Evaluation of the Potential for Drug-Induced Liver Injury Based on in Vitro Covalent Binding to Human Liver Proteins\textsuperscript{\textregistered}

Toru Usui, Masashi Mise, Takanori Hashizume, Masashi Yabuki, and Setsuko Komuro

Pharmacokinetics Research Laboratories, Dainippon Sumitomo Pharma Co., Ltd., Kasugade-naka, Konohana-ku, Osaka, Japan

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ABSTRACT:
Prediction of idiosyncratic drug-induced liver injury (DILI) is difficult, and the underlying mechanisms are not fully understood. However, many drugs causing DILI are considered to form reactive metabolites and covalently bind to cellular macromolecules in the liver. The objective of this study was to clarify whether the risk of idiosyncratic DILI can be estimated by comparing in vitro covalent binding (CB) levels among the positive (12 compounds) and negative (12 compounds) groups. Although toxic candidate compounds are mostly screened out by discovery during preclinical safety studies, in some cases, toxicity is detected only in late clinical phases or in postmarketing evaluation. Often the problem is caused by what is known as idiosyncratic drug reactions (IDRs). IDRs refer to adverse reactions with a low frequency of occurrence (<0.1%) that do not involve the known pharmacological properties of the drug, do not occur in most patients at any dose of the drug, and typically have a delayed onset of weeks to months after initial exposure (Uetrecht, 1999). Drug-induced liver injury (DILI), one of the IDRs, is the most frequent reason for withdrawal of a drug candidate during drug development (Lee, 2003). Examples of drugs withdrawn from the market because of idiosyncratic DILI include nefazodone, troglitazone, ibuprofen, paroxetine, pioglitazone, rosiglitazone, sertraline, theophylline, venlafaxine, and zolpidem. After incubation with human liver microsomes in the presence of NADPH, there was a large overlap in the distribution of CB amounts between the positive and negative groups. On addition of UDP-glucuronic acid (UDPGA) as a cofactor for glucuronidation, the CB levels of bromfenac and diclofenac were increased. With addition of nucleophilic glutathione (GSH), values for most compounds were decreased. However, separation of the two groups on the basis of CB could not be improved by UDPGA or GSH. Furthermore, CB with human hepatocytes also failed to discriminate positive from negative compounds. Therefore, the CB amount alone is not sufficient for risk assessment of DILI. In contrast, when the CB amount was multiplied by the maximum daily dose, which may reflect maximum hepatic exposure, the two groups did become discriminated. Taken together, our findings suggest that the combination of CB amount and daily dose can estimate the risk of idiosyncratic DILI.

Although toxic candidate compounds are mostly screened out by discovery during preclinical safety studies, in some cases, toxicity is detected only in late clinical phases or in postmarketing evaluation. Often the problem is caused by what is known as idiosyncratic drug reactions (IDRs). IDRs refer to adverse reactions with a low frequency of occurrence (<0.1%) that do not involve the known pharmacological properties of the drug, do not occur in most patients at any dose of the drug, and typically have a delayed onset of weeks to months after initial exposure (Uetrecht, 1999). Drug-induced liver injury (DILI), one of the IDRs, is the most frequent reason for withdrawal of an approved drug from the market and also a major cause of attrition in drug development (Lee, 2003). Examples of drugs withdrawn from the market because of idiosyncratic DILI include nefazodone, troglitazone, and bromfenac. Therefore, idiosyncratic DILI is of great concern to the pharmaceutical industry and is the focus of the present study. The mechanisms are not fully understood, but several hypotheses have been proposed. Because a high proportion of drugs involved in IDRs are capable of generating reactive metabolites, which covalently bind to various target macromolecules by nucleophilic substitution, it is thought that metabolic activation of a drug to reactive metabolites might be a necessary first step in the generation of IDRs in many cases (Uetrecht, 1999; Walgren et al., 2005).

It is therefore important to avoid chemical functional groups that are known to cause toxicity during drug design. To detect reactive metabolites as adducts with nucleophilic trapping agents, many in vitro trapping assays featuring a hepatic microsomal enzyme system (one of the most commonly used techniques is the GSH trapping assay) are available for application during the early stages of drug discovery. However, with use of these assays false-negative and false-positive results cannot be avoided (Gan et al., 2009). Therefore, trapping assays may not be adequate for judgment of whether a compound should advance to clinical development, and a higher-precision approach is required. To minimize the risk of reactive metabolites, the U.S. Food and Drug Administration mentioned in the draft guidance document “Drug-Induced Liver Injury: Premarketing Clinical Evaluation” (Food and Drug Administration, 2007, http://www.fda.gov/downloads/Drugs/GuidanceCompliance RegulatoryInformation/Guidances/UCM072278.pdf) that radiochemical in vitro methods are available to detect and quantify covalent binding (CB) to liver proteins for a drug or its metabolites. In addition, Evans et al. (2004) have also provided a rationale for compound evaluation based on CB. However, it has not been confirmed that positive and negative compounds can actually be distinguished on the basis of in vitro CB data. Therefore, to clarify whether the risk of idiosyncratic DILI can be estimated by CB, we here selected 12 positive and 12 negative
compounds with regard to idiosyncratic DILI and determined levels of CB to human liver proteins under the following conditions: 1) with microsomes in the presence of NADPH [for cytochrome P450 (P450)-mediated metabolism]; 2) with microsomes in the presence of NADPH and UDP-glucuronic acid (UDPGA) [for mixed P450/UDP-glucuronosyltransferase (UGT)-mediated metabolism]; 3) with microsomes in the presence of NADPH, UDPGA, and GSH (for possible scavenging reactive metabolites); and 4) with hepatocytes (for more complete metabolism). In addition, whether pharmacokinetic parameters ($C_{\text{max}}$ and daily dose) are useful for prediction of DILI was investigated.

Materials and Methods

Materials. $[^{14}C]$Alpidem, $[^{14}C]$carbamazepine, $[^{14}C]$tacrine, $[^{14}C]$ticlopidine, $[^{14}C]$losartan, $[^{14}C]$sertraline, $[^{14}C]$venlafaxine, and $[^{14}C]$zolpidem were synthesized by BlyChem Ltd. (Billingham, UK). $[^{14}C]$Bromfenac and $[^{14}C]$nefazodone were synthesized by GE Healthcare (Little Chalfont, Buckinghamshire, UK). $[^{14}C]$Tienilic acid was synthesized by Sekisui Medical Co., Ltd. (Tokyo, Japan). $[^{14}C]$Troglitazone, $[^{14}C]$pioglitazone, and $[^{14}C]$rosiglitazone were synthesized in-house. $[^{14}C]$Acetaminophen, $[^{14}C]$Acetylsalicylic acid, $[^{14}C]$caffeine, $[^{14}C]$Chlorpheniramine, and $[^{14}C]$theophylline were purchased from American Radiolabeled Chemicals (Brea, CA), and $[^{3}H]$imipramine and $[^{3}H]$paroxetine from PerkinElmer (Waltham, MA). Radiolabeling positions and chemical structures of all compounds are shown in Fig. 1. Unlabeled alpidem, troglitazone, and rosiglitazone were synthesized in-house. Unlabeled acetaminophen, carbamazepine, diclofenac, flutamide, imipramine, nefazodone, tacrine, ticlopidine, acetylsalicylic acid, caffeine, dexethasone, ibuprofen, paroxetine, sertraline, venlafaxine, and zolpidem were purchased from Sigma-Aldrich (St. Louis, MO). Unlabeled bromfenac and tienilic acid were from AKos Consulting and Solutions Deutschland GmbH (Steinen, Germany) and Cypex Ltd. (Dundee, Scotland, UK), respectively. Unlabeled losartan and paroxetine were from PerkinElmer Life and Analytical Sciences (Waltham, MA). Radiolabeled and unlabeled compounds were combined to make the substrate solutions (1 mM, $\sim 0.7$ GBq/nmol).

Human liver microsomes (mixed gender pool of 50 individuals) were obtained from XenoTech, LLC (Lexena, KS), and human pooled cryopreserved hepatocytes (mixed gender pool of 10 individuals) were purchased from In Vitro Technologies, Inc. (Baltimore, MD). NADPH and reduced GSH were from Oriental Yeast Co., Ltd. (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. UDPGA and alamethicin were purchased from Sigma-Aldrich. All other reagents and solvents were of the highest grade available commercially.

Incubation. To estimate the potential for CB to human liver proteins, the following four-arm assays were conducted. In the first arm, radiolabeled compounds (final concentration 10 $\mu$M) were incubated with 1 mg/ml pooled human liver microsomes and 1 mM NADPH for 60 min at 37°C in 500 $\mu$L of a reaction mixture consisting of 50 mM phosphate buffer (pH 7.4) and 10 mM MgCl$_2$. The second arm assay was performed as for the first arm using the same mixture fortified with 2 mM UDPGA as a cofactor. The third arm incubation was performed in the presence of a nuclease (1 mM GSH) under the second arm conditions. Microsomes used in second and third arms were preincubated with alamethicin (50 $\mu$g/mg microsomal protein) for 10 min on ice before use. Incubation in the absence of NADPH and UDPGA served as a blank control. In the fourth arm, radiolabeled compounds (10 $\mu$M) were incubated with human cryopreserved hepatocytes ($1 \times 10^6$ cells/ml) for 4 h at 37°C under an atmosphere of 95% air/5% CO$_2$ on 24- well plates in 300 $\mu$L of hepatocyte incubation medium (XenoTech, LLC). Reactions were stopped by adding 2 ml of ice-cold methanol. All experiments were conducted in duplicate.

Measurement of CB Amounts. CB amounts were measured according to the method of Day et al. (2005) with some modifications. For measurement of radioactivity bound to proteins and analysis of unbound metabolites, the reaction mixtures after precipitation were loaded onto GF/C glass fiber filters (Whatman International Ltd., Maidstone, UK) in a 1225 sampling manifold (Millipore, Billerica, MA) using aspiration and washed seven times with 80% (v/v) methanol containing 5% (w/v) trichloroacetic acid, seven times with acetonitrile, and then 10 times with 80% (v/v) methanol to remove unbound radioactivity. The first filtrate was used for radio liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of metabolites unbound to proteins as described below. The filter was transferred to a scintillation vial with 3 ml of 10% SDS and incubated overnight at 55°C to dissolve proteins. An aliquot of the protein solution was mixed with a scintillation cocktail, Clear-sol 1 (Nacalai Tesque), and radioactivity was measured using a liquid scintillation counter (Tri-Carb 2700TR; PerkinElmer Life and Analytical Sciences). Protein determination was conducted using the BAC Protein Assay Kit (Pierce Chemical, Rockford, IL). The CB amount was calculated from the following equation:

\[
\frac{\text{radiocactivity in the protein solution (dpm/ml)}}{\text{specific radioactivity of substrate (dpm/pmol)}} \times \text{protein concentration in the protein solution (mg/ml)}
\]

Radio-LC-MS/MS Analysis. The first filtrate from the sampling manifold was collected and evaporated to dryness in a centrifugal evaporator (CC-105; Tomy, Tokyo, Japan). The residue was dissolved in mobile phase and loaded onto a column (Inertsil ODS-3V, 5 $\mu$m, 4.6 i.d. $\times$ 250 mm; GL Science, Inc., Tokyo, Japan) with a column temperature of 40°C. The LC system consisted of an Agilent 1200 (Agilent Technologies, Inc., Santa Clara, CA) pump set at a flow rate of 1 ml/min. The mobile phase consisted of a linear gradient of solvent A (10 mM ammonium acetate) and solvent B (acetonitrile) in the appropriate ratio (Fig. 2). Radioactivity and mass analyses were conducted in parallel using a microsplitter. Radioactivity was detected with a flow scintillation detector (Radiomatic 610TR; PerkinElmer Life and Analytical Sciences), using Ultima Flo-M scintillation cocktail (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 radioactive peaks detected, each molecular mass was determined by precursor ion survey scans using the enhanced mass scan method at a mass range from 100 to 850 atomic mass units under positive and negative ion modes. Metabolite structures of bromfenac and diclofenac were speculated from the molecular mass and metabolite information obtained as reported previously [for bromfenac (Kirkman et al., 1998) and for diclofenac (Kenny et al., 2004; Wang et al., 2004)] (Fig. 2). When metabolite structures could not be determined from the above information, MS/MS analysis was conducted with the enhanced product ion scan method. Furthermore, important ions were confirmed by exact mass measurement using a hybrid ion-trap time-of-flight mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ion source.

Results

Determination of CB Amounts and Metabolite Profiling. The interday reproducibility of CB determination using compound A (first, second, third, and fourth arm) was examined for 3 days. The average (picomoles per milligram of protein) and the coefficient of variation (percent) of interday precision were 289.5 and 5.3 (first arm), 287.8 and 5.1 (second arm), 56.8 and 5.1 (third arm), and 47.1 and 13.0 (fourth arm), respectively. These results suggest that the CB determination method applied in this study was reproducible.

One of the most representative metabolite profiles were obtained from filtrates after incubation of $[^{14}C]$troglitazone (m/z 440 of the deprotonated molecular ion) with human liver protein (Supplemental Fig. 14). As reported previously (Kawai et al., 1997; He et al., 2004), an NADPH-dependent oxidative metabolite (troglitazone quinone, m/z 456 of the deprotonated molecular ion), a UDPGA-dependent glucuronide conjugate (troglitazone glucuronide, m/z 616 of the deprotonated molecular ion), a GSH conjugate (troglitazone GSH adducts, m/z 745 of the deprotonated molecular ion), and a sulfate conjugate (troglitazone sulfate, m/z 520 of the deprotonated molecular ion) were obtained from the same reaction mixture.
Positive compounds for DILI

\[^{14}C\]-Acetaminophen  \[^{14}C\]-Alpidem  \[^{14}C\]-Bromfenac  \[^{14}C\]-Carbamazepine  \[^{14}C\]-Diclofenac

\[^{3}H\]-Flutamide  \[^{3}H\]-Imipramine  \[^{14}C\]-Nefazodone  \[^{14}C\]-Tacrine

\[^{14}C\]-Ticlopidine  \[^{14}C\]-Tienilic acid  \[^{14}C\]-Troglitazone

Negative compounds for DILI

\[^{14}C\]-Acetylsalicylic acid  \[^{14}C\]-Caffein  \[^{3}H\]-Dexamethasone  \[^{14}C\]-Ibuprofen

\[^{14}C\]-Losartan  \[^{3}H\]-Paroxetine  \[^{14}C\]-Pioglitazone  \[^{14}C\]-Theophylline

\[^{14}C\]-Rosiglitazone  \[^{14}C\]-Sertraline  \[^{14}C\]-Venlafaxine  \[^{14}C\]-Zolpidem

FIG. 1. Chemical structures of positive and negative compounds for DILI. *, position of the radiolabel.
ion) were characteristically detected in the first, second, third, and fourth arm assays, respectively. Moreover, GSH-dependent peaks were distinctly found in radiochromatograms of diclofenac, flutamide, nefazodone, and ticlopidine, all positive compounds for DILI. GSH-dependent peak(s) of other compounds were not detected clearly on addition of GSH. Because chemically reactive acylglucuronides of diclofenac are considered to form covalent adducts (Kretz-Rommel and Boelsterli, 1994), metabolite profiles of diclofenac and bromfenac, which also has a carboxyl group and causes DILI, were investigated (Fig. 2) in the presence of UDPGA. Diclofenac lactam, which has been detected in human hepatocytes (Bort et al., 1999), was not detected in this study. Radiochromatogram and MS data in the first, second, third, and fourth arms for 14 compounds (alpidem, bromfenac, carbamazepine, diclofenac, flutamide, imipramine, losartan, nefazodone, pioglitazone, rosiglitazone, tacrine, ticlopidine, tienilic acid, and troglitazone) are shown in Supplemental Figs. 1 to 14.

CB in Human Liver Microsomes and Human Hepatocytes. The positive compounds have the potential to cause severe or idiosyncratic DILI in clinical use and are metabolically activated by P450 and/or...
Uptake. Acetaminophen, which is not considered an idiosyncratic hepatotoxicant, was here included in positive compounds as a typical hepatotoxicant, to which reactive metabolites relate, for comparison with other reports. The negative compounds chosen are considered much safer drugs despite having metabolic pathways similar to those of positive compounds, high prescription rates, and/or high dosage. Amounts of radioactivity derived from the compounds CB to human liver proteins are shown in Table 1.

Table 1. CB, maximum daily dose, and Cmax, of the tested compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>CB</th>
<th>Daily Dose</th>
<th>Cmax</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st</td>
<td>2nd</td>
<td>3rd</td>
<td>4th</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>179</td>
<td>124</td>
<td>28</td>
<td>16</td>
</tr>
<tr>
<td>Alpidem</td>
<td>440</td>
<td>192</td>
<td>88</td>
<td>101</td>
</tr>
<tr>
<td>Bromfenac</td>
<td>51</td>
<td>107</td>
<td>44</td>
<td>22</td>
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<tr>
<td>Carbazepine</td>
<td>119</td>
<td>90</td>
<td>35</td>
<td>18</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>120</td>
<td>173</td>
<td>59</td>
<td>49</td>
</tr>
<tr>
<td>Flutamide</td>
<td>477</td>
<td>471</td>
<td>225</td>
<td>35</td>
</tr>
<tr>
<td>Imipramine</td>
<td>271</td>
<td>258</td>
<td>63</td>
<td>10</td>
</tr>
<tr>
<td>Nefazodone</td>
<td>1364</td>
<td>997</td>
<td>306</td>
<td>254</td>
</tr>
<tr>
<td>Tacrine</td>
<td>264</td>
<td>201</td>
<td>47</td>
<td>14</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>1286</td>
<td>1136</td>
<td>249</td>
<td>60</td>
</tr>
<tr>
<td>Tienilic acid</td>
<td>538</td>
<td>487</td>
<td>124</td>
<td>28</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>1170</td>
<td>967</td>
<td>273</td>
<td>118</td>
</tr>
<tr>
<td>Negative compounds</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>3</td>
</tr>
<tr>
<td>Caffeine</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>445</td>
<td>272</td>
<td>262</td>
<td>41</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2</td>
</tr>
<tr>
<td>Losartan</td>
<td>105</td>
<td>105</td>
<td>40</td>
<td>19</td>
</tr>
<tr>
<td>Paroxetine</td>
<td>235</td>
<td>182</td>
<td>24</td>
<td>19</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>236</td>
<td>302</td>
<td>59</td>
<td>11</td>
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<tr>
<td>Rosiglitazone</td>
<td>1189</td>
<td>894</td>
<td>229</td>
<td>11</td>
</tr>
<tr>
<td>Sertraline</td>
<td>49</td>
<td>42</td>
<td>26</td>
<td>16</td>
</tr>
<tr>
<td>Theophylline</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Zolpidem</td>
<td>23</td>
<td>14</td>
<td>8</td>
<td>6</td>
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N.D., CB for test compounds was lower than that for the control without NADPH and UDPGA.

Cmax value normalized by dose.

Cmax value normalized by body weight.

Table 1 continues with further parameters related to CB.

EVALUATION OF DRUG-INDUCED LIVER INJURY BY COVALENT BINDING

Test compounds (10 μM) were incubated with human liver microsomes in the presence of NADPH (first arm), NADPH and UDPGA (second arm), or NADPH, UDPGA, and GSH (third arm) or with hepatocytes (fourth arm). The CB amounts were determined with duplicate incubations. Daily dose and Cmax represent the maximum daily dose and the maximum concentration at the maximum daily dose, respectively. Daily dose and Cmax are from the package inserts or the following references: 1) Maass et al., 1982; 2) Durand et al., 1992; 3) Boni et al., 1997; 4) Greene and Barbhaiya, 1997; 5) Fontana et al., 1998; 6) Kalgutkar and Soglia, 2005; 7) Walgren et al., 2005; and 8) Lammert et al., 2008.

In the first arm assay, CB of reactive metabolites occurred under the influence of P450 (as a main catalyst) in the presence of NADPH. The CB amounts of all positive compounds were larger than 50 pmol/mg protein (51–1364 pmol/mg). Nefazodone, ticlopidine, and troglitazone had particularly large values of 1364, 1285, and 1170 pmol/mg protein, respectively. The CB amounts of negative compounds varied. Although protein adducts of acetylsalicylic acid, ibuprofen, and theophylline were not detected, 5 of 12 negative compounds had levels greater than 50 pmol/mg protein. In particular, metabolites of rosiglitazone and dexamethasone bound highly to microsomal proteins (1189 and 445 pmol/mg protein, respectively).

In the second arm assay, CB mediated by glucuronidation as well as oxidative reactions was investigated in the presence of NADPH and UDPGA. The CB amounts of alpidem and dexamethasone were strongly decreased by addition of UDPGA. In contrast, the values for bromfenac, diclofenac, and pioglitazone were increased by 2.0-, 1.4-, and 1.2-fold, respectively, suggesting occurrence of UDPGA-dependent metabolic activation.

In the third arm assay, the effects of a nucleophile GSH on CB of reactive metabolites was investigated. The protein adduct amounts were significantly decreased (except with dexamethasone) compared with the second arm assay, suggesting that GSH is an efficient scavenger of electrophilic metabolites from a variety of compounds. As shown in Fig. 3, the GSH scavenging effects on protein adducts (difference between the second and third arm values) were significantly correlated with the second arm value (r = 0.92), except with dexamethasone data.

In the fourth arm assay, CB in hepatocytes capable of other metabolic activation/inactivation as well as NADPH- and UDPGA-dependent activation showed good correlation with that in microsomes (r = 0.81) (Fig. 4).

Evaluation of the Potential for DILI Based on CB. There was much overlapping of CB levels between positive and negative groups for DILI so that discrimination between the two was not possible through the first arm to fourth arms (Fig. 5). CB in hepatocytes tended to improve the discrimination between positive and negative compounds, but many values for positive compounds still overlapped with those for negative compounds (Fig. 5D). For further analysis to investigate whether other parameters related to CB can discriminate positive from negative compounds, the following parameters were used in microsomes and hepatocytes: CB × Cmax and CB × dose (maximum daily dose). The higher of the two values for the first and second arms was used as the value for CB in microsomes. CB × Cmax, which may reflect systemic exposure, tended to be higher for positive than for negative compounds, but discrimination was still insufficient in microsomes (Fig. 6A). CB × Cmax in hepatocytes did not improve the discrimination (Fig. 6B). In contrast, CB × dose, which may reflect maximum hepatic exposure, demonstrated greater contrast between positive and negative compounds and a bimodal distribution with...
minimal overlapping in microsomes and hepatocytes (Fig. 6, C and D). However, the maximum daily dose alone could not discriminate positive from negative compounds (Fig. 7).

**Discussion**

In the present first arm study of CB with 12 DILI positive and 12 DILI negative compounds, values for all positive compounds exceeded 50 pmol/mg microsomal protein, Evans’s target threshold (under the same experimental conditions as the first arm) (Evans et al., 2004). However, the CB of many negative compounds also exceeded 50 pmol/mg so that positive compounds could not be distinguished from negative compounds on this basis (Fig. 5A).

We expected bioactivation by acylglucuronide or a decrease in oxidative metabolism by an increase in glucuronidation to change CB and to improve the overlapping of CB between positive and negative compounds. The CB amount with diclofenac did increase on addition of UDPGA. It is thought that diclofenac, which has a carboxyl group, is metabolized to an acylglucuronide, which is reported to correlate with CB (Kretz-Rommel and Boelsterli, 1994). Two diclofenac glucuronides and two hydroxyclofenac glucuronides were here detected on addition of UDPGA (Fig. 2). Isomerization of acylglucuronide conjugates may cause peaks for these glucuronides as reported previously (Akira et al., 2002). Bromfenac, which also has a carboxyl group and causes DILI, is also considered to form an acylglucuronide (Skjodt and Davies, 1999). However, the acylglucuronide of unchanged bromfenac has not been detected in practice (Kirkman et al., 1982).

**Fig. 3.** Correlation between CB amounts in microsomes (second arm value) and GSH scavenging effects (difference between the second and third arm values). The second arm value is CB amount in microsomes in the presence of NADPH and UDPGA. The third arm value is the CB amount in microsomes in the presence of NADPH, UDPGA, and GSH. CB amounts of acetylsalicylic acid, ibuprofen, and theophylline were lower than those for the control without NADPH and UDPGA. GSH scavenging effects with venlafaxine could not be calculated. DEX, dexamethasone.

**Fig. 4.** Correlation between CB amounts in microsomes (second arm value) and hepatocytes (fourth arm value). The second arm value is the CB amount in microsomes in the presence of NADPH and UDPGA. The fourth arm value is the CB amount in hepatocytes. Note that for acetylsalicylic acid, ibuprofen, and theophylline, CB amounts in the second arm are lower than that for the control without NADPH and UDPGA. These compound values are not included in this correlation analysis. APAP, acetaminophen; ALP, alpidem; BRO, bromfenac; CAR, carbamazepine; DIC, diclofenac; FLU, flutamide; IMI, imipramine; NEF, nefazodone; TAC, tacrine; TIC, ticlopidine; TA, tienilic acid; TRO, troglitazone; ASA, acetylsalicylic acid; CAF, caffeine; DEX, dexamethasone; IBU, ibuprofen; LOS, losartan; PAR, paroxetine; PIO, pioglitazone; ROSI, rosiglitazone; SER, sertraline; THEO, theophylline; VEN, venlafaxine; ZOL, zolpidem.
and the basis for DILI is unclear. Although the CB amount of bromfenac increased on addition of UDPGA, the metabolite formed was a bromfenac-lactam structure rather than an acylglucuronide (Fig. 2). It would appear that the acylglucuronide of bromfenac was formed and bound to proteins but could not be detected because of instability and ready transformation to bromfenac-lactam. The reason that bromfenac-lactam was detected and diclofenac-lactam was not detected may be the difference in reactivity between primary and secondary amine. On the other hand, CB with acetylsalicylic acid and ibuprofen, which also have carboxyl groups, was not detected despite addition of UDPGA. These results suggest that the cause of hepatotoxicity with carboxylic acids may be acylglucuronide formation. The reasons for the decrease in CB on addition of UDPGA may include decreasing oxidative metabolism and increasing conjugation. Alpidem, which is known to be metabolized by aliphatic or aromatic oxidative pathways (Padovani et al., 1987), is a typical example, and, in fact, oxidative metabolites decreased and glucuronides derived from alpidem were detected on addition of UDPGA (Supplemental Fig. 1). It is unfortunate that, despite addition of UDPGA, CB amounts for positive compounds with microsomes still overlapped negative compounds in the second arm (Fig. 5B).

GSH, which is not contained in microsomes but is constitutively present in hepatocytes, is commonly believed to be responsible for scavenging of reactive metabolites. However, it is known that some reactive metabolites are not trapped by GSH (Argoti et al., 2005). We expected that addition with GSH to microsomes (the third arm condition) might mimic the in vivo liver condition and improve the ability to distinguish between positive and negative compounds. However, addition of GSH did not remove the overlapping (Fig. 5C). GSH scavenging effects (second arm to third arm values) did show a significant correlation with the following second arm value, except in the case of dexamethasone ($r = 0.92$) (Fig. 3). This result also indicates that detection and quantification of GSH adducts are available as surrogate methods for CB quantification, and reactive metabolites, which were not trapped by GSH, were generated in the dexamethasone assay. Although dexamethasone is not known to form any kind of electrophilic species, reactive metabolites were earlier found to be trapped by the hard nucleophilic trapping agent, cyanide, and CB...
was thereby decreased (Meneses-Lorente et al., 2006). For compounds whose reactive metabolites are not trapped by GSH, like dexamethasone, other trapping assays [such as iminium ion trapping with cyanide (Gorrod et al., 1991) and aldehyde trapping with semicarbazide (Xu et al., 2005), among others] should be conducted to help predict the structure of reactive metabolites.

In an attempt to overcome the problem with overlapping, hepatocytes, which have more complete metabolic activation/inactivation system than microsomes, were here used. Moreover, uptake to hepatocytes may mimic the exposure of CB in vivo in the liver. However, our values with hepatocytes significantly correlated with those using microsomes (Fig. 4), and positive compounds still overlapped negative compounds (Fig. 5D). This finding can be attributed to the fact that test compounds metabolized by P450 and UGT were selected for the present study. Because P450-mediated bioactivation has been reported to be the first step in generation of many IDRs (Hess and Rieder, 1997), the microsomal approach could be useful for estimation of idiosyncratic DILI potential. The CB amounts in hepatocytes would be expected to have importance, if extramicrosomal metabolism is involved in activation/inactivation. In this study, rosiglitazone, which demonstrated a relatively low CB amount in hepatocytes (fourth arm) compared with that of microsomes (second arm), may have undergone extramicrosomal inactivation of reactive metabolites (Fig. 4). One bioactivation pathway of valproic acid, which does induce DILI, is known to be mitochondrial oxidation (Baillie, 1988). For such compounds, hepatocyte assays may be more suitable to determine CB. Further verification of our assay conditions is required for evaluation of more compounds known to be metabolized by extramicrosomal enzymes.

By our methods, the metabolite profiles with CB data could be confirmed by radio-LC-MS/MS analysis. One of the most representative and interesting profiles was obtained for troglitazone (Supplemental Fig. 14). In the first arm, an oxidative metabolite, a quinone form, was detected, and the CB was very high. In the second arm, glucuronides were detected, but the CB was still high. In the third arm, GSH adducts were detected, and the CB decreased markedly. In addition, GSH-dependent peaks were distinct in radiochromatograms from diclofenac, flutamide, nefazodone, and ticlopidine, which are all positive compounds for DILI (Supplemental Figs. 4, 5, 8, and 12). At the same time, GSH-dependent
peaks were not detected clearly with other compounds. For example, a GSH adduct of rosiglitazone was not detected by radiochromatography, even though the CB of rosiglitazone was decreased markedly by GSH (Supplemental Fig. 10). These results suggest that detection of GSH adducts itself could be a possible factor for DILI and inactivation caused by GSH might not be reflected in GSH adduct levels and that all GSH adduct is not detectable because of the instability and the difficulty in detection by mass spectrometry. Thus, not only CB amounts but also confirmation of metabolite profiles may be useful to understand the relation between CB and DILI. However, it is not easy to distinguish positive from negative compounds with metabolite profiling data. Evaluation purely on the basis of the CB value or detection of GSH adducts would give many false-positive or false-negative results and therefore cannot accurately estimate the risk of DILI.

It is considered that exposure of liver to reactive metabolites must be proportional to liver injury (Li, 2002). Therefore, in an attempt to remove false-positive results, we multiplied the in vitro CB amount by the $C_{\text{max}}$ (CB × $C_{\text{max}}$) related to systemic exposure or the maximum daily dose (CB × dose) related to the theoretical maximum exposure of liver to reactive metabolites. On evaluation by CB × $C_{\text{max}}$, there was still a great deal of overlap between the two groups in microsomes and hepatocytes and discrimination was not possible (Fig. 6, A and B). The $C_{\text{max}}$ might not reflect exposure to the liver, which is a main site of first-pass metabolism. In contrast, the combination of CB and daily dose did allow good distinction between the positive and the negative compounds in microsomes and hepatocytes (Fig. 6, C and D). With retrospective findings, daily dose is one of the most important factors for IDRs, and very low-dose drugs (<10 mg/day) seem to be devoid of DILI induction potential (Uetrecht, 2000). Our in vitro data are consistent with this epidemiological speculation. It is interesting to note that in the negative compounds, drugs with high amounts of CB had low daily doses (dexamethasone and rosiglitazone), and drugs with high daily doses had low amounts of CB (acetylsalicylic acid, caffeine, ibuprofen, and theophylline). Because daily dose alone could not discriminate positive compounds from negative compounds (Fig. 7), the CB amount is still very important for evaluation of DILI.

Earlier investigations of whether CB can estimate DILI were performed with positive drugs (Evans et al., 2004; Masubuchi et al., 2007; Takakusa et al., 2008), but only a few researchers have made comparisons with examples of safe drugs. However, it was recently reported that the total daily dose of drugs improves the discrimination between hepatotoxic and nontoxic drugs based on in vitro CB intrinsic clearance (Obach et al., 2008; Bauman et al., 2009). Nevertheless, false-positive and false-negative results could still not be avoided. Although our study was limited to compounds that are metabolized by P450 or UGT in microsomes, positive and negative compounds could be distinguished. It is now necessary to validate our evaluation methods with more compounds.

During drug development, CB is determined after radiolabeled compound synthesis, and the daily dose is usually estimated before clinical trials based on animal data and human in vitro data. Therefore, if we can set a criterion for the CB × dose using positive and negative compounds with regard to DILI, it should be possible to estimate the potential for idiosyncratic DILI and to develop drug candidates that are active within the doses estimated as low risk for DILI. Taken together, our findings suggest that the combination of CB amount and daily dose can estimate the risk of idiosyncratic DILI and provide a basic understanding that should aid further studies for minimizing the likelihood of IDRs.

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Fig. 7. Comparison of negative (○) and positive (●) compounds for DILI solely on the basis of maximum daily dose. For abbreviations, see the legend to Fig. 4.


Address correspondence to: Dr. Takanori Hashizume, Pharmacokinetics Research Laboratories, Dainippon Sumitomo Pharma Co., Ltd., 3-1-98, Kasugadenaka, Konohana-ku, Osaka, 554-0022, Japan. E-mail: takanori-hashizume@ds-pharma.co.jp