Evaluation of the potential for drug-induced liver injury
based on in vitro covalent binding to human liver proteins

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Non-standard abbreviations
DILI, drug induced liver injury; CB, covalent binding; UDPGA, UDP glucuronic acid;
IDRs, idiosyncratic drug reactions; P450, cytochrome P450; UGT,
UDP-glucuronosyltransferase; $C_{\text{max}}$, maximum plasma concentration; LC, liquid chromatography; MS/MS, tandem mass spectrometry; m/z, mass-to-charge ratio
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 12 positive compounds (acetaminophen, alpidem, bromfenac, carbamazepine, diclofenac, flutamide, imipramine, nefazodone, tacrine, ticlopidine, tienilic acid, troglitazone) for DILI and 12 negative compounds (acetylsalicylic acid, caffeine, dexamethasone, losartan, ibuprofen, paroxetine, pioglitazone, rosiglitazone, sertraline, theophylline, venlafaxine, 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 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.
Introduction

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 post marketing. 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 following 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 due to 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. Since 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 screen) are available for application during the early stages of drug discovery. However, these assays cannot avoid false negative and false positive (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, U.S. Food and Drug Administration mentioned in the draft guidance document “Drug-Induced Liver Injury: Premarketing Clinical Evaluation” (Food and Drug Administration, 2007) that radiochemical in vitro methods are available to detect and quantify covalent binding (CB) to liver proteins for a drug or its metabolites. Additionally, 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 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.
Methods

Materials

$[^{14}\text{C}]$Alpidem, $[^{14}\text{C}]$carbamazepine, $[^{14}\text{C}]$tacrine, $[^{14}\text{C}]$ticlopidine, $[^{14}\text{C}]$losartan, $[^{14}\text{C}]$sertraline, $[^{14}\text{C}]$venlafaxine and $[^{14}\text{C}]$zolpidem were synthesized by BlyChem Ltd. (Billingham, England). $[^{14}\text{C}]$Bromfenac and $[^{14}\text{C}]$nefazodone were synthesized by GE Healthcare (Buckinghamshire, UK). $[^{14}\text{C}]$Tienilic acid was synthesized by Sekisui Medical Co., Ltd. (Tokyo, Japan). $[^{14}\text{C}]$Troglitazone, $[^{14}\text{C}]$pioglitazone and $[^{14}\text{C}]$rosiglitazone were synthesized in-house. $[^{14}\text{C}]$Acetaminophen, $[^{14}\text{C}]$acetylsalicylic acid, $[^{14}\text{C}]$caffeine, $[^{14}\text{C}]$ibuprofen and $[^{14}\text{C}]$theophylline were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO), $[^{3}\text{H}]$dexamethasone and $[^{14}\text{C}]$diclofenac from GE Healthcare, $[^{3}\text{H}]$flutamide from Moravek Biochemicals (Brea, CA) and $[^{3}\text{H}]$imipramine and $[^{3}\text{H}]$paroxetine from PerkinElmer, Inc (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, dexamethasone, ibuprofen, paroxetine, sertraline, venlafaxine, and zolpidem were purchased from SIGMA (St. Louis, MO). Unlabeled bromfenac and tienilic acid
were from AKos Consulting & Solutions Deutschland GmbH (Steinen, Germany), and Cypex Ltd (Dundee, Scotland, UK), respectively. Unlabeled losartan and theophylline were from LKT Laboratories, Inc (University Avenue West St. Paul, MN) and unlabeled pioglitazone was from KEMPROTEC Limited (Middlesbrough, UK). Radiolabeled and unlabeled compounds were combined to make the substrate solutions (1 mM, ca. 0.7 GBq/mmol).

Human liver microsomes (mixed gender pool of 50 individuals) were obtained from XenoTech, LLC (Lenexa, 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. UDP glucuronic acid (UDPGA) and alamethicin were purchased from SIGMA. All other regents and solvents were of the highest grade commercially available.

Incubation

To estimate the potential for CB to human liver proteins, the following 4-arm assays were conducted. In the first arm (1st arm), radio-labeled compounds (final concentration: 10 µM) were incubated with 1 mg/mL pooled human liver microsomes
and 1 mM NADPH for 60 min at 37°C in 500 µL of a reaction mixture consisting of 50 mM phosphate buffer (pH 7.4) and 10 mM MgCl₂. The second arm (2nd arm) assay was performed as for the 1st arm using the same mixture fortified with 2 mM UDPGA as a cofactor. The third arm (3rd arm) incubation was performed in the presence of a nucleophile (1 mM GSH) under the second arm conditions. Microsomes used in 2nd and 3rd arms were pre-incubated with alamethicin (50 μg/mg of 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 (4th arm), radio-labeled compounds (10 μM) were incubated with human cryopreserved hepatocytes (1×10⁶ cell/mL) for 4 hr at 37°C under an atmosphere of 95% air/5% CO₂ on 24-well plates in 300 µL 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 7 times with 80% (v/v) methanol containing 5% (w/v) trichloroacetic acid, 7 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 I (Nacalai Tesque, Inc., Kyoto, Japan), and radioactivity was measured using a liquid scintillation counter, Tri-Carb 2700TR (PerkinElmer Inc.). Protein determination was conducted using the BAC Protein Assay kit (Pierce, Rockford, IL). The CB amount was calculated from the following equation:

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\text{CB (pmol equivalent of drug/mg protein)} = \frac{\text{radioactivity in the protein solution (dpm/mL)}}{\text{specific radioactivity of substrate (dpm/pmol) × 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 μm, 4.6 ID x 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., 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 (see Fig. 2). Radioactivity and mass analyses were conducted in parallel using a micro splitter. Radioactivity was detected with a flow-scintillation detector, a Radiomatic 610TR (PerkinElmer, Inc.), using Ultima Flo-M scintillation cocktail (PerkinElmer Inc.). Mass analysis was conducted on a 4000 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, CA) equipped with an electrospray ion source. For detected radio active peaks, 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 obtained molecular mass and metabolite information as previously reported (bromfenac: Kirkman et al., 1998, 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 LCMS-IT-TOF, Kyoto, Japan) equipped with an electrospray ion source.
Results

Determination of CB amounts and metabolite profiling

The inter-day reproducibility of CB determination using compound A (1st, 2nd, 3rd and 4th arm) was examined for 3 days. The average (pmol/mg protein) and the coefficient of variation (%) of inter-day precision were 289.5 and 5.3 (1st arm), 287.8 and 5.1 (2nd arm), 56.8 and 5.1 (3rd arm), 47.1 and 13.0 (4th 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 [14C]troglitazone (m/z 440 of the deprotonated molecular ion) with human liver protein (Supplemental figure 14). As previously reported (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 characteristically detected in 1st, 2nd, 3rd and 4th arm assays, respectively. Moreover, GSH-dependent peaks were distinctly found.
in radio-chromatograms of diclofenac, flutamide, nefazodone and ticlopidine, all positive compounds for DILI. GSH-dependent peak(s) of other compounds were not detected clearly on addition GSH. Since 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. For diclofenac, glucuronide conjugates, including acylglucuronides, were formed in the 2nd arm assay, in addition to the hydroxy metabolites generated in the 1st arm assay. On the other hand, bromfenac was metabolized to a unique metabolite, bromfenac lactam, and no acylglucuronide was detected in the presence of UDPGA. Diclofenac-lactam, which has been detected in human hepatocytes (Bort et al., 1999), was not detected in this study.

Radio-chromatogram and MS data in 1st, 2nd, 3rd and 4th arm about 14 compounds (alpidem, bromfenac, carbamazepine, diclofenac, flutamide, imipramine, losartan, nefazodone, pioglitazone, rosiglitazone, tacrine, ticlopidine, tienilic acid, troglitazone) are shown in supplemental figure 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 UGT. 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 similar metabolic pathways to positives, high prescription rates, and/or high dosage. Determined amounts of radioactivity derived from the compounds CB to human liver proteins are shown in Table 1.

In the 1st 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 over 50 pmol/mg protein. In particular, metabolites of rosiglitazone and dexamethasone highly bound to microsomal proteins (1189 and 445 pmol/mg protein, respectively).
In the 2nd 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 3rd 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 2nd 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 2nd and 3rd arm values) were significantly correlated with the 2nd arm value ($R=0.92$), except with dexamethasone data.

In the 4th arm assay, CB in hepatocytes capable of other metabolic activation/inactivation as well as NADPH- and UDPGA-dependent activation showed a 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 no possible through the 1st arm to 4th arms (Fig. 5). CB in hepatocytes tended to improve the discrimination between positives and negatives but many values for positive compounds still overlapped with those for negatives (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×C\text{max}, CB×dose (maximum daily dose). The higher of the two values for the 1st and 2nd arms was employed as the value for CB in microsomes. The CB×C\text{max}, which may reflect systemic exposure, tended to be higher for positive than for negative compounds but discrimination was still insufficient in microsomes (Fig. 6A). The CB×C\text{max} in hepatocytes did not improve the discrimination (Fig. 6B). In contrast, the 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. 6C, D). However, the maximum daily dose alone could not discriminate positive from negative compounds (Fig. 7).
Discussion

In the present 1st arm study of CB with 12 DILI positive and 12 DILI negative compounds, values for all positives exceeded 50 pmol/mg of microsomal protein, the Evans’s target threshold (under the same experimental conditions as the 1st arm, Evans et al., 2004). However, the CB of many negative compounds also exceeded 50 pmol/mg so that positives could not be distinguished from negatives on this basis (Fig. 5A).

We expected bioactivation by acylglucuronide or decrease of oxidative metabolism by increase of glucuronidation to change CB and to improve the overlapping of CB between positive and negatives. 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 hydroxy-diclofenac-glucuronides were here detected on addition of UDPGA (Fig. 2). Isomerization of acylglucuronide conjugates may cause peaks for these glucuronides as previously reported (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., 1998; Osman et al., 1998) 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 why bromfenac-lactam was detected and diclofenac-lactam was not detected may be the difference of 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 might be acylglucuronide formation. The reasons for decrease of 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 figure 1). Unfortunately, despite addition of UDPGA, CB amounts for positives with microsomes still overlapped negatives in the 2nd 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 3rd arm condition) might mimic the in vivo liver condition and improve the ability to distinguish between positives and negatives. However, addition of GSH did not remove the overlapping (Fig. 5C). GSH scavenging effects (2nd arm – 3rd arm values) did show a significant correlation with the following 2nd 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), 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 positives still overlapped negatives (Fig. 5D). This
can be attributed to the fact that test compounds metabolized by P450 and UGT were
selected for the present study. Since P450 mediated bioactivation has been reported
to be the first step in generation of many IDR s (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
extra-microsomal metabolism is involved in activation/inactivation. In this study,
rosiglitazone, which demonstrated a relatively low CB amount in hepatocytes (4th
arm) as compared with microsomes (2nd arm), may have undergone extra-microsomal
inactivation of reactive metabolites (Fig. 4). One bioactivation pathway of valproic
acid, which does induce DILI, is known to be mitochondrial β-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 extra-microsomal 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 figure 14). In the 1st arm, an oxidative metabolite, a quinone form, was detected and the CB was very high. In the 2nd arm, glucuronides were detected but the CB was still high. In the 3rd arm, GSH adducts were detected and the CB decreased markedly. Additionally, GSH-dependent peaks were distinct in radio-chromatograms from diclofenac, flutamide, nefazodone and ticlopidine, which are all positive compounds for DILI (Supplemental figure 4, 5, 8, 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 radio-chromatography, even though the CB of rosiglitazone was decreased markedly by GSH (Supplemental figure 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 due to the instability and the difficulty in detection by MS. 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 compounds from negative with metabolite profiling data. Evaluation purely on the basis of the CB value or detection of GSH adducts would give many false-positives/negatives 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 positives, we multiplied the in vitro CB amount by the $C_{\text{max}}$ ($CB \times C_{\text{max}}$) related to systemic exposure, or the maximum daily dose ($CB \times \text{dose}$) related to the theoretical maximum exposure of liver to reactive metabolites. On evaluation by $CB \times C_{\text{max}}$, there was still a great deal of overlap between the two groups in microsomes and hepatocytes and discrimination was not possible (Fig. 6A, 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. 6C, D). With retrospective findings, daily dose is one of the most important factors for IDR, and very low dose drugs (<10 mg/day) appear to be void of DILI induction potential (Uetrecht, 2000). Our in vitro data are consistent with this epidemiologic speculation. Interestingly, in the negative compounds, drugs with high amounts of CB had low
daily doses (dexamethasone, rosiglitazone), and drugs with high daily doses had low amounts of CB (acetysalicylic acid, caffeine, ibuprofen, theophlline). Since daily dose alone could not discriminate positives from negatives (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 few 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 negatives could still not be avoided. Although our study was limited to compounds which are metabolized by P450 or UGT in microsomes, positives and negatives could be distinguished. It is now necessary to validate our evaluation methods with more compounds.

During drug development, CB is determined after labeled 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 which 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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Footnotes

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Legends for figures

Figure 1. Chemical structures of positive and negative compounds for DILI.

The asterisks indicate the positions of the radio-label.

Figure 2. Effects of UDPGA on radio-chromatograms obtained from filtrates after incubation of bromfenac and diclofenac with human liver microsomes

$[^{14}C]$Bromfenac (10 μM) and $[^{14}C]$diclofenac (10 μM) were incubated with human liver microsomes under the following conditions: 1st arm, added cofactor NADPH; 2nd arm, added cofactors NADPH and UDPGA. The elutes were analyzed by radio-LC-MS/MS and radio-chromatograms and characteristic metabolites are shown. The mobile phase consisted of a linear gradient of solvent A (10 mM ammonium acetate) and solvent B (acetonitrile) according to the following program B (%): 5 (0 min)-35 (20 min)-60 (35 min)-100 (45 min). $[M+H]^+$, protonated molecule

Figure 3. Correlation between CB amounts in microsomes (2nd arm value) and GSH scavenging effects (2nd arm value – 3rd arm value)
The 2nd arm value is the CB amount in microsomes in the presence of NADPH and UDPGA. The 3rd arm value is the CB amount in microsomes in the presence of NADPH, UDPGA and GSH. CB amounts of acetyl salicylic 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.

Figure 4. Correlation between CB amounts in microsomes (2nd arm value) and hepatocytes (4th arm value)

The 2nd arm value is the CB amount in microsomes in the presence of NADPH and UDPGA. The 4th arm value is the CB amount in hepatocytes. 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, Acetyl salicylic acid; CAF, Caffeine; DEX, Dexamethasone; IBU, Ibuprofen; LOS, Losartan; PAR, Paroxetine; PIO, Pioglitazone; ROSI, Rosiglitazone; SER, Sertraline; THEO, Theophylline; VEN, Venlafaxine; ZOL, Zolpidem. Note that ASA, IBU, THEO: CB amounts in 2nd arm.
are lower than that for the control without NADPH and UDPGA. These compound values are not included in this correlation analysis.

Figure 5. Comparison of negative (○) and positive (●) compounds for DILI by CB amounts in human liver protein.

Tested compounds (10 μM) were incubated under the following conditions: 1st arm, liver microsomes – added cofactor NADPH (A); 2nd arm, liver microsomes – added cofactors NADPH and UDPGA (B); 3rd arm, liver microsomes – added cofactors NADPH, UDPGA and GSH (C); 4th arm, cryopreserved hepatocytes (D).

Abbreviations correspond to the same compound names in the caption to Fig. 4. Note that ASA, IBU, THEO: CB amounts are lower than that for the control without NADPH and UDPGA.

Figure 6. Comparison of negative (○) and positive (●) compounds for DILI by CB amounts and pharmacokinetic parameters with regard to exposure to liver

The CB×C_{max} is CB multiplied by the corresponding C_{max} in table 1 (A and B). The CB×dose is CB multiplied by the corresponding maximum daily dose in table 1 (C and D). The CB in microsomes amount is the higher of the values for the 1st and 2nd
arms. Abbreviations correspond to the same compound names in the caption to Fig. 4.

Note that ASA, IBU, THEO: CB amounts are lower than that for the control without NADPH and UDPGA.

Figure 7. Comparison of negative (○) and positive (●) compounds for DILI solely on the basis of maximum daily dose.

Abbreviations correspond to the same compound names in the caption to Fig. 4.
Table 1. *CB, maximum daily dose and C*$_{\text{max}}$ *of the tested compounds*

Test compounds (10 μM) were incubated with human liver microsomes in the presence of NADPH (1st arm), NADPH and UDPGA (2nd arm), NADPH, UDPGA and GSH (3rd arm), or with hepatocytes (4th arm). The CB amounts were determined with duplicate incubations. Daily dose and C$_{\text{max}}$ represent the maximum daily dose and the maximum concentration at the maximum daily dose, respectively. Daily dose and C$_{\text{max}}$ 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; 8) Lammert et al., 2008

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Reference, Reference for $C_{\text{max}}$ and daily dose; ND, The CB for test compounds was lower than that for the control without NADPH and UDPGA; *, $C_{\text{max}}$ value normalized by dose; **, $C_{\text{max}}$ value normalized by body weight.
Figure 1

Positive compounds for DILI


$[^{3}H]$-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

Figure 3

A scatter plot showing the relationship between the 2nd arm value (pmol/mg protein) and the difference between 2nd arm value and 3rd arm value (pmol/mg protein). The data points are categorized as negative (open squares) and positive (filled squares). A regression line is fitted to the data with an R value of 0.92 and a P value of less than 0.001.
Figure 4

CB value in hepatocytes (pmol/mg protein) vs. CB value in microsomes (pmol/mg protein)

- Positive compounds:
  - NEF
  - TRO
  - TIC
  - FLU
  - TA
  - PAR
  - TAC
  - IMI
  - ROSI

- Negative compounds:
  - ASP
  - IBU
  - ZOL
  - VEN
  - CAF
  - ASA
  - SERO
  - LOS
  - DIC
  - DEX
  - BRO
  - APAP

Correlation coefficients:
- R = 0.81
- P < 0.001