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# Markers of Electrophilic Stress Caused by Chemically Reactive Metabolites in Human Hepatocytes.

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Non-standard abbreviations

ABT: aminobenzotriazole; BHA: Butylated hydroxylanisole; BSO: L-buthionine-(S,R)-sulfoximine;

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CES: carboxyesterase; CYP: cytochrome-P450 enzyme; DMSO: dimethyl sulfoxide; FBS: Fetal bovine serum; GAPDH: glyceraldehyde 3-phosphate dehydrogenase (EC: 1.2.1.12); GCS: glutamylcysteine synthetase (EC: 6.3.2.2); GSH: glutathione; GST: glutathione-S-transferase (EC: 2.5.1.18); HO: heme oxygenase (EC: 1.14.99.3) ; IDT: idiosyncratic drug toxicity; LC-MS/MS: liquid chromatography/tandem mass spectrometry; LDH: lactate dehydrogenase (EC: 1.1.1.27); NQO: NAD(P)H:quinone oxidoreductase (EC: 1.6.5.2); Nrf2: NF-E2-related factor 2; Keap1: Kelch-like ECH-associated protein 1; tBHQ: *tert*-butyl hydroquinone, UGT: UDP-glucuronosyltransferase (EC: 2.4.1.17).

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**ABSTRACT**

The metabolic activation of a drug to an electrophilic reactive metabolite and its covalent binding to cellular macromolecules is considered to be involved in the occurrence of idiosyncratic drug toxicity. As a cellular defense system against oxidative and electrophilic stress, phase II enzymes are known to be induced through a Keap1/Nrf2/ARE system. We presumed that it is important for the risk assessment of drug-induced hepatotoxicity and IDTs to observe the biological responses evoked by exposure to reactive metabolites, and then investigated the mRNA induction profiles of phase II enzymes in human hepatocytes after exposure to problematic drugs associated with IDTs, such as ticlopidine, diclofenac, clozapine and tienilic acid, as well as safe drugs such as levofloxacin and caffeine. According to the results, the problematic drugs exhibited inductive effects on HO-1, which contrasted with the safe drugs, and therefore the induction of HO-1 mRNA seems to be correlated with the occurrence of drug toxicity including IDT due to electrophilic reactive metabolites. Moreover, GSH-depletion and CYP inhibition experiments have demonstrated that the observed HO-1 induction was triggered by the electrophilic reactive metabolites produced from the problematic drugs through CYP-mediated metabolic bioactivation. Taken together with our present study, this suggests that HO-1 induction in human hepatocytes would be a good marker of the occurrence of metabolism-based drug-induced hepatotoxicity and IDT due to the formation of electrophilic reactive metabolites.

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The occurrence of idiosyncratic drug toxicity (IDT) is major problem in drug development and is of great concern to the pharmaceutical industry. A number of drugs have been withdrawn from the market or severely restricted in their use due to unexpected toxicities that become apparent only after the launch of new drug entities (Kaplowitz, 2005). Currently, it is suggested that the metabolic activation of a drug to a reactive metabolite and its covalent binding to cellular macromolecules is involved in the occurrence of IDTs (Walgren *et al*., 2005; Zhou *et al*., 2005). Bioanalytical techniques, including the detection of glutathione (GSH) adducts to reactive metabolites by LC-MS/MS and covalent binding assessment using radiolabeled chemical entities, have been utilized during lead optimization in order to minimize chemically reactive metabolite formation (Evans *et al*., 2004; Baillie *et al*., 1993; Gan *et al*., 2005). Since reactive metabolite formation and covalent binding to macromolecules are thought to be necessary yet insufficient processes for the generation of an idiosyncratic reaction, it is also important to observe the biological responses evoked by exposure to reactive metabolites for the risk assessment of IDTs (Liebler *et al*., 2005).

The electrophilic stress derived from reactive metabolites has been reported to cause an immediate adaptive defense response in cells. This involves various mechanisms, including the nuclear translocation of redox-sensitive transcription factors such as NF-E2-related factor 2 (Nrf2), which sense electrophilic stress, oxidative stress and protect against cellular damage via Kelch-like ECH-associated protein 1 (Keap1) (Wakabayashi *et al*., 2004; Dinkova-Kostova *et al*., 2005). An increasing number of studies have identified the genes regulated by Nrf2. These include genes

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involved in phase II drug-metabolism, such as heme oxygenase-1 (HO-1), γ-glutamylcysteine synthetase (γ-GCS), glutathione-S-transferases (GSTs), NAD(P)H:quinone reductase-1 (NQO-1), and UDP-glucuronosyltransferases (UGTs) (Talalay *et al*., 2003). The cooperative activity of these enzymes serves as a cellular defense against electrophiles and oxidative stress products. In addition, the comparative analysis of gene expression changes using *keap1* or *Nrf2*-deficinet mice has also facilitated the identification of numerous new genes regulated by Nrf2 such as CES1 (Thimmulappa *et al*., 2002). We postulate in this report that the induction of Nrf2-related genes would be a good indicator of electrophilic cell stress by reactive metabolites and would be useful as a risk marker for metabolism-based drug toxicity and IDTs that might not be expressed during drug development. Although intensive studies on Nrf2-mediated induction have been carried out using human cultured cell lines such as HepG2 or rodent hepatocytes (Cantoni *et al*. 2003; Nioi *et al*., 2003; Shinkai *et al*., 2006), there are only a few reports regarding this induction in human hepatocytes (Keum *et al*., 2006). For a better assessment of the potential risks of metabolism-based drug toxicities, it is necessary to detect the cellular responses to electrophilic stresses generated through drug metabolism in human hepatocytes.

In the present study, we have investigated the induction profiles of Nrf2-related genes in human hepatocytes after they were exposed to known ‘problematic’ drugs associated with IDTs, such as ticlopidine, diclofenac, clozapine and tienilic acid. The induction profiles after exposure to safe drugs such as levofloxacin and caffeine were also examined for the purposes of comparison.



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**METHODS**

Materials

Butylated hydroxylanisole (BHA), *tert*-butyl hydroquinone (tBHQ), L-buthionine-(S,R)-sulfoximine (BSO), aminobenzotriazole (ABT), diclofenac, clozapine, furosemide, acetaminophen, acetylsalicylic acid, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and caffeine was purchased from Fluka (Buchs, Switzerland). Ticlopidine and levofloxacin were synthesized at Daiichi Pharmaceutical Co., Ltd. $^{14}$C-labeled acetylsalicylic acid and caffeine were purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO, USA). Pooled human (n = 50, mix gender) and male Sprague-Dawley rat liver microsomes were purchased from XenoTech (Lenexa, KS, USA). β-NADP$^+$ and glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Oriental Yeast Co. Ltd, (Tokyo, Japan) and glucose-6-phosphate (G6P) was from SIGMA. Fetal bovine serum (FBS), TRIzol® and custom primers were purchased from Invitrogen (Carlsbad, CA, USA). TaqMan® Gold RT-PCR kits, TaqMan® Universal PCR Master Mix and custom TaqMan® probes were purchased from Applied Biosystems (Foster City, CA, USA). All other chemicals and reagents were of analytical grade and were available from commercial sources.

Human hepatocyte culture

Cryopreserved human hepatocytes (Lot No. FEP, KCT and ZCA) were obtained from In Vitro Technologies (Baltimore, MD, USA). Hepatocytes were seeded onto 48-well collagen I-coated



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plates (ASAHI TECHNO GLASS; Chiba, Japan) in Lanford medium containing 5% *v*/*v* FBS at a density of 3.0 × $10^5$ viable hepatocytes/wells/0.5 mL. They were placed into a 37ºC, 5% $CO_2$, saturated humidity incubator and incubated for 4 h to allow the cells to attach. Then, the medium containing dead or unattached cells was removed from each well and replaced with 0.5 mL of warmed (37ºC) Lanford medium. The hepatocyte cultures were maintained for 48 hours after plating, with incubation medium changed daily. Stock solutions of the test compounds were prepared in DMSO and stored at -20°C until dilution with incubation medium to the final concentration. The final concentration of DMSO in each solution was 0.1% *v*/*v*. Plated hepatocytes were treated in triplicate for each treatment for 24 h at 37ºC, 5% $CO_2$, saturated humidity with incubation media containing each test compound. For a negative control, plated hepatocytes were also incubated with the medium containing DMSO (0.1% *v*/*v*). The hepatocyte cultures were treated with 0.25 mL of ice-cold TRIzol® and harvested into separate 1.5-mL centrifuge tubes on ice. Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method. For each RNA sample, the total RNA extracted was diluted to 5 ng/µL with diethyl pyrocarbonate-treated water and added to the reaction mixture. The concentrations and purity of the isolated RNA samples were determined using UV spectrophotometry conducted on a GeneQuant pro (Amersham Biosciences; Piscataway, NJ, USA).

Reverse transcription reaction

The reaction mixture for reverse transcription was prepared with TaqMan® Gold RT-PCR kits. The reverse transcription was performed on a GeneAmp® PCR System 9700 (Applied

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Biosystems) for 10 min at 25°C, 30 min at 48°C and 5 min at 95°C.

Quantitative real-time PCR

The PCR reaction mixture was prepared with TaqMan® PCR Universal Master Mix. An Inventoried Taqman® MGB probe and primer mixture (Applied Biosystems) were used in the PCR reaction for CES1, γ-GCS, GSTA1, GSTP1, HO-1, NQO1, GAPDH and β-actin. The codes of the TaqMan MGB probes used were as follows: Hs00275607_m1 (CES1), Hs00155249_m1 (γ-GCS), Hs00272272_m1 (GST1A), Hs00168310_m1 (GSTP1), Hs00157965_m1 (HO-1), Hs00168547_m1 (NQO1), 4316317E (GAPDH) and 4326315E (β-actin). The primers and probe for UGT1A1 (Accession No. NM000463) were obtained from Invitrogen and the positions of their sequences are as follows: 490-516 (forward primer), 603-627 (reverse primer) and 453-522 (probe). Quantitative real-time PCR was performed in a MicroAmp Optical 96well Reaction Plate™ (Applied Biosystems) with an ABI PRISM® 7000 (Applied Biosystems). An initial step occurred for 2 min at 50°C, subsequently followed by heating to 95°C for 10 min, and followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The relative quantification of the gene expression levels was determined by a standard curve generated from a dilution series (from 0.0061 to 400 ng/μL) of a standard RNA sample obtained from the control hepatocytes, and normalized to the amount of GAPDH or β-actin mRNA. The results are expressed as mean ± S.D. and significant differences were evaluated using *t*-test.

Lactate dehydrogenase (LDH) release

To determine the toxic effects of the compounds, the levels of lactate dehydrogenase (LDH) in the cell

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culture medium were analyzed according to the recommendations of the manufacturer of the Cytotoxicity Detection Kit (LDH) (Roche Diagnostics Corporation; Indianapolis, IN, USA).

*In vitro* covalent binding assay

The experimental procedure was based on that previously reported (Masubuchi *et al*., 2007). Briefly, the complete incubation of the $^{14}$C-labeled test compounds with HLMs contained the following: 10 μM $^{14}$C-labeled test compound (substrate), 2 mg/mL HLMs, 100 mM potassium phosphate buffer pH 7.4, 25 mM G6P, 2 units/mL G6PDH and 10 mM $MgCl_2$. This mixture was preincubated for 3 min at 37 °C. The reaction was initiated by the addition of β-$NADP^+$ to reach final concentration of 2.5 mM. The final incubation volume was 0.5 mL. As the substrates were dissolved in acetonitrile, the final incubation mixture contained acetonitrile at the concentration of 1%. After 1 h incubation, the reaction was terminated by the addition of 0.5 mL ice-cold acetonitrile. After voltexing and centrifugation, precipitated protein was washed with 80% (*v/v*) aqueous methanol containing 10% (*w/v*) trichloroacetic acid, diethyl ether - methanol (1:1, *v/v*) and 80% (*v/v*) aqueous methanol (twice for each solvent). The resulting precipitated protein was dissolved in 0.5 mL of 1.0 N NaOH, and the aliquots were taken for protein assay by DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and determination of radioactive content by liquid scintillation counting with scintillation fluid cocktail Hionic-fluor (PerkinElmer, Wallesley, MA, USA). The amount of test compounds covalently bound to the microsomal protein was determined and covalent binding [pmol/min/mg of protein] was calculated. As a background, radioactivity of the samples without incubation was also

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determined.

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**RESULTS**

In order to examine the cellular responses to electrophilic stress by a Keap1/Nrf2/ARE system in human hepatocytes, the induction profiles of typical Nrf2-responsive genes after exposure to BHA, a classical activator of Nrf2, were observed. The mRNA expression levels of HO-1, γ-GCS, UGT1A1, CES1, NQO1, GSTA1 and GSTP1 after 24 h-exposure of human hepatocytes (Lot FEP, KCT and ZCA) to 100 μM BHA are shown in Table 1. The concentration and time of exposure were set according to the previous report (Keum *et al*., 2006)). The mRNA expression levels of HO-1, CES, UGT1A1 and NQO1 were increased after 24 h-exposure to BHA. The ratios of BHA-induced mRNA levels relative to the vehicle control were: HO-1, 2.02~4.77; CES1, 1.67~4.49; UGT1A1, 1.77~10.31; NQO1, 1.32~3.88. Lot KCT hepatocytes exhibited the highest induction levels of these enzymes among the three lots of hepatocytes used. The γ-GCS and GSTA1 mRNA expression levels were barely affected by the exposure to BHA, accounting for 0.85~1.22 and 1.16~1.46 fold changes relative to the vehicle control, respectively. GSTP1 mRNA expression levels were not determined because they were below the lower limit of quantification of real time PCR.

We next investigated the induction profiles of Nrf2-related genes by problematic drugs that are known to be associated with severe IDTs, as well as safe drugs. The structures of the drugs tested are shown in Figure 1. Human hepatocytes (Lot KCT) were exposed for 24 h to increasing concentrations (30, 100 and 300 μM) of ticlopidine, clozapine, diclofenac, acetaminophen and tienilic acid as the problematic drugs, and caffeine, levofloxacin, furosemide and acetylsalicylic acid as the

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safe drugs, and BHA as a positive control, followed by the determination of the mRNA expression levels of HO-1, UGT1A1, CES1 and NQO1, which were all observed to be responsive to BHA (Figure 2). Hepatocytes exposed to 300 μM BHA, ticlopidine and clozapine exhibited increased LDH release (>2 fold compared with the vehicle control) in the culture medium, demonstrating the cellular toxicity by these treatments. The mRNA levels of HO-1 increased after exposure to ticlopidine, clozapine, diclofenac, tienilic acid and BHA in a concentration-dependent manner, and the induction ratios relative to the vehicle control were determined to be 2.56 for 100 μM ticlopidine, 3.90 for 100 μM clozapine, 2.71 for 300 μM diclofenac, 2.95 for 300 μM tienilic acid and 4.77 for 100 μM BHA. In contrast, the increase in HO-1 mRNA levels after exposure to the safe drugs were determined to be less than 2-fold relative to the control, suggesting that HO-1 is not induced by safe drugs. These results showed a good contrast in HO-1 mRNA induction between the problematic and safe drugs. UGT1A1 was highly induced by exposure to the problematic drugs, showing a more than 10-fold increase in the mRNA level. Since the mRNA levels were also increased 2.90~6.94-fold after exposure to the safe drugs, the contrast in UGT1A1 induction between the problematic and safe drugs was not clear. In the cases of CES1 and NQO1, neither the problematic nor safe drugs caused significant increase in their mRNA expression levels. The BHA treatment as a positive control exhibited mRNA induction (> 2 fold) in HO-1, UGT1A1, CES1 and NQO1. Next the observed fold changes of induction of the drugs were compared with their intrinsic covalent binding yields in human liver microsomal systems reported previously (Masubuchi *et al.*, 2007). Furthermore, we



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additionally conducted the covalent binding study of acetylsalicylic acid and caffeine using their $^{14}C$-labelled tracers in order to obtain the covalent binding data of the safe drugs by the same method. As a result, the safe drugs, acetylsalicylic acid and caffeine exhibited the trace levels of covalent binding (0.07 and 0.16 pmol/min/mg of protein, respectively) in a human liver microsomal system. The fold changes of induction of the drugs were plotted against their covalent binding yields as shown in Figure 2 (E). HO-1 and UGT1A1 showed weak positive correlations between the induction and covalent binding, and their correlation coefficient were calculated to be 0.43 and 0.57, respectively. On the other hand, no correlation was observed in case of CES1 and NQO1.

In order to investigate whether or not HO-1 induction in human hepatocytes by BHA and problematic drugs was affected by depletion of GSH, a major endogenous scavenger of electrophiles, the mRNA levels of HO-1 were determined after co-treatment of hepatocytes with BSO, a specific inhibitor of GSH synthesis. When human hepatocytes (Lot KCT) were exposed to 100 μM BHA, 100 μM ticlopidine, 300 μM diclofenac and 300 μM acetaminophen, they were co-treated with increasing concentrations (0, 10 and 50 μM) of BSO for 24 h, followed by the determination of HO-1 mRNA levels, as shown in Figure 3a. According to the results, co-treatment with BSO enhanced the HO-1 mRNA induction by BHA, ticlopidine, diclofenac and acetaminophen in a concentration-dependent manner. Control incubations ((-) substrate) of hepatocytes with BSO and without substrate showed that mRNA levels of HO-1 were not changed solely by BSO at the concentrations tested. The effect of co-treatment with BSO and the safe drugs on the mRNA level of



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HO-1 was also examined, as shown in Figure 3b. The mRNA levels of HO-1 after exposure to 300 μM levofloxacin and caffeine with BSO were not different from those of the (-) substrate control, whereas those after exposure to acetylsalicylic acid were slightly enhanced under GSH depletion conditions.

Because BHA and the problematic drugs tested are known to be metabolized into electrophilic reactive species by cytochrome-P450 enzymes (CYPs), metabolism via CYPs is presumed to contribute the the HO-1 induction by these drugs. Then, during 24 h-exposure of human hepatocytes (Lot KCT) with 100 μM BHA, ticlopidine, clozapine and 300 μM diclofenac, they were co-treated with increasing concentrations (0, 300 and 1000 μM) of ABT, a nonspecific and irreversible inhibitor of CYPs (Mico *et al.*, 1988). In this experiment, the hepatocytes were also challenged with 50 μM BSO in order to detect the changes in HO-1 mRNA level with a wider dynamic range. As shown in Figure 4a, ABT treatment significantly suppressed the HO-1 induction by BHA and problematic drugs in a concentration-dependent fashion. The mRNA levels in the control hepatocytes not exposed to any substrate were not changed by ABT, demonstrating that ABT itself has no effect on HO-1 induction. In addition, we also examined the effect of ABT on HO-1 induction by tBHQ (Figure 4b), which is a demethylated metabolite of BHA and is known to be an activator of Nrf2 (Keum *et al.*, 2006). Hepatocytes exposed to 30 μM tBHQ for 8 h exhibited an approximately 2-fold increased mRNA level, and which was not suppressed by the addition of ABT.

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**DISCUSSION**

Several *in vitro* studies using human cultured cell lines such as HepG2 or rodent hepatocytes as well as *in vivo* studies in rodents have shown that Phase II enzymes are induced by electrophiles and that some drugs are known to produce electrophiles via a Keap1/Nrf2 system (Cantoni *et al*. 2003; Nioi *et al*., 2003; Shinkai *et al*., 2006), whereas there are only a few studies examining the Keap1/Nrf2-mediated enzyme induction profiles by the electrophilic stress in human hepatocytes (Keum *et al*., 2006). Induction studies of typical Nrf2-related genes by BHA have shown that the mRNA level induction of HO-1, UGT1A1, CES and NQO-1 is readily observable in human hepatocytes and that the mRNA levels induced were varied among the enzymes observed. This observed variation in the inductive response to electrophilic stress would be due to the fact that there are different regulatory features in the gene expression process after Nrf2 activation. For instance, the chromatin-remodeling molecule BRG1 has recently been identified as a specific coactivator of HO-1 gene expression which interacts with Nrf2 to selectively mediate HO-1 induction in response to oxidative and electrophilic stress (Zhang *et al*. 2006).

In the comparative induction study between the problematic and safe drugs shown in Figure 2, we chose clozapine, ticlopidine, tienilic acid, diclofenac and acetaminophen as the problematic drugs because these drugs are reported to be associated with IDT including hepatotoxicity due to the formation of the electrophilic reactive metabolites and covalent binding to cellular proteins. The proposed structures of the reactive metabolites generated from the problematic drugs are shown in



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Figure 1. Clozapine causes fatal neutropenia, aguranulocytosis and hepatotoxicity, and received a warning regarding its use (Alvir *et al*., 1993; Macfarlane *et al*., 1997). Clozapine forms reactive metabolites such as nitrenium ion by CYPs, activated neutrophils and bone marrow cells (Pirmohamed *et al*., 1995). Ticlopidine also received a warning and is restricted in its use due to severe hepatotoxicity and agranulocytosis (Skurnik *et al*., 2003). The thiophene moiety of ticlopidine is mainly oxidized by CYP2C19 with the formation of electrophilic reactive metabolites, including thiophene S-oxide and thiophene epoxide, which can form covalent binding to cellular proteins including CYP2C19 (Ha-Duong *et al*., 2001). Tienilic acid was withdrawn from the market in 1980 due to severe and fatal liver injury (Zimmerman *et al*., 1984). Tienilic acid has a thiophene moiety as well as ticlopidine and is metabolized mainly by CYP2C9 to form thiophene-derived electrophilic reactive metabolites (Lopez-Garcia et al., 1994). Diclofenac is associated with hepatotoxicity, which is considered to be due to the formation of electrophilic reactive metabolites, including acylglucuronide by UGTs and benzoquinone-imines by CYP2C9 and 3A4, followed by their covalent binding to liver proteins (Boelsterli, 2003). As shown in Figure 2, these problematic drugs exhibited inductive effects on HO-1, which contrasted with the safe drugs, and therefore the induction of HO-1 mRNA seems to be correlated with the occurrence of drug toxicity including IDT due to electrophilic reactive metabolites. Acetaminophen was categorized as a problematic drug, but did not increase the mRNA level of HO-1 in the concentration range tested. Since acute and fatal liver injury of acetaminophen due to the formation of an electrophilic reactive metabolite,

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N-acetyl-benzoquinone-imine, is caused by its overdose (e.g. 4000 mg/day) (Hinson *et al.*, 1981), higher exposure concentrations of acetaminophen may be necessary for the induction of HO-1. Although acetaminophen is the most thoroughly studied problematic drug and there is some body of evidence of toxicity due to bioactivation in particular in mice, it is known that human hepatocytes are less responsive to acetaminophen than mice (Chueng *et al.*, 2005: Tee *et al.*, 1987). This species difference in hepatotoxic effects of acetaminophen is considered to be due to difference in the rate of CYP2E1-mediated formation of reactive metabolites. Thus, the no induction of HO-1 by acetaminophen observed could be explained by the low sensitivity of human hepatocytes to acetaminophen. The parallel gene induction studies with hapatocytes from animal species would be helpful to understand the species deference in metabolic bioactivation and toxicity.

UGT1A1 was induced by exposure to the safe drugs as well as the problematic drugs, as shown in Figure 2. Previous studies have shown that the regulation of UGT expression is targeted by a number of xenobiotic and steroid receptors such as PXR, CAR and PPAR in response to xenobiotics, carcinogens, stress signals and hormones other than the Keap1/Nrf2 signaling pathway (Soars *et al.*, 2004). Therefore, the observed inductive effects of the safe drugs on UGT1A1 are thought to be due to the induction through these receptors and UGT1A1 induction would not be a specific marker for electrophilic stress responses.

The fold changes of induction for HO-1 were shown to be positively and weakly correlated with the covalent binding yields. Nrf2 activation is known to be regulated by the covalent modification pattern of Keap1, which is dependent on the structures of the electrophilic reactive species (Hong *et al.*,

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2005). Therefore, the Nrf2-mediated gene induction would be dependent not only on the quantity of covalent binding but also on the structures of reactive metabolites and the pattern of covalent binding. This may be one reason for no strong correlation between covalent binding and Nrf2-mediated gene induction. For the further consideration, however, it would be necessary to determine the covalent binding yields in the induction experiments using human hepatocytes. In case of UGT1A1, the drugs with the trace levels of covalent binding yield showed inductive effects, supporting the idea that the observed induction of UGT1A1 was, at least partially, mediated by the induction pathways other than Keap1/Nrf2 pathway.

Because electrophilic reactive species can be scavenged by GSH, a major intracellular nucleophile, the GSH depletion experiment using BSO represents that the formation of electrophilic reactive species should be involved in the HO-1 induction by BHA and the problematic drugs. Furthermore, GSH is also a major antioxidant and therefore it was suggested that the hepatocellular redox status could affect the HO-1 induction by electrophilic stress. Though acetaminophen did not affect the mRNA level of HO-1 up to 300 μM under normal condition as shown in Figure 2a, it induced HO-1 under GSH-depleted condition (Figure 3a), indicating that acetaminophen also has a potential to induce HO-1 via reactive metabolite formation and which would be masked by GSH under normal condition. The safe drugs levofloxacin and caffeine had no inductive effect on HO-1 even under the GSH-depleted conditions, showing that GSH depletion can sharpen the contrast in HO-1 induction between the problematic and safe drugs. Acetylsalicylic acid is believed to be relatively



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safe at therapeutic doses and is thus categorized as a safe drug in this study. However, it has been reported that its overdose results in hepatotoxicity, probably due to the formation of electrophilic acyl glucuronide and thus it can be regarded as a potentially problematic drug (Bjorkman, 1998; Dickinson *et al*., 1994). The slight induction by acetylsalicylic acid observed under GSH-depleted conditions may represent its potential risk of hepatotoxicity.

Moreover, CYP inhibition experiments using ABT demonstrate that CYP metabolism is an essential pathway for the HO-1 induction by BHA and the problematic drugs. As mentioned above, ticlopidine, clozapine and diclofenac are transformed to electrophilic reactive metabolites by CYP enzymes. BHA is also known to be typically metabolized by CYPs to a demethylated metabolite, tert-butylhydroquinone (tBHQ), which is in turn converted to the electrophilic reactive species *tert*-butylquinone through autooxidation, as shown in Figure 1 (Cummings *et al*., 1985). Thus, the observed HO-1 induction is considered to be triggered by the electrophilic reactive metabolites produced through CYP metabolism. The lack of an effect of ABT on HO-1 induction by tBHQ suggests that tBHQ is capable of inducing HO-1 without CYP-mediated metabolic bioactivation, which seems to be in reasonable agreement with this conclusion because tBHQ can be autooxidized to *tert*-butylquinone.

HO-1 catalyzes degradation of heme, resulting in production of biliverdin and carbon monoxide (CO). Biliverdin can suppress oxidative stress by scavenging reactive oxygen species (Baranano *et al*., 2002). CO has strong anti-inflammatory effects and protective effects against immune-mediated liver injury (Otterbein *et al*.,

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2000). Because HO-1 has some defensive effects against cellular damages caused by electrophilic stress, HO-1 induction by the problematic drugs would be reasonable from a functional point of view. Future studies to investigate changes in level of HO-1 activity and yields of HO-1 products after mRNA level induction will provide further support for the concept in this study.

In summary, we have investigated the induction profiles of typical Nrf2-related genes after the exposure of human hepatocytes to BHA and a series of the marketed drugs and have revealed that the mRNA levels of HO-1 can be increased specifically by problematic drugs associated with severe hepatotoxicity including IDT. Moreover, GSH-depletion and CYP inhibition experiments have demonstrated that the observed HO-1 induction was triggered by the electrophilic reactive metabolites produced from the problematic drugs through CYP-mediated metabolic bioactivation. Taken together, our present study suggests that HO-1 induction in human hepatocytes would be a good marker for the cellular stress response to electrophilic reactive metabolites and metabolism-based drug toxicity and IDT.

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**FOOTNOTES**

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**LEGENDS TO FIGURES**

Figure 1. Chemical structures of the compounds tested and their proposed reactive metabolites.

Figure 2. Induction profiles of HO-1, UGT1A1, CES1 and NQO1 after exposure of human hepatocytes to the compounds tested. Human hepatocytes (Lot KCT) were exposed for 24 h to increasing concentrations (30, 100 and 300 μM) of ticlopidine, clozapine, diclofenac, acetaminophen, tienilic acid, caffeine, levofloxacin, furosemide, acetylsalicylic acid and BHA, followed by the determination of the mRNA expression levels of HO-1 (A), UGT1A1 (B), CES1 (C) and NQO1 (D). Bar graphs represent the mean ± SD of three determinations. *, $p < 0.01$ compared with control. (E) Comparison of the fold changes of induction of the drugs with their intrinsic covalent binding yields in human liver microsomal systems.

Figure 3. Effect of BSO on the HO-1 mRNA induction in human hepatocytes. (A) Human hepatocytes (Lot KCT) were exposed to 100 μM BHA, 300 μM diclofenac, 100 μM ticlopidine and 300 μM acetaminophen in the presence of BSO (0, 10 and 50 μM) for 24 h. (B) Human hepatocytes (Lot KCT) were exposed to 300 μM acetylsalicylic acid, 300 μM caffeine and 300 μM levofloxacin in the presence of BSO (0, 10 and 50 μM) for 24 h. Bar graphs represent the mean ± SD of three determinations. *, $p < 0.01$ compared with 0 μM BSO.

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Figure 4. Effect of ABT on the HO-1 mRNA induction in human hepatocytes. (A) Human hepatocytes (Lot KCT) were treated with the increasing concentrations (0, 300 and 1000 μM) of ABT during 24 h-exposure to 300 μM diclofenac, 100 μM BHA, ticlopidine and 30 μM clozapine in the presence of 50 μM BSO. (B) Human hepatocytes (Lot ZCA) were treated with the increasing concentrations (0, 300 and 1000 μM) of ABT during 8 h-exposure to 30 μM tBHQ in the presence of 50 μM BSO. Bar graphs represent the mean ± SD of three determinations. *, $p < 0.01$ compared with 0 μM ABT.

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**Table 1. BHA-induced mRNA expression profiles in human hepatocytes.**

| Enzyme | mRNA expression level ratio* (Mean ± SD) | | |
|---|---|---|---|
| | Lot. FEP | Lot. KCT | Lot. ZCA |
| HO-1 | 2.59 ± 0.18 | 4.77 ± 0.18 | 2.02 ± 0.25 |
| γ-GCS | 0.85 ± 0.12 | 0.89 ± 0.02 | 1.22 ± 0.23 |
| UGT1A1 | 2.15 ± 0.16 | 10.31 ± 0.59 | 1.77 ± 0.20 |
| CES1 | 1.67 ± 0.35 | 4.49 ± 0.72 | 2.57 ± 0.56 |
| NQO1 | 1.97 ± 0.29 | 3.88 ± 0.68 | 1.32 ± 0.30 |
| GSTA1 | 1.31 ± 0.17 | 1.46 ± 0.23 | 1.16 ± 0.06 |
| GSTP1 | ND | ND | ND |

Hepatocytes were exposed to 100 μM BHA at 37°C for 24 h.

* Relative to the vehicle control, n=3.

ND: Not determined due to lower mRNA expression level than the quantification limits.

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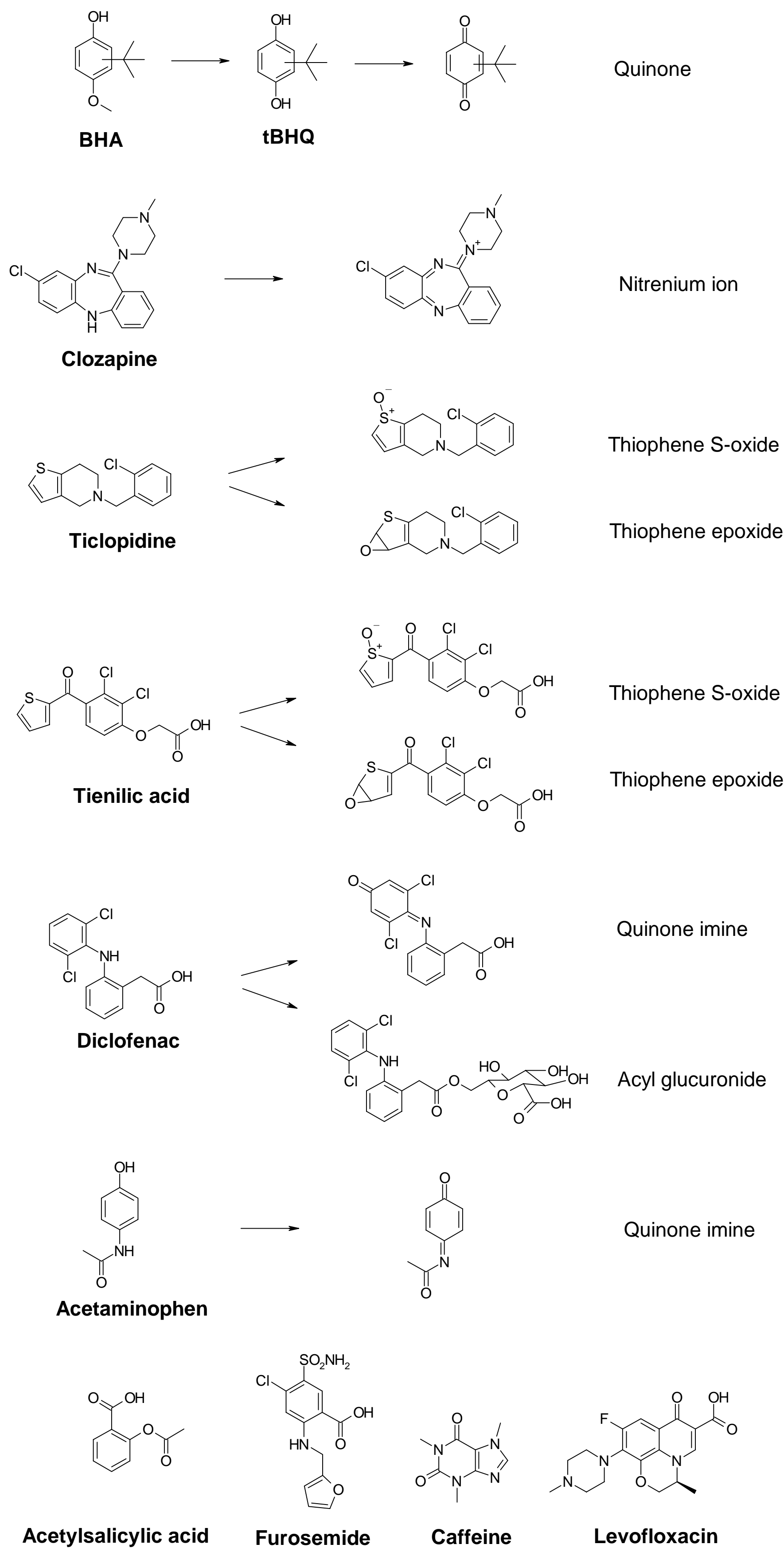


Figure 1

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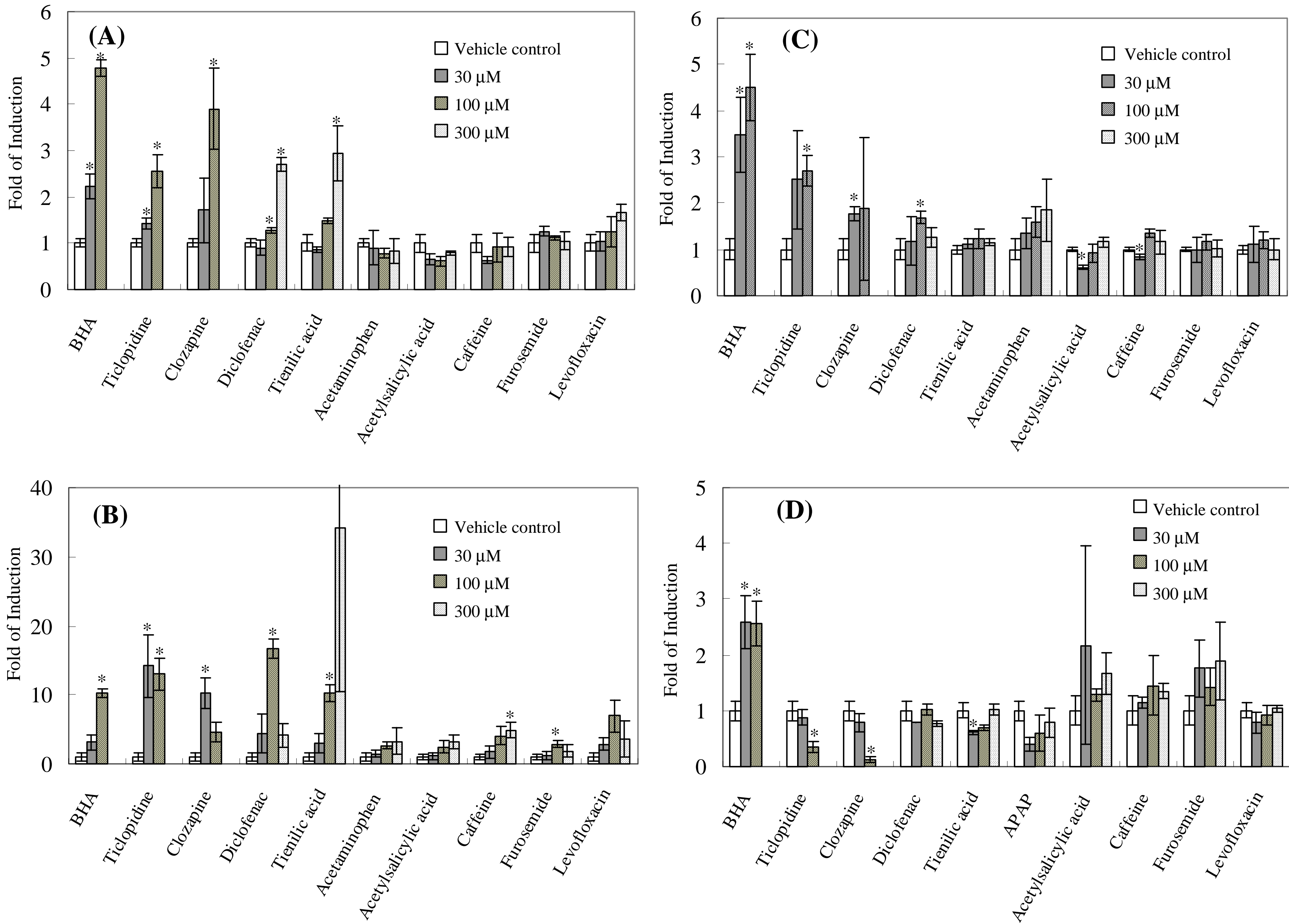


Figure 2

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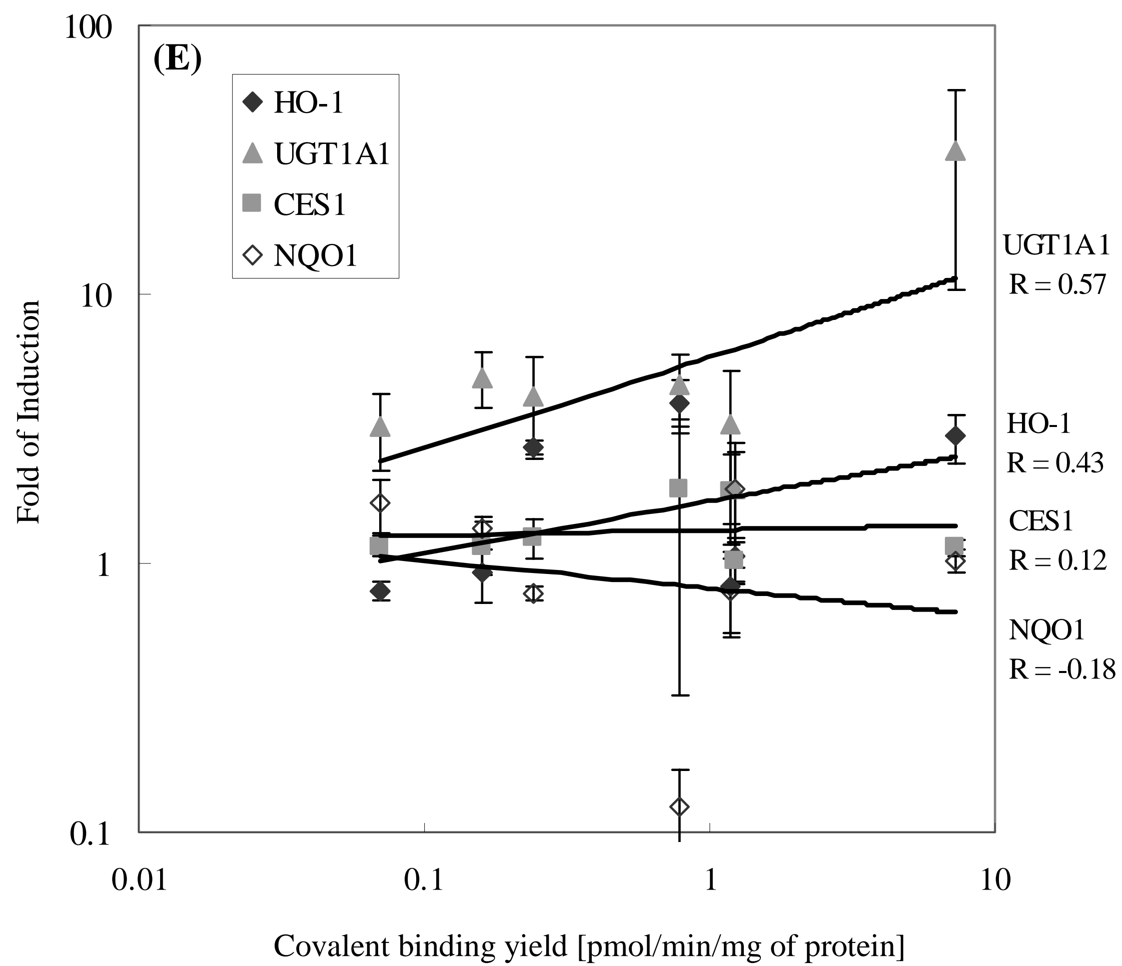


Figure 2

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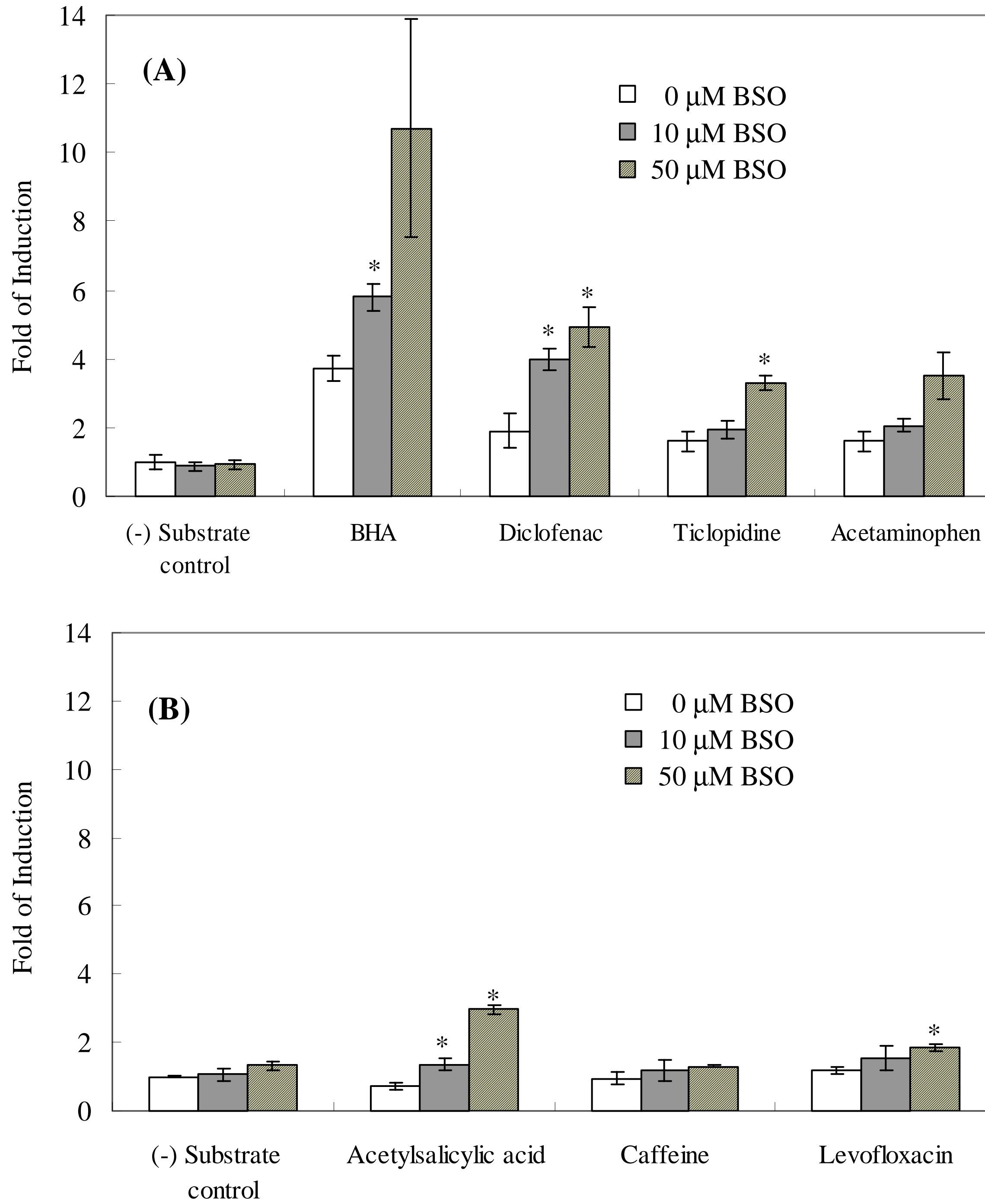


Figure 3

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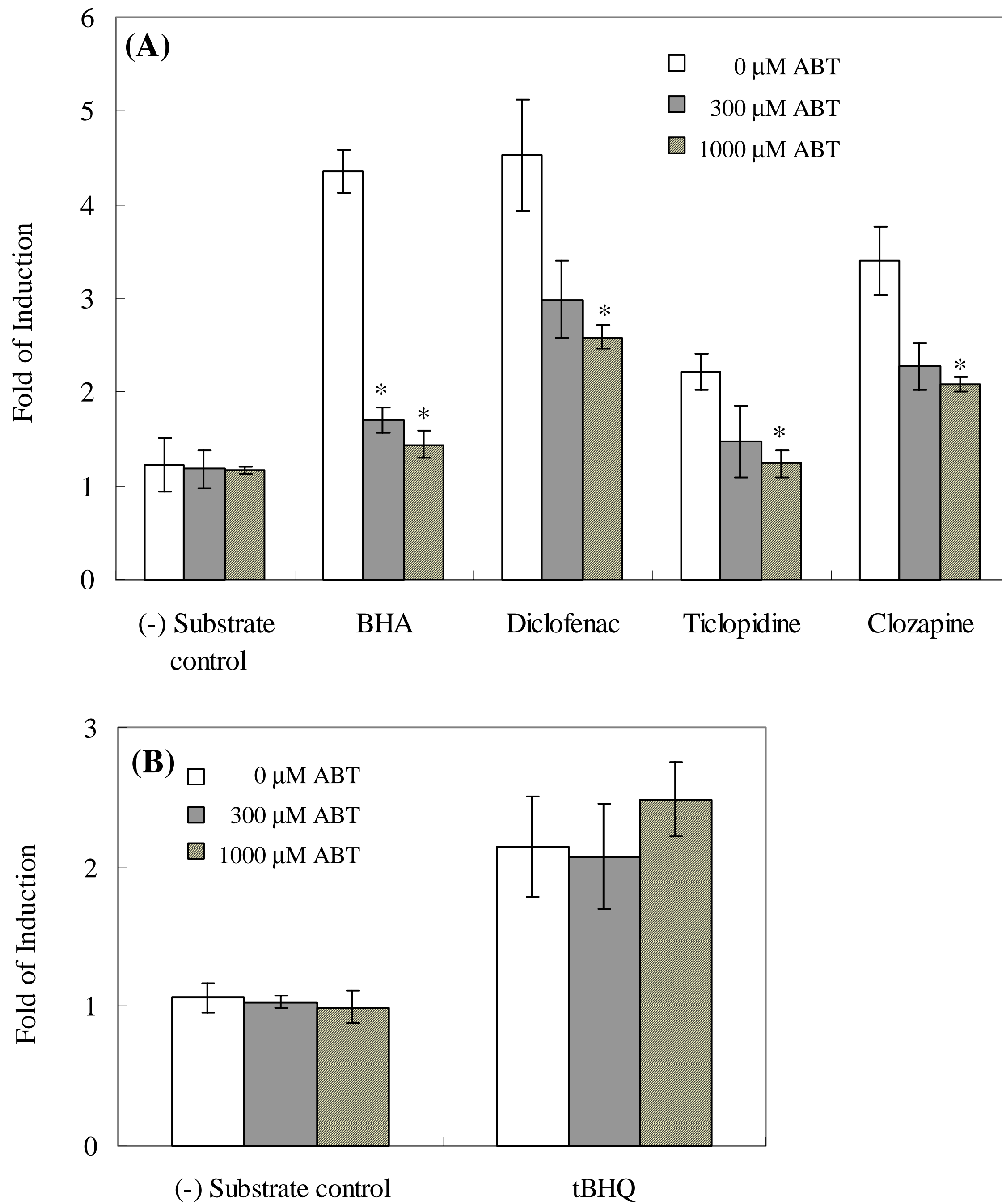


Figure 4

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