neutrophils, NK/NKT cells) important in enhancing liver injury caused by APAP (Liu et al. 2004, 2006). On the other hand, during apoptosis, cells break down into membrane bound apoptotic bodies that are consumed by neighboring cells, minimizing release of inflammatory factors (Kaplowitz 2000). However, it should be noted that apoptosis and necrosis represent two opposite ends in the cell death morphology spectrum, and cell death that displays morphology intermediate between the two forms may also occur during APAP-induced liver injury. In addition, the importance of autophagy, another form of cell death involving formation of autophagosomes (Yin et al. 2008), in APAP-induced hepatotoxicity has yet to be determined.

Although necrosis is the predominant form of hepatocyte death during APAP hepatotoxicity, the importance of signaling pathways modulating APAP-induced hepatotoxicity cannot be discounted. Recent studies have also suggested that even necrotic cell death, which has been traditionally viewed as a passive process resulting from overwhelming cell injury, involves activation and/or inhibition of key signaling pathways that modulate cell death or survival (Proskuryakov et al. 2003). Cells undergoing apoptosis may often switch to necrosis if ATP levels needed for caspase activity are not available. In primary cultured hepatocytes, we observed that H_2O_2-induced necrosis, which has generally been attributed to overwhelming injury due to oxidative damage, was inhibited by PKC inhibitors and AMPK activators (Saberi et al. 2008). The latter may be important in activation of energy generating pathways (e.g., glucose transport, fatty acid oxidation, etc.) that help cell repair and increase survival. When cells are injured, signaling pathways that modulate energy production, ion transport, cell repair systems, and mitochondria bioenergetics could all potentially alter the extent of cellular necrosis. However,

"programmed" necrosis may involve activation of prodeath pathways such as c-Jun kinase (JNK) and bax that directly trigger hepatocyte death, which will be the focus of discussion in the next section.

3.2 NAPQI Formation and Reactive Oxygen Species Generation

APAP hepatotoxicity can be attributed to *N*-acetyl-*p*-benzo-quinoneimine (NAPQI) formation, a reactive metabolite generated during metabolism of APAP by CYP, primarily by the CYP2EI isoform (Dahlin et al. 1984). NAPQI is a highly reactive molecule that forms covalent bonds with protein and nonprotein thiols (Hinson et al. 2004; Jollow et al. 1973). In hepatocytes, NAPQI is mainly detoxified by glutathione (GSH), the major antioxidant and nonprotein thiol in cells. At hepatotoxic doses of APAP, GSH is overwhelmed and becomes severely depleted in cytoplasm and in mitochondria, which contain a separate GSH pool (Hirayama et al. 1983; James et al. 2003). Hepatotoxic doses of APAP (>250 mg kg^{-1} in C57BL/6 mice) deplete over 90% of GSH in the cytoplasm and mitochondria (Hanawa et al. 2008). The metabolism of APAP to NAPQI, is a minor pathway in APAP clearance; consequently, large doses of APAP are generally required to produce sufficient levels of NAPQI to deplete GSH in cytoplasm and mitochondria. Once GSH becomes depleted, NAPQI will covalently bind with thiol residues in proteins, which can potentially affect protein activity (Burcham and Harman 1991; Esterline et al. 1989).

The depletion of mitochondrial GSH caused by NAPQI has a profound effect on reactive oxygen species (ROS) generation by mitochondria. GSH is important in detoxifying H_2O_2 in the mitochondrial matrix and cytoplasm, where GSH peroxidase uses the reducing power of GSH to reduce H_2O_2 to H_2O (Han et al. 2003b). An increase in mitochondrial H_2O_2 production was observed in isolated mitochondria as early as 1 h following APAP treatment (Hanawa et al. 2008; Lores Arnaiz et al. 1995). The increased ROS may be a direct consequence of loss of GSH (GSH peroxidase) as well as an indirect consequence of loss of GSH allowing NAPQI to impair the electron transport chain to promote increase ROS generation (Burcham and Harman 1991; Esterline et al. 1989; Han et al. 2003b). There is also some evidence that suggests NAPQI may redox cycle to generate ROS in hepatocytes. In addition to increased ROS generation, APAP treatment has been shown to cause increase nitric oxide (NO) generation in liver through upregulation of inducible NO synthase (iNOS) and endothelial nitric oxide synthase (eNOS) (Gardner et al. 1998; Ito et al. 2004). A portion of NO formed following APAP treatment reacts with superoxide ($O_2^{\cdot -}$) to form peroxynitrite ($ONOO^-$), a strong oxidant that oxidizes proteins and other macromolecules (Jaeschke et al. 2003). Consequently, APAP hepatotoxicity is associated with increase oxidative stress, due to GSH depletion and increased ROS generation, as well as to increased nitrosative stress caused by increase NO and $ONOO^-$ generation in liver.

3.3 *Protein Redox Changes During APAP-Induced Liver Injury*

The depletion of GSH, as well as increases in ROS and reactive nitrogen species (RNS; NO, $ONOO^-$) caused by NAPQI, has a profound impact on cellular and mitochondrial redox status (Han et al. 2006b; Yap et al. 2008). Hepatocytes, like most mammalian cells, have a very reduced intracellular environment with GSH: GSSG (oxidized glutathione) being greater than 100:1, and sulfhydryls in proteins being mainly in the reduced thiol form (Kaplowitz et al. 1985). Mitochondria, which contain a separate GSH pool, are believed to have an even more reduced environment than cytoplasm (Hansen et al. 2006). A major consequence of reduced GSH levels and/or increase oxidative stress and nitrosative stress caused by NAPQI is an alteration of protein redox status and possibly function (Fig. 1). As previously mentioned, NAPQI can covalent bind with protein thiols and alter protein function when GSH levels are depleted. Increased H_2O_2 caused by GSH depletion can also react with protein thiols and cause disulfide bond formation, sulfenic acid, and other redox changes to protein thiols (Han et al. 2006b). Similarly, NO can cause protein thiol nitrosylation, while $ONOO^-$ can cause thiol oxidation to sulfenic and sulfonic

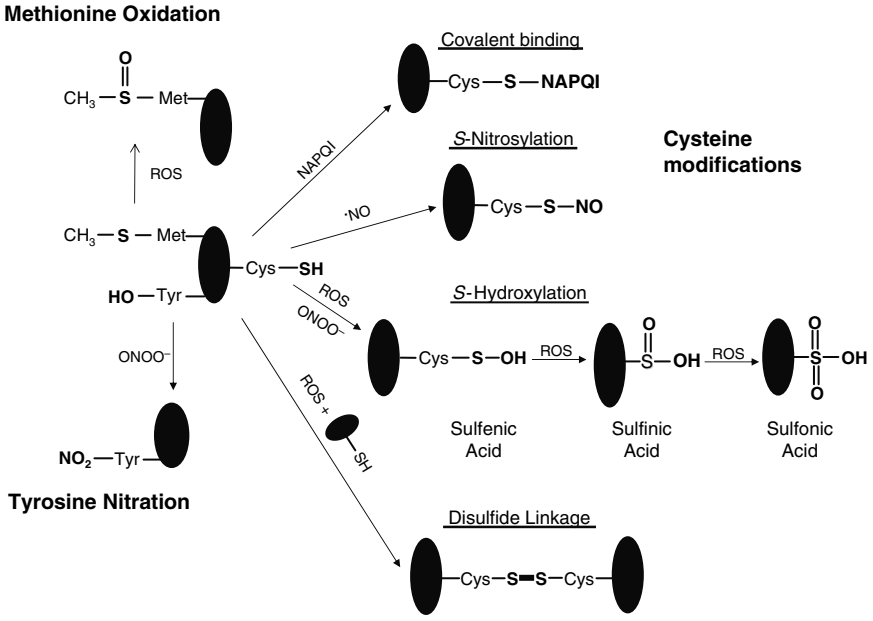

Fig. 1 Posttranslational redox modifications to amino acids in proteins. Many amino acids can undergo various posttranslational redox modifications in the presence of NAPQI, oxidative stress, and nitrosative stress. Thiols in cysteine can undergo covalent binding, mixed disulfide formation, nitrosylation, and become oxidized to sulfenic, sulfinic, and sulfonic acids. Tyrosine can become nitrated by peroxynitrate, and methionine can be oxidized by ROS to methionine sulfoxide. Not shown are many other oxidations that can occur to other amino acids such as proline, histidine, etc.

acids, as well as cause tyrosine nitration (Klatt and Lamas 2000). In addition to cysteine, many other amino acids such as methionine, proline, and histidine may also be oxidized by ROS and RNS (Han et al. 2000; Stadtman et al. 2003). All these redox modification can potentially affect protein activity, depending on the importance of amino acid modified by NAPQI, ROS, and RNS. During APAP-induced liver injury, extensive covalent binding, protein oxidation, and nitrotyrosine, a marker of $ONOO^-$, have been observed (Jaeschke et al. 2003; Michael et al. 2001).

The posttranslational redox modifications of proteins may also be an important mechanism of activating or inhibiting many signaling pathways in hepatocytes following APAP treatment. For example, signaling proteins such as NF-κB and protein phosphatases can be inhibited by redox modifications (Kamata et al. 2005; Klatt and Lamas 2000). On the other hand, proteins such as NF E2-related factor 2 (Nrf-2) are activated through protein redox modifications (Fig. 2). Nrf-2 is an important transcription factor that binds to the antioxidant response element (ARE), a DNA sequence in the promoter of antioxidant enzymes such as glutamylcysteine ligase (GCL), heme oxygenase (HO-1), and microsomal epoxide hydrolase (Itoh et al. 2004). Under normal conditions, Nrf-2 is found in the cytoplasm attached to

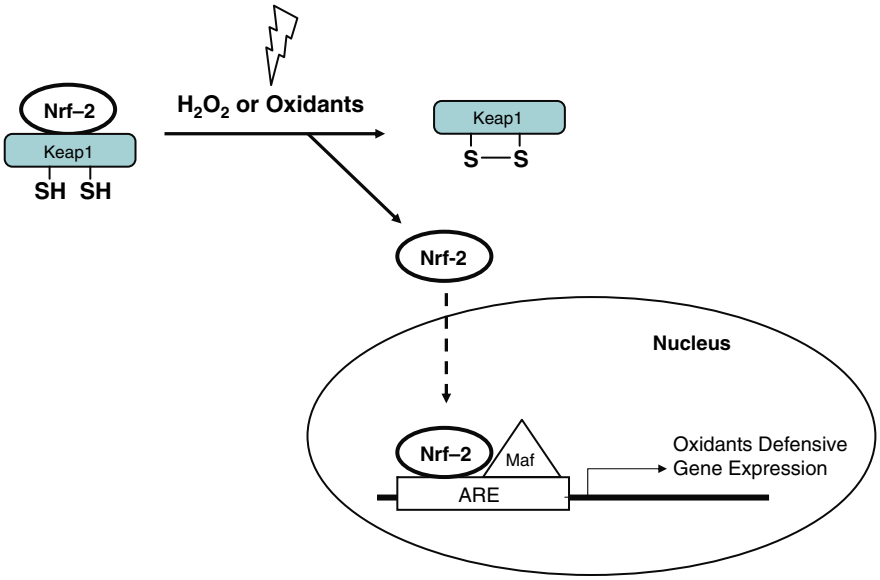

Fig. 2 Activation of Nrf-2 by redox changes. Nrf-2 is an important transcription factor that binds to the antioxidant response element (ARE), a DNA sequence in the promoter of antioxidant enzymes such as glutamylcysteine ligase (GCL), heme oxygenase (HO-1), and microsomal epoxide hydrolase. Under normal conditions, Nrf-2 is found in the cytoplasm attached to Keap1. Keap1 contains critical thiols essential in binding and retaining Nrf-2 in the cytoplasm. NAPQI can directly, through covalent binding, or indirectly, through GSH depletion and/or increase ROS, cause the oxidation of thiols in Keap1. The oxidation of Keap1 causes Nrf-2 to detach and translocate to the nucleus. Nfr-2 in the nucleus binds to the ARE and transactivates antioxidant genes such as GCL to counter the redox disturbance caused by NAPQI

Keap1 (Itoh et al. 2003). Keap1 contains critical thiols essential in binding and retaining Nrf-2 in the cytoplasm (Dinkova-Kostova et al. 2002; Itoh et al. 2004). NAPQI can directly, through covalent binding, or indirectly, through GSH depletion and/or increased ROS, cause the oxidation or modification of thiols in Keap1. The oxidation of Keap1 releases Nrf-2, which then translocates to the nucleus. Nrf-2 in the nucleus will bind to the ARE and activate transcription of antioxidant genes such as GCL to counter the redox disturbance caused by NAPQI (Goldring et al. 2004). Other proteins that have been demonstrated to undergo posttranslational redox modifications following APAP treatment include 3-hydroxy-3-methylglutaryl coenzyme A synthase 2 (HMG-CoA synthase), a key regulatory enzyme in ketogenesis, and catalase, a key enzyme involved in H_2O_2 detoxification (Andringa et al. 2008). Redox modulation of proteins has been identified as an important mechanism of activating and inactivating many signaling proteins in response to ROS and RNS (Han et al. 2006b; Yap et al. 2008).

3.4 Mitochondria Injury and Permeability Transition Caused by NAPQI

There is a certain amount of debate about whether covalent binding or GSH depletion is the key component in causing hepatocyte injury following APAP. However, because GSH depletion and covalent binding occur within a similar time frame, the relative contribution of these factors in causing hepatocyte injury is difficult to ascertain. What is clear though is that covalent binding and/or GSH depletion must occur in mitochondria for APAP-induced hepatotoxicity to occur (Kaplowitz 2005, 2008). The importance of mitochondrial GSH depletion and covalent binding in APAP hepatotoxicity was confirmed by Nelson's laboratory, using *N*-acetyl-*m*-aminophenol (AMAP), a regioisomer of APAP (Rashed et al. 1990). AMAP, like APAP, is metabolized by CYP2E1 to form a reactive metabolite. However, AMAP, unlike APAP, does not cause liver injury even at high doses. The difference between AMAP and APAP is that AMAP treatment does not cause mitochondrial GSH depletion or covalent binding. AMAP treatment leads to cytoplasmic GSH depletion and covalent binding, but due to its high reactivity, the reactive metabolite formed from AMAP metabolism has limited diffusion, and mitochondria are spared (Rashed et al. 1990). The experiments with AMAP illustrate the importance of redox perturbation of mitochondria in mediating hepatocyte death. GSH depletion and covalent binding can inhibit mitochondria bioenergetics through redox changes to respiratory complex and TCA cycle proteins. Consequently, inhibition of mitochondria bioenergetics is an early event (beginning at ~2 h) during APAP-induced liver injury (Donnelly et al. 1994).

Covalent binding and GSH depletion in mitochondria may create a condition that favors induction of mitochondria permeability transition (MPT), which inhibits mitochondria bioenergetics (Hanawa et al. 2008; Masubuchi et al. 2005). MPT is

the permeabilization of the mitochondria inner membrane to protons and other ions that results in dissipation of the proton gradient necessary for oxidative phosphorylation (Boelsterli and Lim 2007). MPT can be both regulated, through the MPT pore, or unregulated, a consequence of severe damage to the mitochondrial inner membrane. The MPT pore is a ~2.3 nm megachannel that allows solutes up to ~1,500 Da to cross the inner mitochondrial membrane, which is normally impermeable to ions. The MPT pore is believed to be composed of (1) adenine nucleotide translocase (ANT), which functions in shuttling ATP and ADP across the inner membrane, (2) voltage dependent anion channel (VDAC), the main channel in the outer membrane, (3) peripheral benzodiazepine receptor on the outer membrane, (4) cyclophin D in the matrix, which binds cyclosporine A that prevents pore opening, and (5), in some cells, hexokinase and creatine kinase in the cytoplasm (Crompton 1999). The opening of the MPT pore is regulated by many factors such as Ca^{2+} and oxidized pyridine nucleotides. The MPT pore also contains critical thiols, whose oxidation promotes opening of the pore (Kowaltowski et al. 1997). Oxidants and redox modifying agents have been shown to cause pore opening in isolated mitochondria. NAPQI may therefore induce pore opening directly through binding to thiols of the pore, or indirectly through GSH depletion and increased ROS. MPT has been suggested to occur in vivo and is considered an important event in APAP-induced liver injury (Hanawa et al. 2008; Masubuchi et al. 2005).

The MPT pore in mitochondria is also regulated by members of the bcl-2 family of proteins (Kaplowitz 2002). The bcl-2 family members are composed of proapoptotic proteins (i.e., bax, bak, bid) and antiapoptotic members (i.e., bcl-2, bcl-xl). Proapoptotic bcl-2 members such as bax have been shown to bind to the MPT pore and favor opening (Sharpe et al. 2004). However, bax can also oligomerize to form channels on the outer membrane that causes the release of cytochrome c and other apoptotic factors from the intermembrane of mitochondria, independent of MPT (Kaplowitz 2002; Sharpe et al. 2004). Antiapoptotic proteins such as bcl-2 and bcl-xl, which reside primarily on the mitochondria outer membrane, inhibit MPT or bax/bak channels on the outer membrane by binding and oligomerizing with proapoptotic bcl-2 members such as bax. The MPT pore remains an important regulatory site in signaling pathways that determine cell death/survival. Consequently, redox changes, prodeath and prosurvival proteins converge at mitochondria to regulate survival. The regulation of MPT in hepatocytes during APAP hepatotoxicity will be discussed in Sect. 3.5 (d).

3.5 The JNK-Mitochondria Signaling Loop in APAP-Induced Liver Injury

The first signaling pathway identified to modulate APAP-induced liver injury was c-Jun N-terminal protein kinases (JNK) (Gunawan et al. 2006; Henderson et al. 2007; Latchoumycandane et al. 2007). JNK are a family of serine/threonine kinases

important in responding to environmental stresses, and growth factors, as well as to cytokines (Johnson and Nakamura 2007). Although JNK activation is an important component of stress response in cells, when JNK activity is sustained, it is believed to promote cell injury and death (Liu et al. 2002; Singh and Czaja 2007). Work from our laboratory first demonstrated that treatment of mice with JNK inhibitor and knocking down JNK (using antisense or knockout mice) was able to markedly protect aganist APAP-induced liver injury, without affecting APAP metabolism (i.e., GSH depletion and covalent binding) (Gunawan et al. 2006; Hanawa et al. 2008). Thus, even in the presence of significant cytoplasmic and mitochondrial GSH depletion and covalent binding, hepatocyte death did not occur without JNK. This finding represented a paradigm shift from the notion that hepatocyte necrosis was a *passive* death due to mitochondrial GSH depletion and covalent binding, and demonstrated that APAP-induced hepatotoxicity requires the *active* participation of a death kinase such as JNK (Kaplowitz et al. 2008).

The mechanism by which JNK induces hepatocyte death following APAP involves a complex JNK-mitochondria signaling loop involving several steps: (a) mitochondrial GSH depletion and ROS generation (b) redox changes and ASK-1 activation of JNK and (c) JNK modulation of bcl-2 family members (d) JNK translocation to mitochondria and inhibition of mitochondrial bioenergetics (Fig. 3).

(a) *Mitochondrial GSH depletion and ROS generation.* JNK is activated by many stresses including oxidative stress caused by ROS in a wide number of cells (Hanawa et al. 2008; Zhou et al. 2007). As previously mentioned, GSH is an important substrate for GSH peroxidase in mitochondria, needed to detoxify H_2O_2 generated in mitochondria (Han et al. 2003b). We observed that depleting of mitochondrial GSH by NAPQI following APAP treatment results in increased H_2O_2 release from mitochondria, probably due to a decrease in GSH peroxidase activity or impaired electron transport (Hanawa et al. 2008). Increased H_2O_2 released from mitochondria may be a key factor in the activation of JNK in cytoplasm. The notion that mitochondrial ROS is important in activating JNK has been supported by experiments showing: (1) treatment of primary cultured hepatocytes with exogenous H_2O_2 or mitochondrial respiratory chain inhibitors (i.e., antimycin, rotenone), which increase ROS generation, caused JNK activation, (2) AMAP, which does not depleted mitochondria GSH but depletes cytoplasmic GSH does not activate JNK, and (3) mitochondrial GSH depletion precedes JNK activation in vivo (Hanawa et al. 2008). A detailed time course in vivo revealed that mitochondrial GSH depletion (maximum ~2 h), precedes JNK activation in liver (maximum ~4 h). Taken together, the evidence suggests that increase in H_2O_2 released by mitochondria, due to mitochondrial GSH depletion caused by NAPQI, activates JNK in cytoplasm.

(b) *Redox changes and ASK-1 activation.* JNK can be activated by several kinases including apoptosis signaling-regulating kinase 1 (ASK-1). In the cytoplasm, ASK-1 is inhibited by an association with thioredoxin, which contains critical redox sensitive thiols (Saitoh et al. 1998). The oxidation of these critical thiols on thioredoxin by H_2O_2 or other oxidants will cause thioredoxin to disassociate from

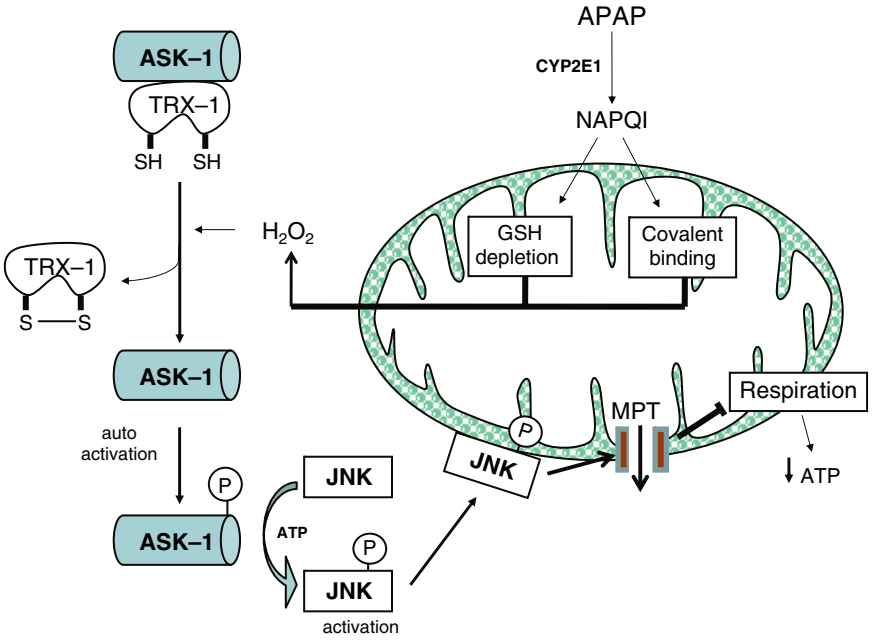

Fig. 3 The JNK-mitochondria signaling loop important in mediating APAP-induced liver injury. APAP is metabolized by CYP2E1 to the reactive metabolite, NAPQI. At hepatotoxic doses of APAP, NAPQI covalently binds to GSH and proteins in mitochondria, as well as in the cytoplasm, causing dramatic redox changes in hepatocytes. The depletion of GSH, utilized by GSH peroxidase to detoxify H_2O_2, results in increased release of H_2O_2 from mitochondria. Also, direct effects of NAPQI (covalent binding) on respiratory complexes may contribute to ROS production. H_2O_2 released from mitochondria oxidize redox sensitive proteins, such as thioredoxin, which contain critical thiols important in binding and inhibiting ASK-1. The oxidation of thiols in thioredoxin results in the disassociation of thioredoxin from ASK-1. Once free, ASK-1 (MAP3K) self-activates and phosphorylates and activates MAP2K (not shown), which then activates JNK. The newly activated JNK translocates to mitochondria and induces MPT and inhibits mitochondrial bioenergetics, which initiates hepatocyte necrosis. JNK will only induce MPT in mitochondria that are redox modified by NAPQI and not in healthy mitochondria

ASK-1, which subsequently self-activates. Once activated, ASK-1 will phosphorylate MAP2K, which then activates JNK. Consequently, the increased release of mitochondrial H_2O_2 caused by GSH depletion may be activating JNK through oxidation of thioredoxin and ASK-1 disassociation. The importance of ASK-1 in APAP hepatotoxicity was supported in recent studies by Nakagawa et al., who demonstrated that knocking out ASK-1 blunted sustained JNK activation and prevented APAP-induced liver injury (Nakagawa et al. 2008). Interestingly, ASK-1 was not the only factor involved in JNK activation following hepatotoxic doses of APAP. Nagakawa et al. observed that JNK activation had two phases, an ASK-1 independent phase (~1.5 h) and a JNK dependent phase (~3 h). JNK itself may be released from inhibitory binding by redox sensitive proteins such as GSH S-transferase Pi (Adler et al. 1999), and this type of JNK activation may represent

the first phase of JNK activation. The possibility that JNK itself may self-amplify its own activation by modulating mitochondrial ROS leading to ASK-1 activation also exists. Nevertheless, ASK-1 activation is necessary for sustained JNK activation following APAP, and thus essential in mediating hepatocyte necrosis. Another factor that may contribute to sustained JNK activity is decreased JNK phosphatase activity. Phosphatase needed to dephosphorylate and inactivate JNK has been shown to contain critical thiols that can be oxidized to sulfenic acids by ROS (Kamata et al. 2005). Whether the oxidation of thiols in phosphatases occurs and contributes to sustained JNK activation in APAP hepatotoxicity remains to be investigated.

(c) *JNK modulation of bcl-2 family members*. Once activated, JNK modulates several signaling pathways including members of the bcl-2 family of proteins. APAP treatment in vivo has been shown to cause bax activation and translocation to mitochondria (Gunawan et al. 2006), and phosphorylation and inactivation of bcl-xl on the mitochondria outer membrane (Latchoumycandane et al. 2007). These signaling pathways were inhibited by JNK inhibitor treatment, suggesting a direct involvement of JNK in the regulation of bcl-2 family members. Previous studies have established that JNK can activate bax, through phosphorylation of bax anchoring protein, 14-3-3 (Tsuruta et al. 2004), or phosphorylation of bax directly (Kim et al. 2006). Both types of phosphorylation will induce bax translocation to mitochondria. JNK has also been shown to directly phosphorylate and inhibit bcl-xl in mitochondria (Fan et al. 2000). The accumulation of bax in mitochondria (which can form channels or become part of the MPT pore that inhibit mitochondrial bioenergetics), and inactivation of bcl-xl (which can counteract bax and other proapoptotic bcl-2 proteins), correlate with a decline in mitochondrial bioenergetics early during APAP hepatotoxicity in vivo. This would suggest an important role of JNK-activated bax and inactivation of bcl-xl in mitochondria dysfunction during APAP-induced liver injury. However, the central role of bax translocation to mitochondria during APAP-induced liver injury seems unlikely. The amount of cytochrome *c* released from mitochondria to cytoplasm during APAP injury is minimal, suggesting there is not a great deal of bax channel formation in mitochondria (Hanawa et al. 2008). In addition, bax knockout mice display the same amount of liver injury following APAP treatment as wild-type mice, although there is some delay in injury (Bajt et al. 2007). These findings do not rule out bax as playing a contributing role, but unlike JNK, bax does not play a central role in APAP hepatotoxicity. Bax may be one of many other bcl-2 proapoptotic family and apoptotic protein members involved in the inhibition of mitochondrial bioenergetics during APAP-induced liver injury. Because of the antiapoptotic properties of bcl-xl, its inactivation by JNK may also be contributing to mitochondria dysfunction, but its importance in APAP-induced liver injury has not yet been determined.

(d) *JNK translocation to mitochondria and inhibition of mitochondrial bioenergetics*. JNK is one of several kinases that have been shown to translocate to mitochondria and regulate MPT. In cell culture studies, various stresses such as UV light have been shown to cause JNK activation and translocation to mitochondria

(Tournier et al. 2000). JNK translocation to mitochondria has been shown to induce MPT and release of apoptotic factors such as cytochrome c, SMAC, and AIF (Aoki et al. 2002; Chauhan et al. 2003). During APAP-induced hepatotoxicity, we similarly observed that, following JNK activation by ASK-1, JNK translocated to mitochondria (Hanawa et al. 2008). Pretreatment with JNK inhibitor, which inhibited JNK translocation to mitochondria, partially protected against a decline in mitochondrial bioenergetics that occurs following APAP treatment (~70% inhibition for APAP alone and ~40% inhibition with APAP plus JNK inhibitor treatment). This finding suggests that loss of mitochondrial bioenergetics following APAP treatment has a JNK-dependent and JNK-independent component. The JNK-independent pathway is likely due to mitochondrial GSH depletion and covalent binding, which cause protein redox changes that partially inhibit mitochondrial bioenergetics. Many proteins in the mitochondrial respiratory chain and matrix contain critical thiols that if oxidized can inhibit activity. The inhibition of mitochondrial bioenergetics caused by GSH depletion and covalent binding, when JNK is not present, is not sufficient to cause hepatocyte death, since JNK inhibitor pretreatment completely protects against APAP-induced liver injury. The JNK-dependent inhibition of mitochondria respiration may be due to a direct effect of JNK on mitochondria following translocation. This notion was confirmed when we observed that addition of active purified JNK caused loss of mitochondrial bioenergetics in mitochondria isolated from mice treated with APAP plus JNK inhibitor (mitochondria with GSH depleted and covalent binding but no JNK translocation) (Hanawa et al. 2008). However, the addition of purified active JNK to mitochondria isolated from control mice had no effect on mitochondrial respiration, suggesting JNK only affects redox-modified mitochondria. The inhibitory effect of active JNK on mitochondrial bioenergetics in redox-modified mitochondria (APAP plus JNK inhibitor-treated mice) was prevented by cotreatment with cyclosporine A, an inhibitor of MPT. This suggests that JNK may be inhibiting mitochondria respiration by inducing MPT in redox-modified mitochondria, but not in healthy mitochondria. Taken together, APAP-induced liver injury involves two hits to mitochondria: (1) covalent binding and GSH depletion that decreases mitochondrial bioenergetics and sensitizes to a second hit; and (2) JNK translocation to mitochondria, which induces MPT that further inhibits mitochondrial bioenergetics. The two hits to mitochondria are essential in causing hepatocyte death and liver injury following APAP.

Taken together, these studies suggest that mitochondria are the central points where redox modifications (e.g., GSH depletion and covalent binding) and prodeath signaling pathways such as JNK and bax converge to initiate MPT and hepatocyte death. The levels and activity of bcl-xl, bcl-2, and other prosurvival proteins in mitochondria may counteract the prodeath pathways and determine the extent of MPT and hepatocyte survival. Overall hepatocyte injury is determined by the balance of redox modifications (i.e., GSH depletion, ROS, covalent binding), prodeath signaling (i.e., JNK, bax, bak) and prosurvival signaling (i.e., bcl-xl, bcl-2) that occurs in mitochondria. The downstream target of JNK in mitochondria has not yet been identified. Work by Zhou et al. (2007) suggests that, in isolated brain mitochondria, JNK does not enter

the outer membrane of mitochondria and only affects proteins on the mitochondrial outer membrane or at contact junctions between the outer and inner membrane. Addition of purified active JNK to isolated brain mitochondria was shown to inhibit pyruvate dehydrogenase (PDH) activity in the matrix, suggesting JNK induces signals from the outer membrane to the matrix (Zhou et al. 2007). The mitochondrial targets of JNK, which are important in mediating the effects on mitochondrial respiration and MPT, need to be further investigated.

3.6 *Time Course of JNK Activation During APAP-Induced Hepatotoxicity*

The sequence of events involved in APAP hepatotoxicity is (1) NAPQI formation and GSH depletion (maximal GSH depletion occurs at 2 h), (2) JNK activation and translocation to mitochondria (starting ~1 h with maximal translocation occurring at 2–4 h), and (3) liver injury (ALT levels begin to rise significantly after ~4–6 h) (Hanawa et al. 2008). This time course is greatly affected by DMSO which is necessary to dissolve JNK inhibitor and often used to solubilize APAP. DMSO is an inhibitor of CYP2E1, and consequently slows and blunts NAPQI formation and GSH depletion (Jaeschke et al. 2006; Park et al. 1988). Since JNK activation and translocation is not an immediate event, JNK inhibitor treatment, even hours after APAP treatment, can significantly reduce APAP-induced liver injury. When APAP was dissolved in DMSO, JNK inhibitor treatment given even 6 h following APAP could significantly protect mice from APAP hepatotoxicity (Gunawan et al. 2006). When APAP was dissolved in warm PBS, JNK inhibitor provided 2 h following APAP was able to protect against APAP injury (Hanawa et al. 2008). At present, the major therapy for APAP hepatotoxicity is *N*-acetylcysteine (NAC), which acts as a cysteine source to replenish liver GSH. Unfortunately, for NAC to be effective in treating APAP hepatotoxicity, it must be administered very early, to prevent GSH depletion. Since JNK activation occurs at a later stage than GSH depletion, JNK inhibitors may be more effective than NAC at later time points for treatment of APAP hepatotoxicity. Consequently, the therapeutic use of JNK inhibitors to treat APAP hepatotoxicity at time points when NAC is no longer effective, remains an intriguing possibility.

3.7 *The Involvement of Different Isoforms of JNK*

Two isoforms of JNK, JNK1 and JNK2, are expressed in liver (Bogoyevitch 2006). JNK1 has been suggested to play an important role in insulin resistance and fatty liver disease (Schattenberg et al. 2006), while JNK2 has been shown to be involved in TNF-induced apoptosis and ischemia reperfusion (Theruvath et al. 2008; Wang

et al. 2006). In the APAP model, both JNK1 and JNK2 appear to be involved in hepatotoxicity, although JNK2 may play a slightly more preferential role. When APAP is delivered in warm PBS, knocking down either JNK1 or JNK2 alone could not protect against APAP-induced liver injury. Only when both JNK1 and JNK2 were simultaneously knocked down was protection against APAP observed (Hanawa et al. 2008). On the other hand, when APAP is given with DMSO, which blunts and slows the rate of progression of the liver injury due to DMSO inhibition of CYP2E1, knocking down JNK2 significantly protected against APAP, while knocking down JNK1 alone had no protective effects (Gunawan et al. 2006). Maximal protection was again observed when both JNK1 and JNK2 were simultaneously knocked down in mice (Hanawa et al. 2008). Why there might be a slight preference for JNK2 over JNK1 when APAP is dissolved in DMSO is not clear. It is, however, clear that for maximal protection both JNK1 and JNK2 need to be knocked out, which may be due to the fact that both JNK isoforms translocate to mitochondria following APAP treatment.

Recent evidence from our laboratory also suggests that there may be a range where the inhibition of JNK may protect hepatocytes from APAP (manuscript in preparation). When doses of APAP far exceed hepatotoxic doses (> 500 mg kg^{-1}, without DMSO present), the protective effects of knocking down both JNK1 and JNK 2 wane (unpublished results). We similarly observed a range of protection in primary hepatocytes, where the protective effects of PKC inhibitor against H_2O_2-induced necrosis occurred in a dose range (0–400 μM for H_2O_2) (Saberi et al. 2008). It is likely that very high doses of APAP in mice and very high dose of H_2O_2 in primary hepatocytes cause such overwhelming oxidative injury that prodeath signaling pathways no longer become relevant. Consequently, very high doses of APAP cause overwhelming oxidative damage to lipids, proteins, and DNA that severely disrupt cell function to trigger necrosis. JNK and other prodeath pathways may themselves be inhibited by the extensive oxidative damage. However, the doses of APAP where prodeath signaling pathways no longer contribute to hepatocyte necrosis are extremely high, and it is uncertain if this is encountered in the clinical setting.

3.8 Other Signaling Changes in Hepatocytes that Mediate APAP-Induced Hepatotoxicity

The JNK signaling pathway appears to be the *primary* prodeath pathway mediating hepatocyte death following hepatotoxic doses of APAP. However, APAP also induces many other signaling pathways, including pathways involved in cell stress response, repair, and regeneration. While these other signaling factors do not directly mediate hepatocyte death, they modulate factors (i.e., ROS levels, protein folding, repair pathways) important in APAP hepatotoxicity, and thus will modulate APAP-induced liver injury to some extent. Some of these signaling pathways

are likely activated by redox changes to proteins induced by NAPQI such as Nrf-2. Other signaling pathways activated/inhibited during APAP hepatotoxicity may be a consequence of oxidative and nitrosative damage to lipids, DNA, and other macromolecules, which causes hepatocyte stress and injury. Our discussion of cell signaling changes activated by hepatotoxic doses of APAP in liver will be arbitrarily divided into five categories: (a) antioxidant response, (b) stress response, (c) survival and repair, (d) nitric oxide, and (e) metabolic signaling (Fig. 4).

(a) *Antioxidant response*. As previously discussed, redox changes caused by NAPQI lead to oxidation of Keap1 leading to Nrf-2 translocation to the nucleus and transactivation of antioxidant gene expression including: upregulation of heme oxygense, microsomal epoxide hydrolase, and GCL, the rate limiting enzyme in GSH synthesis (Goldring et al. 2004). The upregulation of GCL is important in increasing the synthesis of GSH needed to detoxify NAPQI. Consequently, Nrf-2 knockout mice are much more susceptible to APAP injury than normal mice

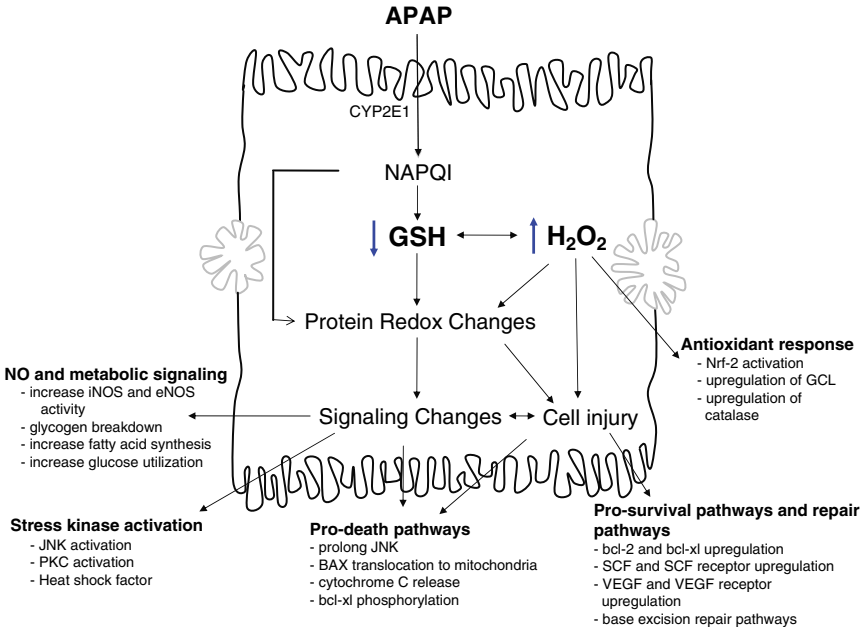

Fig. 4 Signaling changes in hepatocytes caused by hepatotoxic doses of APAP. Hepatotoxic doses of APAP cause redox changes to proteins, through GSH depletion and increased H_2O_2, which activate many proteins such as Nrf-2. Other signaling pathways may be activated/inhibited as a result of cell stress and injury caused by oxidative and nitrosative damage to lipids, DNA, and other macromolecules. The signaling pathways activated/inhibited by APAP can be divided into five categories: (a) antioxidant response, (b) stress response, (c) survival and repair, (d) prodeath pathways, and (e) nitric oxide and metabolic signaling. Some signaling pathways, such as JNK, play an essential role in APAP-induced liver injury, while other pathways probably play a minor role in this model but may be of greater importance in other situations

(Goldring et al. 2004). In addition to Nrf-2, other transcription factors that regulate antioxidants enzymes are also likely to be activated by APAP treatment, since proteomic studies have shown antioxidant enzymes such as catalase are upregulated by APAP treatment (Thome-Kromer et al. 2003). Because of the importance of ROS in APAP-induced liver injury, proteins involved in the antioxidant defense system such as GCL and catalase may be important in modulating APAP-induced liver injury. Not surprisingly, the overexpression of GCL protected against APAP-induced liver injury (Botta et al. 2006).

There are some counter-intuitive reports suggesting that mice lacking antioxidant enzymes such as Cu,Zn-SOD, the major enzyme response for superoxide detoxification in cytoplasm, are more resistant to APAP-induced hepatotoxicity. This protection against APAP in Cu,Zn-SOD knockout mice was also associated with a lack of JNK activation (Zhu et al. 2006). However, work by Lei et al. (2006) demonstrated that resistance of Cu,Zn-SOD knockout mice to APAP was due to a downregulation of CYP2E1, a major source of superoxide cytoplasm in cells. The lack of Cu,Zn-SOD in mice presumably caused adaptive changes to decrease cytoplasmic levels of superoxide including a downregulation CYP2E1 in cytoplasm. Thus Cu,Zn-SOD knockout mice form less NAPQI due to less CYP2E1 in the ER, consequently there is less GSH depletion and no JNK activation following APAP treatment. These findings are supported by other studies suggesting that overexpression of Cu,Zn-SOD protects against APAP (Mirochnitchenko et al. 1999). The Cu,Zn-SOD knockout mice illustrates the pitfalls associated with using transgenic animals, which often develop new genetic adaptations in response to genes being knocked out.

(b) *Stress response*. GSH depletion and covalent binding in hepatocytes causes cellular stress that can activate a wide range of adaptive, protective stress responses. Many stress kinases activated during oxidative stress such as JNK, protein kinase C, and Akt are activated by APAP (unpublished results). JNK may be important in stress responses, but prolonged increases in JNK induce cell death through pathways outlined above. Akt generally promotes survival pathways, while PKC can either promote survival or death pathways, depending on isoforms activated and the cellular context (Jeon et al. 2002; Saberi et al. 2008). The potential role of PKC and Akt in mediating APAP-induced liver injury is being presently investigated in our laboratory. In addition, as with most stress models, APAP treatment results in activation of a number of heat shock proteins including heat shock protein 25 (HSP25) and 70i (HSP70i) (Salminen et al. 1997; Sumioka et al. 2004). HSP70i knockout mice were more susceptible to APAP hepatotoxicity, suggesting HSP70i plays an important protective role (Tolson et al. 2006). In addition, pretreatment of mice with transient hyperthermia, which upregulates heat shock proteins, also protected against APAP-induced liver injury, suggesting heat shock proteins play important protective roles against APAP hepatotoxicity (Tolson et al. 2006). Heat shock proteins may help proper protein folding, which may be altered by covalent binding and redox changes, to protect hepatocytes from APAP-induced injury. Along these lines, some data suggest that the unfolded protein response and ER

stress occur in APAP toxicity, but the contribution of this pathway in protection (UPR) or injury (ER stress response) needs to be better defined.

(c) *Repair and survival genes*. Cell stress and injury caused by ROS and RNS following APAP upregulates many repair and regeneration pathways. Liver injury caused by APAP induces cell proliferation pathways including cell nuclear antigen protein and cyclin D gene expression. Repair and survival genes can be divided into (A) DNA repair in hepatocytes and (B) liver repair and regeneration.

(A) *DNA repair*. DNA fragmentation and 8-hydroxy-deoxyguanosine (8-OH-dG), a marker of DNA oxidation, has been observed in hepatocytes as early as 6 h following APAP treatment (Powell et al. 2006). Calcium, endonuclease D, apoptosis inducing factor, and deoxyribonuclease 1 (DNASE1) are believed to contribute to DNA damage during APAP hepatotoxicity (Jaeschke and Bajt 2006; Napirei et al. 2006). DNASE1 release from dying hepatocytes has also been shown to spread APAP injury, beginning with the pericentral hepatocytes and outwards to neighboring hepatocytes (Napirei et al. 2006). Necrosis of pericentral hepatocytes, where APAP injury initially starts, results in release of DNASE1 that is taken up by neighboring hepatocytes, where it initiates DNA damage. DNASE1 knockout mice had the same amount of hepatocyte injury in pericentral hepatocytes but less injury in surrounding hepatocytes following APAP (Napirei et al. 2006). DNA damage caused by DNASE1 and other factors can activate base excision repair (BER) pathways including proliferating cell nuclear antigen (PCNA), poly ADP-ribose polymerase (PARP), and AP endonuclease 1 (Powell et al. 2006), as well as an increased expression of p53 (Ray et al. 2001). Excessive PARP activity can result in severe NADH depletion that causes an energy crisis (ATP consumption), which results in cell death (Heeres and Hergenrother 2007). 3-aminobenzamide, a PARP inhibitor, has been shown to reduce APAP-induced liver injury in mice, suggesting a possible role of PARP in hepatotoxicity (Ray et al. 2001). However, other PARP inhibitors, such as 5-aminoisoquinolinone, did not attenuate APAP-induced hepatotoxicity, nor were PARP knockout mice protected from APAP (Cover et al. 2005). These findings suggest that protective effects of 3-aminobenzamide against APAP may be nonspecific and that PARP activation is not essential in APAP-induced liver injury.

(B) *Liver injury, repair, and regeneration*. Several growth factors and other hormones are released in response to hepatocyte death and liver injury following APAP. The factors include: (1) stem cell factor, (2) vascular endothelial growth factor, and (3) plasminogen activator inhibitor-1.

(1) Stem cell factor (SCF) and its receptor c-kit play an important role in hepatocyte regeneration and proliferation. APAP treatment results in increase SCF and the c-kit receptor expression in liver beginning at 6 h. Pathways activated by SCF include PI-3 kinase, Src family members, JAK/STAT, and Ras-Raf-MAPK cascade (Hu and Colletti 2008). SCF administration hours after APAP treatment resulted in increase hepatocyte proliferation and a reduction of APAP-induced liver injury. SCF treatment increased bcl-xl and bcl-2 expression in liver mitochondria,

which was associated with a decrease in hepatocyte apoptosis and liver injury. The role of bcl-2 in liver is controversial, with some reports suggesting that it does not exist in hepatocytes (Tzung et al. 1997), while other reports suggest its presence in hepatocytes, particularly following stress (Hu and Colletti 2008; Kovalovich et al. 2001). Whether bcl-2 may be countering the effects of JNK in mitochondria remains to be fully investigated. However, one report suggests that transgenic mice with overexpression of human bcl-2 exhibited enhanced APAP-induced liver injury (Adams et al. 2001), through an unknown mechanism.

(2) Vascular endothelial growth factor (VEGF), an important regulator of angiogenesis and tissue repair, increases in liver starting at 8 h following APAP treatment. The increase in VEGF release in liver is associated with expression of VEGF receptors 1, 2, 3. Pretreatment of mice with SU5416, a synthetic VEGF inhibitor, decreased cell proliferation, suggesting VEGF plays an important role in liver regeneration following APAP treatment (Donahower et al. 2006).

(3) Plasminogen activator inhibitor-1 (PAI) is the principal inhibitor of plasminogen, a major regulator of fibrinolysis. PAI levels increase in liver with APAP treatment. PAI knockout mice were more sensitive to APAP, suggesting PAI promotion of fibrin clots in liver has a protective role against APAP-induced liver injury (Bajt et al. 2008).

(d) *Nitric oxide*. NO is an important signaling molecule that plays important roles in cell death and survival (Boyd and Cadenas 2002). NO signaling often entails binding and activation of guanylyl cyclase, which regulates protein kinase G. However, as previously shown, NO can also cause nitrosylation of proteins to modulate many signaling pathways (Han et al. 2006b; Klatt and Lamas 2000). The role that NO plays in APAP hepatotoxicity is somewhat controversial. Both endothelial NO synthase (eNOS) and inducible nitric oxide synthase (iNOS) have been shown to be induced and/or activated following APAP treatment in liver (Gardner et al. 1998; Ito et al. 2004). Since NO rapidly reacts with superoxide to form $ONOO^-$, which has been shown to damage proteins in liver (nitrotyrosine formation) during APAP hepatotoxicity (Jaeschke et al. 2003), NO has traditionally been viewed to play a deleterious role in APAP-induced liver injury. However, hepatic necrosis was similar in iNOS knockout mice and in wild type mice, suggesting that NO was not essential in mediating cell damage caused by APAP treatment. iNOS knockout mice had less nitrated tyrosine, but hepatocyte injury was similar suggesting cell injury can occur through nitrotyrosine-dependent (through $ONOO^-$) and nitrotyrosine-independent pathways (superoxide-mediated damage) (Michael et al. 2001). Some studies have suggested that NO may play a protective role against APAP. NO delivered using NCX-1000, which releases NO in the liver, protected against APAP-induced liver injury in mice. NCX-1000 also prevented MPT induced by APAP treatment in cultured hepatocytes (Fiorucci et al. 2004). Work with NOS inhibitors also suggests that eNOS exerts a protective role in liver microcirculation to reduce APAP-induced liver injury (Ito et al. 2004). It has been suggested that iNOS may play a slight deleterious role against APAP-induced liver injury, while eNOS may help to protect liver from APAP (Ito et al. 2004). However, since

NO is freely diffusible in liver, how different isoforms affect hepatocytes differently remains to be determined. Although nitrotyrosine levels correlate with injury and reflect both oxidative and nitrosative stress, there is no definitive proof this is specifically involved in APAP-induced cell death. One interesting possibility that needs to be examined is whether mitochondrial NO is important in hepatotoxicity and if it is regulated by JNK.

(e) *Metabolic signaling*. APAP treatment causes many metabolic changes to occur in the liver. APAP administration initially causes a rapid depletion of glycogen in the liver suggesting dramatic changes in metabolic signaling (Hinson et al. 1983). Metabolomic studies have shown that both glucose and glycogen are reduced in liver following APAP, suggesting an increased utilization of energy, probably for repair and detoxification pathways (Coen et al. 2003). In addition, there is an increase in triacylglycerol synthesis in liver following APAP, suggesting a slight increase in fatty liver following APAP (Coen et al. 2003). AMP-activated kinase (AMPK) is a key regulator of glycogen and fatty acid synthesis, and alterations in AMPK signaling may be contributing to glycogen and fatty acid changes during APAP-induced hepatotoxicity (Hardie 2007). Overall, the signaling changes that cause metabolic effects in liver or their importance in modulating APAP hepatotoxicity have not been characterized.

3.9 Overview of Signaling Pathways Involved in APAP-Induced Liver Injury

Hepatocyte stress and injury caused by NAPQI activate numerous signaling pathways that are important in modulating liver injury. These signaling pathways act at different stages and different levels in hepatocytes. Redox changes caused by NAPQI activate Nrf-2 and other antioxidant enzymes important in protecting hepatocytes. At low doses of APAP, Nrf-2-induced GCL can sufficiently replenish GSH levels to prevent JNK activation and hepatotoxicity. However, when NAPQI overwhelms GSH levels, redox changes activate JNK, which translocates to mitochondria to induce MPT and hepatocyte death. The discovery that APAP hepatotoxicity requires the active recruitment of JNK signaling was an important paradigm shift in understanding DILI. Traditionally, APAP-induced liver injury was believed to be due to overwhelming injury caused by GSH depletion, oxidative damage and covalent binding to mitochondria. Our data suggest MPT in mitochondria not only requires GSH depletion and covalent binding, but the requirement of JNK activation and translocation to mitochondria. This suggests that, even in necrotic cell death, there is an active signal involving JNK that is required to trigger hepatocyte death. The prosurvival signaling pathways, which may counteract cell death induced by JNK during APAP hepatotoxicity, remain to be fully identified. Members of the bcl-2 family such as bcl-xl may play an important role in

modulating mitochondria and counteracting the induction of MPT by JNK. Since mitochondria are the central target where redox changes, prodeath pathways, and prosurvival pathways converge, closer examination of mitochondria changes caused by JNK and bcl-2 family members need to be further explored. Whether other signaling pathways that modulate hepatotoxicity (e.g., NO, heat shock proteins) can modulate JNK signaling or work through other pathways remain to be determined. Finally, JNK-induced death of hepatocytes activates many regeneration and cell proliferation pathways (e.g., SCF, VGF, PAI) that limit the extent of liver injury. There are likely a host of other signaling pathways important in modifying APAP hepatotoxicity that have not yet been identified.

3.10 Sublethal Doses of APAP Sensitize Hepatocytes to the Cytotoxic Effects of Tumor Necrosis Factor

Thus far, we have focused on signaling pathways in hepatocytes that are modulated by hepatotoxic doses of APAP. However, even nontoxic doses of APAP perturb the redox status of cytoplasm in hepatocytes, which can potentially affect important survival/death pathways in cells, particular in signaling proteins containing critical cysteines. Nontoxic doses of APAP, for example, have been shown to sensitize hepatocytes to tumor necrosis factor (TNF)-induced apoptosis (Matsumaru et al. 2003; Nagai et al. 2002). TNF is an important cytokine, which is released by macrophages and other immune cells during inflammation. Hepatocytes, like most nontransformed cells, are normally resistant to TNF due to the activation of NF-κB, an important transcription factor that promotes transcription of survival genes (i.e., IAP, iNOS, bcl-xl) (Han et al. 2006a). However, NF-κB and proteins involved in NF-κB activation (e.g., IκB-α kinase, IKK) contain critical cysteines necessary for proper function. Nonhepatotoxic doses of APAP alter the redox environment of hepatocytes to inhibit NF-κB activation, resulting in the sensitization of primary hepatocytes to TNF-induced apoptosis (Nagai et al. 2002). Other agents that alter the redox status of cells (DEM, H_2O_2, mitochondrial inhibitors, diamide) were also found to sensitize primary hepatocytes to the cytotoxic effects of TNF (Han et al. 2006a; Matsumaru et al. 2003). Strong oxidative or redox stress induces an IKK-dependent upstream effect to inhibit NF-κB activation, whereas milder redox stress inhibits in an IKK-independent fashion by interfering with transcriptional activity of NF-κB, which is bound to κB sites in DNA in response to TNF (Lou and Kaplowitz 2007). The role of TNF and NF-κB in APAP hepatotoxicity is controversial. However, most studies do not support an important role of TNF in liver injury caused by excessive doses of APAP (Dambach et al. 2006; Gunawan et al. 2006). On the other hand, APAP-induced sensitization of hepatocytes to the cytotoxicity of TNF may be a factor that contributes to APAP hepatotoxicity that occurs at therapeutic doses or low level overdose of APAP. Our data suggest that sublethal doses of APAP may

modulate NF-κB signaling and sensitize hepatocytes to TNF released during inflammation that occurs in various liver pathologies (e.g., alcoholic liver disease, liver ischemia) in vivo. Thus, drugs that cause nonlethal oxidative stress or redox perturbation may sensitize to TNF-toxicity in the setting of mild liver injury. It is intriguing to speculate that this mechanism may contribute to the potentiating effect of lipopolysaccharide (LPS) and hepatotoxins as exemplified in the Roth Model of hepatotoxicity (see below).

3.11 Relevance of the APAP Model to Other Drugs that Cause Drug-Induced Liver Injury

APAP shares many features with other drugs that cause hepatotoxicity, but also has some unique characteristics. Consequently, some facets of APAP hepatotoxicity may be generalized to other drugs that cause liver injury, while certain attributes are not applicable. The extensive GSH depletion caused by NAPQI is somewhat unique to APAP, and most drugs that cause liver injury do not cause extensive GSH depletion. However, many drugs are associated with increased oxidative stress (Kaplowitz 2002), and thus are likely to involve some redox perturbation. Consequently, many signaling pathways activated/inhibited by oxidative stress during APAP-induced liver injury may be similarly activated/inhibited by other drugs that cause oxidative stress. The importance of JNK in modulating APAP hepatotoxicity has not been observed with all liver toxins. For example, JNK inhibition or ASK knockout mice are protected from APAP, but not protected against carbon tetrachloride (CCl_4)-induced liver injury (Gunawan et al. 2006; Nakagawa et al. 2008). This is surprising, since CCl_4 generates a reactive intermediate important in initiating damage and is associated with JNK activation, as observed with APAP (Iida et al. 2007; Trudell et al. 1982). However, our experiments with isolated mitochondria demonstrated that JNK only inhibits mitochondria bioenergetics in redox-altered mitochondria (GSH depletion and covalent binding caused by APAP and JNK inhibitor) and not in normal mitochondria (Hanawa et al. 2008). Thus, it is possible that CCl_4-induced liver injury may not involve redox alteration to mitochondria, and consequently JNK may not mediate injury. Further studies analyzing the mechanistic differences between APAP and CCl_4, particularly differences in mitochondria, would add greater insight into why JNK modulates liver injury in one case but not in another. However, JNK is an important mediator of liver injury in other models of liver injury including during liver ischemia reperfusion and transplantation. In addition, JNK has been suggested to play an important role in other pathologies such as neurodegeneration and ischemia reperfusion injury in the heart, thus representing a common mechanism of cellular injury in many diseases (Johnson and Nakamura 2007).

4 Other Animal Models to Study Drug-Induced Liver Injury

Besides APAP, several new animal models to study DILI have been developed. These new models have moved away from healthy animals and more towards a "stress" animal model (Dixit and Boelsterli 2007). Most toxicology studies that use animal models to screen for potential adverse effect of drugs generally use healthy animals, including studies with APAP. However, the use of healthy animals to screen for drugs that cause DILI has drawn major criticism and has been suggested as the underlying reason for failure of animal models to detect drugs that cause idiosyncratic drug hepatotoxicity in humans (Dixit and Boelsterli 2007). Generally, patients who take drugs have at least one preexisting medical conditions. Since idiosyncratic DILI is more likely to occur in stressed or weakened individuals (i.e., increased age, infections, etc.), healthy animals may not be the appropriate model for prediction of idiosyncratic drug hepatotoxicity. Cancer researchers have used animal models that have a genetic predeposition to cancer (i.e., p53 heterozygous, ras-overexpressing mice), to identify chemicals that can potentially promote tumor formation (Sills et al. 2001). Consequently, to study DILI, researchers have recently turned to models that do not involve "healthy animals" but rather to ones that involve certain stresses or genetic polymorphisms. Two new animal models to investigate liver injury have received particular attention: (1) cotreatment of drugs with lipopolysaccharides (LPS) that simulate inflammation, and (2) Mn-SOD (+/−) heterozygous mice. Both models not only provide insights into DILI but may also be able to potentially identify drugs that cause idiosyncratic DILI.

4.1 LPS Costimulation

Work by Roth's laboratory has demonstrated that normally nonhepatotoxic drugs in mice, which have been associated with idiosyncratic DILI in patients, can become hepatotoxic if administered after low, nontoxic doses of lipopolysaccharide (LPS; bacteria endotoxin that promotes inflammation) (Ganey et al. 2004). Chlorpromazine (an antipsychotic drug), trovafloxacin (a fluoroquinolone antibiotic), and ranitidine (a histamine 2 receptor antagonist) are known to cause idiosyncratic DILI in humans but not in animals. However, the pretreatment of rats with nontoxic doses of LPS followed by chlorpromazine, trovafloxacin, or ranitidine caused liver injury associated with hepatocyte necrosis (Buchweitz et al. 2002; Luyendyk et al. 2003). The mechanism underlying LPS sensitization of liver to drugs is believed to involve the hemostatic system, fibrin deposition, and the innate immune system consisting of neutrophil activation and TNF release (Deng et al. 2007; Luyendyk et al. 2005). Blocking TNF production or activity using various TNF inhibitors prevented liver injury caused by cotreatment of LPS and trovafloxacin, suggesting an essential role of TNF in hepatocyte injury (Shaw et al. 2007). Ganey et al. have suggested that inflammation sensitizes hepatocytes to drugs to promote liver injury (Ganey et al. 2004). However,

it is alternatively possible that the drugs themselves are sensitizing hepatocytes to the cytotoxic effects of TNF and other cytokines. As previously discussed, subtoxic doses of APAP can sensitize primary hepatocytes to TNF-induced apoptosis, through redox changes that disturb NF-kB signaling. Similarly, drugs may be sensitizing hepatocytes to the cytotoxic effects of TNF (whose secretion increases in response to LPS) to promote liver injury. Further studies are needed to understand the signaling pathways involved in hepatocyte death when rats are treated with both LPS and drugs. The LPS-drug costimulation model demonstrates the synergistic action of cytokines, inflammation, and drugs to cause liver injury. Overall, these studies illustrate that inflammation or a genetic predisposition to a greater TNF response may be an important risk factor for idiosyncratic DILI in humans.

4.2 *Mn-SOD $^{(+/-)}$ Heterozygous Mice*

Mn-SOD (SOD2) is essential in detoxifying superoxide generated in the mitochondrial matrix and protecting mitochondria from ROS (Cadenas and Davies 2000). Homozygous Mn-SOD$^{-/-}$ mice are embryonic lethal. On the other hand, heterozygous Mn-SOD$^{+/-}$ mice do not have any gross phenotypic abnormalities, but develop a higher incidence of tumors compared to wild-type with age (Van Remmen et al. 2003). Mn-SOD$^{+/-}$ mice are under chronic oxidative stress due to decreased Mn-SOD activity (~30–80% decrease), thus gradually accumulating oxidative damage manifested in mtDNA oxidation, decreased aconitase activity, and reduction of complex I activity resulting in decreased mitochondrial function and ATP generation. Young Mn-SOD$^{+/-}$ mice can be considered an animal model with subclinical chronic oxidative and mitochondrial stress.

As previously mentioned, troglitazone was a promising antidiabetic drug that was removed from the market due to unacceptable idiosyncratic liver injury. Troglitazone is normally well tolerated in animals, and liver injury is not observed. Work from Boelsterli's laboratory demonstrated that troglitazone treatment for 4 weeks caused liver injury in Mn-SOD$^{+/-}$ mice but not in wild-type mice (Ong et al. 2007). In Mn-SOD$^{+/-}$ mice, troglitazone caused increased serum ALT and hepatic necrosis in mid-zonal areas of liver, which was accompanied by significant decline in mitochondrial function (i.e., reduction in aconitase activity, increase protein carbonyls in mitochondria, and decreased complex I activity) in liver. Mitochondrial damage took 4 weeks to develop in Mn-SOD$^{+/-}$ mice compared to 4 h commonly observed with APAP, but the role of mitochondrial oxidative stress and stress kinases is strongly supported in both models, the difference apparently related to the rate of development. Whether JNK or other cell death signaling pathways participate in troglitazone-induced liver injury in Mn-SOD$^{+/-}$ mice remains to be determined. Human endothelial cells (HUVEC) studies have, however, suggested that activation of the Trx/ASK-1 pathway in mitochondria may be important in mediating hepatotoxicity of troglitazone (Zhang et al. 2004), and

previous studies have identified the important role of JNK in the culture model of troglitazone toxicity (Bae and Song 2003).

Treatment of mice with another drug, nimesulide, an NSAID used as an analgesic and antiinflammatory agent, also caused increase mitochondria injury in Mn-SOD$^{+/-}$ mice compared to control (Ong et al. 2006). However, nimesulide did not cause significant liver damage in Mn-SOD$^{+/-}$ mice compared to wild-type, as determined by ALT and hepatic necrosis. Why the increase in mitochondria stress caused by nimesulide did not translate into liver injury in Mn-SOD$^{+/-}$ mice is not known. Overall, the increased sensitization of Mn-SOD$^{+/-}$ mice to certain drugs supports the notion that oxidative stress and mitochondrial injury are an important component of liver injury caused by drugs. These studies also support the notion that genetic mutations that effect mitochondria, such as Mn-SOD$^{+/-}$ (a genetic polymorphism in a small percentage of the population), or acquired mitochondria injury (e.g., exposure to mitochondria toxins such rotenone used as an insecticide), may be an important risk factor for idiosyncratic DILI. Both the Mn-SOD$^{+/-}$ mice model and the LPS plus drug model emphasize that idiosyncratic DILI requires the drug plus additional risk factors (Dixit and Boelsterli 2007).

5 Overview of Drug-Induced Liver Injury

Taken together, studies from animal models as well as studies from cultured primary hepatocytes have shown drugs that cause nonallergic liver injury share many similar effects. Although each drug may cause some unique features involved in liver injury, many drugs that cause nonallergic liver injury likely share the recruitment of a common set of pathways important in causing hepatocyte death and liver injury. The key steps involved in DILI can be roughly divided into: (1) drug metabolism and reactive metabolite formation in hepatocytes, (2) covalent binding, (3) reactive oxygen species generation, (4) activation of signal transduction pathways that modulate cell death/survival in hepatocytes, and (5) mitochondrial damage. In most cases, hepatocyte injury and death is the critical step leading to the clinical manifestations of DILI. However, in certain circumstances, cholangiocytes or endothelial cells may be the principle target cell (e.g., ductopenic cholestasis and sinusoidal obstruction syndrome).

5.1 Drug Metabolism and Reactive Metabolite Formation in Hepatocytes

Most drugs are designed to be lipophilic so they can cross intestinal cells and be readily absorbed into circulation. Drug clearance, therefore, involves conversion of the drug to a more hydrophilic molecule for excretion into bile or urine, which

generally occurs through conjugation with hydrophilic carriers (e.g., glucuronide, sulfate, GSH). However, for many drugs, the conjugation with hydrophilic carriers is not possible until the drugs has been biotransformed into a more reactive molecule by CYP or other phase I enzyme. Consequently, reactive intermediates are generated during drug metabolism for conjugation with hydrophilic carriers, catalyzed by phase II enzymes, to form hydrophilic molecules that can be excreted into urine or bile. Each drug produces unique reactive metabolites when metabolized by CYP and other phase I enzymes. As previously mentioned, metabolism of APAP by CYP2E1 generates NAPQI, a highly reactive intermediate that binds to protein thiols and GSH (Hinson et al. 2004; Jollow et al. 1973). Similarly, the metabolism of troglitazone forms an unstable troglitazone-sulfoxide that forms disulfide bonds with GSH (Alvarez-Sanchez et al. 2006). The formation of a reactive metabolite is probably the initial important step that triggers DILI in most cases. However, what determines whether reactive metabolites are innocuous (e.g., AMAP) or detoxified or initiate liver injury is complex and undoubtedly involves genetic and environmental influences on reactive metabolite exposure in the individual patient, the chemical nature of the metabolite, and its distribution and targets in cells.

5.2 Covalent Binding

Since reactive intermediates are generated by CYP for conjugation with hydrophilic carriers, reactive intermediates have an innate ability to covalently bind. Reactive metabolites generated during drug metabolism not only conjugate with hydrophilic carriers, but they can also form covalent bonds with many different macromolecules including protein. The chemical nature of the metabolite determines if it binds to thiols or other moieties. The covalent binding of reactive intermediates with protein is an important step in causing hepatocyte injury by many drugs (Kaplowitz 2005). As previously mentioned, covalent binding of NAPQI to protein can affect protein activity, and covalent binding of other reactive drug metabolites is likely to have similar effects on protein activity to disturb hepatocyte function and signaling. In a small number of cases, drug metabolites do not form covalent bonds but form products that inhibit key proteins. For example, the metabolism of valproate, a drug that causes idiosyncratic liver injury, forms a metabolite that sequesters important cofactors (e.g., carnitine or CoA) to inhibit mitochondria fatty acid oxidation (Eyer et al. 2005; Granneman et al. 1984).

5.3 Oxidative Stress and ROS Generation

The APAP model and Mn-SOD$^{+/-}$ model demonstrate the importance of ROS in DILI. Many reactive metabolites can undergo redox cycling to directly generate ROS, which can oxidize proteins, lipids, DNA, and other macromolecules to disrupt

cell processes and cause hepatocyte injury. Diclofenac, a widely prescribed nonsteroidal antiinflammatory drug (NSAID), is metabolized by CYP to form a quinine imine, which can redox cycle to generate ROS and causes hepatocyte injury (Boelsterli 2003). On the other hand, reactive metabolites such as NAPQI may also indirectly cause ROS generation through GSH depletion in mitochondria as previously described (Hanawa et al. 2008). ROS species can activate/inhibit signaling pathways through redox changes to proteins (Fig. 1) or through cell injury caused by free radical damage to proteins, lipids, and DNA (Cadenas and Davies 2000; Han et al. 2006b). Covalent binding or oxidative effects on electron transport can further enhance ROS production. Because oxidative damage can accumulate with time, the long latency observed for many drugs that cause nonallergic idiosyncratic DILI may be due to a continuous buildup of oxidative damage in hepatocytes. ROS are important activators of cell death signals such as JNK and important damaging agents that mediate liver injury caused by drugs.

5.4 Activation of Signal Transduction Pathways that Modulate Cell Death/Survival

In APAP hepatotoxicity, the JNK signaling pathway plays an essential and active role in mediating hepatocyte death and liver injury. Without JNK activation, NAPQI causes GSH depletion and covalent binding, but no hepatocyte death occurs unless extremely high doses of APAP are given (Hanawa et al. 2008). These findings suggest the notion that some types of necrosis may be "programmed" and involve the active recruitment of signaling molecules to mediate cell death, as similarly observed in apoptosis. Whether this type of active death program is essential in liver injury caused by other drugs remains to be examined. However, JNK activation is essential in liver injury caused by ischemia and during transplantation, as well as a number of pathologies in other tissues (Schattenberg et al. 2006; Uehara et al. 2005). Therefore, activation of programmed cell death pathways may be a common theme in liver injury caused by drugs and may represent important therapeutic targets to reduce DILI. In addition to prodeath signaling pathways, hepatocyte death following drug intake can be modulated by many signaling pathways involved in cell regeneration, metabolism, protein unfolding (heat shock proteins), and repair pathways. In the APAP model, signaling pathways involving SCF, heat shock proteins, antioxidant enzymes, and a host of other pathways modulate liver injury. Many of these signaling pathways also represent potential areas for treatment of liver injury caused by drugs.

Drugs such as APAP that cause redox changes may also promote liver injury by altering signaling pathways that sensitize hepatocytes to TNF and other cytokines. Nontoxic doses of APAP inhibit NF-κB signaling that sensitizes hepatocytes to the cytotoxic effects of TNF (Nagai et al. 2002). Similarly, drugs such as trovafloxacin may sensitize hepatocytes to TNF, stimulated by cotreatment with LPS in vivo.

Since NF-κB is essential in protecting hepatocytes from the cytotoxic effects of TNF, drugs that interfere with NF-κB signaling could promote hepatocyte death and liver injury. The redox changes induced by drugs may be an important component in modulating signaling pathways important in hepatocyte survival/death.

5.5 *Mitochondria: A Central Target of Drugs*

Both the APAP and Mn-SOD$^{+/-}$ models demonstrate the potential central role of mitochondria in mediating DILI. Mitochondria have taken center stage in most types of cell death, both apoptotic and necrotic. The importance of mitochondria in cell survival and death are underscored by the fact that mitochondria are the major generators of ATP, as well as being the major sources of ROS in cells (Cadenas and Davies 2000; Han et al. 2003a). Mitochondria also house apoptotic factors such as cytochrome c and AIF, whose release into cytoplasm triggers apoptosis. Damage or injury to mitochondria can have profound effects on ATP and ROS levels, as well as release of prodeath proteins such as cytochrome c, that affect both necrotic and apoptotic death (Kaplowitz 2002). Mitochondria are also the battleground where prodeath and prosurvival signaling pathways converge to decide the fate of a cell. Prodeath kinases such as JNK and prodeath bcl-2 family members such as bax converge on mitochondria to promote MPT and hepatocyte death. Prosurvival bcl-2 family members such as bcl-xl and bcl-2 reside in mitochondria to counteract prodeath bcl-2 family members to prevent MPT. Whether prodeath or prosurvival pathways prevail probably depends on the extent of mitochondria injury. In the APAP model, without depletion of mitochondrial GSH and covalent binding, prodeath pathways such as JNK appear to do little damage (Gunawan et al. 2006; Hanawa et al. 2008). Thus, the convergence of redox changes and prodeath pathways in mitochondria are necessary for MPT and hepatocyte death in the APAP model. Overall, most, if not all, types of liver injury involve mitochondrial injury and MPT, induced either through signaling pathways (regulated) or induced by damage to the mitochondrial membranes and proteins (nonregulated).

An important component of DILI is that drugs or reactive metabolites may directly target and disrupt mitochondrial bioenergetics (Boelsterli and Lim 2007; Kass 2006). Drugs, either in the parent form (e.g., troglitazone?) or in the form of reactive metabolites, may accumulate in mitochondria and either alter redox status of electron transport complexes directly (e.g., GSH depletion or redox cycling) or, by covalent binding to proteins in these complexes, cause interruption of electron flow and promote ROS production, which can feed forward to further impair electron transport leading to more ROS (Fig. 5a, b). The inhibition of mitochondrial respiration by drugs or reactive metabolites leads to oxidative and metabolic changes which, after a certain threshold is crossed, activate key prodeath signaling pathways such as JNK as previously described. Consequently, inhibition of mitochondria by drugs and reactive metabolite may be a key upstream event in triggering signals leading to hepatocyte death. Therefore, in DILI, mitochondria may be

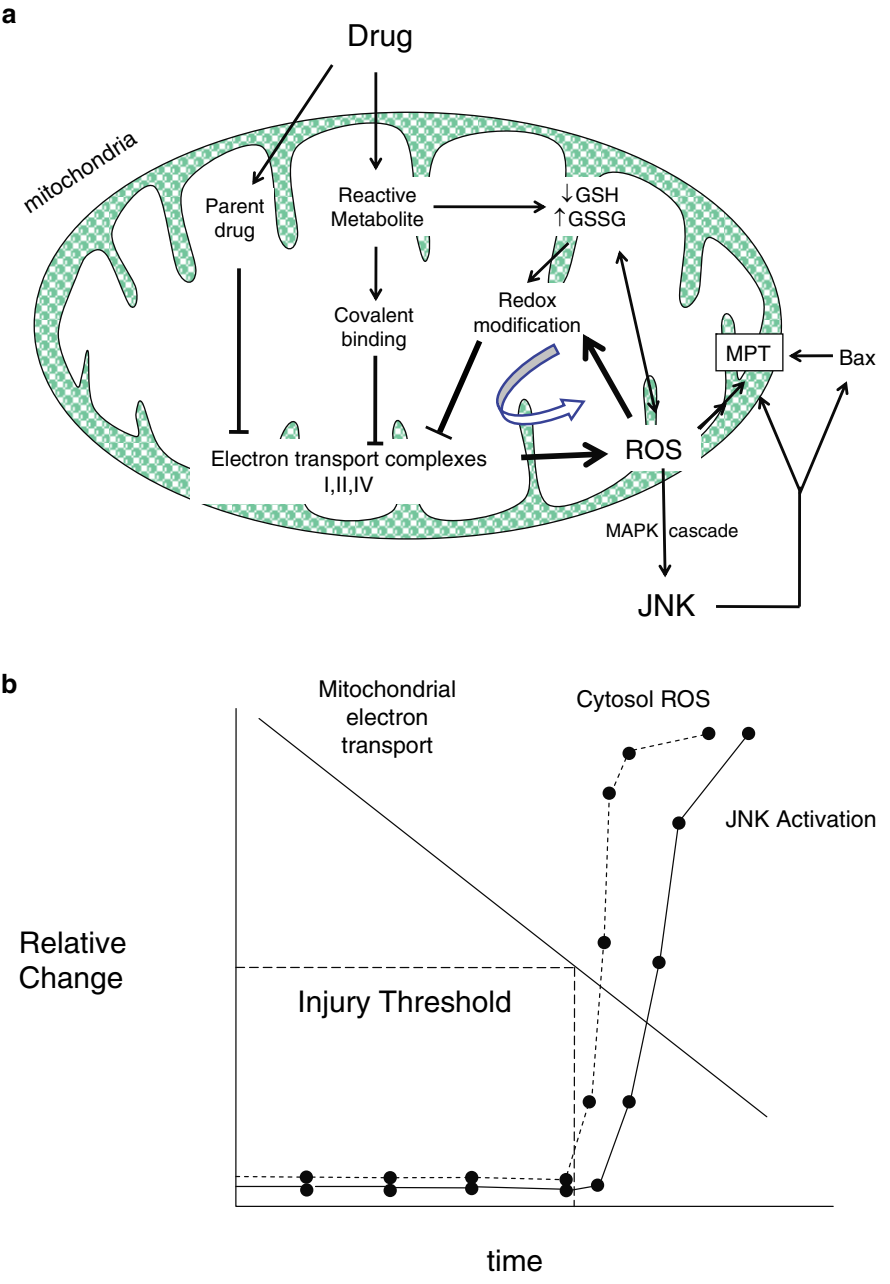

Fig. 5 Mitochondria as focal points in drug-induced liver injury. (**a**) There are two levels of mitochondrial participation in DILI: (1) final common pathway of cell death in liver – bax, bak, bim, tbid versus bcl-xl, mcl-1, bcl2 regulating mitochondrial outer membrane permeabilization and apoptosis (possibly also MPT and necrosis) which responds in external signals (e.g., TNF or FasL)

Table 1 Drugs associated with mitochondrial dysfunction

Drugs	Model system	References
Acetaminophen	In vivo mice liver, cultured rat hepatocytes	Donnelly et al. (1994); Hanawa et al. (2008)
Amiodarone	Isolated rat mitochondria, rat hepatocytes	Varbiro et al. (2003); Waldhauser et al. (2006)
Aspirin	Reye's syndrome – human liver	Iancu et al. (1977); Osterloh et al. (1989)
Benzarone, Benzbromarone	Rat hepatocytes	Kaufmann et al. (2005)
Diclofenac	Rat and human hepatocytes, isolated kidney mitochondria	Boelsterli (2003); Gomez-Lechon et al. (2003)
Diflunisal	Isolated rat liver mitochondria	McDougall et al. (1983)
Fialuridine	In vivo woodchuck and rat liver mitochondria, HepG2 cells	Lewis et al. (1997); Lewis et al. (1996)
Isoniazid	HepG2 cells, in vivo mice liver when cotreated with rifampicin	Chowdhury et al. (2006); Schwab and Tuschl (2003)
Mefenamic acid	Isolated kidney or liver mitochondria	McDougall et al. (1983); Mingatto et al. (1996)
Nefazodone	Human hepatocytes, isolated rat liver mitochondria	Dykens et al. (2008)
Nimesulide	Isolated rat liver mitochondria, rat hepatocytes	Berson et al. (2006); Mingatto et al. (2002)
Perhexiline	Rat hepatocytes	Berson et al. (1998); Deschamps et al. (1994)
Stavudine	HepG2 cells	Setzer et al. (2008); Velsor et al. (2004)
Tolcapone	Isolated rat liver, human neuroblastoma	Haasio et al. (2002); Korlipara et al. (2004)
Trazodone	Human hepatocytes	Dykens et al. (2008)
Troglitazone	HepG2 cells, OUMS-29 cells	Shishido et al. (2003); Tirmenstein et al. (2002)
Valproic acids	In vivo mice liver, rat hepatocytes	Sobaniec-Lotowska (1997); Turnbull et al. (1983)

involved in *upstream* events (i.e., drug-induced mitochondrial impairment leading to decrease ATP and increase in ROS) that trigger signaling pathways important in hepatocyte death as well as participating in the final *downstream* pathway (i.e., MPT, permeabilization of the mitochondrial outer membrane) that leads to

Fig. 5 (continued) or internal stress (not shown); and (2) conditions in which mitochondrial function (e.g., electron transport and ROS) are directly affected by drug, its metabolite, or the consequences of the metabolites in mitochondria. In this scenario, drugs rapidly or gradually cause accumulation of mitochondrial damage including a possible self-amplifying mechanism in which drugs or metabolites directly interfere with electron transport causing ROS generation, which then further interfere with electron transport (redox effects) and deplete antioxidant mechanisms leading to more ROS. (**b**) When sufficient mitochondrial impairment occurs (after acute or chronic drug effects), a threshold is crossed where the cytoplasm is exposed to sufficient ROS to activate the MAPK cascade targeting effects directly (e.g., JNK translocation) and indirectly (e.g., bax translocation) leading to MPT, more ROS generation and bioenergetic collapse

hepatocyte death. In the APAP model, mitochondria were involved very upstream (e.g., NAPQI-induced inhibition of mitochondrial respiration and increased ROS that activate JNK) and participated in the final downstream event (e.g., MPT) that caused hepatocyte death.

Table 1 lists many drugs shown to inhibit mitochondrial bioenergetics or cause MPT in primary cultured hepatocytes or in vivo. A recent study by Xu et al. (2008) also demonstrated that a majority of drugs that cause idiosyncratic liver injury also caused mitochondria changes in cultured primary human hepatocytes. In testing 300 drugs that cause idiosyncratic liver injury, 50–60% of the drugs were found to cause some type of mitochondrial alteration (i.e., GSH, ROS, membrane potential). Even more interesting was the fact that drugs that do not cause DILI (e.g., tacrine, fluoxetine, raloxifene) did not affect mitochondrial function and had a 0–5% false positive rate. Although the applicability of mechanistic insights gained from cultured hepatocytes at high doses of drugs is often questioned, taken as a whole, the evidence suggests a relationship between DILI and mitochondrial damage by drugs or reactive metabolites. In addition, a limited amount of human data suggests mitochondrial abnormalities occur in liver following idiosyncratic hepatotoxic drug intake. Tolcapone-induced DILI was associated with mitochondria swelling and decreased density in the liver of affected patients (Spahr et al. 2000). Similarly, electron microscopy studies have demonstrated that aspirin-induced Reye's syndrome is associated with abnormal mitochondria in the liver (Iancu et al. 1977; Osterloh et al. 1989). Whether mitochondria participate as a downstream final common pathway for cell death or are the principle upstream targets (i.e., drug-induced mitochondrial impairment is a specific upstream component of the pathophysiology) or both, as observed with APAP, remains to be further characterized for most drugs.

The accumulation of mitochondrial damage caused by drugs may help to explain the long latency period of drugs that cause nonallergic idiosyncratic liver injury. Mitochondrial damage (oxidative injury, covalent binding, etc.) may accumulate with drug intake, but considering that there are hundreds of mitochondria per cell, mitochondria damage during the early phase of drug intake will probably have little clinical impact. However, the interruption of electron flow and increased ROS production can feed forward to further impair electron transport leading to more ROS. When a critical threshold of mitochondria damage is reached, then hepatocyte death may ensue, resulting in the clinical manifestations of DILI. This threshold of mitochondrial impairment may expose cells to sufficient ROS to activate signal transduction mechanisms that then acutely further impair mitochondria function and promote cell death.

JNK is one of many kinases that has been reported to target mitochondria. Many studies have shown that prosurvival kinases such as Akt and prodeath signaling pathways such as glycogen synthase kinase-3β(GSK-3β) translocate and regulate mitochondria. The importance of protein kinase translocation and regulation of mitochondrial bioenergetics is a growing theme in pathophysiology. In the ischemia-reperfusion heart model, GSK-3β has been shown to translocate to mitochondria to regulate MPT and promote cell death (Juhaszova et al. 2004; Nishihara et al. 2007). GSK-3β is believed to bind and regulate VDAC in the MPT

pore in mitochondria and also inactivate the antiapoptotic bcl2 family member, Mcl-1 (Das et al. 2008; Maurer et al. 2006). Other studies have demonstrated that during ischemia-reperfusion PKC-δ translocates to mitochondria, inhibits mitochondrial bioenergetics, and promotes cytochrome c release, an effect counteracted by PKC-ε, which also translocates to mitochondria (Churchill and Mochly-Rosen 2007). Like many models of cell injury, translocation of prodeath and prosurvival kinases are likely to be an important component of liver injury caused by drugs. The mechanisms by which kinases such as JNK interact with and modulate mitochondrial proteins and function is an important area of research in liver injury.

6 Perspective

Based primarily on work done with APAP and other drugs, a hypothesis of events important in DILI can be outlined (Fig. 5). Formation of reactive metabolite, covalent binding, ROS, signaling pathways, and mitochondrial injury are key factors in DILI. Conceptually, the notion that drug injury requires cell death signaling pathways represents a new understanding of how drugs cause hepatocyte death. Drug injury may not be a passive event due to overwhelming injury caused by reactive metabolites in hepatocytes, but may require the activation and participation of prodeath proteins such as JNK that trigger death. Mitochondria are the central point where oxidative injury, prodeath proteins, and prosurvival proteins converge to determine the fate of cells. Mitochondrial damage and consequent hepatocyte death depends on the extent of redox modifications, and the balance of prodeath proteins, and prosurvival proteins that converged on mitochondria.

For most drugs, the underlying mechanism of why DILI, depicted in Fig. 5, occurs idiosyncratically in a very small percent of the population remains an important field of research. Most drugs probably have some dose dependence, with higher drug doses causing greater levels of reactive metabolite formation and injury in hepatocytes. APAP is the classic example of a drug with strong dose dependence in causing liver injury. Higher APAP intake is associated with greater NAPQI formation in liver, which causes extensive GSH depletion and greater covalent binding and oxidative stress. However, for some drugs, the dose dependence is not very linear, as observed with troglitazone in diabetic patients, and other contributing factors are likely involved. Because hepatocyte injury during DILI involves multiple steps, genetic polymorphism or environmental factors that modulate steps outlined in Fig. 5 may contribute to idiosyncratic drug hepatotoxicity. For example, the levels of CYP2E1, which metabolizes APAP to NAPQI, determine the extent of NAPQI formation and hepatocyte injury. Alcohol intake is known to upregulate CYP2E1, and thus alcohol intake increases susceptibility to APAP-induced liver injury (Zimmerman and Maddrey 1995). There may be genetic polymorphisms in important signaling pathways such as JNK or mitochondria bioenergetics in the population that may contribute to idiosyncratic DILI. Idiosyncratic DILI may be likened to a “perfect storm”,

a situation where many variable factors become simultaneously favorable to form a situation where liver damage occurs. Idiosyncratic drug hepatotoxicity represents the convergence of many factors (e.g., genetic, environmental, nutrient, infections) which creates a condition that causes liver injury following drug intake. Animals and cell culture studies suggest that certain drugs and TNF, secreted during inflammation, may work synergistically to promote idiosyncratic liver DILI. With the advancement of genomic, proteomic, and metabolomic profiling technologies, it may be possible in the future to identify and screen patients for possible risk factors that increase susceptibility to idiosyncratic drug hepatotoxicity and gain greater insights into pathogenesis. Better understanding of signaling pathways that modulate cell death in hepatocytes will lead to greater insights in idiosyncratic DILI, as well as other liver pathologies.

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Mitochondrial Involvement in Drug-Induced Liver Injury

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Contents

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Abstract Mitochondrial dysfunction is a major mechanism of liver injury. A parent drug or its reactive metabolite can trigger outer mitochondrial membrane permeabilization or rupture due to mitochondrial permeability transition. The latter can severely deplete ATP and cause liver cell necrosis, or it can instead lead to apoptosis by releasing cytochrome *c*, which activates caspases in the cytosol. Necrosis and apoptosis can trigger cytolytic hepatitis resulting in lethal fulminant hepatitis in some patients. Other drugs severely inhibit mitochondrial function and trigger extensive microvesicular steatosis, hypoglycaemia, coma, and death. Milder and more prolonged forms of drug-induced mitochondrial dysfunction can also cause macrovacuolar steatosis. Although this is a benign liver lesion in the short-term, it can progress to steatohepatitis and then to cirrhosis. Patient susceptibility to drug-induced mitochondrial dysfunction and liver injury can sometimes be explained by genetic or acquired variations in drug metabolism and/or elimination that increase the concentration of the toxic species (parent drug or metabolite). Susceptibility may also be increased by the presence of another condition, which also impairs mitochondrial function, such as an inborn mitochondrial cytopathy, β-oxidation defect, certain viral infections, pregnancy, or the obesity-associated metabolic syndrome. Liver injury due to mitochondrial dysfunction can have important consequences for pharmaceutical companies. It has led to the interruption of clinical trials, the recall of several drugs after marketing, or the introduction of severe black box warnings by drug agencies. Pharmaceutical companies should systematically investigate mitochondrial effects during lead selection or preclinical safety studies.

Keywords Mitochondria · Drug-induced liver injury · Hepatotoxicity · Steatosis · Hepatitis

1 Introduction

About 1.5 billion years ago, a precursor of present day eukaryotes began a parasitic/symbiotic partnership with a wild bacterium (Yang et al. 1985). The precursor allowed the bacterium to reside and divide in its cytoplasm, while in exchange, the bacterium generated large amounts of energy from the oxidation of fat and other substrates.

The relationship may have been unstable at first, because wild bacteria tend to proliferate when food is plentiful (Pessayre 2007). However, eventually, the host may have found ways to disable overly proliferating bacteria, while the bacteria may have answered by killing any host that took advantage of this control mechanism (Pessayre 2007). The present day sequel of this warfare is a complex mechanism of cell death, where the host can permeabilize mitochondrial membranes, but this permeabilization can cause its death (Pessayre 2007).

In addition to this mutual deterrence, a mutual dependence also ensured the stability of this odd couple. Most of the ancient bacterial genes have been lost or

have migrated to the nucleus (Mourier et al. 2001), including all the genes required for the stability, replication, transcription, and repair of the bacterial/mitochondrial genome, thus transforming wild bacteria into tame mitochondria (Pessayre 2007). Nevertheless, mitochondria still retain a small, compact, circular genome in the mitochondrial matrix (Taanman 1999). Only the mother transmits this precious DNA, which encodes for 13 critical polypeptides of the respiratory chain (Taanman 1999). Although other proteins necessary for mitochondrial energy generation and all the proteins required for fatty acid oxidation are encoded by nuclear DNA and synthesized in the cytoplasm, these proteins are then imported into the mitochondria, which thus fully retain their ancient role as energy providers for the symbiont (Pessayre 2007). Because mitochondria are our main energy source, they are also our Achilles' heel. When drugs damage mitochondria, the accumulation of non-metabolized lipids in the liver can cause steatosis; the lack of energy can result in cell dysfunction while the permeabilization of mitochondrial membranes can trigger cell death. Indeed, mitochondrial dysfunction is primarily or secondarily involved in most forms of drug-induced liver injury (DILI) (Pessayre and Larrey 2007; Pessayre et al. 2001, 2007, 2008). Even when liver injury is initially triggered by reactive metabolites (Pessayre 1995), mitochondrial membrane disruption still occurs as a final mechanism of cell death (Haouzi et al. 2000).

In the present chapter, we will briefly summarize the physiological roles of mitochondria in cell life and cell death and the clinical manifestations of diverse forms of drug-induced mitochondrial dysfunction. The mechanisms leading to drug-induced mitochondrial dysfunction and DILI will then be reviewed as well as genetic and acquired factors that cause a predisposition to these adverse effects. Finally, the need to evaluate mitochondrial effects during preclinical studies (Labbe et al. 2008) will be discussed before concluding.

2 Physiological Roles of Mitochondria

2.1 Fuel Oxidation and Energy Production

Mitochondria play an important role in pyruvate oxidation, fat oxidation, and energy production (Fig. 1) (Pessayre 2007). Although the glycolysis of glucose into pyruvate occurs in the cytosol, the mitochondrial enzyme, pyruvate dehydrogenase, then transforms NAD^+, CoA, and pyruvate into NADH and acetyl-CoA, which is further oxidized by the mitochondrial tricarboxylic acid cycle (Pessayre 2007). Moreover, mitochondria are the main organelles in fat oxidation (Pessayre 2007). The entry of long-chain fatty acyl-CoAs into mitochondria requires a carnitine shuttle and is modulated by carnitine palmitoyltransferase I (Fig. 1). Inside the mitochondria, the fatty acyl-CoA is regenerated from acyl-carnitine and is split by successive ß-oxidation cycles into acetyl-CoA subunits, which either condense into ketone bodies or, like other fuels, are degraded to CO_2 by the tricarboxylic acid cycle (Fig. 1).

Fatty acid ß-oxidation, pyruvate oxidation, and the final oxidation of acetyl-CoA by the tricarboxylic acid cycle generate $FADH_2$ and/or NADH. These reduced cofactors then transfer their electrons to the mitochondrial respiratory chain (Fromenty and Pessayre 1995), regenerating the NAD^+ and FAD necessary for other fuel oxidation cycles (Fig. 1). The electrons donated by NADH or $FADH_2$ migrate all along the respiratory chain up to cytochrome *c* oxidase, where four electrons react in quick succession with oxygen and protons to safely form water, with no release of reactive oxygen species (ROS) at this site (Fig. 1).

The transfer of electrons across respiratory chain complexes I, III, and IV is coupled with the extrusion of protons from the mitochondrial matrix into the intermembrane mitochondrial space (Fig. 1) (Wallace 1999), creating a large electrochemical potential across the inner membrane.

When ADP is high, protons reenter the matrix through the F_0 portion of ATP synthase, causing rotation of a molecular rotor within the F_1 portion of ATP synthase and conversion of ADP into ATP (Fig. 1) (Wallace 1999). The adenine nucleotide translocator then extrudes the formed ATP in exchange for cytosolic ADP (Wallace 1999), while at the same time the ADP-driven reentry of protons

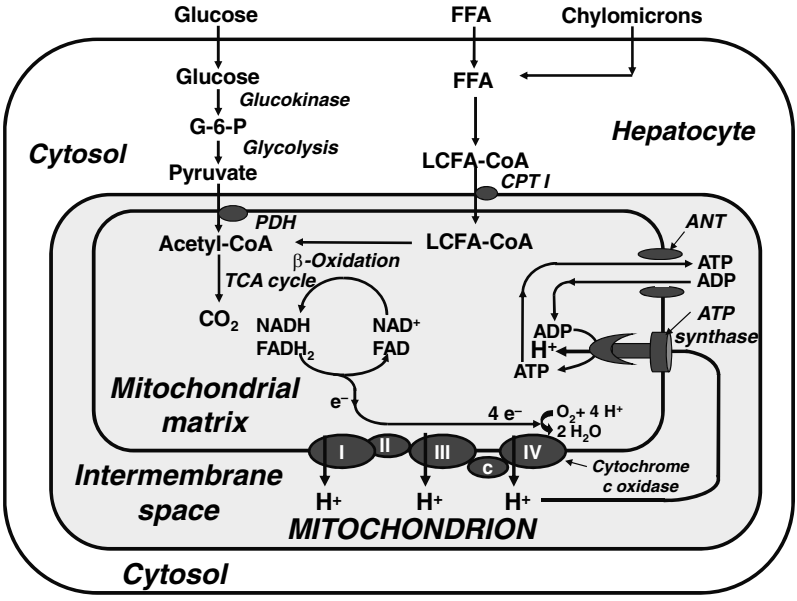

Fig. 1 Oxidative metabolism and energy production by mitochondria. The oxidation of pyruvate and free fatty acids (FFA) inside mitochondria produces NADH and $FADH_2$, which transfer their electrons to the mitochondrial respiratory chain. The flow of electrons in mitochondrial complexes I, III, and IV is coupled with the extrusion of protons from the mitochondrial matrix into the intermembrane space. When energy is needed, these protons reenter the matrix through ATP synthase, to generate ATP from ADP. The adenine nucleotide translocator (ANT) then exchanges the formed ATP for cytosolic ADP. *G-6-P* Glucose 6-phosphate, *PDH* pyruvate dehydrogenase, *LCFA-CoA* long-chain fatty acyl-CoA, *CPT I* carnitine palmitoyltransferase I, *TCA cycle* tricarboxylic acid cycle, *c* cytochrome *c*

decreases the mitochondrial membrane potential, unleashing the flow of electrons in the respiratory chain and increasing the reduction of oxygen into water.

In contrast, when cells are full of ATP, the reentry of protons into the matrix is minimal, and the high mitochondrial membrane potential slows down the flow of electrons and mitochondrial respiration.

Thus, mitochondria can turn fat and other fuels into CO_2 and water to provide ATP for the cell as needed. However, the price of this energy is increased ROS formation contributing to aging and cancer (Wallace 1999).

2.2 *Reactive Oxygen Species, Aging, and Cancer*

A few of the electrons going through complexes I and III react with oxygen to form the superoxide anion radical ($O_2^{-}\bullet$) (Fig. 2) (Shigenaga et al. 1999). This radical has little reactivity alone, except that it can dislodge iron from iron–sulfur complexes. However, $O_2^{-}\bullet$ reacts with nitric oxide (NO•) to form the highly reactive,

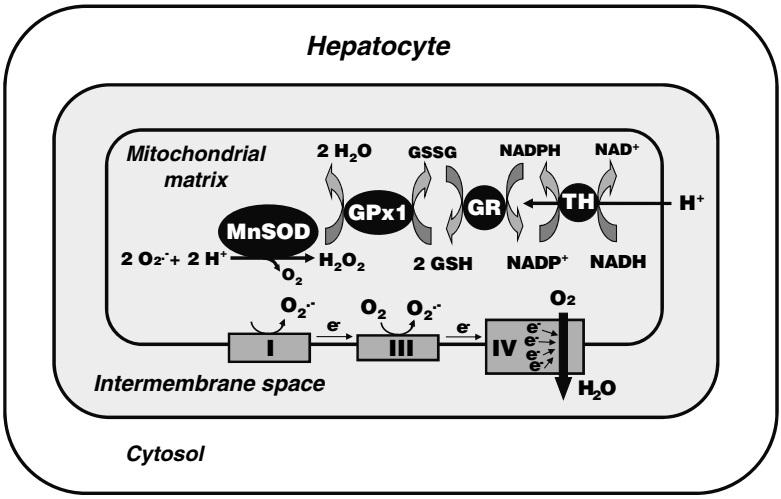

Fig. 2 Formation and inactivation of the superoxide anion radical in mitochondria. Most of the electrons entering the respiratory chain end up in cytochrome *c* oxidase (complex IV of the respiratory chain) where four electrons are added quickly to oxygen so that reactive oxygen species are not released, but only water is safely formed. However, a few of the electrons, which migrate through the respiratory chain, can react with oxygen within complex I and complex III to form the superoxide anion radical ($O_2^{-}\bullet$). In the mitochondrial matrix, manganese superoxide dismutase (MnSOD) dismutates two molecules of the superoxide anion into one oxygen molecule and one molecule of hydrogen peroxide (H_2O_2). Glutathione peroxidase 1 (GPx1) then reduces H_2O_2 into water while oxidizing two reduced glutathione (GSH) molecules into one glutathione disulfide (GSSG). Glutathione reductase (GR) then regenerates GSH, at the expense of NADPH. Finally, an energy-linked $NAD(P)^+$ transhydrogenase (TH) uses both NADH and the mitochondrial membrane potential to regenerate NADPH from $NADP^+$

peroxynitrite anion ($ONOO^-$). $O_2^-\bullet$ is also dismutated by manganese superoxide dismutase into oxygen and hydrogen peroxide (Fig. 2), which can form the hydroxyl radical (•OH) in the presence of iron. The highly reactive •OH can trigger lipid peroxidation which releases reactive lipid peroxidation products.

Peroxynitrite, the hydroxyl radical, and lipid peroxidation products damage DNA. Mitochondrial DNA (mtDNA) is particularly sensitive to ROS-induced damage due to its proximity to the inner membrane (the main cellular source of ROS), the absence of protective histones, and the nonrepair of bulky DNA lesions (LeDoux et al. 1999) such as those induced by reactive lipid peroxidation products. Oxidative lesions of mtDNA bases can cause errors during mtDNA replication (Kuchino et al. 1987) or repair (Pinz et al. 1995) and result in point mutations. Furthermore, ROS-mediated mtDNA strand breaks can occasionally lead to mtDNA deletions (Berneburg et al. 1999).

The accumulation of point mutations and deletions in the mtDNA of heart and muscles can decrease mtDNA-encoded polypeptide synthesis and the transfer of electrons along the respiratory chain during old age (Shigenaga et al. 1999). If the electron flow is partially blocked along the respiratory chain, respiratory chain complexes located above the block become overly reduced and can directly transfer their electrons to O_2 to form $O_2^-\cdot$ (Esposito et al. 1999). The resulting increase in ROS formation further damages mtDNA causing more respiratory impairment and ROS formation. Finally, as the mitochondrial membrane potential decreases, the potential-driven import of DNA repair enzymes into the mitochondria becomes impaired (Szczesny et al. 2003). These vicious circles could explain why mtDNA deletions and point mutations, which are uncommon before the age of 40, exponentially accumulate during old age (Cortopassi et al. 1992; Michikawa et al. 1999).

Part of the hydrogen peroxide generated by mitochondria leaves the mitochondria and can therefore form hydroxyl radicals elsewhere. The radical and the lipid peroxidation products that it generates can damage nuclear DNA, contributing to aging and the development of cancer.

2.3 Mitochondrial Membrane Alterations and Cell Death

Mitochondria also participate in the control of cell life and death by summing up prodeath and antideath signals (Kroemer and Reed 2000). Signals coming from death receptors on the plasma membrane, from damaged nuclear DNA, permeabilized lysosomes, or a stressed endoplasmic reticulum, all converge on the mitochondria (Fig. 3a). These signals activate Bcl-2-associated x protein (Bax), which translocates from the cytoplasm to the mitochondria.

The mitochondria then summarize this information to make a yes or no decision (Fig. 3b). When there are only a few activated Bax and Bak molecules, they can be sequestered by binding to B cell leukemia X long form (Bcl-X_L) and myeloid cell leukemia-1 (Mcl-1) leaving the outer mitochondrial membrane intact so that the cell can live (Finucane et al. 1999) (Fig. 3b). In contrast, when there are too many

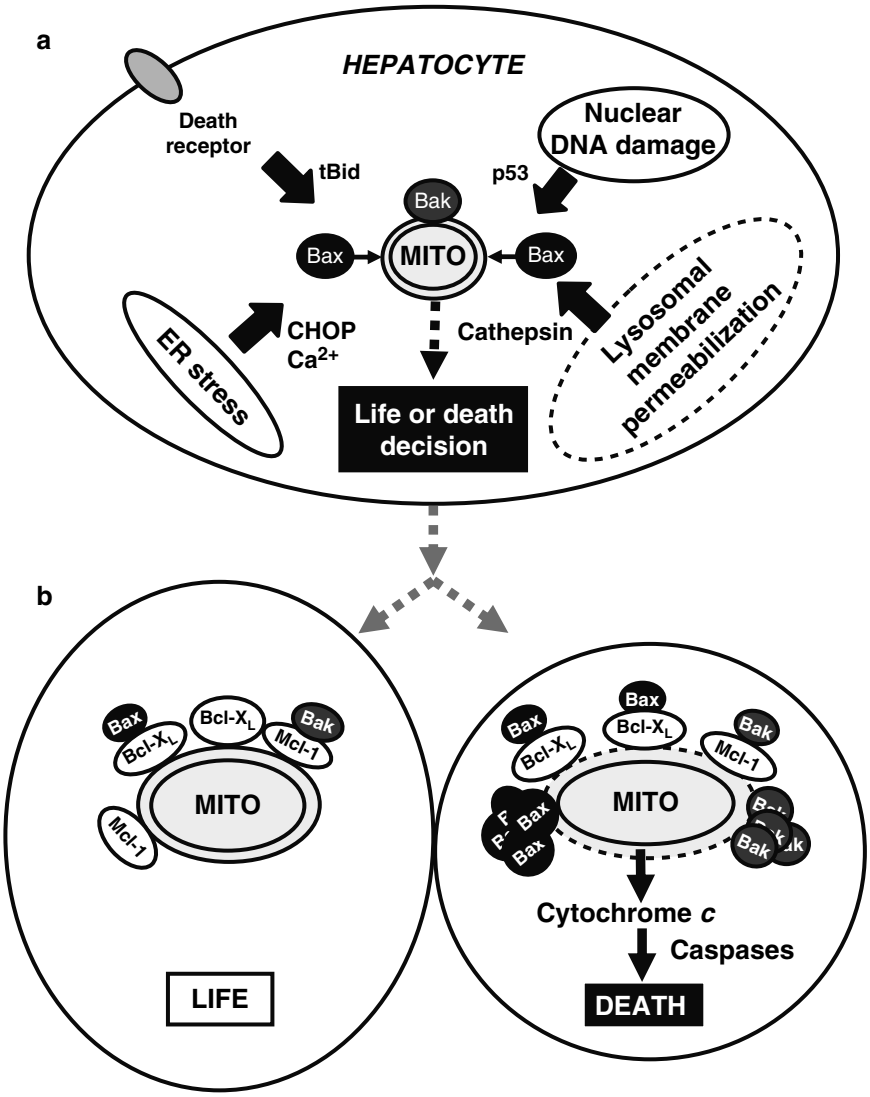

Fig. 3 Mitochondria at the center of cell life or death. (**a**) All proapoptotic signals converge on the mitochondria, including the stimulation of death receptors, which cleaves BH3-interacting domain death agonist (Bid) into truncated Bid (tBid), or the occurrence of nuclear DNA damage, which stabilizes p53, or the permeabilization of the lysosomal membrane, which releases cathepsins, or the occurrence of an endoplasmic reticulum (ER) stress, which induces C/EBP homologous protein (CHOP) and releases calcium from the ER. All these initial events can activate Bcl-2-associated X protein (Bax) to cause its translocation from the cytosol to the mitochondria. They also activate Bcl-2 homologous antagonist/killer (Bak), which is already present on the mitochondria. (**b**) Mitochondria then sum up this information to take a yes or no decision. When only moderate amounts of activated Bax and Bak are formed, Bax and Bak can be sequestered by the antiapoptotic proteins B cell leukemia X (long form) ($BclX_L$) and myeloid cell leukemia-1 (Mcl-1), thus allowing the cell to live. In contrast, when too much Bax and Bak are activated to be sequestered by $BclX_L$ and Mcl-1, Bax and Bak then form large oligomers that permeabilize the outer mitochondrial membrane, thus allowing the egress of cytochrome *c*, which activates caspases in the cytosol to trigger apoptosis.

activated Bax and Bak to bind to Bcl-X_L and Mcl-1, they form large oligomers on the outer mitochondrial membrane which then becomes permeable (Nechushtan et al. 2001) (Fig. 3b). This allows cytochrome *c* to move from the mitochondrial intermembrane space into the cytosol, where it interacts with apoptosis protease-activating factor 1 (apaf-1) and procaspase-9, which auto-activates into caspase-9. The latter activates caspase-3 and other effector caspases that cut several cytosolic and nuclear proteins to trigger apoptosis.

Cytosolic cytochrome *c* also migrates to the endoplasmic reticulum where it binds to and activates inositol (1,4,5) triphosphate receptors triggering an efflux of calcium. Part of this cytosolic calcium then enters the mitochondria (Boehning et al. 2003). The translocation of cytochrome *c* out of the mitochondria also causes partial blocking of electron flow and increased mitochondrial ROS formation. The increase in mitochondrial calcium and ROS can trigger a second type of mitochondrial membrane alteration called mitochondrial permeability transition (MPT). The MPT pore, a multi-protein pore structure present at contact sites between the outer and inner membrane (Pessayre et al. 1999), can be opened by diverse stimuli, including ROS, an increase in the ratio of oxidized glutathione (GSSG) over reduced glutathione (GSH), or an increase in cell calcium (Pessayre et al. 2000). While pore closure allows the cell to survive, pore opening causes cell death (Pessayre et al. 1999). Indeed, pore opening results in massive reentry of protons through the inner membrane, collapse of the mitochondrial membrane potential, and interruption of mitochondrial ATP synthesis (Fig. 4a). Pore opening also causes matrix expansion, rupture of the outer membrane, herniation of the inner membrane and matrix through the outer membrane gap, and release of cytochrome *c* from the intermembrane space into the cytosol (Fig. 4a) (Feldmann et al. 2000). Pore opening can therefore trigger either necrosis or apoptosis. If the pore opens quickly in all mitochondria, then severe ATP depletion prevents apoptosis (an energy-requiring process) and causes necrosis (Fig. 4b) (Leist et al. 1997). In contrast, if the pore only opens in certain mitochondria, unaffected mitochondria continue to synthesize ATP (avoiding necrosis), while disrupted mitochondria release cytochrome *c*, which activates caspases in the cytosol to trigger apoptosis (Fig. 4b) (Leist et al. 1997).

Thus, both the permeabilization of the outer mitochondrial membrane by Bax and Bak and the rupture of the outer membrane from MPT and matrix swelling release mitochondrial cytochrome *c* and cause cell death.

3 Clinical Consequences of Impaired Mitochondrial Function

3.1 Consequences of Outer Membrane Permeabilization or Rupture

As mentioned above, the egress of cytochrome *c* from the mitochondria can cause caspase activation and apoptosis, whereas severe ATP depletion can trigger

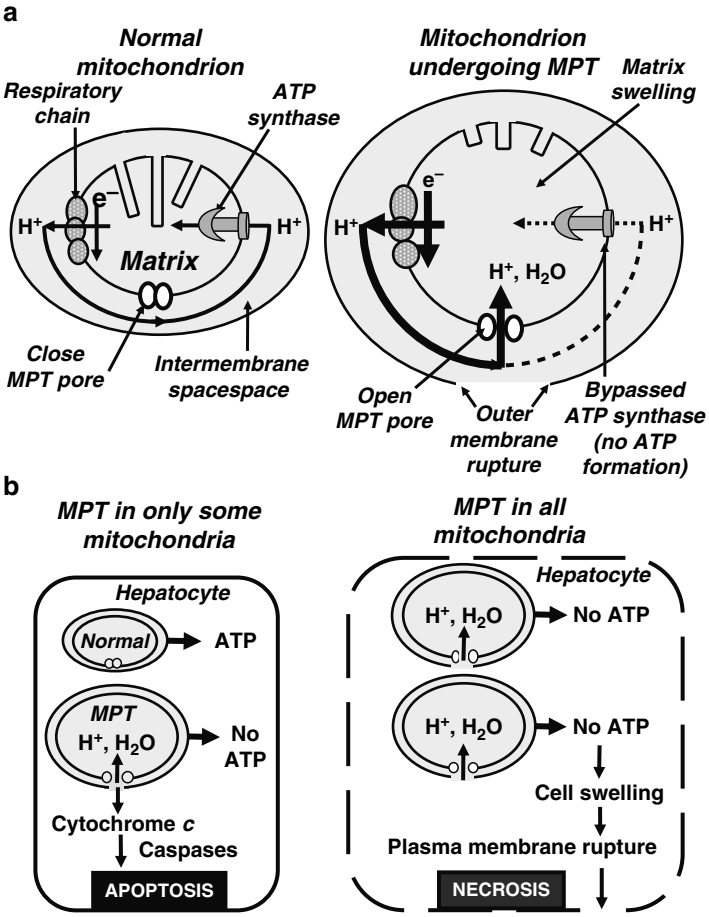

Fig. 4 Mitochondrial permeability transition (MPT) can cause either severe ATP depletion and cell necrosis, or caspase activation and apoptosis. (**a**) Opening of the MPT pore allows a reentry of protons through the pore, thus bypassing ATP synthase and preventing mitochondrial ATP generation. MPT also causes an influx of water driven by the oncotic pressure of matrix proteins. The outer membrane ruptures from matrix swelling. (**b**) When MPT only occurs in some mitochondria, the unaffected organelles synthesize enough ATP to prevent necrosis, while the affected mitochondria release cytochrome *c*, which activates caspases to trigger apoptosis. However, when MPT occurs in all mitochondria, severe ATP depletion causes cell swelling, rupture of the cell plasma membrane and necrosis

cell necrosis. The extensive death of hepatocytes leads to jaundice and can even cause hepatic encephalopathy and death in the most severe cases. When prolonged, the destruction of hepatocytes can also lead to chronic hepatitis, which may progress to liver fibrosis and even cirrhosis.

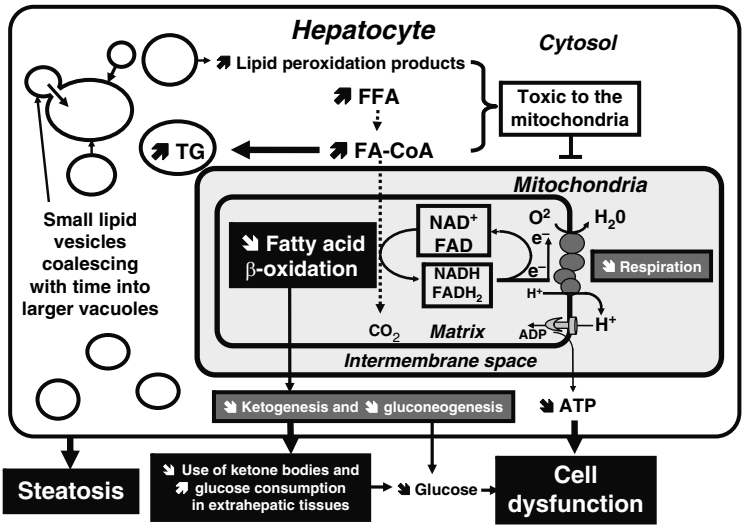

Fig 5 Consequences of a primary impairment of fatty acid oxidation. The impairment of fatty acyl-CoA (FA-CoA) β-oxidation in mitochondria increases free fatty acids (FFA) and triglycerides (TG), which accumulate as small lipid vesicles that can progressively coalesce into larger vacuoles. The impairment of fat oxidation deprives the cell of an important source of energy during fasting episodes. The decreased ketogenesis forces extrahepatic tissues to instead use glucose. Furthermore, the impairment of β-oxidation also inhibits gluconeogenesis, so that hypoglycemia may occur during fasting. In extrahepatic organs, this limits pyruvate oxidation as an alternative source of energy. Finally, the toxic effects of lipid peroxidation products, FFA and FA-CoA on mitochondria further decrease energy formation, which may cause cell dysfunction in liver and extrahepatic organs

3.2 Consequences of a Primary Impairment of β-Oxidation

Only severe impairment of fatty acid oxidation triggers hepatic steatosis (Fig. 5); mild inhibition of mitochondrial β-oxidation is not enough (Fromenty and Pessayre 1995). In the former case, the free fatty acids taken up or synthesized by the liver are insufficiently oxidized by deficient mitochondria and are esterified into triglycerides that accumulate in the cytoplasm of hepatocytes as small vesicles or larger vacuoles. Acute impairment of fatty acid ß-oxidation typically causes microvesicular steatosis (Fromenty and Pessayre 1995). In this peculiar form of steatosis, numerous tiny lipid vesicles leave the nucleus in the center of the cell and give the hepatocyte a “foamy”, “spongiocytic” appearance. In contrast, when ß-oxidation impairment is more chronic, mixed forms of steatosis can occur. Certain hepatocytes fill with tiny lipid vesicles, while others exhibit large fat vacuoles or both small vesicles and larger vacuoles. These associations and transitions suggest that tiny lipid vesicles progressively coalesce into larger vacuoles. Indeed longer-term causes of steatosis tend to result in macrovacuolar steatosis (Fromenty and Pessayre

1995), with hepatocytes distended by a single, large fat vacuole, displacing the nucleus to the cell periphery.

Primary impairment of fatty acid oxidation also secondarily impairs mitochondrial energy production (Fig. 5) (Fromenty and Pessayre 1995). Indeed, fatty acid oxidation is the main source of cellular energy between meals, and patients with impaired mitochondrial ß-oxidation cannot tolerate fasting (Saudubray et al. 1999). The inhibition of β-oxidation slightly suppresses hepatic gluconeogenesis and markedly increases the clearance of glucose by decreasing the formation of ketone bodies, thus forcing extra-hepatic tissues to instead use glucose (Derks et al. 2008). Furthermore, β-oxidation inhibition also secondarily inhibits gluconeogenesis (Fromenty and Pessayre 1995). Fasting may therefore trigger hypoglycemia in patients with impaired β-oxidation (Saudubray et al. 1999) and hamper energy production from this still oxidizable fuel. Fasting also causes massive adipose tissue lipolysis, flooding the liver with free fatty acids that are not oxidized by deficient mitochondria and accumulate in the hepatocytes. Accumulated fatty acids and their dicarboxylic acid derivatives can have toxic effects because they inhibit and uncouple mitochondrial respiration, further decreasing energy production (Fromenty and Pessayre 1995). Finally, steatosis leads to lipid peroxidation, whose reactive products damage the respiratory chain and mtDNA (Pessayre and Fromenty 2005). All these effects can cause an energy deficit in the cells (Fig. 5) leading to cell dysfunction in different organs. Patients with inhibited mitochondrial β-oxidation may develop liver failure (sometimes with renal failure and pancreatitis) and severe brain dysfunction, resulting in coma and death (Saudubray et al. 1999).

3.3 Consequences of a Primary Impairment of Respiration

Impaired mitochondrial respiration decreases energy formation, which can cause either cell dysfunction or death depending on the severity of the deficit (Fig. 6) (Pessayre and Fromenty 2005). While moderate impairment only causes cell dysfunction, severe impairment can cause liver cell death, cholestasis, and fibrosis (Bioulac-Sage et al. 1993; Morris et al. 1998; Morris 1999).

Impaired respiration also blocks the transfer of electrons along the respiratory chain, causing reduction of upstream respiratory chain components, which then react with oxygen to form the superoxide anion radical (Fig. 6). Increased ROS formation can damage mtDNA and respiratory polypeptides thus further impairing respiration. ROS may also play a role in necroinflammation and fibrosis (Pessayre and Fromenty 2005).

Finally, severe impairment of respiration impairs mitochondrial ß-oxidation (Watmough et al. 1990). Normally the NADH formed by ß-oxidation is then reoxidized by the mitochondrial respiratory chain regenerating the NAD^+ required for fatty acid ß-oxidation. During severe impairment of respiration, NAD^+ regeneration cannot sustain ß-oxidation (Watmough et al. 1990), which impairs ß-oxidation

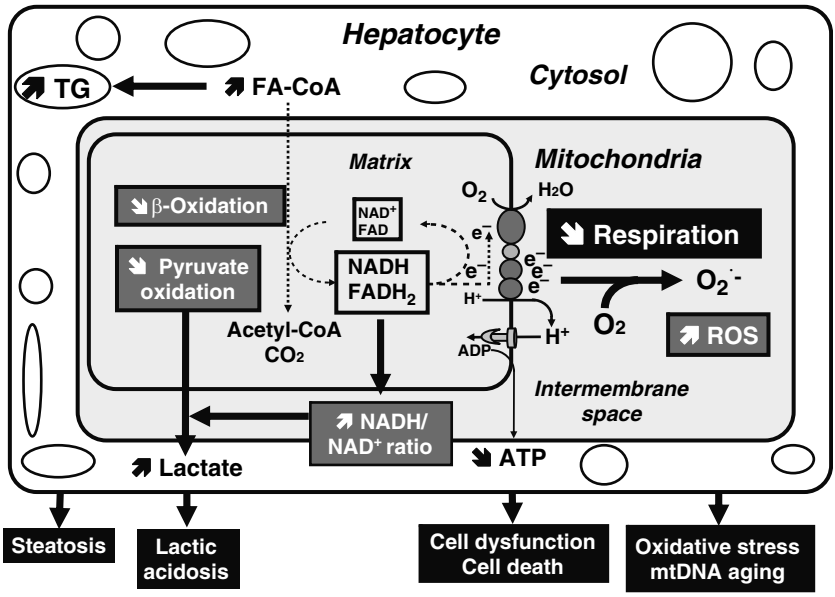

Fig. 6 Consequences of a primary impairment of mitochondrial respiration. The block in the flow of electrons in the respiratory chain decreases ATP formation, thus causing cell dysfunction or cell death. It also causes the accumulation of electrons in upstream respiratory chain complexes, thus increasing ROS formation, which can cause oxidative stress and aging of mitochondrial DNA (mtDNA). Finally, the block in electron flow also decreases the reoxidation of NADH into NAD^+. A first consequence of the lack of NAD^+ is to decrease the β-oxidation of fatty acids, thus causing steatosis. A second consequence is to decrease the oxidation of pyruvate by the pyruvate dehydrogenase complex. Pyruvate is not degraded and, due to the high NADH/NAD^+ ratio, pyruvate is instead reduced into lactate, which can trigger lactic acidosis

and causes microvesicular steatosis (Fig. 6). The lack of NAD^+ also inhibits pyruvate oxidation by the pyruvate dehydrogenase complex. Instead, because of the high NADH/NAD^+ ratio, pyruvate is reduced to lactate which can accumulate and trigger lactic acidosis (Fig. 6).

3.4 Common Features

Thus, primary ß-oxidation impairment causes both microvesicular steatosis and secondary energy deficiency (causing cell dysfunction), while primary impairment of respiration causes both an energy deficiency (leading to cell dysfunction or death) and secondary impairment of ß-oxidation (causing microvesicular steatosis). Thus, whatever the initial defect, drug-induced mitochondrial dysfunction may associate features of both steatosis and cell dysfunction. However, depending on the initial mechanism, one of these features can predominate.

4 Mechanisms of Drug-Induced Mitochondrial Dysfunction

4.1 Metabolite-Mediated Mitochondrial Membrane Permeabilization or Rupture

A frequent mechanism of DILI is the metabolic activation of drugs by cytochrome P450 (CYP) into chemically reactive, electrophilic metabolites, which react with and covalently bind to hepatic proteins and glutathione (Pessayre 1995). These reactive metabolites can trigger hepatitis through direct toxicity or immune reactions (Robin et al. 1997; Pessayre et al. 1999).

4.1.1 Direct Toxicity of Reactive Metabolites

When reactive metabolites are formed in large amounts (for example, after acetaminophen overdoses) several biochemical events may be triggered which all affect the mitochondria, disrupting the outer mitochondrial membrane and leading to cell death (Fig. 7) (Pessayre et al. 1999; Haouzi et al. 2000).

Thus, extensive formation of reactive metabolites can cause severe ATP depletion and necrosis, or caspase activation and apoptosis (Fig. 7). The same compound can cause either of these two types of liver lesions depending on the circumstances. Indeed, the metabolic activation of the diterpenoids from the germander plant caused liver cell necrosis in treated mice (Loeper et al. 1994), but apoptosis in isolated rat hepatocytes (Fau et al. 1997).

4.1.2 Immune Reactions Triggered by Reactive Metabolites

When only small amounts of reactive metabolites are formed, severe toxic hepatitis does not occur. However, mild toxicity may still occur in metabolically susceptible patients, as shown by a clinically silent increase in serum transaminase activity. This mild toxicity causes the release of hepatic proteins, which have been modified by covalent binding with reactive metabolites (Pessayre and Larrey 2007).

The immune system can recognize macromolecules that differ from the unmodified Self. This usually involves foreign proteins but may also include modified Self proteins. The release of metabolite-bound hepatic proteins into the circulation can therefore trigger humoral and cellular immune responses. Some of the elicited antibodies recognize the region of the covalently bound metabolite (neoantigenic target), while others recognize a normal epitope of the protein (autoimmune target) (Robin et al. 1996, 1997). One possible mechanism for the appearance of these two types of antibodies involves the stimulation of previously quiescent B lymphocytes expressing a plasma membrane immunoglobulin that traps the modified hepatic protein either by binding to the metabolite-modified moiety of the protein or to a normal, nonmodified, epitope (Fig. 8) (Beaune et al. 1994). After internalizing the

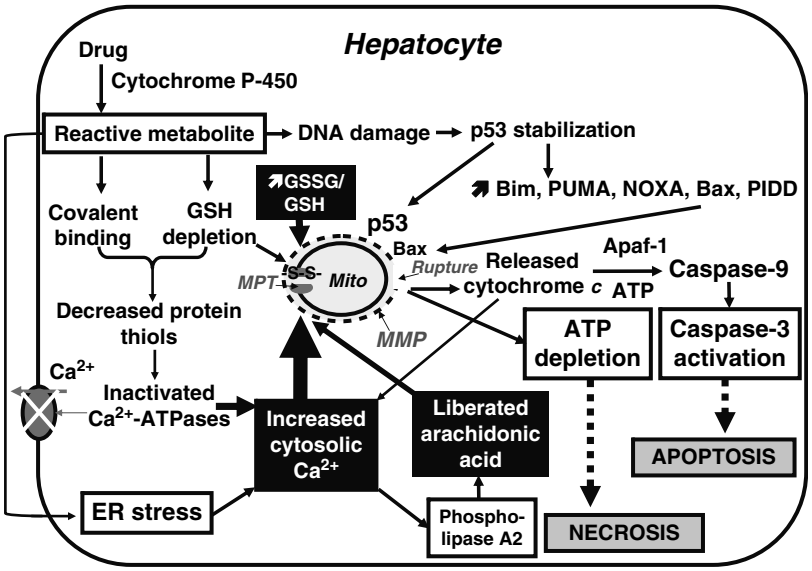

Fig. 7 Metabolite-mediated mitochondrial membrane permeabilization (MMP), mitochondrial permeability transition (MPT) and cell death. Cytochrome P-450-generated reactive metabolites can damage DNA and stabilize p53. The latter can induce outer MMP by migrating to the mitochondria, and by inducing Bcl-2 interacting mediator of cell death (Bim), p53-upregulated modulator of apoptosis (PUMA), NOXA ("damage"), Bcl-2-associated x (Bax) and p53-induced protein with a death domain (PIDD). Reactive metabolites can also covalently bind to proteins and GSH. This binding depletes GSH and increases the GSSG/GSH ratio, which may lead to the formation of disulfide bridges in the MPT pore structure. A decrease in protein thiols is associated with the inactivation of plasma membrane-located Ca^{2+}-ATPase, thus increasing cytosolic Ca^{2+}. Cytosolic calcium can also increase as a consequence of its release from the endoplasmic reticulum due to a reactive metabolite-mediated ER stress and/or to the activation of inositol (1,4,5) triphosphate receptors by the released cytochrome *c*. Calcium then enters the mitochondrial matrix, and it also activates phospholipase A2, which liberates arachidonic acid. The formation of disulfide bridges in the MPT pore proteins, the increase in matrix Ca^{2+} and the increase in arachidonic acid all trigger MPT leading to outer membrane rupture. The resulting decrease in ATP, when severe, can cause necrosis. Otherwise, the egress of cytochrome *c* and the activation of caspases can trigger apoptosis

protein, these quiescent B cells may express modified peptides recognized by helper T cells, leading to the activation of B cells into plasmocytes secreting the antibody or autoantibody (Fig. 8).

Using sensitive techniques, a mild humoral response has been detected in many patients receiving drugs transformed into reactive metabolites, even if they had no liver abnormalities. Indeed, anti-diclofenac-protein adduct antibodies were found in 60% of patients treated with this drug without any liver test abnormalities (Aithal et al. 2004). Related findings have been observed with halothane, which is transformed by CYP2E1 into a chemically reactive metabolite that covalently modifies the CYP2E1 protein. Anti-CYP 2E1 autoantibodies were found in all 106 pediatric anesthesiologists who were occupationally exposed to halothane, although only one

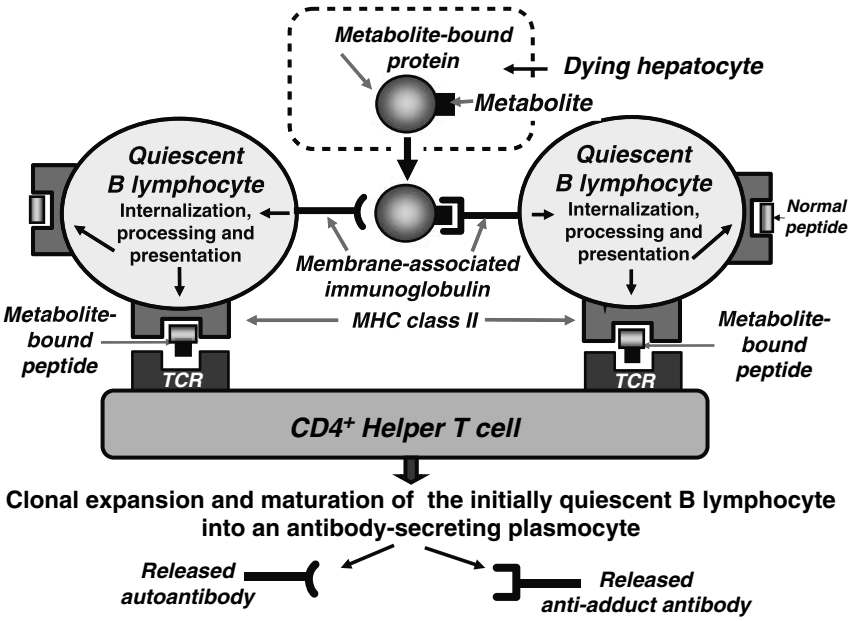

Fig. 8 Suggested mechanism for the humoral immune responses triggered by reactive metabolites. The covalent binding of reactive metabolites modifies hepatic proteins. After the death of a hepatocyte, a haptenized hepatic protein may be taken up by a quiescent B lymphocyte expressing a membrane immunoglobulin able to bind the normal protein. The haptenized protein may also be taken up by another quiescent B lymphocyte with a membrane immunoglobulin able to bind the metabolite-protein adduct. Internalization of the protein and its processing into peptides can lead to the presentation of both normal peptides and haptenized peptides on the major histocompatibility complex (MHC) class II molecules of the B cell. Unlike normal peptides, metabolite-bound peptides differ from the Self, and can be recognized by the T cell receptor (TCR) of helper T cells. The help provided by helper T cells can then lead to the clonal expansion of the B cells, and their maturation into plasmocytes, which now secrete the immunoglobulin into the blood instead of expressing it on the plasma membrane

of them had liver disease of an unknown etiology (Njoku et al. 2002). These clinical data indicate that a mild infra-pathological humoral immune response may occur frequently but only plays a limited role, if any, in clinical hepatitis.

In contrast, the lymphocyte proliferation test was reported to be frequently positive in patients with drug-induced liver injury but constantly negative in patients receiving the same drugs without liver injury (Maria et al. 1994). This suggests that the cellular immune response probably plays a major role in the immune destruction of hepatocytes.

How T lymphocytes are stimulated during drug-induced hepatitis is not completely understood, although the process probably begins with the phagocytosis of modified hepatic proteins by antigen-presenting cells, leading to activation of helper T cells, which then stimulate cytotoxic T lymphocytes recognizing modified peptides on the surface of hepatocytes (Fig. 9) (Pessayre and Larrey 2007).

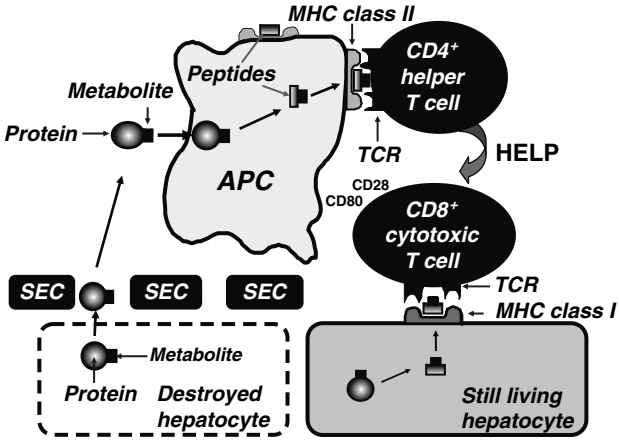

Fig. 9 Possible mechanisms for cell-mediated immune responses triggered by reactive metabolites. The covalent binding of the reactive metabolite modifies hepatic proteins. The death or alteration of one hepatocyte due to mild direct toxicity may lead to the extrusion of a modified protein through the fenestrae of sinusoidal endothelial cells (SEC). The uptake of this haptenized protein by an antigen-presenting cell (APC), such as a Kupffer cell, may lead to the presentation of a metabolite-bound peptide on a major histocompatibility (MHC) class II molecule of the APC, and the recognition of this modified peptide by the T cell receptor (TCR) of a helper T cell. The latter may then provide help to a cytotoxic T lymphocyte recognizing modified peptides presented on the MHC class I molecules of hepatocytes

Cytotoxic T lymphocytes use four main mechanisms that all target the mitochondria to kill target cells (Fig. 10). They express Fas ligand on their surface; they express tumor necrosis factor-α (TNF-α) on their surface and release it at contact sites; they express TNF-α-related apoptosis-inducing ligand (TRAIL); and finally, they release granzyme B and perforin. The latter creates holes in the plasma membrane as well as in the membrane of endocytic vesicles allowing granzyme B to enter into the cytoplasm.

The interaction of Fas ligand, TNF-α, or TRAIL with their cognate receptors activates caspase-8, which cuts the BH3-interacting domain death agonist (Bid) into truncated Bid (tBid) (Fig. 10). Granzyme B also cuts Bid into tBid, and cleaves the antiapoptotic protein myeloid cell leukemia-1 (Mcl-1), liberating Bcl-2 interacting mediator of cell death (Bim) from its sequestering interaction with Mcl-1 (Fig. 10). Bim and tBid cause a conformational change in Bax, which migrates and inserts into the mitochondrial outer membrane forming large Bax aggregates that permeabilize the outer mitochondrial membrane and allow cytochrome *c* and other proapoptotic proteins to escape from the intermembrane space of the mitochondria into the cytosol (Fig. 10).

The loss of cytochrome *c* partly blocks electron flow between complex III and complex IV of the mitochondrial respiratory chain, thus causing the accumulation of electrons within complex I and complex III. The accumulated electrons react with oxygen to form the superoxide anion radical and other ROS, triggering the

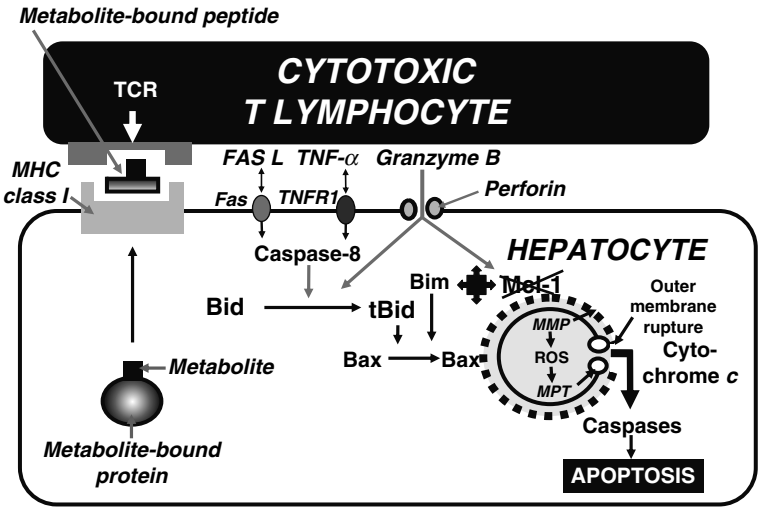

Fig. 10 Cytotoxic T lymphocytes kill target hepatocytes by permeabilizing their mitochondria. The covalent binding of the reactive metabolite to hepatic proteins may lead to the presentation of metabolite-bound peptides on major histocompatibility (MHC) class I molecules on the surface of hepatocytes. These modified peptides may be recognized by the T cell receptor (TCR) of cytotoxic T lymphocytes, which kill target cells by expressing Fas ligand (Fas L) and tumor necrosis factor-α (TNF-(α) and by releasing granzyme B and perforin at contact sites. Fas L and TNF-α bind to Fas and the TNF-α receptor 1 (TNFR1), respectively to activate caspase-8, which cuts BH3-interacting domain death agonist (Bid) into truncated Bid (tBid) that causes a conformational change in Bcl-2-associated x (Bax). Perforin makes holes in the plasma membrane, allowing the entry of granzyme B, which cuts Bid and also cuts myeloid cell leukemia-1 (Mcl-1) thus releasing Bcl-2-interacting mediator of cell death (Bim), which also activates Bax. Activated Bax translocates to the mitochondria to trigger mitochondrial membrane permeabilization (MMP), increased formation of reactive oxygen species (ROS), mitochondrial permeability transition (MPT), outer mitochondrial rupture, cytochrome *c* release, caspase activation and apoptosis

opening of the MPT pore in some mitochondria and causing outer membrane rupture. This rupture further releases cytochrome *c*, which activates caspases in the cytosol to trigger apoptosis (Fig. 10) (Feldmann et al. 2000).

4.2 Parent Drug-Mediated Mitochondrial Membrane Permeabilization or Rupture

4.2.1 Anionic Uncouplers

The flow of electrons along the respiratory chain is coupled with the extrusion of protons from the mitochondrial matrix into the intermembrane space. Once a high membrane potential is achieved, this high-energy state slows both the flow of electrons in the respiratory chain and the associated oxygen consumption.

Carboxylic and other acidic compounds including several nonsteroidal antiinflammatory drugs (NSAIDs) can have a protonophoric effect in mitochondria (Fig. 11). In the acidic intermembrane space, these drugs are present as uncharged species (e.g., R-COOH), which can easily cross the inner mitochondrial membrane. Once the uncharged molecule is in the more alkaline matrix, it dissociates into the anionic form ($R\text{-}COO^-$) and a proton. The mitochondrial membrane potential then drives the translocation of the anionic form back into the intermembrane space through anion transporters of the inner membrane (Wieckowski and Wojtczak 1997). The repetition of these back-and-forth movements translocates protons across the inner membrane and decreases the mitochondrial membrane potential, unleashing the flow of electrons in the respiratory chain, and increasing oxygen consumption (Fig. 11). However, the reentry of protons into the matrix bypasses ATP synthase so the increased respiration produces heat instead of ATP. Severe uncoupling can therefore decrease cell ATP.

Mild ATP depletion due to anionic uncouplers can be further aggravated by MPT. This has been shown with carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone (Costantini et al. 1996; Catisti and Vercesi 1999), diclofenac (Masubuchi et al. 2002), and nimesulide, at least when the latter is incubated without albumin, which otherwise sequesters nimesulide in the medium and prevents cellular toxicity (Berson et al. 2006). Likewise, the anionic uncouplers salicylic acid and valproic acid facilitate MPT in isolated mitochondria incubated with calcium (Trost and Lemasters 1996).

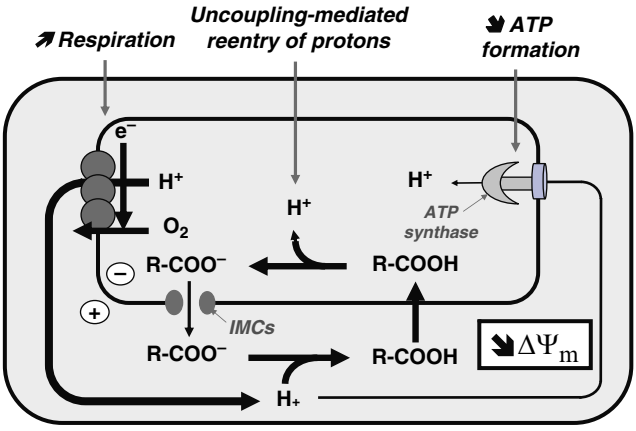

Fig. 11 Anionic uncouplers increase mitochondrial respiration, but decrease ATP formation. Anionic uncouplers, such as drugs with a carboxylic group (R-COOH) can translocate protons across the inner membrane, and may then dissociate into a proton (H^+) and the anionic form ($R\text{-}COO^-$) in the more alkaline matrix. The negatively charged $R\text{-}COO^-$ is then pushed back through diverse inner membrane carriers (IMCs) into the intermembrane space by the mitochondrial membrane potential ($\Delta\psi_m$), ready for another cycle of proton translocation. The reentry of protons into the mitochondrial matrix decreases the $\Delta\psi_m$, thus unleashing the flow of electrons in the respiratory chain and increasing mitochondrial respiration. However, ATP synthase is bypassed, so that the increased respiration produces heat instead of ATP

To understand how anionic uncouplers can trigger MPT it should be remembered that the superoxide anion formed by the respiratory chain is detoxified by the successive action of manganese superoxide dismutase, glutathione peroxidase 1, glutathione reductase, and NADP transhydrogenase (Fig. 2). The latter enzyme consumes both NADH (which is oxidized to NAD^+) and the mitochondrial membrane potential (which partially decreases) to reduce $NADP^+$ into NADPH (Fig. 2). The latter is needed to regenerate GSH from GSSG for the detoxification of H_2O_2 into water by glutathione peroxidase 1 (Fig. 2).

Uncouplers can decrease mitochondrial NADP transhydrogenase activity by decreasing the mitochondrial membrane potential and increasing mitochondrial respiration and the associated consumption of NADH, thus depleting NADH (Fig. 12) (Berson et al. 2006). Impaired NADP transhydrogenase activity may retro-inhibit the regeneration of GSH from GSSG and the detoxification of hydrogen peroxide. The resulting increases in the GSSG/GSH ratio and ROS can then trigger MPT (Fig. 12) (Berson et al. 2006).

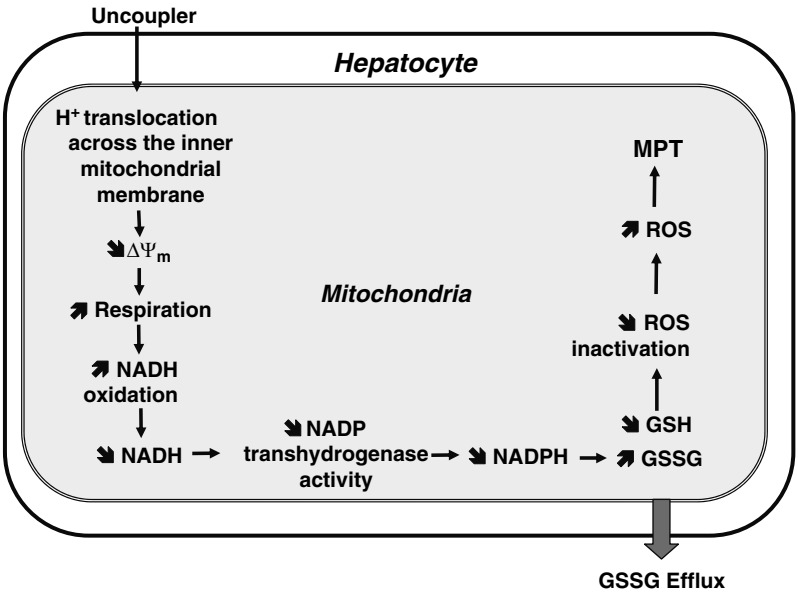

Fig. 12 Anionic uncouplers can trigger mitochondrial permeability transition (MPT). Uncouplers translocate protons into the mitochondrial matrix, thus decreasing the mitochondrial membrane potential ($\Delta\psi_m$). The decreased $\Delta\psi_m$ unleashes the flow of electrons in the respiratory chain, which increases mitochondrial respiration and the reoxidation of NADH, thus decreasing NADH. Decreases in both $\Delta\psi_m$ and NADH may decrease the activity of NADP transhydrogenase, thus causing NADPH depletion and decreased NADPH-supported reduction of GSSH into GSH. The increased cellular levels of GSSG lead to its extrusion from the cell, which further decreases cell GSH. Decreased GSH levels decrease the inactivation of reactive oxygen species (ROS) and increase their concentrations. Both the increase in ROS and the increase in the GSSG/GSH ratio may trigger MPT in some mitochondria

4.2.2 Peripheral Benzodiazepine Receptor Ligands

The peripheral benzodiazepine receptor (PBR) is located on the outer mitochondrial membrane and interacts with the MPT pore. PBR ligands have been shown to either inhibit or enhance MPT and apoptosis in different circumstances. At low concentrations, the PBR ligand alpidem was not toxic alone in hepatocytes but increased TNF-α-mediated toxicity (Berson et al. 2001a). Although PBR ligands were not toxic alone in fibroblasts, they increased TNF-α-mediated MPT and cell death (Pastorino et al. 1996). In hepatic stellate cells, 4′-chlorodiazepam and another selective PBR ligand decreased the mitochondrial membrane potential and triggered apoptosis (Fisher et al. 2001).

4.2.3 Troglitazone

Troglitazone, an agonist of peroxisome proliferator-associated receptor-γ, was taken off the market because of potential hepatotoxicity. One possible mechanism is CYP-mediated metabolic activation (Kassahun et al. 2001) while another involves mitochondrial proapoptotic effects. Indeed, troglitazone triggers c-Jun N-terminal protein kinase (JNK) activation, Bid truncation, MPT, mitochondrial membrane potential collapse, mitochondrial cytochrome *c* release, ROS formation, and apoptosis in liver cells (Bae and Song 2003; Shishido et al. 2003). These effects occur in a human hepatic cell line with negligible CYP expression (Bae and Song 2003), thus excluding a role for metabolic activation in these mitochondrial proapoptotic effects.

4.2.4 Other Drugs Causing Outer Membrane Permeabilization or Rupture

Several other parent compounds can trigger mitochondrial membrane permeabilization. Some of them, such as the anticancer drugs betulinic acid, lonidamine, and arsenite, directly trigger mitochondrial membrane permeabilization/disruption (Fulda et al. 1998; Ravagnan et al. 1999; Belzacq et al. 2001). Other compounds first act on another organelle, which then sends signals that activate Bax to target the mitochondria.

Nuclear DNA damage. Topoisomerase inhibitors and other DNA-damaging anticancer drugs first damage nuclear DNA, thus stabilizing p53. The latter can trigger mitochondrial membrane permeabilization by translocating to the mitochondria and by inducing proapoptotic genes (Erster et al. 2004).

Lysosomal permeabilization. The lysosomotropic drugs, ciprofloxacin, hydroxychloroquine, and chloroquine (Boya et al. 2003a,b; Zhao et al. 2005), the topoisomerase inhibitor, camptothecin (Paquet et al. 2005), and the microtubule stabilizing drug, paclitaxel (Bröcker et al. 2004), can permeabilize the lysosomal membrane. This releases cathepsins, which activate Bax that target the mitochondria.

Endoplasmic reticulum stress. Alcohol, DNA-damaging drugs, NSAIDs, and antihuman immunodeficiency virus (HIV) protease inhibitors can trigger endoplasmic reticulum stress (Ji et al. 2005; Hägg et al. 2004; Tsutsumi et al. 2004; Zhou

et al. 2006). This stress induces CHOP, which leads to the activation of Bax and JNK, which phosphorylates and activates Bax (Oyadomari and Mori 2004; Urano et al. 2000). Severe ER stress can also lead to calcium release from the endoplasmic reticulum, thus increasing cytosolic calcium, which enters the mitochondria to trigger MPT (Deniaud et al. 2008).

Multiple initial targets. Finally, it should be remembered that there is extensive cross-talk between these diverse organelles. For example, p53 and p73 induce scotin, which triggers ER stress (Terrinoni et al. 2004). Activated Bax permeabilizes both the mitochondrial outer membrane and the lysosomal membrane, thus releasing cathepsins, which further activate Bax (Kågedal et al. 2005; Feldstein et al. 2006). These diverse connections may explain why DNA-damaging drugs can trigger DNA damage and p53 stabilization as well as ER stress and lysosomal permeabilization, which can all play a role in mitochondrial membrane permeabilization (Hägg et al. 2004; Paquet et al. 2005).

4.3 Degradation of Mitochondrial DNA

4.3.1 Alcohol

As the most frequently consumed and abused drug, alcohol must be discussed in this chapter. Alcohol abuse increases cellular ROS through diverse mechanisms. First, the metabolism of ethanol to acetaldehyde and acetate transforms NAD^+ into NADH, thus increasing the NADH/NAD^+ ratio. This increases the NADPH/$NADP^+$ ratio, causing the reduction of ferric iron into ferrous iron, a potent generator of the hydroxyl radical (Kukielka and Cederbaum 1989). Ethanol also increases the mitochondrial formation of ROS (Kukielka et al. 1994) and decreases mitochondrial ROS detoxification by decreasing both mitochondrial glutathione import (Fernandez-Checa et al. 1991) and glutathione peroxidase activity (Polavarapu et al. 1988). Moreover, ethanol stabilizes the ROS-generating CYP2E1 (Dai et al. 1993) and can increase the permeability of the gut to endotoxin, which acts on Kupffer cells to activate the ROS-generating NADPH oxidase (Uesugi et al. 2001).

This significant ROS formation damages proteins, lipids, and mtDNA (Nordmann et al. 1992; Lettéron et al. 1993; Wieland and Lauterburg 1995). The intragastric administration of a single, large dose of ethanol (5 $g kg^{-1}$) to mice degrades hepatic mtDNA, which is maximally depleted 2 h after ethanol administration (Mansouri et al. 1999). mtDNA is also depleted in skeletal muscles, heart, and brain (Mansouri et al. 2001). mtDNA depletion can be prevented by 4-methylpyrazole, which blocks ethanol metabolism, or by melatonin, vitamin E, or ubiquinone, three antioxidants (Mansouri et al. 1999, 2001).

After a single alcohol binge, damaged mtDNA molecules are quickly repaired and resynthesized de novo from intact mtDNA templates. mtDNA levels are quickly restored, with even an overshoot phenomenon at 24 h (Mansouri et al. 1999). In contrast, after 4 days of a once-a-day binge, the accumulation of

nonrepaired, bulky lesions (possibly due to lipid peroxidation products) on mtDNA limits the number of intact mtDNA templates and impairs the re-synthesis of mtDNA (Demeilliers et al. 2002). mtDNA depletion lasts for several days after alcohol deprivation (Demeilliers et al. 2002). Repeated mtDNA strand breaks during chronic alcoholism in humans can result in diverse mtDNA deletions (Fromenty et al. 1995; Mansouri et al. 1997; Tsuchishima et al. 2000).

Alcohol-induced mtDNA lesions contribute to the impairment of mitochondrial function and hepatic steatosis after repeated binges in mice (Demeilliers et al. 2002). However, other steatogenic mechanisms are also involved. Firstly, alcohol-generated ROS directly damage mitochondrial proteins and lipids (Nordmann et al. 1992; Lettéron et al. 1993; Wieland and Lauterburg 1995). Secondly, excessive reduction of NAD^+ into NADH during ethanol metabolism can lower NAD^+, whose decreased availability then slows down mitochondrial ß-oxidation and the tricarboxylic acid cycle (Grunnet and Kondrup 1986). Thirdly, in animals receiving alcohol, there is increased hepatic expression of sterol regulatory element-binding protein-1 and increased hepatic fatty acid synthesis (You et al. 2002). Finally, decreased hepatic expression of MTP may relatively blunt the expected adaptive increase in hepatic lipoprotein secretion in a fat-laden liver (Lin et al. 1997).

4.3.2 Acetaminophen

The inadvertent or deliberate ingestion of a large dose of acetaminophen (paracetamol) leads to extensive *N*-acetyl-*p*-benzoquinone-imine formation. This electrophilic metabolite depletes hepatic glutathione and protein thiols, increases cell calcium, damages mitochondria, increases the formation of ROS, induces the expression of inducible NO synthase (thus increasing peroxynitrite formation), and activates c-Jun N-terminal kinase, to finally trigger MPT and liver cell necrosis (Bajt et al. 2003; Masubuchi et al. 2005; Reid et al. 2005; Gunawan et al. 2006). Hepatic mtDNA is rapidly depleted after an acetaminophen overdose in mice, possibly due to the mtDNA damage caused by peroxynitrite and other ROS (Cover et al. 2005).

4.4 Decreased Replication of Mitochondrial DNA

4.4.1 Dideoxynucleosides and Abacavir

Several 2′,3′-dideoxynucleosides are given to patients with HIV. These analogs include 3′-azido-2′,3′-dideoxythymidine (zidovudine, AZT), 2′,3′-dideoxycytidine (zalcitabine, ddC), 2′,3′-dideoxyinosine (didanosine, ddI), 2′,3′-didehydro-3′-deoxythymidine (stavudine, d4T), and (-)-2′-deoxy-3′-thiacytidine (lamivudine, 3TC). A related molecule is abacavir (ABC), which contains a cyclopentene-methanol moiety instead of the dideoxyribose moiety of the above-mentioned

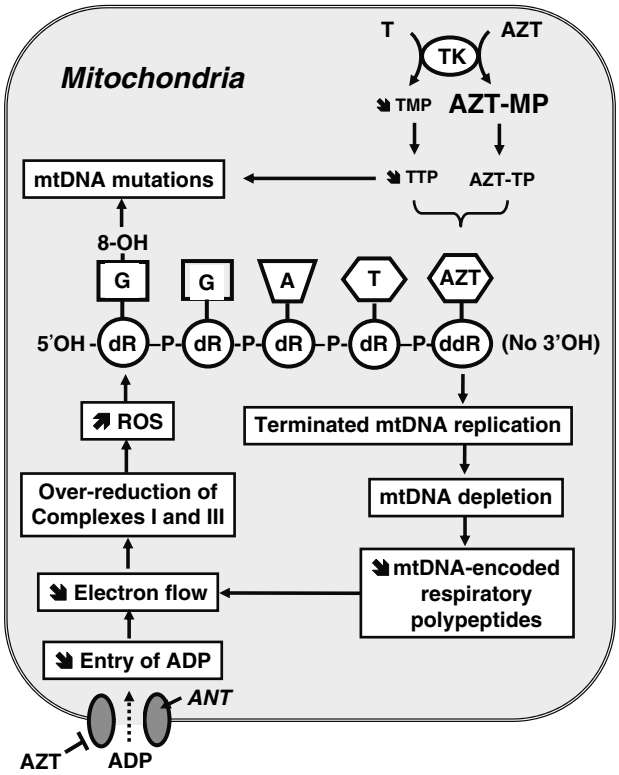

Fig. 13 Possible mitochondrial effects of zidovudine (AZT). AZT competes with thymidine (T) for phosphorylation by thymidine kinase (TK). A high formation rate of AZT monophosphate (AZT-MP) may decrease the formation of TMP and then TTP. The decreased TTP together with the formation of some AZT triphosphate (AZT-TP) can lead to the incorporation of an AZT pseudo-nucleotide into a growing chain of DNA. The DNA now lacks a 3′-hydroxyl group, because the dideoxyribose (ddR) present in AZT, unlike the normal deoxyribose (dR), has no 3′-hydroxyl group. Unless the AZT pseudo-nucleotide can be removed by the proofreading activity of polymerase γ, no other nucleotide can be incorporated. This may decrease mtDNA replication and lead to mtDNA depletion, which can decrease the synthesis of mtDNA-encoded respiratory polypeptides. Concomitantly, AZT may inhibit the adenine nucleotide translocator (ANT), thus decreasing the entry of ADP into the mitochondria. Together with the decreased levels of respiratory polypeptides, the decreased ADP may slow down the flow of electrons along the respiratory chain, thus causing over-reduction of respiratory complexes and increased formation of reactive oxygen species (ROS) that can damage mtDNA bases. The formation of 8-hydroxy-deoxyguanosine (8-OH-G-dR), together with the imbalance in the deoxyribonucleoside-triphosphate pools may finally cause mtDNA mutations

drugs. These analogs can impair mtDNA replication and/or integrity through several mechanisms (Fig. 13).

Chain termination. Because the 5′-hydroxyl group of deoxyribose is present in these analogs, the triphosphate derivative can be formed and the analog can be incorporated into a growing chain of DNA. However, the normal 3′-hydroxyl group

of deoxyribose is absent. If a single molecule of the analog is incorporated, the DNA molecule lacks a 3′-hydroxyl group and no other nucleotide can be incorporated (Fig. 13) (Mitsuya and Broder 1986; Yarchoan et al. 1989). Thus, the effects of these compounds depend on the capability of diverse polymerases to incorporate the analogs into DNA. HIV reverse transcriptase achieves this, thus impairing reverse transcription of HIV RNA (Mitsuya and Broder 1986). In contrast, DNA polymerases acting in the nucleus barely incorporate the analogs into nuclear DNA, which is why these drugs can be used for treatment (Yarchoan et al. 1989). However, DNA polymerase γ incorporates the nucleoside analog into mtDNA. Unless the nucleotide analog can be removed by the proofreading 3′-5′-exonuclease activity of polymerase γ (Johnson et al. 2001), its presence at the end of DNA terminates mtDNA replication (Fig. 13) (Chen and Cheng 1989; Lewis and Dalakas 1995). Marked slowing of mtDNA replication can result in a progressive decrease in mtDNA levels (Fig. 13). For reasons that are not yet fully understood, different dideoxynucleosides tend to have differential effects on mtDNA in various organs. Although AZT occasionally depletes hepatic mtDNA (Chariot et al. 2000), the so-called "D-drugs", namely ddC ddI, and d4T, seem to be more likely to deplete hepatic mtDNA than AZT, 3TC, or ABC (Walker et al. 2004). mtDNA depletion in turn decreases mitochondrial respiratory complex expression, except complex II, which is only encoded by nuclear DNA (Brivet et al. 1999).

Thymidine triphosphate (TTP) depletion. AZT and thymidine (T) compete with each other for phosphorylation by thymidine kinase into AZT-monophosphate (AZT-MP) and thymidine-monophosphate (TMP), respectively (Fig. 13) (Lynx and McKee 2006). AZT can therefore decrease the formation of TMP and TTP, whose relative deficiency can then slow mtDNA replication (Lynx and McKee 2006). Interestingly the administration of uridine in animals and perhaps also in humans can prevent AZT, ddC, and d4T toxicity (Walker and Venhoff 2005; Banasch et al. 2006). Uridine administration may provide an alternate route for TTP synthesis, thus preventing TTP depletion and the impairment of mtDNA replication (Lynx and McKee 2006). Furthermore, the uridine-induced restoration of mtDNA levels and respiratory chain function could improve the activity of dihydroorotate dehydrogenase, a key mitochondrial enzyme involved in pyrimidine synthesis. Thus, a virtuous circle is initiated by uridine supplementation (Setzer et al. 2008).

ROS formation. The impaired synthesis of mtDNA-encoded respiratory chain polypeptides can partially block the flow of electrons in the respiratory chain (Lewis et al. 2001) so that they accumulate in complex I and complex III where they react with oxygen to form the superoxide anion radical. With AZT, this effect is further aggravated by the inhibitory effect of AZT on the ADP/ATP translocator (ANT) (Fig. 13) (Barile et al. 1997). The inhibition or knockout of the ANT prevents the exchange of mitochondrial ATP for cytosolic ADP (Esposito et al. 1999). Because the entry of ADP into the mitochondrial matrix is impaired, protons cannot reenter through ATP synthase, causing a high mitochondrial potential (Esposito et al. 1999). This blocks the flow of electrons in the respiratory chain and causes the overreduction of respiratory chain complexes increasing mitochondrial ROS formation and triggering mtDNA deletions (Esposito et al. 1999).

Indeed, AZT administration increased the urinary excretion of 8-hydroxydeoxyguanosine in patients (de la Asuncion et al. 1998), and increased peroxide formation by hepatic mitochondria and the amount of 8-hydroxydeoxyguanosine present in mouse liver mtDNA (de la Asuncion et al. 1999).

mtDNA mutations. Both oxidative damage to mtDNA and imbalances in deoxyribonucleotide pools cause occasional mtDNA point mutations and mtDNA deletions (Fig. 13) (Song et al. 2003). In patients receiving nucleoside reverse transcriptase inhibitors, heteroplasmic point mutations and deletions can accumulate in mtDNA (Martin et al. 2003; Bartley et al. 2001). Once these develop, mitochondrial dysfunction can occur even though the total level of mtDNA (normal and mutated) remains normal (Bartley et al. 2001).

Insufficient stimulation of mitochondrial biogenesis. When mtDNA is decreased, damaged, or mutated, there is a compensatory increase in mitochondrial biogenesis in certain tissues. In human volunteers treated for 2 weeks with d4T/3TC or AZT/3TC, a decrease in mtDNA-encoded messenger RNAs was associated with increased expression of the peroxisome proliferator receptor gamma coactivator 1 (PGC-1), nuclear respiratory factor 1, and mitochondrial transcription factor A in adipose tissue (Mallon et al. 2005). All three factors are master regulators of mitochondrial biogenesis in adipocytes and myocytes, which may help limit the adverse effects of nucleoside reverse transcriptase inhibitors. Indeed, an increase in the number of muscle mitochondria is often observed in patients with AZT-induced myopathy (Lewis and Dalakas 1995). An adaptive increase in mitochondrial biogenesis can also occur in the liver (Mandel et al. 2001; Kamal and French 2004). However, there is usually less mitochondrial proliferation in the liver than in muscles.

4.4.2 Other Nucleoside Analogs

Fialuridine. This compound was being developed for the treatment of chronic hepatitis B, but clinical trials were interrupted because several patients developed microvesicular hepatic steatosis and severe lactic acidosis associated with pancreatitis, neuropathy, or myopathy in some cases (McKenzie et al. 1995). Fialuridine possesses both a 5′-hydroxyl group and a 3′-hydroxyl group, so that the incorporation of a single molecule of fialuridine into DNA should not immediately terminate mtDNA replication. However, when several adjacent molecules of fialuridine are successively incorporated, they decrease DNA polymerase γ activity, mtDNA replication, and mtDNA levels (Lewis et al. 1996b).

Ganciclovir. The nucleoside analog ganciclovir is mainly used in the treatment of cytomegalovirus infections (Thust et al. 2000). The viral kinase converts ganciclovir into ganciclovir-monophosphate, which is then activated by cellular kinases into ganciclovir-triphosphate, thus leading to the possible incorporation of ganciclovir nucleotides into growing chains of DNA (Thust et al. 2000). Ganciclovir is incorporated into mtDNA and can trigger mtDNA depletion, ultrastructural mitochondrial lesions, steatosis, and apoptosis (Herraiz et al. 2003). The acyclic, pseudosugar analog of ganciclovir has two hydroxyl groups, so the incorporation of a

ganciclovir nucleotide into DNA does not terminate DNA replication. However, the incorporated ganciclovir molecules may distort the DNA helix and block the next round of DNA replication when the ganciclovir-modified DNA strand serves as a replication template (Thust et al. 2000).

4.4.3 Drugs Inhibiting Topoisomerases and/or Binding to DNA

Drugs that intercalate between DNA bases or bind strongly to a DNA groove can directly inhibit DNA replication (Rowe et al. 2001). Intercalating drugs can also inhibit and/or poison DNA topoisomerases, further impairing DNA replication (Rowe et al. 2001). Topoisomerases play an important role in DNA replication and transcription (Schneider et al. 1990). Topoisomerases transiently cut the phosphodiester backbone of DNA by forming a covalent bond between the liberated phosphorus of DNA and a tyrosine of the enzyme. Topoisomerases then reseal the DNA gap. Although a few inhibitors prevent topoisomerases from cutting DNA initially, most topoisomerase-interfering drugs inhibit DNA resealing. These topoisomerase "poisons" increase the number of enzyme-bound DNA complexes.

Both the inhibition and "poisoning" of topoisomerases are deleterious to cells. The collision of a transcription complex or a replication fork against a topoisomerase-associated DNA break interrupts RNA or DNA synthesis, and can lead to real (nontopoisomerase-bound) double-strand breaks and to gene translocations, which can trigger apoptosis and/or cancer (Li and Liu 2001).

Mitochondria contain both a type I topoisomerase (Zhang et al. 2001) and a bacterial-like type II topoisomerase (Lin and Castora 1991). Topoisomerase inhibitors or poisons can therefore affect the replication of mtDNA. Indeed, mtDNA rather than nuclear DNA is selectively targeted with certain drugs that are electrophoretically concentrated in the mitochondrial matrix.

Tacrine. The reversible cholinesterase inhibitor, tacrine has been given to patients with Alzheimer's disease although it increased ALT activity in 50% of recipients (Watkins et al. 1994). Because of the first-pass metabolism of tacrine in the liver, other organs are less exposed and the liver is selectively injured (Berson et al. 1996). Tacrine, being a weak base, is taken up and concentrated within mitochondria (Berson et al. 1996), thus selectively targeting mtDNA (but not nuclear DNA) (Fig. 14) (Mansouri et al. 2003). Tacrine intercalates between mtDNA bases, poisons topoisomerases, and decreases mtDNA synthesis in mice (Mansouri et al. 2003). This leads to progressive depletion of hepatic mtDNA in mice, eventually followed by the death of a few hepatocytes by necrosis or apoptosis (Mansouri et al. 2003).

Tamoxifen. The antiestrogenic drug, tamoxifen, is used in the treatment of advanced breast cancer, although it frequently causes steatosis in overweight women (Bruno et al. 2005). This cationic amphiphilic drug accumulates electrophoretically in mitochondria, where it directly inhibits mitochondrial respiration and mitochondrial β-oxidation, thus causing steatosis (Larosche et al. 2007).

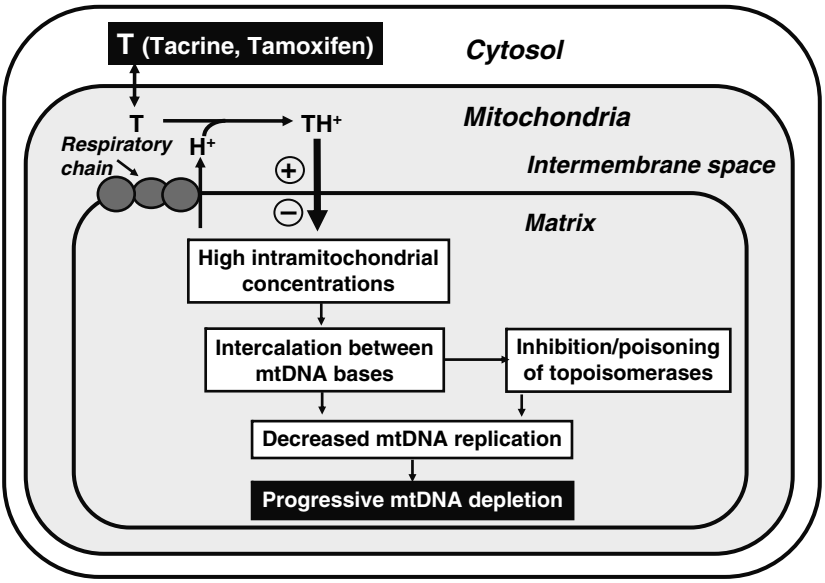

Fig. 14 Effects of tacrine and tamoxifen on mtDNA. The weak bases tacrine and tamoxifen are protonated in the acidic intermembrane space of mitochondria and electrophoretically concentrated into the mitochondrial matrix. At these high concentrations, they significantly intercalate between DNA bases, thus inhibiting mtDNA replication both directly and by inhibiting topoisomerases. The decreased synthesis of mtDNA can lead to progressive mtDNA depletion

In addition, tamoxifen intercalates between DNA bases, inhibits topoisomerases, and decreases mtDNA synthesis, leading to progressive hepatic mtDNA depletion in mice (Fig. 14) (Larosche et al. 2007).

Ciprofloxacin and nalidixic acid. The 4-quinolone antibiotics, ciprofloxacin and nalidixic acid, inhibit gyrase (a bacterial type II topoisomerase) and the type II topoisomerase present in mitochondria (Lawrence et al. 1993, 1996). In cultured cells, nalidixic acid and ciprofloxacin progressively decrease mtDNA and impair mitochondrial respiration and cell growth (Lawrence et al. 1993). However, it is unknown whether the marked hepatotoxicity of ciprofloxacin in humans is due to mtDNA depletion or to another mechanism, for example, altered expression of mitochondrial proteins (Liguori et al. 2005), lysosomal membrane permeabilization leading to MPT (Boya et al. 2003a), or the metabolic activation of ciprofloxacin to reactive metabolites (Sun et al. 2008) possibly leading to immune reactions (Lazarczyk et al. 2001).

Pentamidine. Pentamidine prevents and treats *Pneumocystis carinii* infections, and it may inhibit mitochondrial translation (Zhang et al. 2000). It also binds to the minor groove of duplex DNA and can deplete mtDNA in cultured mammalian cells (Rowe et al. 2001).

Polyamine analogs. Methylglyoxal bis(guanine hydrazone) and several polyamine analogs also progressively deplete mtDNA in cultured cell lines (Nass 1984; Bergeron et al. 1988). The polycationic polyamines accumulate electrophoretically in the mitochondrial matrix, where they strongly interact with the phosphate groups on the major groove of DNA causing conformational changes (Rowe et al. 2001).

Ethidium bromide and related compounds. Ethidium bromide, ditercalinium, and dequalinium are also cationic drugs which concentrate electrophoretically into the mitochondrial matrix, where they intercalate between mtDNA bases, inhibit mtDNA synthesis, and progressively deplete mtDNA (Wiseman and Attardi 1978; Esnault et al. 1990; Schneider Berlin et al. 1998).

1-Methyl-4-phenylpyridinium. A Parkinson's disease-causing toxin is oxidized to the positively charged, 1-methyl-4-phenylpyridinium (MPP^+), which accumulates in mitochondria (Miyako et al. 1997). MPP^+ destabilizes the D loop (Iwaasa et al. 2002), a triple-stranded mtDNA structure that plays a key role in transcription and replication, and it inhibits mtDNA synthesis thus causing mtDNA depletion (Miyako et al. 1997).

4.5 Decreased Synthesis and Stability of Mitochondrial Transcripts

Interferon-α. Interferon-α is used to treat chronic viral hepatitis and some forms of cancer. Interferons induce 2′,5′-oligoadenylate synthases, which, in the presence of double-stranded RNAs, synthesize 2′,5′-oligoadenylates from ATP (Samuel 2001). These 2′,5′-oligoadenylates activate RNase L which is also induced by interferon-α and -β (Samuel 2001). The activation and induction of RNase L by interferon can affect mitochondrial transcripts in two ways (Fig. 15). First, RNAse L may cleave the nuclear DNA-encoded mRNA of mitochondrial transcription factor A (mtTFA or TFAM) (Inagaki et al. 1997). This factor is imported into the mitochondrial matrix after being synthesized in the cytoplasm where TFAM binds to enhancer sequences on mtDNA to increase mtDNA transcription. Thus, by decreasing TFAM, RNase L decreases mitochondrial mRNA synthesis (Shan et al. 1990). Second, RNase L, also present inside the mitochondria, is targeted to the mitochondrial 28 S-mRNA complex by binding to the mitochondrial translation initiation factor (IF2mt), which itself may bind to the 28 S subunit (Le Roy et al. 2007). RNase L can then degrade mitochondrial mRNAs (Fig. 15) (Le Roy et al. 2001). Overall interferon-α decreases both the synthesis and the stability of mitochondrial transcripts (Shan et al. 1990; Le Roy et al. 2001). In cultured cells, these dual effects may decrease mtDNA-encoded respiratory chain polypeptides and mitochondrial respiration (Lewis et al. 1996a). Although it is not known if these effects also occur in treated patients, several of the adverse effects of interferon-α, including minor blood dyscrasia, myalgia, paresthesia, convulsions, depression (Okanoue et al. 1996), and hepatic steatosis (Castéra et al. 1999), resemble the clinical manifestations of mild forms of inborn mitochondrial cytopathies.

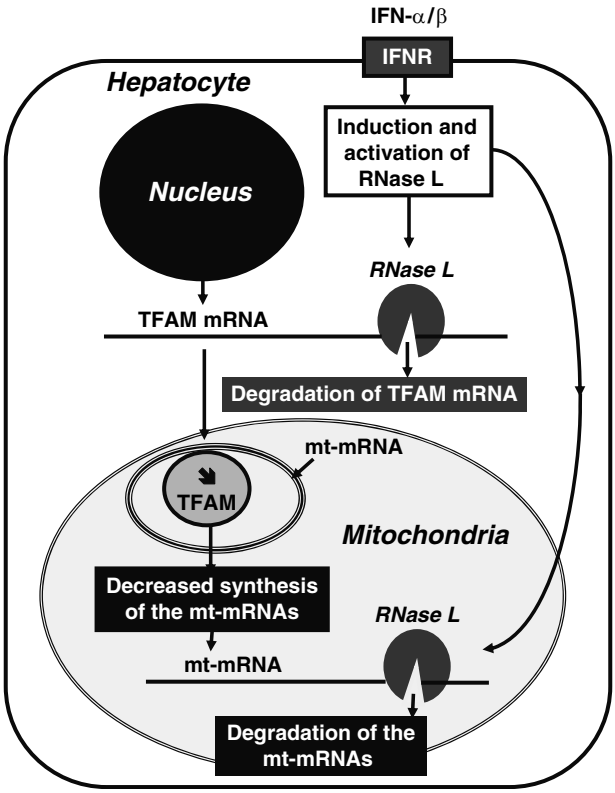

Fig. 15 Effects of interferon-α or -β (IFN-α/β) on mitochondrial transcripts. Through its membrane receptor (IFNR), IFN-α/β both induces and activates RNase L. This endonuclease cleaves the mRNA of mitochondrial transcription factor A (TFAM), thus decreasing the TFAM protein and the synthesis of mitochondrial mRNAs (mt-mRNAs). RNase L is also present within mitochondria, where it cleaves the mt-mRNAs. Therefore, IFN-α/β decreases both the synthesis and the stability of mitochondrial transcripts

4.6 Decreased Protein Synthesis

Several antibiotics that decrease protein synthesis in bacteria also decrease protein synthesis in mitochondria.

Linezolid. The antibiotic linezolid inhibits bacterial protein synthesis and is used against drug-resistant, Gram-positive pathogens (De Vriese et al. 2006). However, linezolid also inhibits mitochondrial protein synthesis, and can decrease the activity of respiratory chain complexes that contain mtDNA-encoded proteins (De Vriese et al. 2006). This drug can trigger lactic acidosis and neuropathy in humans (De Vriese et al. 2006).

Chloramphenicol and thiamphenicol. These two antibiotics bind to a ribosomal subunit to inhibit protein synthesis in both bacteria and mitochondria (Kroon and

de Vries 1969). Mitochondrial dysfunction may be involved in the reversible bone-marrow suppression induced by chloramphenicol (Yunis 1989). However, other adverse effects of chloramphenicol, such as aplastic anemia or cholestatic hepatitis, could be related to reactive metabolite formation instead (Yunis 1989).

Erythromycins. Erythromycins are amphiphilic cationic drugs, which accumulate in acidic compartments, including lysosomes, where they can inhibit phospholipases and cause phospholipidosis (Montenez et al. 1999). Erythromycins bind to the 50 S ribosomal subunit of bacteria to inhibit the transfer of amino acids from the aminoacyl-tRNA to the peptide chain (Oleinick and Corcoran 1969). Erythromycins can also inhibit mitochondrial protein synthesis (Anandatheerthavarada et al. 1999) causing megamitochondria (Karbowski et al. 1999) and sensorineural hearing loss (Ress and Gross 2000). In contrast, hepatitis seems to occur because erythromycins are transformed into reactive metabolites, which may covalently bind to proteins and form neoantigens triggering immunoallergic hepatitis (Danan et al. 1981; Pessayre et al. 1985).

4.7 Inhibition of ATP Synthase

Organotin compounds (Matsuno-Yagi and Hatefi 1993) and several natural toxins such as apoptolidin (Salomon et al. 2001), aurovertin (Ebel and Lardy 1975), citreoviridin (Linnett et al. 1978), efrapeptins (Cross and Kohlbrenner 1978), oligomycin, and venturicidin (Qian et al. 1999), are potent ATP synthase inhibitors. These toxins block aerobic ATP formation by mitochondria and can damage cells that cannot synthesize enough ATP through glycolysis. ATP synthase activity is also inhibited by high, supra-physiological concentrations of estrogens (Zheng and Ramirez 1999), and by high concentrations of several phenolic phytochemicals present in the human diet, such as resveratrol, curcumin, genistein, or quercetin (Zheng and Ramirez 2000).

4.8 Primary Impairment of Both β-Oxidation and Respiration

4.8.1 Amiodarone, 4,4'-Diethylaminoethoxyhexestrol and Perhexiline

Amiodarone, 4,4′-diethylaminoethoxyhexestrol, and perhexiline are cationic amphiphilic drugs. They have both a lipophilic moiety and an amine function, which can become protonated and thus become positively charged. This cationic amphiphilic structure interferes with both lysosomal and mitochondrial function, explaining why the three drugs can cause both phospholipidosis and steatosis or steatohepatitis (Pessayre et al. 2001).

Lysosomes. The uncharged, lipophilic form of these three drugs easily crosses the lysosomal membrane (Kodavanti and Mehendale 1990). The drug is then protonated inside the acidic lysosomal milieu and is trapped there because the

charged species cannot cross back through the lysosomal membrane. Therefore, there are much higher concentrations of the protonated drug inside the lysosomes than in the cytosol. At these very high concentrations, the cationic amphiphilic drug hampers phospholipase action by forming noncovalent but tight complexes with phospholipids (Kodavanti and Mehendale 1990). Phospholipids are not degraded and the phospholipid-drug complexes progressively accumulate in the form of myelin-like figures in grossly enlarged lysosomes (Kodavanti and Mehendale 1990). Although phospholipidosis is frequent in patients receiving these drugs, it may have only limited clinical consequences and often occurs without clinical symptoms or marked biochemical disturbances (Guigui et al. 1988).

Mitochondria. However, these drugs also interfere with mitochondrial function (Fig. 16) (Fromenty et al. 1990a, b, 1993; Deschamps et al. 1994; Berson et al. 1998). The unprotonated, lipophilic form of the drug crosses the mitochondrial outer membrane and is protonated in the acidic intermembrane mitochondrial space. The positively charged, protonated form is electrophoretically "pushed" inside the mitochondrial matrix by the high electrochemical potential existing across the inner mitochondrial membrane. This crossing may occur through the aqueous channels of some inner membrane carrier(s) or, more probably, directly across the lipid bilayer thanks to charge delocalization. Whatever the route, the active electrophoretic uptake of the drug leads to much higher concentrations inside the mitochondria than in the cytosol. At these high concentrations, the drug inhibits

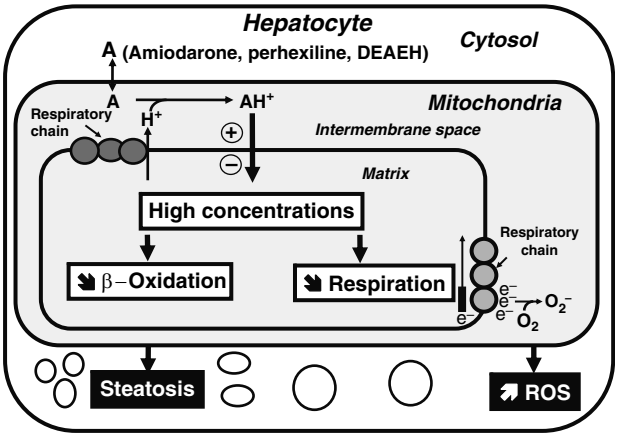

Fig. 16 Effects of amiodarone, perhexiline, and diethylaminoethoxyhexestrol (DEAEH) on mitochondrial function. After crossing the outer membrane, the uncharged secondary or tertiary amine (A) of amiodarone, perhexiline, or diethylaminoethoxyhexestrol (DEAEH) is protonated in the acidic intermembrane space. The positively charged molecule (AH^+) is then electrophoretically "pushed" by the mitochondrial membrane potential into the matrix. High intramitochondrial concentrations inhibit both ß-oxidation (causing steatosis) and oxidative phosphorylation, thus causing the accumulation of electrons in the respiratory chain and increasing the mitochondrial formation of ROS. The latter oxidize fat deposits, causing lipid peroxidation, which, together with ROS-induced cytokine production, could cause steatohepatitis.

mitochondrial fatty acid ß-oxidation causing steatosis, and also partially hampers electron transfer along the respiratory chain (Fromenty et al. 1990a, b, 1993; Deschamps et al. 1994; Berson et al. 1998). Upstream respiratory chain components become overly reduced and directly transfer their electrons to oxygen to form the superoxide anion radical and other ROS (Fig. 16) (Berson et al. 1998).

Other molecular lesions. Increased mitochondrial ROS formation causes lipid peroxidation, which together with cytokines can trigger necroinflammation and fibrosis (Berson et al. 1998). In isolated rat hepatocytes treated with amiodarone, perhexiline, or diethylaminoethoxyhexestrol, calcium-activated transglutaminase polymerizes cytokeratin 8, a process that may initiate the formation of Mallory–Denk bodies (Robin et al. 2008). In addition to initial steatosis, patients may develop Mallory–Denk bodies, necrosis, a mixed inflammatory cell infiltrate, fibrosis, and even cirrhosis (De la Iglesia et al. 1974; Pessayre et al. 1979; Poucell et al. 1984).

4.8.2 Other Drugs Impairing Respiration and β-Oxidation

Buprenorphine. The morphine analog, buprenorphine, is used as a substitution drug in heroin addicts. The sublingual route prevents extensive first pass metabolism of buprenorphine in the liver. At high concentrations, buprenorphine inhibits both mitochondrial ß-oxidation and respiration in rat hepatocyte mitochondria (Berson et al. 2001b). In humans, buprenorphine concentrations are lower, and the drug is usually well tolerated. However, cytolytic hepatitis and steatosis have been observed in a few patients (Berson et al. 2001c). Predisposing factors may include intravenous buprenorphine misuse (resulting in higher concentrations), and concomitant exposure to viruses, ethanol, or other drugs, which, together with buprenorphine, could additively impair mitochondrial function (Berson et al. 2001c).

Antimalarial drugs. Chloroquine and most other antimalarial drugs are cationic compounds, which accumulate in the acidic vacuole of the malaria parasite to alkalinize the vacuole and disrupt its function (Ginsburg and Geary 1987). Chloroquine also accumulates in host lysosomes, which can cause phospholipidosis (Hostetler et al. 1985). Chloroquine, primaquine, and quinine may also interfere with mitochondrial function, as they have been shown to impair respiration in rat liver mitochondria (Katewa and Katyare 2004). Finally, the antimalarial drugs, primaquine (Vásquez-Vivar and Augusto 1992) and amodiaquine (Maggs et al. 1987), form reactive metabolites, which may be involved in amodiaquine-induced agranulocytosis and hepatitis (Larrey et al. 1986).

Tamoxifen. The antiestrogen, tamoxifen, is a cationic amphiphilic drug which is electrophoretically transported into the mitochondrial matrix where it reaches high concentrations that directly inhibit both mitochondrial β-oxidation and mitochondrial respiration, and also deplete mtDNA in mice, as mentioned above (Larosche et al. 2007).

Benzarone and benzbromarone. Despite their structural analogy with amiodarone, benzarone and benzbromarone are not cationic drugs but phenolic compounds. These two drugs uncouple and inhibit respiration at low concentrations (Kaufmann

et al. 2005), and can cause hepatocellular liver injury in humans (Babany et al. 1987; van der Klauw et al. 1994). Although both drugs also impair mitochondrial β-oxidation, this effect requires higher concentrations (Kaufmann et al. 2005) that may not be clinically relevant because steatosis does not seem to be an adverse effect of these two drugs.

4.9 Primary Impairment of β-Oxidation

4.9.1 Valproic Acid

Valproic acid is a branched-chain fatty acid used to prevent seizures. Although valproate administration frequently increases serum ALT activity, it rarely causes a Reye's-like syndrome, which mainly occurs in very young children (Farrell 1994). Valproate-induced liver lesions can be associated with microvesicular steatosis with necrosis and sometimes cirrhosis (Zimmerman and Ishak 1982). These lesions may be related to the diverse effects of valproic acid on mitochondria. Indeed, as an anionic uncoupler, valproic acid can favor MPT and trigger cell death (Fig. 17) (Trost and Lemasters 1996). Furthermore, like all natural medium-chain and short-chain fatty acids, valproic acid can enter the mitochondria. Inside the matrix, extensive formation of valproyl-CoA then sequesters intramitochondrial CoA decreasing the formation of natural fatty acyl-CoA thioesters and their mitochondrial β-oxidation (Fig. 17) (Turnbull et al. 1983; Ponchaut et al. 1992). The lack of CoA may also inhibit pyruvate dehydrogenase (Silva et al. 1997), which requires CoA as a cofactor. Another mechanism for valproate-induced steatosis involves CYPs 2C9 and 2A6. These CYPs dehydrogenate the two outer carbons of valproate to form 4-ene-valproate (Fig. 17) (Sadeque et al. 1997). Inside the mitochondria, 4-ene-valproate is activated into 4-ene-valproyl-CoA and then 2,4-diene-valproyl-CoA (Bjorge and Baillie 1985; Kassahun et al. 1991). The latter is a chemically reactive metabolite that may inactivate β-oxidation enzymes (Kassahun et al. 1991, 1994). The concomitant administration of phenytoin and carbamazepine can induce CYPs and increase the formation of 4-ene-valproate leading to an increase in the hepatotoxicity of valproate (Levy et al. 1990).

4.9.2 Aspirin

Aspirin is hydrolyzed into salicylic acid, which is activated into salicylyl-CoA on the outer mitochondrial membrane (Killenberg et al. 1971). Extensive salicylyl-CoA formation sequesters extramitochondrial CoA so there is not enough CoA to activate long-chain fatty acids, preventing their entry into the mitochondria and β-oxidation (Deschamps et al. 1991). Another effect of salicylate is to uncouple mitochondrial respiration (Deschamps et al. 1991) and favor MPT and cell death (Trost and Lemasters 1996; Oh et al. 2003). The latter effect could be involved in the spotty liver cell death observed in patients receiving high therapeutic doses of

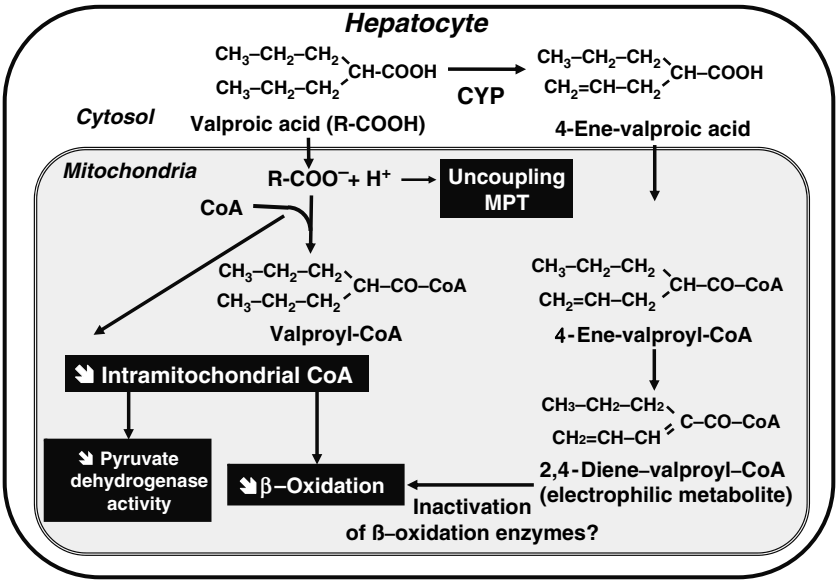

Fig. 17 Mitochondrial effects of valproic acid. Valproic acid freely enters mitochondria, and thus translocates protons into the mitochondrial matrix. This protonophoric effect can slightly uncouple mitochondrial respiration, and can help trigger mitochondrial permeability transition (MPT). Inside the matrix, valproate is extensively transformed into valproyl-CoA, thus sequestering intramitochondrial CoA. The lack of CoA impairs both mitochondrial fatty acid ß-oxidation and pyruvate oxidation. Valproate is also dehydrogenated by cytochrome P450 (CYP) into 4-ene-valproate, which then forms 4-ene-valproyl-CoA and 2,4-diene-valproyl-CoA within mitochondria. The latter is an electrophilic metabolite, which may inactivate ß-oxidation enzymes

aspirin (Zimmerman 1981), and could also contribute to the development of Reye's syndrome, as discussed later.

4.9.3 Tetracyclines

At present oral doses, tetracycline and its derivatives produce mild hepatic steatosis in humans at worst. However, fatal cases of microvesicular steatosis have occurred in the past, usually after 4–10 days of intravenous administration of high doses of either tetracycline or diverse tetracycline derivatives (Zimmerman 1978). Predisposing conditions (Zimmerman 1978) included preexisting renal failure, which decreases tetracycline elimination, or pregnancy, which can impair mitochondrial function.

In experimental animals, tetracycline and the various tetracycline derivatives lead to extensive microvesicular steatosis of the liver (Fréneaux et al. 1988; Labbe et al. 1991). This marked steatogenic effect is due to the dual effects of the tetracyclines, which inhibit both the mitochondrial ß-oxidation of fatty acids (Fréneaux et al. 1988; Labbe et al. 1991) and also MTP activity, thus decreasing the hepatic secretion of very low-density lipoproteins (Lettéron et al. 2003).

4.9.4 Other Drugs That Inhibit β-Oxidation

Glucocorticoids. Glucocorticoids impair mitochondrial β-oxidation by inhibiting acyl-CoA dehydrogenases (Lettéron et al. 1997), and can cause steatosis and even steatohepatitis in humans (Itoh et al. 1977).

Nonsteroidal antiinflammatory drugs. Pirprofen, naproxen, ibuprofen, and ketoprofen can occasionally cause microvesicular steatosis in humans (Bravo et al. 1997; Victorino et al. 1980; Danan et al. 1985; Dutertre et al. 1991). These NSAIDS have a 2-arylpropionate structure, with an asymmetric carbon, and exist as either the S(+)- or the R(−)-enantiomers. Only the S(+)-enantiomer inhibits prostaglandin synthesis, whereas only the R(−)-enantiomer is converted into the acyl-CoA derivative. However, both the S(+)-enantiomer and the R(−)-enantiomer of ibuprofen inhibit the β-oxidation of medium- and short-chain fatty acids (Fréneaux et al. 1990). Pirprofen, tiaprofenic acid, and flurbiprofen also inhibit mitochondrial β-oxidation (Genève et al. 1987a).

Amineptine and tianeptine. The French antidepressant drugs, amineptine and tianeptine, have both a tricyclic moiety and a heptanoic side chain. The tricyclic moiety undergoes metabolic activation by CYPs (Genève et al. 1987b; Larrey et al. 1990), explaining why amineptine and tianeptine cause rare immunoallergic hepatitis. The heptanoic side chain of these two drugs undergoes mitochondrial ß-oxidation, which shortens it to the 5-carbon and 3-carbon derivatives (Sbarra et al. 1981; Grislain et al. 1990). In patients receiving these drugs, mitochondria are thus exposed to C7, C5, and C3 analogs of natural fatty acids, which reversibly inhibit β-oxidation of medium-chain and short-chain fatty acids (Le Dinh et al. 1988; Fromenty et al. 1989), explaining why amineptine or tianeptine can cause rare mild hepatic steatosis from impaired β-oxidation (Pessayre and Larrey 2007).

Calcium hopantenate. The administration of the pantothenic acid antagonist, calcium hopantenate (also called calcium homopantothenate), can decrease CoA and inhibit mitochondrial β-oxidation, and it has caused several cases of Reye's-like syndrome in Japan (Noda et al. 1988).

Panadiplon. The development of panadiplon as an anxiolytic drug was interrupted due to elevated transaminases (Ulrich et al. 2001). The drug is converted into cyclopropane carboxylic acid, which sequesters coenzyme A and carnitine and inhibits β-oxidation (Ulrich et al. 2001).

Pivampicillin. The administration of pivampicillin results in extensive pivaloylcarnitine formation, thus depleting free carnitine and inhibiting fatty acid oxidation (Melegh et al. 1997).

4.10 Multiple Mechanisms

Some drugs impair mitochondrial function through a combination of mechanisms. A good example is valproic acid, which inhibits mitochondrial fatty acid oxidation by sequestering coenzyme A (a cofactor necessary for fatty acid activation and

oxidation), and possibly also through inactivation of β-oxidation enzymes by an electrophilic valproic acid metabolite. In addition, valproic acid is an anionic uncoupler of mitochondrial respiration and can induce MPT, which may explain why valproate-induced microvesicular steatosis can be associated with liver cell death.

5 Individual Susceptibility

When a drug being developed causes frequent adverse effects in humans it rarely reaches the market. As a result, when marketed drugs are used at recommended therapeutic doses, very few cases of DILI occur. Except for cases from overdoses, most cases of DILI can be considered idiosyncratic. The reasons for the unique susceptibility of a few individuals are not completely understood. However, both variations in drug metabolism/excretion and the presence of medical conditions that also impair mitochondrial function can be involved.

5.1 Drug Metabolism and/or Excretion

When the parent drug (rather than a metabolite) directly impairs mitochondrial function, any factor decreasing drug elimination can be expected to increase the risk of DILI. For example, perhexiline maleate inhibits mitochondrial fat oxidation and energy production, but it is detoxified through the formation of water-soluble metabolites by CYP2D6 (Morgan et al. 1984). Patients who are genetically deficient in CYP2D6 are at increased risk of developing perhexiline-induced liver injury (Morgan et al. 1984). Another example is chloramphenicol, which inhibits mitochondrial protein synthesis, but is detoxified by glucuronide formation. The mitochondrial toxicity of high doses of chloramphenicol was increased in premature or newborn babies whose capacity for drug glucuronide formation is still immature (Weiss et al. 1960). Finally, renal insufficiency, which decreases tetracycline elimination, was a risk factor for severe microvesicular steatosis after the intravenous administration of high doses of tetracycline (Zimmerman 1978).

In other instances, a nontoxic parent compound is transformed by CYP into a reactive metabolite that is toxic to the mitochondria. This is seen with acetaminophen, which is transformed by CYP2E1 to the chemically reactive, N-acetyl-p-benzoquinone imine. The hepatic toxicity of acetaminophen is increased in alcoholics (Seef et al. 1986). Ethanol abuse increases CYP2E1 in the endoplasmic reticulum and in the mitochondria (Robin et al. 2005). The mitochondrial localization of CYP2E1 may lead to the in situ generation of reactive metabolites of acetaminophen in the mitochondria, where the metabolite may trigger MPT (Weis et al. 1992; Masubuchi et al. 2005). Mitochondria also contain other inducible CYPs, such as CYP1A1 and CYP2B1 (Anandatheerthavarada et al. 1997; Sepuri et al. 2007). The concomitant administration of CYP-inducers, phenobarbital or phenytoin increases the hepatotoxicity of valproic acid, which is transformed by microsomal and mitochondrial CYPs and then β-oxidation enzymes into a reactive

metabolite that can inactivate mitochondrial β-oxidation enzymes (Kassahun et al. 1991, 1994).

5.2 *Preexisting Mitochondrial Dysfunction*

Severe mitochondrial dysfunction is required to trigger liver injury. Obviously drugs that are released on the market do not impair mitochondrial function enough to cause liver injury in most recipients. However, these drugs can still trigger mitochondrial dysfunction-associated DILI in a few patients whose mitochondrial function is already impaired by preexisting conditions, such as an inborn mitochondrial cytopathy, a mitochondrial β-oxidation defect, a viral infection, pregnancy, and/or the mitochondrial alterations associated with the metabolic syndrome. In these subjects, the hepatotoxic drug and the mitochondria-impairing condition (s) can additively impair mitochondrial function to trigger liver disease.

5.2.1 Inborn Mitochondrial Cytopathies

Inborn mitochondrial cytopathies can be caused by defects in nuclear genes or by mtDNA mutations (Schon et al. 1997; DiMauro and Schon 1998). There may be about 50 different pathological point mutations and 200 different mtDNA deletions (Schon et al. 1997). Point mutations are usually transmitted by the mother, while most mtDNA deletions are acquired during oogenesis or embryogenesis. Point mutations can be either homoplasmic (affecting all mtDNA genomes) or heteroplasmic (affecting some of the mtDNA genomes), whereas mtDNA deletions are usually heteroplasmic (since the homoplasmic state is usually not viable).

Although the overall prevalence of these inborn mitochondrial cytopathies remains unknown, the frequency of the A3243G mtDNA point mutation alone is estimated to be 1.6 in 10,000 in a Finnish population (Majamaa et al. 1998). Overall, clinically patent mitochondrial cytopathies could exist with a frequency of around 1 in 5,000 children (Haas et al. 2007).

Mitochondrial cytopathies affect mitochondrial respiration, which may secondarily inhibit β-oxidation, as explained above. Mitochondrial cytopathies may therefore be revealed during the administration of drugs that have mitochondrial effects. Thus, the administration of valproate, which inhibits mitochondrial β-oxidation and pyruvate-supported respiration, may reveal a previously latent mitochondrial cytopathy (Chabrol et al. 1994; Lam et al. 1997; Krähenbühl et al. 2000). For the same reasons, valproate administration can also reveal an inborn β-oxidation defect (Njølstad et al. 1997; Kottlors et al. 2001).

5.2.2 Inborn β-Oxidation Defects

Inborn defects in β-oxidation enzymes may affect the various enzymes involved in fatty acid transport and β-oxidation. The most frequent defect is medium-chain

acyl-coenzyme dehydrogenase (MCAD) deficiency. A German ancestor of present day Europeans had a specific RFLP haplotype in the nuclear MCAD locus and developed an A985G point mutation in the MCAD gene (Zhang et al. 1993). This point mutation transforms a lysine residue into a glutamate residue (Kelly et al. 1990), and the substitution impairs the normal association of the enzyme monomer into the active homotetramer, resulting in the rapid degradation of the monomer (Yokota et al. 1992). This German ancestor was astoundingly efficient at transmitting this gene to his/her descendants (suggesting that the heterozygous carrier state may offer some unknown advantage). Indeed, about 1 out of 50 Europeans (and North Americans of European descent) are now carriers of this initial RPLP haplotype (or derived haplotypes) and the A985G point mutation (Miller et al. 1992; Fromenty et al. 1996). This results in an estimated frequency of about 1 in 10,000 for the homozygous state, making it one of the most prevalent, potentially fatal, existing genetic diseases.

5.2.3 Viral Infections

The additive adverse effects of drugs and viral infections on mitochondrial function are well documented in children treated with aspirin. Although lethal overdoses of aspirin frequently cause microvesicular steatosis (Partin et al. 1984), therapeutic doses do not, although they can trigger Reye's syndrome in a few children with viral infections. Indeed, in some children with an initially benign viral infection such as varicella or influenza, protracted vomiting can suddenly occur, followed by obnubilation, increased liver enzymes, hyperammonemia, hypoglycemia, a hyperechogenic liver on ultrasound indicating steatosis, and finally, coma and death. This postinfectious disease, which is known as Reye's syndrome, is thought to be due to an acquired mitochondrial dysfunction.

Interferon-α, tumor necrosis factor-α (TNF-α), and nitric oxide, which are released during viral infections, can all impair mitochondrial function. Interferon-α decreases the synthesis and stability of mitochondrial transcript as previously mentioned. Nitric oxide reversibly inhibits mitochondrial respiration (Borutaité and Brown 1996) and may trigger MPT (Susin et al. 1998). TNF-α can also inhibit respiration and trigger MPT (Pessayre et al. 2000). Nevertheless, viral infections rarely cause Reye's syndrome, suggesting that these endogenous substances usually do not impair enough mitochondrial function to trigger the disease. However, if children take aspirin during a viral illness, the added effects of salicylate and infection may sufficiently impair mitochondrial function to trigger the syndrome in a few patients. The following evidence supports the role of aspirin in the development of Reyes's syndrome. In the past, 93% of children with Reye's syndrome had received aspirin during the initial acute viral illness (Hurwitz et al. 1985). Children with Reye's syndrome had received aspirin more frequently than those with similar viral diseases not followed by Reye's syndrome (Forsyth et al. 1989). Finally, when recommendations against the use of aspirin in feverish children have been issued, there was a parallel decline in the use of aspirin and the incidence of Reye's syndrome in the USA (Remington et al. 1986).

Now that the use of aspirin has been curtailed, the few residual cases of Reye's syndrome that still occur are mainly in children with another potentiating factor, in particular a previously latent genetic defect in mitochondrial ß-oxidation enzymes (Rowe et al. 1988). In these children, the deficit is suddenly revealed because the fever increases energy demands, while the viral infection further damages the mitochondria. Furthermore, the anorexia may result in insufficient nutrition, which triggers adipose tissue lipolysis, thus flooding the liver with free fatty acids that are not oxidized by deficient mitochondria.

Another example of the increased susceptibility of patients with certain viral infections for drug-induced mitochondrial dysfunction is the increased hepatotoxicity of highly active antiretroviral therapy (HAART) in HIV/HBV or HCV coinfected patients (Sulkowski et al. 2002; Wit et al. 2002). Certain viral proteins such as the HCV core protein or the HBV X protein disturb mitochondrial function (Rahmani et al. 2002; Korenaga et al. 2005; Piccoli et al. 2007), and, together with cytokines, could impair mitochondrial function and make HBV- or HCV-coinfected subjects more susceptible to HAART-induced liver toxicity.

5.2.4 Pregnancy

One in 13,000 pregnant women develop microvesicular steatosis during the last trimester of pregnancy (Kaplan 1985). Untreated, the disease progresses to coma, kidney failure, and hemorrhage, and leads to the death of the mother and child in 75–85% of cases. In contrast, if the pregnancy is terminated rapidly, a healthy child is usually born and the mother rapidly recovers (Ebert et al. 1984).

Both pregnancy itself (Grimbert et al. 1993) and the administration of estradiol and progesterone (Grimbert et al. 1995) slightly alter mitochondrial ultrastructure and function in mice. However, the mitochondrial ß-oxidation of fatty acids is only slightly impaired, and microvesicular steatosis does not develop in these mice. Similarly, most human pregnancies do not cause fatty liver. Therefore, additional factors probably help trigger the syndrome in a few pregnant women. One factor may be the administration of a drug that also impairs mitochondrial function. Indeed, pregnancy was a predisposing factor for the development of microvesicular steatosis in women receiving high intravenous doses of tetracycline (Zimmerman 1978).

In other women, acute fatty liver of pregnancy is triggered by an inborn β-oxidation defect in the fetus. Indeed, partial deficiency of long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD), which is part of the trifunctional membrane-bound β-oxidation enzyme, has been reported in some women with acute fatty liver of pregnancy (Ibdah et al. 1999). Mothers with a single defective LCHAD allele who marry a heterozygous carrier and conceive a fetus with two defective alleles develop the disease, while those with an unaffected child usually have uncomplicated pregnancies. Although the fetus itself may not use fatty acids for energy production, the placenta metabolizes fatty acids (Shekhawat et al. 2003). During LCHAD-negative conceptions, the placenta may release

toxic 3-hydroxy fatty acids, which could trigger hepatic steatosis in the mother (Shekhawat et al. 2003).

5.2.5 Obesity and Nonalcoholic Steatohepatitis

Obesity can cause insulin resistance and hepatic steatosis, which can lead to nonalcoholic steatohepatitis (NASH) in some patients (Pessayre and Fromenty 2005). Patients with NASH have an impaired ability to resynthesize ATP after a fructose challenge (Cortez-Pinto et al. 1999). Their hepatic mitochondria exhibit ultrastructural lesions with paracristalline inclusions in megamitochondria (Caldwell et al. 1999; Sanyal et al. 2001). Patients with NASH have decreased protein expression of several mtDNA-encoded polypeptides and lower activity of respiratory complexes (Pérez-Carreras et al. 2003). Thus, obesity-associated NASH combined with the administration of drugs impairing mitochondrial β-oxidation may additively damage mitochondria to aggravate steatosis and steatohepatitis. Indeed, obesity has been shown to increase the risk of tamoxifen-induced steatosis and steatohepatitis in women (Bruno et al. 2005).

6 Conclusions

Drugs or their reactive metabolites can trigger MPT causing severe ATP depletion and necrosis, or leading to cytochrome *c* release, caspase activation, and apoptosis. Necrosis can also occur from drug-induced uncoupling or inhibition of mitochondrial respiration causing ATP depletion. Drugs can cause hepatic steatosis by sequestering coenzyme A and/or by inhibiting mitochondrial ß-oxidation enzymes or by first impairing mitochondrial respiration, which secondarily inhibits β-oxidation. These two effects can occur with drugs that deplete mtDNA by damaging mtDNA and/or by inhibiting mtDNA replication, or that decrease the synthesis and stability of mtDNA transcripts and/or inhibit mitochondrial protein synthesis. When ß-oxidation is directly or secondarily impaired, fatty acids are poorly oxidized by mitochondria and are instead esterified into triglycerides, which initially accumulate as small lipid vesicles that may progressively coalesce into larger vacuoles. The inability to oxidize fatty acids, together with the mitochondrial toxicity of free fatty acids, dicarboxylic acids, and lipid peroxidation products, and, in some instances, the associated impairment of respiration, may all decrease mitochondrial energy formation. This could explain the apparent severity of extensive microvesicular steatosis, which can be associated with liver failure, coma, and death. Milder and more prolonged forms of drug-induced mitochondrial dysfunction can also lead to macrovacuolar steatosis. Although this is a benign liver lesion in the short-term, macrovacuolar steatosis can evolve towards steatohepatitis, which can slowly progress to liver fibrosis and even cirrhosis.

Drugs and other associated medical conditions that also impair mitochondrial function may have an additive effect to damage mitochondria and trigger liver injury.

The presence of diverse comorbid factors that also impair mitochondrial function (such as inborn mitochondrial cytopathies, inborn β-oxidation defects, viral infections, obesity-associated NASH, or pregnancy) may play an important role in the idiosyncratic occurrence of these drug-induced adverse effects.

Mitochondrial effects are rarely investigated during the preclinical development of new drug molecules. However, over the last 20 years, the development of several drugs has been stopped during clinical trials due to mitochondrial dysfunction-associated DILI (fialuridine, panadiplon); other drugs were withdrawn temporarily or permanently from the market (alpidem, perhexiline, pirprofen, troglitazone), or have been given severe "black box" warnings by drug agencies that have partly restricted their use (amiodarone, benzbromarone, buprenorphine, several NRTIs, tamoxifen, tolcapone, and valproic acid) (Labbe et al. 2008). We therefore recommend systematic screening of all new drug molecules for possible mitochondrial effects during lead selection or preclinical studies (Labbe et al. 2008).

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Part III
Examples/Models

Mechanisms of Acetaminophen-Induced Liver Necrosis

Jack A. Hinson, Dean W. Roberts, and Laura P. James

Contents

Abstract Although considered safe at therapeutic doses, at higher doses, acetaminophen produces a centrilobular hepatic necrosis that can be fatal. Acetaminophen poisoning accounts for approximately one-half of all cases of acute liver failure in the United States and Great Britain today. The mechanism occurs by a complex sequence of events. These events include: (1) CYP metabolism to a reactive metabolite which depletes glutathione and covalently binds to proteins; (2) loss of glutathione with an increased formation of reactive oxygen and nitrogen species in hepatocytes undergoing necrotic changes; (3) increased oxidative stress, associated with alterations in calcium homeostasis and initiation of signal transduction responses, causing mitochondrial permeability transition; (4) mitochondrial permeability transition occurring with additional oxidative stress, loss of mitochondrial membrane potential, and loss of the ability of the mitochondria to synthesize ATP; and (5) loss of ATP which leads to necrosis. Associated with these essential events

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there appear to be a number of inflammatory mediators such as certain cytokines and chemokines that can modify the toxicity. Some have been shown to alter oxidative stress, but the relationship of these modulators to other critical mechanistic events has not been well delineated. In addition, existing data support the involvement of cytokines, chemokines, and growth factors in the initiation of regenerative processes leading to the reestablishment of hepatic structure and function.

Keywords Acetaminophen · Liver · Glutathione · Covalent binding · Mitochondria · Oxidative stress · JNK

1 Introduction

Acetaminophen (paracetamol, N-acetyl-*p*-aminophenol; APAP) is a widely used over-the-counter analgesic and antipyretic drug (Bessems and Vermeulen 2001; James et al. 2003b; Prescott and Critchley 1983). At therapeutic doses, it is believed to be safe, having analgesic and antipyretic effects similar to those of aspirin and ibuprofen. Unlike these other drugs, acetaminophen has only weak antiinflammatory properties.

Acetaminophen was originally introduced as an analgesic by von Mering in 1893, but was not widely used until the 1960s, following the recognition that the structural analog phenacetin was nephrotoxic in chronic abusers (Hinson 1980). More recently, concern about aspirin-mediated gastrointestinal bleeding and Rye's syndrome has further increased its popularity. According to the US Food and Drug Administration, each week approximately 50 million adults in the United States take acetaminophen-containing products.

Although considered safe at therapeutic doses, at higher doses, acetaminophen produces a centrilobular hepatic necrosis that can be fatal. Acetaminophen poisoning accounts for approximately one-half of all cases of acute liver failure in the United States and Great Britain today (Larson et al. 2005; Ostapowicz et al. 2002) Annually, it accounts for a very high percentage of inquiries to poison control centers and deaths (Litovitz et al. 2002). The direct costs of acetaminophen overdose have been estimated to be as high as US$87 million annually (Bond and Novak 1995).

2 Acetaminophen-Induced Hepatotoxicity

Davidson and Eastham were the first to report that acetaminophen was hepatotoxic in overdose (Davidson and Eastham 1966). They described two individuals who developed hepatotoxicity following acetaminophen overdose and died on the third day following the overdose. Microscopic examination of liver sections from these

individuals indicated fulminating hepatic necrosis. The necrosis was primarily in the centrilobular areas. Eosinophilic degeneration of the cells together with pyknosis of nuclear material was observed in these hepatocytes. Vacuolization and early degenerative changes were observed in the more peripheral cells surrounding the portal areas. A mild polymorphonuclear leukocytic infiltration occurred in both cases. These changes indicated fulminating necrosis confined primarily to the hepatocytes in the centrilobular regions of the liver. Necrosis in the cells of the proximal tubules of the kidney was observed in one of the patients.

Subsequent to this initial report, many cases of acetaminophen overdose were reported. Boyer and Rouff described the principal clinical symptoms as development of nausea and vomiting within 2–3 h of ingestion followed by abdominal pain in the right upper quadrant. Liver dysfunction occurred within 24 h and reached a maximum approximately 3–4 days after ingestion (Boyer and Rouff 1971). Prescott et al. summarized the clinical and biochemical changes to be a dramatic increase in serum alanine aminotransferase (ALT) and asparatate aminotransferase (AST) levels, mild hyperbilirubinemia, and increased prothrombin time (Dixon et al. 1971). In conjunction with these clinical symptoms, the elimination half-life of acetaminophen may be delayed (Schiodt et al. 2002). Also, some patients may develop nephrotoxicity in addition to hepatotoxicity (Boyer and Rouff 1971; Prescott et al. 1971).

Hepatotoxicity of acetaminophen was also observed in rodents treated with large doses of acetaminophen (Boyd and Bereczky 1966; Mitchell et al. 1973a). Whereas the rats were not very sensitive to the hepatotoxicity, both mice and hamsters proved to be very sensitive (Davis et al. 1974). Histologically the toxicity was characterized by glycogen loss and vacuolization of centrilobular hepatocytes by 2 h, resulting in a clear demarcation of the centrilobular areas from the rest of the liver. By 3 h, nuclear changes were observed in centrilobular hepatocytes and single cell necrosis with pycnotic cells. By 6 h, gross necrosis of the entire centrilobular areas were observed (Mitchell et al. 1973a). Similar changes were subsequently reported by Walker and coworkers (Walker et al. 1980). These investigators also reported the changes observed by electron microscopic analysis. In addition, they reported that toxicity was associated with hepatic congestion.

The role of apoptosis in acetaminophen liver injury is controversial. Ray and coworkers reported that following a toxic dose of acetaminophen to mice as many as 40% of the dead hepatocytes were apoptotic and 60% necrotic (Ray et al. 1996). However, in subsequent research, Gujral and coworkers (Gujral et al. 2002) used standard morphological criteria of apoptosis (cell shrinkage, chromatin condensation and margination, and apoptotic body formation) and were unable to confirm that acetaminophen caused apoptosis in livers of mice treated with toxic doses. They found that massive necrosis occurred in livers of mice treated with a toxic dose of acetaminophen (3–24 h). The number of cells meeting the morphological criteria for apoptosis was less than 1% of all parenchymal cells. Moreover, levels of caspase 3, an effector of apoptosis, were not increased in the livers of the acetaminophen-treated mice. These data support the postulation that acetaminophen toxicity in mice occurs almost exclusively by necrosis. This review will focus on acetaminophen-induced necrosis since this is the primary toxicity in humans. However, intracellular signaling

and regulatory mechanisms support the concept that there is a close relationship between apoptosis and necrosis, and alterations in mitochondria may be the key to understanding these differences (Kon et al. 2004, 2007; Malhi et al. 2006).

3 Metabolism in Acetaminophen Toxicity

In a series of four publications, Mitchell, Jollow, Potter, Gillette, and Brodie examined the role of metabolism in the hepatotoxicity in mice (Jollow et al. 1973; Mitchell et al. 1973a, b; Potter et al. 1973). Their research showed that acetaminophen was converted by drug metabolizing enzymes to a reactive metabolite that covalently bound to proteins. At nontoxic doses, the metabolite was efficiently detoxified by glutathione forming an acetaminophen-glutathione conjugate (Jollow et al. 1974). However, at toxic doses, the metabolite depleted hepatic glutathione by as much as 80–90% (Jollow et al. 1974; Mitchell et al. 1973a, b) and subsequently covalently bound to protein. The amount of covalent binding correlated with the relative hepatotoxicity (Jollow et al. 1973). Since diethylmaleate depleted hepatic glutathione without causing toxicity, it was postulated that glutathione depletion per se was not the mechanism of toxicity (Mitchell et al. 1973a, b).

Subsequently, the reactive metabolite of acetaminophen was identified to be N-acetyl-*p*-benzoquinone imine (NAPQI). It was found to be formed by cytochrome P-450 (CYP) by a direct two electron oxidation of acetaminophen, a previously unrecognized mechanism of CYP (Dahlin et al. 1984; Gillette et al. 1981; Potter and Hinson 1987). The CYP isoforms important in acetaminophen metabolism have been shown to be CYP2E1, CYP1A2, CYP3A4, and CYP2D6 (Dong et al. 2000; Raucy et al. 1989; Snawder et al. 1994; Thummel et al. 1993). Reaction of NAPQI with glutathione occurs by conjugation to form 3-glutathion-*S*-yl-acetaminophen and by reduction to acetaminophen (Dahlin and Nelson 1982; Hinson et al. 1982). The second order rate constant for the reaction of NAPQI with glutathione was found to be ($3.2 \times 10^4 M^{-1} s^{-1}$). Moreover, the reaction could be catalyzed by glutathione transferase pi, and NAPQI is one of the best substrates ever described for this enzyme (Coles et al. 1988). Thus, detoxification of NAPQI is extremely rapid, and the rapid rate may explain why covalent binding to proteins was not observed in hepatocytes until glutathione was almost completed depleted (Mitchell et al. 1973a, b).

In initial work describing the importance of hepatic glutathione in acetaminophen-induced hepatotoxicity in mice, Mitchell et al. (1973a, b) showed that administration of cysteine prevented hepatotoxicity. This finding led to the development of N-acetylcysteine (available as Mucomyst®) as the preferred antidote (Peterson and Rumack 1977; Piperno and Berssenbruegge 1976; Prescott et al. 1977). Rumack and coworkers (Rumack et al. 1981; Rumack and Peterson 1978) analyzed toxicity data from a large number of acetaminophen overdose patients treated with N-acetylcysteine. Treatment of acetaminophen-poisoned patients by 10 h after the overdose was effective at decreasing the toxicity (Prescott et al. 1977; Rumack et al. 1981). Patients treated after 10 h of the overdose had a fourfold elevation in ALT values. The plot of serum

acetaminophen levels versus time lapsed since overdose, and clinical outcome, led to the development of a treatment nomogram. This nomogram has been very effective at predicting those individuals who may be most susceptible to development of toxicity and are candidates for N-acetylcysteine treatment (Rumack et al. 1981; Smilkstein et al. 1988). The mechanism by which N-acetylcysteine inhibits acetaminophen toxicity has been postulated to be increased detoxification of NAPQI by a direct conjugation or through increased glutathione synthesis (Corcoran et al. 1985).

Covalent binding of acetaminophen to protein was found to correlate with acetaminophen-induced hepatotoxicity (Jollow et al. 1973). Covalent binding was ascertained utilizing radiolabeled drug. Subsequently, immunochemical approaches were developed by Roberts et al. (1987) and Bartolone et al. (1987) for analysis of acetaminophen covalently bound to cysteine groups in proteins. Western blot analyzes of liver proteins from mice treated with toxic doses of acetaminophen indicated that a limited number of proteins contained acetaminophen adducts (Bartolone et al. 1989; Pumford et al. 1990). Competitive ELISA indicated that maximum levels of adducts occurred in liver at 1–2 h with subsequent lysis of hepatocytes. Acetaminophen-protein adducts were observed in the serum and immunoblot assays indicating that these adducts were of hepatic origin (Pumford et al. 1990). The appearance of acetaminophen-protein adducts in serum correlated with increases of ALT and AST in serum (Pumford et al. 1989). These data indicated that the presence of acetaminophen-protein adducts in serum was a biomarker for the formation of hepatic acetaminophen-protein adducts and acetaminophen toxicity.

Immunohistochemical analysis of liver sections from treated mice revealed a high correlation between the presence of acetaminophen-protein adducts and toxicity (Cohen and Khairallah 1997; Roberts et al. 1991). Figure 1 is a time course for formation of the acetaminophen protein adducts and development of toxicity in individual murine hepatocytes (Roberts et al. 1991). In this assay, the adducts are stained red. Adducts are visible in the liver sections within 15 min of dosing. By 1 h, staining intensity is maximal and adducts are confined to the centrilobular hepatocytes. Adducts do not occur in the periportal hepatocytes. At 2–6 h, hepatocytes containing adducts are undergoing necrotic changes as evidenced by vacuolization and pycnotic changes in the nuclei. Note that the only hepatocytes observed to develop necrosis were those containing acetaminophen-protein adducts. By 24 h, all the necrotic cells contained adducts. The majority of hepatocytes that had acetaminophen-protein adducts were reported to develop necrosis (Roberts et al. 1991). The covalent binding data suggest that the primary determinant leading to toxicity is metabolism, and that evidence for progression of toxicity subsequent to metabolism as has been suggested by other investigators (Limaye et al. 2003; Liu et al. 2004; Liu et al. 2006) was not observed in these studies (Roberts et al. 1991).

These data supported the hypothesis that acetaminophen-induced liver toxicity is mediated by covalent binding to critical proteins. In an attempt to further understand the mechanism of hepatotoxicity of acetaminophen, specific proteins to which acetaminophen was covalently bound were isolated and sequenced by our laboratory and by Cohen's laboratory (Cohen et al. 1997). The proteins that were identified by this approach were: glutamine synthase, glutamate dehydrogenase,

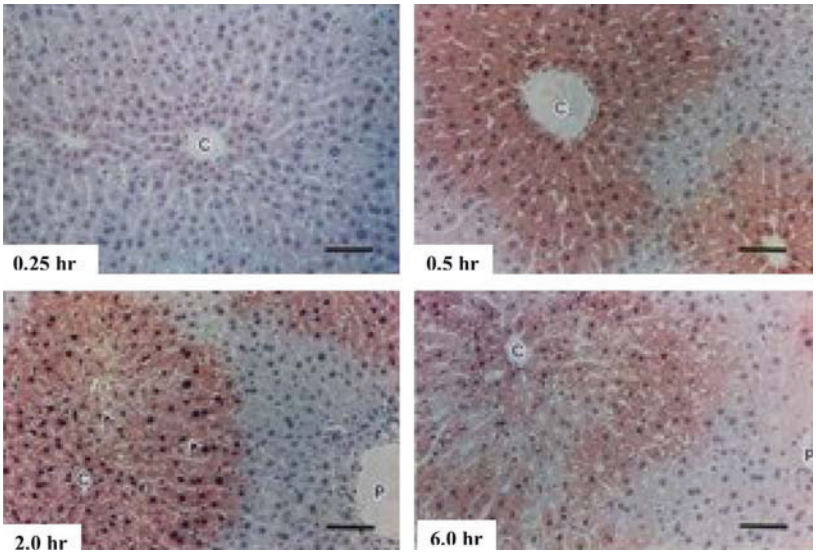

Fig. 1 Immunohistochemical time course for acetaminophen protein adduct formation and hepatic necrosis in mice treated with a toxic dose of acetaminophen. The liver sections were immunochemically stained using antiacetaminophen-cysteine antiserum. The hepatocytes containing the adducts are stained *red*. Note the appearance of valcuoles and pycnotic nuclei indicative of necrosis only in the hepatocytes containing acetaminophen-protein adducts at 2 and 6 h. Staining was not observed in livers of saline treated mice (data not shown) (Roberts et al. 1991)

aldehyde dehydrogenase, selenium (acetaminophen) binding protein, and N-10 formyltetrahydrofolate dehydrogenase. A toxic dose of acetaminophen to mice decreased the catalytic activity of the hepatic enzymes glutamate dehydrogenase and N-10 formyltetrahydrofolate dehydrogenase by approximately 25%. Subsequently, proteomic analyzes using mass spectral methods identified a number of additional proteins: glutathione peroxidase, thioether S-methyltransferase, aryl sulfotransferase, pyrophosphatase, topomyosin 5, proteasome subunit C8, methionine adenosyl transferase, protein synthesis initiation factor 4A, ATP synthase α subunit, carbonic anyhydrase III, urate oxidase, 2,4-dienyl Co-A reductase, osteoblast specific factor 3, glutathione transferase π, sorbitol dehydrogenase, glycine N-methyltransferase, and 3-hydroxyanthranilate 3,4-dioxygenase (Qiu et al. 1998). Unfortunately, the percent covalent binding to the proteins and the possible effect on enzyme activities are not known. The role of these adducts in the development of acetaminophen-induced liver toxicity is unclear.

Clinical data also support the association of covalent binding and toxicity. Early studies utilized immunoassays to detect adducts in the blood samples of patients with acetaminophen overdose (Hinson et al. 1990). The highest levels of adducts were found in the patients with the most severe toxicity. The recent development of a highly sensitive and specific HPLC-EC assay for detection of acetaminophen protein adducts (3-cysteine-acetaminophen in proteins) has allowed for further

study of adducts in various clinical settings. Using this assay, it has been shown that adduct levels in serum correlate with hepatic transaminase values in adults with acetaminophen-related liver failure (Muldrew et al. 2002). In addition, the assay may have value in the diagnostic examination of patients with acute liver failure of unknown etiology (Davern et al. 2006). Approximately 20% of patient serum samples from adults with acute liver failure of unknown etiology were positive for adducts, strongly suggesting that acetaminophen was the etiology of the liver failure. These patients had been previously tested for other known causes of acute liver failure. In addition, adducts were recently shown to persist in serum for at least 12 days after severe acetaminophen overdoses in adults (James et al. 2009).

Even though there is an excellent correlation between covalent binding of acetaminophen to protein and development of hepatotoxicity, there is significant evidence that suggest that covalent binding per se is not the mechanism of toxicity. Henderson and coworkers (Henderson et al. 2000) examined acetaminophen-induced glutathione depletion and hepatotoxicity in mice glutathione S-transferase Pi knockout mice, the transferase that catalyzes the conjugation of NAPQI with GSH (Coles et al. 1988). Following a toxic dose of acetaminophen, hepatic glutathione was depleted by greater than 90% in the wild-type mice but only by approximately 70% in the knockout mice. Unexpectedly, the knockout mice were much less sensitive to acetaminophen-induced hepatotoxicity than the wild-type mice; however, both groups of mice had similar levels of covalent binding. These data appear to separate covalent binding of acetaminophen to protein from development of the toxicity and are consistent with a hypothesis that 90% glutathione depletion in hepatocytes is critically necessary for the development of necrosis.

Additional data obtained using hepatocyte suspension assays suggest that covalent binding per se is not the mechanism of toxicity. Boobis and coworkers (Boobis et al. 1986; Tee et al. 1986) found that acetaminophen toxicity in freshly isolated hamster hepatocytes occurred in two phases. In these experiments, incubation of the hepatocytes with acetaminophen (2.5 mM) for 90 min resulted in glutathione depletion and covalent binding, but no toxicity. Subsequent washing of the hepatocytes to remove acetaminophen and reincubation of the hepatocytes with media alone resulted in significant toxicity in the reincubation phase. Addition of N-acetylcysteine or dithiothreitol to the reincubation media protected the hepatocytes against development of toxicity. Acetaminophen toxicity in mouse hepatocytes was similarly found to occur by a two phase mechanism (Grewal and Racz 1993; Rafeiro et al. 1994; Reid et al. 2005). These data do not rule out a role for covalent binding in toxicity but suggest that mechanism(s) downstream from GSH depletion such as oxidative stress play a role in development of toxicity.

4 Alterations in Hepatic Blood Flow in Acetaminophen Toxicity

Acetaminophen-induced hepatotoxicity has been reported to occur with hepatic congestion in humans (Rose 1969; Thompson et al. 1972) and rodents (Dixon et al. 1971; Walker et al. 1980, 1985). In mice, it occurs early and before the appearance

of necrosis. Morphological studies in mice by Walker and coworkers revealed that the congestion results from the accumulation of red blood cells within endocytic vacuoles and the Space of Disse with a collapse of the sinusoidal lumens (Walker et al. 1980, 1983). They examined changes in liver weight relative to blood flow and toxicity in a time course study design. In their studies, they found that at 1.5 h after a very toxic dose of acetaminophen to mice there was a significant increase in liver weight. The liver weight continued to increase, and at 6 h it was approximately twofold over baseline levels and subsequently decreased by 24 h. Associated with the increase in liver weight was a very large increase in liver hemoglobin (approximately fourfold at 6 h) with a subsequent decrease by 24 h. ^{125}I-albumin studies indicated a blockade of blood flow (Walker et al. 1985). The decrease in liver weight that occurred between 6 and 24 h was associated with a large increase in serum ALT and AST levels indicative of lysis of hepatocytes (Roberts et al. 1991). Associated with the large increase in liver weight and liver hemoglobin level there was a dramatic decrease in intrahepatic pressure and portal vein pressure. Administration of N-acetylcysteine at 3 h after acetaminophen administration ameliorated these observed effects (Walker et al. 1985). Subsequent work by Lim and coworkers (Lim et al. 1995) using a vascular casting technique indicated that acetaminophen toxicity in rats occurred with microvascular injury in the centrilobular areas.

DeLeve and coworkers (DeLeve et al. 1997) examined the possibility that sinusoidal hepatic endothelial cells may metabolize acetaminophen in vitro and that this may be important in toxicity. Previous data indicated that hepatic endothelial cells contain CYP enzymes (Oesch and Steinberg 1987; Steinberg et al. 1990), and that activation of acetaminophen by CYP enzymes in endothelial cells may produce toxicity. Endothelial cells were isolated from two strains of mice. Acetaminophen was not toxic to cultured endothelial cells from Swiss Webster mice but was toxic to cultured endothelial cells from C3H-HEN mice. Glutathione was depleted in the sensitive endothelial cells before the development of toxicity whereas glutathione was not depleted in endothelial cells from the Swiss Webster mice. Addition of glutathione to the incubation or the CYP inhibitor aminobenzotriazole inhibited development of toxicity in the C3H-HEN cells. However, the two strains of mice appeared to be equally sensitive to the centrilobular hepatic necrosis produced by acetaminophen *in vivo*.

McCuskey and coworkers reexamined the role of microvascular injury in acetaminophen toxicity (Ito et al. 2003; McCuskey 2006). In support of the reports by Walker and coworkers (Walker et al. 1985), they found that acetaminophen produces damage to the hepatic microvasculature (sinusoidal endothelial cells) and that these effects precede hepatocellular injury. *In vivo* microscopy indicated that the injury consisted of swelling of the endothelial cells and penetration of erythrocytes into the extrasinusoidal Space of Disse (Ito et al. 2003). There was a significant decrease at 2 and 6 h in the hepatic sinusoids containing blood (Ito et al. 2004). Utilization of an assay for the functional integrity of the endothelial cells (uptake of formaldehyde treated serum albumin) indicated impairment of function in the endothelial cells in the centrilobular regions but not in the periportal regions. These findings indicated that acetaminophen toxicity occurred with altered function

of the sinusoidal endothelial cells in the centrilobular regions and confirmed the previous findings that acetaminophen toxicity is accompanied by reduced sinusoidal perfusion. These findings suggest that endothelial cell damage may play a role in the toxicity and the biochemical events associated with toxicity (Ito et al. 2003; Walker et al. 1985); however, the exact role altered blood flow plays in acetaminophen toxicity is unknown.

5 Oxidative Stress in Acetaminophen Toxicity

Early research on understanding oxidative stress in acetaminophen toxicity focused on iron-mediated oxidative stress (Fenton mechanism). This mechanism is initiated by cellular superoxide formation and its dismutation to form increased hydrogen peroxide. Superoxide may be formed by multiple mechanisms including uncoupling of cytochrome P-4502E1 or other enzymes (Koop 1992) and mitochondria (Brand et al. 2004; Casteilla et al. 2001), or activation of NADPH oxidase (Sies and de Groot 1992). Since glutathione is depleted by the metabolite NAPQI in acetaminophen-induced hepatotoxicity and glutathione is the cofactor for glutathione peroxidase detoxification of peroxides, a major mechanism of peroxide detoxification is compromised in acetaminophen-induced toxicity. Thus, glutathione depletion may be expected to lead to increased intracellular peroxide levels and increased oxidative stress via a Fenton mechanism. This mechanism involves the reduction of peroxide by ferrous ions forming the highly reactive hydroxyl radical which may in turn oxidize lipids leading to initiation of lipid peroxidation as well as oxidation of proteins and nucleic acids. This mechanism has been implicated in various toxicities (Aust et al. 1985). In early work, Wendel and coworkers (Wendel et al. 1979) reported that acetaminophen administration to mice was accompanied by increased levels of exhaled ethane, a measure of lipid peroxidation. Younes et al. (1986) reported that acetaminophen administration to mice did not cause lipid peroxidation (ethane exhalation), but coadministration of ferrous sulfate caused an increase in lipid peroxidation without an increase in toxicity. Subsequently, Gibson et al. (1996) examined hepatic protein aldehydes in acetaminophen toxicity in mice. As with lipid peroxidation, protein aldehyde formation is also mediated by a Fenton mechanism. No evidence of increased hepatic protein aldehyde formation was observed. Thus, early findings as to the role of oxidative stress in acetaminophen-induced toxicity in animals were unclear.

However, work in hepatocytes suggested that acetaminophen toxicity may involve iron-mediated oxidative stress. Albano and coworkers (Albano et al. 1983) reported that incubation of acetaminophen with cultured mouse hepatocytes or with polycyclic aromatic hydrocarbon-induced rat hepatocytes produced oxidative stress as indicated by peroxidation of lipids (malondialdehyde formation). Moreover, the importance of iron in the toxicity of acetaminophen has been shown in both rat and mouse hepatocytes by numerous investigators (Adamson and Harman 1993; Ito et al. 1994; Kyle et al. 1987). Collectively, these data

indicated that an iron chelator such as deferoxamine inhibited development of toxicity whereas addition of iron back to the incubation restored the sensitivity of the hepatocytes to acetaminophen toxicity. These data are consistent with Fenton mechanism-mediated oxidative damage playing a role in the hepatotoxicity of acetaminophen; however, the data do not rule out involvement of chelatable iron associated with a critical enzyme function or other critical protein as a mechanistic step in development of toxicity.

The discovery of nitric oxide as an important signaling molecule has led to a more in depth understanding of mechanisms of oxidative stress. Oxidative stress not only includes the classical Fenton-mediated mechanism but also involves nitric oxide. Nitric oxide reacts with superoxide at an extremely rapid rate ($\sim 9 \times 10^9 M^{-1} s^{-1}$) to form peroxynitrite. Peroxynitrite is both an oxidizing agent and a nitrating agent. It is detoxified by glutathione (Sies et al. 1997) which is depleted by NAPQI in acetaminophen-induced hepatotoxicity (Mitchell et al. 1973a,b). Peroxynitrite nitrates tyrosine, leading to formation of the unique biomarker 3-nitrotyrosine, and nitrated proteins have been used as unique biomarkers of nitrogen stress (Beckman and Koppenol 1996). Livers from mice treated with toxic doses of acetaminophen develop 3-nitrotyrosine in the hepatic proteins of the centrilobular regions. Nitrated tyrosine occurs in the same cells that contain acetaminophen-protein adducts, and the development of the nitrated protein correlates with the development of necrosis (Hinson et al. 1998). Figure 2b depicts an immunohistochemical analysis of livers of acetaminophen-treated mice for 3-nitrotyrosine in proteins. Note that nitrated proteins do not occur in the periportal areas of the livers of the acetaminophen-treated mice or in the livers of saline-treated mice (Fig. 2c). Figure 2a is a section adjacent to the section in Fig. 2b but it is immunochemically stained for acetaminophen-protein adducts. Immunohistochemical comparison of slides stained for acetaminophen-protein adducts (Fig. 2a) to slides stained for 3-nitrotyrosine adducts (Fig. 2b) indicate that all cells that contained acetaminophen protein adducts also contained nitrated proteins. These were the only cells with necrotic changes (Hinson et al. 1998). These data indicate that oxidative stress is occurring in the liver following a toxic dose of acetaminophen, and that the site of oxidative stress correlates with site of the toxicity. In a previous publication, we reported that the only cells that undergo necrotic changes contained acetaminophen-protein adducts (Fig. 1) (Roberts et al. 1991). Collectively, these data are consistent with a hypothesis that the only hepatocytes that develop necrosis are those where oxidative stress is occurring, and further suggest that reactive oxygen and nitrogen species are critical for development of acetaminophen toxicity.

The role of hepatic-inducible nitric oxide synthase (iNOS) has been investigated in the rat and in the mouse. Gardner and coworkers (Gardner et al. 1998) reported that toxic doses of acetaminophen to rats induced iNOS in the centrilobular hepatocytes. The development of toxicity correlated with the expression of iNOS. Moreover, treatment of rats with the iNOS inhibitor, aminoguanidine, decreased hepatotoxicity. The role of iNOS in the progression of acetaminophen toxicity was evaluated in iNOS knockout mice. Whereas ALT levels in acetaminophen-treated iNOS knockout mice were approximately 50% of those observed in wild-type mice,

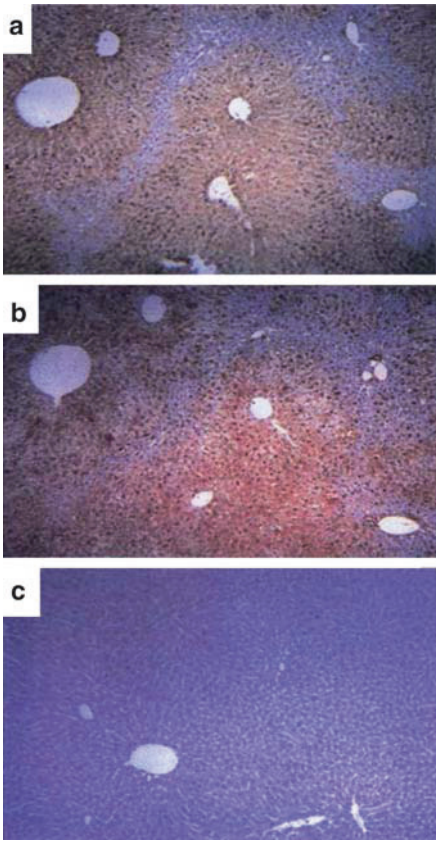

Fig. 2 Immunohistochemical comparison of cellular localization of acetaminophen-protein adducts to nitrotyrosine in hepatic proteins of mice treated with a toxic dose of acetaminophen. Mice were treated with acetaminophen (300 mg kg^{-1}) or saline and livers removed at 4 h. (**a**) Liver section was immunochemically stained for acetaminophen protein adducts using an antiacetaminophen antiserum. (**b**) Liver section was immunochemically stained for nitrotyrosine in protein using an antinitrotyrosine antiserum. Note that the liver sections in (**a**) and (**b**) were adjacent sections from the same liver. (**c**) Liver section from a saline-treated mouse stained for nitroytrosine in protein using antinitrotyrosine antiserum (Hinson et al. 1998)

there was no difference in histological scoring of the toxic injury to the liver (Michael et al. 2001). Similar results were reported by Bourdi et al. (2002). Also, the iNOS inhibitor aminoguanidine did not alter acetaminophen toxicity in mice (Hinson et al. 2002). Protein nitration was decreased but not eliminated in iNOS knockout mice (Michael et al. 2001). These data suggested that iNOS-mediated protein nitration was not an important factor leading to acetaminophen toxicity (Michael et al. 2001). However, Gardner reported that acetaminophen toxicity was decreased in iNOS knockout mice compared to the wild-type mice (Gardner et al. 2002). Thus, iNOS appears to play a role in nitration but the role in toxicity is unclear.

The mechanism of increased reactive oxygen/nitrogen species in acetaminophen toxicity has been investigated extensively. Three different mechanisms have been suggested to account for the increased level of reactive oxygen: uncoupled CYP2E1 or other enzymes (Koop 1992), activated NADPH oxidase (Sies and de Groot 1992), and mitochondrial uncoupling (Brand et al. 2004; Casteilla et al. 2001). By each mechanism, it is envisioned that increased superoxide production is the

critical event. The increased levels of superoxide can lead to increased hydrogen peroxide and peroxynitrite formation in the cell.

Work by Gonzalez and coworkers utilizing CYP2E1 null mice support the hypothesis that CYP2E1 plays a role in acetaminophen toxicity in vivo (Chen et al. 2008; Gonzalez 2007). CYP2E1 is a major CYP contributing to in the metabolism of acetaminophen to NAPQI; however, other CYPs including CYP1A2 and CYP3A4 also metabolize acetaminophen to NAPQI. CYP2E1 catalytic activity with characteristic uncoupling may be a source of increased oxidative stress in the hepatocyte. Uncoupling of oxygen consumption with NADPH oxidation produces superoxide leading to other reactive oxygen species such as hydrogen peroxide or peroxynitrite (Cederbaum 2006; Cheung et al. 2005; Gonzalez 2007). The importance of CYP2E1 in acetaminophen toxicity was demonstrated by the finding that CYP2E1 null mice were much less sensitive to acetaminophen hepatotoxicity than the wild-type mice or CYP1A2 null mice. The double null mice ($CYP2E1^{-/-}$; $CYP1A2^{-/-}$) were only mildly sensitive to the toxic effects of acetaminophen. Moreover, in CYP2E1 null mice in which the human *CYP2E1* gene was introduced as a artificial chromosome genomic clone of bacterial origin, the hepatotoxic effects of acetaminophen were restored to a significant extent (Cheung et al. 2005; Gonzalez 2007). The importance of CYP2E1-mediated oxidative stress in acetaminophen toxicity was supported in metabolomic studies where urine from acetaminophen-treated wild-type and CYP2E1 null mice were examined for the relative amounts of metabolites derived from NAPQI (acetaminophen-cysteine conjugate, acetaminophen-N-acetylcysteine conjugate, and acetaminophen-glutathione conjugate). In these studies, CYP2E1 null mice were much less sensitive to the toxic effects of acetaminophen; however, the urinary NAPQI derived metabolites at toxic doses of 200 and 400 mg kg^{-1} were not significantly different from those in wild-type mice. Moreover, maximal acetaminophen-induced hepatic depletion of glutathione was not different between the wild-type mice and the CYP2E1 null mice. These data suggested that metabolic formation of the toxic metabolite NAPQI was not different between wild-type and CYP2E1 null mice. However, there was a substantial difference at a therapeutic dose (10 mg kg^{-1}) which indicated the importance of CYP2E1 in metabolism at therapeutic doses. Importantly, it was shown that administration of the toxic doses of acetaminophen to the wild-type mice resulted in a significant increase in hepatic hydrogen peroxide concentrations. Thus, CYP2E1 appears to be a significant mechanism leading to increased reactive oxygen species in acetaminophen toxicity. These data suggest that Fenton-mediated oxidative stress may be an important event in acetaminophen toxicity and may explain why iron chelators block toxicity (Adamson and Harman 1993; Hinson et al. 2004; Ito et al. 1994; Kyle et al. 1987; Sakaida et al. 1995; Schnellmann et al. 1999).

Available data suggest that NADPH oxidase does not play a role in acetaminophen toxicity. NADPH oxidase is the major respiratory burst enzyme that generates superoxide formation in activated Kupffer cells. Mice that were deficient in gp91*phox*, the primary subunit of NADPH, were shown to have comparable toxicity to acetaminophen as wild-type mice (James et al. 2003c). Also, the mice

had similar levels of nitrated protein. In addition, treatment with the NADPH oxidase inhibitors diphenylene iodonium chloride or apocynin did not reduce toxicity in mice treated with acetaminophen (Cover et al. 2006).

Another mechanism of oxidative stress that has been investigated in acetaminophen toxicity is neutrophil-induced oxidant stress. Hypochlorite (hypochlorous acid) is produced by neutrophils by a mechanism involving myeloperoxidase utilization of hydrogen peroxide and chloride ions. The resultant hypochlorite reacts with tyrosine residues to form 3-chlorotyrosine. It was recently reported that chlorotyrosine adducts are not detected in the livers of acetaminophen-treated mice (Hasegawa et al. 2005), but these adducts are readily detected in endotoxemia, a known neutrophil-mediated hepatotoxic injury (Gujral et al. 2004). Nonetheless, Liu and coworkers reported that depletion of neutrophils in mice by treatment with anti-Gr-1 antibody (RB6-8C5) significantly protected mice against acetaminophen-induced liver injury, as evidenced by markedly reduced serum ALT levels, centrilobular hepatic necrosis, and improved mouse survival (Liu et al. 2006). However, the role of neutrophils in the development of acetaminophen toxicity has been questioned because substantial recruitment does not occur until after acetaminophen-induced liver injury in the mouse (Jaeschke and Hasegawa 2006; Lawson et al. 2000).

6 Mitochondrial Injury as a Critical Alteration in Acetaminophen Toxicity

A number of studies have examined the importance of mitochondrial dysfunction in acetaminophen toxicity. Electron microscopic examination of livers from acetaminophen treated livers by Racz's laboratory indicated alterations in mitochondrial morphology (Walker et al. 1980). Jollow et al. reported that mitochondria were a target for the acetaminophen-reactive metabolite (Jollow et al. 1973), and a number of arylated proteins were found in mitochondria (Bulera et al. 1996; Pumford et al. 1990). Functional alterations in the ability to sequester calcium have been reported (Tirmenstein and Nelson 1989). Inhibition of mitochondrial respiration at complexes I and II, but not at complex III was reported in isolated rat hepatocytes (Burcham and Harman 1991) and in vivo (Donnelly et al. 1994). In addition, ATP levels decrease in vivo and in treated hepatocytes (Burcham and Harman 1991; Vendemiale et al. 1996). Similar changes have been shown by adding NAPQI to hepatocytes (Andersson et al. 1990). Moldeus and Orrenius's laboratory reported that addition of NAPQI to isolated rat liver mitochondria caused release of sequestered calcium (Weis et al. 1992, 1994).

Mitochondrial permeability transition (MPT) has emerged as a likely mechanism in acetaminophen-induced hepatotoxicity (Kon et al. 2004; Masubuchi et al. 2005; Reid et al. 2005). Briefly, MPT represents an abrupt increase in the permeability of the inner mitochondrial membrane to ions and small molecular weight solutes. Oxidants such as peroxides and Ca^{++} promote MPT, and hepatocyte levels

of peroxides (Chen et al. 2008) have been reported to increase in acetaminophen toxicity as well as alterations in calcium homeostasis (Corcoran et al. 1987; Nicotera et al. 1989; Tirmenstein and Nelson 1989; Tsokos 1989). Associated with the permeability change are inner mitochondrial membrane depolarization, uncoupling of oxidative phosphorylation, release of intramitochondrial ions and metabolic intermediates, mitochondrial swelling, and decreased ATP synthesis. As established above, MPT is promoted by oxidative stress and MPT is in turn associated with a very large increase in oxidative stress. Cyclosporine A specifically blocks MPT in a saturable manner. The block is believed to occur at a protein channel or pore that transports both anionic and cationic solutes of masses less than 1,500 Da, which may be the same structure as the voltage-dependent anion channel (VDAC), an essential element of the pore. Three proteins are believed to be in the MPT pore: the adenine nucleotide translocator, cyclophilin D, and the voltage-dependent anion channel. Whereas the adenine nucleotide translocator does not appear to be essential for development of MPT, cyclophilin D is a necessary component (Baines et al. 2005; Kokoszka et al. 2004). A number of oxidants can lead to pore opening including t-butylhydroperoxide (t-BuOOH) (Nieminen et al. 1997) and peroxynitrite (Packer et al. 1997). Addition of t-BuOOH to hepatocytes results in oxidation of pyridine nucleotides and generation of reactive oxygen species (ROS) within the cell. Subsequently, onset of MPT occurs, mitochondria depolarize, ATP levels decrease, and hepatocytes lose viability. Under normal conditions, mitochondria produce small amounts of ROS. However, after t-BuOOH treatment, mitochondrial ROS increase 15-fold as determined by confocal microscopy studies monitoring the conversion of dichlorodihydrofluorescein ($DCFH_2$) to the fluorescent product dichlorofluorescin (DCF) (Myhre et al. 2003; Nieminen et al. 1997). A major source of mitochondrial ROS may be the reaction of ubisemiquinone (generated in the cytochrome bc1 complex by the Q cycle) with oxygen leading to formation of superoxide. Importantly from an experimental perspective, Cyclosporine A, which binds avidly to cyclophilin D, will block MPT (Kowaltowski et al. 2001).

Blockade of acetaminophen toxicity both in vitro and in vivo by MPT inhibitors has been reported. Kon and coworkers (Kon et al. 2004, 2007) showed that acetaminophen toxicity in cultured mouse hepatocytes was inhibited by cyclosporine A and by the nonimmunosuppressive Cyclosporine A analog NM811. Cyclosporine A was shown not to alter acetaminophen-induced glutathione depletion indicating that the prevention of toxicity did not occur by inhibition of metabolism of acetaminophen to NAPQI. Toxicity was shown to occur with loss of mitochondrial membrane potential by using tetramethylrhodamine methyl ester (TMRM) and fluorometric analysis. Reid and coworkers (Reid et al. 2005) examined the effect of MPT inhibitors in freshly isolated mouse hepatocytes using the approach of Boobis and coworkers (Boobis et al. 1986; Tee et al. 1986). In these studies, acetaminophen was incubated with the hepatocytes for 2 h, the hepatocytes were washed free of acetaminophen, and subsequently the hepatocytes were incubated with media alone. This approach clearly separated direct metabolic effects from effects occurring downstream of acetaminophen metabolism. As previously

reported, toxicity occurred in the reincubation phase (3–5 h). Inclusion of the MPT inhibitors Cyclosporine A, trifluoperazine, or dithiothreitol in the reincubation phase completely inhibited toxicity. Also, addition of N-acetylcysteine in the reincubation phase completely inhibited toxicity. Toxicity was shown to correlate with loss of mitochondrial membrane potential by utilizing the dyes TMRM and JC-1. The loss of mitochondrial membrane potential was prevented by addition of cyclosporine A and N-acetylcysteine in the reincubation phase. Utilization of the redox sensitive dye dichlorodihydrofluorescin indicated that toxicity occurred with a large increase in reactive oxygen species in the reincubation phase. The large increase in oxidative stress was eliminated by addition of Cyclosporine A or N-acetylcysteine to the reincubation phase. These data are consistent with acetaminophen metabolism leading to glutathione depletion and covalent binding occurring in the initial 2 h incubation. Subsequently, MPT occurs with loss of mitochondrial membrane potential, a large increase in oxidative stress, and toxicity (Reid et al. 2005).

McLean and coworkers have reported that inhibitors of MPT (cyclosporine A and trifluoperazine) inhibited acetaminophen toxicity in rat liver slices and in vivo when administered as a cocktail with fructose (Beales and McLean 1996; Nieminen et al. 1997). Also, Dimova et al. (1995) reported that the MPT inhibitor trifluoperazine decreased acetaminophen-induced hepatotoxicity in the mouse. Masubuchi et al. (2005) reported that Cyclosporine A decreased acetaminophen toxicity in mice. Since hepatic glutathione depletion was the same in acetaminophen-treated and acetaminophen plus cyclosporine A-treated mice , it was concluded that the decrease in toxicity was not mediated by inhibition of NAPQI formation. Moreover, they observed that acetaminophen caused a swelling of liver mitochondria and a decrease in mitochondrial membrane potential, both of which were eliminated by cotreatment with Cyclosporine A. Collectively, the data indicate that MPT is an important mechanism leading to acetaminophen toxicity.

Peroxynitrite may be an important oxidant produced in acetaminophen-induced MPT. As discussed above, acetaminophen-induced MPT occurred with increased oxidation of the redox-sensitive dye $DCFH_2$. This dye is readily oxidized by peroxynitrite but not by superoxide, hydrogen peroxide, or hypochlorous acid; however, it may be oxidized by peroxide plus a peroxidase or a Fenton mechanism (ferrous ions plus peroxide) (Crow 1997; Myhre et al. 2003). Peroxynitrite is known to rapidly react with thiols such as N-acetylcyteine (Crow 2000), and N-acetylcysteine prevented acetaminophen-induced MPT and $DCFH_2$ oxidation (Reid et al. 2005). The finding that nitration was predominantly in mitochondria of acetaminophen-treated mice supports the hypothesis that peroxynitrite formation occurred in that organelle (Cover et al. 2005). As pointed out above, the NOS isoform was probably not iNOS, which suggests involvement of another NOS species such as mitochondrial nitric oxide synthase (mtNOS) (Ghafourifar and Cadenas 2005).

Whereas necrosis appears to be mediated by opening of the MPT pore in the inner mitochondrial membrane leading to loss of ability to produce ATP, apoptosis may also be mediated by opening of a pore or channel in the outer mitochondrial membrane, the mitochondrial apoptosis-induced channel. This pore is believed to

release proapoptotic factors including cytochrome C, endonuclease G, Smac/Diablo, and apoptosis-inducing factor (AIF) from the membrane space into the cytosol (Dejean et al. 2006b; Kinnally and Antonsson 2007). The development of mitochondrial apoptosis-induced channels appear to be sensitively regulated by Bcl-2 family proteins, and consists of proapoptotic proteins such as Bax and anti-apoptotic proteins such as Bcl-2. In particular, the proapoptotic protein Bax is normally in the cytosol. Activation results in translocation of Bax to mitochondria, insertion into the outer membrane, and oligomerization to form the channels (Antonsson et al. 2000; Dejean et al. 2005, 2006a). These channels release apoptotic proteins (Boelsterli and Lim 2007).

The relative amount of ATP appears to be an important factor relative to whether the hepatocytes dies by necrosis or apoptosis. Low ATP levels are associated with necrosis whereas adequate ATP levels favor apoptosis. With acetaminophen, Kon and coworkers showed that cultured mouse hepatocytes died primarily by necrosis. However, inclusion of fructose, an ATP generating glycolytic substrate, and glycine, a membrane stabilizer, in the media decreased necrosis and promoted apoptosis (Kon et al. 2004).

7 Inflammation, Cytokines and Chemokines in Development of Acetaminophen Toxicity

The complex role of inflammatory cells and cytokines in the mediation of acetaminophen toxicity has been investigated for over 20 years. Initial investigations to understand inflammatory responses that occur in the liver during acetaminophen toxicity were performed by Laskin and coworkers. They reported that acetaminophen toxicity occurred with activation of Kupffer cells (hepatic macrophages) (Laskin and Pilaro 1986; Laskin et al. 1986). Subsequent research indicated that Kupffer cell activation led to increases in both pro-inflammatory and anti-inflammatory cytokines. Cytokines have important functions in immunity, inflammation, cell proliferation, differentiation, and cell death (Shen and Pervaiz 2006). TNF-α has been linked to increased oxidative stress, (increased formation of reactive oxygen species and reactive nitrogen species) and is known to recruit and activate other inflammatory cells (Gardner et al. 2003). Blazka and coworkers showed dramatic increases in serum levels of TNF-α and IL-1α in mice treated with acetaminophen. (Blazka et al. 1995b). Moreover, they reported that treatment of acetaminophen-intoxicated mice with either anti-TNF-α or anti-IL-1α partially prevented hepatotoxicity (Blazka et al. 1996). In further studies, it was shown that the Kupffer cell inactivators, gadolinium chloride and dextran sulfate, decreased acetaminophen toxicity in the rat (Laskin et al. 1995) and the mouse (Blazka et al. 1995a; Chen et al. 1999). These data, coupled with the proinflammatory cytokine data, suggested that Kupffer cells mediated proinflammatory responses in acetaminophen toxicity. However, more recent work brings into

question the mechanistic role of Kupffer cell activation in acetaminophen toxicity. Ju et al. (2002) treated mice with liposome-encapsulated chlodronate (dichloromethylene disphosphonate) to more completely eliminate Kupffer cells. Subsequent, treatment of the mice with acetaminophen resulted in reduced mRNA levels for TNF-α and other cytokines (IL-10, Il-6, COX-2, Il-18Bp, Clq) and there was an *increase* in liver toxicity as measured by serum ALT. Ju and colleagues postulated that these findings suggest alternative roles for Kupffer cells in the toxicity, and suggest that Kupffer cells may counteract inflammation or have a role in liver repair. Further studies are needed to elucidate the role of Kupffer cells and other cells of the innate immune system in the regulation of inflammation or the initiation of liver repair.

Other data have not supported a role for TNF-α in the development of acetaminophen toxicity. Wild-type and TNFα knockout mice had equal sensitivity to acetaminophen (Boess et al. 1998), and treatment with anti-TNF antibody or soluble TNF receptor did not alter acetaminophen toxicity in mice (Simpson et al. 2000). Subsequent work by James reported no difference in toxicity between TNFR1 knockout mice and wild-type mice (James et al. 2005). Conflicting data from these studies, compared to the findings of Blaska (Blazka et al. 1995b, 1996), may be related to strain or dose differences, or variations in repair processes among mice strains.

Other proinflammatory cytokines such as interleukin one beta (IL-1β) and interferon gamma (IFNγ) have also been examined in acetaminophen toxicity (Blazka et al. 1995b; Gardner et al. 2003; James et al. 2003d). Ishida et al. (2002) and Liu et al. (2004) reported that IFNγ mRNA was induced in livers of acetaminophen-treated mice. The importance of IFNγ in acetaminophen toxicity was investigated in wild-type and IFNγ knockout mice (Ishida et al. 2002). IFNγ knockout mice were shown to have reduced toxicity compared to wild-type mice, as indicated by serum ALT and AST levels and by histopathological evaluation of necrosis. In addition, the IFNy knockout mice had reduced mortality. The IFNγ knockout mice had significant attenuation of the mRNA transcripts for inflammatory cytokines, chemokines, adhesion molecules, Fas, and inducible nitric oxide synthase (iNOS) compared with wild-type (WT) mice. (Ishida et al. 2002). Ishida et al. further showed that treatment of mice with an anti-IFNγ antibody lowered ALT values in APAP-treated mice. Liu and coworkers examined the role of natural killer cells (NKT) and natural killer T cells, and reported these were the primary cell type important in IFNγ production in acetaminophen toxicity. However, other investigators did not find IFNγ induction in livers of acetaminophen treated mice (Gardner et al. 2003). Pohl's laboratory reported that acetaminophen administered in a saline solution did not increase hepatic IFNγ mRNA; however, when administered with DMSO there was an induction of IFNγ (Masson et al. 2008). DMSO alone was shown to increase the number of hepatic NKT cells and to activate both NKT and NK cells to produce IFNγ and granzyme B. Thus, as pointed out by Masson et al. (2008), the utilization of DMSO to facilitate dissolution may be a confounding factor.

Interleukin-6 (IL-6) has also been examined in acetaminophen toxicity. While it is known to be important in hepatocyte regeneration (discussed below), one study

showed that depletion of IL-6 resulted in increased sensitivity to acetaminophen (Masubuchi et al. 2003). IL-6 knockout mice were found to have increased toxicity to acetaminophen and reduced formation of heat shock proteins (HSP) 25, 32, and 40, as well as inducible HSP70. HSPs are induced in cells by exogenous stressors, and the presence of nonnative proteins is thought to be a trigger for their induction. Salminen et al. (1998) showed that the combined treatment of mice with acetaminophen and N-acetylcysteine, protected from toxicity, and attenuated but did not prevent adduct formation or HSP induction. Pretreatment with diallyl sulfide, a CYP2E1 inhibitor, abolished HSP25 and HSP70i induction and toxicity. These data suggest that CYP2E1-mediated oxidative stress played a mechanistic role in HSP induction in acetaminophen toxicity.

The role of anti-inflammatory cytokines in acetaminophen toxicity has also been examined. Interleukin 10, 11, and 13 are anti-inflammatory cytokines that are known to modulate the pro-inflammatory response in hepatic injury (Bourdi et al. 2002; Louis et al. 1997a, b; Yee et al. 2007; Zingarelli et al. 2001). The liver is a major source of IL-10 and many cell types including activated Kupffer cells, and T and B lymphocytes produce IL-10. Bourdi et al. (2002) reported that IL-10 knockout mice had increased toxicity to acetaminophen compared to wild-type mice. In addition, IL-10 knockout mice had increased mRNA transcripts for the pro-inflammatory cytokines TNF-α and IL-1, as well as increased mRNA transcripts for iNOS and increased serum nitrate plus nitrite, a marker of NO. The anti-inflammatory cytokine IL-11 is known to be protective in a number of organ injury models (Fiore et al. 1998; Maeshima et al. 2004; Trepicchio et al. 2001) and may mediate protection by decreasing pro-inflammatory cytokine production or decreasing macrophage activation. Trepicchio et al. (2001) showed that pretreatment of mice with recombinant human IL-11 lowered ALT values and TNF-α levels by approximately one-half the values of non-IL-11-treated mice. In addition, histologic measures of hepatotoxicity were improved. More recently, IL-13 has been examined for its hepatoprotective role in acetaminophen toxicity. Pretreatment of mice with IL-13 reduced acetaminophen toxicity- and IL-13 knockout mice were sensitive to the toxic effects of acetaminophen. In further studies, IL-13 was shown to modulate IFN-γ, nitric oxide, and inflammatory cells, including neutrophils, NK cells, and NKT cells (Yee et al. 2007).

A recent study examined acetaminophen toxicity in two strains of mice that had distinct inflammatory and cytokine profiles (Cover et al. 2006). Despite the greater predominance of neutrophils and pro-inflammatory gene expression in C3Heb/FeJ mice, and the predominance of anti-inflammatory gene expression in C57BL/6 mice, biochemical markers of toxicity and histopathology of the livers were equivalent in the two strains of mice. Cumulatively, available data suggest that a balance of pro- and anti-inflammatory cytokines is maintained in acetaminophen toxicity and that no one single pro-inflammatory or anti-inflammatory cytokine is *critical* to the mediation of the toxicity. While the animal data support the postulation that genetic variability in cytokine expression may be a contributing factor to acetaminophen susceptibility in man (Bourdi et al. 2002; Welch et al. 2006), few clinical studies have been performed in this area.

Chemokines also play a role in acetaminophen-induced toxicity. These low molecular weight cytokines were initially recognized for their role in the chemotaxis of lymphocytes. In addition, some classes of chemokines may have angiogenic, wound healing, cell proliferative, or antiinflammatory properties. Multiple laboratories have shown that chemokines are upregulated in acetaminophen toxicity (Bone-Larson et al. 2001b; Hogaboam et al. 1999a, 2000b; James et al. 2001 ; Osawa et al. 2002).

The prototype chemokine, macrophage inhibitor protein 2 (MIP-2), is a member of the CXC chemokine family and is produced by many cell types in response to the pro-inflammatory cytokines, TNF-α and IL-1β. Jaeschke's laboratory showed that chemokine upregulation (MIP-2 and KC) and neutrophil accumulation followed the onset of acetaminophen toxicity in mice, and inactivation of B_2 integrins with an anti-CD18 antibody (e.g., neutrophil inactivation) did not alter toxicity (Lawson et al. 2000). Hepatocytes exposed to acetaminophen develop toxicity in the absence of neutrophils (Moldeus 1978; Reid et al. 2005). Mouse strains with differing degrees of neutrophil accumulation had similar toxicity to acetaminophen, suggesting that neutrophils are not mechanistically important in acetaminophen toxicity (Cover et al. 2006), but this is a controversial area. While it has been postulated that the primary role of neutrophil influx in acetaminophen toxicity is that of removal of damaged cells and cellular debris (Lawson et al. 2000), a more recent study using the anti-Gr-1 antibody (RB6-8C5) to neutrophils, showed that toxicity was significantly attenuated with neutrophil depletion in acetaminophen-treated mice (Liu et al. 2006). Several lines of evidence support the potential role of the chemokine MIP-2 as a hepatoprotective factor in acetaminophen toxicity. Hogaboam's laboratory (Hogaboam et al. 1999a) reported that treatment with MIP-2 was more effective as a "late therapy" given to acetaminophen-treated mice than the antidote N-acetylcysteine. In vitro data showed that MIP-2 maintained hepatocyte proliferation in cells exposed to acetaminophen. In addition, adenoviral vector delivery of MIP-2 reduced toxicity in acetaminophen-treated mice (Hogaboam et al. 1999a, b).

The mechanism of the protective effects of the CXC chemokines in acetaminophen toxicity is poorly understood. Some data suggest that MIP-2 may lead to the increased nuclear localization of the transcription factor signal transducer and activator of transcription 3 (STAT3), a major signal transduction factor important in hepatocyte regeneration (Hogaboam et al. 1999a, 2000a; Ren et al. 2003). Another CXC chemokine, IP-10 (Interferon – inducible protein), does not have direct mitotic effects in vitro, but may be able to induce hepatocyte growth factor (HGF), a known mitogen (Koniaris et al. 2001). Bone-Larson et al. (2001a) found that IP-10 was protective in acetaminophen toxicity and that this protection was associated with induction of the MIP-2 receptor (CXCR2) on hepatocytes. Thus, the available data suggest that MIP-2 and IP-10 are important in cell proliferation (hepatocyte regeneration) in response to acetaminophen toxicity. One study suggested that chemokines may also dampen pro-inflammatory cytokine production in acetaminophen toxicity. Mice deficient in the primary receptor for the chemokine monocyte chemoattractant protein-1 (MCP-1) were found to have increased levels

of TNFα and IFNγ. Neutralization of these pro-inflammatory cytokines resulted in attenuation of toxicity to acetaminophen (Hogaboam et al. 2000b).

Very few studies have examined the role of cytokines and chemokines in acetaminophen toxicity in the clinical setting. Interleukin 8 (the human homologue of MIP-2) was shown to be increased in patients with acetaminophen toxicity and to correspond with markers of hepatic injury (James et al. 2001). Further investigation in this area may help to identify potential early determinants of severe cases of acetaminophen toxicity.

Another anti-inflammatory mechanism that has been reported to play a role in acetaminophen toxicity is cyclooxygenase-2 (COX-2) (Reilly et al. 2001). Whereas COX-1 is constitutively expressed and produces low levels of prostaglandins, COX-2 is inducible and plays a role in anti-inflammatory processes. COX-2 derived prostaglandins are recognized for their critical role in female reproduction, bone resorption, renal function, and mucosal defense. Importantly, prostaglandins have been reported to play a protective role in various hepatotoxicities (Quiroga and Prieto 1993). Reilly and coworkers showed that COX-2, but not COX-1, was induced in livers of acetaminophen-treated mice. Moreover, acetaminophen was more toxic with high lethality in COX-2 knockout mice compared to wild-type mice. Also, the COX-2 inhibitor celecoxib (Celebrex®), was found to increase acetaminophen toxicity (Reilly et al. 2001). These data are consistent with COX-2 playing an anti-inflammatory role in acetaminophen-induced hepatotoxicity.

8 Intracellular Signaling Mechanisms in Acetaminophen Toxicity

The c-Jun N-terminal kinases (JNKs), a subfamily of the mitogen-activated protein (MAP) kinases, have been shown to be activated by phosphorylation early in acetaminophen toxicity both in vitro and in vivo (Gunawan et al. 2006; Henderson et al. 2007; Latchoumycandane et al. 2006, 2007; Matsumaru et al. 2003). JNK activation may be mediated by reactive oxygen species as well as by TNF-α (Shen and Pervaiz 2006). Kaplowitz's laboratory (Gunawan et al. 2006; Matsumaru et al. 2003) reported that incubation of acetaminophen with mouse hepatocytes leads to induction of JNK activity, and that the induction of activity could be blocked by a specific JNK inhibitor (SP600125). *In vivo*, the inhibitor protected mice from the toxic effects of acetaminophen without altering acetaminophen-reactive metabolite formation. Acetaminophen-induced glutathione depletion and protein covalent binding were not altered by inhibitor treatment (Gunawan et al. 2006). Henderson and coworkers (Henderson et al. 2007) reported similar results and found that late administration of the SP600125 inhibited hepatic necrosis and was more effective than the antidote N-acetylcysteine in limiting the injury. In agreement with a role for JNK activation in acetaminophen toxicity, Latchoumycandane et al. (2007) found that the antirheumatic drug leflunomide inhibited acetaminophen-induced hepatic JNK activation and blocked development of acetaminophen toxicity in mice.

JNK activation may be a mechanism that is associated with the initiation of mitochondrial permeability transition (MPT) (Hanawa et al. 2008; Latchoumycandane et al. 2006, 2007). As discussed above, both JNK activation (Matsumaru et al. 2003) and MPT (Lemasters 1998) are known to occur as a result of increased oxidative stress. MPT leads to additional oxidative stress with loss of mitochondrial membrane potential and loss of the ability of the hepatocyte to synthesize ATP. Latchoumycandane et al. (2006, 2007) found that leflunomide protected mice from mitochondrial permeabilization. Direct evidence for a role of JNK activation in acetaminophen-induced MPT was recently reported by Hanawa et al. (2008). A time course of events indicated GSH depletion by 1–2 h, JNK activation in liver homogenate by 2–4 h, JNK translocation to mitochondria by 4 h, and increased toxicity (serum ALT by 6 h). The JNK inhibitor did not alter GSH depletion but blocked JNK activation in homogenate, JNK translocation to mitochondria, and toxicity. Mitochondria from liver of acetaminophen-treated mice showed decreased State III respiration and decreased respiratory control ratios, whereas mice treated with acetaminophen plus JNK inhibitor were partially protected from these losses. Addition of activated JNK1 or JNK2 to mitochondria from acetaminophen-treated mice plus JNK inhibitor showed a decrease in State III respiration and decreased respiratory control ratio. Addition of the MPT inhibitor cyclosporine A prevented these decreases. It was hypothesized that activated JNK is an important mediator of acetaminophen-induced MPT (Hanawa et al. 2008).

Acetaminophen-induced hepatotoxicity has also been examined in knockout mice. JNK1 knockout mice and wild-type mice were found to be equally sensitive to the toxic effects of acetaminophen in three different laboratories (Gunawan et al. 2006; Henderson et al. 2007; Bourdi et al. 2008). However, data on the role of JNK-2 are confusing. Initially, Gunawan et al. (2006) found JNK-2 knockout mice to be less sensitive to the toxicity than wild-type mice. They suggested that JNK acts downstream of metabolism in acetaminophen toxicity. However, Henderson et al. (2007) found that disruption of either JNK1 or JNK2gnenes did not protect against acetaminophen-induced liver toxicity in mice. Since administration of a JNK inhibitor blocked both JNK-1 and JNK-2, it was suggested that inhibition of both may be important in toxicity. However, Bourdi et al. recently reported JNK-2 knockout mice to be more sensitive to acetaminophen toxicity (Bourdi et al. 2008; Gunawan et al. 2006; Henderson et al. 2007). Bourdi suggested that JNK-2 modulated hepatocellular regeneration and repair. Thus, understanding the role of JNK in acetaminophen toxicity will require additional research.

DNA fragmentation is another mechanism that has been implicated in acetaminophen-induced hepatotoxicity (Salas and Corcoran 1997). Genomic DNA fragmentation in liver (TUNEL assay and DNA laddering) following hepatotoxic doses of acetaminophen in the mouse was originally reported by Corcoran's laboratory (Ray et al. 1990, 1993). They found that the rate of DNA fragmentation paralleled the rate of development of hepatotoxicity and was associated with an increase in nuclear calcium levels. Similarly, acetaminophen-induced cytotoxicity in cultured mouse hepatocytes was found to occur with DNA fragmentation and nuclear calcium accumulation (Shen et al. 1991). The presence of ladder-like DNA fragments

were observed indicating the involvement of a calcium-dependent endonuclease. Aurintricarboxylic acid, a general calcium-endonuclease inhibitor, and EGTA, a chelator of calcium required for endonuclease activation, significantly decreased DNA fragmentation and toxicity (Shen et al. 1992). The calcium-calmodulin antagonist chlorpromazine and the calcium channel blocker verapamil decreased acetaminophen-induced hepatic necrosis and deceased DNA fragmentation in acetaminophen-treated mice (Ray et al. 1993).

Subsequently, it was reported that endonuclease G was important in the acetaminophen-induced nuclear fragmentation. This endonuclease is present in the mitochondria and is released under conditions of outer mitochondrial membrane permeabilization. Whereas endonuclease G was found in control mitochondria, incubation with acetaminophen resulted in trafficking of the protein to the nucleus, and the relative rate of trafficking correlated with rate of development of acetaminophen-induced loss of mitochondrial membrane potential and nuclear DNA fragmentation (Bajt et al. 2006). Subsequently, they investigated the role of Bax in acetaminophen-induced endonuclease G and apoptosis-inducing factor (AIF) trafficking to the nucleus (Bajt et al. 2008). Bax had been reported to localize in the mitochondria in acetaminophen toxicity (Adams et al. 2001; El-Hassan et al. 2003) and is well recognized to induce apoptosis-induced channels in the mitochondria with release of apoptotic proteins including endonuclease G (Antonsson et al. 2000; Dejean et al. 2005, 2006a). In Bax knockout mice, it was shown that the rate of development of acetaminophen toxicity in mouse hepatocytes was slower than in wild-type hepatocytes. At 6 h, toxicity and DNA fragmentation were much less in the Bax knockout mice than in the wild-type mice, but protein nitration was similar. However, at 12 h toxicity, DNA fragmentation and protein nitration were not different. These data suggested that in the acetaminophen toxicity model, Bax was playing a role in mitochondrial outer membrane permeabilization with formation of mitochondrial apoptosis-induced channels and release of intermembrane proteins (Bajt et al. 2008). The relationship between development of the mitochondrial apoptosis-induced channel with release of apoptotic proteins and mitochondrial permeability transition in development of necrosis is poorly understood and will require further investigations.

9 Mechanisms of Repair of Acetaminophen Liver Toxicity

Following liver injury, in an attempt to restore homeostasis, a complex series of events occur in the liver. Proliferation of all existing mature cellular populations occurs, beginning with hepatocytes (Michalopoulos and DeFrances 1997). Entry into and progression of hepatocytes through the early G_1 phase of the cell cycle, or *priming*, is mediated by TNF-α and interleukin 6 (IL6) (Diehl et al. 1994; Li et al. 2002). TNF-α has potent mitotic effects in vitro and in vivo (Gallucci et al. 2000). Diehl showed that TNF-α neutralizing antibodies administered to rats prior to partial

hepatectomy decreased DNA synthesis and the activation of signaling pathways involved in hepatocyte regeneration (Diehl et al. 1994). Mice deficient in the TNF receptor one (TNFR1) have reduced DNA synthesis after partial hepatectomy and reduced activation of signal transduction factors (STAT 3) (Li et al. 2001, 2002; Yamada et al. 1997).

Several laboratories have examined the role of the TNF-α receptor 1 (TNFR1) in acetaminophen toxicity. Toxicity was increased in the TNFR1 knockout mice compared to the wild-type mice in three studies (Chiu et al. 2003a, b; Gardner et al. 2002; James et al. 2005). Chiu et al. showed that TNFR1 knockout mice had alterations in antioxidant expression following acetaminophen toxicity (Chiu et al. 2003a). Restoration of hepatic glutathione was delayed and heme oxygenase-1 and CuZn superoxide dismutase expression were reduced in the knockout mice. Two laboratories showed that TNFR1 was important in hepatocyte regeneration following acetaminophen toxicity in the mouse (Chiu et al. 2003b; James et al. 2005). In addition, TNFR1 knockout mice had higher levels of chemokines (MIP-2, IP-10) (James et al. 2005), which have been implicated to have a role in hepatocyte regeneration following acetaminophen toxicity (Hogaboam et al. 1999a, b). The data illustrate the pleotropic properties of TNF-α in acetaminophen toxicity and also suggest a redundancy of pathways for hepatocyte regeneration and recovery following acetaminophen toxicity in the mouse.

IL-6 is closely related to TNF-α, and is known to have promitotic effects on various cells types. TNF-α upregulates IL-6 via the signal transduction factor NF-kB, and IL-6 activates STAT3. STAT3 is signal transduction factor that activates a large number of genes important in hepatocyte regeneration (Li et al. 2001, 2002). IL-6 knockout mice had reduced hepatocyte regeneration, as measured by the expression of proliferating cell nuclear antigen (PCNA), following acetaminophen toxicity (James et al. 2003a). PCNA is an auxiliary protein for DNA polymerase delta and a biomarker of increased cellular proliferation (Essers et al. 2005). Treatment of the knockout mice with murine IL-6 normalized PCNA expression following acetaminophen toxicity. Similar findings were reported by Bajt et al. (2003). Decreased activation of a number of genes important in liver regeneration (e.g., c-Fos, c-Myc, LRF-1, STAT3, and JunB) have been shown in IL-6 knockout mice (Cressman et al. 1996). In addition to these studies, it is likely that multiple mechanisms for repair of the liver following acetaminophen toxicity are operative. For example, TNF-α may be linked to hepatocyte regeneration via mechanisms other than IL-6 activation. TNF-α can increase the expression of transforming growth factor-β (Kalthoff et al. 1993; Tamura et al. 1993), a known mitogen for hepatocytes and other liver-derived cells.

Growth factors are closely related to cytokines and may have overlapping effects. Vascular endothelial growth factor (VEGF) is expressed by endothelial cells and is a critical mitogen and survival factor for endothelial cells. It is also the major regulator of angiogenesis during organ development and differentiation during embryogenesis (reviewed by Ferrara 2001) and a critical mediator of angiogenesis in cancer. VEGF induces the expression of antiapoptotic proteins

in human endothelial cells, suggesting that it promotes the survival of these cells. VEGF mRNA transcripts and protein are expressed by other cell types as well, including hepatocytes (Mochida et al. 1996) and hepatic stellate cells (Ankoma-Sey et al. 2000). A number of factors may regulate VEGF, including hypoxia, cytokines, iNOS, and hyperglycemia (Ankoma-Sey et al. 2000; Yamada et al. 2003). VEGF has two primary receptors, VEGFR1 and VEGFR2, that have distinct roles. VEGFR2 appears to mediate the angiogenic properties of this growth factor, whereas emerging data suggest that VEGR1 may have promitotic effects.

VEGF has been shown to have mitogenic effects on hepatocytes by orchestrating interactions between endothelial cells and hepatocytes. LeCouter et al. (2003) showed that mice injected with a VEGF-producing cell line (Chinese hamster ovarian cells) had greater liver weights and increased mitotic counts compared to mice treated with vehicle control (Kim et al. 2002). However, VEGF itself had no direct effect on hepatocyte mitosis in vitro. Activation of VEGFR1 in vitro resulted in significant increases in the release of mitogens by endothelial cells (LeCouter et al. 2003). These mitogens included the known hepatocyte mitogens: hepatocyte growth factor (HGF) and IL-6. Treatment of mice with VEGFR1 and VEGFR2 ligands was shown to be protective in carbon tetrachloride toxicity; however, treatment with the VEGR2 ligand resulted in increased endothelial cell proliferation.

The role of VEGF in acetaminophen toxicity and repair has been examined in both the mouse and rat (Donahower et al. 2006; Papastefanou et al. 2007). Hepatic VEGF levels were increased 30-fold in mice treated with acetaminophen and followed the onset of toxicity (Donahower et al. 2006). Peak elevation of hepatic VEGF was apparent at 8 h and remained increased until 48 h. Induction of VEGFR1 peaked at 48 h and induction of VEGFR2 peaked at 8 h. Similar time course data were demonstrated in acetaminophen-treated rats. In further studies, mice were treated with SU5416, an inhibitor of VEGFR2-mediated signaling. Treatment of mice with SU5416 did not alter the metabolism of acetaminophen, but lowered ALT values and increased PCNA expression (hepatocyte regeneration) (Donahower et al. 2006). In further studies, the role of exogenous treatment with VEGF in acetaminophen toxicity in the mouse was examined. Treatment with VEGF lowered ALT values at 8, 18, 24, and 36 h and reduced the relative area of necrosis (Donahower et al. 2007). VEGF had no effect on glutathione depletion or covalent binding. In addition, VEGF enhanced the expression of PCNA in the late stages of acetaminophen toxicity (18, 24, 36 h). The mechanism for the hepatoprotection of VEGF in acetaminophen toxicity is unclear. It is possible that VEGF may have improved hepatic blood flow or altered hepatic microcirculation but further studies are needed to assess this potential effect in vivo. In addition, the VEGF-treated mice had reduced production of IL-6, possibly indicating internal regulation of the expression of this promitotic cytokine. Further studies are needed to elucidate the mechanisms of VEGF hepatoprotective effects and to test the growth factor further as a potential late phase therapy for acetaminophen toxicity.

10 Conclusion

A significant impediment to a more in-depth understanding of the mechanisms of acetaminophen toxicity has been conflicting data from different laboratories regarding various modulators of toxicity. A major problem is that hepatic CYP enzymes or glutathione levels important in metabolic activation of acetaminophen and detoxification may be altered by treatments or genetic modification of the animal. For example, CYP2E1 may be inhibited by solvents such as DMSO, propylene glycol, or ethanol used in the administration of inhibitors. This results in decreased formation of NAPQI, less glutathione depletion, less covalent binding, and less toxicity. Figure 1 is included to show the relationship between acetaminophen metabolism (covalent binding) and development of toxicity. The only hepatocytes that developed necrosis had acetaminophen-protein adducts. Since covalent binding occurs only under conditions of glutathione depletion, the affected hepatocytes are believed to have very low concentrations of glutathione. This predisposes them to increased oxidative stress which is shown in Fig. 2. The only hepatocytes that had increased oxidative stress (nitrated proteins indicating peroxynitrite formation) contained acetaminophen-protein adducts. Thus, understanding how modulators affect toxicity requires an understanding of their effects on metabolism and oxidative stress.

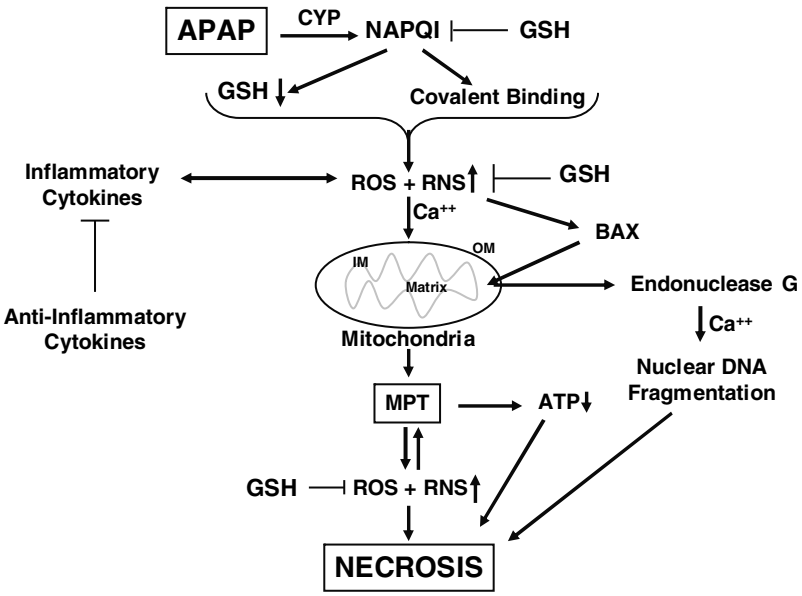

Fig. 3 Mechanistic determinants in acetaminophen-induced hepatic necrosis. *APAP* Acetaminophen, *NAPQI* N-Acetyl-*p*-benzoquinone imine, *CYP* cytochrome P-450, *GSH* reduced glutathione, *ROS* reactive oxygen species, *RNS* reactive nitrogen species, *om* outer membrane, *im* inner membrane, *MPT* mitochondrial permeability transition, *BAX* Bcl-2-associated X protein

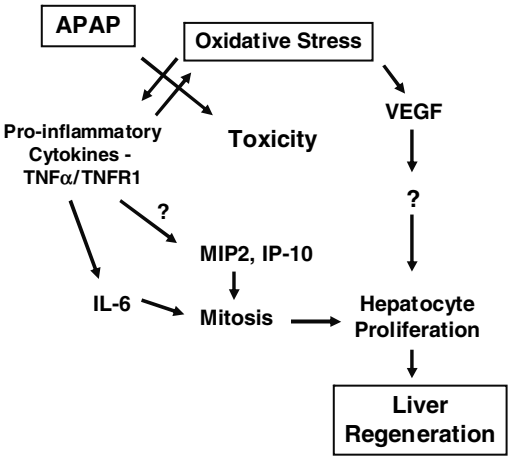

Fig. 4 Mechanisms determinants in repair of acetaminophen-induced hepatic necrosis. *VEGF* Vascular endothelial growth factor, *TNF-α* tumor necrosis factor alpha, *TNR1* tumor necrosis factor alpha receptor 1, *MIP-2* macrophage inflammatory protein, *IP-10* Interferon-γ-inducible protein, *IL-6* Interleukin-6

In summary, the hepatotoxicity of acetaminophen appears to occur by a complex mechanistic sequence (Fig. 3). These events include: (1) CYP metabolism to the reactive metabolite NAPQI which depletes glutathione by a conjugation reaction and covalently binds to proteins; (2) loss of glutathione causing an increased oxidative stress response (decreased detoxification of reactive oxygen and nitrogen species); (3) increased oxidative stress, possibly associated with alterations in calcium metabolism, initiation of signal transduction responses and mitochondrial permeability transition; (4) mitochondrial permeability transition occurring with an even larger increase in oxidative stress, loss of mitochondrial membrane potential, and loss of the ability of the mitochondria to synthesize ATP; and (5) loss of ATP which causes necrosis. Associated with these essential events there appears to be a number of modulators of inflammatory responses that can alter the severity of liver injury following the initiation of toxicity. Further study of the interactions of these mediators with each other and the interplay of the immune cells that produce them will help to elucidate the significance of their roles in acetaminophen toxicity. In addition, studies are needed to examine the effect of inflammatory mediators on oxidative stress and/or signal transduction responses in acetaminophen toxicity. In conjunction with these inflammatory events, apoptotic responses occur; however, the loss of ability of the hepatocyte mitochondria to produce ATP may be the single most important event causing necrosis. Finally, the liver has a very great capacity to regenerate and various cytokines and growth factors are major initiators of this process (Fig. 4). Future investigations to elucidate the signaling for these pathways may help to identify novel targets for the treatment of acetaminophen toxicity.

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Drug-Induced Liver Injury in Humans: The Case of Ximelagatran

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Contents

Abstract Ximelagatran was the first orally available direct thrombin inhibitor under clinical development that also reached the market. Ximelagatran was tested in an extensive clinical programme. Short-term use (<12 days) in humans including the phase III clinical trials did not indicate any hepatotoxic potential. Increased hepatic enzyme levels were first observed at a higher frequency when evaluating the long-term (>35 days) use of ximelagatran (incidence of >3× upper limit of normal (ULN) plasma ALT was 7.9%). The frequency of elevated total bilirubin levels was similar in the ximelagatran and the comparator groups. However, the combination of ALT > 3×ULN and total bilirubin > 2×ULN was 0.5% among patients treated with ximelagatran and 0.1% among patients in the comparator group. Symptoms such as fever and rash potentially indicating hypersensitivity (immunologic type of reaction) were low and did not differ between ximelagatran and the comparators. The withdrawal of ximelagatran from the market and termination of the ximelagatran development program was triggered by safety data from a 35-day study, indicating that severe hepatic injury in a patient could develop after exposure to the drug has been completed and that regular liver function monitoring may not

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mitigate the possible risk of severe hepatic injury. As for many drugs causing liver injury, the standard preclinical toxicological studies provided no indication that ximelagatran affected hepatic functions. In addition, extensive investigations using human-based in vitro models have not been able to define mechanisms explaining the pattern of hepatic injury observed in long-term clinical trials. A pharmacogenomic study provided evidence that the ALT increases were associated with major histocompatibility complex (MHC) alleles DRB1'07 and DQA1*02 suggesting a possible immunogenic pathogenesis. This example provides important clues to the mechanism of idiosyncratic drug-induced liver toxicity.

Keywords Ximelagatran · Thrombin inhibitor · Pharmacogenetics · Transaminases · Bilirubin · In vitro liver toxicity models

1 The Clinical Picture

1.1 Background

New oral antithrombotic agents are under development with the aim to replace warfarin, a very effective anticoagulant but with serious side effects subject to slow onset of action, and an interaction profile including numerous foods and drugs. Patients on warfarin are also subject to frequent anticoagulation monitoring and dose adjustments (Hirsch et al. 2001; Ansell et al. 2001). The development of new antithrombotic agents has been facilitated by an understanding of the molecular basis of the interaction between coagulation factors and the nature of their substrates, mainly thrombin and factor Xa. The clinical benefit of any new such agent will depend critically on its safety and simplicity for patients to use.

Ximelagatran was the first orally available direct thrombin inhibitor under clinical development that also reached the market.

1.2 The Ximelagatran Development Program

Ximelagatran was tested in an extensive development program including the following indications/patient categories.

"Short-term treatment" (up to12 days; 35 days)

- Prevention of venous thromboembolic events after total hip and knee replacement; up to 12 days of treatment (also marketed) (Eriksson et al. 2003a,b; Francis et al. 2002, 2003).
- Extended prophylaxis of VTE following elective hip replacement and surgery for hip fracture; planned treatment length: 35 days (Agnelli et al. 2009).

"Long-term treatment"

- Stroke prevention in patients with atrial fibrillation (SPORTIF Executive Steering Committee for the SPORTIF V Investigators 2005; Executive Steering committee on behalf of the SPORIF III Investigators 2003).
- Secondary prevention of venous thromboembolism in patients with DVT/PE after initial (6 months) treatment with vitamin K antagonists; planned treatment length 18 months (Schulman et al. 2003).
- Treatment of deep vein thrombosis; planned treatment length 6 months (Fiessinger et al. 2005).
- Secondary prophylaxis after myocardial infarction; planned treatment length 6 months (Wallentin et al. 2003).

In the overall long-term population, 6,841 patients started ximelagatran treatment. The number of patients exposed for 3 months, 6 months, and 15 months were 6,581, 5,987, and 3,630, respectively. The median exposure time in the long-term trials was approximately 1 year. Longest exposure time exceeds 5 years. Data from patients treated for up to 5 years for stroke prevention with nonvalvular atrial fibrillation have been published (AstraZeneca Clinical Trials: http://www.astrazenecaclinicaltrials.com/article/527374.aspx).

1.2.1 Detection and Incidence of a Hepatic Signal

It is standard practice during clinical trials to monitor hepatic laboratory tests, such as serum aminotransferases (ALT; AST), total bilirubin (Bili), alkaline phosphatase (ALP), and to investigate their potential clinical significance as indicators of hepatotoxicity.

No evidence of hepatotoxicity was noted/observed with ximelagatran during routine preclinical assessment, or at the later extensive investigation of the potential mechanisms for the hepatic injury observed during human exposure (for a detailed description of the preclinical examinations, see later).

Short-term use in humans including the phase III clinical trials for prevention of VTE in elective hip or knee replacement and the experience from marketed use (approximately 12,000 to 15,000 patients) did not indicate any hepatotoxic potential/signal. In fact, at the end of the 7- to 12-day prophylaxis period in the combined studies, the incidence of ALT elevations $>3\times$ upper limit of normal (ULN) was small, e.g., 0.91% in the ximelagatran group and 0.70% in the warfarin group of patients who underwent total knee replacement. ALT level increases were consistently smaller in patients with ximelagatran than those treated with low-molecular weight heparin (the main comparator in the short-term orthopedic surgery studies conducted in Europe).

Increased hepatic enzyme levels were first observed at a higher frequency (4.3%) than in the control group in a study evaluating the long-term use of ximelagatran (i.e., planned treatment>35 days) in comparison with warfarin (Petersen et al. 2003). Following this observation, the frequency of hepatic laboratory

testing was increased in all long-term clinical trials; strict criteria for inclusion and exclusion of patients with raised hepatic enzymes were introduced, and patients who developed an increased hepatic enzyme value were investigated and followed according with a prespecified protocol and algorithm. The frequency of ALT elevations $>3\times$ ULN was 7.9% (6–13.2%) in the ximelagatran-treated group. In the majority of patients, the daily dose of ximelagatran was 36 mg $\times 2$, but doses from 24 mg $\times 2$ to 60 mg $\times 2$ have been tested. Overall, a dose relationship to the ALT elevations could not be established.

A frequency of ALT elevation $>3\times$ ULN of 0–2% was observed among patients treated with the comparators (placebo, warfarin, LMWH/warfarin, aspirin) (Lee et al. 2005). Among 233 patients treated with ximelagatran and 35 patients treated with placebo, the pattern of the hepatic enzyme elevations was evaluated. Of these, 76% (150) and 43% (15) were judged to have hepatocellular injury, while 24% (48) and 57% (20) were judged to have mixed or cholestatic injury (Lewis et al. 2008).

1.2.2 Other Clinical Features of the Hepatic Signal

Data from the 546 patients with ALT $>3\times$ ULN noted among the ximelagatran-exposed patients were analyzed to evaluate the time course of the first occurrence of an increase in the levels of ALT $>3\times$ ULN; 93% of the elevations occurred within the first 6 months and 98% within the first 12 months (Fig. 1). It was possible to document data on recovery in 96% of the 546 ximelagatran-treated patients with ALT $>3\times$ ULN. Subdividing recovery data according to whether patients still received or discontinued ximelagatran treatment could be demonstrated in 93% of the 342 patients who stopped treatment, while their ALT was elevated to $>3\times$ ULN, and in all 204 patients who continued treatment during the period of the ALT elevation (Fig. 2).

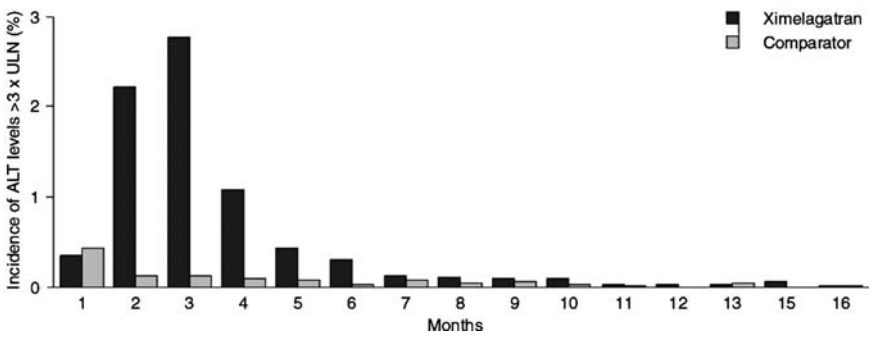

Fig. 1 Histogram of the number of patients with ALT levels of $>3\times$ ULN for the first time per month in each treatment group. *ALT* Alanine aminotransferase, *ULN* upper limit of normal (Lee et al. 2005).

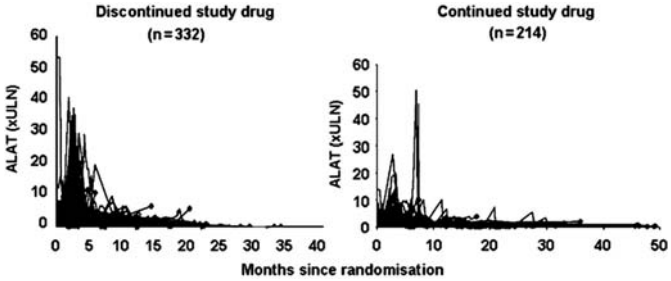

Fig. 2 ALAT elevations >3×ULN in patients treated with ximelagatran with and without discontinuation of study drug. Data from 546 out of 6,948 patients randomized to ximelagatran long-term (in studies SH-TPA-0002, SH-TPA-0003, SH-TPA-0004, SH-TPA-0005, SH-TPV-0003, SH-TPV-0002, SH-TPV-0005, SH-TPC-0001; see the AstraZeneca Clinical trials registry at http://www.astrazenecaclinicaltrials.com/). (Kindmark et al. 2008)

1.2.3 Mortality, Frequency of Bilirubin Increase and Symptoms Indicating Hepatic Disorder (Lee et al. 2005)

All-cause mortality in the total long-term treatment population was similar between the ximelagatran- and comparator-treated groups (3.9 vs 4.4%). The percentage of ximelagatran-treated patients with ALT >3× ULN who subsequently died did not differ compared with the percentage of those with ALT levels of >3× ULN who died in the comparator group (4.0 and 3.9%, respectively). The frequency of elevated total bilirubin levels was similar in both treatment groups: 4.1% in the ximelagatran-treated group and 3.6% in the comparator-treated group for bilirubin >1.5× ULN; the corresponding frequencies were 1.2 and 1.1% for bilirubin >2× ULN.

Concurrent elevations of ALT >3× ULN and total bilirubin>2× ULN under certain circumstances (in the absence of other explanations and with a hepatocellular pattern) is used as a potential indicator for more severe hepatic injury (FDA Clinical White Paper, November 2000, http://www.fda.gov/cder/livertox/clinical.pdf). In the long-term ximelagatran trial program *regardless of etiology*, the frequency of patients with the combination of ALT >3× ULN and total bilirubin >2× ULN was 0.5% among patients treated with ximelagatran and 0.1% among patients in the comparator group.

An analysis in the ximelagatran clinical trial program showed no difference in the number of adverse events "possibly associated with a hepatic disorder" (abdominal pain, nausea, fatigue, jaundice) between the two groups. Such symptoms were noted among 20.2% of the ximelagatran-treated patients and 19.2% of the comparator-treated patients. As expected, in both the ximelagatran and the comparator groups, patients with ALT levels of >3× ULN had a higher incidence of adverse events "possibly associated with a hepatic disorder" than those without ALT level elevations. Symptoms such as fever and rash potentially indicating hypersensitivity (immunologic type of reaction) were low (rash: ximelagatran/comparator: 4.3/4.7%; fever: ximelagatran/comparator: 1.7% in both groups) and did not differ between ximelagatran and the comparators (data on file; AstraZeneca).

1.2.4 Reintroduction

Overall, 18 patients who had an ALT level of >3×ULN and discontinued ximelagatran treatment temporarily subsequently resumed treatment. The duration of reexposure was >2 months in 16 of the 18 patients, and ALT was checked at least monthly during reintroduction. Seventeen patients had no further ALT elevation. One patient developed an ALT elevation of >3× ULN 65 days after reintroduction of ximelagatran treatment without any signs or symptoms of hypersensitivity; after discontinuation of ximelagatran treatment, the patient's ALT level returned to normal (Lee et al. 2005).

1.2.5 "Extended Prophylaxis" (35 Days) Following Elective Hip Replacement or Surgery for Hip Fracture

When 1,158 patients had been randomized and 641 patients had completed the treatment period in this study, it was prematurely stopped due to the increased liver enzymes.

Overall, 58 patients (31 treated with enoxaparin and 27 treated with ximelagatran) had developed ALT >3× ULN. Eleven of the ximelagatran-treated patients showed an ALT increase after the end of the study treatment, all detected at the routine safety screening on day 56 (i.e., 3 weeks after the end of drug treatment). Three patients also showed symptoms of liver injury (nausea, fatigue, weight loss). One of these patients developed an ALT elevation of up to 46×ULN, bilirubin elevation of 17× ULN and INR of 1.8. She received vitamin K and no other specific treatment.

All patients with ALT elevations, including the patients with symptoms, recovered (Agnelli et al. 2009).

The withdrawal of ximelagatran from the market and termination of the ximelagatran development program was triggered by the new safety data from the 35-day study described above, indicating that severe hepatic injury in a patient could develop after exposure to the drug has been completed, and that regular liver function monitoring may not mitigate the possible risk of severe hepatic injury.

2 The Pharmacogenetic Study (EXGEN)

The higher incidence of ALT cases in the ximelagatran-treated patients in northern Europe compared with Asia was a signal that there may be an underlying genetic factor. A retrospective case-control pharmacogenetic study was conducted based on 74 cases and 130 matched controls and included both a genome-wide tag single nucleotide polymorphism (SNP) and a large-scale candidate gene analysis (EXGEN study) (Kindmark et al. 2008). The results indicated a genetic association between patients carrying the major histocompatibility complex (MHC) class II

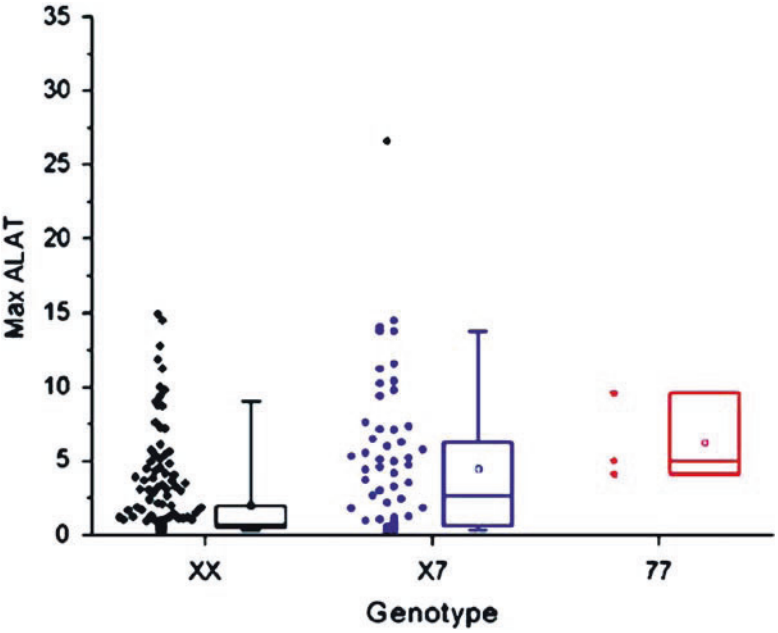

Fig. 3 DRB1*0701 genotypes versus maximum ALAT (in units of xULN) in all samples from EXGEN. Subjects designated as *XX* carry two alleles of DRB1 that are not DRB1*0701, as *X7* are heterozygous for DRB1*0701, and as *77* are homozygous for DRB1*0701. The scatter plots show the maximum ALAT in each genotype group, demonstrating the overlap in ALAT measurements between individuals in different genotype groups. The box-whisker plots (in which the box represents the 25th–75th percentiles of the distribution, the *whiskers* extend to 5th and 95th percentiles, the *central line* the median and the *small square* the mean) demonstrate that the means and distributions of maximum ALAT clearly differ between the genotype groups, with a trend towards increased maximum ALAT with increased copy number of DRB1*0701. (Kindmark et al. 2008).

alleles DRB1*07 and DQA1*02 and ALT increases during ximelagatran treatment (Fig. 3). This result was replicated in a restricted number of individuals, 10 cases and 16 controls. SNP analysis was done for the 20 top-ranked genes, and both DRB1*07 and DQA1*02 showed significant evidence for replication. Interestingly enough, the DRB1*0701 has a broadly similar geographic distribution to the ALT observations with a carrier frequency of approximately 11% in Scandinavia versus 0.3% in Japan (Berlin et al. 1997; Gibert and Sanchez-Mazas 2003). The results suggest that ximelagatran may induce an immunological response resulting in liver injury. However, the clinical picture does not suggest a classical immunological response because patients who experienced an increased ALT did not show evidence of fever, rash, or eosinophilia. The preclinical studies did not provide evidence for a possible immunological response upon exposure to ximelagatran. However, allergenicity studies had shown that guinea pigs could be sensitized by subcutaneously administered ximelagatran, and skin reactions were reported in 7 of approximately 20 individuals exposed to ximelagatran during manufacturing.

After the discovery of the genetic association of elevated ALT with genes involved in the immune system, a retrospective study was initiated to investigate the possible presence of drug-specific immune cells. Despite the fact that patients included in the earlier clinical trials might not have been exposed to the drug for over 4 years, 2 of 21 patients did demonstrate a weak positive lymphocyte transformation test (LTT). Both of these patients had ALAT $>4\times$ULN and one was heterozygous for DRB1*0701.

Drug-induced adaptive responses can arise because the drugs are metabolized into reactive metabolites that bind to proteins, which act as neoantigens (the hapten hypothesis). However, there is no evidence from the preclinical investigative program for the formation of reactive metabolites which could support such a mechanism. Drugs may also activate an immune response by forming a low-affinity association directly with the MHC molecules (Pichler 2002). Using a proprietary competitive binding assay (De Groot 2006), the company EpiVax (Providence, US) provided evidence that ximelagatran and its intermediate metabolite, melagatran-ethyl, at high concentrations, were able to inhibit the binding of peptides to HLA-DRB1*0701 and not to some other common DRB1 protein alleles (Kindmark et al. 2008). A low affinity binding is likely to result in an immune reaction restricted to a T cell but not a B cell response, which could be consistent with the apparent absence of classical signs of hypersensitivity in patients treated with ximelagatran. The sensitivity and specificity of the DRB*07 test in the EXGEN study were 47 and 83%, respectively, which indicates that other factors than HLA-DR1*07 contribute to the observed liver injury.

3 Preclinical Toxicological Studies

3.1 Summary of the Standard Preclinical Toxicological Studies

During the development programme, routine preclinical and toxicological studies showed no indications of any hepatic effects of ximelagatran in any species studied. After the detection of ALT elevations in the first clinical long-term treatment studies, further animal studies were initiated. In these studies, rats, dogs, and cynomolgus monkeys were exposed to high doses of ximelagatran, and blood sampling was performed more frequently than in previous studies. The results were negative: no ALT elevations related to ximelagatran treatment were observed.

3.2 Extended Mechanistic Studies

An extensive effort to investigate whether ximelagatran perturbed cellular functions that possibly could be involved in liver injury was initiated when the first indication of ALT elevations in the clinical study program appeared (Kenne et al.

2008). A very broad approach was adopted since the preclinical studies did not indicate any toxicity that could be related to liver injury, and the clinical study program at the time when the mechanistic studies were planned did not indicate any specific mechanism. Later the pharmacogenetic investigation observed a strong genetic association between patients who experienced ALT elevations and MHC alleles suggesting a possible immunological response.

Many different mechanisms can contribute to DILI (Lee 2003; Navarro and Senior 2006; Park et al. 2005). One of the most common causes of hepatotoxicity is the cytochrome P450 (CYP)-dependent formation of reactive metabolites that are either directly hepatotoxic or form adducts with hepatic proteins, potentially triggering an immune response. Other potential mechanisms of DILI include disruption of mitochondrial functions, inhibition of drug metabolism pathways, and inhibition of bile acid transport (Navarro and Senior 2006). Orphan receptors regulating drug-metabolizing enzymes and several vital functions in the cell, such as cholesterol and bile acid homeostasis, may be targets for hepatotoxic compounds (Repa and Mangelsdorf 2000; Wang and LeCluyse 2003). After oral administration, ximelagatran is rapidly absorbed and bioconverted to the active form, melagatran, in a two-step process involving ester cleavage and reduction of the amidoxime group (Eriksson et al. 2003c). None of the major human CYP isoenzymes appear to be involved in either of these steps (Bredberg et al., 2003; Clement and Lopian 2003). Ximelagatran, melagatran, and the intermediate metabolites, ethyl-melagatran and hydroxy (OH)-melagatran, have also been shown not to inhibit CYP isoenzymes in vitro (Bredberg et al. 2003). Ximelagatran has a predictable and reproducible pharmacokinetic/pharmacodynamic profile, with low inter- and intraindividual variability (Wolzt et al. 2005). In patients receiving oral ximelagatran 36 mg twice daily, the mean peak plasma concentration (C_{max}) was 0.3 μM for ximelagatran and 0.5 μM for melagatran (Wolzt et al. 2003). Concentrations of ethyl-melagatran and OH-melagatran were consistently below 0.1 μM. The concentrations of ximelagatran and its metabolites have not been determined in human liver tissue in vivo. Experimental systems were selected to investigate the potential effects of ximelagatran at the cellular, subcellular, and molecular level. Cell systems used included fresh and cryopreserved human hepatocytes, as well as human-derived hepatoma cell lines (HepG2 and HuH-7). A limitation with all these systems is that, for technical reasons, only short-term exposure can be studied. However, drugs with known clinical hepatotoxicity generally show cytotoxic effects during short-term exposure, even if clinical signs and symptoms only develop after several months of treatment (Gomez-Lechon et al. 2003).

The studies performed determined the effects of ximelagatran and its metabolites across a broad range of concentrations (up to 300 μM) in order to include higher concentrations than those found in plasma at therapeutic dosing (Table 1).

An alternative mechanism for transient increases of serum AST and ALT in patients treated with fibrates has been suggested to involve induction of gene transcription (Edgar et al. 1998). However, ximelagatran (1–30 μM) did not induce transcriptional activation of the human ALT1, ALT2, or AST1 promoter constructs in either HuH-7 or HepG2 cells.

Table 1 Summary of results from the mechanistic in vitro studies

Function tested	Results
Cell viability	Human hepatocytes tolerate ximelagatran well up to at least 200 μM (ATP content)
Apoptosis	An effect was first observed after exposure of HepG2 cells at 100 μM ximelagatran for 24 h
Calcium homeostasis	No effects on calcium homeostasis were observed after 15 min exposure of HepG2 cells up to 300 μM of ximelagatran
Mitochondrial functions	No effects in isolated mitochondrial preparations on: state 4 respiration, ADP-stimulated or 2,4-dinitrophenol-stimulated respiration, the respiratory control ratio, the adenosine diphosphate/oxygen ratio, the rate of calcium-induced mitochondrial swelling at 50 μM. Loss of MMP was seen only at the highest concentration of ximelagatran tested, 300 μM, in mitochondria exposed for 24 h. No effects on β-oxidation of fatty acids up to 300 μM
Reactive oxygen species (ROS)	No stimulation of intracellular ROS in primary human hepatocytes up to 100 μM
Inhibition of biliary transporter proteins	Neither a substrate nor an inhibitor of human bile salt export protein (BSEP)
Reactive metabolites	No indication for a reactive metabolite pathway. No glutathione adducts identified. No indication of protein adducts in human hepatocytes or subcellular fractions at concentrations up to 300 μM
Nuclear receptors	No indication of activation of human PXR and PPARα nuclear receptors or activation of TRβ, LXRα, LXRβ, and FXR coactivator recruitment

4 Concluding Remarks

As for many drugs causing liver injury, the standard preclinical toxicological studies provided no indication that ximelagatran affected hepatic functions. In addition, extensive investigations using human-based in vitro models have not been able to define mechanisms explaining the pattern of hepatic injury observed in long-term clinical trials. ALT elevations, mostly asymptomatic, transient, and occurring during the first 6 months of treatment, were observed in 7.9% of the patients treated with ximelagatran in the long-term (>35 days) clinical trial program. Elevated ALT levels were also noted in some patients 3 weeks after the end of drug treatment in a 35-day study. A few patients developed severe and symptomatic hepatic injury; in one patient, the injury developed weeks after the end of 35 days of ximelagatran exposure. Despite extensive analyses, we found no way to predict which patients are prone to develop severe hepatic injury or to devise a monitoring scheme that would allow early identification and withdrawal of patients from treatment before they developed severe hepatic injury.

The pharmacogenomic study provided evidence that the ALAT increases were associated with MHC DRB1'07 and DQA1*02 suggesting a possible immunogenic pathogenesis. However, further regulatory studies would have been needed before this test could have been implemented as a screening tool in the clinic. Genetic

associations have, however, the potential to be important risk mitigation components in future drug development programs.

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Troglitazone

Tsuyoshi Yokoi

Contents

Abstract Troglitazone was the first thiazolidinedione antidiabetic agent approved for clinical use in 1997, but it was withdrawn from the market in 2000 due to serious idiosyncratic hepatotoxicity. Troglitazone contains the structure of a unique chroman ring of vitamin E, and this structure has the potential to undergo metabolic biotransformation to form quinone metabolites, phenoxy radical intermediate, and epoxide species. Although troglitazone has been shown to induce apoptosis in various hepatic and nonhepatic cells, the involvement of reactive metabolites in the troglitazone cytotoxicity is controversial. Numerous toxicological tests, both *in vivo* and *in vitro*, have been used to try to predict the toxicity, but no direct mechanism has been demonstrated that can explain the hapatotoxicity that occurred in some individuals. This chapter summarizes the proposed mechanisms of troglitazone hepatotoxicity based *in vivo* and in vitro studies. Many factors have been proposed to contribute to the mechanism underlying this idiosyncratic toxicity.

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1 Introduction

Thiazolidinediones (Fig. 1) are a class of oral antidiabetic agents, which are a synthetic ligand for the peroxisome proliferator-activated receptor γ (PPARγ) (Lehmann et al. 1995). Troglitazone (Rezulin®, (±)-5-[[4-[(3,4-dihydro-6-hydroxy-2,5,7,8-tretramethyl-2H-1-benzopyran-2-yl)methoxy]phenyl]methyl]-2,4-thiazolidinedione) was the first thiazolidinedione antidiabetic agent approved for clinical use by the US in 1997. Troglitazone lowers blood glucose levels through increased glucose uptake by skeletal muscle, decreased hepatic glucose production, and increased insulin sensitivity of the target tissue in animal models of metabolic impairment (Ciaraldi et al. 1990; Fujiwara et al. 1995). These pharmacological effects are exerted through PPARγ-dependent transcription of genes involved in glucose and lipid metabolism and energy homeostasis (Lehmann et al. 1995; Saltiel and Olefsky 1996; Spiegelman 1998). Based on the pharmacological advantages and the apparent absence of serious toxic effects, troglitazone was thought likely to become a promising treatment for type II diabetes mellitus in patients with insulin resistance.

In the combined North American clinical trials, elevations of serum alanine aminotransferase (ALT) more than three times the upper limit of normal were observed in 48 out of 2,510 patients (1.9%) treated with troglitazone as compared

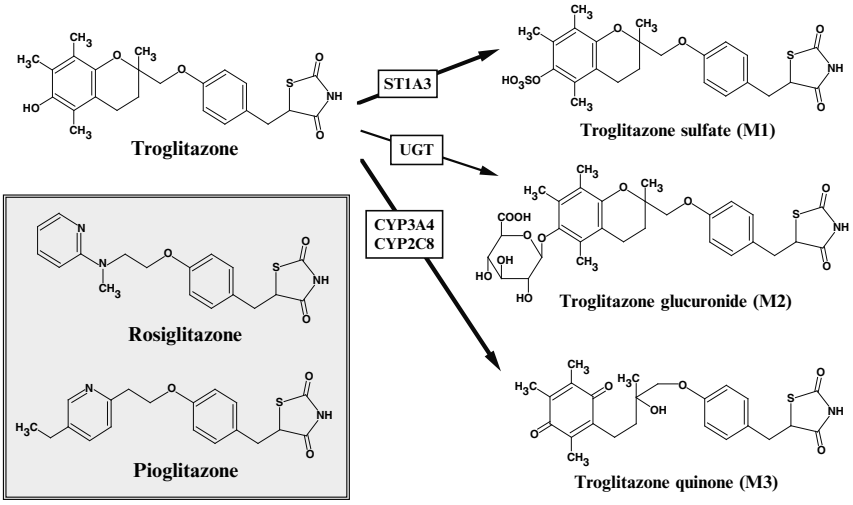

Fig. 1 Structures of thiazolidinediones and pathways of troglitazone metabolism to relatively stable metabolites

to 0.6% in patients who received placebo. Two patients were confirmed to have suffered serious hepatocellular injury from an idiosyncratic drug reaction (Watkins and Whitcomb 1998). Meanwhile, troglitazone was concomitantly reported to be associated with idiosyncratic hepatotoxicity with some patients showing severe or fatal liver damage (Gitlin et al. 1998; Neuschwander-Tetri et al. 1998; Shibuya et al. 1998). Consequently, it was withdrawn from the market in the US and Japan in March 2000. The hepatotoxic effects of troglitazone were not predicted in conventional experimental animals (Watanabe et al. 1999) or in cynomolgus monkeys (300–1,200 mg/kg/day for 52 weeks), a primate model having similar metabolic profiles to humans (Rothwell et al. 2002). Two other thiazolidinediones which are now on the market, rosiglitazone and pioglitazone, were introduced in 1999, and they appear not to exhibit the hepatotoxic effects of troglitazone (Freid et al. 2000; Isley and Oki 2000; Lebovitz et al. 2002), although an association with hepatotoxicity has been reported in very rare instances (Nagasaki et al. 2002). It should also be noted that the clinical dosage regimen for improvement of the fasting glucose level differs among these thiazolidinediones. The recommended dose for troglitazone was 200–600 mg/day, for rosiglitazone 4–8 mg/day, and for pioglitazone 15–45 mg/day (Hanefeld 2001; Loi et al. 1999; PDR 1999, 2005a, b). The Cmax and AUC of troglitazone are 0.90–2.82 mg ml^{-1} and 7.4-22.1 mg-h ml^{-1}, respectively, whereas those of rosiglitazone are 0.076–0.598 mg ml^{-1} and 0.358–2.971 mg-h ml^{-1}, respectively. The plasma elimination half-life and biliary excretion of troglitazone are 16–34 h and 85%, respectively, and those of rosiglitazone are 3–4 h and 23%, respectively. Although the dosage for sufficient pharmacological efficacy could be related to the hepatotoxic potential, the mechanism of troglitazone toxicity is still controversial. Numerous toxicological tests, both *in vivo* and in vitro, have been attempted, but no direct mechanism has been successfully demonstrated that can explain the hepatotoxicity that occurred in some individuals.

Troglitazone represents a model of an idiosyncratic drug reaction that led to withdrawal from the market and to attempts to understand the mechanisms of such adverse drug reactions. This review summarizes the proposed molecular mechanisms of troglitazone hepatotoxicity based on both *in vivo* and in vitro studies. However, so far, there is no direct evidence indicating the precise mechanism of the toxicity. Many factors have been proposed to contribute to this idiosyncratic toxicity.

2 Metabolism of Troglitazone into Stable Metabolites

The bioavailability of troglitazone is 40–50%, which can be affected by food and other factors (Loi et al. 1999). The plasma protein binding is more than 99%, and the distribution into red blood cells is low (Kawai et al. 1997). In humans, there is no evidence that troglitazone accumulates in the liver. However, troglitazone is absorbed in isolated perfused livers and cultured hepatocytes within minutes, even in the presence of albumin or serum (Preininger et al. 1999; Haskins et al. 2001;

Yamamoto et al. 2001). In humans, troglitazone is predominantly metabolized by three pathways: sulfation, glucuronidation, and oxidation, to form a sulfate conjugate (M1), a glucuronide conjugate (M2), and a quinone metabolite (M3), respectively (Fig. 1). The main metabolite, troglitazone sulfate (M1), is catalyzed by phenol sulfotransferase, ST1A3 (Honma et al. 2002), and accounts for about 70% of the metabolites detected in human plasma (Loi et al. 1999), exceeding that of the parent drug. Troglitazone sulfate undergoes enterohepatic circulation after biliary excretion resulting in a long half-life *in vivo* in humans (Kawai et al. 1998), which may be involved in cholestatic liver injury through inhibition of bile acid transport as described below.

A relatively minor metabolite, troglitazone glucuronide (M2), is catalyzed by UGT (Yoshigae et al. 2000). The glucuronidation of troglitazone in human intestine is threefold higher than that in human liver. In the liver, the reaction is likely mediated by UGT1A1, while in the intestine it is mediated by UGT1A8 and UGT1A10 (Watanabe et al. 2002). Furthermore, in enterocytes, it may also be converted to glucuronide by UGTs such as UGT1A8 and UGT1A10.

In human liver, CYP3A4, CYP2C8, and CYP2C19 mainly catalyze troglitazone to a quinone-type metabolite (M3). The chroman ring of vitamin E can be oxidized to a quinone. Kinetic analysis of the troglitazone oxidation (M3 formation) by recombinant P450 enzymes showed that CYP3A4, CYP2C8, and CYP2C19 had relative clearance values of 0.4, 1.6, and 0.9 ml $min^{-1}nmol^{-1}$ P450, respectively (Yamazaki et al. 1999). Considering the relative P450 enzyme contents in human liver, CYP3A4 may be expected to play a major role in the formation of a quinone-type metabolite from troglitazone even at a low concentration. The quinone metabolite M3 is relatively stable and exhibited weaker cytotoxicity than troglitazone (Yamamoto et al. 2001). In addition, troglitazone has been shown to induce CYP3A in human and rat hepatocytes, which stimulates the formation of the quinone metabolites (Ramachandran et al. 1999; Sahi et al. 2000). Therefore, the large interindividual variability of CYP3A4 activities in human liver may be related to the risk of troglitazone-induced hepatotoxicity.

3 Reactive Metabolites and Cytotoxicity

Differing from other thiazolidinediones, troglitazone contains a chroman ring of the vitamin E moiety. This structure accounts for the effective antioxidant property of troglitazone and suggests an advantage in preventing diabetic vascular complications in addition to its hypoglycemic and hypolipidimic effects (Inoue et al. 1997). This structure, however, has the potential to undergo metabolic activation to form the troglitazone quinone metabolite (M3). As mentioned above, although the quinone metabolite M3 is relatively stable, by the action of CYP3As, troglitazone yields several reactive intermediates (Kassahun et al. 2001; Tettey et al. 2001; He et al. 2001) (Fig. 2). The formation of an epoxide of troglitazone quinone was also identified in vitro in humans (Yamamoto et al. 2002) and is likely to be a potent

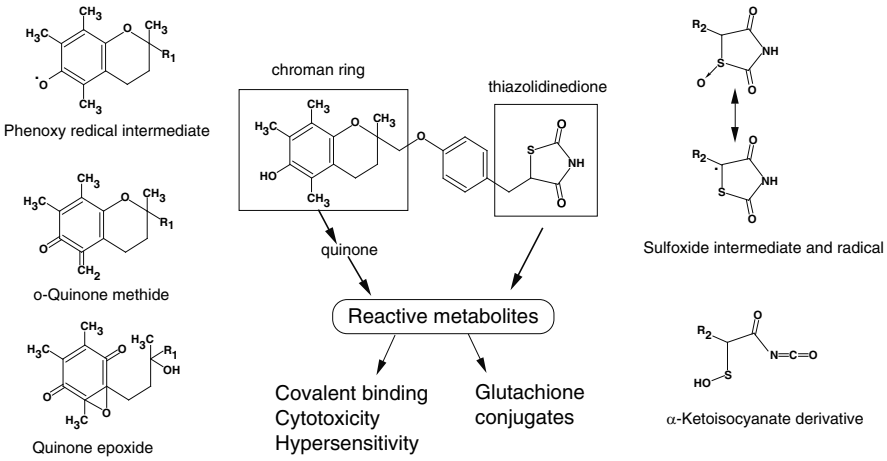

Fig. 2 Reactive metabolites of troglitazone catalyzed by CYP3A potentially leading to toxicity

electrophile. Although the troglitazone quinone does not react directly with GSH, it can be further metabolized to an *o*-quinone methide or undergo ring opening to produce additional highly electrophilic intermediates (Kassahun et al. 2001). Such electrophilic intermediates are toxicologically active, which can result in acute cytotoxicity and immunotoxicity as well as carcinogenesis (Bolton et al. 2000).

Cytotoxicity assays of troglitazone and its metabolites were performed in various types of cells, such as HepG2 cells and hepatocytes from human and experimental animals. The maximum plasma concentrations in patients taking troglitazone at a dose of 600 mg/day only reached about 2.82 μg ml^{-1} or 6.3 μM (Loi et al. 1999). However, a study in rats demonstrated that the concentration of troglitazone in liver tissues was 10- to 12-fold higher than that in the plasma (Sahi et al. 2000). Therefore, the troglitazone levels in human liver might allow the formation of these reactive intermediates, and their accumulation may lead to the hepatotoxicity. In the cytotoxicity assay, the estimated IC50 values of troglitazone and the quinone metabolite, M3, were 34 and 66 μM, respectively, in HepG2 cells incubated in a 5% FBS-containing culture medium (Yamamoto et al. 2001). These reports suggested that the troglitazone levels in human livers could reach such concentrations, which may cause the observed cytotoxicity *in vivo*.

The reactive metabolite(s) covalently binds to cellular macromolecules, but the role of the protein adduct on troglitazone-induced cytotoxicity is still controversial. Using cryopreserved human hepatocytes from 27 individuals, none of the individual phase I or II enzyme activities correlated with the EC50 values of troglitazone cytotoxicity (Hewitt et al. 2002). However, a combination of high CYP3A4 and UGT activities was associated with low toxicity while low CYP3A4 with high ST activity was associated with higher toxicity, which suggested that troglitazone sulfate might act as direct toxicant, and CYP3A4 and UGT were involved in detoxification (Hewitt et al. 2002). On the other hand, chemical inhibitors of drug metabolizing enzymes were employed to elucidate their involvement in the

cytotoxicity to HepG2 cells. Ketoconazole (an inhibitor of CYP3A4), quercetin (an inhibitor of CP2C8), and DCNP (2,4-dichloro-4-nitrophenol, an inhibitor of sulfation) did not successfully attenuate the cytotoxicity in HepG2 cells (Yamamoto et al. 2001). However, inhibition of troglitazone sulfation by DCNP and pentachlorophenol resulted in aggravation of cytotoxicity in human hepatocytes (Kostrubsky et al. 2000), indicating a result opposite to that of Hewitt et al. (2002). The use of cultured cell lines in cytotoxicity assays requires careful interpretation because the activities of drug metabolizing enzymes in such cells are very low. However, Vignati et al. (2005) reported that HepG2 cells, together with microsomes expressing human CYPs or HepG2 cells transfected with CYP3A4, were able to metabolize troglitazone resulting in increased cytotoxicity. Established cell lines expressing the same level of drug metabolizing enzymes as those in human liver would be useful for troglitazone-induced cytotoxicity assays.

4 Biomarkers of Susceptibility to Troglitazone Hepatotoxicity

As mentioned above, troglitazone can undergo metabolic biotransformation by CYP3A4 to form quinone and epoxide metabolites (Izumi et al. 1997a, b; Kawai et al. 1998; Loi et al. 1999; Yamamoto et al. 2002). Quinones are well-established cytotoxic agents and can produce toxicity by redox cycling with molecular oxygen to produce superoxide anion radical and subsequent oxidative stress (Schultz et al. 1996; Bolton et al. 2000). Quinones can also react readily with sulfur nucleophiles such as glutathione (GSH) or cysteine residues on proteins (Bolton et al. 2000). However, little information is available about enzymatic detoxification of these reactive metabolites. The toxic effects of troglitazone have been thought to be mediated by the depletion of GSH, covalent binding to cellular macromolecules, or oxidative stress. In cryopreserved human hepatocytes, large variations in the sensitivity to troglitazone were observed, and sensitive donors were demonstrated to form significantly lower amounts of GSH conjugates and glucuronides than resistant donors (Kostrubsky et al. 2000; Prabhu et al. 2002). It is known that GSH conjugation is catalyzed by the action of glutathione *S*-transferase (GST). A study in rats has shown that GSH adducts of troglitazone are formed and the reaction is enhanced by CYP3As (Tettey et al. 2001). An epoxide of troglitazone quinone catalyzed by CYP3A4 might also be eliminated by GSTs and epoxide hydrolase (Yamamoto et al. 2002). These findings indicate an association between metabolic activation by CYP and detoxification by GSTs. In a key report concerning this aspect, Watanabe et al. (2003) investigated the genetic factors responsible for troglitazone hepatotoxicity *in vivo* in humans. Among 110 patients prescribed troglitazone, 25 had an abnormal increase in ALT or AST levels to at least nine times or five times the upper limit of the normal range, respectively, while 85 control patients showed no significant increase in ALT levels during more than 6 months of treatment. Interestingly, they found that this abnormal elevation of liver enzymes caused by troglitazone treatment was highly associated with the double

null genotype of *GSTM1* and *GSTT1* (odds ratio, 3.692; 95% confidence interval, 1.354–10.066; $P = 0.008$) (Watanabe et al. 2003). A similar association study regarding hepatotoxicity observed in patients treated with tacrine, a drug used for Alzheimer's disease, was reported by Simon et al. (2000). Thus, interindividual differences in detoxification ability appears to contribute to the susceptibility and individual risk for troglitazone hepatotoxicity. Taking into consideration the double null genotype of *GSTM1* and *GSTT1* in clinical practice, the risk for hepatotoxicity could theoretically be reduced by half.

Recently, we established a GSH-knockdown rat model for the prediction of human hepatotoxicity (Akai et al. 2007). An adenovirus vector with short hairpin RNA against rat γ-glutamylcysteine synthetase (GCS) heavy chain subunit was constructed and used to knockdown GSH synthesis. This rat model, with an 80% decreased hepatic GSH level, demonstrated a high sensitivity for acetaminophen-induced hepatotoxicity. With the advance of molecular biology, novel animal models will be established and applied to drug development in the near future.

5 Inhibition of Hepatic Drug Transporters by Troglitazone Metabolites

Troglitazone sulfate (M1, the main metabolite) undergoes biliary excretion and accounts for up to 85% of the dose in humans (Loi et al. 1999). In patients with hepatic impairment, troglitazone sulfate was found to accumulate about fourfold in plasma with a threefold increased half-life (Ott et al. 1998; Loi et al. 1999). This metabolite also inhibited the canalicular bile salt export pump (Bsep), organic anion transporting polypeptide (OATP) transporters as well as drug transporters, suggesting it contributes to the hepatotoxicity.

Troglitazone sulfate inhibits the ATP-dependent taurocholate transport mediated by Bsep in isolated canalicular rat liver plasma membrane (IC_{50} 0.4–0.6 μM) about ten times more strongly than the parent compound (IC_{50} 3.9 μM) (Funk et al. 2001a, b). When troglitazone sulfate accumulates in hepatocytes at high concentrations, it may disturb the hepatobiliary export of bile acids by the inhibition of Bsep leading to intrahepatic cholestasis in humans. Evidence of cholestasis has also been described in a patient with troglitazone hepatotoxicity (Gitlin et al. 1998).

Troglitazone sulfate was also reported as a substrate of organic anion transporting polypeptide (OATP) transporters with higher affinity to OATP-C (SLC01B1) than OATP8 (SLC01B3). Estrone-3-sulfate was demonstrated to be a potent inhibitor for OATP-C and OATP8 (Nozawa et al. 2004). Both OATP-C and OATP8 are members of the organic anion transporting polypeptides, which are expressed in the basolateral membrane of hepatocytes (Hagenbuch and Meier 2003; Kreb 2006). They play important roles in the hepatic handling of endogenous compounds and xenobiotics. Some types of genetic polymorphisms with functional alterations of OATP-C have been reported, and such alterations may lead to the accumulation of troglitazone sulfate in the liver, resulting in troglitazone-associated hepatotoxicity

(Kreb 2006, Michalski et al. 2002). Taking such information into account, the failure of hepatic excretion of troglitazone sulfate might lead to hepatotoxicity, although troglitazone sulfate itself is pharmacologically inactive and did not exhibit cytotoxicity in human hepatoma cells (Loi et al. 1999; Yamamoto et al. 2001)

Using knockout rats lacking multidrug resistant associated protein-2 (Mrp2), it has been demonstrated that troglitazone glucuronide is a substrate for Mrp2 (Kostrubsky et al. 2001). Therefore, the troglitazone glucuronide formed in enterocytes might be excreted to the intestinal lumen via transporters such as Mrp2 expressed in the brush border membrane. Then, the glucuronide would again be converted to troglitazone by β-glucuronidase and the troglitazone might be reabsorbed. There has been no reported evidence that M2 is responsible for the hepatotoxic effects of troglitazone.

6 Hypersensitivity Reaction Associated with Troglitazone Hepatotoxicity

Idiosyncratic adverse reactions are difficult to study because of their rare occurrence, dose-independence, and lack of reproducibility in experimental animal models. Many idiosyncratic drug reactions have an immunological (hypersensitivity) basis, whereas some are due to a metabolic abnormality of the host (Pohl et al. 1988; Ju and Uetrecht 2002). Idiosyncratic drug-induced hepatitis has been assumed to be mediated by immunogens formed by covalent interaction of a reactive drug metabolite with cellular macromolecules (Park et al. 1998). The bioactivated immunogens may not only lead to an immune response directed against the haptenic epitope and the neoantigen, but also against autoantigenic determinants, which is characterized by the formation of autoantibodies (Pohl et al. 1988). A number of hepatotoxic drugs have been reported to produce autoantibodies. For example, antiprotein disulfide isomerase, antimicrosomal carboxyesterase, anticalreticulin, anti-ERp72, anti-GRP78, anti-GRP94, and anti-CYP2E1in halothane hepatitis (Bourdi et al. 1996; Gut et al. 1993; Kenna et al. 1993; Pumford et al. 1993), anti-CYP2C9 in tienilic acid-induced hepatitis (Homberg et al. 1984; Robin et al. 1996), anti-CYP1A2 in dihydralazine-induced hepatitis (Bourdi et al. 1990), and anti-CYPs in aromatic anticonvulsant-induced hypersensitivities (Leeder et al. 1992) have been reported. However, it is not fully understood whether the autoantibodies are the causes or consequences of hepatotoxicity. Studies to clarify the possible involvement of autoantibodies in drug-induced hepatitis are limited because the appearance of autoantibodies can usually be seen only in humans. We recently reported that aldolase B, which is an enzyme predominantly localized in the liver and kidney (Penhoet et al. 1966), was detected as an autoantigen that reacted with antibodies in the sera from two patients with type II diabetes mellitus and troglitazone-induced liver dysfunction (Maniratanachote et al. 2005b). The titer of antialdolase B remained high for several weeks after stopping troglitazone administration. This finding supported the idea that troglitazone hepatotoxicity may have an immunological basis.

However, autoantibodies to aldolase B were also detected in the sera of patients with chronic hepatitis as well as liver cirrhosis (Brown et al. 1987; Maniratanachote et al. 2005b). There are several reactive metabolites generated by troglitazone (Fig. 2) (Kassahun et al. 2001; Tettey et al. 2001; Yamamoto et al. 2002). Aldolase B, which is an enzyme predominantly localized in the liver (Penhoet et al. 1966), may be one of the target proteins that interact with those reactive species and trigger the immune response. This study suggested that liver dysfunction might cause the appearance of autoantibodies to aldolase B, which may then aggravate the hepatitis. In addition, the antialdolase B titer might indicate the severity of liver dysfunction. Further studies will be needed to clarify the mechanisms of hypersensitivity reactions.

7 Mechanisms of Troglitazone-Induced Hepatotoxicity

Troglitazone has been shown to induce apoptosis in various hepatic (Bae and Song 2003; Tirmenstein et al. 2002; Yamamoto et al. 2001) and nonhepatic (Shiau et al. 2005) cell types depending on the concentration and duration of exposure. Unlike its pharmacological effects, the toxicity of troglitazone seems to be a PPAR γ-independent mechanism, and the higher affinity PPARγ agonists such as rosiglitazone and pioglitazone possess much lower toxic effects (Lehmann et al. 1995; Shiau et al. 2005). In addition, Shiau et al. (2005) demonstrated that a synthetic counterpart of troglitazone, which lacks PPARγ activation activity, was also able to induce apoptosis in cultured cells. A PPARγ-independent mechanism is also possible in human hepatocytes because the expression of PPARγ in normal human liver cells is very low (Green 1995), and rosiglitazone does not induce apoptosis (Toyoda et al. 2001). Troglitazone was shown to inhibit equally the proliferation of both $PPAR\gamma^{-/-}$ and $PPAR\gamma^{+/+}$ mouse embryonic stem cells (Palakurthi et al. 2001).

As mentioned above, M1, M2, and M3 metabolites are relatively stable, and the quinone metabolite, M3, has been suggested to be associated with troglitazone hepatotoxicity in humans (Neuschwander-Tetri et al. 1998). Although these metabolites showed lower toxic effects compared to the parent compound, troglitazone, when mammalian hepatocytes and hepatoma cell lines were treated directly (Tettey et al. 2001; Tirmenstein et al. 2002; Yamamoto et al. 2001, 2002), the possibility that the metabolites are toxic was not excluded due to the shortage of CYPs and other enzyme activities in the cells. In addition, when exposing the cells to these metabolites, they are unlikely to enter the cells in significant concentrations. On the other hand, it is most likely that troglitazone causes hepatic cell death via apoptosis. Caspase-3 was activated by troglitazone treatment, and pharmacological inhibition of caspase blocked troglitazone-induced cell death (Jung et al. 2007). Apoptosis is a normal physiologic form of cell death and plays a prominent role in liver pathogeneses such as autoimmune liver diseases, viral hepatitis, and drug-induced hepatitis. From this point of view, the cellular, molecular, and *in vivo* responses to troglitazone toxicity will be reviewed in the following sections.

7.1 Mitochondria-Mediated Toxicity

Mitochondria are known to be a source of reactive oxygen species (ROS), suggesting that a direct effect of troglitazone on mitochondrial physiology may play a role in hepatotoxicity (Narayanan et al. 2003). The development of troglitazone-induced toxicity in liver cells could be caused by a reduction of the mitochondrial membrane potential with a concomitant depletion of cellular ATP concentration (Bova et al. 2005; Tirmenstein et al. 2002). Subsequently, it increases the mitochondrial membrane permeability transition and calcium ion (Ca^{2+}) efflux (Masubuchi et al. 2006). The result of these effects on mitochondria is the release of cytochrome c into the cytoplasm and activation of caspases leading to apoptosis (Bova et al. 2005). Using immortalized human hepatocytes, Lim et al. (2008) found that troglitazone rapidly dissipated the mitochondrial inner transmembrane potential, followed by a shift of the redox ratio of mitochondrial thioredoxin-2 (Trx2) toward the oxidized state, and subsequent activation of apoptosis signal-regulating kinase 1 (Ask1). Ong et al. (2007) established heterozygous superoxide dismutase 2 hetero-knockout [Sod2(+/−)] mice as an experimental animal model of silent mitochondrial stress. They found that troglitazone caused liver injury in the high-dose (30 mg/kg/day, i.p.) group, manifested by an approximately twofold increase in serum ALT in Sod2(+/−) but not in wild-type mice. This mouse model could be useful to analyze the dynamics of mitochondrial changes *in vivo* and to investigate the involvement of reactive metabolites in mitochondrial toxicity. Thus, mitochondrial abnormalities could be one of the useful biomarkers of troglitazone-induced idiosyncratic hepatotoxicity.

7.2 Kinase-Mediated Cell Toxicity Pathway

The three well-characterized mammalian mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (Erk), c-Jun N-terminal kinase (JNK), and p38 are regulated by phosphorylation and play important roles in a variety of cellular processes including growth, differentiation, and apoptosis (Johnson and Lapadat 2002). Erk is generally activated by mitogens, while JNK and p38 are preferentially activated by stress and inflammatory cytokines. The most obvious effect of troglitazone on apoptosis is likely via the promotion of JNK, which in turn activates c-Jun by phosphorylation as well as by activation of p38 (Bae and Song 2003). Gardner et al. (2005) and Jung et al. (2007) reported that calcium/calmodulin-dependent kinase II (CaMKII) is a critical regulator of double-stranded RNA-activated protein kinase (PKR)-dependent p38 and eukaryotic initiation factor 2α (eIF2α) phosphorylation in response to endoplasmic reticulum (ER) calcium depletion by troglitazone. Activation of these kinase-signaling pathways is PPAR γ-independent. In addition, troglitazone also causes the induction of Bax, Bad, the cleavage of Bid, and the release of cytochrome c. Moreover, the mitogen-activated protein kinase (MEK) 1/2-ERK1/2 signaling pathway may be implicated in the

growth inhibitory effect by troglitazone in human cancer cell lines (Motomura et al. 2005; Jung et al. 2007). JNK is characterized as a stress-activated protein kinase based on its activation in response to the inhibition of protein synthesis. These reports suggest that troglitazone induces apoptosis via a caspase-dependent mechanism associated with the downregulation of MEK/ERK and upregulation of p38.

Cyclin-dependent kinases (CDKs) are serine-threonine protein kinases that regulate cell cycle progression. These kinases are activated by various cyclins, inhibited by natural inhibitors such as p21, p27, and p18, and are tightly controlled by transcriptional and posttranscriptional modifications (Sherr and Roberts 1999). Bae et al. (2003) reported that troglitazone-induced cell cycle arrest by this pathway, and apoptosis of hepatoma cell lines ware caused G1 cell cycle arrest through the induction of p53 related proteins and the reduction of cyclin D1, phospho-RB and CDK activities.

7.3 Protein Translation-Associated Toxicity

The endoplasmic reticulum (ER) is a major site of protein synthesis, and its inside or lumen is a major site of protein folding (Gething and Sambrook 1992). In mammalian cells, naturally the rate of protein synthesis is rapidly reduced following the induction of apoptosis. The phosphorylation of eIF2α is important in the regulation of selective translation during ER stress and the unfolded protein response (Holcik and Sonenberg 2005). Troglitazone was shown to promote Ca^{2+} release from the ER leading to PERK and PKR activation, phosphorylation of eIF2α, translation inhibition, and growth arrest (Fan et al. 2004; Gardner et al. 2005).

It is known that the ER is a major cellular storage site of Ca^{2+} in the cell, and that ER chaperones play important roles in Ca^{2+} accumulation and release. Any disturbance in the ER homeostasis causes the release of Ca^{2+}, which in turn blocks ER protein processing. This results in the accumulation of incompletely folded proteins and activates the transcription of ER chaperone genes (Liu et al. 1998; Lodish and Kong 1990). We found that troglitazone treatment of hepatoma cell lines led to overexpression of immunoglobulin heavy chain binding protein (BiP), an abundant chaperone protein in the ER (Maniratanachote et al. 2005a). The important role of this chaperone protein was indicated by the phenotypic change in cell viability when BiP expression was inhibited by small interference RNA (Maniratanachote et al. 2005a). This condition rendered cells more susceptible to the toxic effects of troglitazone. Collectively, it might be postulated that troglitazone acts as a chemical stress signal that causes the release of Ca^{2+} from the ER, and that BiP expression is one of the cellular defense mechanisms of the ER in response to troglitazone-induced toxicity.

Ribosomal protein P0 (P0) was found to be one of the targets of troglitazone cytotoxicity in HepG2 cells (Maniratanachote et al. 2006). P0 is known as a phosphoprotein that functions in the protein translation process (Gonzalo et al. 2001). It was found that, rather than its overexpression, dephosphorylation of P0, which could not be prevented by caspase inhibition, occurred in troglitazone-induced cytotoxicity

(Maniratanachote et al. 2006). Although the dephosphorylation enzyme involved was not identified, a posttranslational modification, dephosphorylation, of P0 was suggested to be associated with the troglitazone-induced toxicity. Proteomics and system biology studies will provide new insights into troglitazone-induced toxicity.

8 Conclusions

Factors affecting the susceptibility to drug-induced hepatic injury include age, sex, co-administered drugs, genetic polymorophism, and enzyme activities catalyzing metabolic activation pathways. Idiosyncratic hepatotoxicity in human is usually unpredictable, pharmacologically independent, very rare, and not reproducible in experimental animal models, which makes it difficult to study (Lee 2003). Troglitazone is known as a typical cause of idiosyncratic hepatotoxicity and has been extensively studied for the past decade. Although a number of toxicological tests, both *in vivo* and in vitro, have been performed, no direct mechanism has been found that can explain why troglitazone hepatotoxicity occurred in only some individuals. We have learned from previous reports that the mechanism of troglitazone hepatotoxicity is PPARγ-independent, that the molecular mechanisms of apoptotic cell death are most likely involved in the hepatotoxicity, and that its idiosyncratic nature may be genetically determined.

Recent findings concerning the miRNA functions in specific tissues has enabled better understanding of the molecular mechanisms of various pathologies and diseases. Among several hundred miRNAs, we first reported the involvement of miRNA on the posttranscriptional regulation of CYPs (Tsuchiya et al. 2006). The decreased expression of miR-27b is one of the causes of the high expression of CYP1B1 protein in humans (Tsuchiya et al. 2006). In addition, we found that miR-148a posttranscriptionally regulated human hepatic pregnane X receptor, resulting in a modulation of the inducible and/or constitutive levels of CYP3A4 in human liver (Takagi et al. 2008). Therefore, studies on miRNAs and their targets could contribute to elucidating the mechanism of troglitazone-induced idiosyncratic hepatotoxicity.

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Nevirapine Hypersensitivity

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Contents

Abstract Treatment of HIV-1 infections with nevirapine is associated with skin and liver toxicity. These two organ toxicities range from mild to severe, in rare cases resulting in life-threatening liver failure or toxic epidermal necrolysis. The study of the mechanistic steps leading to nevirapine-induced skin rash has been facilitated by the discovery of an animal model in which nevirapine causes a skin rash in rats that closely mimics the rash reported in patients. The similarity in characteristics of the rash between humans and rats strongly suggests that the basic mechanism is the same in both. The rash is clearly immune-mediated in rats, and partial depletion of $CD4^+$ T cells, but not $CD8^+$ T cells, is protective. We have demonstrated that the rash is related to the 12-hydroxylation of nevirapine rather than to the parent drug. This is presumably because the 12-hydroxy metabolite can be converted to a reactive quinone methide in skin, but that remains to be demonstrated. Although the rash is clearly related to the 12-hydroxy metabolite rather than the parent drug, cells from rechallenged animals respond ex vivo to the parent drug by producing cytokines such as interferon-γ with little response to the 12-hydroxy metabolite, even when the rash was induced by treatment with the metabolite rather than the parent drug. This indicates that the response of T cells in vitro cannot be used to determine what caused an immune response. We are now studying the detailed steps by which the 12-hydroxy metabolite induces an immune

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response and skin rash. This animal model provides a unique tool to study the mechanistic details of an idiosyncratic drug reaction; however, it is likely that there are significant differences in the mechanisms of different idiosyncratic drug reactions, and therefore the results of these studies cannot safely be generalized to all idiosyncratic drug reactions.

Keywords Reactive metabolites · Skin rash · Idiosyncratic drug reactions · T cells · Brown Norway rat

1 Nevirapine-Induced Skin Rash in Patients

In June 1996, the US Food and Drug Administration (FDA) approved nevirapine (marketed as Viramune; Fig. 1) for the treatment of human immunodeficiency virus (HIV) 1 infection (Kubic 1997). Nevirapine was the first in a class of nonnucleoside reverse transcriptase inhibitor drugs that was marketed for use together with a nucleoside reverse transcriptase inhibitor, or protease inhibitor (Kubic 1997). Nevirapine was not approved as monotherapy because short-term treatment resulted in resistance (Havlir et al. 1996). The most common mutation leading to resistance was observed at the enzyme residue 181, from tyrosine to cytosine (Havlir et al. 1996), tyrosine 181 and tyrosine 188 representing nevirapine binding sites on the HIV-1 reverse transcriptase (Cohen et al. 1991).

Prior to marketing nevirapine, Boehringer-Ingelheim performed a series of clinical trials, which concluded that nevirapine can lead to both skin rash and liver toxicity in patients (Pollard et al. 1998). At a dose of 400 mg/day, the incidence of rash ranged from 32 to 48% (Taiwo 2006). A lower lead-in dose of 200 mg/day for the first 2 weeks of treatment followed by 400 mg/day reduced the frequency of skin rash to 17%, with 0.3% of rashes being severe, i.e., toxic epidermal necrolysis (TEN) or Steven's Johnson Syndrome (SJS) (Pollard et al. 1998). Liver toxicity was detected in 1% of the tested patient population (Pollard et al. 1998). The postmarketing reports collected by Boehringer-Ingelheim demonstrated that the prevalence of skin rashes was actually lower than observed prior to approval (decreased from 17 to 9%), but also that the incidence of liver toxicity was greater than observed prior to approval (increased from 1 to 3%; Nevirapine

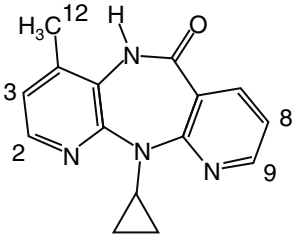

Fig. 1 Nevirapine

product insert). The first 6 weeks of nevirapine therapy are the most critical for the onset of adverse drug reactions (Pollard et al. 1998), hence, during the first 12 weeks, physicians are urged to provide close clinical and laboratory monitoring of the patients so that any life-threatening drug reactions are promptly identified and addressed (Antiviral Briefs 2001).

A number of risk factors have been associated with the onset of nevirapine-induced adverse events as outlined in Table 1.

Some of the above risk factors may be associations rather than being causally linked. For example, the substantial decrease in risk resulting from a 2-week lower lead-in dose is compelling evidence that a dose within the therapeutic range is a significant risk factor and that a low body mass index may represent a higher dose/weight ratio. If the rash is due to a reactive metabolite, the apparent lack of correlation with blood level may represent differences in the balance of metabolic pathways leading to different amounts of reactive metabolite, but the same level of parent drug. The increased risk associated with viral hepatitis and cotreatment with agents such as alcohol or isoniazid may represent an increased incidence of elevated transaminases associated with these conditions/drugs rather than an increased incidence of nevirapine-induced liver damage. Of note, nevirapine is not a drug of choice for postexposure prophylaxis because healthy patients have a higher propensity to develop liver and skin toxicity than HIV-infected individuals (Taiwo 2006), presumably because of the higher number and activity of $CD4^+$ T cells in a healthy patient population.

Tolerance induction treatments have been used for both nevirapine naïve and nevirapine-sensitized patients. Nevirapine naïve patients are started on a daily 100 mg dose during the first week of treatment followed by a weekly incremental dose increase by 100 mg until the final 400 mg dose is reached (Antón et al. 1999). In nevirapine-sensitized patients (those who have previously experienced nevirapine-related skin rash and/or liver toxicity at a 400 mg dose), a total of 12 nevirapine doses are administered starting with a tiny 2.5 μg dose, followed by a stepwise dose increase over a period of 5.5 h to reach a final top dose of 100 mg (Hosein 1999; Demoly et al. 1999; Messaad et al. 2002). Both tolerance treatments are partially successful, the former resulted in 2.1% of patients developing skin rash instead of the previously reported 9% (Antón et al. 1999), while the latter enabled two of three previously nevirapine-sensitized patients to continue with nevirapine treatment (Demoly et al. 1999). Although antihistamine or corticosteroid use are not advised in patients with nevirapine-induced skin rash and liver toxicity, in some patients antihistamine use may have prevented nevirapine-induced skin rash (Hosein 1999), while in others, corticosteroid use ameliorated TEN, SJS, and severe liver toxicity symptoms (Johnson et al. 2002). A combination of an intravenous immunoglobulin and N-acetylcysteine treatment was shown to be effective in a treatment of a single patient with a nevirapine-induced TEN and hepatitis, but no controlled trials were performed to confirm these results (Claes et al. 2004).

Despite significant research in the field of adverse drug reactions, the understanding of their mechanisms is still rudimentary. Most adverse drug reactions are characterized as type A or "augmented" and occur because the dose was too high, or the

Table 1 Risk factors for nevirapine-induced liver and skin toxicity

Predisposing factors	Details	References
Sex and weight	Skin rash and/or liver toxicity are more common in females with body mass index <18.5 and serum albumin level $<35\ gL^{-1}$	Taiwo (2006); Sanne et al. (2005); Bersoff-Matcha et al. (2001)
Ethnic background	Skin rash is more prevalent in the Chinese	Ho et al. (1998)
Genetic background	Combination of hepatitis, fever, or rash is associated with the HLA-DRB*0101 haplotype and high $CD4^+$ T cell count (>25%), whereas no association was found for skin rash alone	Martin et al. (2005)
	In Sardinians, HLA-Cw8 and HLA-B14(65) haplotypes are associated with increased incidence of skin rash	Littera et al. (2006)
$CD4^+$T cell count	Treatment initiation is contraindicated in males with $CD4^+$ T cell counts >400 cells mm^{-3} and in females >250 cells mm^{-3}	Nevirapine Product Insert
Liver enzyme levels	An abnormal baseline transaminase level is a risk factor for liver toxicity	Maniar et al. (2006)
Nevirapine plasma levels	Findings with regard to the role of nevirapine plasma levels are contradictory: (a) Nevirapine plasma concentrations greater than 6 μg mL^{-1} are associated with a 92% incidence of liver toxicity (b) Plasma nevirapine concentrations are not predictive of hepatotoxicity	González de Requena et al. (2002); Núñez et al. (2003); De Maat et al. (2003) De Maat et al. (2002)
Infection	Hepatitis B/C coinfection potentiates liver toxicity	Bonjoch et al. (2006); González de Requena et al. (2002); Manfredi and Calza (2007)
Diabetes, dyslipidemia	Increases risk of hepatotoxicity	Buyse et al. (2006)
Concomitant drug use	Alcohol, isoniazid, methadone, cocaine or metamphetamine use has been correlated with increased liver toxicity	Taiwo (2006); Buyse et al. (2006)
Antiallergenic drugs	Antihistamine and corticosteroid cotreatment increase skin rash incidence	Antinori et al. (2001); Montaner et al. (2003); Launay et al. (2004)
Pregnancy	Higher incidence of hepatotoxicity observed in women in their third trimester	Joy et al. (2005); Manfredi and Calza (2007)

patient had a somewhat greater response to the usual dose (Pirmohamed et al. 2002). This type of toxicity is usually reversed by decreasing the dose. Less frequent drug reactions belong to the type B "idiosyncratic" group, which represents reactions that are unpredictable because they do not occur in most patients at any dose. However, when they do occur, they can result in severe events including skin, blood, and liver toxicity. Circumstantial evidence supports the role of reactive metabolites and the host immune system in being the key factors in many idiosyncratic drug reactions (Pirmohamed et al. 2002). Nevirapine-induced skin rash and liver toxicity belong to this latter group of adverse drug reactions (Shenton et al. 2003). Ideally, mechanistic studies addressing the question of nevirapine-induced toxicity would be performed in patients previously sensitized with nevirapine; however, such an approach would be both unethical and potentially life-threatening. Therefore, alternative approaches are required such as valid animal models; nevertheless, development of animal models has proven to be very challenging. Luckily, in the case of nevirapine, an animal model of mild skin rash has been successfully accomplished, enabling further investigation of the steps leading to the skin rash onset in vivo (Shenton et al. 2003). This chapter addresses the discovery and characterization of a rat model of nevirapine-induced skin rash, highlights the findings the model has provided to date, and outlines the importance of animal models in general in elucidating the mechanistic steps leading to idiosyncratic drug-induced reactions in patients.

2 Animal Model of Nevirapine-Induced Skin Rash

In 2003, Shenton et al. reported a novel animal model of a drug-induced idiosyncratic reaction: nevirapine-induced skin rash in the female Brown Norway rat. This animal model does not reproduce severe skin rashes such as TEN and SJS, nor does it reproduce the liver toxicity observed in some nevirapine-treated patients. However, the skin rash that develops in nevirapine-treated rats closely resembles the mild erythematous rash observed in patients (Shenton et al. 2003).

2.1 Characteristics of the Skin Rash in Humans and Rats

Comparison of the rash characteristics in rats and humans demonstrated a number of parallels between the two species, emphasizing the similarity in the mechanisms leading to the rash, and strengthening the usefulness and validity of the new rat animal model. The full list of the characteristics is outlined below.

2.1.1 Time to Rash Onset

Patients have the highest risk of developing skin rash within the first 6 weeks of therapy, mostly between weeks 1 and 3 (Pollard et al. 1998). In Brown Norway rats,

Fig. 2 Rat model of nevirapine-induced skin rash

the situation is similar: nevirapine treatment results in an onset of red ears by day 7 and skin rash between weeks 2 and 3 (Fig. 2). All female Brown Norway rats develop a skin rash within the first 3 weeks of the treatment, while 20% of female Sprague–Dawley rats develop a rash at a later time point: between weeks 4 and 6 (Shenton et al. 2003).

2.1.2 Rash Severity Range

In patients, a range of rashes occur that vary from mild erythematous to blistering skin eruptions such as erythema multiforme, SJS and TEN (Pollard et al. 1998). In female rats, the rash starts with mild erythematous lesions, which over time progress to a more severe phenotype. No blistering skin reactions are observed in rats, partially because of their thin epidermis (Shenton et al. 2003).

2.1.3 Sex Predisposition

In both humans and rats, females are at a higher risk of developing the rash than males. In humans, the rash is often more severe in females leading to their discontinuation of the treatment (Bersoff-Matcha et al. 2001).

2.1.4 Skin Histology

In skin lesions of patients with nevirapine rash (erythema multiforme), the dermis is populated with a perivascular lymphocytic infiltrate and is associated with endothelial cell swelling (Havlir et al. 1995). In patients with SJS and TEN, mononuclear cells are observed in the upper dermis, lining the dermal-epidermal junction, and in the epidermis. In rats, a mononuclear infiltrate is observed in the dermis. Apoptotic keratinocytes are present in the epidermis and at the dermal-epidermal junction, mimicking the findings in the SJS and erythema multiforme patients (Shenton et al. 2003).

2.1.5 Dose-Dependency

At a nevirapine dose of 400 mg/day, 32–48% of patients develop skin rash versus 9% with a dose of 200 mg (Taiwo 2006). In female Brown Norway rats treated with nevirapine at a dose of 150 mg/kg/day, all developed a skin rash, while only half the rats developed the rash when dosed at 100 mg/kg/day. No rat developed a rash at doses of $\leq$75 mg/kg/day (Shenton et al. 2003).

2.1.6 Tolerance Induction

To decrease the incidence of skin rash in patients, a tolerance induction regime was established in patients: 200 mg of nevirapine is administered daily for the first 2 weeks, followed by the full therapeutic dose of 200 mg twice daily. This regime successfully decreases the rash incidence by about 50% (Nevirapine product insert). In rats, daily treatment with 40 or 75 mg/kg/day of nevirapine for the first 2 weeks followed by 150 mg/kg/day (a dose that otherwise leads to a 100% incidence of rash) completely prevents the skin rash (Shenton et al. 2005).

2.1.7 Rechallenge

Although controlled studies have not been conducted in which patients with severe nevirapine-induced skin rash are rechallenged, it appears that the onset of rash can be accelerated and the severity increased on rechallenge (Gangar et al. 2000). With mild rashes, it is sometimes possible to treat through them, i.e., the rash sometimes resolves despite continued treatment (Taiwo 2006; Gangar et al. 2000). In Brown Norway rats, primary challenge results in onset of red ears by day 7 and skin rash by day 14–21 of treatment, while rechallenge ranging from a month to a year post primary sensitization results in red ears within 24 hours and skin rash accompanied by malaise by day 4–7 of treatment (Fig. 2; Shenton et al. 2003). Rechallenged rats experience severe systemic illness not observed on the primary exposure, with as little as one 30th of the initial sensitizing dose (Chen et al. 2008). The rapid rash onset on rechallenge in both humans and rats suggests an amnestic response of the immune system.

2.1.8 T Cell Involvement

In the skin lesions of both patients and rats with nevirapine-induced skin rash, T cells have been observed. Furthermore, patients with low $CD4^+$ T cell counts have a significantly lower incidence of rash than those with normal counts, and in rats, partial depletion of these cells also decreases the incidence of skin rash (Shenton et al. 2005). Most drug-induced skin rashes are believed to be immune-mediated and dependence of the rash on $CD4^+$ T cells in both humans and rats supports this hypothesis.

2.2 *Immune Component of the Skin Rash in Rats*

2.2.1 Sequence of Events Resulting in the Skin Rash

To determine the chain of events that precede the onset of red ears and skin rash in nevirapine-treated rats, auricular lymph nodes and ear and neck skin sections were examined by immunohistochemistry and flow cytometry. By the end of the first week of treatment, an increase in the total mononuclear cells was observed in the auricular lymph nodes. One-third of these cells expressed either intercellular adhesion molecule (ICAM)-1 or major histocompatibility complex (MHC) II cell surface activation markers, in comparison to one-tenth and one-fifth in the controls, respectively (Popovic et al. 2006). Specifically, macrophages and B cells expressed the MHC II marker and likely acted as antigen-presenting cells in the course of skin rash development (Popovic et al. 2006). In the neck and ear skin, macrophage and eosinophil infiltration of the dermis and increased ICAM-1 expression were observed on day 7 of dosing, the time point at which rats have red ears but no skin rash. The infiltration by macrophages and eosinophils preceded lymphocyte infiltration into the skin, which was evident between days 14 and 21 of dosing and corresponded to the onset of skin rash (Fig. 2). This sequence of events outlines the importance of macrophages in the early stages of the immune response, and of lymphocytes (presumably T cells) in the later stages. Once present in the skin, macrophages may act as antigen-presenting cells, processing and presenting antigen to naïve infiltrating T cells. In rats presenting with skin lesions, overall MHC I and MHC II expression is significantly increased in the skin. Additionally, elevated interleukin (IL)-1β, IL-2, IL-4, IL-6, IFN-γ, and tumor necrosis factor (TNF)-α cytokine levels were detected in the sera of the treated rats (Baban et al., unpublished results). Interferon gamma plays a crucial role in MHC upregulation (Steiniger et al. 1988; Hao et al. 1989), while IL-1, IL-6, and TNF-α are proinflammatory cytokines that act as propagators of the immune response onset (Bernot et al. 2005). In combination, these cytokines promote a clinically evident immune response.

2.2.2 The Role of $CD4^+$ T and $CD8^+$ T Cells in the Skin Rash

Starting with the second week of nevirapine treatment, progressive infiltration of T cells into the rat skin dermis was observed. To assess the specific role of the T cell populations in triggering the onset of skin rash, Shenton et al. transferred splenocyte T cells from rechallenged rats into naïve recipients. Spleen $CD4^+$ T and $CD8^+$ T cells were isolated from the nevirapine-rechallenged rats, purified, and intravenously injected into naïve recipients, which were then started on a full (150 mg/kg/day) nevirapine dose (Shenton et al. 2005). Recipients of $CD4^+$ T cells developed skin rash 9 days later, while $CD8^+$ T cell recipients behaved as nevirapine naïve rats, only developing red ears by day 7 and skin rash by day 21

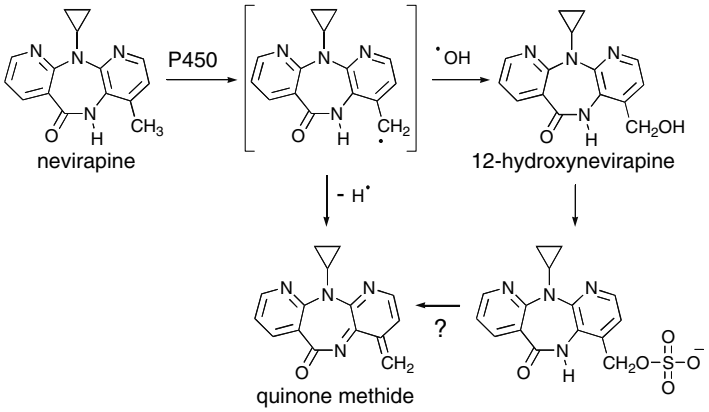

Fig. 3 Nevirapine metabolic scheme (Chen et al. 2008)

of the treatment (Shenton et al. 2005). Furthermore, a delayed onset of skin rash in naïve rats partially depleted of $CD4^+$ T cells confirmed the key role of $CD4^+$ T cells in the development of rash, while an almost complete depletion of $CD8^+$ T cells led, if anything, to a more severe rash (Shenton et al. 2005). It is important to consider the role of both effector and regulatory T cells in the aforementioned experiments. Examination of the auricular and mesenteric lymph nodes of nevirapine-treated rats revealed increased FoxP3 expression, a marker of regulatory T cells (Cosmi et al. 2003) in both $CD4^+$ T and $CD8^+$ T cell populations, with higher expression in the $CD8^+$ T cell population (Abdulla, unpublished observation). In addition, rat sera showed elevated levels of the antiinflammatory cytokine IL-10, indicator of the regulatory $CD8^+$ T cell role (Filaci and Suciu-Foca 2002). Hence, the apparent increase in rash severity in $CD8^+$ T cell-depleted animals may have been due to a decrease in a population of $CD8^+$ T regulatory cells.

2.2.3 Mechanism of Tolerance Induction

Partial depletion of $CD4^+$ T cells successfully delayed the onset of skin rash; however, it did not completely prevent it. Due to the key role of the immune system in the onset of rash, studies were conducted to determine if the rats could be made immunologically tolerant to nevirapine. A 2-week low-dose nevirapine treatment prior to the full dose resulted in long-term tolerance to continued nevirapine dosing. Similar findings were reported in the D-penicillamine model of drug-induced lupus in male Brown Norway rats (Masson and Uetrecht 2004). In these rats, 2 weeks of a lower dose followed by the full dose of D-penicillamine resulted in long-term tolerance even if D-penicillamine dosing was stopped and restarted. In the case of nevirapine, absence of continued nevirapine dosing resulted in the loss of tolerance, and rats rechallenged with nevirapine developed skin rash following the time

course observed in naïve rats (Popovic et al. 2006). Unlike in the D-penicillamine rat model, the tolerance induced by low-dose nevirapine treatment did not have immunologic memory; in D-penicillamine-treated rats, tolerance was transferable with splenocytes isolated from the tolerant donor rat to a naïve recipient rat (Masson and Uetrecht 2004), which was not the case with the nevirapine model. Nevirapine is a known cytochrome P450 inducer; therefore, the low dose treatment leads to lower nevirapine levels when the dose is increased, and this could result in tolerance to the higher dose. In support of this hypothesis, cotreatment with aminobenzotriazole, a general P450 inhibitor, eliminated the tolerance induced by low dose treatment and all of the animals developed a rash. Thus, the tolerance can be termed "metabolic" rather than immune (Shenton et al. 2005). To test whether immune tolerance could be induced in nevirapine-treated rats using other means than dose escalation, various cotreatments with nevirapine and immunosuppressants, such as tacrolimus and cyclosporine, or antiallergic drugs such as a combination of cromolyn, astemizole, and ketanserin were performed. Cotreatment with antiallergic drugs did not result in tolerance; however, 5 weeks of cotreatment with nevirapine and immunosuppressant prevented skin rash not only during the cotreatment phase but also post immunosuppressant withdrawal with continued nevirapine dosing (Shenton et al. 2005). Furthermore, when nevirapine dosing was stopped and restarted in rats already tolerized by cotreatment with immunosuppressant, the rats remained partially tolerant to nevirapine as would be expected for immune tolerance. It is possible that full immune tolerance may occur after a longer period of immunosuppressant treatment during initial nevirapine dosing. In the case of nevirapine rechallenge, cotreatment with immunosuppressants did not prevent the rash in previously sensitized rats, nor did immunosuppressant use post-nevirapine termination decrease the time to rash recovery.

2.2.4 Nevirapine Hydroxylation is Required for Rash Induction

An important question is whether the rash is caused by the parent drug or a reactive metabolite? Most idiosyncratic drug reactions are believed to be caused by reactive metabolites of the drug rather than the parent drug (Naisbitt et al. 2001). A case report was published on a patient who suffered nevirapine-induced hepatitis and had peripheral mononuclear cells that were activated by nevirapine (Drummond et al. 2006). In addition, a poster by Keane et al. (2007) reported patients which a history of nevirapine hypersensitivity reactions whose peripheral mononuclear cells responded to nevirapine with the production of IFN-γ. This is consistent with the pharmacological interaction (p–i) hypothesis (Pichler 2002).

In the animal model, based on variations between strains and males versus females, there was a correlation between the blood level of nevirapine and the incidence of rash (Chen et al. 2008). In addition, inhibition of metabolism by aminobenzotriazole led to higher nevirapine blood levels and rash at a lower dose, with lower levels of most metabolites. However, there was one metabolite

that was not decreased by aminobenzotriazole: 12-hydroxynevirapine. It appears that this is because P450 is responsible for both the formation of this metabolite and its further oxidation to a carboxylic acid. We proposed that this metabolite could be sulfated in the skin and that elimination of the sulfate would form a reactive quinone methide. If this is true, treatment of animals with 12-hydroxynevirapine should produce a rash at a lower dose than required for nevirapine. This was found to be true, but this observation did not prove that the 12-hydroxylation was required to induce a rash. Substitution of the methyl hydrogen atoms of nevirapine with deuterium should decrease the rate of 12-hydroxylation by a factor of 2–8 (deuterium isotope effect; Nelson and Trager 2003) without changing any other properties of the drug, and if oxidation is required for the rash, the deuterated analog should not cause a rash at the same dose as nevirapine. The only other change should be somewhat higher blood levels of nevirapine because of the decrease in 12-hydroxylation, which is one of the three major metabolic pathways. Although the deuterated analog did not cause a rash, the very surprising result was that the blood concentrations of the deuterated analog, instead of being slightly higher than those of nevirapine, were markedly lower. There are now several lines of evidence that the reason for this discrepancy is that the P450-generated free radical precursor to 12-hydroxynevirapine can also lose another hydrogen atom to directly form the same quinone methide that would be formed from elimination of sulfate from the 12-sulfate. This quinone methide binds to P450 leading to its inhibition, and because less of the quinone methide is formed from the deuterated analog, there is less P450 inhibition and therefore faster oxidation of the deuterated analog through the other oxidative pathways. To overcome this difference, both the nevirapine- and deuterated nevirapine-treated animals were cotreated with aminobenzotriazole. This led to very similar blood levels of the parent drug in nevirapine and the deuterated analog, and yet, compared to the nevirapine-treated animals where the rash incidence was 100%, only one out of five animals treated with the deuterated analog developed a rash (Chen et al. 2008). These experiments provide conclusive evidence that 12-hydroxylation of nevirapine is required in order to produce a rash; however, it remains to be determined whether sulfation of this metabolite and formation of the quinone methide is also required. The fact that the 12-hydroxy metabolite causes a rash proves that the rash cannot be caused by direct oxidation of nevirapine to the quinone methide, because the 12-hydroxy metabolite is not converted back to nevirapine.

2.2.5 T Cell Response to Nevirapine and Its Metabolites

The fact that 12-hydroxylation of nevirapine is required in order to induce a rash appears to be in conflict with the observation that peripheral mononuclear blood cells from patients with a history of nevirapine-induced hypersensitivity reactions were activated by the parent drug in the absence of a metabolizing system. When the response of cells from the cervical lymph nodes of sensitized rats was studied, they also responded to nevirapine by producing IFN-γ analogous to the human study, but

there was very little response to the 12-hydroxy metabolite (Chen et al. 2009). Depletion of $CD4^+$ T cells but not $CD8^+$ T cells abolished the response. Furthermore, when the rash was induced by treatment with the 12-hydroxy metabolite and the animals had never been exposed to nevirapine, their T cells still responded to nevirapine with little response to the 12-hydroxy metabolite. Thus, there is a disconnect between what induces the rash and what the T cells from the affected animals respond to. At this point it is not known what role the T cell response to the parent drug plays in the pathogenesis of the rash, we only know that the parent drug cannot induce the rash. This calls into question the basis for the p–i hypothesis, which is based on the implied assumption that what the T cells from an affected person respond to is what initiated the adverse reaction in the first place.

3 Conclusion and Future Directions

Understanding the mechanisms of rare, unpredictable, idiosyncratic drug reactions is of great importance for the successful development of new and safe pharmaceutical compounds. As with virtually every area of biomedical research, valid animal models are essential to test hypotheses and to investigate the detailed steps involved. The fact that there are very few valid animal models for the study of idiosyncratic drug reactions has been a major handicap in the study of these complex adverse reactions. The nevirapine model has provided significant insight into the mechanism of nevirapine-induced skin rash. For example, there are at least six potential reactive metabolites of nevirapine, and without the animal model it would be impossible to determine which pathway is involved or if the rash is caused by the parent drug. The animal model conclusively identified the 12-hydroxylation pathway as being responsible for the rash, and it would be virtually impossible to determine this any other way. It also uncovered the direct oxidation of nevirapine to a reactive metabolite. Furthermore, it led to insight into the basis for the p–i hypothesis and led to the unexpected finding that what induces an immune response is not necessarily what activates T cells in vitro. Further studies are ongoing to determine exactly what chemical species induces the rash and how it induces an immune response. Again, such studies simply are not possible without an animal model. An important question is how well the mechanism of this idiosyncratic reaction reflects the mechanism of other idiosyncratic drug reactions. It is likely that there are significant differences in the mechanisms of idiosyncratic drug reactions caused by different drugs and possibly even the same drug in different patients; therefore, it would be dangerous at this time to extrapolate the findings from the nevirapine model to infer the mechanism of idiosyncratic reactions to other drugs. We need more valid animal models to determine the range of possible mechanisms. If we had a better mechanistic understanding, it would probably be easier to develop such models.

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Mechanisms of Adverse Drug Reactions to Biologics

Janet B. Clarke

Contents

Abstract Biologics encompass a broad range of therapeutics that include proteins and other products derived from living systems. Although the multiplicity of target organs often seen with new chemical entities is generally not seen with biologics, they can produce significant adverse reactions. Examples include IL-12 and an anti-CD28 antibody that resulted in patient deaths and/or long stays in intensive care units. Mechanisms of toxicities can be categorized as pharmacological or nonpharmacological, with most, excepting hypersensitivity reactions, associated with the interaction of the agent with its planned target. Unexpected toxicities generally arise as a result of previously unknown biology. Manufacturing quality is a significant issue relative to the toxicity of biologics. The development of recombinant technology represented the single biggest advance leading to

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humanized products with minimal or no contaminants in comparison to products purified from animal tissues. Nevertheless, the type of manufacturing process including choice of cell type, culture medium, and purification method can result in changes to the protein. For example, a change to the closure system for erythropoietin led to an increase in aplastic anemia as a result of changing the immunogenicity characteristics of the protein. Monoclonal antibodies represent a major class of successful biologics. Toxicities associated with these agents include those associated with the binding of the complementary determining region (CDR) with the target. First dose reactions or infusion reactions are generally thought to be mediated via the Fc region of the antibody activating cytokine release, and have been observed with several antibodies. Usually, these effects (flu-like symptoms, etc.) are transient with subsequent dosing. Although biologics can have nonpharmacologic toxicities, these are less common than with small molecule drugs.

Keywords Biologics · Mechanisms · Pharmacological · Monoclonal antibodies · Cardiotoxicity · Superagonism

1 Introduction

Biologics (biotherapies, biotechnology-derived pharmaceuticals) are a rapidly growing sector of pharmaceuticals that are approved for a variety of therapeutic areas, including the treatment of cancer, rheumatoid arthritis, multiple sclerosis, and many others. Although definitions of biologics can vary somewhat, for the purpose of this chapter they encompass products derived from living systems and include endogenous proteins and peptides, monoclonal antibodies, fusion proteins, and vaccines. Among the first biologics were proteins isolated from animal tissues, such as porcine insulin for diabetes or horse antiserum to treat diphtheria. Following the advent of technology to allow sequencing of human proteins and recombinant DNA technology to enable their production, the development of drugs such as insulin, Factor VIII, and others represented a major advance in the ability to treat serious diseases. The breakthrough of using cell fusion for the production of monoclonal antibodies from immunized mice (Kohler and Milstein 1975) was the first step that has led to the plethora of monoclonal antibodies as therapeutics, arguably the most successful class of biologics. Table 1 lists a selection of currently approved biologics in the US. Most of these biologics are manufactured through the growth of genetically modified cells in vitro that have the appropriate insertion of a sequence of DNA and a promoter sequence such that the cells secrete the desired protein into the culture supernatant. The culture of the cells and subsequent purification process to obtain the protein are extremely important and relevant in the consideration of potential adverse reactions to biologics.

Table 1 Selected marketed biologics and associated adverse events

Generic name	Target/ligand	Indication	Adverse reactions
Monoclonal antibodies			
Alemtuzumab	CD52	B-cell chronic lymphocytic leukemia	Infections, hematologic toxicity, infusion reactions, hypersensitivity
Adalimumab	TNFα	Rheumatoid arthritis, Crohn's disease	Infection, neurological events, lymphoma, hypersensitivity, infusion reactions
Abciximab	IIbIIa	Adjunct to percutaneous coronary intervention	Bleeding, thrombocytopenia
Basiliximab	CD25	Organ transplant	Hypersensitivity
Bevacizumab	VEGF	Colorectal cancer	Gastrointestinal perforations, wound healing inhibition, hemorrhage, hypertension, protein urea, infusion reactions
Cetuximab	EGFr	Colorectal cancer	Infusion reactions, skin rash, pulmonary toxicity
Daclizumab	CD25	Organ transplant	Hypersensitivity
Efalizumab	CD11a	Psoriasis	Infection, thrombocytopenia, hypersensitivity, infusion reactions
Infliximab	TNFα	Rheumatoid arthritis, Crohn's disease	Infection, neurological events, lymphoma, hypersensitivity, infusion reactions
Trastuzumab	HER2	Breast cancer	Cardiomyopathy, hypersensitivity
Rituximab	CD20	Non-Hodgkins lymphoma	Fever, infusion reactions, tumor lysis syndrome, infections, progressive multifocal leukoencephalopathy (PML)
Palivizumab	F protein of respiratory syncytial virus	Prophylaxis of serious lower respiratory tract disease	Hypersensitivity
Omalizumab	IgE	Asthma	Malignant neoplasms, anaphylaxis
Natalizumab	4 integrin	Multiple sclerosis	Hypersenisitivy, progressive multifocal leukoencephalopathy (PML)
Recombinant endogenous proteins			
Alteplase	Tissue plasminogen activator	Restoration of function to central venous access devices	Bleeding, infection
Anakinra	rhIL receptor antagonist	Rheumatoid arthritis	Infections, injection site reactions

(*continued*)

Table 1 (continued)

Generic name	Target/ligand	Indication	Adverse reactions
Betaseron	Interferon γ 1b	Multiple sclerosis	Injection site reactions, flu-like symptoms
Rebif	Interferon β 1a	Multiple sclerosis	Hepatotoxicity, flu-like symptoms, suicide and depression, anaphylaxis
Insulin aspart	Insulin	Diabetes	Hypoglycemia, injection site reaction, systemic allergy
Laronidase	a-L-iduronidase	MPS I	Infusion reaction, rash, infection
Rasburicase	Recombinant urate-oxidase enzyme	initial management of plasma uric acid levels in pediatric patients expected to result in tumor lysis	Hypersensitivity, methemaglobinemia, hemolysis
Agalisdase β		Fabry disease	Infusion reactions
Epoetin α	Erythropeitin	Anemia	Infection, hypertension, cardiovascular events, hypertension, seizures, pure red cell aplasia
Dornase α	DNase	Cystic fibrosis	Voice alteration, rash
Imiglucerase		Gaucher disease	Infusion reactions
Oprelvekin	rhIL-11	Thrombocytopenia in chemotherapy	Hypersensitivity, fluid retention, pulmonary edema, ventricular arrhythmias
Aldesleukin	rhIL-2	Metastatic renal cell carcinoma	Capillary leak syndrome, infection, sepsis, CNS, pulmonary and cardiac effects
Reteplase	Rh plasminogen activator	Myocardial Infarction	Bleeding, arrhythmias
Mecasermin	rhIGF-1	Growth failure secondary to severe primary IGF-1 deficiency	Hypoglycemia, intracranial hypertension, transaminas elevations, otitis, cardia murmur
Drotrecogin α	Rh activated protein C	Severe sepsis	Bleeding
Follitropin	Hu follicle stimulating hormone	Infertility	Ovarian enlargement, ovarian hyperstimulation syndrome, pulmonary embolism, pulmonary infarction
Tenecteplase	Tissue plasminogen activator	Acute myocardial infarction	Bleeding, arrhythmias, cholesterol embolization
Teriparatide	Parathyroid hormone	Osteoperosis	Nausea, injection site reactions
Pegfilgrastim	Pegylated-GCSF	Neutropenia	Splenic rupture, respiratory distress

(*continued*)

Table 1 (continued)

Generic name	Target/ligand	Indication	Adverse reactions
Becaplermin	rhPDGF	Diabetic ulcers	Increased mortality secondary to malignancy, erythmatous rashes
Fusion proteins			
Abatacept	Anti-CTLA4-Fc fusion	Rheumatoid arthritis	Infusion reactions, infections, malignancies, hypersensitivities
Alefacept	LFA-3 Fc fusion	Psoriasis	Malignancy, infection
Etanercept	TNF receptor Fc fusion protein	Rheumatoid arthritis	Serious infection, malignancies, hepatotoxicity, hypersensitivity, infusion reactions
Rilonacept	IL-1 receptor Fc fusion	Cryopyrin-Associated Periodic Syndromes	Injection site reactions, infections
Vaccines			
BCG	NA	NA	Lymphadenitis, osteomyletis (rare, 1 in one million), disseminated BCG infection (1-10 per 10 million)
Diptheria, pertussis, hepatitis B and tetanus toxoid	NA	NA	Injection site pain, fever
Hepatitis A	NA	NA	Injection site pain, headache, allergic reaction
Influenza	NA	NA	Injection site reaction, muscle ache, autoimmune hemolytic anemia, nervous system and respiratory disorders
Measles, mumps and rubella	NA	NA	Thrombocytopenia, hypersensitivity to egg components or neomyecin, fever, vasculitis, arthritis, pneumonitis
Polio	NA	NA	Injection site reaction, fever, anaphylaxis (rare)

Although a focus on adverse events mechanisms with biologics can leave the impression that these types of therapeutics have substantial toxicities, as a class, they are often generally well tolerated. Preclinical studies rarely identify dose-limiting toxicities, and dose range finding pilot studies are rarely necessary to progress from shorter-term toxicology testing to chronic studies. Since biologics can have highly species-specific activities, the selection of the appropriate, pharmacologically relevant species for investigating mechanisms of toxicity is crucial.

2 Mechanisms Overview

Broadly speaking, the mechanism of toxicity associated with biologics can be grouped into similar categories as those for new chemical entities (NCE); that is, those that are pharmacologically mediated and those that are not (Fig. 1). Pharmacologically mediated is defined as the interaction of the biologic with its intended target, such as the binding of anti-vascular endothelial growth factor (anti-VEGF) antibody to VEGF or the interaction of an endogenous protein such as interleukin 12 (IL-12) with its relevant receptor. Interaction with this intended target can result in an anticipated biological effect, such as the reduction in blood glucose by insulin, or in a previously unanticipated effect such as cardiotoxicity secondary to HER2 downmodulation on myocytes after the administration of anti-HER2 antibody. Nonpharmacological effects are those unrelated to the interaction with the intended target and include, for example, hypersensitivity reactions secondary to an immune response to the protein or acute phase reactions due to the Fc region of a therapeutic antibody. From a purely quantitative perspective, the most frequent adverse events in patients taking biologics are hypersensitivity reactions, thus falling into the nonpharmacological category. However, in terms of the diversity of types of adverse events, by far the majority are pharmacologically mediated. In fact, excepting the two examples cited above (hypersensitivity and acute phase reactions), almost all adverse events to biologics, whether initially understood or not, turn out to be pharmacologically mediated. Why is this?

Proteins are catabolized into amino acids that are indistinguishable from endogenous amino acids, which are then recycled into other proteins via new protein synthesis or are excreted. There are therefore no metabolites per se, no production

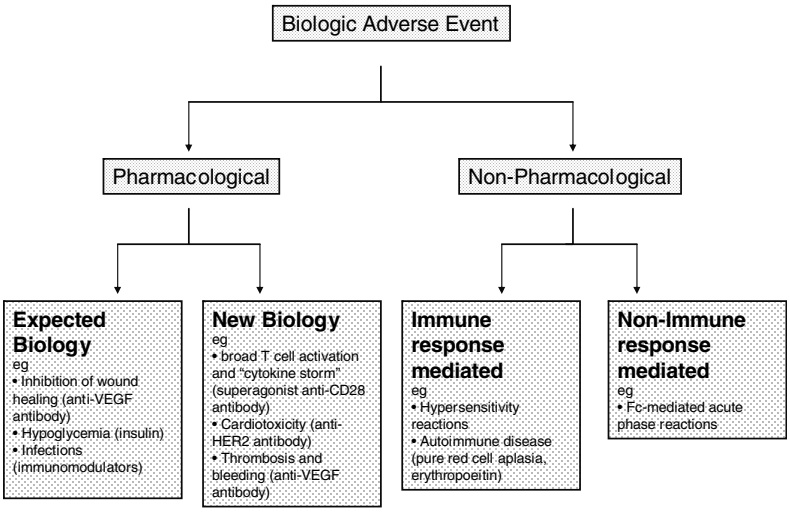

Fig. 1 Categories for mechanisms of toxicity associated with biologics

of new chemical entities, no xenobiotic that may have a previously unpredicted and non-pharmacological interaction with a cell system. Proteins are so critically dependent on primary (sequence), secondary (folding), and tertiary structure for their proper interaction in the in vivo system that catabolism results in a biologically inert molecule (with the exception as noted above of potentially serving as an immunogen). It therefore follows that the majority of the toxicological effects of an exogenously administered protein reside from its interaction with the relevant ligand or receptor in the in vivo system.

It does not, however, follow that all effects of biologics are relatively benign. Although many are well tolerated, there are examples of serious and even life-threatening toxicities occurring through pharmacological interactions, including two, IL-12 and an anti-CD28 superagonists, that are reviewed in this chapter. The examples selected for discussion in this chapter cover monoclonal antibodies, including those that are designed to inhibit the target (anti-VEGF antibody, anti-HER2 antibody), and those designed to agonize the target (anti-CD28 antibody). Although monoclonal antibodies and endogenous proteins share similar issues and mostly common mechanisms, a unique mechanism relative to the latter can involve the inadvertent removal of the native endogenous protein as a result of an autoimmune response to the exogenously administered therapeutic. Erythropoietin is an example of this and is discussed relative to the implications for product quality and the implications of a change in how the material is vialed. Finally, combination toxicity is relatively rare with biologics. They are not metabolized by the cytochrome P450 system, as are many NCEs, and therefore do not present interaction concerns with other NCEs based on cytochrome P450 induction or inhibition. However, though uncommon, it is possible for an interaction to occur on the basis of intersecting biological effects, and the anti-HER2 antibody toxicity in combination with anthracyclines is discussed as an example of this.

3 Pharmacologically Mediated Toxicities

3.1 Cardiotoxicity with a Monoclonal Antibody: Trastuzumab

Trastuzumab is a humanized monoclonal antibody against HER2 (ErbB2) and is used for the treatment of breast cancer in patients whose tumors overexpress this tyrosine kinase receptor on their cell surface. Trastuzumab treatment as a single agent resulted in overall response rates of 15–30% with substantially increased benefits in combination with other chemotherapies including anthracyclines. However, beginning in late phase clinical trials, cardiotoxicity was noted in a percentage of treated patients. Congestive heart failure occurs in 1–4% of treated patients, and 10% of patients have decreased cardiac function. The incidence of cardiac dysfunction increases in combination with exposure to anthracylines, over and above the known cardiotoxicity associated with these latter drugs as single agents.

Thus trastuzumab will serve in our discussion, both as a relatively rare example of a combination toxicity associated with a biotherapeutic, as well as an example of what appears to be a pharmacologically mediated mechanism of toxicity (a more common category with biotherapeutics).

Although a complete understanding of the desired pharmacological mechanism of action of trastuzumab is not yet established, it is thought to have activity through several mechanisms, all involving sequelae following binding to tumor surface HER2. Trastuzumab can kill tumor cells as a result of antibody-mediated cellular cytotoxicity (ADCC) through the activation of natural killer cells expressing the Fc gamma receptor. Trastuzumab may also downmodulate the expression of HER2 on the surface of the tumor cell removing or reducing the tumorigenic effects of HER2 overexpression such as the promotion of angiogenesis. Other possible mechanisms as well as the above have been reviewed recently in an article by Valabrega and colleagues (Valabrega et al. 2007). Of most relevance to the discussion of the mechanisms of cardiotoxicity is the inhibition and/or downmodulation of HER2 expression, for, as it turns out, HER2 likely has a role in the survival of normal healthy myocytes under situations of stress.

Before discussing this, it should be noted that the traditional range of safety studies to support the registration of the drug did not detect cardiotoxicity in normal healthy animals, either as a single agent or in combination with anthracyclines. Tissue cross-reactivity studies did not detect binding of the antibody to the heart using immunohistochemistry techniques.

Studies with murine knockouts have demonstrated that ErbB2, its coreceptor ErB4, and the ligand for the latter, neuregulin, are essential for the normal development of the heart. However, prior to the clinical cardiotoxicity observed with trastuzumab, there was little knowledge about the role of HER2 in the adult heart. A conditionally mutated mouse with a deficiency of ErbB2 only in the ventricle was developed to study this. These mice, although viable, developed cardiomyopathy as adults, including chamber wall thinning, decreased contractility, and chamber dilation (Crone et al. 2002). Cardiomyocytes prepared from these mice were more susceptible to cytotoxicity following incubation with anthracyclines than myocytes from normal mice. Taken together, this and other work has led to the overall hypothesis that the ErbB2/ErbB4 complex provides a survival mechanism to myocytes in the face of cellular stresses that could otherwise lead to cell death. With the reduction in ErbB2 (HER2) levels associated with trastuzumab treatment, the cells have less protection against a subsequent challenge with a stressful stimulus such as anthracycline exposure, leading to cell death.

An understanding of this two hit hypothesis led to the question of whether the clinical cardiotoxicity could be ameliorated by avoiding coadministration of trastuzumab with anthracylines by staggering the treatment regimen, thereby allowing ErbB2 levels to have recovered to a sufficient degree to protect the myocytes against anthracyline toxicity. Indeed, a small clinical trial to test this hypothesis showed promising results by maintaining the overall benefits of trastuzumab and chemotherapy treatment while greatly reducing the incidence of cardiotoxicity (Joensuu et al. 2006).

3.2 Interleukin-12

Interleukin-12 (IL-12) is a heterodimeric cytokine, which regulates cell-mediated immunity (Trinchieri 1993). This cytokine is produced by monocytes/macrophages, B cells, neutrophils, and dendritic cells in response to stimuli produced during infections. IL-12 has been studied for its therapeutic potential in the treatment of cancer and infectious diseases (Gollob et al. 2000) and included an evaluation in a phase II trial for the treatment of renal cell carcinoma. Administration of IL-12 in the phase II trial resulted in serious adverse events resulting in hospitalization and deaths (Leonard et al. 1997). The mechanism of this toxicity was investigated by analyzing the clinical data and by investigative toxicology studies in several animal models. The toxicity appears to have been associated with an exaggerated pharmacological activity of IL-12.

IL-12 has been found to have several biological activities, principally enhancing natural killer (NK)-mediated cytotoxicity and promoting T-helper cell type I immune responses. The latter occurs through the action of IL-12 on Th naïve cells to promote differentiation to Th1 cells. Although IL-12 stimulates the production of a variety of cytokines by cells in the immune system, the increased production of IFN-γ by NK and T cells is considered a principal mediator of its pharmacologic activity. IL-12 treatment has been shown to have anti-tumor and anti-metastatic activities in mice in vivo which involve direct and indirect T cell effector mechanisms including IL-12-induced secretion of IFN-γ (Weiss et al. 2007). The biology of IL-12 has been reviewed elsewhere (Trinchieri 1993; Del Vecchio et al. 2007).

IL-12 (500 ng kg^{-1}) administered to patients in a phase II trial resulted in serious adverse events that involved the liver (elevated transaminases, hyperbilirubinemia) and hematopoietic system (leucopenia, thrombocytopenia), and included severe fatigue, dyspnea, and stomatitis (Leonard et al. 1997). Twelve patients were hospitalized and two died, even though the same dose was tolerated in a phase I trial (Gollob et al. 2000). The investigation of this toxicity focused on several areas including: patient characteristics, test material differences, pharmacokinetics, regimen comparison, and animal models. The patient population in the two trials was similar, with similarity in age and race, the proportion who had previously received IL-12 treatment, and cancer status. A change in the manufacturing process, described as minor, was made between the phase I material and phase II material. However, in vitro biological activity and biochemical characterization, including amino acid composition, N-terminus sequencing, gel electrophoresis, peptide mapping, carbohydrate analysis, in vitro stimulated proliferation assays, and peripheral blood lymphocyte IFN-γ induction assay, were all comparable between the materials used and were not sufficient to account for the dramatic toxicities seen in phase II patients. Although only limited data were available due to the abbreviated nature of the trial, the pharmacokinetics of IL-12 were not reported to be significantly different between the patient groups. The regimen employed between the two trials was different: in phase I, patients were administered a single dose of

IL-12 14 days prior to subsequent consecutive doses whereas in Phase II, patients were administered consecutive doses without a pretreatment dose.

The mechanism of toxicity that was elucidated with animal models and confirmed by analysis of the clinical data was found to be associated with the expected induction of IFN-γ by IL-12 and was significantly affected by the regimen employed. Animals including mice and nonhuman primates demonstrated similar toxicities associated with IL-12 treatment as seen in patients (Car et al. 1999). Using recombinant murine IL-12, mice administered IL-12 at 500 ng/day had a high incidence of mortality that was ameliorated when the same dose was administered 7 days prior to consecutive daily dosing. This reduction in toxicity correlated with reductions in serum IFN-γ concentrations. Interestingly, different strains of mice were found to have differing sensitivities in terms of severity of toxicities observed as well as qualitative differences, including the absence of muscle toxicity in the strain of mice known to be deficient in inducible isoenzymes, phospholipase A2 (Car et al. 1999). Muscle toxicity was not reported in humans. Additional experiments in mice included coadministration with neutralizing antibodies to IFN-γ as well as administering IL-12 to a strain of mice deficient in the IFN gamma receptor. These studies confirmed that the toxicity seen in mice is associated with the overproduction of IFN-γ. The analysis of the clinical trial data further confirmed that the patients in the phase II trial had higher levels of IFN-γ even though the pharmacokinetics of IL-12 were similar when compared to patients in the phase I trial. Furthermore, the direct administration of IFN-γ in humans results in some of the same effects, although less severe, as those observed with IL-12 treatment. The mechanism by which an earlier "priming" dose of IL-12 blunts the subsequent IFN-γ response is not understood. However, because IFN-γ production by IL-12 was established as a necessary and desired component of the antitumor pharmacological activity of IL-12, this toxicity appears to be in the class of exaggerated pharmacological activity.

3.3 Multiple Adverse Events with a Monoclonal Antibody, Bevacizumab

Bevacizumab is a recombinant humanized IgG1 antibody that binds to and inhibits the biologic activity of human vascular endothelial growth factor (VEGF). VEGF is an endothelial-specific survival factor (Gerber et al. 1998b), mitogen, and angiogenic factor, which also has significant effects on vascular permeability (Ferrara et al. 2004). VEGF activity is mediated through its interaction with two receptors on endothelial cells, KDR and Flt1, which are high affinity tyrosine kinase receptors. Bevacizumab is indicated for first- or second-line treatment of patients with metastatic carcinoma of the colon or rectum. Bevacizumab was the first in the class of angiogenesis inhibitors that, by inhibiting the formation of new vessels from preexisting vasculature, proved the potential of this therapeutic approach to cancer treatment originally proposed by Judah Folkman more than 30 years ago. In

combination with chemotherapy, treatment with bevacizumab has extended survival by 4.5 months. Adverse events associated with bevacizumab treatment in patients have included gastrointestinal perforations, wound healing complications, arterial thromboembolic events, proteinurea, hemorrhage, and hypertension. At first glance, at least some of these events would seem to comprise mechanisms that are unlikely to be related to the pharmacological action of inhibiting angiogenesis; however, as the story has unfolded, many of them have been found to have an association with biological activities of VEGF that were previously unknown or due to a complex biological process that led unexpectedly to toxicity.

Angiogenesis is known to play a role in a range of physiological processes including wound healing, embryogenesis, and corpus luteum formation. It is therefore not surprising that bevacizumab administered to cynomolgus monkeys at doses up to 50 mg/kg led to physeal dysplasia in both sexes and an absence of corpora lutea in females (Ryan et al. 1999). Longtitudinal bone growth occurs at the physis and is characterized by an organized sequence of chrondocyte differentiation through zones of resting, proliferating, and hypertrophied chrondocytes. Vascular invasion of the hypertrophied chrondocytes and subsequent mineralization is a critical component in the net lengthening of the bone. Both physical disruption of the blood supply, as well as disruption through bevacizumab administration, results in a significant increase in the width of the physis also characterized by disorganization of the chrondocyte columns and absence of vascular invasion. More recently, VEGF has also been shown to be a survival factor for chrondocytes (Zelzer et al. 2004). The net result in these adolescent monkeys was the disruption in the normal growth and closure of the bone plate. Ovarian and uterine weights were significantly reduced in females by administration of bevacizumab at doses of 10 and 50 mg/kg (but not 2 mg/kg). Microscopically, decreased ovarian weights correlated with an absence of corpora lutea in the high dose group (Ryan et al. 1999). Cyclical angiogenesis is a necessary part of the life cycle of a healthy ovary. The development of corpora lutea is associated with the proliferation of vessels in the theca interna, which invade the ruptured follicle after release of the egg, to form a capillary network around the luteal cells. VEGF expression has been demonstrated to be time- and location-specific relative to the corpora lutea cycle. Other means of VEGF inhibition in rats have likewise demonstrated inhibition of corporal lutea formation. In summary, both effects on bone growth and corpora lutea formation appear to be directly related to the inhibition of new vessel formation through inhibition of VEGF by bevacizumab treatment. What then of the other adverse events associated with bevacizumab treatment?

The label for bevacizumab describes an increased incidence of arterial thromboembolic events, such as cerebral infarction, transient ischemic attacks, and others, when combined with chemotherapy compared with chemotherapy alone. Although the potential mechanisms underlying these events have not been fully elucidated, two main hypotheses involve either endothelial cells lining the vessels becoming prothrombotic or direct activation of platelets. Given the complexity of the mechanisms maintaining a patent vasculature, these may not indeed be mutually exclusive. Under normal physiological situations, endothelial cells lining the blood vessels

play an important role in preventing coagulation. These cells produce a variety of molecules that: prevent platelet aggregation (NO, ecto-ADPase, PGI2), inhibit thrombin formation (TFPI, thrombomodulin), or breakdown fibrin should a thrombus be formed (tPA). Incubation of endothelial cells with VEGF has been shown to shift them to a prothrombotic state through increased expression of tissue factor and other proteins. Under these circumstances, they are then able to activate platelets. This suggests that elevated levels of VEGF can produce a prothrombotic state, and that, therefore, reduced levels of VEGF via bevacizumab treatment should have an antithrombotic effect. However, as noted earlier, as well as promoting new vessel growth, VEGF is also a survival factor for endothelial cells (Gerber et al. 1998a). If levels of VEGF become too low, perhaps in combination with other factors, endothelial cells may become apoptotic. Apototic endothelial cells are procoagulant. This, then, describes a U-shaped curve in the relationship between VEGF concentration and prothrombotic conditions on the endothelial surface of blood vessels (Fig. 2), with both high levels and low levels producing factors consistent with a prothrombotic state. Other hypotheses providing a potential link between bevacizumab and arterial thrombosis include direct affects on platelets. Platelets are known to play an important role in arterial thrombosis. In an in vitro system, incubation of bevacizumab, heparin, and VEGF in the right proportions with platelets resulted in platelet activation. Further research is needed to determine whether this could occur in an in vivo system and to further understand the relative contributions of the endothelial and platelet interaction of VEGF and their relationship to bevacizumab-mediated arterial thrombotic events. For further reading on these and other mechanisms of toxicity associated with bevacizumab treatment,

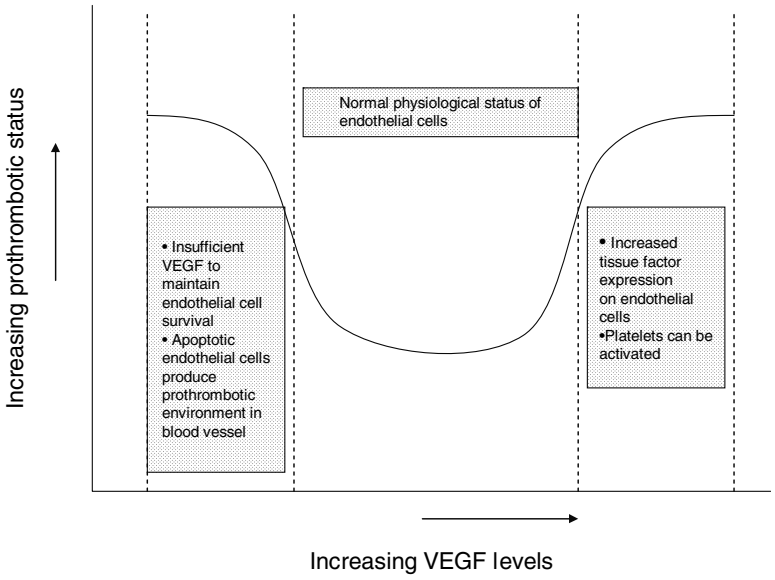

Fig. 2 Relationship between VEGF concentration and prothrombotic activity

Drs Verheul and Pinedo have published a comprehensive overview (Verheul and Pinedo 2007).

In summary, many of the adverse events associated with bevacizumab treatment appear to be related to its pharmacological action to inhibit VEGF. Some of the adverse events such as inhibition of wound healing were expected pharmacologically mediated toxicities, whereas others (such as arterial thrombosis) were unexpected as both the knowledge of the biology of VEGF and its impact on complex balanced systems developed in parallel with the clinical experience with bevacizumab.

3.4 Superagonist Anti-CD28 Monoclonal Antibody

In 2006, a phase I trial with a superagonist monoclonal antibody to CD28 (TGN1412) in six healthy volunteers produced life-threatening adverse events that required intensive care (Suntharalingam et al. 2006). Although all the volunteers survived, the event resulted in focused regulatory and scientific investigation to understand the mechanism of toxicity and to improve the manner in which phase I trials are evaluated and conducted.

Normal T cell stimulation requires two simultaneous signals: one that is antigen-dependent via the T cell receptor, and a second that is antigen-independent via costimulatory receptor stimulation, CD28, which is one of the costimulatory partners in T cell activation. Monoclonal antibody agonists to CD28, therefore, require a second antigen-dependent signal to result in T cell activation. However, about 10 years ago, a family of agonist anti-CD28 antibodies were found that did not require a costimulatory signal for T cell activation and were designated "superagonists". These antibodies could stimulate T cell activation without the need for any other factor. Although the presence of a T cell receptor was necessary, it did not need to be ligated (Schraven and Kalinke 2008). The pharmacologic rationale for TGN1412 was based on the observation that, when a superagonist anti-CD28 antibody was administered to rodents, there was a preferential expansion and activation of a regulatory T cell subset, and thus this approach might have promise in the treatment of autoimmune disease. Within 90 min of a single intravenous dose of 0.1 mg kg^{-1} TGN1412, the volunteers developed headaches closely followed by myalgia, nausea, diarrhea, vasodilation, lymphopenia, and hypotension. After 12–16 h, they became critically ill with lung injury, renal failure, and disseminated intravascular coagulation. These changes were associated with a rapid increase in proinflammatory cytokines and a significant depletion in lymphocytes and monocytes (Suntharalingam et al. 2006). Preclinical safety studies were conducted with TGN1412 prior to the trials, in cynomolgus monkeys and rhesus monkeys, and they did not identify any toxicity.

Subsequent studies have not answered all of the questions surrounding the mechanisms of toxicity; however, they have indicated that the toxicity is mediated through the pharmacological activity of the antibody to activate T cells by CD28 agonism, producing a proinflammatory state of sufficient magnitude to result in

high circulating levels of proinflammatory cytokines known as a "cytokine storm". This should not be confused with a first dose reaction or acute phase reactions to be discussed below, which result in transient and more modest effects and are associated with the induction of much lower levels of cytokines. The latter is mediated via the Fc portion of the antibody molecule whereas the TGN1412 effects were produced via the intended interaction of the antibody with its target, albeit resulting in unintended effects.

When human PBMCs were incubated with TGN1412 in the aqueous phase in vitro at a wide range of concentrations, no cytokine release or lymphocyte proliferation was produced. This was consistent in studies conducted prior to the trial or subsequently. However, the manner in which the TGN1412 antibody was presented to the PBMCs proved crucial because when the antibody was first air dried onto the plate, wet coated on top of anti-Fc antibodies, or coated on top of endothelial cells, all resulted in the release of cytokines into the medium and lymphocyte proliferation (Stebbings et al. 2007). It was thus apparent that, when the antibody was presented to human lymphocytes in a fashion more similar to the in vivo situation, it was able to stimulate a pro-inflammatory activity. Therefore, a clustering of the antibody on the surface of the lymphocytes appeared to be needed to produce activation. A primary role for the Fc portion of the antibody was ruled out by the observation that a Fab fragment of the antibody lacking the Fc portion was likewise able to stimulate cytokine release and lymphocyte proliferation (Stebbings, personal communication). The dose-response relationship observed in vitro was bell-shaped, with a peak effect at concentrations that were in the range of extrapolated estimates with the starting dose used in the trial. Incubation of cynomolgus monkey whole blood with TGN1412 using the same conditions resulted in the activation of lymphocytes (demonstrated by increased expression of IL-2R and blast transformation), but it did not lead to cytokine release or lymphocyte proliferation (Stebbings et al. 2007). Therefore, TGN1412 does bind to and activate CD28 in nonhuman primates, but appears to have a qualitatively different cellular response to the CD28 activation. Although the extracellular domain of CD28 is 100% identical between nonhuman primates and humans, there are three amino acid differences in the transmembrane region that may have a role in this differing signal.

Further supporting a pharmacologically mediated role for TGN1412-mediated toxicity, studies with a superagonist CD28 antibody (JJ316) in rats demonstrated an analogous response with initial T cell activation, acute lymphopenia, and cytokine release when the superagonist is administered to rats (Muller et al. 2008). However, this is followed by a subsequent second phase of activation that is predominantly associated with regulatory T cells. During the initial phase, there was a dramatic redistribution of T lymphocytes to secondary lymphoid organs from the periphery. Proadhesive changes on the cell surface were accompanied by strong activation including upregulation of CD25, CD69 and pro-inflammatory mediators. Therefore, the pharmacological activity of CD28 superagonism does include general T cell activation; however, in humans, the effect appears to be much more pronounced than in rats, and where this proceeds quickly to an expansion of regulatory T cells in rats, this was not apparent in humans or was overwhelmed

by the potent initial activating event. In summary, while there are many aspects of the TGN1412 mechanism of toxicity still to be understood, it does appear to fall in the category of a pharmacologically mediated toxicity albeit one that involved new understanding of the immunoregulatory role of CD28.

4 Non-pharmacologically Mediated Toxicities

4.1 Acute Phase Reactions

An adverse reaction often associated with the initial administration of some biologics, and particularly monoclonal antibodies, is an immune stimulation that is termed an acute phase response. The symptoms include fever, flu-like symptoms, fatigue, and anorexia. Usually, these symptoms diminish with subsequent injections. In MS patients who experience this reaction, they have, in addition, an exacerbation of their neurological symptoms presumably as a result of increasing the autoimmune response resulting in worsening brain lesions (Moreau et al. 1996). Under normal physiological circumstances, this response is associated with conditions of inflammation, tissue damage, or infection, and functions to remove infectious organisms and activate tissue repair processes (Gribble et al. 2007). Biologics associated with an acute phase response or "first-dose-effect" include monoclonal antibodies to CD52 (alemtuzumab), to CD3 (muromonab) and CD20 (rituximab). Although a number of different proteins either increase or decrease during an acute phase response, the cytokines IL-1, IL-6, and TNFα are considered the initiators (Baumann and Gauldie 1994). The main sources of these cytokines in an acute phase response can be macrophages via toll-like receptor activation or, as discussed below, NK cells.

The exact mechanism by which some biologics produce a significant acute phase response while others do not is unclear, but has been investigated in more detail for alemtuzumab (Wing et al. 1996). Using ex-vivo whole blood and nonadherent mononuclear cell cultures, these investigators examined the mechanism and time-course of IL-6, TNFα, and IFN-γ release upon incubation with alemtuzumab. Cytokine release in vitro was found to have the same time course (TNFα and IFN-γ first, IL-6 second) as measured in patients, and was not due to the presence of endotoxin. The postulated mechanism involved the interaction of the IgG1 isotype with the low affinity Fc receptor (FcγR) on the surface of immune cells. This was supported by the 40–50% reduction in cytokine release when an anti-CD56 IgG1 was used with a mutation such that it does not bind the FcR. An antibody to the FcγR1 (CD16) inhibited the cytokine release which further supported the involvement of this receptor. Cytokine release was not found to be a consequence of complement activation as a C1q- mutant antibody stimulated equivalent levels of TNFα as the unchanged antibody. Although isotype was found to be important (IgG1 antibodies with their capacity to bind the FcR having the highest likelihood of stimulating an acute phase response), the target to which

the antibody binds was also found to be important. An IgG1 anti-CD4 antibody was found to induce only a modest cytokine release, and the authors speculate that this may be related to the antigen density since CD52 expression is about 20 times higher than CD4 on the cell surface. This is supported by studies that show IgG1 antibodies induce cytokine release in whole blood cultures in proportion to the antigen density on the lymphocytes (Wing et al. 1995).

In summary, acute phase responses are due to the release of cytokines, particularly TNFα, IFN-γ, and IL-6. This can occur through direct stimulation of immune cells by the biologic, or indirectly via binding of the Fc portion of the antibody to the low affinity Fc receptor on other immune cells. The density of the antibody's target also plays a role as well as the antibody isotype and therefore its FcR binding affinity. Although this might be avoided by changing the isotype of the antibody, in some cases the desired mechanism of efficacy requires FcR activity such as is the case with alemtuzumab. In these cases, pretreatment of patients with methylprednisone, a steroid that inhibits cytokine synthesis, has been found to be a successful approach to mitigating the acute phase response.

4.2 Immunogenicity

Biologics are sufficiently large and complex as to elicit immune responses directed to the protein. For the most part, the principal response elicited is a T cell-dependent humoral response. The development of an antibody response to a biologic in most cases has no adverse consequences (Schellekens 2002a; Shankar et al. 2006). An antibody response is not an adverse event in itself. However, an antibody response to a biologic can have consequences that fall into three main categories: hypersensitivity reactions, reduction in efficacy, and the induction of autoimmune disease (Schellekens 2002b).

A review of the labeling for marketed biologics demonstrates that the majority contain warnings with regard to potential idiosyncratic reactions or hypersensitivity reactions (Table 1). The incidence of these types of reactions, though, has dramatically reduced from the original biologic products that were based on isolated animal proteins (e.g., porcine insulin) and murine monoclonal antibodies. This was made possible by the application of recombinant DNA technology and the movement towards proteins with fully human sequences. Serum sickness is a result of the deposition of antigen–antibody complexes in the tissues, where normal clearance mechanisms for such complexes have become overwhelmed, and is associated with fever, skin lesions, gastrointestinal symptoms, lymphadenopathy, and proteinuria. Tissue damage occurs as a result of inflammatory processes stimulated via interaction with the complement system, activation of macrophages, and direct interaction with basophils and platelets. Serious cases of "serum sickness" or immune-complex disease are now rare, and only isolated cases have been reported with recombinant therapeutic proteins (D'Arcy and Mannik 2001).

Immune responses can ablate or reduce efficacy, either by binding directly to the biologically active site of the molecule (e.g., the CDR on an antibody) and inhibiting

its pharmacological activity, or by increasing the rate of clearance of the protein, thus reducing exposure levels to below those needed for efficacy. Good examples of this are proteins derived from *E. coli* or plant origin such as streptokinase trichosanthin that produce strong immune responses. Other examples include Factor VIII, GMCSF, and IFNβ. How "adverse" this is for the patient depends on the nature of the disease and the availability of alternative therapies.

Some biologics are exogenous proteins that are intended to replace an absence of the endogenous protein or to supplement a deficit in production of the endogenous protein. It would therefore be theoretically possible for antibodies generated in response to the exogenously administered protein to cross-react with the endogenous protein and ablate its biological activity. Depending on the role of the endogenous protein and whether it is the sole contributor to a biologically important system, a serious autoimmune reaction can result. Fortunately, despite the relatively large number of endogenous proteins marketed or in development, this has occurred with only two products; erythropoietin and megakaryocyte-derived growth factor (MDGF). The mechanisms associated with aplastic anemia as a result of epoetin treatment are discussed in more detail below. Pegylated MDGF was in development for the increase of platelet yields in blood donors as well as in cancer patients. In normal healthy volunteers, thrombocytopenia was observed and correlated with the development of antibodies to MDGF, resulting in the cessation of clinical development (Wire 1998). Antibodies to peg-MDGF apper to have inhibited the endogenous production of this factor, impairing the ability of megakaryocytes in these individuals to produce normal levels of platelets.

The reasons why some proteins induce no or low titer antibodies, while others in rare instances result in autoimmune-inducing antibodies, is not well understood. Certainly, sequence homology plays a significant role as evidenced by the immunogenicity of murine monoclonal antibodies as compared to chimeric (human constant region, murine variable region) and humanized antibodies (mouse complementarity region grafted onto human variable and constant regions; Clark 2000; Presta 2006). However, as hopes of eliminating immunogenicity entirely have faded with the realization that fully human sequenced proteins can still be immunogenic, other factors that can influence immunogenicity are apparent (Schellekens 2002a). These include glycosylation (reduced or no glycosylation being more immunogenic), the effector status off an antibody, host cell products, contaminants and process-related impurities (see section below), route of administration (IM and SC routes are generally more immunogenic than IV), formulation (freeze drying can increase aggregation and thus immunogenicity), dose and regimen, and of course, patient factors.

4.3 Manufacturing and Product Quality

Biologic products are derived from living systems, such as the culture of mammalian or bacterial cells that secrete the desired product into the culture medium; as such, their production constitutes a complex manufacturing process. Subsequent steps isolate and purify the product. Due to this very different process from the

synthesis of chemicals, different quality issues arise that, if not properly controlled, can cause adverse effects. These can include the presence of aggregates, host cell proteins, and viruses. The protein molecules themselves are also complex; differences such as in folding and glycosylation can have a significant effect on the activity of the drug (Schellekens 2002a). These complexities in generating a reproducible drug product have led to substantial discussions regarding the requirements needed to generate follow-on biologics or biosimilars (Covic and Kuhlmann 2007). The principal mechanism of toxicity associated with these product changes is via an induction or enhancement of immunogenicity to the drug substance which can lead to an immune-mediated toxicity (Kromminga and Schellekens 2005). These can include hypersensitivity reactions, immediate or delayed, or even induction of an antibody directed to self-antigens where an endogenous protein is the target of the immune response. An example of this is erythropoietin α used in patients with chronic renal failure. Erythropoietin is a growth factor for red blood cells and in some patients antibodies develop capable of cross-reacting with and neutralizing endogenous erythropoietin leading to pure red cell aplasia (PRCA). Cases of PRCA increased dramatically beginning in 1998 which appeared to coincide with the removal of human serum albumin from the formulation and its replacement by polysorbate 80 and glycine (Locatelli et al. 2007). The likely mechanism of toxicity was an interaction of the polysorbate 80 with the uncoated rubber stopper of the prefilled syringe, leading to the release of a leachate that acted as an adjuvant. This was supported by studies that demonstrated the rate of PRCA was far lower with the same formulation delivered using syringes with coated stoppers versus uncoated stoppers (4.61/10,000 patient years vs 0.26/10,000 patient years; Boven et al. 2005). Since the introduction of prefilled syringes with coated stoppers, with the same formulation, the rate of PRCA has returned to very low levels.

From the earliest types of biologics that were isolated from animal or human tissues or plasma, the possibility of infection as an adverse effect of treatment was a significant concern. A well-known example of this was the high incidence of HIV and hepatitis C infection in patients receiving factor VIII to treat hemophilia. Since then, biologics made from recombinant technology and those that are human plasma-derived have had good viral safety records by employing controls on source materials and the mode of purification (Farshid et al. 2005). More recently, concern has shifted to defense against potential infection by transmissible spongiform encephalopathy such as scrapie and bovine spongiform encephalopathy. Unfortunately, these agents are difficult to detect and remove (Rohwer 1996), although robust purification procedures such as used in the preparation of a product from bovine lung was found to clear a spiked contamination of a mouse-adapted scrapie (Kozak et al. 1996). To date, there have been no reports of TSE as a result of a contaminated biotechnology-derived therapeutic. Ironically, the formulation changes made for erythropoietin described above, which indirectly led to many cases of PRCA, was made as part of the effort to remove human-derived products from recombinantly derived therapeutics from concern of TSE.

5 Vaccines

Vaccines represent such a distinctive type of biologic, with a patient population (including infants), regimen, formulation, and production method very different from recombinantly produced biologics that they are here assigned their own section. The mechanisms involved in adverse reaction to vaccines has been recently reviewed in detail (Siegrist 2007) from which much of the following information is summarized. Although a distinctive type of biologic, adverse reactions to vaccines can nevertheless be categorized as associated with the intended effect of the vaccine (to induce a protective immune response to an antigen), i.e., pharmacological, and those unrelated to the intended effect, i.e., nonpharmacological.

5.1 Pharmacological Effects

Excess replication of a live vaccine can lead to disease and can be the result of an immune deficient individual, or due to the selection of a too virulent strain, or because of reversion of a live strain to wild-type. The development of aseptic meningitis as a result of administering the live mumps vaccine is one example of this. In the case of the polio vaccine where both live and inactivated vaccines are available, there is a 1:750,000 risk of the development of polio as a result of reversion of the live virus strain. The inactivated vaccine does not carry this risk, but it is not as effective as the live vaccine.

Other adverse reactions with an association with the intended effect of the vaccine are represented by local inflammation and systemic inflammatory reactions. Local inflammatory reactions are caused by the large local deposit of antigen and the subsequent infiltration of macrophages. This is thought to be mediated via Toll-like receptor signaling. Since different formulations containing the same antigen component induce different degrees of local reaction, it is clear that there is both an antigen- and adjuvant-driven element to the nature and severity of the local reaction. Systemic inflammatory reactions, characterized by fever, nausea, and myalgia are a consequence of the release of cytokines from the liver following immune cell activation. These cytokines then have a systemic effect to produce fever, myalgia, and vascular effects. Host factors are a significant driver of these reactions: age (the young and the old are likely to have weaker reactions), gender (more common in females than males), genetics, and previous vaccine dose (stronger reaction with subsequent doses due to stronger amnestic cytokine responses.

5.2 Non-pharmacological Effects

Fainting as a result of the vagal reaction, shortly before or after injection, is an adverse reaction that can, of course, occur with some of the biologics, which are likewise injectables. Allergic reactions can occur to the formulation components of

the vaccine, this has, for example, been reported with thiomersal. It is possible for the vaccine to induce antibodies that, through molecular mimicry, can react with self-antigens. An example is the induction of antibodies cross-reactive to platelet surface glycoproteins which result in thrombocytopenia. This has occurred with both the measles (1:6,000) and rubella (1:3,000) vaccines. The production of antibodies that autoreact with antigens in the myelin sheath of peripheral nerves can result in peripheral neuropathies secondary to inflammation of the myelin and sometimes further axon loss. The mechanism for how these self-antibodies result from vaccination with, for example, the influenza vaccine remains unknown.

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Part IV
Risk Factors/Screening Drug Candidates

Pharmacogenetics of Idiosyncratic Adverse Drug Reactions

Munir Pirmohamed

Contents

Abstract Idiosyncratic adverse drug reactions are unpredictable and thought to have an underlying genetic etiology. With the completion of the human genome and HapMap projects, together with the rapid advances in genotyping technologies, we have unprecedented capabilities in identifying genetic predisposing factors for these relatively rare, but serious, reactions. The main roadblock to this is the lack of sufficient numbers of well-characterized samples from patients with such reactions. This is now beginning to be solved through the formation of international consortia, including developing novel ways of identifying and recruiting patients affected by these reactions, both prospectively and retrospectively. This has been led by the research on abacavir hypersensitivity – its association with *HLA-B*5701* forms the gold standard of how we need to identify associations and implement them in clinical practice. Strong genetic predisposing factors have also been identified for hypersensitivity reactions such as are associated with carbamazepine, allopurinol, flucloxacillin, and statin-induced myopathy. However, for most other idiosyncratic adverse drug

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reactions, the genetic effect sizes have been low to moderate, although this may partly be due to the fact that only small numbers have been investigated and limited genotyping strategies have been utilized. It may also indicate that genetic predisposition will be dependent on multiple genes, with complex interactions with environmental factors. Irrespective of the strength of the genetic associations identified with individual idiosyncratic adverse drug reactions, it is important to undertake functional investigations to provide insights into the mechanism(s) of how the drug interacts with the gene variant to lead to a phenotype, which can take a multitude of clinical forms with variable severity. Such investigations will be essential in preventing the burden caused by idiosyncratic reactions, both in healthcare and in industry.

Keywords Hypersensitivity · Pharmacogenetics · Drugs · HLA · Polymorphisms

1 Introduction

Adverse drug reactions can be divided into two basic types: type A and type B (Rawlins and Thompson 1991) (Table 1). Idiosyncratic drug reactions fall into the category of type B or bizarre adverse reactions – these reactions cannot be predicted from the known pharmacology of the drug, do not show a simple dose–response relationship, and importantly, affect a minority of patients taking the drug. The factors predisposing individuals to type B reactions with individual drugs are in most cases unknown. Genetic factors have long been postulated to be important, and this area of research is only now beginning to bear fruit. Although type B reactions are proportionately more severe than type A reactions, it is important to note that type A reactions can also be severe, lead to patient deaths, and show genetic predisposition; for example, warfarin-related dose requirements and predisposition to bleeding risk (Wadelius and Pirmohamed 2007). However, these will not be covered in this chapter.

Table 1 Characteristics of type A and type B adverse drug reactions

Characteristics	Type A	Type B
Dose-dependency	Usually shows a good relationship	No simple relationship
Predictable from known pharmacology	Yes	Not usually
Host factors	Genetic factors may be important	Dependent on (usually uncharacterized) host factors
Frequency	Common	Uncommon
Severity	Variable, but usually mild	Variable, proportionately more severe
Clinical burden	High morbidity and low mortality	High morbidity and mortality
Animal models	Usually reproducible in animals	No known animal models

Idiosyncratic adverse reactions are thought to account for up to 20% of all adverse reactions (Pohl et al. 1988), but this may be an overestimate, with 5% being closer to reality (Pirmohamed et al. 2004). Our large epidemiological study showed that 6.5% of all hospital admissions were caused by adverse drug reactions (Pirmohamed et al. 2004) – while 95% of these were type A reactions, type B reactions still remain a clinical problem accounting for 1 in 3,000 hospital admissions. Clearly, this represent the extreme of the severity spectrum, i.e., the reaction was severe enough to warrant hospital admission. Many reactions may not be severe enough to warrant admission, but nevertheless can lead to significant deterioration in quality of life for the patient, require assessment by doctors, and sometime warrant treatment. For instance, with phenytoin, skin rashes occur in up to 16% of patients at initiation of therapy (Chadwick et al. 1984), while hypersensitivity syndrome is much less common, occurring in between 1 in 5,000–10,000 patients (Shear et al. 1988). Similarly, while abnormal liver function tests are common with drug therapy, the more severe hepatitis is seen in a minority (Pirmohamed and Park 1996). For example, with isonazid, abnormal liver function is seen in 10% of patients, but hepatitis occurs in less than 1%. Whether genetic factors are important in determining the severity and pattern of clinical manifestations is unknown, and will require investigation through large well-executed studies.

Another way of estimating the burden of idiosyncratic adverse drug reactions is by evaluating their effects on drug development by the pharmaceutical industry (Lesko and Woodcock 2004). The numbers of new drugs being brought to market has shown a steady decline – many drugs never make it to the market because of problems encountered in the early phases, for example, QT interval prolongation. Some that do make it, and get a product license, are later withdrawn because it is felt that the harm exceeds the benefits of the compound (Giacomini et al. 2007).

Idiosyncratic adverse drug reactions can basically be divided into two categories (Pirmohamed et al. 1998):

- Immune-mediated, where the reaction has either the clinical or laboratory features which suggest that the immune system is involved in the pathogenesis; and
- Nonimmune-mediated reactions, or "metabolic idiosyncrasy", where nonimmune, often poorly characterized, pathways are affected by the drug leading to the reaction.

Both categories are covered in this chapter, but only a few of the most significant examples have been highlighted.

2 Familial Occurrence of Idiosyncratic Adverse Drug Reactions

Twin studies, where the incidence of a disease is compared between monozygotic and dizygotic twins, have long been used to the ascertain the heritability estimates of many diseases (Strachan et al. 2001). However, this would be extremely

difficult to accomplish with idiosyncratic reactions, as it would require an enormously large study (which would probably be economically unfeasible) comprising twins with the same disease that had the same drug treatment, and were then compared with respect to the incidence of hypersensitivity. In the absence of such studies, evidence of genetic predisposition to drug hypersensitivity can be gleaned from case reports that describe the occurrence of similar reactions in different family members treated with the same drug (Edwards et al. 1999; Gennis et al. 1991; Johnson-Reagan and Bahna 2003; Pellicano et al. 1992; Peyrieere et al. 2001). For example, hypersensitivity syndrome has been reported in identical twins, both of whom were being treated with carbamazepine for primary generalized epilepsy (Edwards et al. 1999).

3 Genetic Contribution to Idiosyncratic Adverse Drug Reactions

The genetic contribution to the occurrence of idiosyncratic adverse drug reactions is likely to vary with the drug, ethnic group, and the clinical phenotype of the adverse reaction (this is illustrated below with respect to carbamazepine). Resolution of this complexity will therefore require that phenotyping of the patients in terms of clinical manifestations, drug utilization, and ancestry is as accurate as possible, although for the latter, it may be possible to discern ancestry through genotyping for unlinked genetic markers, and evaluating the sample for population stratification (Cardon and Palmer 2003). For some reactions, for example abacavir hypersensitivity, it is now becoming clear that a single gene locus can provide adequate predictive accuracy to allow the gene test to be used in clinical settings (Alfirevic and Pirmohamed 2008). Recently, an increasing number of genetic predispositions with large genetic effect sizes have been reported, and these are highlighted in the individual sections below. Alternatively, and perhaps more commonly, the genetic effect size will be small or moderate with an interaction with environmental factors, akin to that seen in complex polygenic diseases (Fig. 1). Thus, each gene will contribute to the risk of developing the idiosyncratic reaction, but each individual gene is neither necessary nor sufficient by itself to cause the reaction (Pirmohamed and Park 2001). The genetic factors may reside in either the pharmacokinetic (for example, the drug metabolism and transporter genes responsible for drug disposition) or pharmacodynamic (genes coding for drug targets, immune response genes, cytokines, etc.) pathways. Greatest focus has been placed on the former largely because these pathways have been more extensively characterized than the pharmacodynamic genes, but the recent evidence with drug hypersensitivity reactions clearly shows the importance of pharmacodynamic pathways. The advent of unbiased approaches, for example genome-wide screening, will provide us with the power to identify genetic variants in all pathways, as long as the patient samples are available.

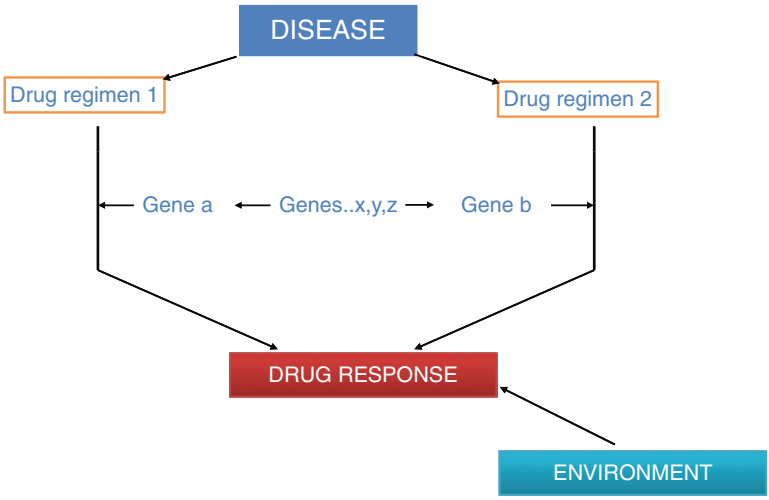

Fig. 1 The multigenic and multifactorial nature of idiosyncratic adverse drug reactions. The occurrence of an idiosyncratic reactions is dependent on the interaction between multiple genes and environmental factors

3.1 Abacavir Hypersensitivity

The use of abacavir, a potent HIV-1 reverse transcriptase inhibitor, has been hampered by the occurrence of a hypersensitivity reaction in about 5% of patients (Hetherington et al. 2001). These reactions are characterized by skin rash and gastrointestinal and respiratory manifestations, and can be fatal, particularly on rechallenge. An immunological etiology was suggested by the clinical manifestations and the finding of drug-reactive T cells. In an extensive investigation of the MHC, Mallal et al. (2002) found a strong association between abacavir hypersensitivity and the haplotype comprising *HLA-B*5701, HLA-DR7, and HLA-DQ3* with an odds ratio of over 100. This association has now been shown in two other cohorts by GlaxoSmithKline Pharmaceuticals, the manufacturer of the drug, and independently in a cohort of patients from the UK (Hetherington et al. 2002; Hughes et al. 2004a,b). Subsequent studies have shown that this haplotype resides on the ancestral haplotype 57.1, and that the combination of *HLA B*5701* and polymorphism in *HSP-Hom* (heat shock protein-Hom) has greater predictive accuracy than *HLA B*5701* by itself (Martin et al. 2004). More recently, a large randomized controlled trial (PREDICT-1) has demonstrated the utility of preprescription genotyping in preventing abacavir hypersensitivity – immunologically confirmed hypersensitivity reactions were not seen in the prospective-screening group but were seen in 2.7% of the comparator control group, providing a negative predictive value of 100% and a positive predictive value of 47.9% for *HLA-B*5701* testing (Mallal et al. 2008). The association of *HLA-B*5701* with immunologically confirmed abacavir hypersensitivity reactions has also been shown in black patients (Saag et al. 2008).

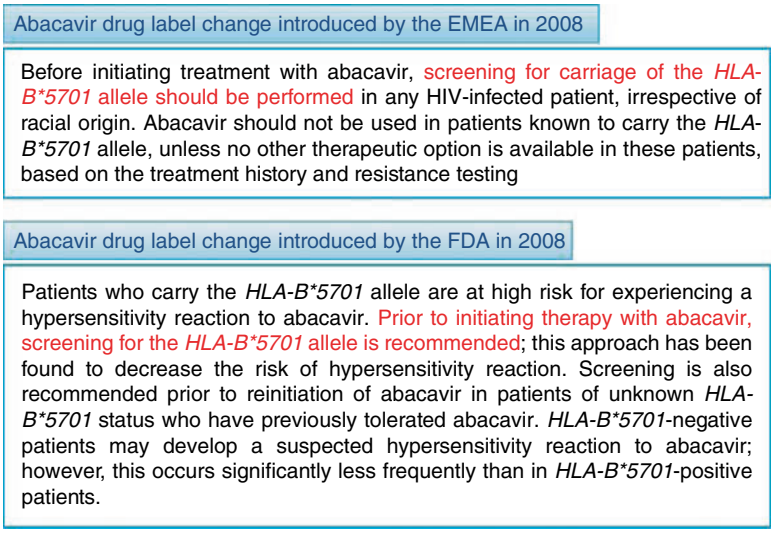

Fig. 2 The changes in the drug label for abacavir introduced by the EMEA and FDA in 2008. Note the slight differences in wording relating to the need to undertake testing for *HLA-B*5701* testing prior to the use of abacavir

Even before PREDICT-1 was undertaken, many HIV clinics, particularly those in Australia and the UK, had started using *HLA-B*5701* testing to identify susceptible patients. Our study showed that this would be a cost-effective approach (Hughes et al. 2004b), which has recently been confirmed by other workers (Schackman et al. 2008). The use of *HLA-B*5701* in clinical settings has been shown to reduce the frequency of hypersensitivity in Australia (Rauch et al. 2006), UK (Waters et al. 2007), and France (Zucman et al. 2007), showing that the genetic testing can have a powerful influence in reducing the burden associated with serious adverse drug reactions. In 2008, the evidence was evaluated by the regulators, and both the FDA and EMEA changed the drug label for abacavir. In Europe, it is now mandatory to undertake *HLA-B*5701* testing before the prescription of abacavir (Fig. 2).

3.2 *Carbamazepine Hypersensitivity*

Carbamazepine (CBZ) is a widely used anticonvulsant that can cause rashes in up to 10% of patients, and in occasional cases this may be the precursor to the development of a hypersensitivity syndrome characterized by systemic manifestations such as fever and eosinophilia (Leeder 1998; Vittorio and Muglia 1995). Rarely, CBZ can induce blistering skin reactions such as Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis, two conditions associated with a high fatality rate (Rzany et al. 1999). There is now increasing laboratory evidence to show that

CBZ hypersensitivity is a T cell-mediated disease (Mauri-Hellweg et al. 1995; Naisbitt et al. 2003).

Initial investigations to identify genetic predisposing factors concentrated on the pharmacokinetic genes, in particular microsomal epoxide hydrolase (Leeder 1998; Pirmohamed et al. 1991; Shear et al. 1988). However, extensive investigations by at least two groups showed no association with genes determining drug disposition (Gaedigk et al. 1994; Green et al. 1995). Focus then turned towards the immune genes. This has led to the finding that *CBZ hypersensitivity syndrome*, but not mild *maculopapular eruptions*, is associated with the haplotype *TNF2-DR3-DQ2* (Pirmohamed et al. 2001). This has also been borne out in more recent studies in an extensive analysis of the heat shock protein (HSP) locus, which has shown that severe, but not mild, CBZ hypersensitivity reactions, are associated with three SNPs in the HSP-70 locus, two in *HSP70-1,* and one in *HSP-Hom* (Alfirevic et al. 2005). From these studies, we have concluded that the causal variant for *CBZ hypersensitivity syndrome* in Caucasians resides on the ancestral haplotype 8.1 (Pirmohamed 2006).

Interestingly, a study in a Han Chinese population from Taiwan has shown that *HLA-B*1502* may act as a genetic marker for CBZ-induced *SJS*, with an odds ratio that was greater than 2504 (Chung et al. 2004). All patients with SJS carried the *HLA-B*1502* allele, but this was only observed in 3% of tolerant patients. However, this association was not shown in Han Chinese patients with *CBZ hypersensitivity syndrome* (Hung et al. 2006). Furthermore, recent studies have shown that *HLA-B*1502* does not act as a predisposing factor to CBZ-induced *SJS* or *hypersensitivity syndrome* in Caucasian patients (Alfirevic et al. 2006; Lonjou et al. 2006). These findings prompted the FDA to change the drug label for CBZ stating that it should be tested in "most patients of Asian ancestry". This has led to debate amongst epileptologists as to what constitutes "Asian" and which groups should be tested prior to the use of CBZ because (1) there is an enormous amount of genetic diversity in Asians, and (2) the association has not been investigated in most Asian populations.

The complexity has been further underlined by two recent studies. First, a study in Japanese patients failed to show an association between *HLA-B*1502* and CBZ-induced SJS (Kaniwa et al. 2008). This is perhaps not surprising given that the population frequency of the *HLA-B*1502* allele is low in the Japanese. Second, a study in Thai patients has also shown an association between CBZ-induced SJS and *HLA-B*1502* (OR 25.5, 95% CI 2.68–242.61) (Locharernkul et al. 2008). Interestingly, the same association was also demonstrated with phenytoin-induced SJS (OR 18.5, 95% CI 1.82–188.40). Confusingly, however, some patients, who were *HLA-B*1502* and suffered from CBZ-induced SJS, were tolerant to phenytoin and vice versa, suggesting that *HLA-B*1502* is either not the causative allele or other factors in addition to *HLA-B*1502* are necessary to result in SJS.

The recent data with CBZ-induced idiosyncratic toxicity illustrate the complexity of genetic predisposition – the genetic factors seem to vary with the clinical phenotype and ethnicity. Unlike abacavir, a formal cost-effective analysis for pre-prescription genotyping for CBZ-induced SJS has not been performed, and data on the uptake of testing are lacking.

3.3 Flucloxacillin-Induced Cholestatic Hepatitis

Flucloxacillin is an antistaphylococcal β-lactam antibiotic that is widely used in the UK and Australia. It can cause a cholestatic hepatitis, which can either occur in isolation or may be accompanied by a rash (Olsson et al. 1992). It is more common in the elderly or with prolonged therapy, and occurs at an incidence of 8.5 per 100,000 users (Russmann et al. 2005). The DILIGEN (drug-induced liver injury genetics) network in the UK has recently shown a strong association between *HLA-B*5701,* the same allele associated with abacavir hypersensitivity, and flucloxacillin hepatitis (OR 80) (Daly et al. 2009). The same allele was also identified using a genome-wide scanning approach in only 56 cases, with a genome-wide significance value of 10^{-30}. The mechanistic basis for the association with the *HLA-B*5701* allele is not clear, but recent studies in our laboratory have shown that flucloxacillin preferentially binds covalently to two main lysine residues in human serum albumin (Jenkins et al. 2009). Whether such preferential binding is also important for *HLA-B*5701* requires further study. Unlike abacavir, it is unlikely that we will be able to use *HLA-B*5701* typing prospectively for flucloxacillin-induced cholestatic hepatitis since its incidence is low, and therefore it will have a low positive predictive value and may not be cost-effective. However, this test may be useful for diagnosis in patients where there is a need to distinguish between drug-induced disease and nondrug disease because its negative predictive value approaches 100%.

3.4 Statin-Induced Muscle Toxicity

Statins are now amongst the most widely used drugs worldwide. They are highly effective in lowering cholesterol levels, and have been shown to be effective in the primary and secondary prevention of ischaemic heart disease. Statins, however, can cause muscle toxicity, which most commonly manifests as an asymptomatic rise in CPK, and in the most severe cases, can cause rhabdomyolysis and death (Fig. 3) (Laaksonen 2006). Cerivastatin was withdrawn in 2001 because of its propensity to

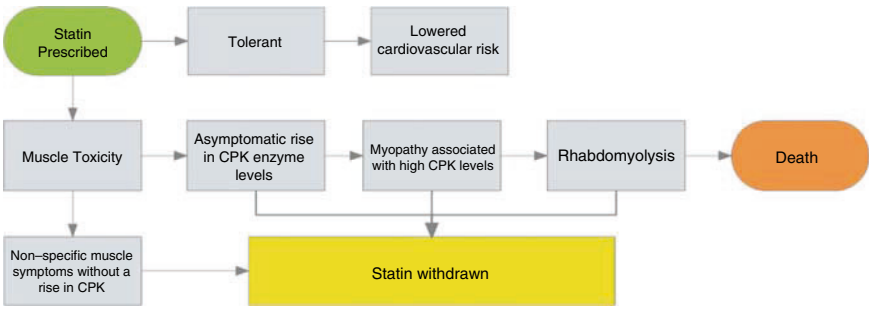

Fig. 3 The spectrum of muscle toxicity induced by statins

Table 2 Genetic predisposing factors for statin-induced muscle toxicity

Drug	Gene(s)	Form of toxicity
Cerivastatin	*CYP2C8, OATP2, OATP1B1* (*SLCO1B1*)	Rhabdomyolysis
Pravastatin	*OATPC, OATP1B3* (*SLCO1B3*)	Myopathy
Simvastatin	*ABCB1* (*MDR1*)	Myalgia
Atorvastatin	*CYP3A5*	Myalgia
Multiple statins, including cerivastatin	*CPT 2, PYGM*	Myopathy
Simvastatin	*OATP1B1* (*SLCO1B1*)	Myopathy
Multiple statins, including rosuvastatin and atorvastatin	*COQ2*	Myopathy

Adapted from Wilke et al (2007). *CYP* Cytochrome P450, *OATP* organic anion transporter protein, *ABCB1* ABC transporter protein, *CPT2* carnitine palmitoyltransferase II, *PYGM* glycogen phosphorylase, muscle, *COQ2* coenzyme Q2

cause rhabdomyolysis – approximately 100 deaths and 1,600 injuries were linked to use of the drug (Marwick 2003).

The mechanism of the muscle damage is unclear (Laaksonen 2006), and various hypotheses have been proposed, including most recently that atrogin-1, a gene involved in muscle atrophy, is involved in the pathogenesis of statin-induced muscle toxicity (Hanai et al. 2007). Various candidate gene studies have been performed (Wilke et al. 2007), but most findings either showed a low odds ratio or have not been replicated (Table 2). However, more recently, a genome-wide association study was able to show a strong association of simvastatin-induced myopathy with the OATP transporter gene, *SLCO1B1,* and in particular with a noncoding SNP rs4363657 (Link et al. 2008). The odds ratio for myopathy was high (4.5 per copy of the C allele and 16.9 in CC homozygotes), with more than 60% of the myopathy cases being attributed to the C variant. Mechanistically, this noncoding SNP is in linkage disequilibrium with the nonsynonymous SNP rs4149056, which has been shown to affect the disposition of statins (Pasanen et al. 2007). Although the authors suggested that this SNP could be used in patients being prescribed statins, this may be premature because it needs to be replicated in another cohort. It was only shown with 40 and 80 mg simvastatin (20 mg cases were not available), and we also do not know whether this is a class-effect or a drug-specific effect.

3.5 Drug-Induced QT Interval Prolongation

Drug-induced QT interval prolongation, and its more serious complication, torsades de pointes, is one of the most common reasons for drug withdrawal (Wilke et al. 2007). Many drugs are known to cause QT-interval prolongation (Roden 2008), and genetic studies have largely focused on single cases or small case series using candidate gene approaches. Although QT interval prolongation is relatively common, torsades de pointes is rare, and QT prolongation represents a poor biomarker for the latter more serious condition. It is not known whether the genetic

predisposing factors for QT interval prolongation are the same as those required for torsades de pointes. This will require larger numbers of well-phenotyped patients. Our knowledge of the mechanisms of QT-interval prolongation has improved recently (Roden 2008), and most candidate gene studies have focused on the sodium (Makita et al. 2002) and potassium channel genes (Mank-Seymour et al. 2006), as well as the P450 isoforms such as *CYP2D6* (Ford et al. 2000) determining drug disposition. There are now some genome-wide association studies being undertaken which will provide an unbiased investigation of possible predisposing loci, which will subsequently require mechanistic studies for further evaluation.

4 Biopharmaceutical Products and Idiosyncratic Adverse Reactions

Approximately 24% of all new entities approved by the FDA between 2002 and 2006 were biological compounds (Giezen et al. 2008). Given the nature of these compounds, their high specificity for biological targets, and potency, there are worries about possible serious adverse effects – this was witnessed dramatically in six healthy volunteers given TGN1412 in the UK (Suntharalingam et al. 2006). While the TGN1412-related toxicity cannot be described as being idiosyncratic in nature, there are many reactions that do have features of idiosyncratic adverse drug reactions, i.e., they occur in a small proportion of the patients prescribed the drug. We cannot predict who will develop these reactions, and there may be genetic predisposing factors. For instance, a particular worry with protein-based agents is their potential immunogenicity, which can lead to the development of antibodies leading to neutralization (thereby reducing efficacy), and in some cases lead to hypersensitivity. A recent study in patients with multiple sclerosis who had received interferon-beta showed that *HLA-DRB1*0401* and *HLA-DRB1*0408* (odds ratio: 5.15) were strongly associated with the development of binding and neutralizing antibodies to IFN-beta (Hoffmann et al. 2008).

5 Future Perspectives and Conclusion

As our knowledge of the human genome improves, we will undoubtedly identify novel genetic predisposing factors for idiosyncratic adverse drug reactions. Most of the studies to date have utilized a candidate gene approach, but more recently, with the advances in genome-wide scanning (2007), this powerful technique is being used in the investigation of idiosyncratic adverse drug reactions, as outlined above.

Further progress in this area is going to be limited through the lack of well-phenotyped cases. Given the relative rarity of idiosyncratic adverse drug reactions, it is unlikely that a single center, let alone a single country, will be able to identify a sufficient number of cases, and therefore multicenter multinational collaborations

Table 3 Consortia that have been formed to investigate the genetic basis of idiosyncratic adverse drug reactions

Consortium	Area of interest	Website
DILIGEN	Drug-induced liver injury	http://www.diligen.org
DILIN	Drug-induced liver injury	http://www.dilin.dcri.duke.edu
EUDRAGENE	Various	http://www.eudragene.org
SAEC	Various	http://www.saeconsortium.org

are going to be essential. This is now beginning to happen with many consortia having been formed (Table 3). One of these, the Serious Adverse Event Consortium (http://www.saeconsortium.org/), is a nonprofit organization comprised of pharmaceutical companies, academic institutions, and regulatory agencies, which is looking at several areas including SJS-TEN and DILI. Although power calculations for complex diseases have suggested that a minimum of 2,000 cases are required to show minor gene effects, it is very unlikely that we will ever be able to collect such large numbers of patients with idiosyncratic drug reactions. Fortunately, recent data with abacavir (Nelson et al. 2009), flucloxacillin (Daly et al. 2009), and simvastatin (Link et al. 2008) have shown that the genetic effect sizes are much greater than those seen in complex diseases and hence smaller numbers are required.

When cases are identified, it is essential to ensure that the phenotype is as accurate as possible. Gold standards of phenotypes need to be developed. An important point to consider with respect to phenotype is whether the varying manifestations represent the same disease process, or different diseases altogether, as this will impact on the statistical power to identify the genetic predisposing factors. Clearly, it is also possible that the genetic predisposition to the phenotypic manifestations may be drug-specific. Thus, in the studies in Han Chinese, while *HLA-B*1502* acts as a marker for CBZ-induced SJS only (Chung et al. 2004), with allopurinol, the genetic predisposing factors seem to be relatively similar irrespective of whether the patient developed hypersensitivity syndrome or SJS (Hung et al. 2005). As part of the phenotypic characterisation, details of ancestry need to be obtained. This is important because genetic factors seem to vary according to ethnicity, as discussed above with carbamazepine.

The ultimate aim of pharmacogenetics is to predict the response to the drug before the patient takes the drug. With respect to idiosyncratic drug reactions, the aim would therefore be to prevent these reactions through preprescription genotyping. Two important aspects need to be considered here. First, what level of predictive accuracy will be acceptable to patients and clinicians before the test is used? It is unlikely that we will achieve 100% predictive accuracy with any pharmacogenetic test (Lindpaintner 2002). Second, given that predisposition is likely to be multigenic, is it going to be cost-effective to screen patients prior to starting the drug, especially when the background incidence of the hypersensitivity reaction is low? Such information will be needed to convince those responsible for holding scarce health-care resources to invest in such testing.

In conclusion, there is increasing evidence to show that idiosyncratic adverse drug reactions are genetically determined. Identification of such factors is important, not

only to realize the prospect of developing preventive strategies but also to learn about the mechanisms of these reactions, which may ultimately lead to other preventive strategies through better drug design, and to better treatment strategies for patients who develop the reactions. The success of this area of research is going to be critically dependent on the identification and careful phenotyping of patients with such reactions. This is only likely to be achieved through international collaborations.

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The Danger Hypothesis Applied to Idiosyncratic Drug Reactions

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Contents

Abstract The danger hypothesis has had a profound effect on the way immunologists view the immune response. This hypothesis proposes that the major determinant of whether an immune response is mounted against some agent is determined by whether that agent causes some type of cell damage. Assuming that most idiosyncratic drug reactions (IDRs) are immune-mediated, this hypothesis also has the potential to explain many aspects of the mechanism of these adverse drug reactions. For example, most IDRs appear to be caused by chemical metabolites rather than the parent drug, but not all drugs that form reactive metabolites are associated with a significant incidence of IDRs. Therefore, using the danger hypothesis, one feature of a drug candidate that may predict whether it causes an IDR is whether the drug, or more likely its reactive metabolites, cause cell damage. Although the range of molecules that can act as danger signals is unknown, the most

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attractive candidates are high mobility group box 1 protein (HMGB1), heat shock proteins, and S100 proteins. These molecules act through the same receptors (toll-like receptors) as pathogen-associated molecules that stimulate the immune system. Therefore, other environmental factors such as infections or trauma might determine which patients would be at increased risk for IDRs. Although there are examples where this appears to be the case, in most cases there are no obvious environmental factors that determine IDR risk. In addition, in animal models of immune-mediated reactions, stimulation of toll-like receptors often does not increase the immune response, and depending on the timing, it can actually be protective. Therefore, there may be additional unknown control mechanisms that are involved. A better understanding of these fundamental immune mechanisms has the potential to have a significant impact on many areas of medicine.

Keywords HMGB1 · Heat shock proteins · S100 proteins · Reactive metabolites · Immune-mediated

1 Review of Immunity Models

Safety is a vital issue for any form of life. Over the long span of evolution, biological systems have evolved a set of elaborate, dynamic, and well-regulated machinery called the immune system to closely guard organisms and to defend against any substance that could potentially damage it. In-depth understanding of this sophisticated system would provide a solid basis for dealing with a wide range of immune-related problems that influence virtually all areas of medicine. Rigorous studies in the past few decades have significantly expanded our knowledge of the immune system; however, it has become routine for new data to overthrow long-standing concepts, and there remain many unknowns.

1.1 Self-Nonself Models

The first immunological model to address the specificity of the immune system, known as the self-nonself (SNS) model, was proposed by Burnet in 1959 (Billingham et al. 1953; Burnet 1961). Ever since, it has been widely accepted as one of the most fundamental theories of modern immunology. Simply stated, it suggested that an immune response is mediated by lymphocyte surface antibodies specific to foreign substances, and negative selection is programmed early in life to delete self-reactive lymphocytes to differentiate self from nonself. The key principle of the SNS model is that the exclusive determinant of what the immune system responds to is the recognition of nonself by immune cells. The SNS model was accepted until immunologists began to realize that T cell responses depend on two

indispensable activation signals delivered by other cells known as antigen-presenting cells (APCs). Several major modifications were made to the original SNS model eventually resulting in the birth of Janeway's infectious-nonself (INS) model in 1989 (Janeway 1989). The two activation signals for T cells were defined as signal 1, which consists of processed antigen presented by major histocompatibility complex II molecules (MHC II) on APCs to T cell receptors (TCRs), and signal 2, which consists of costimulatory interaction between B7 molecules of APCs and CD28 of T cells, respectively. The gate-keeping step suggested by the INS model is the recognition of a particular pathogen-associated molecular pattern (PAMP) on pathogens by pathogen recognition receptors (PRRs) on APCs. This recognition activates APCs and upregulates their surface expression of B7 and other costimulatory molecules. On receiving two activation signals from APCs, T cells are activated and differentiate into specific types of helper cells to facilitate either cell-mediated (Th1 or Th17) or antibody-mediated (Th2) immune reactions. Each pathway is characterized by the cytokines and chemokines that are released. Although Janeway proposed that it is PRRs on APCs instead of lymphocytes that discriminate between self and nonself, both the SNS and INS models are based on the recognition of foreignness. Over more than 50 years, the self-nonself concept dominated immunology. It is true that lymphocytes with a high affinity for self molecules are deleted in the thymus making it more difficult to mount a strong immune response against self molecules. However, this hypothesis does not address several other issues such as what causes autoimmunity and why there is no immune response to tumors even though they express neoantigens, etc. Thus, further refinement of this concept was needed.

1.2 Danger Model

In 1994, Polly Matzinger proposed the danger model that posits it is cell damage rather than nonself that determines whether an immune response will occur (Matzinger 1994). Injured cells (i.e., stress, necrosis) release danger/alarm signals that activate APCs resulting in increased expression of costimulatory molecules. The danger signals are also referred to as signal 3. According to this model, the immune system is more concerned with potential danger than foreignness. This can explain why a wide variety of nonself exposures do not trigger an immune response in the absence of significant cell damage. In addition, the danger model offers an explanation of how endogenous molecules can induce immune reactions. Therefore, independent of whether a molecule is an exogenous pathogen, chemical, or endogenous intracellular molecule released from necrotic cells, they all must cause damage or cell stress in order to elicit an immune response. Although the danger model is difficult to rigorously test, and it was quite controversial at first, it now appears to have become part of accepted immune theory. Figure 1 illustrates the progression of immunological models from the original SNS model to the current danger model.

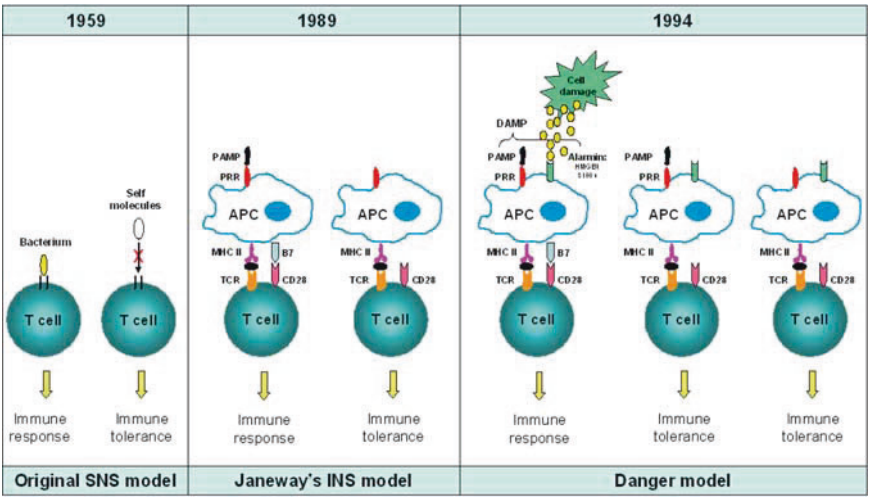

Fig. 1 Progression of immunological models

2 Implications of the Danger Model

Although it must be considered just a theory at present, the danger model has significantly changed our perspective on what is involved in the induction of an immune response, and it has obvious implications for idiosyncratic drug reactions, most of which are likely immune-mediated.

2.1 Vaccines

The practical use of various vaccines in the past has enabled us to control many different types of human diseases including chicken pox, influenza, hepatitis A and B, measles, fowl cholera, anthrax, swine erysipelas, rabies, and pneumonia. The concept of vaccination is being incorporated into management of more diseases, for example, cancer treatment (Benton and Kennedy 1998; Curigliano et al. 2007; Daskalakis et al. 2004; Giarelli 2007; Hung et al. 2007). However, sometimes vaccines do not work efficiently or at all, largely because the host's immune system does not respond adequately (Kimman et al. 2007). Although coadministration of vaccines with adjuvants (Ozpolat and Lachman 2003) is always employed to boost the recipient's immune response, insufficient immunogenicity still remains the primary cause of vaccine failure, particularly in the case of DNA vaccination. If the danger hypothesis is correct, the use of an adjuvant capable of causing cellular damage should enhance the efficacy of vaccines. In fact, an increasing number of studies have begun to introduce damage-causing reagents to optimize the efficacy of vaccines (Bergmann-Leitner and Leitner 2004; Schirrmaker 2003).

2.2 Maternal/Fetal Immunity

According to the SNS model, theoretically, a mother's immune system should be activated by fetus because it is foreign or nonself. The reason it is not is because the mother's immune system responds with tolerance instead. The major supporting evidence came from studies identifying high-level expression of many immunosuppressive molecules in pregnant women, such as progesterone, prostaglandins, tumor growth factor-β and interleukin-10, Fas ligand, and HLA-G, etc. (Gorczynski et al. 2002; Le Bouteiller and Tabiasco 2006; Power et al. 2002). A recent finding that indoleamine-2,3-dioxygenase (IDO) (an enzyme responsible for the catabolism of amino acid tryptophan to kynurenine; Grohmann et al. 2003) is increased in the placenta provides another possible mechanism for this tolerance. Tryptophan is a key amino acid for T cell proliferation; therefore, without sufficient tryptophan, maternal T cells cannot proliferate sufficiently to attack the fetus (Sedlmayr 2007). However, Matzinger disputed the role of IDO in maintaining immune tolerance at three major aspects: (1) IDO is not exclusively expressed in placental cells; (2) IDO is an unspecific inhibitor of T cells; and (3) immune responses often progress in spite of the presence of IDO (Bonney and Matzinger 1998). Other suspected immunosuppressive molecules involved in normal pregnancy have similar problems; in contrast, the danger model suggested that whether the fetus is attacked by maternal immune system depends on whether it is healthy or not rather than the efficacy of immunosuppression. The production of danger signals by an abnormal fetus appear to activate the mother's immune system and be responsible for conditions such as preeclampsia (Bonney 2007).

2.3 Autoimmunity

With a focus on self versus nonself, neither the SNS model nor the INS model explain the common occurrence of autoimmunity. Everyone has autoreactive lymphocytes that escaped clonal deletion; however, only about 5–7% of the population develops classic autoimmune diseases (Coutinho and Avrameas 1992; von Herrath et al. 2001). It is likely that there are many mechanisms acting in concert to establish immune tolerance including CD4+Foxp3+ regulatory T cells, expression of IDO, and regulation of the expression of B cell receptors (BCR) and T cell receptors (TCR) on autoreactive cells (Goodnow et al. 2005; Ochs et al. 2007; Romagnani 2006; Tarner and Fathman 2006; Thompson and Thomas 2002). In the danger model, Matzinger proposed that cellular stress or abnormal cell death caused by mutations or environmental factors such as pathogens or chemicals can induce immune reactions towards damaged self and lead to autoimmunity (Matzinger 2007). In this model, it is the damaged cells that control the immune response, not the immune system.

2.4 Adverse Drug Reactions

Accounting for 3–7% of all hospital admissions, adverse drug reactions (ADRs) represent a major clinical problem and also an important issue for the pharmaceutical industry. Many ADRs are a simple extension of the drug's pharmacological effects. For example, a person taking a drug to reduce high blood pressure may feel dizzy if the drug reduces blood pressure too much. This type of ADRs is usually predictable. Another group of ADRs results from mechanisms that are not currently well understood. Also known as idiosyncratic drug reactions, these ADRs are unpredictable. Relatively common IDRs include skin rashes, idiosyncratic liver failure, and agranulocytosis; however, IDRs can affect any organ. The clinical features of IDRs suggest an immune mechanism, and therefore models such as the danger model have implications for the mechanisms of IDRs.

3 Idiosyncratic Drug Reactions (IDRs)

IDRs refer to a specific group of adverse drug reactions that do not occur in most patients within the therapeutic dose range and cannot be explained by the known pharmacological properties of the drugs (Park et al. 1998; Uetrecht 2007). In general, the incidence of IDRs associated with an individual drug is low; however, given the wide variety of drugs that can cause such reactions and the number of patients taking drugs, such reactions are not rare. IDRs can be severe and even life-threatening in some cases (Goldstein and Patterson 1984). In spite of many studies done to elucidate the mechanisms involved, limited progress has been made. Hence, it is still impossible to accurately predict which drug candidates are likely to cause IDRs or which patients will develop such reactions. A much better mechanistic understanding is required in order to effectively deal with this problem.

The clinical characteristics of IDRs provide some mechanistic clues. First of all, the delay between starting the drug and the onset of the adverse reactions is most easily explained by an immune mechanism because it requires time for the lymphocytes that are specific for a specific immunogen to be activated and to proliferate to sufficient numbers to cause a clinically evident response (Park et al. 1998; Uetrecht 2007). Secondly, there is a large amount of circumstantial evidence to suggest that most IDRs are caused by reactive metabolites rather than by the parent drug (Uetrecht 2007). Therefore, most IDR mechanistic studies involve reactive metabolites and how they may lead to an immune response.

3.1 Working Hypotheses of IDRs

At present, there are three major working hypotheses proposed to explain the interaction between drugs and/or reactive metabolites and the immune system

causing pathogenic immune reactions: the hapten hypothesis, the danger hypothesis, and the pharmacological interaction hypothesis (Uetrecht 2007). Although suggesting different triggering events, all three hypotheses center on an immunological mechanism. They are not mutually exclusive and one or more might be useful in explaining a specific IDR.

3.1.1 Hapten Hypothesis

A basic principle of immunology postulated by Landsteiner over 70 years ago (Landsteiner and Jacobs 1935) is that small molecules with a molecular mass of less than 1,000 Da are unable to induce an immune response unless they are bound to a macromolecule such as a protein. The immunological term given to a small molecule that leads to an immune response after binding to a macromolecule is hapten. This provides a good explanation for the allergic reactions caused by penicillin and other ß-lactam antibiotics. The ß-lactam ring is reactive and penicillin binds to proteins. The allergic reactions associated with penicillin are mediated by IgE antibodies against penicillin-modified proteins; thus, penicillin is acting as a hapten (Parker 1981, 1982). Although there are several other examples, such as halothane-induced hepatitis in which a reactive metabolite covalently binds to proteins and induces antibodies against the metabolite-modified proteins (Vergani et al. 1980), it is not clear that these antibodies mediate the liver damage. There are few other examples where the covalent binding of a drug so clearly causes an IDR as in the case of penicillin.

3.1.2 Danger Hypothesis Applied to IDRs

If the danger model is correct, simply binding to proteins to make them foreign would not be sufficient to induce an immune response (Pirmohamed et al. 2002; Seguin and Uetrecht 2003; Uetrecht 1999, 2007). In addition, it would require the activation of the immune system by damaged/stressed cells, which is mediated by proteins or other molecules acting as danger signals by binding to certain receptors on innate immune cells such as macrophages (Miyake 2007; Oppenheim et al. 2007). This hypothesis could explain why many drugs that form reactive metabolites and covalently bind to proteins are not associated with a significant incidence of IDRs. It may be that the drug, or more likely a reactive metabolite, must also cause cell damage in order to cause IDRs. A follow-up question is whether the danger signal must come from the drug or whether other sources of tissue injury such as infection, surgery, or other inflammatory conditions act as risk factors for IDRs. In the past few years, several studies have been done that have implications for the danger hypothesis and IDRs: (1) identification of potential danger/alarm signals released from cells or tissues, and (2) investigation of the correlation between danger molecules and the induction of IDRs.

Identity of Danger Signals

At present, the range of molecules that have the potential to act as danger signals is unknown. As defined by Matzinger, danger signals are endogenous molecules produced by damaged tissue. Therefore, something like a virus could stimulate the immune system in two different ways: viral molecules such as double-stranded RNA (a PAMP) could stimulate APCs via toll-like receptor 3, and damage to cells by the virus could produce danger signals that also stimulate APCs. To some degree, the distinction between the two signals is artificial because it is thought that endogenous danger signals operate through the same pathways, such as toll-like receptors, as exogenous PAMPs (Matzinger 2007). Since danger signals are the defining element of the danger model, identifying and characterizing potential danger signals is critical to understanding what initiates an immune response. In the case of immune-mediated IDRs, they should be endogenous molecules whose expression or location is changed as a result of tissue damage or cell stress caused by parent drug and/or a reactive metabolite. Given the ability of many drugs that are associated with a relatively high incidence of IDRs to cause oxidative stress, it is likely that one type of danger signal involved in IDRs includes molecules involved in the oxidative stress response, such as the Nrf2-mediated antioxidant signaling pathway. In addition, other stress-responsive intracellular molecules from damaged cells that are translocated from nucleus or cytosol to the extracellular space, such as high mobility group box 1 (HMGB1), heat shock proteins HSPs, and S100 proteins, are good candidates to be danger signals. Such molecules have also been referred to as alarmins (Bianchi 2007; Oppenheim et al. 2007). Other possible danger signals include hepatoma-derived growth factor, IL-1, uric acid, cathelicidins, defensins, galectins, thymosins, nucleolin, and annexins (Bianchi 2007).

High Mobility Group Box 1 (HMGB1)

HMGB1 is an abundant nuclear protein that binds to chromatin and regulates DNA-protein interactions by acting as a DNA chaperone. During cell necrosis, HMGB1 is released. In addition, factors such as oxidative stress, LPS, TNFα, and IL-1ß can lead to posttranslation modification of HMGB1 (e.g., hyperacetylation of lysine in the case of LPS or phosphorylation in the case of TNFα) leading to translocation (Lotze and Tracey 2005), first to the cytoplasm and then escape into extracellular milieu. For example, elevated serum levels of HMGB1 have been reported in many disease states such as sepsis, rheumatoid arthritis, and systemic lupus erythematosus (SLE) (Lotze and Tracey 2005). Studies have shown that, after being released, HMGB1 can behave as a chemokine (Dumitriu et al. 2007; Lotze and Tracey 2005) to recruit immune cells to the site of damage or inflammation via interacting with the specific receptor of advanced glycation end products (RAGE) (Kokkola et al. 2005) and toll-like receptors, 2, 4, and 9, probably as a complex with other molecules such as DNA (Klune et al. 2008; Tian et al. 2007). HMGB1 also leads to activation of macrophages (Andersson et al. 2000). The activity of HMGB1 appears to be related to its oxidation state, with oxidation of the thiols leading to

inactivation (Kazama et al. 2008). Such properties make HMGB1 a good candidate as a danger signal.

Heat Shock Proteins

Heat shock proteins (HSPs) and glucose regulated proteins (GRPs), located in the cytoplasm and mitochondria, act as molecular chaperones and have several functions, especially in protein folding. Although originally found to be upregulated by elevated temperature, many other forms of cell stress such as infection, inflammation, oxidative stress, and toxins have been shown to stimulate their production (Osterloh and Breloer 2008). Therefore, HSPs are accepted as essential stress proteins that can transmit danger signals to the immune system. Like HMGB1, HSPs can also be released into extracellular milieu by necrotic or damaged cells and induce production of proinflammatory cytokines through specific receptors including toll-like receptors (Calderwood et al. 2007). An example is Hsp72, an inducible molecule that has gained attention as a danger signal (Williams and Ireland 2008). Its upregulation has been found in many different types of cellular stress. Therefore, increased serum expression of Hsp72 has been used as a biomarker to indicate ongoing cell stress.

S100 Proteins

The S100 protein family, also known as calgranulins, is the largest group of calcium-binding proteins containing more than 20 members (Hofmann et al. 1999). They are involved in a variety of intracellular and extracellular functions, such as protein phosphorylation, Ca^{++} homeostasis, cell growth and differentiation, and the inflammatory response, etc. Out of the family, three members, S100A8, S100A9, and S10012, are found to be associated with immune reactions by interacting with innate immune cells (Foell et al. 2007). They are highly expressed in phagocytes, and like other alarmins, they are actively secreted into the extracellular compartment by damaged cells and are commonly present at sites of inflammation. Mechanistically, S100A8 and S100A9 are released in the form of a heterodimer, S100A8/A9, which acts as a chemokine to stimulate leukocyte migration to sites of inflammation. The receptors thought to be involved in this process are toll-like receptors. In contrast, secreted S10012 binds to RAGE, thus acting as a proinflammatory signal. These three S100 proteins appear to be able to alert the immune system leading to the initiation and promotion of an immune response.

Other Potential Sources of Danger Signals

An important question is, if the danger hypothesis is correct, how closely tied to the drug does a danger signal have to be in order to increase the risk of an IDR? In other words, does the danger signal have to originate from the drug or can

other sources of danger signals unrelated to the drug also increase the risk of an IDR? Strictly speaking, a danger signal must be endogenous; however, since PAMPs appear to act on many of the same receptors as danger signals (DAMPs), molecules originating from pathogens should have similar effects on APCs. There are some examples in which the presence of another factor that can likely act as a danger signal significantly increases the risk of an IDR. For example, the administration of ampicillin or amoxicillin to a patient with mononucleosis almost always results in a skin rash (Pullen et al. 1967). Other examples include the administration of sulfamethoxazole to someone with AIDS which is associated with an almost 50% chance of an IDR (Fischl et al. 1988), and the administration of procainamide to someone immediately after open-heart surgery which appears to increase the risk of agranulocytosis by about tenfold (Ellrodt et al. 1984; Uetrecht 1999). However, it does not appear that most IDRs are associated with risk factors such as surgery or infection, and it is not even clear that such factors commonly increase the risk of an IDR. For example, although regulatory agencies usually specify that drugs that cause idiosyncratic liver toxicity should not be used in patients with preexisting liver disease, it is not clear that people with preexisting liver disease are at increased risk of drug-induced idiosyncratic liver toxicity (Zimmerman 1999). One obvious question is whether a patient with viral hepatitis is at increased risk of hepatotoxicity due to a drug. A common issue is the treatment of patients with tuberculosis who also have viral hepatitis or AIDS. Isoniazid is the most common drug for the treatment of tuberculosis, but it is also associated with a significant risk of liver toxicity and liver failure. Early studies in patients with hepatitis B and AIDS did not show a significant increase in the risk of isoniazid-induced hepatotoxicity (Hwang et al. 1997; Saukkonen et al. 2006). However, it appears that the presence of hepatitis C viral RNA, a measure of active infection as opposed to antiviral antibodies, which was used in earlier studies but only demonstrates an immune response to the virus, is a risk factor for isoniazid-induced liver toxicity (Fernandez-Villar et al. 2007). However, even if viral hepatitis is a risk factor for isoniazid-induced hepatotoxicity, the added risk is small. It may be that the immune system is able to specifically respond to what is causing the danger – in this case, responding to the virus – and ignoring isoniazid if it is not causing significant cell damage. It is also likely that the immune response to something like a virus is quite complex, and depending on how it shifts the balance of immune mediators such as cytokines, it might even decrease the risk of an IDR. Such is the case for patients with AIDS who have a low CD4 T cell count and have a lower incidence of nevirapine-induced IDRs than uninfected individuals for which the drug is used as prophylaxis (Montaner et al. 2003). Timing is also likely to be quite important because the response of the immune system to an acute infection is quite different from that to a chronic infection, and it is also different at different stages of the infection. In animal models of colitis and multiple sclerosis, stimulation of toll-like receptor 9 prior to the stimulus leading to colitis/multiple sclerosis actually prevented the inflammatory condition (Krieg and Vollmer 2007).

Data Consistent with the Danger Hypothesis of IDRs

Tienilic Acid-Induced Hepatotoxicity

One example appearing to provide support for the danger hypothesis is tienilic acid-induced idiosyncratic hepatotoxicity. Tienilic acid is metabolized by CYP 2C9 in humans and by CYP 2C11 in rats to a reactive metabolite that covalently binds to these enzymes leading to inhibition of their activity. Although there is no evidence that they are responsible for the hepatotoxicity, the observation that antibodies against CYP 2C9 have been found in patients who were treated with tienilic acid and developed liver toxicity is evidence of induction of an immune response (Lecoeur et al. 1996). It is also consistent with the hapten hypothesis. Furthermore, if the only target of covalent binding were cytochrome P450, which is not essential for the immediate survival of a cell, it seems unlikely that this would generate a danger signal. If tienilic acid-induced hepatotoxicity did not involve a danger signal it would be evidence that this is not an essential feature of drugs that cause IDRs.

We used global screening of mRNA expression to determine if there were changes consistent with danger signals released from hepatocytes after administration of tienilic acid to Sprague–Dawley rats. We found that gene expression changes indicative of cellular stress occurred shortly after a single dose or two doses (6 and 24 h) of tienilic acid (Pacitto et al. 2007). The genes whose expression changed included those indicating oxidative stress (aldo-keto reductase, glutathione-S-transferase, thioredoxin reductase, epoxide hydrolase), inflammation (IL-1β, interferon regulatory factor 1, macrophage stimulating rotein 1), cytotoxicity (caspase-12), and liver regeneration (p27Kip1,DUSP6, serine dehyratase, spectrinβII, inhibinβA). These diverse danger signals released at a very early stage of drug treatment are likely to alert the immune system and, therefore, suggest their involvement in pathogenesis of TA-induced liver toxicity. It has also been determined that the reactive metabolite of tienilic acid binds to proteins other than P450, which makes it easier to understand its ability to generate danger signals (Koenigs et al. 1999).

Carbamazepine and Phenytoin-Induced IDRs

Carbamazepine and phenytoin are two anticonvulsants that are also associated with a wide variety of IDRs including the aromatic anticonvulsant hypersensitivity syndrome (Shear and Spielberg 1988). These IDRs have characteristics that strongly suggest that they are immune-mediated, and that they are also associated with drug-specific T cells (Beeler et al. 2006). There are several potential reactive metabolites of both drugs, but two metabolites, 3-OH-carbamazepine and 3-OH-phenytoin, appear to cause oxidative stress (Lu and Uetrecht 2008). We also performed microarray analysis of the changes in gene expression induced by these drugs, and they caused changes mostly related to Keap1-Nrf2-ARE signaling pathways, which are important cellular defense mechanisms against oxidative stress. These changes are also consistent with a danger signal.

A related study was also performed on a series of anticonvulsants drugs including felbamate, carbamazepine, phenytoin, phenobarbital, and valproic acid to evaluate their cellular stress induction potential (Leone et al. 2007). Common features shared by these anticonvulsants are that they form reactive metabolites and cause a relatively high incidence of IDRs. Hepatic covalent binding patterns and the toxicogenomic profiles of these drugs were determined. All were found to induce hepatic gene expression changes associated with active metabolite regulation (i.e., aflatoxin B1 aldehyde reductase) and cellular oxidative stress response, which mainly clustered on Nrf2-mediated antioxidative stress pathway. However, no clear association was observed between covalent binding and induction of oxidative stress for the tested drugs, and this may be due to limitations in the metabolizing system that was utilized.

Sulfamethoxazole-Induced IDRs

Sulfamethoxazole is a primary aromatic amine that is also associated with a wide variety of serious IDRs (Rieder et al. 1989). All drugs that are primary aromatic amines given at a dose of more than 50 mg/day are associated with a relatively high incidence of IDRs independent of the rest of their chemical structure (Uetrecht 2002). This is presumably because primary aromatic amines are oxidized to hydroxylamines and nitroso derivatives that can covalently bind to proteins and also redox cycle. In parallel to the tienilic acid study, an analysis of mRNA changes in mice given sulfamethoxazole was performed (Pacitto et al. 2007). However, the results were very different from those from tienilic acid-, carbamazepine- and phenytoin-treated animals. Specifically, the dominant picture was downregulation of gene expression. It is possible that such inhibition of mRNA expression is also a marker of cell stress, and this is a hypothesis that is currently being tested with other aromatic amine drugs. This indicates the mechanistic complexity of IDRs, and it may preclude finding a limited number of patterns of gene expression that can act as biomarkers of IDR risk.

4 Use of the Danger Model to Produce New Animal Models of IDRs

The idiosyncratic nature of IDRs limits our ability to study them and presents a major challenge to elucidating the mechanisms involved. In vitro studies of IDRs cannot possibly mimic all the complex interactions that occur in vivo. Therefore, developing animal models with the potential to provide insight into the mechanisms of IDRs is essential to test mechanistic hypotheses and to study the sequence of events involved. Nevertheless, consistent with low incidence in humans, IDRs are also generally idiosyncratic in animals (Shenton et al. 2004). One explanation of why IDRs only occur in a minority of treated individuals is that in most cases the immune response is tolerance. If the danger hypothesis is correct, it should be

Table 1 A list of potential danger signals

Molecule	Location	Receptor	Activity
HMGB1	Nucleus	RAGE	Stimulatory activity on macrophages
HSPs	Cytoplasm and membrane	CD91, TLR2 and TLR4	Stimulate cytokines production in macrophages
S100s	Cytoplasm	RAGE, TLR2 and TLR4	Inflammatory and chemotactic activity
Defensins	Cytoplasmic granules	TLR4	Chemotactic for leukocytes
Cathelicidin	Cytoplasmic granules	FPRL1 and EGFR	Chemotactic for leukocytes
Uric acid	Cytoplasm	?	Induction of DC maturation, inflammatory effect on macrophages
Hyppo	Membranes	TLRs and scavenger receptors	General inflammation

possible to overcome tolerance by generating a danger signal. Given that danger signals appear to be mediated through toll-like receptors, agents that act through toll-like receptors should markedly increase risk. In the case of one IDR animal model, penicillamine-induced autoimmunity in the Brown Norway rat, poly-IC, which stimulates the immune system through toll-like receptor 3, did increase the incidence and severity of the reaction (Sayeh and Uetrecht 2001). However, in another model, nevirapine-induced skin rash in the rat, poly-IC had no effect even though the rash is clearly immune-mediated (Shenton et al. 2005). Furthermore, our attempts to develop animal models by treating animals with drugs that are associated with a relatively high incidence of IDRs in humans along with cotreatments to stimulate the immune system through toll-like receptors and other mechanisms have not been successful to date (unpublished observations). This is consistent with the fact that in most cases agonists for toll-like receptors do not cause flares in lupus-prone animals or other animal models of autoimmune diseases, and in some cases, such agents are actually protective (Krieg and Vollmer 2007). It also appears to mimic the lack in most cases of an increase in the risk of IDRs in patients with viral infections and other potential sources of danger signals as discussed earlier. A better understanding should lead to an increased ability to produce valid animal models or at least understand why it is so difficult. In some cases, an IDR may require a specific genotype such as a specific MHC molecule required to effectively present a specific hapten; however, this does not appear to be a requirement for most IDRs.

5 Concluding Remarks

The danger hypothesis has had a revolutionary effect on the way that immunologists view what initiates an immune response. Assuming that most IDRs are immune-mediated, this hypothesis has very important implications for the

mechanisms of IDRs and what might act as a risk factor that determines which patients will be affected. However, it has been a difficult hypothesis to rigorously test. Furthermore, stimulation of the immune system through toll-like receptors, which is thought to mediate many of the effects of danger signals, does not appear to reliably increase the risk of an IDR in either humans or animal models. Therefore, there must be aspects of the balance between induction of an immune response and tolerance that we do not understand. This is a fundamental problem that has implications for a wide range of medical problems.

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Approaches for Minimizing Metabolic Activation of New Drug Candidates in Drug Discovery

Sanjeev Kumar, Kaushik Mitra, Kelem Kassahun, and Thomas A. Baillie

Contents

Abstract A large body of circumstantial evidence suggests that metabolic activation of drug candidates to chemically reactive electrophilic metabolites that are capable of covalently modifying cellular macromolecules may result in acute and/or immune system-mediated idiosyncratic toxicities in humans. Thus, minimizing the potential for metabolic activation of new drug candidates during the drug discovery and lead optimization stage represents a prudent strategy to help discover and develop the next generation of safe and effective therapeutic agents. In the present chapter, we discuss the scientific methodologies that currently are available to industrial pharmaceutical scientists for assessing and minimizing metabolic activation during drug discovery, their attributes and limitations, and future

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scientific directions that have the potential to help advance progress in this field. We also propose a roadmap that should help utilize the armamentarium of available scientific tools in a logical way and contribute to addressing metabolic activation issues in the drug discovery-setting in a rapid, scientifically appropriate, and resource-conscious manner.

Keywords Metabolic activation · Bioactivation · Reactive intermediates · Covalent binding · Drug discovery

Abbreviations

GSH	Glutathione
KCN	Potassium cyanide
TZD	Thiazolidinedione
OZD	Oxazolidinedione

1 Introduction

We live in an era where there are tremendous societal demands for low-risk and low-cost medicines to treat unmet medical needs, as well as to improve upon existing therapies in terms of efficacy and safety. One significant challenge to the pharmaceutical industry meeting these demands is the unpredictable nature of most forms of drug toxicity. This lack of predictability frequently leads to failure of new drug candidates during low-throughput and relatively expensive preclinical toxicity testing, with the cost of this failure being directly related to the stage at which the toxicities manifest (e.g., during acute, subchronic, or chronic toxicity testing). Further, this necessitates pharmaceutical companies to invest valuable resources into back-up compounds/programs (many times longer than necessary) to increase the number of "shots on goal" and increase the probability of success. These factors, along with the poor success rate at which new biological targets yield clinical efficacy for human disease conditions, add tremendously to the cost of drug development which, according to some estimates, now exceeds ~US$800 million for each new drug introduced onto the market (DiMasi et al. 2003; Adams and Brantner 2006). An even more serious issue relates to the fact that, while the potential for many target organ toxicities can be identified during preclinical safety evaluation, some adverse effects (that are many times mediated via the immune system) fail to manifest in animals. These toxicities (usually referred to as idiosyncratic toxicities) often have a low incidence (1 in 5,000 or more) and may only become apparent during large-scale clinical trials when a significant resource investment has already been made into the drug candidate or,

even worse, after a compound has been introduced into the market and needs to be withdrawn as a result. This unpredictable nature of drug toxicity represents an issue of paramount importance, both in terms of patient safety and economic loss to the sponsor company, as has been well-illustrated by the recent high profile drug withdrawals for troglitazone (Rezulin®), fenfluramine (Fen-Phen ®), and rofecoxib (Vioxx®).

While there can be many possible mechanisms of drug-induced toxicity, it appears that biotransformation of drugs to reactive, electrophilic metabolites that bind covalently to cellular macromolecules is the initial step in many drug-induced adverse events, including direct target organ damage and immune-mediated idiosyncratic toxicity (Park et al. 2005, 2006; Uetrecht 2006, 2007, 2008; Baillie 2006). There is strong indirect evidence from research conducted over the last 40 years that some, but not all, reactive metabolites can cause cellular toxicity. Most marketed drugs that have been associated with idiosyncratic toxicity are known to form reactive metabolites that are capable of covalently modifying proteins *in vitro* and/or *in vivo* (Kalgutkar and Soglia 2005; Kalgutkar et al. 2005; Zhou et al. 2005; Boelsterli et al. 2006). Although it is now possible, in most cases, to identify the structures of reactive metabolites of drug candidates using modern analytical technologies, it is not possible to predict a priori which of these electrophiles would produce adverse events since the current knowledge on the downstream biochemical/physiological events remains at a very rudimentary stage. In general, it would appear that covalent binding of reactive drug metabolites to critical cellular macromolecules, when combined with host-specific genetic, environmental, and/or disease factors, can render certain individuals more susceptible to drug-induced idiosyncratic toxicity. Since it is not possible to identify these individual-specific factors during preclinical safety testing and map their relationship to drug-induced idiosyncratic toxicity, our ability to predict the potential for these toxicities remains limited. Because of this inability to predict and quantify the risk for idiosyncratic drug toxicities, the strategy of attempting to minimize the formation of reactive metabolites by informed structural modification during the lead optimization stage of drug discovery represents a prudent approach to an otherwise very challenging problem. The relatively modest investment of time and resources early in the lead optimization phase to address this issue has the potential to offer large returns in terms of greater probability of success for development, and an overall reduced risk of eliciting idiosyncratic toxicities in humans via this mechanism. This view is accepted by many pharmaceutical companies, as evidenced by a number of excellent articles from industrial drug metabolism scientists that have focused on the role of metabolic activation in drug-induced toxicity (Nassar and Lopez-Anaya 2004; Kalgutkar and Soglia 2005; Kalgutkar et al. 2005; Erve 2006; Tang 2007; Baillie 2008). In the current chapter, we review the various approaches that are available for addressing metabolic activation and present a logical roadmap to deal with this issue during pharmaceutical lead optimization. We will also discuss the limitations of various approaches and future scientific developments that have the potential to address these limitations.

2 Approaches for Minimizing Metabolic Activation in Drug Discovery

Approaches for addressing bioactivation issues during drug discovery need to rely on both a qualitative and a quantitative assessment of the potential for formation of reactive intermediates. The qualitative assessment entails the identification of the reactive metabolite(s) in question, with particular attention to the substructural motif that is involved in bioactivation so that appropriate chemical modifications can be made by medicinal chemists to block the undesired metabolic pathway(s). Since pharmaceutical lead optimization invariably is a balancing act that strives to achieve the best possible combination of numerous desirable attributes in a drug candidate (e.g., physicochemical properties, potency at the target, on- and off-target pharmacology, pharmacokinetics, and metabolism, to name a few), a quantitative assessment of bioactivation liabilities is also important so that appropriate comparisons among different lead candidates can be made.

Proteins and DNA are believed to be the toxicologically relevant macromolecular targets for chemically reactive intermediates arising from bioactivation of drug candidates. Thus, the most direct approach to assessing the potential (both qualitative and quantitative) for bioactivation is the identification and quantification of the actual adduct of the reactive species with protein or DNA in an appropriate biological system. However, the throughput and speed of currently available technologies for this purpose is not compatible with a fast-paced drug discovery setting. Hence, industrial drug metabolism scientists need to rely on more rapid and higher-throughput surrogate approaches, such as those described below, to identify and measure the bioactivation potential of pharmaceutical lead candidates.

2.1 Chemical Structural Alerts

It has been shown repeatedly that certain chemical substructures are particularly prone to forming reactive electrophilic metabolites that are capable of covalently binding to cellular macromolecules. Examples of these include: anilines (unmasked), hydrazines, nitroarenes, α,β-unsaturated carbonyls, thiophenes, terminal alkenes, or alkynes, etc. Sufficient circumstantial evidence exists in the literature linking bioactivation of these functional groups to various forms of toxicity observed with drugs that contain these substructures (e.g., ticlodipine, tienilic acid, and zileuton for thiophene; carbutamide, procainamide, and tocainide for aniline; phenelzine, hydralazine, dihydralazine, and isoniazid for hydrazine; chloramphenicol, tolcapone, flutamide, and metronidazone for nitroaromatics, etc.) (Kalgutkar and Soglia 2005; Kalgutkar et al. 2005; Boelsterli et al. 2006). At least in some cases, replacement of the *offending* substructure with a more *metabolically benign* one has indeed led to a safer and less toxic second generation agent. For example, the antidiabetic agent, carbutamide, was withdrawn from the market due to severe

bone marrow toxicity; however, replacement of the aniline moiety of carbutamide with a toluene substituent led to the discovery of tolbutamide which is devoid of this toxicity. Similarly, the antiarrhythmics, procainamide and tocainide, both contain an aniline substructure and cause bone marrow aplasis and a lupus-like syndrome, while a closely related congener, flecainide, lacks the aniline motif and is devoid of these toxicities. This subject has been reviewed in depth in several recent articles, and we would point the reader to these papers for additional information (Nelson 2001; Dalvie et al. 2002; Kalgutkar and Soglia 2005; Kalgutkar et al. 2005). In addition to the above more widely appreciated structural alerts, information on novel pathways of bioactivation of other substructures continues to emerge on a constant basis and adds to this list of potentially troublesome functional groups; examples of this include thiazolidinedione (TZD), pyrazinone, furan, pyrazine, and N-substituted piperidines, etc. (Kassahun et al. 2001; Singh et al. 2003; Yin et al. 2003, 2004; Doss et al. 2005). Thus, it should be emphasized that, while awareness of bioactivation structural alerts certainly should be part of the strategy to address this issue during pharmaceutical lead optimization, and replacement with metabolically benign substructures should be explored, an approach that altogether avoids all potential structural alerts would neither be possible nor appropriate; such a strategy would severely limit medicinal chemists' exploration of the full chemical space for SAR, and fails to take into account the fact that metabolism of the offending functionality needs to occur before reactive intermediates are formed. The focus of the strategy should, in fact, be on minimizing the metabolism of the potential structural alert to reactive species, either via *masking* the offending motif within the overall structure of the molecule and/or by incorporating alternative clearance/metabolism pathways into the chemical class.

2.2 *Liquid Chromatography–Mass Spectrometry (LC–MS)-Based Identification of Reactive Metabolites Via "Trapping" Studies*

LC–MS-based approaches likely represent the single most important tool, and the first step for assessing the bioactivation liabilities in a drug discovery setting and for identifying the metabolic pathways that result in formation of reactive species. The latter is usually accomplished via *in vitro* metabolism studies where a select number of drug candidates of interest are incubated with appropriately cofactor-fortified liver preparations (e.g., hepatocytes, microsomes, S9, etc.) from selected preclinical species and humans. The reactive electrophilic species formed from drug candidates generally do not exhibit sufficient stability to allow their direct detection and identification via LC–MS and so need to be detected following adduction to either the endogenous nucleophiles in the biological system or via inclusion of exogenous nucleophiles as "traps" in the incubation; some exceptions to this are acyl glucuronides or CoA thioesters, and rarely some epoxides. As indicated above, however, the technologies for identification of adducts of reactive intermediates with

endogenous nucleophiles (proteins and DNA) currently are not compatible with a fast-paced drug discovery setting. Thus, most drug discovery applications involve inclusion of small molecule nucleophilic traps in the incubation and identification of the resulting adduct of the reactive species with the trapping agent. This approach assumes that the covalent binding of compounds to biological macromolecules and to the surrogate small-molecule trapping agents involves the same bioactivation mechanism. The tripeptide glutathione (γ-glutamylcysteinylglycine, GSH) is the most common trapping agent used in such applications, where its cysteinyl thiol acts as the nucleophilic *war-head* that reacts covalently with the electrophilic reactive intermediate. As an extension of this approach, other thiols, such as *N*-acetylcysteine, cysteine, and 2-mercaptoethanol, have also been used in such studies; however, when the reaction between the thiol nucleophile and the reactive species can be catalyzed enzymatically (e.g., by glutathione-S-transferases), GSH may appear more efficient in trapping the relevant reactive species. Interestingly, cysteine has been shown to react with both its thiol and amine functional groups to trap bifunctional electrophiles. For example, the enedial intermediate from the furan-containing compound, ipomeanine, is trapped by cysteine to form a cyclic adduct (Fig. 1) (Chen et al. 2006). It is to be noted that these thiol derivatives are "soft" nucleophiles and react readily with soft electrophiles (e.g., quinones, epoxides; Fig. 1), but they are not efficient at trapping "hard" electrophilic intermediates such as iminium ions and other reactive species such as electrophilic carbonyls (e.g., aldehydes and ketones). Such reactive intermediates are more efficiently trapped by the nonthiol hard nucleophiles such as cyanide, semicarbazide, methoxylamine, and DNA bases (e.g., guanine).

The LC–MS analysis of adducts of drug candidates with trapping agents typically involves two steps; in the first step, the molecular mass of the adduct is determined via identification of the molecular ion, and, in the second, product ion spectra are generated by collision-induced dissociation (CID) of the parent ion in order to gain insights into the structural motif(s) involved in bioactivation. The challenge in the first step is to detect ions of interest, which frequently have a low

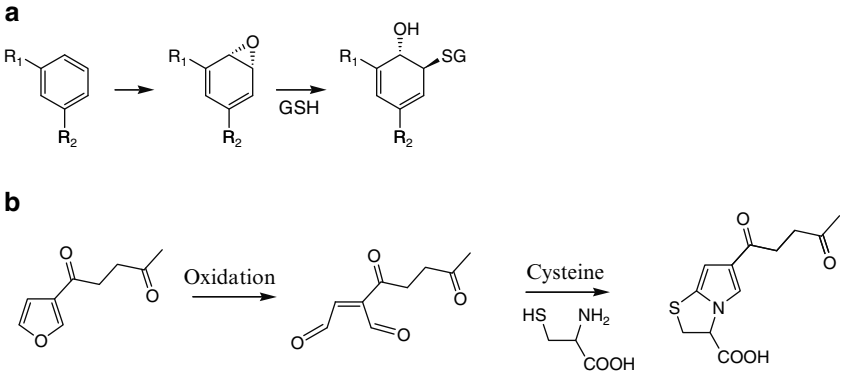


Fig. 1 (**a**) Trapping of an arene oxide by glutathione, (**b**) Trapping of enedial intermediate from ipomeanine by cysteine

abundance, in complex biological matrices; this challenge is compounded further when the adduct(s) arises from unanticipated and complex biotransformations. However, advances in mass spectrometry over the last decade, coupled with developments in separation technologies, have provided drug metabolism scientists with extremely sensitive and reliable tools for detecting low level metabolites in complex matrices. Neutral loss and precursor ion scans are the most commonly employed modes for the detection of molecular ions of adducts. The neutral loss scan mode has found particularly wide applicability for the detection of GSH adducts which frequently lose a neutral fragment of 129 Da (in positive ion mode), corresponding to the loss of pyroglutamic acid from the protonated GSH adduct (Baillie and Davis 1993). This methodology can be adapted to screen for reactive intermediates in a relatively high-throughput assay in a drug discovery setting (Chen et al. 2001). In recent years, a number of refinements of the classical neutral loss assay for the detection of GSH adducts of drug candidates have been published that enhance the reliability of this approach. Soglia and coworkers demonstrated a much greater sensitivity for the detection of thiol adducts via the use of the glutathione ethyl ester as the *in vitro* trapping agent and of micro-bore LC for chromatographic separation of analytes of interest (Soglia et al. 2004). Castro-Perez et al. (2005) reported an LC–MS/MS method that uses a high-resolution Q-Tof mass spectrometer and employs a "pseudo" neutral loss scan corresponding to the exact mass of pyroglutamic acid (129.0426 Da) by alternating low and high collision energy scans and triggering a product ion scan of the relevant precursor ion when a difference of 129.0426 Da is detected (within preset mass error windows). This approach significantly enhances the selectivity of detection and reduces the number of false positives (Castro-Perez et al. 2005). However, it should be appreciated that not all GSH adducts lose a neutral fragment of 129 Da upon CID (e.g., aliphatic and benzylic thioethers frequently eliminate the intact GSH molecule corresponding to a neutral loss of 307 Da), and awareness of bioactivation chemistry is necessary while employing these screening methodologies. Recently, Dieckhaus et al. (2005) investigated the MS/MS behavior of intact GSH and GSH adducts of xenobiotics in the negative ion mode and demonstrated that these produce an abundant anion at *m*/*z* 272 corresponding to deprotonated γ-glutamyl-dehydroalanyl-glycine (loss of the elements of hydrogen sulfide from glutathione). Thus, scanning for precursors of this specific ion led to the sensitive detection of GSH adducts in both *in vitro* and *in vivo* model systems (Dieckhaus et al. 2005). It is important to note that the negative ion MS/MS spectra obtained by fragmentation of the deprotonated molecular ions of the GSH conjugates are characterized primarily by fragments of the GSH moiety, thus affording no structural information on the site of attachment of GSH to the drug candidate. Thus, when an unknown conjugate is detected via the above neutral loss or negative ion precursor scan methods, a positive ion CID experiment needs to be employed to elucidate the structure of the GSH adduct. The neutral loss scans have also been used to detect adducts of drug candidates with other nucleophiles; for example, a high-throughput method that employs the neural loss of 27 Da to detect cyanide adducts of a series of compounds forming iminium ions has been reported (Argoti et al. 2005).

In addition to the above methods, another commonly used approach for the identification of molecular ion(s) of adducts is a knowledge-based search for the expected masses from full scan MS data. This has traditionally been done manually, but more recently the facility of rule-based algorithms has become available within the LC–MS software that allows automated generation of exhaustive lists of masses of expected metabolites, the detection of which, in turn, can be used to trigger further CID scans. As an example of such an approach, Samuel and coworkers used lists of expected masses to trigger MS^n scans on an ion trap mass spectrometer to gain insights into the structures of reactive species involved in GSH adduction (Samuel et al. 2003). A further interesting application of a similar concept is to be found in a recent study where up to 100 possible MRM (multiple reaction monitoring) transitions corresponding to expected GSH adducts were included in a single run on a API4000 Q-Trap® mass spectrometer, and a positive signal in an MRM channel was used to trigger a full product ion scan for that particular precursor ion (Zhu et al. 2007). This approach allowed rapid detection of various GSH adducts with significantly improved sensitivity, and simultaneously afforded product ion spectra on relevant ions to eliminate false positives.

Several investigators have focused their efforts on enhancing the ease and reliability with which the often very small amounts of adducts of various nucleophiles with reactive species generated from the drug candidate(s) can be detected in the presence of a complex biological matrix. For example, the isotope patterns of chlorine or bromine-containing compounds have been used to identify corresponding metabolites; thus, a compound with one or two chlorine atoms displays two molecular ion species in a 3:1 or a 9:6 ratio, respectively, that are separated by 2 Da. Based on the same principle, several studies have used stable-isotope-labeled GSH that incorporates [1,2-$^{13}C_2$, ^{15}N] glycine as a trapping agent in microsomal incubations. LC–MS analysis of incubations of drug candidates with an equimolar mixture of naturally occurring GSH and [1,2-$^{13}C_2$,^{15}N] GSH produced characteristic doublet peaks that are separated by 3 Da, and therefore are readily detectable by their isotopic "signature" (Yan and Caldwell 2004; Yan et al. 2005; Mutlib et al. 2005). Similar results have also been obtained with stable-isotope-labeled cyanide as the trapping agent to detect adducts of iminium ions (Merck Research Laboratories, unpublished data).

The widespread availability of high resolution mass spectrometers in the past 3–4 years has provided new avenues for rapid and unbiased identification of drug-related components in complex biological matrices using so-called mass defect filter-based approaches (Zhu et al. 2006; Bateman et al. 2007; Ruan et al. 2007). These approaches capitalize on the similarity of the mass defects between the parent molecule and its metabolites, and on the fact that most xenobiotics exhibit negative mass defects relative to endogenous materials; thus, by defining preset filter windows, ions that fall outside the defect range can be filtered out and compound-related ions are selected over those from the matrix. Recently, application of the mass defect filtering technique to the identification of the glutathione adducts of reactive intermediates from a series of model compounds was illustrated nicely by scientists at Bristol Myers Squibb (Zhu et al. 2007). These investigators

used specifically defined filter windows (±40 mDa) for the mass defect of the parent drug or the GSH adduct of the parent (parent mass + 305 Da) over a mass range of ±50 Da around the mass of the filter template (parent or parent-GSH adduct); the mass window of ±50 Da for filtering further helps to enhance the selectivity of the approach, but allows for the identification of various biotransformations that might be expected to occur on the parent molecule itself or in combination with GSH adduction. Overall, this approach was highly sensitive, selective, and unbiased in detecting all GSH adducts formed from the model compounds, regardless of their fragmentation pattern, and, in combination with the high resolution MS/MS data, afforded an extremely rich dataset for rapid and reliable elucidation of bioactivation mechanisms for these compounds. The use of such approaches for assessing the formation of reactive metabolites in drug discovery is likely to grow rapidly in the near future as high resolution mass spectrometry instrumentation and software tools for mass defect filtering become more widely available.

In spite of the tremendous advances in LC–MS technology over the past decade, the above trapping assays with GSH generally provide only qualitative information on the formation, or a lack thereof, of a reactive intermediate when a drug candidate is incubated with metabolically competent tissue preparations. Hence, several investigators have made attempts to tailor these approaches to provide at least semiquantitative data for decision-making in drug discovery. One such approach is the use of a quaternary ammonium derivative of GSH that carries a fixed positive charge, thus reducing differences in ionization efficiency between adducted versus nonadducted trapping agent and allowing for the latter to be used as a calibration standard for measuring the amounts of thiol adduct(s) formed (Soglia et al. 2006). Another elegant method was developed by utilizing fluorescent dansylated glutathione (dan-GSH) as the trapping agent and employing fluorescent detection in combination with LC–MS/MS for the detection, structure elucidation, and quantification of relevant thiol adduct peaks (Gan et al. 2005).

Cyanide has been used to trap "hard" electrophiles, e.g., iminium ions resulting from metabolic activation of compounds such as S-nicotine (Fig. 2a) and prolintane (Kalgutkar et al. 2002). For compounds suspected to yield aldehydes as reactive intermediates, the most commonly used trapping agents are semicarbazide and methoxylamine. For example, furan-containing compounds undergo ring opening to form aldehyde intermediates which can be trapped by methoxylamine and semicarbazide (Fig. 2b) (Dalvie et al. 2002; Peterson 2006). An interesting product from semicarbazide trapping of the furan-derived intermediate of pulegone is the tetrahydrocinnoline derivative shown in Fig. 2c. This product is suggested to arise from one molecule of semicarbazide condensing with the γ-ketoenal intermediate of pulegone metabolism (Khojasteh-Bakht et al. 1999). A similar product is also observed from trapping of the reactive intermediate of thiophene oxidation, as illustrated with 2-(4-methoxybenzoyl)thiophene (Dalvie et al. 2002).

The above discussion has focused on the identification of reactive intermediates formed from drug candidates via (P450-mediated) oxidative metabolism. While oxidative metabolism represents the most prolific pathway for the generation of

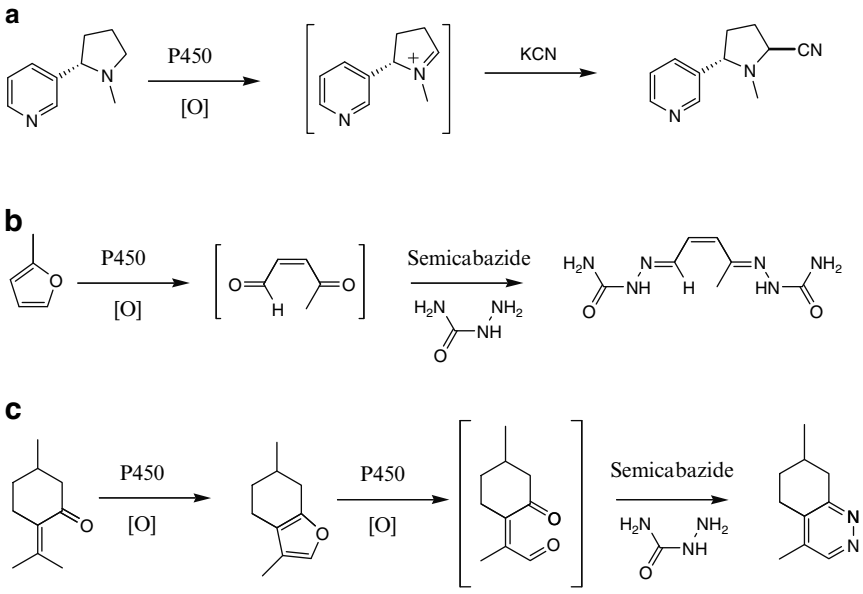


Fig. 2 (**a**) Cyanide trapping of (S)-nicotine, (**b**) semicarbazide trapping of 1-methylfuran, (**c**) semicarbazide trapping of pulegone

reactive species, other metabolic routes can also generate reactive species capable of covalent binding to macromolecules. For example, several drugs containing the carboxylic acid moiety are known to form conjugates with amino acids such as glycine, taurine, and glutamine (Li et al. 2003b; Olsen et al. 2005, 2007). Amino acid conjugation is assumed to occur via activation of the carboxylic acid moiety by coenzyme A (CoA) to form a CoA thioester intermediate which, being an electrophile, reacts with the amine nitrogen of the amino acid to form an amide linkage (e.g., acyl-CoA thioesters of tolmetin and zomepirac, drugs that form amino acid conjugates, have been detected in rat liver) (Olsen et al. 2005, 2007). However, the electrophilic nature of CoA thioesters also makes them potential candidates to covalently modify nucleophilic sites on proteins. The correlation between the formation of CoA adducts and covalent binding to proteins has been demonstrated elegantly *in vivo* in the rat (Li et al. 2003a). When rats were pretreated with trimethylacetic acid, an inhibitor of acyl-CoA thioester formation via depletion of pools of cellular CoA, covalent binding of the model carboxylate, 2-phenylpropionic acid, to liver proteins was significantly decreased (Li et al. 2003a). Thus, it is clear that compounds possessing carboxylate groups may be subject to bioactivation via CoA adduct formation, and there is a need for *in vitro* assays to evaluate bioactivation liabilities via this mechanism. CoA adducts for both tolmetin and zomepirac have been detected in freshly isolated rat hepatocytes (Olsen et al. 2005, 2007). In addition, CoA thioester formation can be investigated readily in hepatic microsomes supplemented with CoA, Mg^{2+} and ATP as cofactors (Li et al. 2003b). While the

LC–MS detection of CoA adducts is relatively straightforward, as the adducts have a relatively large mass (addition of 749 Da to the carboxylic acid) and provide characteristic CID fragmentation, these conjugates tend to be labile, and special precautions need to be taken during sample handling.

In addition to bioactivation via CoA thioester formation, glucuronidation of carboxylate groups can lead to the formation of reactive acyl glucuronide conjugates. It has been suggested that the glutathione thioester conjugate of diclofenac detected in the urine of human subjects dosed with the drug can be formed from reaction of GSH with the acyl glucuronide of diclofenac via a transacylation process (Grillo et al. 2003a, b). There are many other examples in the literature where acyl glucuronide conjugates of carboxylic acid-containing drugs form reactive aldehyde species via migration of the acyl moiety around the hydroxyl groups of the glucuronic acid core (Stachulski 2007). Hepatocytes or liver microsomes supplemented with UDPGA serve as excellent models to screen for the bioactivation of carboxylate-containing drug candidates via acyl glucuronidation, where the key parameters to identify acyl glucuronide reactivity are the stability of the conjugate and its propensity to form isobaric rearrangement products via acyl migration.

While proteins, most likely due to their ubiquitous cellular presence, are the major targets of reactive intermediates formed via metabolism of drug candidates, it has been shown that low molecular weight electrophiles can also bind to DNA, with the potential to result in mutagenicity and carcinogenesis. In fact, some drugs for the treatment of cancer (cyclophosphamide, mitomycin) exert their pharmacological effect through the formation of DNA adducts. DNA modifications generally are detected as adducts to individual bases following digestion of the adducted DNA strands. Common examples of such adducts are from the selective estrogen modulator class of molecules (SERMs), of which tamoxifen is a classic example. Guanine adducts of tamoxifen have been observed in endometrial tissues from patients taking the drug (Shibutani et al. 2000). Several pathways have been proposed for the bioactivation of tamoxifen that involve transient carbocations, quinones, and quinone methides as electrophilic intermediates capable of reacting with DNA. Chemically prepared quinone methides of desmethyl arzoxifene and acolbifene, two newer SERMs, have been shown to form adducts *in vitro* when incubated with deoxynucleosides (Liu et al. 2005a, b). The reactive intermediate from furan metabolism, namely *cis*-2-butene-1,4-dial, has also been shown to form adducts with deoxynucleosides (Peterson 2006). In a drug discovery setting, there are frequent situations when drug candidates turn out be positive in early mutagenicity testing in a metabolism-dependent manner (Ames bacterial mutagenicity assay in the presence of liver S9 fraction). At Merck Research Laboratories, attempts have been made to use DNA bases (e.g., guanine) as trapping agents for the reactive species generated in such systems in order to explore SAR for guanine adduct formation and Ames assay results (Merck Research Laboratories, unpublished data). The limited experience with this strategy suggests that it has the potential to guide rational SAR for eliminating mutagenicity potential, at least in some chemical series, and needs to be further evaluated.

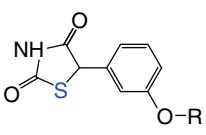
2.3 Covalent Binding Studies with Radiolabeled Drug Candidates

2.3.1 Application of Covalent Binding Studies in Drug Discovery

All the above LC–MS based approaches aimed at evaluating the potential for bioactivation currently provide, at best, semiquantitative information, and are not likely to be applicable universally for trapping all types of reactive intermediates. The current "gold standard" approach for reliably quantifying the extent of bioactivation remains the traditional covalent binding studies that are conducted with radiolabeled analogs of drug candidates. However, the requirement for synthesis of a radiolabeled analog of the drug candidate makes these studies low-throughput, costly, and not amenable to the rapid screening strategies employed in a drug discovery setting. Thus, covalent binding studies generally are conducted as a second step in the lead optimization process following synthesis of radiolabeled analogs of a limited number of more mature lead candidates. At Merck Research Laboratories, the approach that has been adopted involves measuring the extent of covalent binding of drug-related material to rat and human liver microsomal protein *in vitro* (or to hepatocytes when the major metabolic routes involve cytosolic and/or phase II enzymes) and to liver and plasma proteins in rats *in vivo* under standardized conditions (Evans et al. 2004). Standardized, semiautomated methods have been developed to provide an increased throughput for measuring the extent of covalent binding and allow comparison of data across compounds in a drug discovery setting (Day et al. 2005). The covalent binding data (expressed as pmol eq mg^{-1} protein) obtained from these assays are indicative of the propensity of the drug candidate to undergo metabolic activation to reactive species that are capable of covalent adduction to proteins. The *in vitro* covalent binding assay allows assessment of comparative propensity for bioactivation in animal (typically rat) versus human liver preparations over a fixed incubation time period (e.g., 1 h), while the covalent binding studies in rats measure the overall exposure of the rat liver and plasma to electrophilic species formed from the drug candidate under *in vivo* conditions, where factors such as dose, systemic exposure, blood-to-liver partitioning, plasma protein binding, and native protective mechanisms (e.g., GSH conjugation, quinone reductases), that can modulate metabolism and reactive metabolite exposure, are taken into account. It should be stressed that, because of the significance of the aforementioned factors, more emphasis should be placed on the *in vivo* data (which can only be generated routinely in rodents for reasons of practicality) for assessing risk from covalent binding-related liabilities. Furthermore, the key to correct interpretation of the covalent binding data is the qualitative and quantitative understanding of metabolic and bioactivation routes of the drug candidate in liver preparations from the rat and human, and in rats *in vivo*. This information serves to "bridge" the preclinical data to man and helps project exposure of humans to chemically reactive metabolites after administration of the drug candidate at relevant dose. The covalent binding studies, albeit somewhat crude in terms of their toxicological relevance, afford a means during drug discovery to compare different

lead candidates for their potential to generate reactive metabolites in animal safety studies and eventually in humans, and to help advance only those lead candidates into development which are unlikely to be subject to extensive metabolic activation.

An example of the application of the above approach derives from our peroxisome proliferator activated receptor-γ (PPARγ) agonist drug discovery program (Kumar et al. 2008). Following the withdrawal of troglitazone from the market due to recognition of a significant risk of drug-related hepatotoxicity with this agent, it was determined that both the chromane substructure unique to troglitazone and the thiazolidinedione (TZD) ring-system common to all three marketed PPARγ agonists underwent bioactivation to a variety of reactive species (Kassahun et al. 2001; Alvarez-Sanchez et al. 2006). However, the link between chromane or TZD bioactivation and the observed hepatotoxicity of troglitazone remains unclear, and it is possible that multiple other mechanisms play a role in this toxicity (Smith 2003; Masubuchi 2006). It should be noted that both rosiglitazone and pioglitazone, which contain a TZD ring-system, continue to be widely used clinically, with no reported incidences of troglitazone-like hepatotoxicity, although the clinical doses of these agents are at least an order of magnitude lower than those for troglitazone. However, it was now apparent that a common pathway by which TZD-containing PPARγ agonists undergo conversion to reactive metabolites involves cytochrome P450-mediated oxidation of the TZD sulfur atom. Hence, the strategy employed at Merck Research Laboratories was to take advantage of this knowledge to design PPARγ agonists that lacked the metabolic activation-related risk factor. This was achieved relatively easily by replacing the sulfur atom in the TZD ring by oxygen to give corresponding oxazolidinedione (OZD) derivatives. As an example, the TZD derivative compound 1 (Fig. 3) gave rise to a disulfide-linked GSH adduct in "trapping" assays, similar to that described for troglitazone, and was shown to bind covalently to liver microsomal protein (~210 pmol eq mg^{-1} protein under standard *in vitro* conditions; Evans et al. 2004). In contrast, the corresponding OZD analog 2 neither gave rise to any GSH adducts nor displayed significant covalent binding of drug-related material to protein (~25 pmol eq mg^{-1} microsomal protein). Over the past 5 years, a similar strategy has been adopted in multiple drug discovery programs at Merck in order to minimize the potential for metabolic



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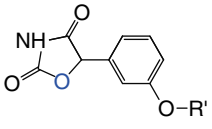

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Fig. 3 General chemical structures of PPARγ agonists 1 and 2 that contain a thiazolidinedione (TZD) or an oxazolidindione (OZD) ring system, respectively. The TZD moiety is prone to bioactivation via P450-mediated oxidation of the sulfur atom, while the OZD ring system is devoid of such liability

activation in lead candidates before they are advanced into development (Chauret et al. 2002; Samuel et al. 2003; Singh et al. 2003; Zhang et al. 2005; Doss et al. 2005; Tang et al. 2005; Levesque et al. 2007).

Since covalent binding data provide quantitative information on the extent of metabolic activation liabilities and afford a means for making comparisons across compounds, they serve as the most logical dataset for decision-making purposes on bioactivation issues in drug discovery. However, metabolic activation obviously is only one aspect of the overall risk/benefit assessment for advancing a particular lead candidate into development. Hence, as has been advocated previously (Evans et al. 2004; Evans and Baillie 2005; Kumar et al. 2008), covalent binding data need to be interpreted in a broader context that includes factors such as: Is the drug intended to treat a disabling or life-threatening disease that represents an unmet medical need? Will the drug be used acutely, chronically or prophylactically? Is the drug aimed at a novel target awaiting clinical proof of concept? Does the mechanism of biological action of the drug involve bioactivation and covalent binding to its target? (e.g., covalent binding studies for antimicrobials from the β-lactam class and for many cytotoxic anticancer agents that act via alkylation of cellular macromolecules would not be relevant for risk/benefit assessment). What is the intended patient population (pediatric, elderly)? Is the clinical dose likely to be low ($\leq$10 mg/day)? How tractable is the chemical lead with respect to modification at the site of bioactivation? Does this modification introduce other undesirable properties (unsuitable pharmacokinetics, P450 inhibition, other off-target activities, etc.)? Because such a diverse range of factors must be taken into account, *trade-offs* invariably need to be made in order to arrive at the drug candidate with the optimum balance of desired properties. Thus, the lead optimization process involves frequent comparison of multiple compounds in regards to their metabolic activation potential (along with other properties) such that a risk/benefit assessment can be made. As a result, a sound understanding of the attributes and limitations of the experimental models and approaches used to measure the potential for metabolic activation become vitally important for making informed and scientifically sound decisions.

2.3.2 Limitations of Covalent Binding Studies

It will be apparent from the above discussion that the currently available approaches/models for measuring metabolic activation potential are relatively crude in that they measure only the overall exposure of the biological system to chemically reactive species under specific experimental conditions, without any regard to the toxicological events that might or might not ensue. This section provides a critique of the attributes and limitations of these models so that the data obtained from them can be placed in context.

There has been much debate within the pharmaceutical industry as to what constitutes an acceptable level of metabolic activation or covalent binding for a drug candidate? Considering the limitations of our understanding of the biochemical mechanisms by which reactive intermediates cause toxicities, a simple answer

to this question obviously does not exist. It has been proposed that a value of 50 pmol eq mg^{-1} protein (under well-defined experimental conditions; Evans et al. 2004) be used as an upper-end target for advancing drug candidates into development. This target is based primarily on the observation that the extent of covalent binding of a number of known hepatotoxins (e.g., acetaminophen, bromobenzene, furosemide, and 4-ipomeanol) in animal liver, under conditions where they cause liver necrosis, is of the order of ~1 nmol eq mg^{-1} protein. Thus, the 50 pmol eq mg^{-1} protein value provides an ~20-fold margin over the levels of binding that typically are associated with frank hepatic necrosis. A second (practical) reason for the selection of $<$50 pmol eq mg^{-1} protein as the target for covalent binding is that this figure is ~tenfold higher than the limit of quantification of the liquid scintillation counting assays when typical levels of radioactivity are employed in the covalent binding study.

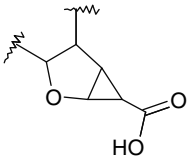
An additional point of debate, and probably an even more difficult one on which to reach a consensus, is the assay conditions under which the extent of covalent binding is measured; this includes considerations such as the concentration of the drug candidate in incubations with liver preparations *in vitro*, and the dose, formulation, bioavailability, and systemic exposure in studies *in vivo*. To a certain extent, optimal conditions to allow estimation of the maximal extent of bioactivation are expected to differ for every drug candidate due to differing kinetics of their metabolism, variable absorption, and pharmacokinetic properties, and tissue (liver) partitioning, etc. However, the fast-paced nature of the drug discovery process does not lend itself to understanding each one of these variables for individual compounds. Further, since a key goal of the lead optimization process is to be able to make comparisons across multiple compounds and advance those with the least number of potential liabilities, it is important to perform measurements under carefully standardized conditions which may be less than optimal for individual compounds or may not be entirely physiologically relevant. For these reasons, we have adopted a strategy of measuring the extent of covalent binding somewhat arbitrarily at a drug concentration of 10 μM in the *in vitro* studies and at an oral dose of 20 mg kg^{-1} in rats *in vivo* (Evans et al. 2004). This strategy allows for better comparisons across various lead candidates from an individual program and also across different programs (especially within the same therapeutic area) in terms of the intrinsic metabolic activation liabilities of leads. However, due to the rather arbitrary nature of the assay conditions chosen, the target value of 50 pmol eq mg^{-1} protein covalent binding value mentioned above should not be used as a cut-off criterion to make decisions about advancing or terminating compounds. Indeed, the optimum use of these data is to identify the bioactivation mechanisms (in combination with LC–MS-based trapping assays) and make appropriate modifications to the chemical structure to minimize the potential for covalent modification of protein. However, if this is not possible, due to tractability issues within the chemical lead class or other competing factors, the covalent binding data should be assessed within a broader context that takes into account the totality of the data available on the compound (see also Evans et al. 2004; Evans and Baillie 2005; Kumar et al. 2008).

2.3.3 Covalent Binding Studies in Drug Discovery: Practical Considerations

In addition to the more fundamental limitations of the covalent binding studies to assess metabolic activation potential outlined above, there are also several practical issues that relate specifically to the lead optimization process in drug discovery that warrant discussion here.

The first of these issues relates to the fact that, for many drug candidates, the extent of metabolic turnover during the incubation time period (1–2 h) is quite low. The number of compounds that fall into this category is substantial because a key goal of many drug discovery programs is, in fact, to decrease rates of metabolism and thereby extend elimination half-life so as to achieve once-a-day dosing for optimal patient compliance and viability in the marketplace. The low metabolic turnover presents multiple challenges during lead optimization in relation to minimizing the potential for bioactivation, including difficulties in accurately measuring bioactivation potential and identifying the "trapped" reactive species, reduced confidence in assessing qualitative and quantitative similarities and differences in various metabolic pathways (including those involved in bioactivation) across species, and less robust "bridging" of preclinical data to the human situation. An extreme example of such a scenario is that of compound 3 and related analogs from a drug discovery program at Merck (Fig. 4) which underwent essentially no detectable metabolism or covalent binding *in vitro* in liver microsomes or hepatocytes from any species over the course of 1–2 h incubations. However, when radiolabeled analogs of these compounds were administered to rats *in vivo* at a dose of 20 mg kg^{-1}, significant amounts of drug-related radioactivity became irreversibly bound to liver protein (ranging from ~50 to 300 pmol eq mg^{-1} protein). To complicate matters further, when the disposition of **[^{3}H]3** was evaluated in rats and dogs, it was found that the compound was eliminated via a combination of different proportions of amino acid conjugation, acyl glucuronidation, and glutathione conjugation (possibly following oxidative bioactivation). Since all these metabolic pathways have the potential to involve the formation of chemically reactive intermediates, it remains unknown as to which pathway might be involved in the covalent binding of radioactivity to liver protein in rats and whether there is the potential for such covalent modification to occur in humans.

A second issue is related to the fact that the rate and quantitative importance of the metabolic pathway(s) that leads to the generation of chemically reactive intermediates may, or may not, correlate with the overall metabolic turnover of the compound in the *in vitro* covalent binding assay systems. Thus, it is not advisable to



3

Fig. 4 General chemical structure of **3** and its analogs

Table 1 Overall metabolic turnover and levels of covalent binding of drug-related material following incubation of compounds 4, 5, or 6 (10 μM) with rat and human liver microsomal preparations in the presence of NADPH at 37°C for 1 h

Compound	Covalent binding (pmol eq mg^{-1} protein)		% Metabolic turnover	
	Human	Rat	Human	Rat
4	169±46	310±24	26	31
5	261±10	571±76	38	55
6	247±27	258±55	~5	22

universally normalize the extent of covalent binding of drug-related material to microsomal/hepatocyte protein to the overall turnover of the compound. This raises interesting challenges while comparing bioactivation liabilities of compounds or lead series with differing rates and extent of metabolic turnover *in vitro*. As an illustration, compounds 4 and 5 in Table 1 belong to the same chemical series and contain a TZD ring that is involved in the generation of chemically reactive intermediates capable of binding to liver microsomal protein. It would appear from the data presented in the Table 1 that the covalent binding of radioactivity to protein for 4 and 5 is closely related to the extent of their overall metabolic turnover (for both compounds, the overall metabolic turnover and covalent binding are greater in rat liver microsomes, and both overall metabolic turnover and covalent binding for 4 are lower as compared to 5). Thus, even though the levels of covalent binding are greater for 5 under the conditions of the assay, it is probably fair to assume that both compounds have the same potential for metabolic activation, and that the risk is similar in rats and humans. This contrasts with the situation for compound 6 in Table 1 where approximately similar levels of covalent binding are observed in both rat and human liver microsomes, but the extent of overall metabolic turnover in human liver microsomes is >fourfold lower. One possible interpretation of these data is that the extent of liability for metabolic activation for 6 is much greater in humans where ~50% of the metabolized parent leads to the formation of chemically reactive metabolites that bind to microsomal protein (as opposed to ~10% in rat liver microsomes). Thus, it is important to consider overall metabolic turnover issues in the interpretation of, and risk-assessment from, the covalent binding data while making comparisons across compounds.

A third issue relates to the fact that species differences in the quantitative contribution of various metabolic pathways to drug disposition, including those involved in the formation of chemically reactive metabolites, are extremely common. Thus, it is important to understand the disposition of the drug candidate(s) and gain a mechanistic view of the pathways involved in bioactivation and covalent binding for accurate assessment of the relevance of covalent binding data generated in rats to humans.

The final issue arises from the fact that the extent of covalent binding observed *in vivo* in most cases likely will depend upon the magnitude of the systemic and liver exposure to drug-related material which, in turn, is dependent upon the physico-chemical properties, formulation, and pharmacokinetic and tissue distribution properties of the drug candidate. Although there is no simple way to precisely

control or correct for these variables across many lead candidates, it is clear that they should be taken into account while interpreting covalent binding data. One possible approach to dealing with this issue is to examine the magnitude of covalent binding at a systemic exposure that is at least 10- to 20-fold higher than the targeted clinical exposure, thus building a minimal "safety cushion" for all compounds.

In summary, the discussion above supports the notion that rigid "cut-off" values for the extent of covalent binding to protein for decision-making in drug discovery are inappropriate and should be avoided. The extent of covalent binding should be interpreted in light of knowledge of the overall metabolic turnover of the compounds and systemic exposure to drug-related material in relation to the target clinical exposure, and species differences in metabolism should be taken into account when extrapolating covalent binding risk to humans.

2.4 Higher-Throughput Surrogate Assays for Quantifying the Potential for Bioactivation

Because of the fact that covalent binding studies with radiolabeled analogs of drug candidates are low-throughput and costly, there is tremendous interest within the pharmaceutical industry to develop higher-throughput approaches for quantitatively assessing bioactivation liabilities during lead optimization. Generic LC–MS-based approaches described earlier (e.g., those based on a neutral loss scan of 129 Da in positive ion mode or a precursor ion scan of *m*/*z* 272 in negative ion mode in incubations with GSH-supplemented liver microsomes) are amenable to higher-throughput implementation, but they provide information of a qualitative nature only. Variations of these LC–MS methods that rely on measuring a specific signal from a chemically modified trapping agent have been developed to provide quantitative (or semiquantitative) data. These include the use of radiolabeled GSH or cyanide (Gorrod et al. 1991) or a fluorescent dansylated analog of GSH (Gan et al. 2005) as traps where the radiolabeled or fluorescent trapped adduct can be quantified following HPLC separation. Another interesting methodology is the use of a quaternary ammonium derivative of GSH that carries a fixed positive charge, thus reducing differences in ionization efficiency between adducted versus non-adducted trapping agent and allowing for the latter to be used as a calibration standard (Soglia et al. 2006). When a correlation has been established between the extent of covalent binding of drug-related material to protein and the amount of adduct(s) trapped with these methods (i.e., when the identity of the reactive species binding to protein and to the trapping agent is presumed to be the same), these approaches can reduce the need to synthesize radiolabeled analogs of individual compounds and provide a much improved throughput and cost-benefit over the traditional covalent binding assays. However, these methods do require sample processing, chromatographic separation, and manual data handling for individual compounds to quantify drug adduct(s) and, as a result, are able to achieve only a *medium* overall throughput. Thus, we and others have explored the use of

radiolabeled trapping agents (such as [^{3}H]GSH, [^{35}S]GSH, [^{35}S]β-mercaptoethanol and [^{14}C]CN) for the capture of reactive intermediates where the resulting radioactive adduct can be separated from the excess trapping agent by appropriate extraction procedures and quantified using plate-based radioactivity detection methods (Meneses-Lorente et al. 2006). This assay format is easily amenable to automation, thus dramatically increasing the speed and throughput of data generation. It is conceivable that, in addition to the above radiolabeled trapping agents, the assay using a fluorescent dansylated GSH derivative (Gan et al. 2005) will also be adaptable to this format. The assumption underlying the use of all these methodologies is that the small-molecule trapping agent will act as a good quantitative surrogate for nucleophilic sites on proteins in the biological system that covalently react with the electrophilic intermediates generated from the drug candidate; this assumption needs to be tested for each series of compounds based on the knowledge of metabolic pathways potentially involved in covalent binding. An example of this validation is provided by Meneses-Lorente et al. (2006) who demonstrated that the extent of covalent binding of a series of piperidine-containing drug candidates measured using radiolabeled analogs of individual compounds was reduced substantially by the inclusion of KCN in incubations. Further, formation of the radiolabeled cyanide adducts of these compounds in liver microsomal incubations supplemented with K^{14}CN correlated reasonably well with the covalent binding of these analogs to liver microsomal protein. These data suggest that, for this series of compounds, iminiun ions are the likely *culprit* species involved in covalent binding of drug-related material to microsomal protein, and a strategy based on screening for metabolic activation potential with radiolabeled cyanide could be a viable option. In contrast, the extent of covalent binding of another class of compounds from a Merck drug discovery program was attenuated significantly with inclusion of GSH (but not cyanide) in incubations, and a number of thiol adducts that were postulated to be formed via trapping of arene oxide and quinone intermediates were identified in these incubations (Samuel et al. 2003). For this class of compounds, we explored the use of [^{35}S]β-mercaptoethanol (as a substitute for [^{35}S]GSH which tends to be have issues with stability) as a trapping agent and compared the extent of covalent binding of the compounds evaluated previously (Samuel et al. 2003) with the amount of reactive species trapped with [^{35}S]*β*-mercaptoethanol upon incubation of these drug candidates in human liver microsomes. The data from these investigations are presented in Table 2 and demonstrate excellent correlation between the two assays. Similarly, Masubuchi and coworkers conducted an elegant study where they attempted to correlate the amount of GSH conjugates formed (using both unlabeled and [^{35}S]-labeled GSH) from a set of model compounds with the extent of covalent binding of radiolabeled drug-related material to human and rat liver microsomal protein (Masubuchi et al. 2007). Ten drugs (acetaminophen, amodiaquine, carbamazepine, clozapine, diclofenac, furosemide, imipramine, indomethacin, isoniazid, and tienilic acid), that are known to form protein adducts via the formation of reactive intermediates, were included in this study. The results showed good correlation between levels of GSH adduct formation and the levels of *in vitro* covalent binding of drug-related material to rat and human liver

Table 2 Comparison of the extent of covalent binding of radioactivity to human liver microsomal protein measured with individual tritiated analogs of compounds I–V with the amount of reactive species trapped using [^{35}S]β-mercaptoethanol under the same incubation conditions

Compound	Covalent binding to human liver microsomal protein (pmol eq mg^{-1} protein/1 h incubation)	Amount of [^{35}S]β-mercaptoethanol adduct(s) trapped upon incubation with human liver microsomes (pmol eq mg^{-1} protein/1 h incubation)
7	1,690	2,236
8	911	1,643
9	303	538
10	88	653
11	17	88

microsomal protein. Also, acceptable correlation was found between the extent of GSH adduction and the maximum extent of covalent binding of radioactivity to rat liver protein *in vivo* following a 20 mg kg^{-1} oral dose of the radiolabeled drug when factors such as systemic exposure (plasma AUC) and plasma-free fraction were taken into account (Masubuchi et al. 2007). These limited number of studies that describe application of variations of trapping assays for quantitative assessment of bioactivation liabilities suggest that a judicious selection of the trapping agent(s) for reactive intermediate screening that is based on a sound understanding of the metabolic pathways involved in bioactivation/covalent binding can help increase the speed, efficiency, and throughput, and lower the overall cost of bioactivation studies by significantly reducing the number of radiolabeled drug candidates that need to be synthesized for this purpose.

2.5 *Proposed Roadmap for Addressing Metabolic Activation in Drug Discovery*

Based on the scientific approaches discussed above that are currently available, we propose a 4-tier roadmap outlined in Fig. 5 as a general strategy to enable quick and rational decision-making for addressing metabolic activation issues in drug discovery. The first step of this strategy (that preferably occurs at an early stage in the program) involves qualitative (or possibly semiquantitative) evaluation of the pathways involved in metabolic activation of several representative chemical lead structures via LC–MS-based trapping assays in liver preparations from appropriate species (usually rat and human), with a judicious selection of the trapping agent based on anticipated bioactivation routes. These studies enable a rapid determination of the sites and mechanism(s) of bioactivation and can be used to drive structural modification, in parallel with overall program SAR, to block these metabolic pathways at an early stage. As the program and the leads mature, and as a tier 2 strategy in the proposed roadmap, radiolabeled analogs of a select number of more advanced lead candidates should be synthesized and *in vitro* and *in vivo* covalent binding and metabolism studies conducted to quantitatively assess the

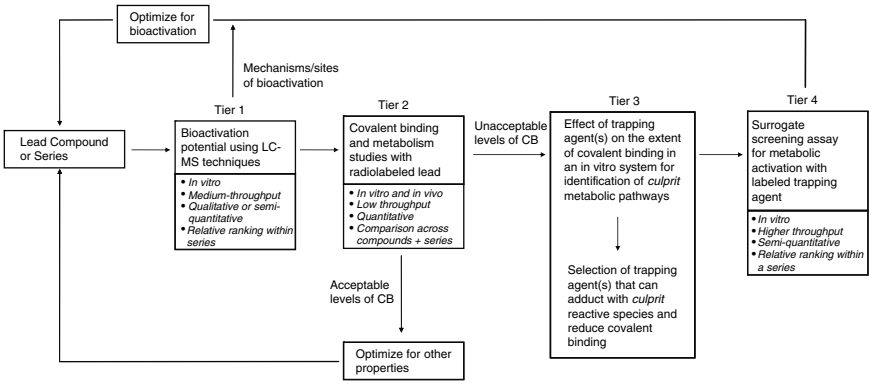


Fig. 5 Proposed roadmap for addressing metabolic activation issues in drug discovery

bioactivation liabilities, and to guard against potential reactive intermediates that may not be efficiently scavenged by the trapping reagent(s) employed. If these studies reveal minimal potential for metabolic activation, the program can progress towards optimization of other desired properties. However, if significant covalent binding is observed in tier 2 studies, effort should be placed on identifying the mechanisms of bioactivation and covalent binding by examining the effect of various trapping agents on the extent of covalent binding (tier 3 studies), and identification of the adducts formed [with the trapping agent(s) that results in reductions in covalent binding] via LC–MS studies similar to those in tier 1. If these studies clearly reveal the identity of the reactive species that are involved in covalent binding, higher-throughput surrogate screening assays that employ appropriate radiolabeled or fluorescent trapping agent can be used as a tier 4 step to develop quantitative SAR for bioactivation. As the chemical lead evolves in response to this screening process, these surrogate screening assays should be interfaced with additional tier 1 studies to monitor for potential switch in the mechanisms of bioactivation. For this 4-tier cycle to be most effective, a close and iterative collaboration between medicinal chemistry and drug metabolism scientists should begin as early as possible in the life-cycle of the drug discovery program.

Although relatively costly and low-throughout, the covalent binding studies to protein using radiolabeled drug candidates currently play a central and indispensable role in this proposed roadmap for quantifying the extent of metabolic activation, and in the selection of appropriate trapping agent(s) for understanding and addressing the mechanisms involved in bioactivation. This is underscored by the following two examples from drug discovery programs at Merck.

In the first case study, a lead candidate 12 (Fig. 6) was assessed for its potential to form reactive metabolites in LC–MS-based trapping assays using a variety of trapping agents. There were no indications of the formation of any thiol or cyanide adducts when GSH, N-acetylcysteine, or cyanide were included in incubations of 12 with human liver microsomes. However, when a tritium-labeled derivative of 12 was incubated with human liver microsomes under standard conditions

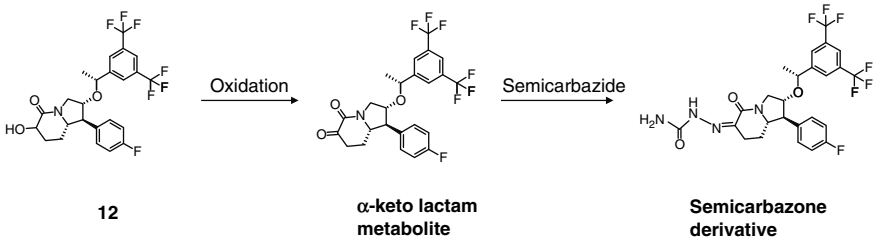


Fig. 6 Metabolism of compound 12 to a α-ketolactam metabolite, which can be trapped as the corresponding semicarbazone derivative by reaction with semicarbazide

(Evans et al. 2004), the extent of covalent binding of radioactivity was estimated to be ~500 pmol eq mg^{-1} liver microsomal protein over a 1-h incubation time. Further, the major metabolite of [^{3}H]12 in human liver microsomal incubations was the oxidized α-keto lactam derivative. This α-keto lactam metabolite underwent facile condensation with semicarbazide and essentially all of the metabolite formed in human liver microsomal incubations could be trapped as the corresponding semicarbazone derivative when semicarbazide was included in the incubation; this finding possibly implicated this metabolite as a culprit in the covalent binding of [^{3}H]**12**-associated radioactivity to human liver microsomal protein via its reaction with basic amino acid residues. However, the inclusion of semicarbazide in the incubations led to very small (<20%) reductions in the extent of covalent binding of [^{3}H]**12**-associated radioactivity to protein, suggesting that the α-keto lactam is likely not the major reactive species of concern. This case study stresses the point that mere detection of an adduct with a trapping agent does not necessarily provide a true indication of the metabolic activation and covalent protein binding liability of a particular chemical substructure, and SAR driven on this basis only, without appropriately interfacing with covalent binding studies, has the potential to lead to misguided medicinal chemistry efforts.

In the second case study, compound 13 contained a spirocyclic motif with tetrahydrofuran and piperidine moieties (Fig. 7). Similar to compound 12 in the previous case study, 13 also did not yield any thiol or cyanide adducts when incubated with human liver microsomes in the presence of thiol or cyanide trapping agents. However, when a radiolabeled analog of this compound ([^{3}H]13) was administered to rats at an oral dose of 20 mg kg^{-1}, the extent of covalently bound radioactivity in liver tissue was measured to be ~50–60 pmol eq mg^{-1} protein at 6 and 24 h postdose. In addition, incubation of [^{3}H]**13** with human liver microsomes under standard assay conditions led to ~400 pmol eq of radioactivity covalently bound per mg of protein over 1 h. The extent of this covalent binding was not reduced significantly by the inclusion of GSH or cyanide in incubations but was suppressed nearly completely (>90%) with methoxylamine. This led to the hypothesis that oxidation at the carbons α- to the oxygen of the tetrahydrofuran ring or nitrogen of the piperidine ring could lead to the formation of corresponding hemiacetal or hemiaminal metabolites, respectively, which could exist in equilibrium with their ring-opened and reactive aldehyde form; one or more of these aldehyde derivatives could then react covalently

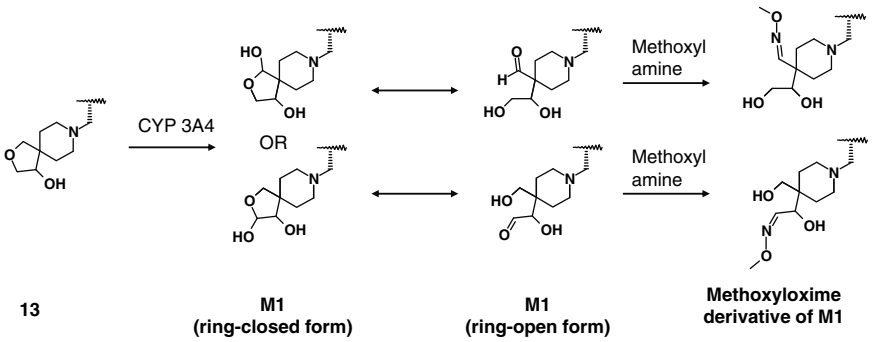


Fig. 7 Metabolism of compound 13 to reactive hemiacetal metabolites via oxidation of the tetrahydrofuran ring and identification of these reactive species via trapping with methoxylamine

with basic amino acid residues and lead to the covalent binding observed with this drug candidate. At least one of these aldehyde intermediates could be trapped by methoxylamine as its corresponding oxime derivative, the mass spectral fragmentation of which corresponded to a condensation product between methoxylamine and a ring-opened aldehyde intermediate formed following oxidation of the tetrahydrofuran ring (Fig. 7). Although two different adducts are possible with this chemical structure, the aldehyde intermediate depicted in the top row of Fig. 7 would bind with amine nucleophiles (e.g., lysine residues) to form an imine which would generally be unstable towards hydrolysis in a biological environment and would not result in detectable covalent binding, whereas the α-hydroxy imine derivative formed from the α-hydroxy aldehyde intermediate depicted in the bottom row would rearrange in an Amadori-like process to form a stable β-keto amine adduct that would result in measurable covalent binding of drug-related material to protein. In agreement with this hypothesis, the corresponding cyclopentane analog of 13, where the oxygen of the tetrahydrofuran ring was replaced by a carbon in order to block ring-opening and formation of aldehyde intermediates, displayed greatly reduced levels of covalent binding to human liver microsomal protein (~100 pmol eq mg^{-1} protein/1 h incubation) under the standard conditions of the assay (Evans et al. 2004). This example highlights the critical role that covalent binding studies play in the selection of the correct trapping agent for understanding the bioactivation/covalent binding mechanism(s) involved so that medicinal chemistry efforts can be focused in a productive fashion.

3 Metabolic Activation, Quality of Drug Candidates, and Probability of Success for Development

Although a major rationale for minimizing metabolic activation during drug discovery is related to the desire to reduce the potential for idiosyncratic toxicities in humans, these efforts can also aid in the interpretation of the preclinical safety

assessment data and in the design of lead compounds that may have reduced liabilities in a number of other aspects as discussed below, thus enhancing the overall quality and probability of success for development candidates.

3.1 Metabolic Activation and Risk Assessment for Species-Specific Toxicity

As opposed to the formidable challenges in correlating metabolic activation with low-incidence and human-specific idiosyncratic toxicities, many of which appear to be mediated by the immune system, it seems likely that a closer relationship exists, at least for certain compounds, between bioactivation and target organ toxicity. For example, studies in animals have established a clear dose–response relationship for acetaminophen hepatotoxicity following depletion of protective GSH stores, and the degree of covalent binding to liver tissues in these experiments correlates well with the severity of the resulting lesions (Potter et al. 1973, 1974; Tarloff et al. 1996). In this regard, efforts to understand mechanisms of metabolic activation can be of value in rationalizing foreign compound-mediated toxicities, especially when these toxicities are species-specific. Such understanding, in turn, can provide a framework for assessing risk of certain toxicities in humans that may be mediated through metabolic activation phenomena. An elegant illustration of this is provided by studies with the nonnucleoside HIV reverse transcriptase inhibitor, efavirenz, which causes renal tubular epithelial cell necrosis in rats but not in cynomolgus monkeys or humans at equivalent or greater systemic exposures (Mutlib et al. 1999, 2000). Efavirenz is metabolized to a cyclopropanol metabolite via hydroxylation at the methine position of the cyclopropane moiety that is linked to an alkyne functionality; this cyclopropanol metabolite likely serves as a substrate for a *rat-specific* glutathione-S-transferase(s) and results in the addition of GSH to the alkyne moiety of efavirenz in rats but not in other species. This GSH conjugate of efavirenz is processed further in the rat kidney to a cysteinylglycine conjugate via γ-glutamyl transpeptidase-catalyzed removal of the glutamic acid residue, and the cysteinylglycine conjugate is either excreted in rat urine or is involved in further bioactivation events that eventually lead to nephrotoxicity in the rat. Strong evidence was obtained for this hypothesis where a decrease in the formation of the cysteinylglycine conjugate, either via interfering with the formation of the cyclopropanol metabolite or via inhibition of γ-glutamyl transpeptidase, led to reductions in the incidence and severity of nephrotoxicity. These data clearly demonstrate the value of understanding bioactivation mechanisms in preclinical safety assessment species so as to rationalize possible mechanisms of species-specific toxicity and assess risk in humans.

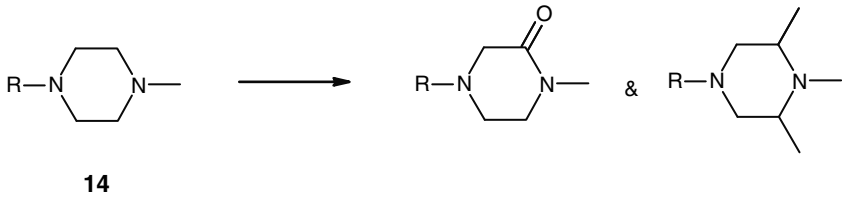
3.2 Metabolic Activation and Genotoxicity

The potential of chemically reactive metabolites to cause genotoxicity and cancer has been well established (Miller 1994, 1998). Thus, efforts at minimizing

metabolic activation can help in screening out candidates that may prove to be mutagenic in downstream preclinical studies. For example, a number of quinone and/or quinone methide reactive intermediates derived from estrogens and tamoxifen have been shown to bind to DNA bases and induce DNA damage (Bolton et al. 2000; Zhang et al. 2001; Liu et al. 2003; Kolbanovskiy et al. 2005). Another recent example in this field is the elegant work of Kalgutkar et al. (2007a) on a drug candidate that was found to be mutagenic in an *in vitro* assay (activated *Ames* test), although in this case the bioactivation studies were conducted retrospectively to elucidate mechanisms of genotoxicity. The authors identified reactive nitrone, aldehyde, and quinone-methide metabolites using cyanide, methoxylamine, and GSH as trapping agents, respectively. Interestingly, the increase in reverse mutations in the *Ames* test was attenuated significantly when methoxylamine was included in the test system, implicating the hard electrophilic nitrone and/or aldehyde derivatives in the alkylation of DNA.

3.3 Metabolic Activation and Mechanism-Based P450 Inactivation

Mechanism-based irreversible inhibition of P450 enzymes represents a serious flaw in any drug candidate because of the potential for clinical drug–drug interactions, as was demonstrated by the withdrawal of mibefradil (Posicor®) from the market. It is a well-known fact that chemically reactive metabolites can cause mechanism-based inhibition of P450 enzymes via alkylation of either the heme or the P450 apoprotein (Masubuchi and Horie 2007; Kalgutkar et al. 2007b). For example, the grapefruit juice constituent, bergamottin, is a mechanism-based inhibitor of CYP3A4, CYP1A2, 2B6, 2C9, 2C19, and 2D6, but not of 2E1, in human liver microsomes (He et al. 1998; Tassaneeyakul et al. 2000; Lin et al. 2005); when [^{14}C]bergamottin was incubated with membrane preparations of individual human recombinant P450 enzymes, the covalent binding of radioactivity, or a lack thereof, correlated well with the inhibitory potential of bergamottin for these enzymes (the covalent binding values were 575, 152, 214, 263, 593, 166, and <5 pmol-eq/nmol P450 over 1 h incubation for CYP3A4, 1A2, 2B6, 2C9, 2C19, 2D6, and 2E1, respectively; S. Kumar, unpublished data). Thus, minimizing the formation of chemically reactive metabolites should, in general, yield benefits in terms of reducing the potential for mechanism-based P450 inhibition. A recent example is illustrated by our work on the *N*-methyl piperazine derivative 14 shown in Fig. 8 (Kumar et al. 2008). This compound was found to be an effective incubation time-dependent inhibitor of CYP3A activity in human liver microsomes (comparable to troleandomycin) and displayed NADPH-dependent covalent binding of drug related material to human liver microsomal protein (~300 pmol eq mg^{-1} protein over 1 h). The extent of covalent binding of radioactivity was reduced by ~10% when potassium cyanide was included in the incubations, and this was accompanied by the detection of trace levels of a cyanide adduct formed via trapping of the iminium species (formed via



14

Incubation time-dependent inhibitor of CYP3A4

No incubation time-dependent inhibition of CYP3A4

Fig. 8 The lactam and methyl substituted derivatives of 14 that do not undergo metabolic activation to reactive iminium species via oxidation of the distal nitrogen and also do not display incubation time-dependent inhibition of CYP3A4 in human liver microsomes

oxidation at either or both of the two nitrogens). This profile appears characteristic of many mechanism-based inactivators of P450 where covalent binding to protein occurs, but is not easily attenuated by nucleophilic scavengers added to the incubation medium; this is likely related to the rapid covalent binding of the highly reactive species within the active site of the enzyme before they have the opportunity to diffuse into the incubation medium and react with nucleophiles. This is consistent with the fact that addition of nucleophilic scavengers in the incubation usually does not protect the enzyme from mechanism-based inhibition (Sahali-Sahly et al. 1996; Jushchyshyn et al. 2003, 2006; Kalgutkar et al. 2007b). Based on this information, lactam and α,α'-dimethyl analogs of the *N*-methyl piperazine at the distal nitrogen were synthesized that did not form cyanide adducts in human liver microsomes and were also devoid of the incubation time-dependent CYP3A inactivation property of 14 (Fig. 8) (Kumar et al. 2008).

Overall, we believe that efforts to minimize metabolic activation during drug discovery can enhance the overall quality of drug candidates that are advanced into development in a number of respects and result in an increased probability of success.

4 Minimizing Metabolic Activation in Drug Discovery: Future Directions

In spite of the progress made in recent years in our ability to address metabolic activation issues in drug discovery, it should be acknowledged that our scientific approach to addressing this issue remains rather crude. This is largely the result of scientific gaps in at least two different areas: the first area relates to our lack of understanding of the biological mechanisms that result in toxic insult following exposure to some, but not all, chemically reactive metabolites, and the second to the experimental models that are used for measuring the extent of metabolic activation and covalent binding and their relevance to human risk assessment. It can perhaps be argued that the inability to quantify risk with specific reactive species formed from individual drug candidates forces the pharmaceutical industry to adopt

a conservative approach, where attempts are made to minimize metabolic activation recognizing that, in the process, many molecules may be eliminated from consideration that could be developed as safe drugs in spite of their metabolic activation potential. In the past 2–3 years, several exciting scientific breakthroughs have occurred that have the potential to address the above gap areas and transform the process of minimizing metabolic activation during drug discovery from a crude empirical approach to a much more rational one. The following section discusses some of these developments and their potential impact on the drug discovery process.

Drug toxicity is a multifactorial process, and it is clear that, at the point of manifestation of toxicity, it is generally no longer possible to identify the original trigger mechanisms. Thus, the systems biology-based "omic" approaches, including toxicogenomics and proteomics that investigate early patterns of change across a broad range of biological networks in response to toxicants, offer tremendous promise in identifying original trigger mechanisms that lead to toxic insult. Microarray-based genomic technologies have already matured to a point where they can be applied routinely in a drug discovery environment (Gatzidou et al. 2007; Woods et al. 2007). In an effort to increase speed and reduce development costs, the pharmaceutical industry is bound to increasingly employ toxicogenomic analyses in preclinical safety assessment studies as a means of understanding the transcriptional networks that may be modulated in response to xenobiotic exposure and differentiate drug candidates at an early stage in short-term toxicity studies. Since these investigations will often have toxicologic correlates in animals, they should aid in the rapid development of predictive tools for assessing the toxicity potential of drug candidates (via metabolic activation or other mechanisms) during drug discovery. From a purely metabolic activation perspective, it is evident that the most promising avenue for predictive differentiation between *benign* versus *toxic* reactive intermediates is likely to come from the identification of the cellular macromolecules that are targeted by various reactive metabolites and the nature of the resulting protein modification for a large number of diverse xenobiotics. However, until recently, progress in this area has been very slow, such that only a limited number of protein targets have been identified for a range of model compounds in over 2-3 decades of research (reactive metabolite target protein database, http://tpdb.medchem.ku.edu/tpdb.html; Hanzlik et al. 2007). This is largely due to the lack of adequate analytical tools that have the ability to rapidly identify drug-protein adducts from a complex biological matrix where the abundance of different proteins can vary by a million-fold or more and where reactive intermediate adduction to target protein invariably is substoichiometric. However, recent progress in the proteomics field as a result of advances in high resolution mass spectrometry technology and intelligent data analysis software tools (e.g., SALSA, P-Mod or OpenSea) shows much promise in tackling this problem (Liebler and Guengerich 2005; Dennehy et al. 2006; Shin et al. 2007; Liebler 2008). In principle, these higher-throughput "shotgun-type" proteomic tools will facilitate our understanding of the identities of protein targets of reactive intermediates and contribute to deciphering the complex protein networks whose function can be modulated via covalent modifications (Liebler and Guengerich 2005; Hanzlik et al.

2007; Koen et al. 2007). Because of the complex and multifactorial nature of drug toxicity, these "shotgun" type genomic and proteomic technologies and powerful network integration and pattern recognition tools offer great promise to provide a detailed elucidation of the biochemical and cellular processes by which certain chemically reactive metabolites trigger toxicity, while others activate cellular defense mechanisms and thus represent a benign form of bioactivation.

As has been discussed earlier in this chapter, the current experimental models for assessing the potential for metabolic activation (liver microsomes and hepatocytes *in vitro*, rodents *in vivo*) are far from ideal and enable only an empirical approach to addressing the issue of metabolic activation. However, significant progress has been made recently in the development of improved preclinical models for predicting human drug metabolism, including the propensity for metabolic activation. These include, on the one hand, better *in vitro* tools such as engineered micropatterned human liver tissue that is closer in three-dimensional structure and function to the native human liver (Sivaraman et al. 2005; Khetani and Bhatia 2006, 2008; Baudoin et al. 2007) and, on the other hand, even more ambitious and promising genetically engineered and chimeric animals that express single, multiple, or the entire complement of human drug metabolizing enzymes and transporters (van Herwaarden et al. 2005; van Waterschoot et al. 2007; Senekeo-Effenberger et al. 2007; Katoh et al. 2007; Azuma et al. 2007; Katoh and Yokoi 2007). These animal models, once validated, will offer unparalleled opportunities to explore a variety of variables of interest to drug discovery scientists, including the potential for metabolic activation as a function of dose and exposure and under "human-like" *in vivo* conditions that take into account important variables such as plasma protein binding, tissue partitioning, and a more complete set of protective and clearance mechanisms than is available in *in vitro* models. It is tempting to speculate that, in the not-too-distant future, one or more of the above humanized *in vitro* and/or *in vivo* models will emerge as good predictors of human drug metabolism and metabolic activation potential. These models will then offer unprecedented opportunities to investigate the link between metabolic activation of established human toxicants (that are suspected to elicit toxicity via covalent binding to macromolecules) to changes in the genomic and the proteomic signature of the target tissues (liver, in particular). The hope is that patterns of change will emerge that will yield predictive biomarkers for human toxicity which, in turn, will aid in assessing the toxicological significance, or lack thereof, of metabolic activation of a particular drug candidate before it is advanced into development or introduced onto the market.

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