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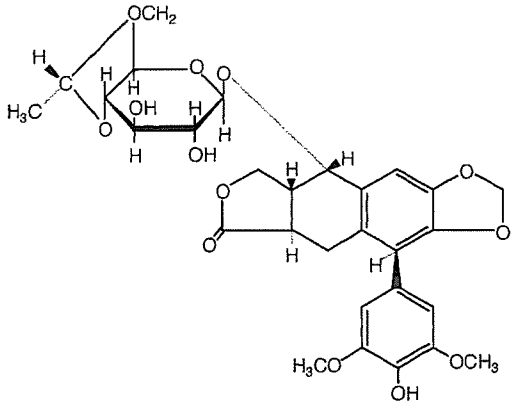

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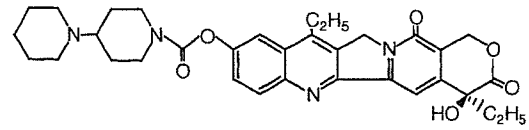

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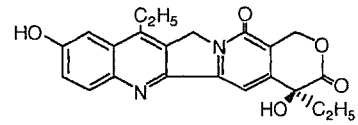
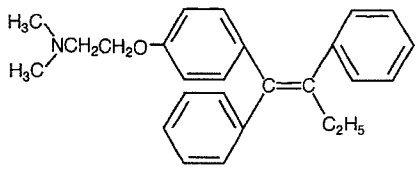


SN-38



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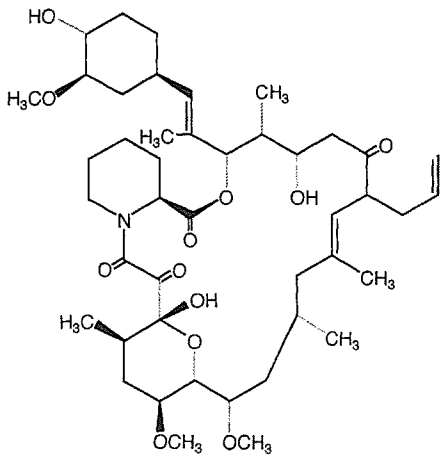
Immunosuppresant



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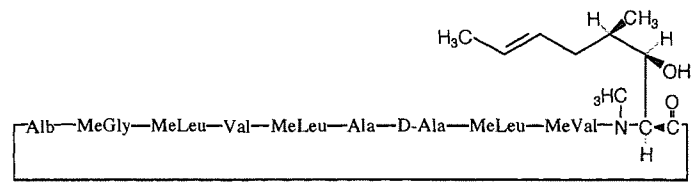

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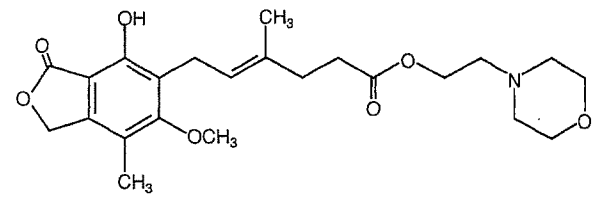
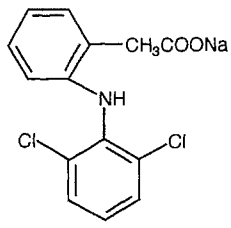


Mycophenolate

Analgetic drug



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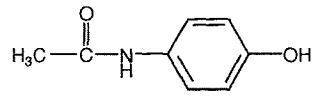

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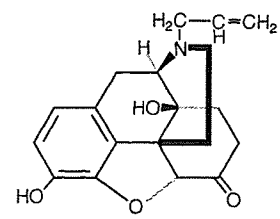

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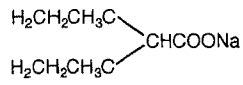
Anticonvulsant



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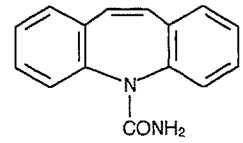
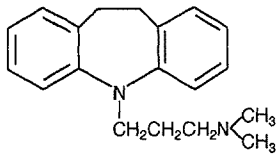
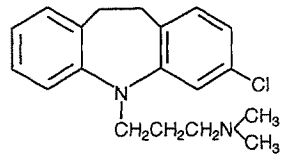


Carbamazepine

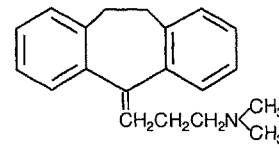
Tricyclic antidepressant



Imipramine



Clomipramine



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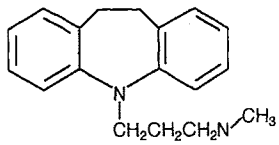
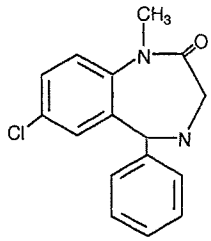


Desipramine

Benzodiazepine agonist



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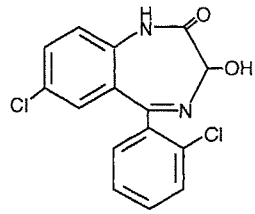

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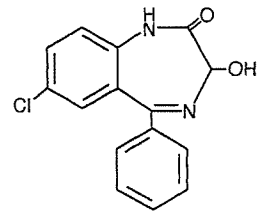
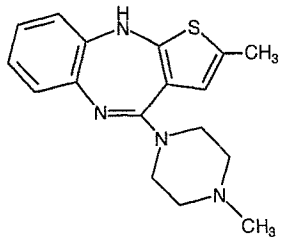
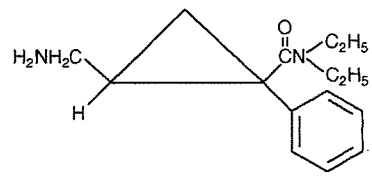


Oxazepam

Antipsychotic drug

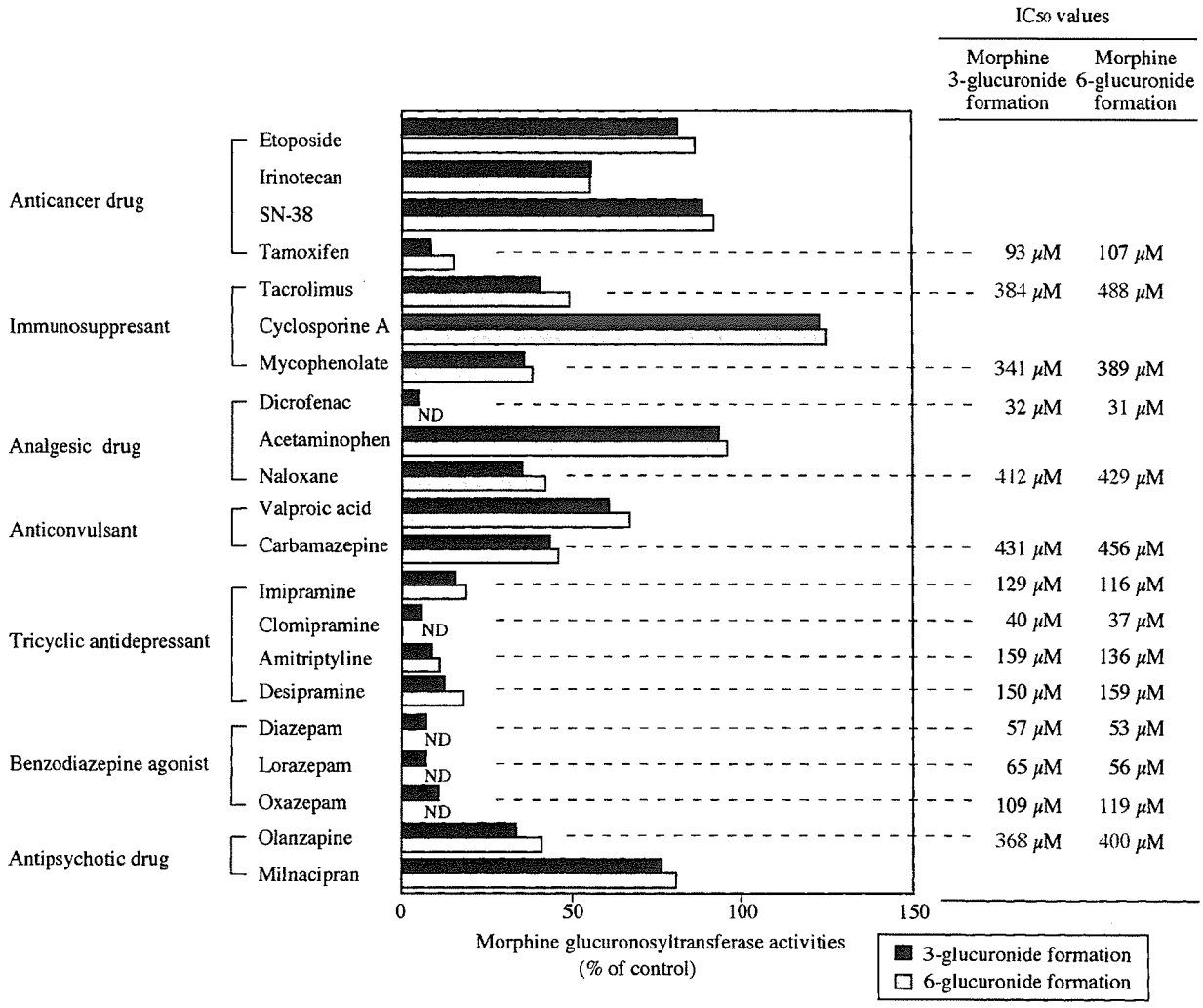


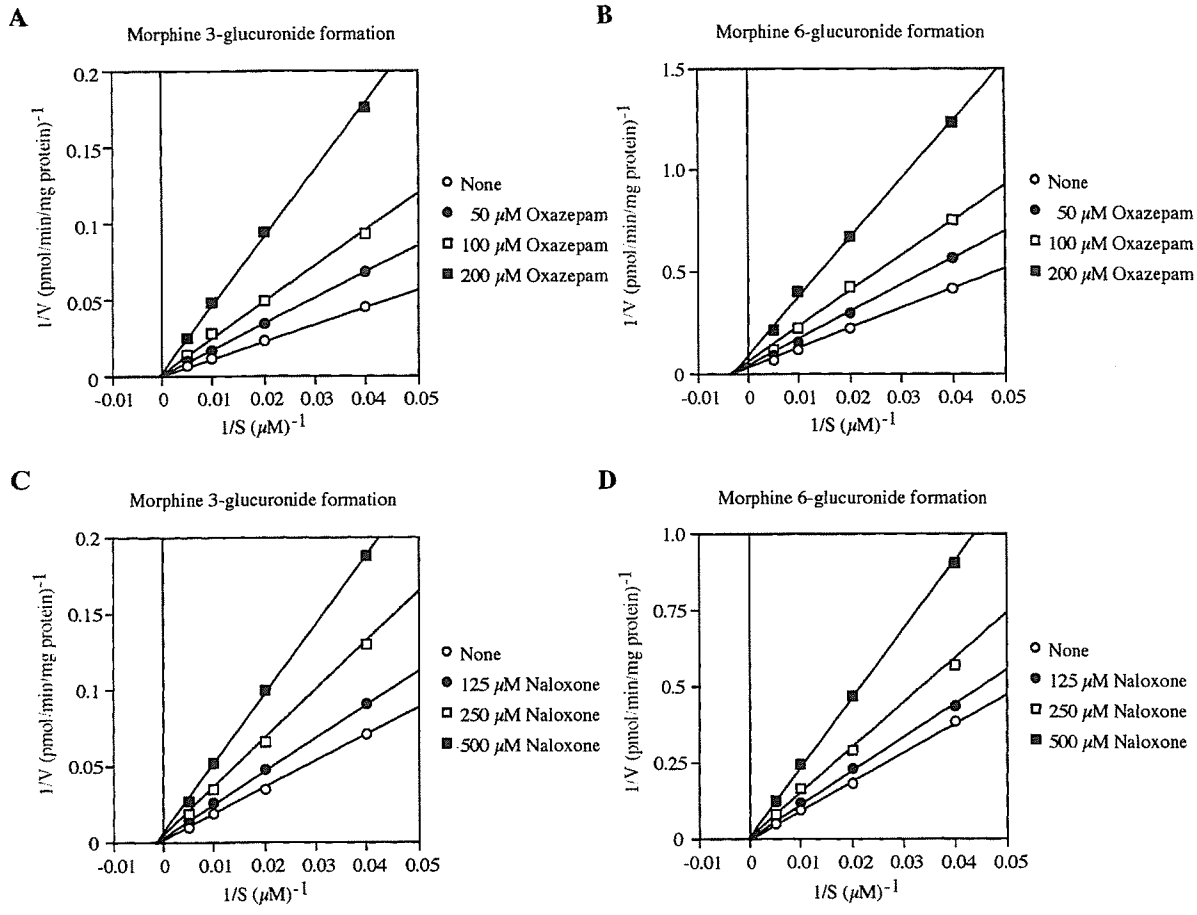
Olanzapine



Milnacipran

Fig. 2. Hara et al.

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1/S (μM)$^{-1}$
None
50 μM Oxazepam
100 μM Oxazepam
200 μM Oxazepam
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1/V (pmol/min/mg protein)$^{-1}$
1/S (μM)$^{-1}$
None
50 μM Oxazepam
100 μM Oxazepam
200 μM Oxazepam
C
Morphine 3-glucuronide formation
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1/S (μM)$^{-1}$
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D
Morphine 6-glucuronide formation
1/V (pmol/min/mg protein)$^{-1}$
1/S (μM)$^{-1}$
None
125 μM Naloxone
250 μM Naloxone
500 μM Naloxone

A Mechanistic View of Troglitazone Hepatotoxicity

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Contents

1. Introduction

Thiazolidinediones (Fig.1) are a class of oral antidiabetic agents and are the 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 U.S. Food and Drug Administration in 1997. Troglitazone lowers the 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., 1988, 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 severe toxic effects, troglitazone was thought likely to become a promising treatment for type II diabetes mellitus in patients with insulin resistance.

However, in the combined North American clinical trials, elevations of serum alanine aminotransferase more than three times the upper limit of normal were observed in 48 out of 2,510 patients (1.9%) treated with troglitazone. Liver biopsies from two patients was confirmed the hepatocellular nature of the injury as an idiosyncratic drug reaction (Watkins and Whitcomb, 1998). Meanwhile, troglitazone had been 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 USA and Japan in March 2000. The hepatotoxic effects of troglitazone were not predicted from conventional animal models (Watanabe et al., 1999) or in cynomolgus monkeys (300-1200 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 in the market, rosiglitazone and pioglitazone, have been introduced in 1999 and they are unlikely share the hepatotoxic effects of troglitazone (Freid et al., 2000; Isley and Oki, 2000; Lebovitz et al., 2002). It should also be noted that the clinical dosage regimen for improvement of fasting glucose is distinguishable among these thiazolidinediones (Table 1). The recommended dose for troglitazone was 200 to 600 mg/day, for rosiglitazone is 4 to 8 mg/day and for pioglitazone is 15 to 45 mg/day

(Hanefeld, 2001; Loi et al., 1999; PDR, 1999; PDR, 2005a, b). The dosage requirement for their efficacy might have reflected their hepatotoxic potential.

This review summarizes the molecular mechanism of troglitazone hepatotoxicity from the studies both *in vivo* and *in vitro*. Even though, there is no direct evidence indicated the precise mechanism of the toxicity so far. Many points of view, however, have been proposed to contribute to the toxic effects of troglitazone.

2. Potential of troglitazone metabolites for hepatotoxicity

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). M1 and M3 are the major metabolites in plasma, while M2 is a minor metabolite (Izumi et al., 1997a, b; Kawai et al., 1998; Loi et al., 1999). The main metabolite, troglitazone sulfate (M1), is formed by the action of phenol sulfotransferase, ST1A3 (Honma et al., 2002). It is account for about 70% of the metabolites detected in human plasma (Loi et al., 1999).

Differing from other thiazolidinediones, troglitazone contains a 6-hydroxy-5,7,8-trimethylchromane moiety (a chroman ring of vitamin E). 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 troglitazone quinone or metabolite M3 by cytochrome P450s (CYPs) 3A4 and 2C8 (Yamazaki et al., 1999). In human, it is likely that CYP3A4 is primarily responsible for this reaction (He et al., 2001). In addition, troglitazone has been shown to induce CYP3A in human and rat hepatocytes, which stimulates the formation of the quinone (Ramachandran et al., 1999; Sahi et al., 2000). By the action of CYP3A, troglitazone yields several reactive intermediates in rats (Kassahun et al., 2001; Tettey et al., 2001) (Fig. 2). *In vitro*, the formation of an epoxide of troglitazone quinone was also identified (Yamamoto et al., 2002). It is known that quinones represent a class of toxicological intermediates, which can result in acute cytotoxicity and immunotoxicity as well as carcinogenesis (Bolton et al., 2000). The maximum plasma concentrations in patients taking troglitazone at the dosage of 600 mg/day reached to only about 2.82 μg/ml or 6.3 μM (Loi et al., 1999). However, a study in rats demonstrated that the concentration

of troglitazone in liver tissues was 10-12 fold higher than that in the plasma (Sahi et al., 2000). Therefore, the troglitazone levels in human liver might allow the formation of these putative reactive intermediates and their accumulation may lead to the hepatotoxicity (see next topic).

A relatively minor metabolite, troglitazone glucuronide (M2), is catalyzed by UGT (Yoshigae et al., 2000). The glucuronidation of troglitazone in human intestine is 3-fold 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). A polar, partially β-glucuronidase-sensitive metabolite with retention properties similar to M2 was found in the profiling of urine samples (Loi et al., 1999). There has been no reported evidence that M2 is responsible for the hepatotoxic effects.

3. Susceptible genetic factors associated with troglitazone hepatotoxicity

As mentioned above, troglitazone can undergo metabolic biotransformation by CYP3A4 to form a quinone metabolite (M3) and epoxide specie (Izumi et al., 1997a, b; Kawai et al., 1998; Loi et al., 1999; Yamamoto et al., 2002). Quinones can react readily with sulfur nucleophiles such as glutathione (GSH) or cysteine residues on proteins (Bolton et al., 2000). 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 the GSH conjugation is formed 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 CYP3A (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 human. Among one hundred and ten patients prescribed troglitazone, 25 case patients had an abnormal increase in ALT or AST levels to at least 9 times or 5 times the upper limit of the normal range, respectively, while 85 control patients

showed no significant increase in the 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* (Watanabe et al., 2003). Hence, interindividual differences in the detoxification ability might contribute to the susceptibility and individual risk for troglitazone hepatotoxicity. However, the complete mechanism is still largely unknown.

4. Implications of canalicular bile salt export pump and drug transporters

The main metabolite of troglitazone, troglitazone sulfate, undergoes biliary excretion and accounts for up to 60% of the dose in rats (Kawai et al., 1997). In patients with hepatic impairment, troglitazone sulfate was found to accumulate about 4 fold in plasma with a 3-fold increased half-life (Ott et al., 1998). This metabolite also showed an inhibition effect on the canalicular bile salt export pump (Bsep) as well as drug transporters, suggesting it contributes to the liver toxicity.

Funk et al. (2001a, b) reported that 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 10 times more strongly than the parent compound (IC_{50} 3.9 μM). The inhibition of Bsep suggests it is one of the possible factors contributing to the hepatotoxicity since the subsequent accumulation of bile salts may lead to intrahepatic cholestasis in humans. Previously, cholestatic signs have also been described in a patient with troglitazone hepatotoxicity (Gitlin et al., 1998).

Another group of researchers reported that troglitazone sulfate is transported by organic anion transporting polypeptide (OATP) transporters with higher affinity to OATP-C than OATP8, and showed a strong inhibitory effect on estrone-3-sulfate transport by these transporters (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. Therefore, any factors that affect or impair the OATP-C levels or activity, for example genetic polymorphisms (Kreb, 2006), may cause an accumulation of M1, leading to troglitazone hepatotoxicity.

5. Hypersensitivity reaction in troglitazone hepatotoxicity

Idiosyncratic toxicity is generally considered to be host dependent, dose-independent, difficult to reproduce in experimental animals and relatively uncommon. Some idiosyncratic drug reactions are due to a metabolic abnormality of the host, but many have an immunological basis or result from immune-mediated hypersensitivity (Pohl et al., 1988). A well-characterized example is the idiosyncratic hepatitis induced by halothane. Sera of these patients contain autoantibodies directed against some trifluoroacetyl (TFA) - protein adducts including protein disulfide isomerase, microsomal carboxyesterase, calreticulin, ERp-72, GRP-78, GRP-94 (review in Gut et al., 1993), as well as CYP2E1 (Bourdi et al., 1996). Our recent report described that aldolase B, which is an enzyme predominantly localized in the liver and kidney (Penhoet et al., 1966; Rutter, 1964), was detected as an autoantigen that reacted with antibodies in the sera from two patients with type II diabetes mellitus with troglitazone-induced liver dysfunction (Maniratanachote et al., 2005b). The titer of anti-aldolase 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). At present, a definitive explanation for the occurrence of aldolase B autoantibodies, whether it is the cause or consequence of the progression of hepatotoxicity, is still lacking. 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; Rutter, 1964), may be one of the target proteins that interact with those reactive species and trigger the immune response. Further investigation may provide better understanding of this mechanism.

6. Molecular mechanism of troglitazone-induced liver toxicity

Troglitazone has been shown to induce apoptosis in various hepatic (Bae and Song, 2003; Tirmenstein et al., 2002; Yamamoto et al., 2001) and non-hepatic (Shiau et al,. 2005) cell types depending on the concentration and time of exposure. Unlike its pharmacological effects, the toxicity of troglitazone seems to be a PPARγ-independent mechanism and the higher affinity PPARγ agonists such as rosiglitazone 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 of PPARγ activation activity, was also able to induce apoptosis in cultured cells. As mentioned above, troglitazone can generate main metabolites M1 and M3 via the action of ST1 and CYP2C8/CYP3A4, respectively. In *in vivo* experiments in rats, M1 showed the potential to inhibit Bsep suggesting it is one of the factors contributing to cholestasis in humans (Funk et al., 2001a; b). In addition, the troglitazone quinone metabolite M3 has been suggested to be associated with troglitazone hepatotoxicity in humans (Neuschwander-Tetri et al., 1998). However, these metabolites showed fewer toxic effects compared to the parent compound, troglitazone, when mammalian hepatocytes and hepatoma cell lines were treated directly (Honma et al., 2002; Kostrubsky et al., 2000; Tettey et al., 2001; Tirmenstain et al., 2002; Yamamoto et al., 2001, 2002).

It is most likely that troglitazone causes hepatic cell death via apoptosis. Apoptosis is a normal physiologic form of cell death and plays a prominent role in liver pathogenesis such as autoimmune liver diseases, viral hepatitis, and drug-induced hepatitis. In this regard, we summarize below the molecular responses to troglitazone toxicity in the cells (Fig. 3).

6.1 MAPK-mediated cell death pathway

The three well-characterized subfamilies of 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 process 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) reported that calcium/calmodulin-dependent kinase II (CaMKII) is a critical upstream activator of p38 phosphorylation in GN4 cells. In addition, troglitazone also causes the induction of Bax, Bad, the cleavage of Bid and release of cytochrome c. However, troglitazone showed a negligible effect on Erk (Bae and Song, 2003; Gardner et al., 2003, 2005). JNK is characterized as a stress-activated protein kinase based on its activation in response to the inhibition of protein synthesis. We will provide the additional information supporting this point in a later section.

6.2 Impairment of mitochondrial functions

Another obvious mechanism of troglitazone-induced toxicity in liver cells is by causing a reduction of the mitochondrial membrane potential with a concomitant depletion of the cellular ATP concentration (Bova et al., 2005; Tirmenstein et al., 2002). Subsequently, it increases the plasma membrane permeability and calcium ion (Ca^{2+}) efflux. The result of these effects on mitochondria is the release of cytochrome c into the cytoplasm and activation of the caspases leading to apoptosis (Bova et al., 2005).

6.3 Induction of cell cycle arrest

Cyclin dependent kinases (CDKs) are serine-threonine protein kinases that regulate the 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 post-transcriptional modifications (Sherr and Roberts, 1999). Bae et al. (2003) reported that troglitazone-induced cell cycle arrest by this pathway and that apoptosis of hepatoma cell lines was caused an elevation of the levels of p53 and its downstream proteins, Gadd45, p21 and p27, as well as by a reduction in the levels of cyclin D1 and phospho-Rb.

7 Effect of troglitazone on the inhibition of protein translation

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 down regulated following the induction of apoptosis. The phosphorylation of eukaryotic initiation factor 2 α (eIF2 α) is important in the regulation of the 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 eukaryotic initiation factor 2 α (eIF2 α), translation inhibition and growth arrest (Fig.3) (Fan et al., 2004; Gardner et al., 2005; Palakurthi et al., 2001). A study using PPAR$\gamma^{-/-}$ and PPAR$\gamma^{+/+}$ mouse embryonic stem cells suggested that these effects were PPARγ-independent (Palakurthi et al., 2001).

It is known that the ER is a major cellular storage site of Ca^{2+} in the cell, and ER chaperones play important roles in Ca^{2+} accumulation and release. Any disturbance in the

ER homeostasis causes a release of Ca^{2+}, which in turn blocks ER protein processing, resulting 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 immunoglobulin heavy chain binding protein (BiP), an abundant chaperone protein in the ER, was overexpressed in hepatoma cell lines by troglitazone treatment (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 (Fig.3).

With respect to the inhibition of translation by troglitazone toxicity, we recently found that ribosomal protein P0 (P0) is also one of the targets of troglitazone cytotoxicity in HepG2 cells (Maniratanachote et al., in press). P0 is known as a phosphoprotein that functions in some processes of protein translation (Gonzalo et al., 2001). It was found that, rather than its overexpression, dephosphorylation of P0 occurred in troglitazone-induced cytotoxicity (Maniratanachote et al., in press). Therefore, dephosphorylation of P0 may play a role in the regulation of protein translation in response to the toxic effects of troglitazone (Fig. 3).

8 Conclusions and perspectives

Troglitazone is a drug that can cause idiosyncratic hepatotoxicity in human. This kind of toxicity is usually unpredictable, pharmacologically independent, rare, and not reproducible in experimental animal models, which makes it difficult to study (Lee, 2003). A number of toxicological tests, both *in vivo* and *in vitro*, have been performed. So far, no direct mechanism has been found that can explain why troglitazone hepatotoxicity occurred in only some individuals. The failure to develop such toxicity in animal models is still inexplicable. However, we have learned from previous reports that the mechanism of troglitazone hepatotoxicity is PPARγ-independent, the molecular mechanisms of apoptotic cell death are most likely involved in the hepatotoxicity, and the idiosyncratic hepatotoxicity might be a consequence of a genetic basis in susceptible individuals.

Recent findings concerning the miRNA functions in specific tissues has enabled better understanding of the molecular mechanisms of various pathologies and diseases (review in Bartel, 2004). Among several hundreds miRNAs, miR-122 is the most abundant and liver-specific (Lagos-Quintana et al., 2002; Baskerville and Bartel, 2005). It has been shown to have various roles, for example, in hepatitis C viral infection (Jopling et al., 2005) and in lipid metabolism (Esau et al., 2006). Therefore, studies on miRNAs and their targets might reveal clues concerning troglitazone idiosyncratic hepatotoxicity.

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