Mechanisms of Troglitazone Hepatotoxicity

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Invited Review

Mechanisms of Troglitazone Hepatotoxicity

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Contents

1. Introduction

In January 1997, the drug Rezulin (TGZ)[1] was approved for use in the treatment of type II diabetes by the Food and Drug Administration. Between one and two million patients were treated with TGZ from 1997 through the end of February 2000, when TGZ was withdrawn from the market (*1*). This withdrawal was based on numerous reports of liver failure associated with TGZ use. For example, Kohlroser et al. reviewed 46 cases of hepatotoxicity among Rezulin users and concluded that "there can be no doubt that, in some patients, TGZ causes severe hepatotoxicity" (*2*). The exact nature of the hepatotoxicity remained controversial, however. Initial reports described TGZ hepatotoxicity as being idiosyncratic, that is, a rare event that occurs without explanation and is not dose-dependent. Usually such hepatotoxins work by an indirect, often immune, allergic type mechanism. However, in clinical trials, more than 1.9% of patients had elevated serum transaminases and other signs of liver toxicity. Thus, the moderate hepatotoxic effects of TGZ were not that rare. Furthermore, few, if any, of the patients with severe liver damage showed signs of fever or other indicators of a cytotoxic lymphocyte-based immune mechanism. Moreover, TGZ often produced a zonal necrosis of the liver, whereas idiosyncratic hepatotoxins usually produce a diffuse, focal necrosis. Zonal necrosis of the centrilobular region is usually consistent with metabolism of a hepatotoxin to reactive intermediates that produce direct toxic effects. Considerable evidence has also emerged that TGZ can be directly toxic to liver cells leading to suggestions that TGZ is "an idiosyncratic direct-acting hepatotoxin" (*3*). Here, I discuss the possible

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[1]Abbreviations: TGZ, troglitazone; P4503A4, cytochrome P4503A4; JNK, c-Jun N-terminal protein kinase; PST, phenol sulfotransferase; TZD, thiazolidinedione; NQO1, NAD(P)H:quinone oxidoreductase 1; PPAR, peroxisome proliferator-activated receptor; BSEP, bile salt export pump; NASH, nonalcoholic steatohepatitis.
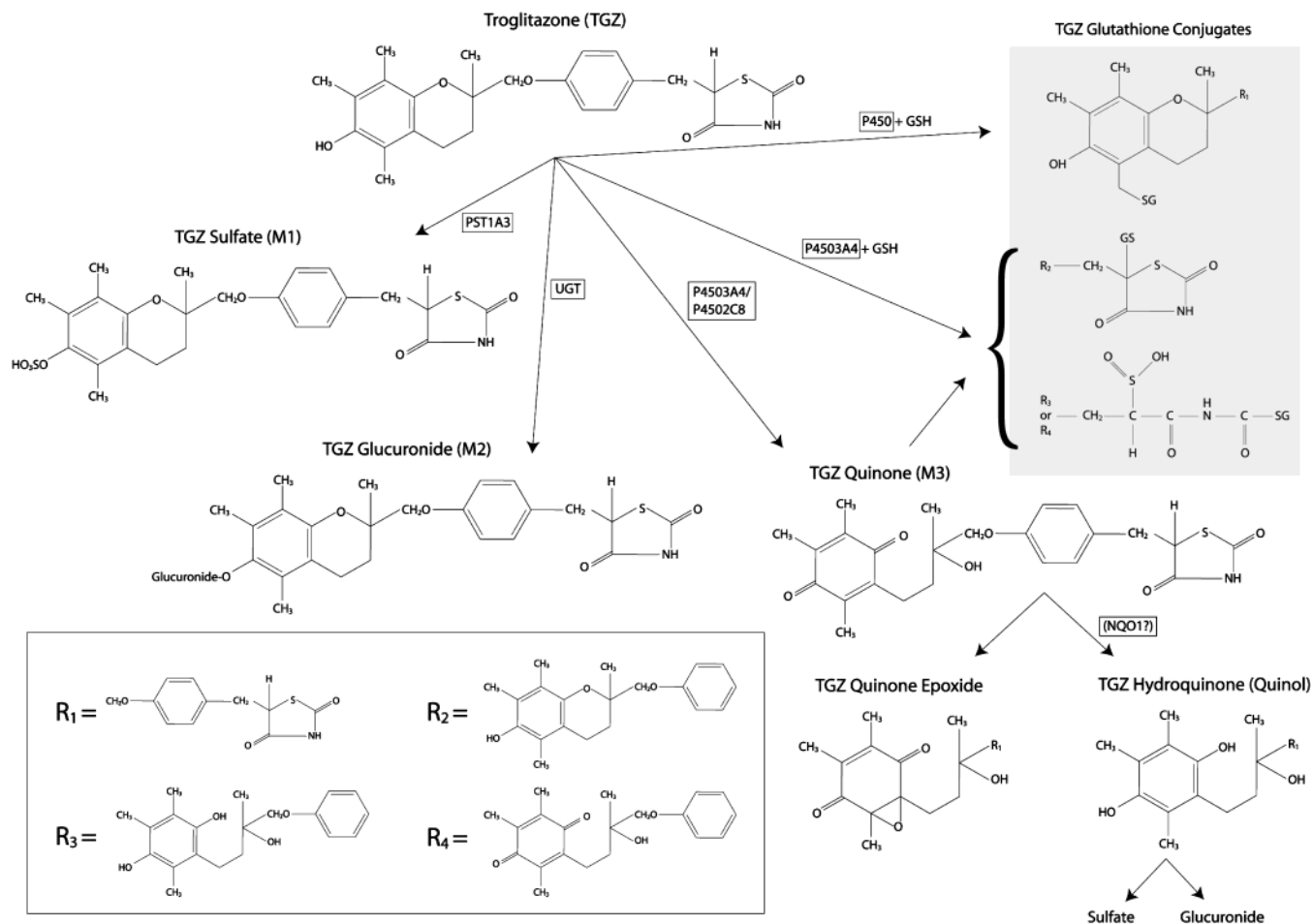
Figure 1. Known pathways in the metabolism of TGZ. Enzymes catalyzing the different pathways are shown in boxes: PST1A3; UGT, uridine diphosphate glucuronyl transferase; P450, cytochrome P450; and NQO1. Information from refs *12, 13, 15, 18,* and *19* was used to construct this figure.

reasons for this confusion and review the likely mechanisms by which TGZ can damage the liver. I conclude that TGZ most likely acts largely through the induction of apoptosis and that the initial stages of the disease process may be silent and difficult to establish in conventional animal models. This has important implications for the design of toxicological tests to predict drug-induced hepatotoxicity. Many questions also remain about why certain individuals were so susceptible to the toxic effects of TGZ. Consideration of TGZ's most likely mechanism of action provides clues as to the reasons underlying this susceptibility and the apparent idiosyncracy of it toxic effects.

2. Metabolism and Bioavailability of TGZ

TGZ, in the form of Rezulin, has a bioavailability of 40−50% with food and other factors affecting overall absorption (*4*). Once in the plasma, TGZ is more than 99% bound to albumin and its distribution into red blood cells is low (*4, 5*). However, it accumulates in the liver and adipose tissue and must readily enter hepatocytes, because effects are seen in isolated perfused livers and cultured hepatocytes within minutes even in the presence of albumin or serum (*6, 7*). Once in the heptocyte, TGZ is metabolized by a number of pathways (Figures 1 and 2). The major metabolite is TGZ-sulfate, called M1 (*5, 8*), that is formed via the action of PST. The PST

isoenzyme 1A3 has recently been identified as the primary form responsible for this metabolic pathway (*9*). TGZ-sulfate plasma concentrations greatly exceed those of the parent drug, and it undergoes enterohepatic circulation after biliary excretion such that it has a long half-life in the body (*5, 8*). TGZ may also be conjugated to a glucuronide metabolite, M2, via UDP-glucuronyl transferase (*10*), but it is relatively minor in comparison to the sulfate conjugate M1 (*4, 5*). M2 is also excreted in the bile and may also, through the action of gut flora glucuronyltransferases, undergo enterohepatic circulation. The extensive biliary excretion of TGZ means that most of it is excreted in the feces and little appears in the urine (*4, 5*).

TGZ was derived from a program to develop agents with antioxidant and antilipidemic properties, and the TGZ molecule has the chroman ring of vitamin E and a TZD ring (*11*). The chroman ring of vitamin E can be oxidized to a quinone, and another major metabolite of TGZ is TGZ-quinone, or metabolite M3 (*5, 12, 13*). This quinone metabolite is relatively stable but can be reduced to its diol or hydroquinone form and excreted as a conjugate (*13*) (Figure 1). As for vitamin E, this reduction could be catalyzed by NQO1 (*14*), but there is no direct proof of this in the literature. The formation of the quinone M3 has been shown to be mediated by two specific isoforms of cytochrome P450, namely, P4503A4
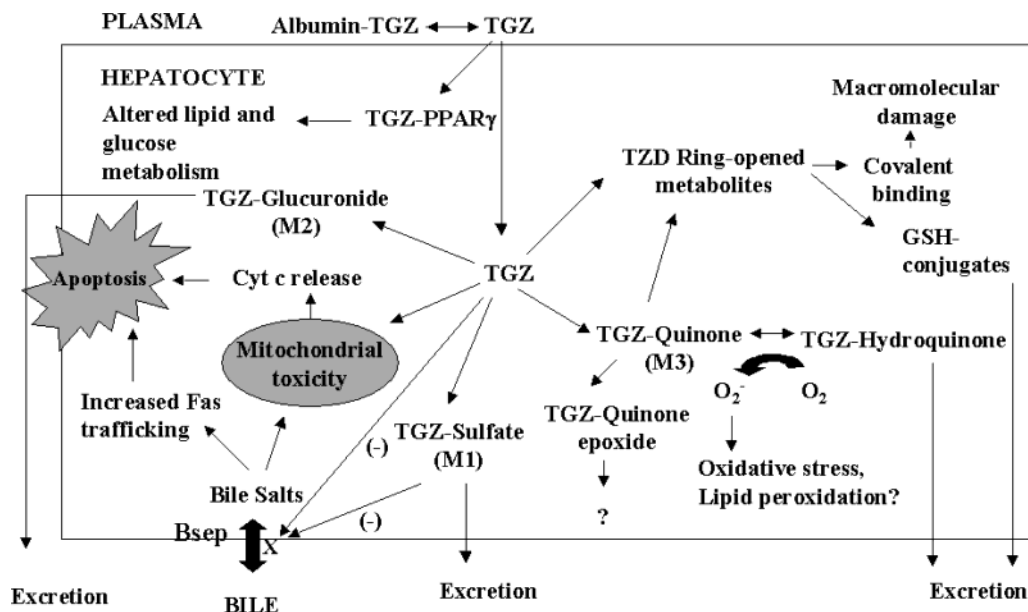
Figure 2. Metabolism and bioeffects of TGZ in the hepatocyte.

and CYP2C8 (*12*), and it is likely that in humans P4503A4 is primarily responsible for this reaction (*15*) (Figure 1). The reaction mechanism leading to quinone formation has been shown to be atypical of P4503A4 where a one electron oxidation mechanism produces a phenoxy radical, which combines with ferryl oxygen to form the quinone (*15*). Exposure to TGZ has also been shown to induce P4503A4 levels in liver cells, which would stimulate the formation of the quinone (*16, 17*).

The TZD ring can also undergo oxidative attack mediated by P4503A4 leading to ring opening and the formation of at least three reactive intermediates, most probably highly electrophilic α-ketoisocyanate and sulfenic acid intermediates, that can react with GSH (*18*) (Figures 1 and 2). Furthermore, although the TGZ-quinone does not react directly with GSH, it can be further metabolized to an o-quinone methide or undergo ring opening to produce two additional highly electrophilic intermediates (*18*) (Figures 1 and 2). This could result in bifunctional reactive intermediates, which could covalently bind to DNA or proteins and continue to redox cycle via the quinone moiety, thereby producing localized oxygen radical production (*18*). The TGZ quinone may also undergo epoxidation to form a quinone epoxide, which again is likely to be a potent electrophile (*19*) (Figures 1 and 2). Such electrophilic intermediates if formed in vivo would, in theory, deplete GSH and covalently bind to important cellular macromolecules such as DNA and protein potentially leading to toxicity and cancer.

3. Potential Mechanisms of Toxicity Involving the Formation of Electrophilic Reactive Intermediates

The findings that TGZ could form a number of reactive intermediates, including quinones and quinone methides, led investigators to hypothesize that TGZ hepatotoxicity was mediated by these intermediates through either a classic GSH depletion/covalent binding mechanism or via oxidative stress caused by redox cycling of the quinone. This would potentially explain the zone 3 centrilobular necrosis produced by TGZ in the liver as P450 activities are higher and GSH levels are lower in this zone than in

others (*20–22*). Quinones are also well-established cytotoxic agents and can produce toxicity by redox cycling with molecular oxygen to produce superoxide anion radical and subsequent oxidative stress (*23–25*). The formation of oxygen radicals has been shown to lead to GSH and protein oxidation and the peroxidation of membrane lipids. Thus, TGZ had the potential to act via classical mechanisms and be a simple, intrinsic hepatotoxin similar to acetaminophen (*26*).

However, several features of TGZ hepatotoxicity and the chemical nature of the quinone metabolite point to a limited role for metabolic activation and this classical mechanism of direct intrinsic hepatotoxicity. First, the quinone metabolite was shown not to be that cytotoxic to human and porcine hepatocytes, whereas the parent compound was quite cytotoxic (*13, 27*). Furthermore, TGZ has potent antioxidant properties, similar to vitamin E (*28*); thus, it was not clear how lipid peroxidation could play much of a role. However, like vitamin E, TGZ's antioxidant potential can be exhausted and lipid peroxidation is seen during TGZ cytotoxicity (*29*). The onset of TGZ hepatototoxicity is typically delayed and does not usually occur within a matter of a day or two as acetaminophen hepatotoxicity does. TGZ also is not particularly efficient at depleting GSH, but it does enhance the hepatocytotoxic effects of acetaminophen (*30*). Finally, it has been shown that high levels of P4503A4 actually protected hepatocytes from cytotoxicity; thus, metabolism/metabolic activation is protective in most circumstances (*31*). These findings show that while it is still possible that metabolic activation of TGZ plays a role in TGZ hepatotoxicity and perhaps cancer induction in some people, it cannot be the primary mechanism of hepatotoxicity in most patients succumbing to the toxic effects of Rezulin.

The "activation to a toxic quinone" hypothesis was attractive initially for another reason. It could potentially explain why TGZ appears to be more cytotoxic and hepatotoxic than other TZD drugs, such as rosiglitazone (Avandia) and pioglitazone (*32*), because TGZ has a chroman ring that can become a quinone whereas the other TZD drugs do not. However, because the TGZ

quinone does not appear to be that cytotoxic, at least to liver cells, this cannot be the explanation for this difference. Furthermore, all TZD drugs possess the TZD ring, and so, ring opening of this part of the molecule to reactive intermediates is unlikely to explain the higher toxicity of TGZ. However, TZD ring opening has not been demonstrated for other approved TZD-containing drugs (rosiglitazone and pioglitazone). Furthermore, rosiglitazone is usually prescribed at much lower doses than TGZ. Thus, the role of TZD ring scission in TGZ hepatotoxicity requires further evaluation.

4. Direct Mechanisms of Toxicity and the Potential Role of PPARγ

If metabolic activation to reactive intermediates is unlikely to be the primary mechanism of toxicity, other more direct mechanisms must be considered for TGZ itself or its conjugates. This shifts our focus from the right-hand side of Figure 2 to the left. TGZ and other TZD drugs are specific ligands for PPARs, especially PPARγ (*33–39*). PPARs are a family of nuclear receptors that regulate gene expression and belong to the nuclear hormone receptor superfamily of proteins (*40, 41*). There can be numerous downstream consequences of binding to PPARγ including altered lipid and glucose metabolism and changes in the expression of genes that control apoptosis, a form of cell suicide or programmed cell death (*40, 41*). Cell death occurring by apoptosis has several characteristic features, including blebbing of the cell surface, cell shrinkage, nuclear and DNA fragmentation, and removal of the dying cell by phagocytosis before it ruptures and releases it contents (*42, 43*). In this manner, it differs from the other form of cell death called necrosis, which is characterized by cell swelling and eventual rupture to release the cell contents. Necrosis can be monitored by blood tests, which detect the presence of enzymes released from the dying cells into the bloodstream, such as transaminases. Apoptosis, on the other hand, does not release proteins into the blood until the phagocytic process has been overwhelmed or a massive inflammatory response has been induced (*44, 45*). The initial stages of apoptotic injury in a tissue may therefore be "silent" both pathologically, because the apoptotic cells are removed, and clinically, because there will be no rise in serum transaminases and other proteins that are used as markers of tissue injury. However, recent evidence suggests that ongoing apoptotic injury in the liver can be a potent inflammatory signal (*45*). Thus, eventually, the apoptotic death will lead to a massive inflammatory response and the killing of surrounding cells releases proteins into the bloodstream.

TGZ has been shown to induce apoptosis in a wide variety of cells including both cancer cells and normal cells (*32, 33, 46, 47*). This may occur by at least two different pathways: the mitochondrial pathway involving cytochrome c release (Figure 2) or via TRAIL/tumor necrosis factor family death receptor signaling (*48*). Neither of these mechanisms is dependent on binding to PPARγ, and in normal human liver cells, the expression of PPARγ is very low (*49*); so, it seems unlikely that apoptosis induction by TGZ in hepatocytes is dependent on binding to PPARγ. Other TZD drugs are also better at binding PPARγ but are less effective at inducing apoptosis in cultured liver cells in vitro (*50*). Thus, non-PPARγ related mechanisms most probably account for the induction of apoptosis in hepatocytes by TGZ.

TGZ and other TZD drugs can, however, induce PPARγ levels selectively in the liver of diabetic murine models (*34, 51*). Furthermore, treatment of diabetic KKA mice with TGZ and rosiglitazone was shown to result in severe microvesicular periacinar steatosis (fatty liver), whereas lean control mice were unaffected (*34, 51*). Thus, under pathophysiological conditions, such as noninsulin-dependent diabetes, the liver may become sensitized to PPARγ activating drugs such as TGZ and severe steatosis may result. The possible consequences of severe and persistent hepatic steatosis are manifold and can include the build up of the drug in the lipid-laden hepatocytes, lipid peroxidation, and the subsequent development of fibrosis. Hence, while not inducing apoptosis by this mechanism, TGZ effects on PPARγ may have profound effects on the pathophysiology of the liver of diabetics and obese individuals. In combination with apoptosis induction by other mechanisms, exposure to TGZ could produce fulminant liver injury in these sensitized individuals.

5. Induction of Cytotoxicity in Liver Cells by TGZ and the Probable Role of Mitochondrial Injury

TGZ has been shown to induce cytotoxicity in hepatocytes from numerous species including humans. In 1999, Ramachandran et al. first reported that TGZ increased cytochrome P4503A activity in human hepatocytes at less than 5 μM but became toxic to the cells above 25 μM (*16*). Researchers at Parke-Davis reported similar findings a few months later in rat and human hepatocytes (*17*). They showed TGZ-induced P4503A activity and that its effects in rat and human hepatocytes were similar, leading the authors to suggest that the rat was a reasonable model for future studies with TGZ. Kostrubsky et al. then reported on the effects of TGZ in human and porcine hepatocytes (*27*). Porcine hepatocytes, from miniature pigs, were chosen because of their low sulfation capacity. Treatment of human hepatocytes for 2 h with $\geq$25 μM TGZ produced an irreversible inhibition of protein synthesis and cytotoxicity, whereas in porcine hepatocytes 100 μM was lethal. Thus, the porcine hepatocytes were actually more resistant to cytotoxicity than human liver cells, probably because of their abilty to rapidly convert TGZ to the nontoxic glucuronide conjugate. Inhibiting sulfation in the human hepatocytes increased the concentration of unmetabolized TGZ and enhanced toxicity, suggesting that formation of the sulfate and its biliary excretion is protective against hepatotoxicity. However, if the TGZ sulfate metabolite built up within the hepatocyte, it could also be toxic (see below). The authors suggested that "diabetic patients with a history of cholestasis may have a decreased capacity to excrete TGZ sulfate into the bile and therefore be at increased risk of developing hepatotoxicity" and that "cholestasis has been observed...in patients experiencing liver failure" after TGZ treatment (*27*). This is important because patients with various forms of hepatic insufficiency were shown to have a 3-fold increase in the half-life of TGZ sulfate and a decreased capacity to clear the parent drug and its metabolites (*38*). Such patients are also likely to be more susceptible to TGZ toxicity. Overall, the data point to the parent drug TGZ itself and/or TGZ sulfate as being the toxic agents responsible.

This idea of TGZ itself and/or TGZ sulfate being the ultimate toxicant is further supported by recent studies

in human hepatocytes from a large number of different individuals. In a study of hepatocytes from 27 different donors, Hewitt et al. (*31*) found that high P4503A4 activity with a predominance for glucuronidation conferred resistance to TGZ cytotoxicity, in agreement with the findings of Kostrubsky et al. (*27*), whereas low P4503A4 activity and extensive conversion to TGZ sulfate was associated with high sensitivity to the hepatotoxic effects of TGZ.

The most likely mechanism by which TGZ is toxic to hepatocytes is via effects on mitochondria producing the depletion of ATP and release of cytochrome c, which induces cell death via apoptosis (Figure 2). Exposure of human hepatocytes to TGZ has been shown to produce a rapid decline in mitochondrial transmembrane potential within 15 min and subsequent ATP depletion (*7*). Increased plasma membrane permeability and calcium ion influx were seen later. Similar effects have been observed in experiments with human hepatoma HepG2 cells, where concentration-dependent decreases in cellular ATP levels and mitochondrial membrane potential were observed and preincubation with the mitochondrial permeability transition inhibitor, cyclosporin A, provided complete protection against TGZ-induced cell death (*52, 53*). The decline in mitochondrial transmembrane potential seen in these experiments is called the mitochondrial permeability transition and causes pores to be opened in the mitochondrial membrane and cytochrome c to be released into the cytoplasm triggering apoptosis (*54*). The proapoptotic protein Bax is translocated to the mitochodrial membrane, and the whole process of cell death is set in motion (*55*). For TGZ-induced liver cell apoptosis, this process has recently been studied in detail by Bae and Song (*56*). They found a critical role for Bax and JNK activation. In HepG2 and Chang liver cells, TGZ but not rosiglitazone, induced apoptosis that was preceded by activation of JNK and p38 kinase and increased levels of Bax, Bad, release of cytochrome c, and cleavage of Bid in a time-dependent manner. Thus, TGZ appears to induce apoptosis in liver cells by the classic JNK-related cell death pathway (*56*). However, the exact nature of its interaction with the mitochondrial respiratory chain needs to be investigated further at the molecular level.

The depletion of the cells main source of chemical energy in the form of ATP is seen later in the hepatocytotoxicity of TGZ, followed by increased plasma membrane permeability and calcium ion influx (*7*). ATP is required for apoptosis, as this is an energy-consuming process, and if ATP is unavailable, then necrosis will result instead of apoptosis (*57, 58*). Thus, Lemasters and co-workers have shown that both apoptotic and necrotic cell death develop after death signals and toxic stresses and they have introduced the term "necrapoptosis" to emphasize the shared pathways leading to both forms of cell death. Chemicals that uncouple mitochondria can produce both forms of cell death depending on the availability of an alternative glycolytic supply of ATP independent of the mitochondrion. If some ATP is available, then they induce apoptosis, but if not necrosis is produced. Thus, TGZ could potentially induce apoptosis and necrosis depending on the availability of ATP in the liver.

TGZ sulfate may produce toxicity by an alternative mechanism that also results in damage to the mitochondria, namely, inhibition of bile salt excretion (*59, 60*), causing the accumulation of bile salts, which possess detergent properties and induce apoptosis through the Fas death receptor (see below).

6. Inhibition of the BSEP as Another Probable Mechanism of Hepatotoxicity

Another probable mechanism of hepatotoxicity for Rezulin is the inhibition of the BSEP by TGZ and/or TGZ sulfate causing the accumulation of toxic bile salts in the liver cells. Bile formation is an essential function of the liver and failure to produce bile is called cholestasis. BSEP plays a key role in removing bile salts from liver cells and uses energy in the form of ATP to pump bile salt molecules out of the hepatocyte (*61*). Retention of bile salts in the liver cells during cholestasis is associated with hepatocyte apoptosis (*62, 63*). Furthermore, studies of an inherited disorder called progressive familial intrahepatic cholestasis show that failure to secrete bile salts into bile results in liver injury, cirrhosis (fibrosis of the liver), and death from liver failure (*64*). This unfortunate human disease is the result of an inherited mutation in the gene encoding the BSEP protein and demonstrates the importance of BSEP in preventing the toxicity of bile salts in humans (*64*). Preininger and co-workers reported in 1999 that TGZ interfered with the hepatobiliary export of bile acids in isolated perfused rat livers (*6*). Upon addition of only 3.15 μM TGZ to the perfusion medium, a strong decline in bile flow by 67% within 1 h was observed. Funk and co-workers of Hoffmann-La Roche in Switzerland went on to show that this cholestatic effect was observed in an in vivo rat model and that both TGZ and TGZ-sulfate inhibited BSEP activity at IC$_{50}$ values of 3.9 and 0.4 μM, respectively (*59, 60*). Thus, marked inhibition of BSEP function is likely at concentrations of TGZ and TGZ-sulfate readily achievable in the plasma and liver of humans given therapeutic doses of TGZ. This inhibition of BSEP will more likely than not cause the build up of bile salts and the subsequent apoptotic death of liver cells in humans given TGZ.

The mechanisms by which bile salts induce apoptosis in liver cells have been established. High intracellular bile salt levels have been shown to induce cell death and mitochondrial dysfunction due to their intrinsic detergent properties (*65, 66*). Apoptosis may, therefore, be induced by the mitochondrial pathway. However, another apoptosis-inducing pathway involving the Fas death receptor appears to predominate (*67, 68*). Fas is one of the major death receptors expressed by hepatocytes, and liver cells from Fas deficient mice are resistant to bile salt-induced apoptosis (*69*). Bile salts promote the transport of cytoplasmic vesicular Fas to the cell surface and in this manner stimulate Fas aggregation at the cell surface, triggering the caspase cascade and subsequent apoptosis (*70*) (Figure 2).

Other drugs can induce liver injury through the inhition of BSEP. These include glyburide, rifampin, and bosentan (*71*). All produce cholestasis and subsequent liver injury. Interestingly, glyburide (INN, glibenclamide) is a hypoglycemic sulfonylurea drug and was coadministered with TGZ. Coadministration of cholestatic drugs with TGZ is likely to enhance its hepatotoxicity. Indeed, if one examines the case reports of TGZ-induced liver injury, it is quite common that the patients were being coadministered cholestatic drugs in addition to TGZ. For example, in 1998, Shibuya and colleagues described a 58

year old man with TGZ-induced fulminant hepatitis and noted that three of four patients with TGZ-induced fulminant hepatitis were also taking glyburide (*72*). Patient 2 in the Kohlroser et al. case report was also concomitantly taking glyburide with TGZ (*2*), as were the patients in the case reports by Booth et al. and Fukano et al. (*73, 74*).

The example of liver injury induced by bosentan is also informative. Bosentan produces similar bile salt increases in the serum of rats as it does in humans but does not produce liver injury, as evidenced by abnormal serum transaminase levels, in rats. Fattinger et al. conclude that "this species difference is most probably the result of the distinct composition of the bile salt pools in rats and humans"(*71*). In humans, a conjugated cytotoxic bile salt comprises 31% of the total pool but in rats the related conjugated cytotoxic bile salt comprises only 2.5% of the bile salt pool. Thus, inhibition of BSEP in humans will lead to a higher accumulation of cytotoxic bile salts than in rats. This could explain why bosentan and TGZ did not induce significant liver injury in rats during drug development and calls into question the use of conventional rat strains in the toxicological testing of drugs that have the potential to produce cholestatic liver injury.

7. Multiple Mechanisms and Varying Host Factors Lead to a Mixed Clinical Picture of Hepatotoxicity from TGZ

From the above, one can see that there are several probable mechanisms by which TGZ can induce hepatotoxicity. The literature to date suggests that TGZ is (i) a direct-acting hepatotoxin that produces necrosis of the liver; (ii) capable of killing liver cells, with apoptosis induction as one mechanism; (iii) able to cause mitochondrial injury and bile salt retention in liver cells, which has been shown to lead to apoptosis; (iv) potentially able to damage the liver silently because apoptosis does not cause elevation of liver enzymes in serum; (v) activated to metabolites that are capable of covalently binding to key cell macromolecules. Furthermore, it is likely that TGZ will have greater effects in diabetics, obese individuals, and other persons with impaired liver function, because there is a decreased ability to eliminate the drug, the liver cells have compromised mitochondrial function, there may be ongoing bile salt retention and steatosis, and/or there is an enhanced activity of the immune system, which enhances cell killing and fibrosis. Thus, host factors may explain the peculiar susceptibility of some individuals to TGZ.

These host factors are not simply genetic in nature, although genetic differences almost certainly play a role. In the case of TGZ, the following host factors more likely than not enhance the hepatotoxicity of this drug: (i) the presence of diabetes with ongoing cholestasis; (ii) the simultaneous ingestion of other cholestatic drugs; (iii) impaired liver function leading to low rates of drug metabolism and clearance; (iv) low P4503A4 activity or inhibition of this activity by other drugs or dietary factors; and (v) obesity/hypertriglyceridemia/insulin resistance syndrome leading to NASH.

Earlier, I described how diabetics with a history of cholestasis are especially at risk from TGZ because they have ongoing bile salt retention. Inhibition of BSEP by TGZ will enhance this further, causing bile salt-mediated apoptosis and hepatotoxicity. Similarly, coadministration

of cholestatic drugs, as discussed earlier, will result in excessive bile salt build-up and subsequent toxicity. The liver plays a central role in the metabolism of many drugs including TGZ. Liver dysfunction may not only reduce the plasma clearance of drugs eliminated by biotransformation and/or biliary excretion, but it can also affect plasma protein binding, which in turn could influence the processes of distribution and elimination (*75, 76*). A variety of medical conditions impair liver function and decrease drug metabolism and elimination, including alcohol-induced liver disease and NASH (*75, 77*). TGZ levels in the livers of patients with alcohol-induced liver disease and NASH will be higher than in normal individuals leading to higher rates of liver injury in these patients. NASH occurs in patients with the obesity/hypertriglyceridemia/insulin resistance syndrome or can be induced by chronic amidodarone, perhexiline, or diethyl aminoethoxyhestrol administration (*78*). Both lipid peroxidation and oxidative stress activate inflammatory cytokines and may contribute to the development of NASH (*78*); thus, TGZ can worsen this condition by also producing oxidative stress and subsequent inflammation. Furthermore, in alcohol-related liver disease and NASH, crystalline deposits are seen in liver mitochondria and the mitochondria are often swollen suggesting ongoing injury to these key organelles (*79*). These swollen and damaged mitochondria do in fact look very similar to those in TGZ-treated cells (*80*). Thus, it seems likely that TGZ would damage these already injured mitochondria to a greater extent and at lower doses than in individuals without NASH or alcohol-related liver injury.

When the liver suffers a direct toxic insult, the human immune system attempts to repair the damage. Macrophages infiltrate into the damaged areas, and an inflammatory process can result. In some cases, such repair is completed without serious adverse consequences. However, in other patients, the immune system response establishes the cellular foundation upon which a series of injurious developments can occur. This sequence typically involves apoptosis or necrosis (liver cell death), followed by fibrosis beginning at the site of the initial injury, and then continues to build upon the initial foundation. Further cell death and fibrosis continue in a cyclical fashion, and the damage can be so widespread that liver failure results. Again, the pathology reports of some patients suffering from TGZ-induced liver failure are consistent with this pattern in a number of published papers (*2, 81*).

8. Reasons for the Failure of Animal Models to Predict TGZ Hepatotoxicity and the Need for a More Specific Approach to Toxicity Testing

Preclinical toxicological testing of TGZ in rodents by the companies developing the drug showed no significant increases in serum transaminases. In mice, there was an increase in liver weight and in hepatocellular vacuolation in males given ≥400 mg/kg and females given ≥800 mg/kg but no overt necrosis (*82*). In rats, diffuse centrilobular necrosis of the liver was observed in both sexes at the high dose, but this was considered to be secondary to effects on the heart (*82*). The only other nonneoplastic effect observed in rat liver was an increase in hepatocellular hypertrophy in both sexes.

In studies at Pfizer, TGZ was given to groups of four cynomolgus monkeys at 300, 600, or 1200 mg/kg daily

by gavage for 52 weeks (*83*). Absolute and relative liver weights increased at all doses in both sexes by 40−71%. However, the only microscopic change attributable to TGZ treatment was minimal to mild bile duct hyperplasia in males at all doses and in females at 600 and 1200 mg/kg. The company scientists concluded that oral administration of TGZ to monkeys for a period of 1 year resulted in significant systemic exposure, with only minimal gastrointestinal, hematologic, and hepatic effects (*83*).

Thus, while some markers of liver injury were increased in the preclinical animal studies, no overt hepatic necrosis or liver failure was observed except in rats given cardiotoxic doses of TGZ. Yet, FDA scientists recently concluded that TGZ is a potent hepatotoxin in humans, conferring a substantially increased risk (up to 240-fold) of acute liver injury, including acute liver failure (*84*). The first factor that may contribute to this apparent discrepancy between the preclinical animal studies and the findings of potent hepatotoxicity in humans may be the failure to examine liver tissue of animals 30−90 days after drug withdrawal. In man, TGZ liver injury frequently progresses or worsens after drug discontinuation. In all animal species studied, hepatic weights increased consistent with liver hypertrophy. In cases of liver enlargement in animals, "After cessation of treatment, enlargement is slowly but fully reversible in 2−4 weeks. Studies have shown that liver regression is associated with massive liver cell death by apoptosis" (*85*). Therefore, a delayed die off of cells and secondary pathophysiologic changes after drug discontinuation would have been missed if liver histology 1−3 months after withdrawal was not obtained. Unfortunately, in conventional safety assessment, it is not standard practice to conduct histological examinations of animals weeks after discontinuation of treatment.

The other major factor that could contribute to this apparent discrepancy may be that the animals employed in the toxicity studies were physiologically normal whereas the humans given the drug were diabetic and usually obese. Indeed, as described earlier, when TGZ was given to diabetic KKAy mice, severe microvesicular periacinar statosis was observed in the animals' livers (*51*). The same effect was not observed in lean control mice given the same doses of TGZ. The authors of the study stated that "the possible consequences of such a severe and persistent hepatic steatosis (following TGZ treatment) can be severalfold. First, lipid-laden hepatocytes may serve as a reservoir of endogenous and exogenous lipophilic agents, including the drug itself. (This would cause the build-up of TGZ and TGZ sulfate in the hepatocytes such that cytotoxic concentrations could be achieved.) Second, under conditions of severe steatosis, lipid peroxidation invariably ensues. Finally, in humans, hepatic steatosis, especially when associated with obesity, is an important precursor to fibrosis. Thus, one can surmise that compound-induced severe hepatic steatosis may sensitize the liver to secondary pathophysiologic changes (*51*).

Recent studies in another mouse model of type 2 diabetes, the obese (NZO × NON)F1 model, have shown even greater hepatotoxicity after treatment with TGZ (*86*). Chronic feeding of TGZ (2 g/kg diet) produced marked hepatic steatosis and subsequent necrosis in this experimental model. Earlier studies employing obese−diabetic rodent models, such as the Zucker diabetic fatty rat, did not report hepatotoxicity, but these studies were focused on metabolic parameters rather than looking at toxic endpoints (*87*, *88*). The fact remains that the humans receiving TGZ treatment were a specific subgroup of the population, in this case obese diabetics. While it is mandated by the FDA that safety assessment of new drugs be conducted with normal, healthy animals, toxicologists should bear in mind the target population for the drug under development and should perhaps also consider utilizing appropriate animal models that reflect this population's unique characteristics. It may be that the failure to see elevated levels of hepatotoxicity in normal experimental strains of rodents and monkeys was due to the fact that these experimental models did not adequately reflect the human target population for the drug, which had a series of host factors making some of them susceptible to TGZ. It may also have been that they were not evaluated sufficiently after discontinuation of the drug such that secondary changes were missed. Thus, standard safety assessment practices may have failed us here and the toxicological community should consider further discussion of these practices.

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