In Vitro Investigation of the Glutathione Transferase M1 and T1 Null Genotypes as Risk Factors for Troglitazone-Induced Liver Injury

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ABSTRACT:
The double null mutation of glutathione transferase, GSTM1 and GSTT1, is reported to influence troglitazone-associated abnormal increases of alanine aminotransferase and aspartate aminotransferase. However, no nonclinical data with a bearing on the clinical outcomes and underlying mechanisms have hitherto been reported. To investigate whether deficiency in GSTM1 and/or GSTT1 is related to troglitazone hepatotoxicity in vitro, the covalent binding level (CBL) (an index of reactive metabolite formation) and cytotoxicity of troglitazone and rosiglitazone, another thiazolidinedione but with low hepatotoxicity, were examined using human liver samples phenotyped for cytochrome P450s and genotyped for GSTM1 and GSTT1. Despite addition of GSH, CBLs of troglitazone and rosiglitazone in human liver microsomes were correlated with CYP3A (or CYP2C8) and CYP2C8 activities, respectively. With addition of recombinant GSTM1, the microsomal CBLs of troglitazone and rosiglitazone decreased. However, the CBLs of troglitazone in GSTM1/GSTT1 wild-type hepatocytes were unexpectedly higher than those in null hepatocytes. Although this discrepancy has not been fully explained, the GSTM1 and GSTT1 null mutations increased the cytotoxicity of troglitazone, independent of CYP3A or CYP2C8 activities. Furthermore, a GSH adduct of troglitazone, M2, limited to GSTM1 wild-type hepatocytes was detected. Of clear interest, GSTM1 and/or GSTT1 null mutation-dependent cytokinetics and higher exposure to the reactive metabolite trapped as M2 as for troglitazone were not observed for rosiglitazone. This result might at least partly explain the findings related to clinical hepatotoxicity, suggesting that measurement of GSH adducts or cytotoxicity using GSTM1- and GSTT1-genotyped hepatocytes might offer an important in vitro system to assist in better prediction of idiosyncratic hepatotoxicity.
ALT and AST after administration of troglitazone on retrospective analysis using clinical samples (Watanabe et al., 2003). However, to our knowledge, no noncl inical data to link with the clinical outcomes and provide more detailed clues to mechanisms have hitherto been reported.

The purpose of the present study was to investigate whether GSTM1 and/or GSTT1 defects are involved in troglitazone hepatotoxicity in vitro. It is clear that metabolic activation of a drug to reactive metabolites and subsequent covalent binding to target macromolecules might be a necessary first step in the generation of idiosyncratic drug reactions in many cases (Walgren et al., 2005). We have already reported that covalent binding is one risk factor for DILI (Usui et al., 2009). Therefore, the covalent binding levels (CBLs) (an index of reactive metabolite formation) of troglitazone and rosiglitazone (as a negative control) were investigated using human in vitro liver samples with a diversity of P450 phenotypes or GST genotypes. Furthermore, cytotoxicity was also investigated to cast light on the idiosyncrasy of troglitazone hepatotoxicity.

Materials and Methods

Materials. [14C]Troglitazone and [14C]rosiglitazone were synthesized in-house. Unlabeled troglitazone and rosiglitazone were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Pooled human liver microsomes (mixed-gender pool of 50 individuals) and 16 individual human liver microsome samples (Reaction Phenotyping Kit, version 7) were obtained from XenoTech, LLC. The activities of major drug-metabolizing enzymes in microsomes and hepatocytes had been measured by the suppliers. Use of human samples in this study was approved by the ethics committee of the Drug Research Division, Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). Recombinant human (rh) GSTA1 and rhGSTM1, used in Fig. 2 (cytosol isolated from Escherichia coli-expressing human GSTA1 and GSTM1, respectively), and control cytosol (isolated from E. coli host strain) were purchased from Cypex Ltd. (Dundee, UK). rhGSTA1, rhGSTM1, and rhGSTP1, used in Supplemental Fig. 2, were purchased from PanVera Corp. (Madison, WI). To prepare the negative control of PanVera rhGST, rhGSTM1 was heat-inactivated at 90°C for 2 min. NADPH and reduced GSH were from Oriental Yeast Co., Ltd. (Tokyo, Japan), and Nacalai Tesque, Inc. (Kyoto, Japan), respectively. All other reagents and solvents were of the highest grade commercially available.

Incubation Using Human Liver Microsomes. In microsomal assays, radiolabeled troglitazone and rosiglitazone (final concentration 10 μM) were incubated with 1 mg/ml pooled human liver microsomes or 16 individual human liver microsome samples phenotyped for P450 activities in the presence of 1 mM NADPH and 1 mM GSH at 37°C for 1 h in 500 μl of a reaction mixture consisting of 50 mM phosphate buffer (pH 7.4), rhGSTA1, rhGSTM1, rhGSTP1, or control cytosol (0.4 mg/ml) as added in an incubation with pooled human liver microsomes and with control cytosol (isolated from E. coli host strain, left bars), rhGSTA1 (recombinant human GSTA1 expressed in E. coli, middle bars), or rhGSTM1 (right bars) in the presence of 1 mM NADPH and 1 mM GSH at 37°C for 1 h followed by determination of CBLs. The eluates were analyzed by radio-HPLC, and the amounts of M1 (the main GSH adduct of troglitazone in this assay) are shown (●). No appreciable GSH adducts of rosiglitazone (10 μM) were incubated with cryopreserved human hepatocytes (1 × 10⁶ cells/ml) genotyped for GSTM1 and GSTT1 at 37°C for 8 h under an atmosphere of 

![Fig. 1. Structures of troglitazone, rosiglitazone, and two postulated troglitazone GSH adducts. –SG, glutathione.](image)

![Fig. 2. Effect of rhGSTA1 or rhGSTM1 on CBLs and a GSH adduct in human liver microsomes. Radiolabeled compounds (10 μM) were incubated with pooled human liver microsomes and with control cytosol (isolated from E. coli host strain, left bars), rhGSTA1 (recombinant human GSTA1 expressed in E. coli, middle bars), or rhGSTM1 (right bars) in the presence of 1 mM NADPH and 1 mM GSH at 37°C for 1 h followed by determination of CBLs. The eluates were analyzed by radio-HPLC, and the amounts of M1 (the main GSH adduct of troglitazone in this assay) are shown (●). No appreciable GSH adducts of rosiglitazone were found with radio-HPLC. The CBL and GSH adduct data are the means ± S.D. from three assays. **, p < 0.01; ***, p < 0.001, significantly different from control cytosol.](image)
95% air- 5% CO₂ in 300-μl suspensions of hepatocytes in incubation medium (XenoTech, LLC).

**Measurement of CBL.** CBL was measured according to the method we reported previously (Usui et al., 2009). Reactions of microsomes and hepatocytes were stopped by addition of ice-cold methanol. For measurement of radioactivity bound to proteins, the reaction mixtures after precipitation were loaded onto glass fiber filters and washed with 80% (v/v) methanol and acetonitrile to remove unbound radioactivity. The first filtrate was used for radio-high-performance liquid chromatography (HPLC) analysis or liquid chromatography (LC)-tandem mass spectrometry (MS/MS) analysis of metabolites unbound to proteins as described below. The buffer was transferred to a scintillation vial with 10% SDS and incubated overnight at 55°C to dissolve proteins. The radioactivity and protein concentration were then measured. The CBL was calculated from the following equation:

Radioactivity in the protein solution (dpm/ml) × Protein concentration in the protein solution (mg/ml)

**Radio-HPLC and LC-MS/MS Analyses.** A first filtrate from the glass fiber filters was collected and evaporated to dryness, and the residue was dissolved in the mobile phase and loaded onto an Inertsil ODS-3 column (3-μm, 2.1 i.d. × 150 mm; GL Science, Inc., Tokyo, Japan) with a column temperature of 40°C. The LC system consisted of an Agilent 1200 (Agilent Technologies, Santa Clara, CA) pump set at a flow rate of 0.25 ml/min. The mobile phase to detect GSH adducts of troglitazone consisted of a linear gradient of solvent A (0.1% formic acid) and solvent B (acetonitrile) according to the following program: 20% B (0 min) to 30% B (5 min) to 35% B (26 min) to 100% B (30 min). The mobile phase to investigate metabolite profiling of troglitazone consisted of a linear gradient of solvent A (0.1% formic acid) and solvent B (acetonitrile) according to the following program: 20% B (0 min) to 30% B (5 min) to 35% B (35 min) to 40% B (55 min) to 100% B (65 min). The mobile phase to investigate metabolite profiling of rosiglitazone consisted of a linear gradient of solvent A (10 mM ammonium acetate) and solvent B (acetonitrile) according to the following program: 5% B (0 min) to 35% B (20 min) to 60% B (35 min) to 100% B (40 min). Radioactivity was detected with a flow scintillation detector (Radiomatic 610TR; PerkinElmer Life and Analytical Sciences, Waltham, MA), using Ultima Flo-M scintillation cocktail (Radiomatic 610TR; PerkinElmer Life and Analytical Sciences). Mass analysis was conducted on a 4000 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA) equipped with an electrospray ion source for detection of GSH adducts of troglitazone, M1 and M2 (Fig. 1). Ionization parameters included an ion spray voltage of −4100 V and source temperature of 200°C. M1 and M2 were identified by means of multiple reaction monitoring (MRM) in the negative ion mode [MRM transitions: M1, [M − H]⁻ (deprotonated molecule) = 745 → 272; M2, [M − H]⁻ = 779 → 272]. MRM parameters of M1 and M2 included a declustering potential of −60 V and collision energy of −35 V. Molecular masses of other metabolites of troglitazone and rosiglitazone were determined as described in our previous article (Usui et al., 2009).

**Measurement of ATP Levels in Hepatocytes.** Troglitazone and rosiglitazone were incubated with cryopreserved human hepatocytes (1 × 10⁵ cells/ml) genotyped for GSTM1 and GSTT1 at 37°C for 2 h under an atmosphere of 95% air-5% CO₂ in 100-μl suspensions of hepatocytes in incubation medium (XenoTech, LLC). ATP levels were measured using a CellTiter-Glo Luminescent cell viability kit from Promega (Madison, WI). This assay generates luminescent signals by luciferase reactions that are proportional to the amount of ATP present.

**Statistical Analysis.** All statistical analyses were performed using SAS Enterprise Guide 4.1 (SAS Institute, Cary, NC). Correlations between CBLs or unchanged drug amounts and specific P450 activities in human liver microsomes or hepatocytes were analyzed using linear regression analysis. The two-way analyses of variance were used to test the effect of the GSTM1 genotype and GSTT1 genotype on CBLs or on remaining unchanged drug amounts. Student’s t test was used to compare the cytotoxicity of troglitazone and rosiglitazone between GSTM1/GSTT1 null and wild-type hepatocytes and to compare the CBL or M1 amounts with addition of rhGSTs and control cytosol.

**Results**

**Correlation between CBLs and P450 Activities of Human Liver Microsomes from Individuals.** CBLs of troglitazone and rosiglitazone with 16 individual human liver microsomes phenotyped for enzymatic activities of P450s were investigated in the presence of 1 mM GSH. The average absolute values were 297 and 435 pmol/mg protein, respectively. Despite addition of GSH as a scavenger, individual microsomes showed large variation in CBLs. Compared with the individual CBLs of troglitazone (152–529 pmol/mg), those of rosiglitazone (141–1013 pmol/mg) varied more widely. The coefficient of variation of CBL was 38% for troglitazone and 46% for rosiglitazone. Correlations between CBLs and P450 activities are shown in Table 1. CBLs of troglitazone were significantly and positively correlated with CYP3A activities (r = 0.89, p < 0.001, testosterone 6β-hydroxylation; r = 0.83, p < 0.001, midazolam 1'-hydroxylation). Furthermore, CBLs of troglitazone were also positively correlated with CYP2C8 activities (r = 0.60, p = 0.01). Meanwhile, CBLs of rosiglitazone were significantly and positively correlated with CYP2C8 activities (r = 0.65, p = 0.005).

**Correlation between CBLs and P450 activities of human liver microsomes from 16 individuals**

<table>
<thead>
<tr>
<th>P450 Activities</th>
<th>CBLs of Troglitazone</th>
<th>CBLs of Rosiglitazone</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2 (7-ethoxyresorufin O-dealkylation)</td>
<td>0.08 (0.76)</td>
<td>0.09 (0.73)</td>
</tr>
<tr>
<td>CYP1A2 (phenacetin O-deethylation)</td>
<td>0.27 (0.30)</td>
<td>0.10 (0.69)</td>
</tr>
<tr>
<td>CYP2A6 (coumaran 7-hydroxylation)</td>
<td>0.22 (0.40)</td>
<td>0.0002 (0.99)</td>
</tr>
<tr>
<td>CYP2B6 (S-mephenytoin N-demethylation)</td>
<td>0.51 (0.05)</td>
<td>0.18 (0.50)</td>
</tr>
<tr>
<td>CYP2B6 (bupropion hydroxylation)</td>
<td>0.31 (0.23)</td>
<td>0.08 (0.76)</td>
</tr>
<tr>
<td>CYP2C8 (paclitaxel 6α-hydroxylation)</td>
<td>0.60 (0.01)*</td>
<td>0.65 (0.005)**</td>
</tr>
<tr>
<td>CYP2C9 (diclefenac 4'-hydroxylation)</td>
<td>0.05 (0.86)</td>
<td>0.11 (0.67)</td>
</tr>
<tr>
<td>CYP2C19 (S-mephenytoin 4'-hydroxylation)</td>
<td>0.16 (0.55)</td>
<td>−0.05 (0.85)</td>
</tr>
<tr>
<td>CYP2D6 (dextromethorphan O-demethylation)</td>
<td>0.37 (0.14)</td>
<td>0.06 (0.82)</td>
</tr>
<tr>
<td>CYP2E1 (chlorozoxazone 6-hydroxylation)</td>
<td>−0.02 (0.94)</td>
<td>0.04 (0.87)</td>
</tr>
<tr>
<td>CYP3A4/5 (testosterone 6β-hydroxylation)</td>
<td>0.89 (&lt;0.001)**</td>
<td>0.24 (0.36)</td>
</tr>
<tr>
<td>CYP3A4/5 (midazolam 1'-hydroxylation)</td>
<td>0.83 (&lt;0.001)**</td>
<td>0.21 (0.43)</td>
</tr>
<tr>
<td>CYP4A11 (laурic acid 12-hydroxylation)</td>
<td>0.07 (0.80)</td>
<td>0.59 (0.13)</td>
</tr>
</tbody>
</table>

* Significant at p < 0.05.
** Significant at p < 0.01.
*** Significant at p < 0.001.
Effects of rhGSTA1 or rhGSTM1 on CBLs in Human Liver Microsomes. The scavenging effects of rhGSTA1 or rhGSTM1 (from Cypex Ltd.) on CBLs of troglitazone and rosiglitazone mediated by pooled human liver microsomes were investigated in the presence of 1 mM GSH (Fig. 2). With addition of rhGSTA1 or rhGSTM1, microsomal CBLs of troglitazone and rosiglitazone were significantly decreased compared with those after addition of control cytosol. Furthermore, the GSH adduct of a troglitazone-reactive metabolite reported as 5-glutathionyl-thiazolidine-2,4-dione (M1, Fig. 1) in a previous publication (He et al., 2004) was increased by addition of rhGSTA1 or rhGSTM1 in radio-HPLC analysis (A representative radiochromatogram of troglitazone is shown in Supplemental Fig. IA.) rhGSTA1 and rhGSTM1 did not affect the decrease in unchanged troglitazone in radio-HPLC analysis (data not shown). Meanwhile, no appreciable GSH adducts of rosiglitazone were found in radio-HPLC.

Correlation between CBLs and GSTM1/GSTT1 Genotypes in Individual Human Hepatocytes. Fifty-nine individual human hepatocytes were genotyped for GSTM1 and GSTT1 null mutations. Three each were selected for each of the four genotypes of GSTM1/GSTT1 (wild/wild, wild/null, null/wild, and null/null) (total 12 lots). With use of the genotyped 12 individual human hepatocytes, CBLs of troglitazone and rosiglitazone were investigated by radio-HPLC analysis from filtrates after an 8-h incubation with 12 individual human hepatocytes. Representative radiochromatograms of troglitazone and rosiglitazone are shown in Supplemental Fig. 1A. The scavenging effects of rhGSTA1 or rhGSTM1 (from Cypex Ltd.) on CBLs of troglitazone and rosiglitazone mediated by pooled human liver microsomes were investigated in the presence of 1 mM GSH (Fig. 2). With addition of rhGSTA1 or rhGSTM1, microsomal CBLs of troglitazone and rosiglitazone were significantly decreased compared with those after addition of control cytosol. Furthermore, the GSH adduct of a troglitazone-reactive metabolite reported as 5-glutathionyl-thiazolidine-2,4-dione (M1, Fig. 1) in a previous publication (He et al., 2004) was increased by addition of rhGSTA1 or rhGSTM1 in radio-HPLC analysis (A representative radiochromatogram of troglitazone is shown in Supplemental Fig. IA.) rhGSTA1 and rhGSTM1 did not affect the decrease in unchanged troglitazone in radio-HPLC analysis (data not shown). Meanwhile, no appreciable GSH adducts of rosiglitazone were found in radio-HPLC.

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TABLE 2
Characterization of individual hepatocytes for drug-metabolizing enzymes in the CBL determination assay (lots A–L) and the cytotoxicity assay (lots A, B, J, and K).

<table>
<thead>
<tr>
<th>Lot</th>
<th>GSTM1/GSTT1 Genotypes</th>
<th>CYP3A</th>
<th>CYP2C8</th>
<th>SULT</th>
<th>UGT</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Wild/wild</td>
<td>152</td>
<td>24</td>
<td>31</td>
<td>129</td>
</tr>
<tr>
<td>B</td>
<td>Wild/wild</td>
<td>100</td>
<td>33</td>
<td>9</td>
<td>109</td>
</tr>
<tr>
<td>C</td>
<td>Wild/wild</td>
<td>223</td>
<td>20</td>
<td>16</td>
<td>432</td>
</tr>
<tr>
<td>D</td>
<td>Wild/null</td>
<td>102</td>
<td>50</td>
<td>9</td>
<td>108</td>
</tr>
<tr>
<td>E</td>
<td>Wild/null</td>
<td>94</td>
<td>16</td>
<td>110</td>
<td>415</td>
</tr>
<tr>
<td>F</td>
<td>Wild/null</td>
<td>99</td>
<td>8</td>
<td>29</td>
<td>444</td>
</tr>
<tr>
<td>G</td>
<td>Null/wild</td>
<td>197</td>
<td>24</td>
<td>33</td>
<td>540</td>
</tr>
<tr>
<td>H</td>
<td>Null/wild</td>
<td>259</td>
<td>31</td>
<td>34</td>
<td>508</td>
</tr>
<tr>
<td>I</td>
<td>Null/wild</td>
<td>85</td>
<td>46</td>
<td>8</td>
<td>86</td>
</tr>
<tr>
<td>J</td>
<td>Null/null</td>
<td>174</td>
<td>24</td>
<td>42</td>
<td>366</td>
</tr>
<tr>
<td>K</td>
<td>Null/null</td>
<td>82</td>
<td>15</td>
<td>14</td>
<td>118</td>
</tr>
<tr>
<td>L</td>
<td>Null/null</td>
<td>188</td>
<td>No data</td>
<td>No data</td>
<td>No data</td>
</tr>
</tbody>
</table>

Activities of CYP3A (formation rate of 6β-hydroxylated testosterone), CYP2C8 (4’-methyldihydroxylation of tolbutamide), SULT (7-hydroxycoumarine sulfate), and UGT (7-hydroxycoumarine glucuronide) were measured by the suppliers.

Fig. 3. Comparison of CBLs in GSTM1/GSTT1 null and wild-type hepatocytes. Radio-labeled troglitazone (A and C) and rosiglitazone (B and D) (10 μM) were incubated with 12 cryopreserved human hepatocytes genotyped for GSTM1 and GSTT1 null mutations and phenotyped for P450 activity at 37°C for 8 h followed by determination of CBLs. Two-way analysis of variance results for GSTM1 genotype and for GSTT1 genotype affecting CBLs were shown in Table 3.
ment Fig. 1, B and C. Remaining unchanged troglitazone amounts in GSTM1 null hepatocytes tended to be higher than those in GSTM1 wild-type hepatocytes (Fig. 4A). As shown by two-way analyses of variance (Table 4), the GSTM1 genotype significantly affected remaining unchanged troglitazone amounts (F = 8.25, p = 0.02) without the interaction term. The remaining unchanged troglitazone amounts between GSTT1 null and wild-type hepatocytes were not significantly different (F = 2.85, p = 0.13). On the other hand, neither the GSTM1 genotype nor the GSTT1 genotype significantly affected remaining unchanged rosiglitazone amounts (F = 0.00, p = 0.99 and F = 2.54, p = 0.15, respectively) without the interaction term (Fig. 4B; Table 4). The stability of [14C]troglitazone and [14C]rosiglitazone under these assay conditions was investigated by radio-HPLC. Troglitazone and rosiglitazone were stable at 10 or 50 μM in hepatocyte incubation medium for 8 h. Saha et al. (2010) reported that troglitazone sulfate was stable under conditions similar to those in our study, and thus troglitazone sulfate was not considered to be deconjugated to unchanged troglitazone.

Troglitazone GSH Adducts (M1 and M2) in GSTM1- and GSTT1-Genotyped Hepatocytes and Substrate Selectivity for the GSH Adducts with the GST Isoforms. GSH adducts of troglitazone were detected by LC-MS/MS analysis from filtrates after incubation with 12 individual human hepatocytes genotyped for GSTM1 and GSTT1 (Fig. 5). The most notable, M1, was identified with all genotypes. However, M2, which is a GSH conjugate of isocyanate or isothiocyanate, the latter involving a novel oxidative scission of the thiazolidinedione ring system (Fig. 1) (Kassahun et al., 2001), was scarcely obtained with GSTM1 null hepatocytes. No appreciable GSH adducts of rosiglitazone were found with any of the genotypes in this study. The substrate selectivity for troglitazone-reactive metabolites trapped as M1 or M2 was verified by incubation of troglitazone with human liver microsomes using rhGSTA1, rhGSTM1, and rhGSTP1 from PanVera Corp. in the presence of 1 mM GSH. LC-MS/MS analysis for detection of GSH adducts, M1 and M2, by means of the MRM method showed that all isoforms (rhGSTA1, rhGSTM1, and rhGSTP1) increased the formation of M1. On the other hand, only rhGSTM1 increased the formation of M2 (Supplemental Fig. 2).

Cytotoxicity Assays in Individual Human Hepatocytes. Data for ATP levels in individual cryopreserved human hepatocytes, genotyped for GSTM1 and GSTT1, after treatment with troglitazone and rosiglitazone are summarized in Fig. 6. CYP3A and CYP2C8 activities, which affect reactive metabolite formation of troglitazone and rosiglitazone (Table 1), were comparable among the hepatocytes in this assay (Table 2). Treatment of GSTM1 and GSTT1 null hepatocytes with 50 μM troglitazone markedly reduced ATP levels compared with those for the no drug control. In contrast, no such reduction was evident in GSTM1 and GSTT1 wild-type hepatocytes exposed to troglitazone. ATP depletion was independent of UDP-glucuronosyltransferase (UGT) and sulforhodamine (SULT) activities (Table 2). Rosiglitazone did not change the cellular ATP concentration with any of the genotypes.

Discussion

“Metabolic idiosyncrasy” and/or “immune idiosyncrasy” are believed to cause DILI (Uetrecht, 2009). Troglitazone-induced liver injury that is not associated with any fever, rash, eosinophilia, or antinuclear antibodies (nonallergic hepatotoxicity) has been considered to belong to the category of metabolic idiosyncrasy. Interindividual differences in drug-metabolizing enzymes derived from polymorphisms that lead to greater exposure to reactive metabolites may be one possible explanation for the idiosyncratic nature of this nonallergic hepatotoxicity (Russmann et al., 2010). Conventional animal experiments would not be expected to be able to reproduce troglitazone hepatotoxicity because of the lack of the genetic variation. In this study, we focused on the metabolic idiosyncrasy of troglitazone hepatotoxicity and tried to investigate the mechanism with human in vitro samples featuring high genetic diversity.

In 16 individual microsomal experiments, CBLs of troglitazone were significantly correlated with CYP3A or CYP2C8 activities despite addition of GSH, which is a scavenger of reactive metabolites (Table 1). Our results are in line with the previous report of formation of troglitazone reactive metabolites through oxidation by CYP3A (Kassahun et al., 2001; Tettely et al., 2001; Yamamoto et al., 2002; He et al., 2004). However, CBLs of rosiglitazone were also significantly correlated with CYP2C8 activities, and both the absolute values and the variability were greater than with troglitazone. Therefore, the risk of reactive metabolite formation alone may not explain the clinical outcomes of troglitazone hepatotoxicity.
It was reported from an earlier clinical study that GSTM1 and GSTT1 null mutations might cause ALT and AST elevation by troglitazone (Watanabe et al., 2003). Therefore, we expected that low activity of detoxification enzymes, GSTs, may be risk factors.

rhGSTT1 is not commercially available, but addition of rhGSTA1 or rhGSTM1 resulted in decreased microsomal CBLs of troglitazone and rosiglitazone (Fig. 2), suggesting that GSTA1 and GSTM1 potentially have a scavenger effect on reactive metabolites. Regarding the main GST isoforms, GSTA1 and GSTP1, null mutation or polymorphism-related clinical hepatotoxicity is unknown. Thus, although rhGSTA1 also potentially had the scavenger effect, CBLs of troglitazone and rosiglitazone in GSTM1- and GSTT1-genotyped hepatocytes were investigated (Fig. 3). Concentrations studied were determined by reference to the estimated unbound maximum concentration of troglitazone in the portal vein ($C_{\text{in, max}, u} = 1.4 \mu M$; calculated in Supplemental 3). The concentrations of troglitazone and rosiglitazone were determined to be 10 μM to estimate an upper limit of hepatic exposure, and thus the concentration in this assay was considered to be reasonable for evaluation of hepatotoxicity. However, CBLs of troglitazone in GSTM1/GSTT1 null hepatocytes were significantly lower than those in GSTM1/GSTT1 wild-type hepatocytes (Fig. 3A; Table 3), independent of CYP3A or CYP2C8 activities (Fig. 3C). GSTs are well known to play crucial roles in detoxification of xenobiotics by preventing the binding of reactive metabolites to cellular proteins and catalyzing the conjugation of electrophilic moieties to GSH (Hayes et al., 2005). Our results were thus contrary to the expectation that GSTs would conjugate and decrease reactive metabolites and subsequent covalent binding, suggesting that microsomal CBL measurements with exogenous rhGSTM1 do not translate to the hepatocyte system with endogenous GSTM1. These are interesting findings because of the lack of CBL differences between GSTM1/GSTT1 null and wild-type hepatocytes with rosiglitazone (Fig. 3B; Table 4).

**TABLE 4**

<table>
<thead>
<tr>
<th>Source</th>
<th>Troglitazone</th>
<th>Rosiglitazone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Square</td>
<td>F p</td>
</tr>
<tr>
<td>GSTM1</td>
<td>242.6 8.25</td>
<td>0.02 0.99</td>
</tr>
<tr>
<td>GSTT1</td>
<td>83.9 2.85</td>
<td>0.13 0.54</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.1 0.00</td>
<td>0.96 0.32</td>
</tr>
</tbody>
</table>

* Significant at p < 0.05.

**Fig. 5.** LC-MS/MS chromatograms of troglitazone GSH adducts, M1 and M2, in GSTM1/GSTT1 null and wild-type hepatocytes. Filtrates after incubation of troglitazone (10 μM) with human hepatocytes genotyped for the GSTM1/GSTT1 null genotypes (lot A–C, wild/wild; lot D–F, wild/null; lot G–I, null/wild; and lot J–L, null/null) at 37°C for 8 h were applied to LC-MS/MS for detection of GSH adducts, M1 and M2, by means of the MRM method in the negative ion mode (MRM transitions: M1, [M – H]− → 745 → 272; M2, [M – H]− → 779 → 272).
observed for rosiglitazone.

expected to decrease the activities of reactive metabolite-forming hepatotoxicity in a clinical context, and this cytotoxicity would be might be supportive evidence for GSTM1- and GSTT1-dependent GSTT1 null hepatocytes specifically (Fig. 6) and independently of sidered to be detected after treatment with troglitazone in GSTM1 and hepatocytes (Hewitt et al., 2002). However, ATP depletion was con-

CYP3A with high SULT activity is linked to higher toxicity in human association between troglitazone and GST genotypes in the M2 might have potential implications for understanding the relationship between troglitazone hepatotoxicity and GST genotypes in the clinical setting. The remaining unchanged troglitazone amounts in GSTM1 null hepatocytes were significantly higher than those in their GSTM1 wild-type counterparts (Fig. 4A; Table 4). Because troglita-

zone was incubated with human cryopreserved hepatocytes geno-
typed for GSTM1/GSTT1 null mutations on cytotoxicity by troglitazone and rosiglitazone. Troglitazone and rosiglita-

zone were matured with human cryopreserved hepatocytes geno-
typed for GSTM1/GSTT1 (□ and ■, wild/wild; ● and ●, null/null) at 37°C for 2 h followed by measurement of ATP levels. Data are means ± S.D. from three assays expressed as percentages of the no drug control values. **, p < 0.01; $$$, p < 0.001, significantly different from no drug controls.

Table 3), but unexplained, suggesting that not only CBL but also unknown factors are involved in the clinical outcomes of DILI.

In an attempt to clarify the discrepancy, metabolites of individual hepatocytes were analyzed by LC-MS/MS. Whereas M1 was found with all genotypes, M2 was scarcely obtained with GSTM1 null hepatocytes (Fig. 5). Because M2 formation is known to be catalyzed by CYP3A (Kassahun et al., 2001) and all hepatocytes used possessed a certain level of basal CYP3A activity (Table 2), it is reasonable to presume that GSTM1 is responsible for scavenging of a reactive metabolite trapped as M2. Verification of substrate selectivity for troglitazone-reactive metabolites trapped as M1 or M2 using rhGSTA1, rhGSTM1, and rhGSTP1 (Supplemental Fig. 2) indicated that M1 is formed by any GST isoforms but that M2 is formed only by rhGSTM1. It would therefore be possible to suggest that the GSH addition to a reactive metabolite to generate M1 might not be catalyzed by GSTM1, but a separate GSH addition to another metabolite to generate M2 could involve GSTM1 in hepatocytes. Specific exposure of GSTM1 null hepatocytes to the reactive metabolite trapped as M2 might have potential implications for understanding the relationship between troglitazone hepatotoxicity and GST genotypes in the clinical case of troglitazone, CBLs of individual human hepatocytes phenotyped for P450s and genotyped for GSTs were not appropriate predictors for clinical hepatotoxicity, because the apparent inverse relationship between CBLs and cytotoxicity (and lack of P450 correlation) was observed in hepatocytes. Therefore, a cytotoxicity testing system using GSTM1- and GSTT1-genotyped hepatocytes may be more useful than a CBL measuring system using microsomes or hepatocytes. GSTM1/GSTT1 defects were found to relate to DILI with
tacrine and carbamazepine in clinical studies (Simon et al., 2000; Ueda et al., 2007). It will now be necessary to investigate our evaluation system using GST-genotyped hepatocytes with more compounds.

In several recent studies, human leukocyte antigen (HLA) haplotypes were reported to be major determinants of DILI (e.g., fluoxoca-
cillin and lumiracoxib) (Daly et al., 2009; Singer et al., 2010), but the association between HLA haplotypes and troglitazone hepatotoxicity in the clinical context has not been reported. Because HLA haplotypes were not investigate the reactive metabolite-forming enzymes and CBLs, providing another possible explanation for the discrepancy in CBL findings. Furthermore, it is interesting that GSTM1 and GSTT1 null mutation-dependent cytotoxicity was not observed for rosiglitazone.

Troglitazone sulfate, the main troglitazone metabolite eliminated into bile, shows competitive bile salt export pump inhibition with an apparent \( K_i \) value of 0.23 \( \mu M \) (Funk et al., 2001b). Treatment of GSTM1 and GSTT1 wild-type hepatocytes with 50 \( \mu M \) troglitazone did not reduce ATP levels, despite formation of a certain level of troglitazone sulfate (speculated from Supplemental Fig. 1B). Therefore, transporter inhibition might not be related to the cytotoxicity in the present study. By our system, however, the transporter activities were not investigated, and it remains to be determined whether clinical hepatotoxicity is caused by cytotoxicity or by transporter inhibition. Meanwhile, unchanged troglitazone has been reported to cause mitochondrial dysfunction (Masubuchi et al., 2006; Lim et al., 2008). Thus, our results cannot preclude the higher levels of unchanged drug in GSTM1/GSTT1 null hepatocytes (Fig. 4) being relevant to mitochondrial dysfunction. However, expression of CYP3A4 with \( \gamma \)-glutamylcysteine synthetase knockdown was shown to produce troglitazone cytotoxicity in a cell-based assay system (Hosomi et al., 2010, 2011), providing supportive information for our finding that high formation of reactive metabolites and low detoxification might be risk factors for troglitazone cytotoxicity.

At present, CBL is considered one of the most reliable tools to evaluate DILI (Evans et al., 2004; Nakayama et al., 2009; Usui et al., 2009). On the other hand, there are certainly some agents falsely identified as hepatotoxic by the CBL approach (Obach et al., 2008). In the case of troglitazone, CBLs of individual human hepatocytes phenotyped for P450s and genotyped for GSTs were not appropriate predictors for clinical hepatotoxicity, because the apparent inverse relationship for CBLs and cytotoxicity (and lack of P450 correlation) was observed in hepatocytes. Therefore, a cytotoxicity testing system using GSTM1- and GSTT1-genotyped hepatocytes may be more useful than a CBL measuring system using microsomes or hepatocytes. GSTM1/GSTT1 defects were found to relate to DILI with
and indicate that measurement of GSH adducts or cytotoxicity using GSTM1- and GSTT1-genotyped hepatocytes might offer an important in vitro system to assist in better prediction of DILI.

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Authorship Contributions
Participated in research design: Usui, Hashizume, and Yokoi.
Conducted experiments: Usui.
Contributed new reagents or analytic tools: Usui.
Performed data analysis: Usui.
Wrote or contributed to the writing of the manuscript: Usui, Hashizume, Katsumata, Yokoi, and Komuro.

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