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Combination of glutathione trapping and time-dependent inhibition assays as a predictive method of drugs generating highly reactive metabolites

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Running title: Minimizing metabolic activation by GSH trapping and TDI assay

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Nonstandard abbreviations:

CB, covalent binding; IDT, idiosyncratic drug toxicity; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; GSH, glutathione; HLM, human liver microsome; RM, reactive metabolite; TDI, time-dependent inhibition; MBI, mechanism-based inhibition; $CL_{int,\ RMs,}$ combined intrinsic clearance for forming RMs; LC/MS; liquid chromatography mass spectrometry

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Abstract

Covalent binding (CB) of reactive metabolites (RMs) is potentially involved in severe adverse drug reactions. Because the CB assay is of low throughput and costly, a qualitative trapping assay using agents such as [^{35}S]glutathione (GSH) is often performed in the early stages of drug discovery. Trapping methods alone, however, cannot replace the CB assay. We hypothesized that the time-dependent inhibition (TDI) assay might be complementary to [^{35}S]GSH trapping assay in detecting RMs. We performed CB assays, [^{35}S]GSH trapping assays, and TDI assays for 42 structurally diverse compounds. First, we showed that the [^{35}S]GSH trapping assay alone does not correlate with the extent of CB. Four compounds that [^{35}S]GSH trapping assay failed to detect but showed high extent of CB were inactivators of the enzyme in the TDI assay. There was a tendency for compounds judged as positive in the TDI assay to show a high degree of CB irrespective of the result of the [^{35}S]GSH trapping assay. Finally, to combine parameters from the two assays, we introduced intrinsic clearance to dscribe the formation of RMs ($CL_{int, RMs}$). The Spearman rank correlation coefficient between the extent of CB and $CL_{int, RMs}$ was 0.77 ($p < 0.0001$), which was better than that for the formation rates of [^{35}S]GS adducts. We therefore demonstrated that a combination of the [^{35}S]GSH trapping and TDI assays is an effective method for detecting compounds potentially capable of generating highly reactive metabolites in the early stages of drug discovery.

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Introduction

The metabolic activation of a compound to form a reactive metabolites (RMs) that undergo covalent binding (CB) to cellular macromolecules is considered to be an undesirable property for a drug (Kaplowitz, 2005; Baillie, 2006; Uetrecht, 2007). Current hypotheses based on retrospective studies suggest that metabolism to form RMs is an initial step in many drug-induced adverse events, such as direct damage to target organs or immune-mediated toxicity, that are involved in idiosyncratic drug toxicity (IDT). IDT occurs in rare cases and is generally manifested as severe hepatotoxicity, agranulocytosis, or other illnesses (Uetrecht, 2001). Many pharmaceutical companies now perform an assay for CB to assess the risk of a compound producing an adverse event (Reese et al., 2010 ; Thompson et al., 2010; Uetrecht, 2001; Evans et al., 2004; Obach et al., 2008; Bauman et al., 2009; Nakayama et al., 2009; Usui et al., 2009).

The current gold-standard approach for reliable quantification of the extent to which RMs covalently bind to proteins is the CB assay using radiolabeled compounds. However, during the process of lead optimization, the CB assay, because of its low throughput and high costs, is unsuitable for assessing whether lead compounds are liable to undergo bioactivation. Pharmaceutical companies have therefore made considerable efforts to develop alternative methods with higher throughputs. Trapping methods using [^{35}S]glutathione (GSH), K^{14}CN, or the fluorescent dansylate analogue of GSH have been established for semiquantitative assessment of the bioactivation potential of compounds (Evans et al., 2004; Inoue et al., 2009; Kumar et al., 2009; Tang and Lu, 2010). Several reports have shown the existence of a positive correlation between the extent of CB and the rate of adduct formation of the trapping agent. For example, Takakusa et al.

demonstrated a good correlation of CB within a single series of derivatives by using [^{35}S]GSH as the trapping agent (Takakusa et al., 2009). Meneses-Lorente et al. obtained a similar result for piperidine-containing compounds by using K^{14}CN trapping (Meneses-Lorente et al., 2006).

However, trapping methods alone cannot replace the CB assay, because methods that use nucleophiles can only detect RMs that have the opportunity to diffuse into the incubation medium from the active site of the enzyme. We hypothesized that mechanism-based inhibition (MBI), which is caused by the binding of short-lived RMs to the active site of the enzymes and is detected in time-dependent inhibition (TDI) assays, could be complementary to trapping methods. There are some reports regarding TDI and the extent of CB. For example, bergamottin is a mechanism-based inhibitor of the enzymes, CYP3A4, 1A2, 2B6, 2C9, and 2C19. When ^{14}C-labeled bergamottin was incubated with membrane preparations of individual human recombinant P450 enzymes, the extent of CB correlated well with the inhibitory potentials of these enzymes (Kumar et al., 2009). On the basis of this result, a TDI assay might be capable of detecting RMs, and might provide a surrogate method for the overall assessment of bioactivation potential in advance of preparing labeled compounds for a CB assay.

In this study, we investigated the relationships between the formation rate of [^{35}S]GS adducts, the enzyme inactivation rate in the TDI assay, and the extent of CB in human liver microsomes (HLMs) for 42 structurally diverse marketed compounds to clarify whether the incorporation of TDI assays would result in improved accuracy in detection of RMs.

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Materials and Methods

Materials

A total of 42 radiolabeled compounds were used. Amodiaquine, benzbromarone, carbamazepine, clozapine, clopidogrel, donepezil, flutamide, furosemide, imipramine, nevirapine, olanzapine, pioglitazone, rosiglitazone, sulfamethoxazole, tienilic acid, tacrine, valsartan, zafirlukast, and zomepirac (all ^{14}C-labeled) were obtained from BlyChem Ltd. (Billingham, UK). Levofloxacin, olmesartan, pravastatin, and ticlopidine (all ^{14}C-labeled) were obtained from Sekisui Medical Co., Ltd. (Ibaraki, Japan). ^{14}C-labeled atorvastatin was obtained from MDS Pharma Services (Montreal, QC, Canada). Celecoxib and warfarin (both ^{14}C-labeled), and propranolol and tamoxifen (both ^{3}H-labeled) were purchased from GE Healthcare (Little Chalfont, UK). Acetaminophen, aminopyrine, caffeine, diclofenac, erythromycin, procainamide, and valproic acid (all ^{14}C-labeled), and ethinylestradiol and fluoxetine (both ^{3}H-labeled) were purchased from American radiolabeled Chemicals, Inc. (St. Louis, MO). Indomethacin and phenytoin (both ^{14}C-labeled), and ^{3}H-labeled verapamil were purchased from PerkinElmer (Boston, MA). ^{14}C-labeled amlodipine and ^{3}H-labeled ritonavir were purchased from Moravek Biochemicals (Brea, CA). The specific activities of ^{14}C-labeled compounds were in the range 13–58 mCi/mmol. ^{3}H-labeled compounds were diluted with their unlabeled compounds to give a final specific activity of 200 mCi/mmol. Unlabeled acetaminophen, amodiaquine, benzbromarone, carbamazepine, clozapine, diclofenac, erythromycin, ethinylestradiol, fluoxetine, flutamide, furosemide, imipramine, indomethacin, phenytoin, propranolol, sulfamethoxazole, tacrine, tamoxifen, ticlopidine, verapamil, warfarin, and zomepirac were obtained from Sigma (St. Louis, MO). Unlabeled aminopyrine, amlodipine, and valproic acid were purchased from Wako Pure Chemical Industries, Ltd.

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(Osaka, Japan). Unlabeled nevirapine, olanzapine, and ritonavir were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Unlabeled caffeine and zafirlukast were obtained from Fluka (Buchs, Switzerland) and Cayman Chemical (Ann Arbor, MI), respectively. Unlabeled atorvastatin, celecoxib, clopidogrel, donepezil, levofloxacin, olmesartan, pioglitazone, pravastatin, procainamide, rosiglitazone, tienilic acid, and valsartan were synthesized by Daiichi Sankyo Co., Ltd. Pooled HLMs (n = 50, mixed gender) were purchased from XenoTech (Lenexa, KS). [^{35}S]GSH was purchased from PerkinElmer (Buchs, Switzerland). Phenacetin was purchased from Wako Pure Chemical (Osaka, Japan). Acetaminophen, paclitaxel, tolbutamide, dextromethorphan, and dextrorphan were purchased from Sigma (St. Louis, MO). Midazolam was purchased from Ultrafine (Manchester, UK). Bupropion, hydroxytolbutamide, (*S*)-mephenytoin, (±)-4'-hydroxymephenytoin, and 1'-hydroxymidazolam were purchased from BD Gentest (Franklin Lakes, NJ). (±)-Hydroxybupropion was purchased from Toronto Research Chemicals Inc (North York, ON, Canada). β-NADP$^+$ and glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan), and glucose-6-phosphate (G6P) was obtained from Sigma (St. Louis, MO). All other reagents and solvents were of the highest grade commercially available.

CB assay using HLMs

The experimental procedure was based on that used in previously reported studies (Evans et al., 2004; Masubuchi et al., 2007). The experiments were performed in triplicate. The incubation mixture consisted of the following: 10 μM radiolabeled test compound (substrate), 2 mg/mL HLMs, 0.1 M potassium phosphate buffer (pH 7.4), 25 mM G6P, 2 units/mL G6PDH, and 10 mM $MgCl_2$. The mixture was preincubated for 3 min at 37 °C.

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Reaction was initiated by the addition of β-$NADP^+$ to reach a final concentration of 2.5 mM, and the final incubation volume was 0.5 mL. As the substrates were dissolved in acetonitrile, the final incubation mixture contained 1% v/v acetonitrile. Radiolabeled compounds of at least 95% purity were used. After incubation of the mixture for 1 h, the reaction was terminated by the addition of ice-cold acetonitrile (0.5 mL). The mixture was then vortexed, sonicated in an ultrasonic bath, and centrifuged. The precipitated protein was washed twice with each of the following solvents in sequence: 80% (v/v) aqueous methanol containing 10% (w/v) trichloroacetic acid, diethyl ether–methanol (1:1, v/v), and 80% aqueous methanol. The resulting precipitated protein was dissolved in 0.5 mL of 1.0 M NaOH. Aliquots were taken for protein assay with a DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) and also for the determination of radioactivity by using a liquid scintillation counter after mixing the aliquot with Hionic-Fluor scintillation cocktail (PerkinElmer, Wellesley, MA). The amount of the test drug-related material as radioactivity covalently bound to the microsomal protein was calculated as a measure of the extent of CB (pmol/mg protein).

[^{35}S]GSH trapping assay

The experimental procedure was based on that used in a previously reported study (Takakusa et al., 2009). Each test compound was dissolved in acetonitrile–methanol (9:1, v/v) or acetonitrile– water (1:1, v/v) to prepare 10 mM stock solution. Each test compound (100 μM) was then incubated at 37 °C with HLMs (2 mg/mL protein), and an NADPH-generating system in 0.1 M potassium phosphate buffer (pH7.4) supplemented with [^{35}S]GSH (0.2 mM, 925 kBq/test). The final reaction mixture contained 25 mM G6P, 2 units/mL G6PDH, and 10 mM $MgCl_2$. Reaction was initiated by the addition of

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β-$NADP^+$ to reach a final concentration of 2.5 mM after a 3-min preincubation at 37 °C. The final incubation volume was 0.5 mL. A sample containing vehicle solution (9:1 acetonitrile–methanol or 1:1 acetonitrile–water) without substrate was used as a negative control. After 30 min incubation, the reaction was terminated by the addition of ice-cold acetonitrile (0.5 mL). The sample was centrifuged at 2000*g* for 15 min at 4 °C and then the supernatant was concentrated to approximately 0.1 mL by using a centrifuge evaporator. The concentrated sample (50 μL) was analyzed by radio-HPLC using a Shimadzu LC-10Avp HPLC system (Kyoto, Japan) with an online radioactivity detector (RAD) (β-RAM model 3, IN/US Systems, Tampa, FL). The HPLC system was equipped with a 4.6 mm I.D. × 150 mm (5 μm) column (TSKgel ODS-100V, TOSOH, Tokyo, Japan) coupled with a 3.2 × 15 mm (5 μm) guard column (TSKguardgel ODS-100V, TOSOH). The sample was separated by using linear-gradient conditions at a flow rate of 1 mL/min. Ecoscint Flow (National Diagnostics, Atlanta, GA) was used as the scintillation fluid cocktail at a flow rate of 1 mL/min. The [^{35}S]GS adduct peaks were identified by visual comparison of radiochromatograms from the samples incubated with the individual test compound and a negative control. The sum of the radioactivity counts of the [^{35}S]GS adduct peaks (AreaGS [counts]) was calculated by using the computer software Laura 3.1 (LabLogic Systems, Sheffield, UK). The formation rate of [^{35}S]GS adducts was calculated by means of the following equation:

formation rate of the [^{35}S]GS adducts [pmol/min/mg protein]

= $Area_{GS}/Area_{total}$ × the total amount of GSH [pmol]/ the reaction time [min]/the amount of HLMs protein [mg protein]

The total amount of GSH was 100,000 pmol, the reaction time was 30 min, and the amount of HLMs protein was 1 mg protein. $Area_{total}$ [counts] expresses the total

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radioactivity counts observed on the radiochromatogram, calculated by means of the following equation:

$Area_{total}$ = Radioactivity injected [dpm] × f [counts/dpm]

The correction constant f [counts/dpm] was calculated by dividing the total radioactivity counts observed on the radiochromatogram of a 1:20 diluted sample by its radioactivity [dpm]. A liquid scintillation counter (TRI-CARB 2900TR, PerkinElmer) was used to measure the radioactivity [dpm] of the sample. The lower limit of quantification in each assay was set at the value of the formation rate of [^{35}S]GS adducts of diclofenac, which was obtained from each assay.

TDI assay

The experimental procedure was based on that used in a previously reported study (Watanabe et al., 2007). The TDI assay was automated by using a Beckman Biomek FX (Beckman Coulter Inc., Fullerton, CA). The preincubation solution contained 0.5 (for CYP3A4), 1.0 (for CYP1A2, 2B6, 2C8, 2C9 and 2D6) or 2.0 (for CYP2C19) mg/mL HLMs. The probe substrate and their final concentration were as follows: 50 μM phenacetin for CYP1A2, 50 μM bupropion for CYP2B6, 10 μM paclitaxel for CYP2C8, 200 μM tolbutamide for CYP2C9, 50 μM (*S*)-mephenytoin for CYP2C19, 30 μM dextromethorphan for CYP2D6, or 2.5 μM midazolam for CYP3A4. The preincubation and incubation solutions contained 0.1 M sodium phosphate buffer. The preincubation solution (178 μL) was transferred into the wells of the assay plate, and then 2 μL of a test compound solution or control solution (10% v/v dimethyl sulfoxide) was added. The concentration of each test compound was initially set at 100 μM. When the reversible inhibition of the compound was strong (percentage of $control_{(0\ min)}$ < 50%), it was diluted

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to a suitable concentration, as listed in Table 1. The preincubation reactions were initiated by the addition of 20 μL of an NADPH-generating system consisting of 25 mM β-$NADP^+$, 250 mM G6P, 20 units/mL G6PDH, and 100 mM $MgCl_2$. The final volume of the preincubation mixture was 200 μL. For the 0-min preincubation, 20 μL of each preincubation mixture was immediately transferred into 180 μL of the incubation solution and then incubated for 10 min. For the 30-min preincubation, each preincubation mixture was diluted ten-fold with the incubation solution after 30 min. At the end of the incubation reactions, 50 μL aliquots of each incubation solution were added to 200 μL of methanol containing an internal standard. The samples were filtered through Captiva (Varian, Inc., Lake Forest, CA). All samples were analyzed by an liquid chromatography mass spectrometry (LC/MS) system consisting of an API 4000 (Applied Biosystems, Foster City, CA) with Shimadzu HPLC pumps and autosampler systems. Chromatographic separation was performed with Capcell Pak C18 MGII (S-5 μm, 2.0 mm I.D. × 100 mm, Shiseido Co., Ltd., Tokyo, Japan). The percentages of the metabolic activity (percentage of $control_{(0\ min)}$ and percentage of $control_{(30\ min)}$) was obtained for each sample after a 0-min or 30-min preincubation with an inhibitor, by comparison with the control sample after a 0 min or 30 min of preincubation without an inhibitor, as follows:

$$\text{Percentage of control}_{(0\ min)} = v_{(0\ min,\ +inhibitor)}/v_{(0\ min,\ -inhibitor)} \times 100$$

$$\text{Percentage of control}_{(30\ min)} = v_{(30\ min,\ +inhibitor)}/v_{(30\ min,\ -inhibitor)} \times 100$$

where, $v_{(0\ min,\ \pm\ inhibitor)}$ is the metabolic activity after a 0-min preincubation with (+) or without (–) an inhibitor, and $v_{(30\ min,\ \pm\ inhibitor)}$ is the metabolic activity after a 30-min preincubation with (+) or without (–) an inhibitor. By using these values, the percentage of enzyme inactivation after 30 min of preincubation relative to that after 0 min of preincubation was calculated as follows:

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Percentage of enzyme inactivation = 100 × [1 – percentage of control$_{(30\ min)}$/percentage of control$_{(0\ min)}$]

The threshold for percentage of enzyme inactivation in each assay was set at 10%.

Calculation of the enzyme inactivation rate

The percentage of enzyme inactivation was transformed into the enzyme inactivation rate by correcting for the incubation time and the contents of each isoform. The following equation was used to calculate the enzyme inactivation rate in each of the tested CYP isoforms:

Enzyme inactivation rate [nmol/min/mg protein]

= percentage of enzyme inactivation × contents of each isoform [nmol/mg protein]/preincubation time [min]

The values for the contents of each isoform were taken from Simcyp version 8.0 (Simcyp Ltd., UK) as follows: CYP1A2: 52, CYP2B6: 17, CYP2C8: 24, CYP2C9: 73, CYP2C19: 14, CYP2D6: 8, and CYP3A4: 142 pmol/mg protein. The preincubation time was 30 min. The sum of the rates of inactivation of all the tested CYP isoforms was calculated.

Calculations of the combined intrinsic clearance for forming RMs based on the formation rate of [^{35}S]GS adducts and the enzyme inactivation rate in the TDI assay

The formation rate of [^{35}S]GS adducts and the enzyme inactivation rate in TDI assay were transformed into an intrinsic clearance for forming RMs by correcting for the concentration of each tested compounds in the test systems by assuming that each enzyme reaction is linear and that the enzyme inactivation rate is equal to the formation rate of the RMs. The following equation was used to calculate the combined intrinsic clearance for

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forming RMs ($CL_{int, RMs}$):

$CL_{int, RMs}$ [μL/min/mg protein]

$= CL_{int, RMs (GSH)} + CL_{int, RMs (TDI)}$

= formation rate of the [^{35}S]GS adducts [pmol/min/mg protein]/substrate concentration [μM] + enzyme inactivation rate in each isoform [pmol/min/mg protein]/tested concentration in preincubation in TDI assay [μM]

where, $CL_{int, RMs (GSH)}$ is the intrinsic clearance for forming RMs calculated from the formation rate of [^{35}S]GS adducts and $CL_{int, RMs (TDI)}$ is the intrinsic clearance for forming RMs calculated from the enzyme inactivation rate in TDI assay.

Classification of the tested compounds on the basis of the results of the [^{35}S]GSH trapping assay and the TDI assay

The compounds were classified into four groups on the basis of the quantitative results of the [^{35}S]GSH trapping assay and the TDI assay. Compounds that show detectable [^{35}S]GS adduct peaks were defined as positive in the [^{35}S]GSH trapping assay. Compounds that showed a percentage of enzyme inactivation of more than 10% in at least one isoform in the TDI assay were defined as positive in the TDI assay. Group 1 consisted of compounds that were judged as negative in both assays, Group 2 consisted of compounds that were judged as positive in the TDI assay only, Group 3 consisted of compounds that were judged as positive in the [^{35}S]GSH trapping assay only, and Group 4 consisted of compounds that were judged as positive in both assays.

Statistical analysis

Statistical analyses were performed with SAS System Release 8.2 (SAS Institute Inc.).

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Spearman rank correlation coefficients were calculated to evaluate the relation between the rates of formation of [^{35}S]GS adducts and the extent of CB (Figure 1), between the enzyme inactivation rates and the extent of CB (Figure 2), and between the values of $CL_{int,RMs}$ and the extent of CB (Figure 3). In relation to the extent of CB, the Wilcoxon rank-sum test was performed for group comparison between Groups 1 and 2, 1 and 3, 1 and 4, 2 and 4, and 3 and 4; a value of $p < 0.05$ was considered to be statistically significant (Table 2).

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Results

The extent of CB in HLMs, the formation rate of [^{35}S]GS adducts, and the percentage of enzyme inactivation in the TDI assay

A summary of the extent of CB in HLMs, the formation rate of [^{35}S]GSH adducts, and the percentage of enzyme inactivation in the TDI assay with the previous reports on GSH adduct formation, MBI or TDI as references is presented in Table 1. With the exception of levofloxacin, all the compounds showed measurable degrees of CB. The extent of CB of the tested compounds ranged from 1.4 pmol/mg protein for valsartan to 937.5 pmol/mg protein for ethinylestradiol. The extent of CB was more than 50 pmol/mg for 19 compounds. 50 pmol/mg was a target maximum value for CB in selecting drug candidates for entry into development as reported by the Merck group (Evans et al., 2004). [^{35}S]GS adduct peaks were detected for 21 compounds, with formation rates ranging from 1.0 pmol/min/mg protein for diclofenac to 43.1 pmol/min/mg protein for ticlopidine. The other 21 compounds did not show measurable [^{35}S]GS adduct peaks in their radiochromatograms. The concentrations of the test compounds ranged from 0.1 to 100 μM in the TDI assay, depending on the strength of reversible inhibition. Enzymes CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4 were inactivated by 9, 4, 1, 2, 5, 3, and 11 compounds, respectively. Twenty-one compounds did not inactivate any isoform of the tested CYP enzyme.

Relationship between the formation rate of [^{35}S]GS adducts and the extent of CB in HLMs

To investigate the relationship between the formation rate of [^{35}S]GS adducts and the extent of the CB in HLMs, the logarithm of the extent of CB was plotted against the logarithm of the formation rate of [^{35}S]GS adducts (Figure 1). The Spearman rank

correlation coefficients (r) between the formation rate of [^{35}S]GS adducts and the extent of the CB was calculated to be 0.50 (p = 0.0008). Four compounds (erythromycin, pioglitazone, ritonavir, and tacrine) that showed an extent of CB of more than 50 pmol/mg protein did not show detectable [^{35}S]GS adduct peaks.

Relationship between the enzyme inactivation rate in the TDI assay and the extent of CB in HLMs

To consider the enzyme inactivation for all the tested CYP isoforms together, the percentage of enzyme inactivation was corrected for the incubation time and the contents of the isoform, and the enzyme inactivation rate was calculated. The logarithm of the extent of CB was plotted against the logarithm of the sum of each enzyme inactivation rate (Figure 2). Erythromycin, pioglitazone, ritonavir, and tacrine, which did not show a detectable [^{35}S]GS adduct peak (Figure 1), did inactivate the CYP enzyme. However, the extent of CB was not correlated with the enzyme inactivation rate (r = 0.14, p = 0.37). Furthermore, six compounds with CB of more than 50 pmol/mg protein did not show inactivation of any of the tested CYP isoforms.

Classification of the tested compounds on the basis of the results of the [^{35}S]GSH trapping assay and the TDI assay

The tested compounds were classified into four groups on the basis of the combined results of the GSH trapping and TDI assays. Compounds that show a detectable [^{35}S]GS adduct peaks were defined as positive in the GSH trapping assay. Compounds that showed more than 10% enzyme inactivation (Table 1) in at least one isoform in the TDI assay were defined as TDI positive. The list of the compounds and the mean values ± standard

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deviations, minima, and maxima in the extent of CB in each group are shown in Table 2. Group 1 consists of 11 compounds that showed negative results in both the GSH trapping and TDI assays (–/–); similarly, Group 2 (–/+), Group 3 (+/–), and Group 4 (+/+) contained 10, 10, and 11 compounds, respectively. Group 1 showed a significantly lower extent of CB than did Group 2 and Group 3 ($p = 0.001$ and 0.02, respectively). Group 4 showed a significantly higher extent of CB than did Group 2 ($p = 0.01$). Although not statistically significant ($p = 0.053$), the average value of the extent of CB in Group 4 (353 pmol/mg protein) was higher than that of Group 3 (104 pmol/mg protein).

Relationship between the combined intrinsic clearance for forming RMs and the extent of CB

The $CL_{int, RMs}$ were calculated based on the formation rates of [^{35}S]GS adducts and CYP enzyme inactivation rates (Table 3). The logarithm of the extent of CB was plotted against the $CL_{int, RMs}$ (Figure 3). The Spearman rank correlation coefficient was 0.77 ($p < 0.0001$).

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Discussion

In a previous study, we established a zone classification system that uses the extent of CB in human hepatocytes and the daily dose to assess the risks of IDT (Nakayama et al., 2009). It is clear from our previous result that both the extent of the CB and the daily dose are risk factors for IDT. From a practical point of view, because the efficacious daily dose cannot be easily determined in the early stages of drug discovery, experimental approaches to minimize the risk of bioactivation should be used to minimize the risk of toxicity. However, because of its low throughput and high costs, the CB assay is unsuitable for use as a bioactivation assay during the process of lead optimization.

The [^{35}S]GSH trapping assay, which is often used to detect RMs, has the advantages that it does not require radiolabeled compounds and that it provides quantitative information on the formation of RMs. However trapping methods alone cannot replace the CB assay. We hypothesized that the TDI assay might be complementary to the [^{35}S]GSH trapping assay in detecting RMs.

First, we showed that the [^{35}S]GSH trapping assay alone failed to show good correlation with extent of CB (Figure 1). Then, as expected from our hypothesis, four compounds (erythromycin, pioglitazone, ritonavir, and tacrine) that the [^{35}S]GSH trapping assay failed to detect, but which showed a high extent of CB, were inactivators of the CYP enzyme (Figure 2). Furthermore, there was a tendency for compounds that were judged as positive in the TDI assay to show a high degree of the extent of CB, irrespectively of the results of the [^{35}S]GSH trapping assay (Table 2). As mentioned in the Introduction, a

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mechanism-based inactivator is not easily attenuated by trapping agents; this is probably the result of rapid CB of highly reactive species at the active site of the enzyme before they have the opportunity to diffuse away from the active site and react with the trapping agent (Silverman, 1995; Kalgutkar et al., 2007). To combine the parameters from the [^{35}S]GSH trapping assay with those from the TDI assay, we introduced a new parameter, $CL_{int,\ RMs}$ (Table 3). As shown in Figure 3, the Spearman rank correlation coefficients between $CL_{int,\ RMs}$ and the extent of CB was 0.77 ($p < 0.0001$), which is a better value than that of the formation rate of [^{35}S]GS adducts. Our results show that we can assess the risk of a compound forming RMs by using a combination of [^{35}S]GSH trapping and TDI assays in the early stages of drug discovery; these methods have a relatively high throughput and do not require the use of radiolabeled compounds.

$CL_{int,\ RMs}$ was calculated by assuming that each enzyme reaction is linear and that the enzyme inactivation rate is equal to the rate of formation of RMs. However, the enzyme inactivation rate is proportional at the low concentration of the inactivator, and it can be saturated at high concentrations. Furthermore, TDI not only occurs by the protein-alkylation mechanism but also by quasi-irreversible or heme alkylation (Riley et al., 2007; Hollenberg et al., 2008). For example, the mechanism of inhibition by some of the tested compounds, such as amlodipine, erythromycin, and verapamil, is reported to be quasi-irreversible inhibition (Grimm et al., 2009). RMs that cause TDI by quasi-irreversible inhibition or heme alkylation are not detected in the CB assay.

And, some type of RMs generated in the incubations are not either bound to the CYP enzyme responsible for their formation (causing TDI) or are trapped by GSH. For example,

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GSH cannot trap some types of "hard" RMs, such as iminium ions, radical species, or aldehydes (Kumar et al., 2008; Kumar et al., 2009). Inoue et al. showed that a combination of the trapping reagents [^{35}S]cysteine and K^{14}CN improved accuracy in the detection of compounds with a high extent of CB (Inoue et al., 2009). Thus, calculating the $CL_{int, RMs}$ may be a rough estimation of the extent of CB. However because our results shows relatively good correlation between the extent of CB and $CL_{int, RMs}$ calculated from the result of [^{35}S]GSH trapping and TDI assays, we consider that the most of RMs could be detected by these two assays. And that this approach is useful in the early stages of drug discovery.

In this study, pioglitazone, tacrine, and erythromycin did not show measurable [^{35}S]GS adduct peaks in their radiochromatograms in [^{35}S]GSH trapping assay in spite of higher extent of CB. The mechanisms of generation of RMs from these compounds are known to be S-oxidation (pioglitazone) or the formation of a quinone methide (tacrine) or a nitroso moiety (erythromycin) (Larrey et al., 1983; Madden et al., 1993; Alvarez-Sanchez et al., 2006). Generally, these RMs could be trapped by GSH, and actually the formation of GSH adduct of pioglitazone and tacrine using LC/MS were reported (Lim et al., 2005, Zhang and Yang, 2008). The reason for the difference in the results seemed to be sensitivity of the detecition, because the information using LC/MS is of qualitative nature only (Kumar et al., 2009). The RMs of pioglitazone and tacrine would be trapped using [^{35}S]GSH, but the amount of the [^{35}S]GS adducts would be lower than the limit of quanitification. On the other hands, based on the result of TDI assays, these RMs would be short-lived to the active site of the enzymes and cause TDI. Thus, we consider that most parts of the RMs of these compounds would be bound to the CYP active site, and a few RMs would diffuse

away from the active site and react with the GSH.

Recently, some studies, including our own previous study, have reported that the CB of drugs in human hepatocytes shows the best correlation with their safety profiles (Bauman et al., 2009; Nakayama et al., 2009; Usui et al., 2009). The results of these studies showed that metabolism, which does not occur in HLMs with NADPH, is also an important factor. Examples involve the acyl glucuronides of zomepirac (Wang et al., 2001) and valproic acid (Kumar et al., 2000), and the β-oxidation of valproic acid (Baillie, 1992). In this study, zomepirac and valproic acid were not assessed as having a positive risk of forming RMs in either the [^{35}S]GSH trapping assay or the TDI assay. It is therefore difficult to make a complete assessment of the risk of formation of RMs by using HLMs. However, in terms of throughput and handling, HLMs provide a suitable enzyme source for the preliminary assessment of the potential risks of generating highly reactive metabolites.

In summary, we have demonstrated that a combination of the [^{35}S]GSH trapping assay and the TDI assay is an effective method for detecting compounds potentially capable of generating highly reactive metabolites in the early stages of drug discovery.

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Authorship Contributions

Participated in research design: Nakayama, Takakusa, Watanabe, and Okazaki

Conducted experiments: Nakayama, Miyaji, Watanabe, and Suzuki

Performed data analysis: Sugiyama, Shiosakai, and Honda

Wrote or contributed to the writing of the manuscript: Nakayama, Okudaira, Izumi, and Okazaki

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Footnotes

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Legends for figures

Figure 1. Log–log plot showing the relationship between the formation rate of [^{35}S]GS adducts and the extent of CB in HLMs.
Abbreviations: ERM, erythromycin, PGZ, pioglitazone; RTV, ritonavir; and TC, tacrine.
For illustrational purposes, the horizontal dotted line is plotted at the 50 pmol/mg protein level in the extent of CB.
r is the Spearman rank correlation coefficient.

Figure 2. Log–log plot showing the relationship between the enzyme inactivation rate of all tested isoforms in the TDI assay.
Abbreviations: ERM, erythromycin, PGZ, pioglitazone; RTV, ritonavir; and TC, tacrine.
For illustrational purposes, the horizontal dotted line is plotted at the 50 pmol/mg protein level in the extent of CB.
r is the Spearman rank correlation coefficient.

Figure 3. Log–log plot showing the relationship between the $CL_{int, RMs}$ and the extent of CB.
For illustrational purposes, the horizontal dotted line is plotted at the 50 pmol/mg protein level in the extent of CB.
r is the Spearman rank correlation coefficient.

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Table 1. The extent of CB in HLMs, the formation rate of the [^{35}S]GS adducts, and the percentage of enzyme inactivation in the TDI assay with the previous reports on GSH adduct formation, MBI or TDI as references

Compound	Extent of CB	Formation rate of [^{35}S]GS adducts	TDI (percentage of CYP enzyme inactivation)							Reference
	(pmol/mg protein)	(pmol/min/mg protein)	1A2	2B6	2C8	2C9	2C19	2D6	3A4	
Acetaminophen	71.3	19.8	-	-	-	-	-	-	- [c]	GSH; 1
Aminopyrine	30.9	N.D.	-	-	-	-	-	-	-	
Amlodipine	7.1	N.D.	-	- [d]	-	- [b]	-	-	42	TDI; 2
Amodiaquin	208.1	2.1	-	-	- [b]	-	-	- [c]	-	GSH; 3
Atorvastatin	352.3	12.7	-	-	-	-	-	-	-	
Benzbromarone	389.9	3	12	- [c]	- [d]	- [e]	-	-	- [c]	GSH; 4
Caffeine	9.9	N.D.	-	-	-	-	-	-	-	
Carbamazepine	3.3	N.D.	-	-	-	-	-	-	- [c]	TDI; 5
Celecoxib	13	N.D.	-	- [b]	-	- [b]	-	- [b]	- [c]	
Clopidogrel	417.4	23.6	-	44 [f]	-	-	10	-	35	GSH; 6, TDI; 7, 8
Clozapine	44.7	14.7	12	-	-	-	-	-	21	GSH; 9, TDI; 2
Diclofenac	14.5	1	-	-	-	-	-	-	-	GSH; 10, TDI; 11
Donepezil	29.7	N.D.	-	-	-	-	-	10	-	
Erythromycin	57.1	N.D.	-	-	-	-	-	-	45	TDI; 2
Ethinylestradiol	937.5	34	18	- [b]	- [c]	27 [b]	- [c]	-	65 [a]	GSH; 12, TDI; 12, 13
Fluoxetine	15	N.D.	12	-	-	-	36 [b]	- [c]	-	TDI; 14
Flutamide	178	2	-	-	-	-	-	-	-	GSH; 15
Furosemide	73.9	7.2	-	-	-	-	-	-	-	GSH; 16
Imipramine	133.8	1.2	-	-	-	-	-	-	-	GSH; 16
Indomethacin	14.7	N.D.	-	-	14	-	-	-	-	GSH; 17
Levofloxacin	N.D.	N.D.	-	-	-	-	-	-	-	
Nevirapine	19.1	1.2	-	-	-	-	12	-	-	GSH and TDI; 18
Olanzapine	138.9	11.3	-	-	-	-	-	63	-	
Olmesartan	3.4	N.D.	-	-	-	-	-	-	-	
Phenytoin	4.4	1.2	-	-	-	-	-	-	-	
Pioglitazone	353	N.D.	-	38	- [b]	-	-	-	61	GSH; 19,TDI;20
Pravastatin	3.7	N.D.	-	-	-	-	-	-	-	
Procainamide	5.1	41.3	-	-	-	-	-	-	-	GSH; 21
Propranolol	70	2.7	25	-	-	-	-	-	-	GSH and TDI;22
Ritonavir	253.3	N.D.	-	- [f]	- [c]	- [d]	- [c]	- [c]	75 [e]	TDI; 23
Rosiglitazone	516.1	2.2	-	-	- [c]	-	-	-	56	GSH; 20,TDI;21
Sulfamethoxazole	3.2	21.3	-	-	-	-	-	-	-	GSH; 24
Tacrine	137	N.D.	28	-	-	-	-	24	-	GSH; 25, TDI, 26
Tamoxifen	11.5	N.D.	-	-	- [c]	-	-	-	-	TDI; 27
Ticlopidine	851.9	43.1	36	35 [g]	-	-	60 [c]	- [c]	26	GSH; 28, TDI; 7, 8
Tienilic acid	436.7	4.2	-	-	-	28 [c]	35	-	-	GSH; 16, TDI; 29
Valproic acid	6.3	N.D.	-	-	-	-	-	-	-	
Valsartan	1.4	N.D.	-	-	-	-	-	-	-	
Verapamil	65.6	4.8	-	10	-	-	-	-	51	GSH; 16, TDI; 2
Warfarin	15.9	N.D.	19	-	-	-	-	-	-	
Zafirlukast	36.4	N.D.	11	- [c]	- [d]	- [c]	-	-	43 [c]	TDI; 30
Zomepirac	6.4	N.D.	-	-	-	-	-	-	-	

In TDI assay, the concentration of each test compound was initially set at 100 μM. For compounds with strong reversible inhibitory effects, the compounds were diluted to a suitable concentration as follows: a: 50 μM, b: 30 μM, c: 10 μM, d: 3 μM, e: 1 μM, f: 0.3 μM, g: 0.1 μM.

N.D.; not determined.

-; percentage of CYP enzyme inactivation < threshold at 10 %.

References; 1: (Coles et al., 1988), 2: (Watanabe et al., 2007), 3: (Naisbitt et al., 1998), 4: (McDonald and Rettie, 2007), 5: (Masubuchi et al., 2001), 6: (Dansette et al., 2009), 7: (Nishiya et al., 2009a), 8: (Nishiya et al., 2009b), 9: (Williams et al., 1997), 10: (Tang et al., 1999), 11: (Masubuchi et al., 2002), 12: (Kent et al., 2006), 13: (Chang et al., 2009), 14: (Stresser et al., 2009), 15: (Kang et al., 2007), 16:

(Masubuchi et al., 2007), 17: (Ju and Uetrecht, 1998), 18: (Wen et al., 2009), 19: (Alvarez-Sanchez et al., 2006), 20: (Lim et al., 2005), 21: (Uetrecht, 1985), 22: (Rowland et al., 1994), 23: (Luo et al., 2003), 24: (Cribb et al., 1991), 25: (Zhang and Yang, 2008), 26: (Kalgutkar et al., 2007), 27: (Yao et al., 2001), 28: (Liu and Uetrecht, 2000), 29: (Jean et al., 1996), 30: (Kassahun et al., 2005).

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Table 2. List of compounds categorized according to the results of the [^{35}S]GSH trapping assay and the TDI assay

Group (GSH trapping/TDI)	1 # (-/-)	2 *# (-/+)	3 * (+/-)	4 * (+/+)
Compound name	Aminopyrine	Amlodipine	Acetaminophen	Benzbromarone
	Caffeine	Donepezil	Amodiaquin	Clopidogrel
	Carbamazepine	Erythromycin	Atorvastatin	Clozapine
	Celecoxib	Fluoxetine	Diclofenac	Ethinylestradiol
	Levofloxacin	Indomethacin	Flutamide	Nevirapine
	Olmesartan	Pioglitazone	Furosemide	Olanzapine
	Pravastatin	Ritonavir	Imipramine	Propranolol
	Tamoxifen	Tacrine	Phenytoin	Rosiglitazone
	Valproic acid	Warfarin	Procainamide	Ticlopidine
	Valsartan	Zafirlukast	Sulfamethoxazole	Tienilic acid
	Zomepirac			Verapamil
Extent of CB (pmol/mg protein) Mean ± S.D.	8.98 ± 8.60	91.9 ± 120	104 ± 115	353 ± 323
(Min - Max)	(N.D. - 30.9)	(7.1 - 253)	(3.2 - 352)	(44.7 - 938)

*, significantly different from group 1 ($p < 0.05$). #, significantly different from group 4 ($p < 0.05$).
N.D.; not determined.

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Table 3. The intrinsic clearance for forming RMs

The intrinsic clearance for forming RMs was calculated from sum of $CL_{int,\ RMs\ (GSH)}$ and $CL_{int,\ RMs\ (TDI)}$. $CL_{int,\ RMs\ (GSH)}$ is the intrinsic clearance for forming RMs based on the formation rate of [^{35}S]GS adducts and $CL_{int,\ RMs\ (TDI)}$ is the intrinsic clearance for forming RMs based on the enzyme inactivation rate in TDI assay.

Compound	$CL_{int,\ RMs\ (GSH)}$ (μL/min/mg protein)	$CL_{int,\ RMs\ (TDI)}$ (μL/min/mg protein)	$CL_{int,\ RMs}$ (μL/min/mg protein)
Acetaminophen	0.198	N.D.	0.198
Aminopyrine	N.D.	N.D.	N.D.
Amlodipine	N.D.	0.020	0.020
Amodiaquin	0.021	N.D.	0.021
Atorvastatin	0.127	N.D.	0.127
Benzbromarone	0.03	0.002	0.032
Caffeine	N.D.	N.D.	N.D.
Carbamazepine	N.D.	N.D.	N.D.
Celecoxib	N.D.	N.D.	N.D.
Clopidogrel	0.236	0.848	1.084
Clozapine	0.147	0.012	0.159
Diclofenac	0.01	N.D.	0.010
Donepezil	N.D.	0.001	0.001
Erythromycin	N.D.	0.021	0.021
Ethinylestradiol	0.34	0.087	0.427
Fluoxetine	N.D.	0.008	0.008
Flutamide	0.02	N.D.	0.020
Furosemide	0.072	N.D.	0.072
Imipramine	0.012	N.D.	0.012
Indometacin	N.D.	0.001	0.001
Levofloxacin	N.D.	N.D.	N.D.
Nevirapine	0.012	0.001	0.013
Olanzapine	0.113	0.006	0.119
Olmesartan	N.D.	N.D.	N.D.
phenytoin	0.012	N.D.	0.012
Pioglitazone	N.D.	0.036	0.036
Pravastatin	N.D.	N.D.	N.D.
Procainamide	0.413	N.D.	0.413
Propranolol	0.027	0.004	0.031
Ritonavir	N.D.	3.550	3.550
Rosiglitazone	0.022	0.028	0.048
Sulfamethoxazole	0.213	N.D.	0.213
Tacrine	N.D.	0.007	0.007
Tamoxifen	N.D.	N.D.	N.D.
Ticlopidine	0.431	2.030	2.461
Tienilic acid	0.042	0.069	0.111
Valproic acid	N.D.	N.D.	N.D.
Valsartan	N.D.	N.D.	N.D.
Verapamil	0.048	0.026	0.074
warfarin	N.D.	0.003	0.003
Zafirlukast	N.D.	0.207	0.207
Zomepirac	N.D.	N.D.	N.D.

N.D.; not determined.

Figure 1

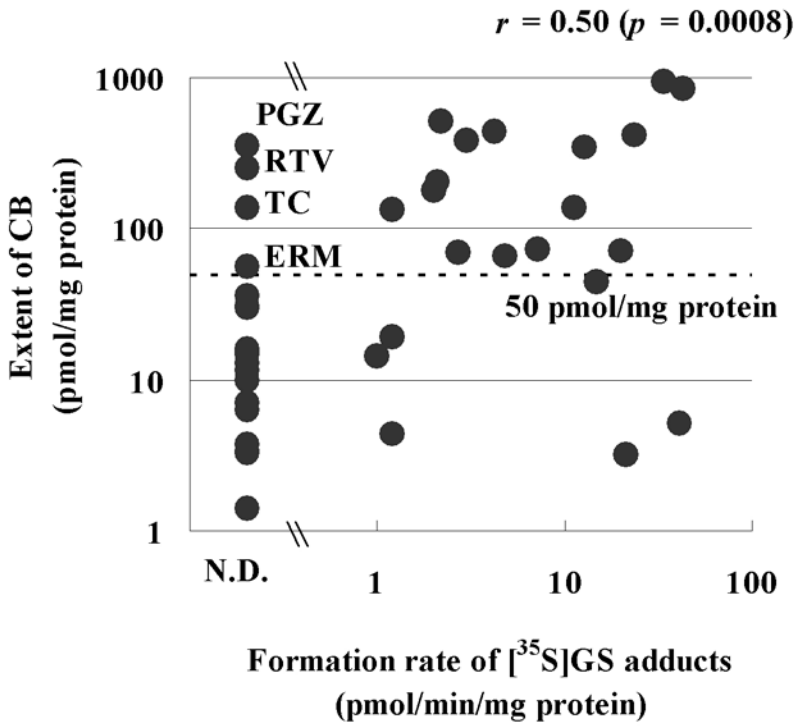

Figure 2

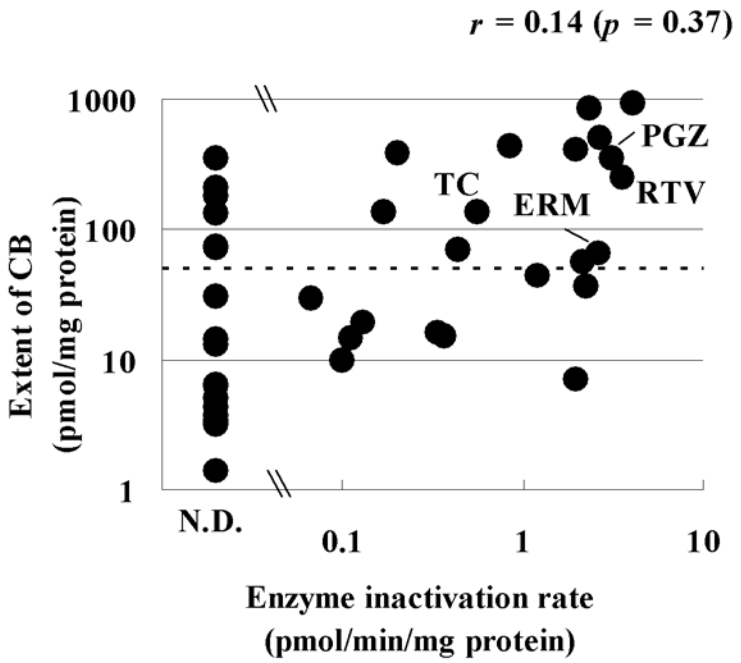

Figure 3