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Predictability of idiosyncratic drug toxicity risk for carboxylic acid-containing drugs based on the chemical stability of acyl glucuronide

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AGs, acyl glucuronides; DILI, drug-induced liver injury; HPLC, high-performance liquid chromatography; HSA, human serum albumin; IDT, idiosyncratic drug toxicity; IS, internal standard; KPB, potassium phosphate buffer; MRM, multiple reaction monitoring; SJS, Stevens-Johnson syndrome; 3-OH MA, 3-hydroxymethylmefenamic acid; 3-COOH MA, 3-carboxymefenamic acid

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Abstract

Acyl glucuronides (AGs) formed from carboxylic acid-containing drugs have been considered to be a cause of idiosyncratic drug toxicity (IDT). Chemical stability of AG is supposed to relate to the reactivity. In this study, the half-lives of 21 AGs of carboxylic drugs in the potassium phosphate buffer (KPB), human serum albumin (HSA) solution and human fresh plasma were analyzed in relation to the IDT risk derived from these drugs. The carboxylic drugs were classified into three safety categories of 'safe', 'warning' and 'withdrawn' in terms of their IDT risk. As for the results, the half-lives of AGs in KPB correlated with the IDT risk better than those in HSA solution or in human fresh plasma with regard to the separation of the 'safe' drugs from the 'warning' drugs or the 'withdrawn' drugs. In KPB, while the half-lives in the 'safe' category were 7.2 h or longer, those in the 'withdrawn' category were 1.7 h or shorter. The classification value of the half-life in KPB which separated the 'safe' drugs from the 'withdrawn' drugs was calculated to be 3.6 h by the regression analysis. In conclusion, this is the first report which clearly shows the relationship between the IDT risk and chemical stability of AG in several *in vitro* systems. The KPB buffer system was considered to be the best for evaluating the stability of AG, and the classification value of the half-life in KPB would serve as a useful key predictor for the IDT risk.

Introduction

Acyl glucuronidation is one of the major metabolic routes of drugs containing carboxylic acid, and phase II metabolism like glucuronidation is generally considered as detoxification pathway. It is well known that acyl glucuronides (AGs) are unstable in physiological conditions, and consequently undergo hydrolysis or intramolecular rearrangement, which occurs by migration of the drug moiety from the 1-O-β position to 2-, 3-, and 4-positions on the glucuronic acid ring (Smith et al., 1990; Benet et al., 1993; Bailey and Dickinson, 2003; Skonberg et al., 2008). As a result, AGs and their isomers potentially bind covalently to cellular macromolecules. Recently, some proteins in liver subcellular fractions and the portion of human serum albumin (HSA) to which AGs bind have been identified for several carboxylic drugs, for example, diclofenac AG covalently bound to the canalicular membrane protein in the rat liver (Seitz et al., 1998). In another case, several proteins of rat liver, which zomepirac AG covalently bound to, were investigated (Bailey and Dickinson, 1999). Further, benoxaprofen AG is reported to bind to Lys-159 in HSA (Qiu et al., 1998). It has been reported that covalent binding with proteins correlates to the risk of idiosyncratic drug toxicity (IDT) (Uetrecht, 2001; Zhou et al., 2007). Many carboxylic acid-containing drugs, such as NSAIDS, fibrates and loop diuretics, are metabolized to AGs in humans. Among them, several drugs, such as benoxaprofen, ibufenac and zomepirac, were withdrawn from the market because of acute hepatotoxicity or anaphylaxis. For these drugs, the formation of reactive AGs is considered to be responsible for their IDT (Castillo and Smith, 1995; Lasser et al., 2002; Bailey and Dickinson, 2003). Hence, a system for predicting the IDT derived from AGs is expected for toxicological assessment.

As a pioneering work, Benet et al. showed good correlation between the extent of covalent binding to HSA and the apparent first order degradation rate of AGs of six drugs (Benet et al., 1993) and the half-life of AG has been regarded as corresponding with its chemical

reactivity. After that, several groups have determined half-lives of AGs in the buffer, HSA solution or human fresh plasma for evaluating reactivity (Sallustio et al., 1997; Boelsterli, 2002; Bolze et al., 2002). Based on these interesting findings, we hypothesized that the chemical stability of AG correlates with the IDT risk because the half-lives of AGs formed from drugs that are reported to induce IDT tended to be shorter than those of safer drugs with no IDT reported. To date, however, there are no systematic investigations on the correlation between the chemical stability of AGs and the IDT risk for adequate numbers of carboxylic drugs to reach a robust conclusion. Besides, a proper incubation material to determine the half-life has not been suggested. It is considered that the classification value of the half-life in the proper material which separates the drugs into the categories of the IDT risk would be applied to an assessment for the IDT risk and would help us select compounds in early drug development. In the present study, therefore, the half-lives of 21 AGs of carboxylic drugs (Fig. 1) were determined in the potassium phosphate buffer (KPB), HSA solution and human fresh plasma. Obtained half-lives of AGs in three *in vitro* systems were analyzed in relation to the IDT risk derived from these drugs, and the classification value of AGs was evaluated.

Materials and Methods

Materials.

Niflumic acid used as an internal standard (IS) for all the AGs except levofloxacin AG, HSA (fatty acid free) and dimethyl sulfoxide (ACS reagent) were purchased from Sigma Chemical Company (St. Louis, MO). Sitafloxacin used as an IS for levofloxacin AG was synthesized in-house. KPB (0.5 M, pH 7.4) was obtained from BD Gentest™ (Franklin Lakes, NJ). Human fresh plasma was prepared by centrifugation from heparinized blood samples collected from healthy subjects without medication under a protocol approved by the Institutional Human Ethical Committee. AGs of gemfibrozil, ibufenac, levofloxacin, meclofenamate, mefenamic acid and repaglinide were prepared in-house by bioconversion, or by chemo-enzymatic synthesis from the corresponding methyl acetyl derivatives. Corresponding AGs of *R*-benoxaprofen, *S*-benoxaprofen, diclofenac, fenclofenac, flufenamic acid, furosemide, ibuprofen, indomethacin, montelukast, *R*-naproxen, *S*-naproxen, probenecid, telmisartan, tolmetin and zomepirac were prepared by synthetic methods in Daiichi Sankyo RD Associe Co., Ltd. Captiva for sample filtration was purchased from Varian Inc. (Palo Alto, CA). Acetic acid, acetonitrile, ammonium acetate, dipotassium hydrogen phosphate, formic acid, potassium dihydrogen phosphate, sodium acetate and tetrahydrofuran were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Incubation of AGs in KPB, HSA solution and human fresh plasma.

Solution for each AG in dimethyl sulfoxide at 1 mM was prepared. Human fresh plasma was buffered to pH 7.4 with 1 M KPB (pH 7.0). 0.1M KPB (pH 7.4), 4% HSA in 0.1 M KPB (pH 7.4) or human fresh plasma (pH 7.4) was preincubated at 37 °C for 5 min. AGs (final conc. 10 μM) were added to 0.1M KPB (pH 7.4), 4% HSA in 0.1 M KPB (pH 7.4) or human fresh plasma (pH 7.4) after preincubation and were incubated at 37 °C. Triplicate incubations

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were performed. The sample aliquots of 50 μl except for those of levofloxacin AG were taken at four or more time points from the start of the incubation, followed by the immediate addition of two-fold volume of 5% formic acid in acetonitrile including IS on ice. The sample aliquots of levofloxacin AG (100 μl) were taken at several time points from the start of the incubation, followed by the immediate addition of four-fold volume of 50 mM acetate buffer (pH 5.0) including IS on ice. The samples were stored at -20 ^{o}C until analysis.

Analytical method.

The samples were filtered with Captiva. Two-fold volume of distilled water was added to the filtered samples. Concentrations of AGs except for levofloxacin AG were determined by liquid chromatography-tandem mass spectrometry, using Quattro Micro tandem mass spectrometry (Waters Corp., Milford, MA) interfaced with an Alliance 2795 (Waters Corp., Milford, MA). The detection of the carboxylic acid, its AG and the isomers of AG was carried out by multiple reaction monitoring (MRM) in the positive ion mode except for gemfibrozil AG. The incubation sample of AG was analyzed for obtaining the MRM transition of the isomers because the authentic reference materials were not available for any of the isomers. Separation of carboxylic acid, its AG and the isomers was achieved using a linear gradient elution mode. Linear gradient elution which was optimized for each AG was carried out from 10:90 (solvent A: solvent B) at a flow rate of 0.5 ml/min at 40°C. The mobile phase consisted of 95% acetonitrile containing 5 mM ammonium acetate and 0.2% formic acid (solvent A) and 5% acetonitrile containing 5 mM ammonium acetate and 0.2% formic acid (solvent B). Chromatography was performed on a CAPCELL PAK C18 MGII (5 μm, 2×50 mm, Shiseido Co., Ltd., Tokyo, Japan). Concentration of levofloxacin AG was determined by high-performance liquid chromatography (HPLC) method with fluorescence detection, using L-7480 fluorescence detector interfaced with L-7100 pump (Hitachi, Ltd., Tokyo, Japan). The

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HPLC separation was carried out on a Symmetry® Shield RP_{18} (3.5 µM, 4.6×150 mm, Waters Corp. Milford, MA). The mobile phase consisted of a mixture of 50 mM potassium phosphate buffer (pH 2.0) (solvent C), and tetrahydrofuran (solvent D), from 99:1 for 5 min, to 80:20 over 0.1 min, maintained at 80:20 for 6.9 min. Analyses are run at a flow rate of 1 ml/min at 40°C. Fluorescence detector was set at 296 nm (excitation) and 504 nm (emission). Calibration curves were prepared by spiking a blank sample with an appropriate amount of working solution to produce the calibration curve points equivalent to 1, 3, and 10 µM of each AG. The concentrations of AGs at each time point were obtained by comparing the ratio of analyte to the IS with that at time zero of degradation. The criteria for acceptability of the data included accuracy within ± 20% of the nominal values. The linearity of the calibration curves was determined in a concentration range of 1-10 µM. The calibration curve showed a coefficient of determination greater than 0.99 for each AG.

Categorization of carboxylic acid-containing drugs.

Information on the safety of all the tested drugs was collected from RxList (http://www.rxlist.com/script/main/hp.asp.), which is the internet drug index for prescription drugs and medications, and Japanese drug labeling. All the tested drugs were classified into 'safe', 'warning' and 'withdrawn' (Table 1). The first safety category of 'safe' included drugs with no warnings in RxList or in Japanese drug labeling. This category included 7 drugs: flufenamic acid, gemfibrozil, levofloxacin, meclofenamate, montelukast, repaglinide and telmisartan. The second safety category of 'warning' included drugs with warnings for IDT in RxList or in Japanese drug labeling. This category included 8 drugs: diclofenac, furosemide, ibuprofen, indomethacin, mefenamic acid, naproxen, probenecid and tolmetin. The third safety category of 'withdrawn' included drugs withdrawn from the market because of IDT, such as hepatotoxicity and anaphylaxis. This category included 4 drugs: benoxaprofen, fenclofenac, ibufenac and

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zomepirac. There was no drug that had a black box warning for IDT in RxList.

Data analysis.

The concentrations at 4 or more time points that AG remained at more than 50% of initial concentration were used to determine the degradation rate constant of AG concentrations after incubation in each material. Degradation rate constant was determined from AG concentration versus time curve by linear regression of the semi-logarithmic plot. The half-life was calculated by the following equation:

$$HL = \ln 2 / K$$

where '*HL*' is the half-life of AGs in each test system and *K* is the degradation rate constant.

Ordinal logistic regression analysis was performed to assess the relationship between the half-life and safety category, or the relationship between the half-life, maximum daily dose and the safety category by the following equation using JMP 5.0.1 statistical software (SAS Institute Inc., Cary, NC):

$$\ln\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 \times \log(HL)$$

$$\ln\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 \times \log(HL) + \beta_2 \times \log(\text{Dose})$$

where, p is the probability of each category, and the left side of the equations is the logit value between the two categories. 'Dose' is the maximum daily dose of the tested drug. β_0, β_1 and β_2 are coefficient values of the equation. When the odds were set in unity between the categories, lines separating the zone were drawn where the logit values were zero and the above equations were rearranged to yield the following:

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$$\log(HL) = -\frac{\beta_0}{\beta_1}$$

$$\log(HL) = -\frac{\beta_0}{\beta_1} - \frac{\beta_2}{\beta_1} \times \log(\text{Dose})$$

Results

Correlation between the risk of IDT and the half-lives of AGs in KPB.

In KPB, while the half-lives of AGs in the 'safe' category were 7.2 h or longer, those in the 'warning' category were 3.2 h or shorter except for mefenamic acid and those in the 'withdrawn' category were 1.7 h or shorter (Table 2). The 'safe' drugs were clearly distinguished from the 'withdrawn' drugs by the logarithmic half-lives of AGs in KPB (Table 3, Fig. 2A). The classification value of the half-life in KPB which separated the 'safe' drugs from the 'withdrawn' drugs was calculated to be 3.6 h by the ordinal logistic regression analysis when the odds were set in unity between the categories. The half-lives in the 'warning' category were less than the classification value except for mefenamic acid. The 'warning' drugs could not be separated from the 'withdrawn' drugs by the logarithmic half-lives of AGs in KPB.

Correlation between the risk of IDT and the half-lives of AGs in HSA solution.

In HSA solution, while the half-lives in the 'safe' category were 7.0 h or longer, those in the 'warning' category except for furosemide and mefenamic acid were 1.3 h or shorter and those in the 'withdrawn' category were 1.8 h or shorter (Table 2). It was difficult to separate the 'warning' drugs from the 'withdrawn' drugs by the logarithmic half-lives of AGs in HSA solution. However, the 'safe' drugs were clearly distinguished from the 'withdrawn' drugs by the logarithmic half-lives in HSA solution as in the case of KPB (Table 3, Fig. 2B). The classification value of the half-life in HSA solution which separated the 'safe' drugs from the 'withdrawn' drugs was calculated to be 3.6 h by the regression analysis when the odds were set in unity between the categories. The half-lives in HSA solution in the 'warning' category were less than the classification value, except for furosemide and mefenamic acid.

Correlation between the risk of the IDT and the half-lives of AGs in human fresh plasma.

The half-lives of AGs in human fresh plasma in the 'safe' category were 1.7 h or longer. On the other hand, the half-lives in human fresh plasma in the 'warning' and 'withdrawn' categories were 0.8 h or shorter except for furosemide and mefenamic acid (Table 2). The logistic regression analysis program failed to converge for separation of the 'safe' drugs from the 'withdrawn' drugs, therefore the classification value of half-life in human fresh plasma could not be calculated (Table 3, Fig. 2C).

Effect of the maximum daily doses on correlation analysis between the risk of IDT and the half-lives of AGs.

In order to distinguish the 'warning' drugs from the 'withdrawn' drugs, the maximum daily doses were added to the correlation analysis between the risk of IDT and the half-lives using ordinal logistic regression analysis (Table 4). In terms of separating the 'safe' drugs from the 'withdrawn' drugs, the ratios of β_2 to β_1 calculated on the analyses between the maximum daily doses and the half-lives in KPB, HSA solution and human fresh plasma were calculated to be -0.1, when the odds were set in unity between the category of 'safe' and that of 'withdrawn'. The ratios of β_0 to β_1 calculated on the analyses between the maximum daily doses and the half-lives in KPB, HSA solution and human fresh plasma were calculated to be -0.1, -0.2 and 0.4, respectively. In each analysis, the 'safe' drugs were clearly separated from the 'withdrawn' drugs, however, the 'warning' drugs were not distinguished from the 'withdrawn' drugs because the maximum daily doses of the 'withdrawn' drugs were similar to the 'warning' drugs.

Discussion

The drugs were classified in terms of IDT according to the definition in the review of Uetrecht that IDT is an adverse drug reactions that does not occur in most patients within the normal therapeutic dose range and does not involve the therapeutic effects of the drug (Uetrecht, 2009). One of the mechanisms of IDT is considered that a drug or reactive metabolite that is acting as a hapten modifies several proteins, and finally the immune response would be induced (Uetrecht, 2009). IDT includes several symptoms, such as Stevens-Johnson syndrome (SJS), anaphylaxis, drug-induced liver injury (DILI), neutropenia, agranulocytosis, and thrombocytopenia. The mechanisms of all IDT are not the same, but they are likely to be immune-mediated (Tesfa et al., 2009; Uetrecht, 2009). The main IDT of the carboxylic acid-containing drugs in the 'warning' or 'withdrawn' category was anaphylaxis, DILI, neutropenia, SJS and thrombocytopenia, which are reported as IDT (Table 1).

In this study, we systematically investigated the relationship between the IDT risk and the half-life of AG using a number of AGs formed from carboxylic acid-containing drugs. The regression analysis was performed for the difference between the 'safe' drugs and the 'withdrawn' drugs because the aim of this study is to make the criterion for selecting a compound with a low IDT risk in early drug development. In the result, the 'safe' drugs were separated clearly from the 'withdrawn' drugs by the half-life. This suggests the possibility that the stability of AG is one of the factors which have some sort of influence on the IDT risks as previously reported (Benet et al., 1993; Boelsterli, 2002). The classification value of the half-life in KPB or HSA solution which separated the 'safe' drugs from the 'withdrawn' drugs was calculated to be 3.6 h. This means that the drug, the AG's half-life of which is longer than the classification value, has more than 50% probability of being in the 'safe' category. The classification value in human fresh plasma could not be calculated because of the failure in

convergence. One of the reasons may be the insufficient difference between the half-lives in the two categories.

In terms of the separation of the 'safe' drugs from the 'warning' drugs by the classification value, the half-lives of AGs of the 'warning' drugs in KPB were less than the classification value except for mefenamic acid, while those in HSA solution were less than the classification value except for mefenamic acid and furosemide. Mefenamic acid is not only glucuronidated, but oxidized by P450 at the 3-methyl position. The 3-hydroxymethylmefenamic acid (3-OH-MA) 1-O-AG is formed after the oxidation. The 3-carboxymefenamic acid (3-COOH-MA) 1-O-AG is also formed after further oxidation of the 3-hydroxymethyl to a carboxyl group occurs. To put it all together, mefenamic acid forms three types of AGs from mefenamic acid, 3-OH-MA and 3-COOH-MA (McGurk et al., 1996). The exposure to all three AGs may cause IDT of mefenamic acid although each of them is chemically stable. Therefore, the KPB buffer system was considered to show a better separation than the HSA solution system.

It is reported that KPB promotes isomerization of AGs, while HSA solution and human fresh plasma catalyze more hydrolysis of AGs than KPB (Georges et al., 2000; Spahn-Langguth et al., 1992). The reactivity of the isomers has also been reported (Smith et al., 1986; Dickinson and King, 1991) and the half-life in KPB is considered to reflect the formation of the isomers more than that in HSA solution or human fresh plasma. On the other hand, the half-life in HSA solution or human fresh plasma is considered to overestimate the reactivity of AGs because the half-life includes the degradation by hydrolysis. Therefore, KPB is considered to be a better material than HSA solution for estimating the reactivity of AG. In this study, we have not assessed the influence of formulation of isomers and hydrolysis on IDT risk, however, we think further examinations are needed on the relationship between the formation of isomers and the

IDT risk as the next step.

There is false-negative drug in the 'warning' category such as mefenamic acid. Therefore, it is important that we continue to pay attention to the IDT risk of AG basically and perform clinical studies and post-marketing surveys carefully even if the reactivity of AG is low, because it is difficult to predict and detect the IDT during drug development (Baillie, 2006).

The 'warning' drugs could not be separated from the 'withdrawn' drugs by the half-life. It is reported that the occurrence of IDT is related to clinical doses (Nakayama et al., 2009; Usui et al., 2009), and IDT is rare with drugs at daily doses of 10 mg or less (Uetrecht, 1999). Therefore, we used the maximum daily dose in the analysis to distinguish the 'warning' drugs from the 'withdrawn' drugs. In the result, however, the 'warning' drugs were not distinguished from the 'withdrawn' drugs because the maximum daily doses of the 'withdrawn' drugs were similar to the 'warning' drugs. The absolute ratio of β_2 to β_1, in other words, the absolute ratio of the weight for the clinical dose to that for the half-life of each incubation material was 0.1 in the regression analysis to determine the difference between the 'safe' drugs and the 'withdrawn' drugs. This indicates that the half-life attributed much more to the IDT risk than the clinical dose in our study. The contribution of the clinical dose was masked by that of the half-life because the half-lives from all the 'safe' drugs were longer than those from all the 'withdrawn' drugs. As a matter of fact, the clinical doses of the 'withdrawn' drugs were 600 mg or more, and the clinical dose is also considered to be one of factors which attribute to IDT risk with regard to AG.

It was reported that a structure effect on the degree of AG reactivity demonstrated the rate order: acetic acid > isopropionic acid> benzoic acid derivatives in covalent binding study using a small peptide (Wang et al., 2004). It was hypothesized that the benzoic acid derivative demonstrates the lowest reactivity due to resonance stabilization provided by the aromatic

moiety, and isopropionic acid derivative displays a lower reactivity than that of acetic acid derivative, possibly due to the higher steric hindrance capacity of the isopropyl group over the acetyl group (Wang et al., 2004). In our study, most AGs from the 'warning' and 'withdrawn' drugs are acetic acid or isopropionic acid derivatives except for furosemide AG, mefenamic acid AG and probenecid AG. The instability of probenecid AG in KPB is speculated to be due to a sulfonamide group in the *para* position which is an electron withdrawing moiety. The AGs from the 'safe' drugs included three benzoic acid derivatives, one acetic acid derivative (montelukast AG) and the other derivatives (gemfibrozil AG and levofloxacin AG). The stability of montelukast AG, gemfibrozil AG and levofloxacin AG is assumed to be due to the steric hindrance in their structures. In terms of structure, it might be suggested that a compound containing benzoic acid substitution at the α-carbon should be considered as NCE, however, it should also be considered that the half-life of AG is affected not only by the electrophilicity of the ester carbonyl carbon of AG, but by the steric hindrance around it and the presence of the electron withdrawing moiety (Baba and Yoshioka, 2009).

In drug development, the selection of compounds with low IDT risks as candidate drugs is expected. From our investigation, the half-life of AG is considered to be one of the good indicators for selecting a compound with a low IDT risk since there was no 'safe' drug among the drugs, the AG's half-life of which was shorter than the classification value. Moreover, calculating the half-life is a reproducible method because the half-lives, that were determined in this study, were similar to those in another report (Boelsterli, 2002). The assessment for IDT risk is able to be performed by comparing the half-life in KPB with the classification value to select a compound with a low IDT risk. In this assessment, it is necessary to elucidate a metabolic pathway to identify the formation of AG, and to synthesize AG by bioconversion, organic synthetic method, or enzymatic method before selecting NCE. However, in

consideration of a risk that clinical study would be forced to be discontinued, or NCE would be forced to be withdrawn from the market because of crucial IDT, we think that it is extremely important to perform this assessment after the identification of metabolic profile and synthesis of AG in early drug development.

In conclusion, the present study is the first report which clearly showed the relationship between the IDT risk and chemical stability in several *in vitro* systems. The KPB buffer system was considered to be best for evaluating the half-lives of AGs, and the classification value of the half-life in KPB would serve as a useful key predictor for the IDT risk, which separated the 'safe' drugs from the 'withdrawn' drugs.

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References

Baba A and Yoshioka T (2009) Structure-activity relationships for degradation reaction of 1-beta-o-acyl glucuronides: kinetic description and prediction of intrinsic electrophilic reactivity under physiological conditions. *Chem Res Toxicol* **22:** 158-172.

Bailey MJ and Dickinson RG (1999) Limitations of hepatocytes and liver homogenates in modeling *in vivo* formation of acyl glucuronide-derived drug-protein adducts. *J Pharmacol Toxicol Methods* **41:** 27-32.

Bailey MJ and Dickinson RG (2003) Acyl glucuronide reactivity in perspective: biological consequences. *Chem Biol Interact* **145:** 117-137.

Baillie TA (2006) Future of toxicology-metabolic activation and drug design: challenges and opportunities in chemical toxicology. *Chem Res Toxicol* **19:** 889-93.

Benet LZ, Spahn-Langguth H, Iwakawa S, Volland C, Mizuma T, Mayer S, Mutschler E, and Lin ET (1993) Predictability of the covalent binding of acidic drugs in man. *Life Sci* **53:** PL141-PL146.

Boelsterli UA (2002) Xenobiotic acyl glucuronides and acyl CoA thioesters as protein-reactive metabolites with the potential to cause idiosyncratic drug reactions. *Curr Drug Meteb* **3:** 439-450.

Bolze S, Bromet N, Gay-Feutry C, Massiere F, Boulieu R, and Hulot T (2002) Development of an in vitro screening model for the biosynthesis of acyl glucuronide metabolites and the assessment of their reactivity toward human serum albumin. *Drug Metab Dispos* **30:** 404-413.

Castillo M and Smith PC (1995) Disposition and reactivity of ibuprofen and ibufenac acyl glucuronides in vivo in the rhesus monkey and in vitro with human serum albumin. *Drug Metab Dispos* **23:** 566-572.

Dickinson RG and King AR (1991) Studies on the reactivity of acyl glucuronides--II. Interaction of diflunisal acyl glucuronide and its isomers with human serum albumin in vitro. *Biochem Pharmacol* **42:** 2301-2306.

Georges H, Presle N, Buronfosse T, Fournel-Gigleux S, Netter P, Magdalou J, and Lapicque F (2000) In vitro stereoselective degradation of carprofen glucuronide by human serum albumin. Characterization of sites and reactive amino acids. *Chirality* **12:** 53-62.

Lasser KE, Allen PD, Woolhandler SJ, Himmelstein DU, Wolfe SM, and Bor DH (2002) Timing of new black box warnings and withdrawals for prescription medications. *JAMA* **287:** 2215-2220.

McGurk KA, Remmel RP, Hosagrahara VP, Tosh D, and Burchell B (1996) Reactivity of mefenamic acid 1-o-acyl glucuronide with proteins in vitro and ex vivo. *Drug Metab Dispos* **24:** 842-849.

Nakayama S, Atsumi R, Takakusa H, Kobayashi Y, Kurihara A, Nagai Y, Nakai D, and Okazaki O (2009) A zone classification system for risk assessment of idiosyncratic drug toxicity using daily dose and covalent binding. *Drug Metab Dispos* **37:** 1970-1977.

Qiu Y, Burlingame AL, and Benet LZ (1998) Mechanisms for covalent binding of benoxaprofen glucuronide to human serum albumin. Studies By tandem mass spectrometry. *Drug Metab Dispos* **26:** 246-256.

Sallustio BC, Fairchild BA, and Pannall PR (1997) Interaction of human serum albumin with the electrophilic metabolite 1-O-gemfibrozil-beta-D-glucuronide. *Drug Metab Dispos* **25:** 55-60.

Seitz S, Kretz-Rommel A, Oude Elferink RP, and Boelsterli UA (1998) Selective protein adduct formation of diclofenac glucuronide is critically dependent on the rat canalicular conjugate export pump (Mrp2). *Chem Res Toxicol* **11:** 513-519.

Skonberg C, Olsen J, Madsen KG, Hansen SH, and Grillo MP (2008) Metabolic activation of carboxylic acids. *Expert Opin Drug Metab Toxicol* **4:** 425-438.

Smith PC, McDonagh AF, and Benet LZ (1986) Irreversible binding of zomepirac to plasma protein in vitro and in vivo. *J Clin Invest* **77:** 934-939.

Smith PC, Benet LZ, and McDonagh AF (1990) Covalent binding of zomepirac glucuronide to

proteins: evidence for a Schiff base mechanism. *Drug Metab Dispos* **18:** 639-644.

Spahn-Langguth H and Benet LZ (1992) Acyl glucuronides revisited: is the glucuronidation process a toxification as well as a detoxification mechanism? *Drug Metab Rev* **24:** 5-47.

Tesfa D, Keisu M, Palmblad J (2009) Idiosyncratic drug-induced agranulocytosis: possible mechanisms and management. *Am J Hematol* **84:** 428-434.

Uetrecht JP (1999) New concepts in immunology relevant to idiosyncratic drug reactions: the "danger hypothesis" and innate immune system. *Chem Res Toxicol* **12:** 387-395.

Uetrecht J (2001) Prediction of a new drug's potential to cause idiosyncratic reactions. *Curr Opin Drug Discov Devel* **4:** 55-59.

Uetrecht J (2009) Immune-mediated adverse drug reactions. *Chem Res Toxicol* **22:** 24-34.

Usui T, Mise M, Hashizume T, Yabuki M, and Komuro S. (2009) Evaluation of the potential for drug-induced liver injury based on in vitro covalent binding to human liver proteins. *Drug Metab Dispos* **37:** 2383-2392.

Wang J, Davis M, Li F, Azam F, Scatina J, and Talaat R. (2004) A novel approach for predicting acyl glucuronide reactivity via Schiff base formation: development of rapidly formed peptide adducts for LC/MS/MS measurements. *Chem Res Toxicol* **17:** 1206-1216.

Zhou SF, Xue CC, Yu XQ, Li C, and Wang G (2007) Clinically important drug interactions potentially involving mechanism-based inhibition of cytochrome P450 3A4 and the role of therapeutic drug monitoring. *Ther Drug Monit* **29:** 687-710.

Footnotes

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Legend for figures

Fig. 1. Structures of the 21 AGs used in this analysis.

Fig. 2. The half-lives of AGs in KPB (A), 4% HSA solution (B) and human plasma (C). Numbers associated with symbols correspond to AGs names as follows: 1, flufenamic acid AG; 2, gemfibrozil AG; 3, levofloxacin AG; 4, meclofenamate AG; 5, montelukast AG; 6, repaglinide AG; 7, telmisartan AG; 8, diclofenac AG; 9, furosemide AG; 10, ibuprofen AG; 11, indomethacin AG; 12, mefenaminc acid AG; 13, *R*-naproxen AG; 14, *S*-naproxen AG; 15, probenecid AG; 16, tolmetin AG; 17, *R*-benoxaprofen AG; 18, *S*-benoxaprofen AG; 19, fenclofenac AG; 20, ibufenac AG; 21, zomepirac AG.

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Table 1. The reported IDT and the maximum daily doses of tested drugs

IDT risk	Drug	Toxicity	Maximum daily dose (mg)
Safe			
	Flufenamic acid		750
	Gemfibrozil		1200
	Levofloxacin		750
	Meclofenamate		400
	Montelukast		10
	Repaglinide		16
	Telmisartan		80
Warning			
	Diclofenac	Anaphylaxis , DILI, SJS	150
	Furosemide	Neutropenia, thrombocytopenia	80
	Ibuprofen	Anaphylaxis, SJS	3200
	Indomethacin	Anaphylaxis, SJS	200
	Mefenamic acid	Anaphylaxis, SJS	1250
	Naproxen	Anaphylaxis, SJS	1000
	Probenecid	Anaphylaxis	1000
	Tolmetin	Anaphylaxis , DILI, SJS	1800
Withdrawn			
	Benoxaprofen	DILI	1200
	Fenclofenac	DILI	1800
	Ibufenac	DILI	4000
	Zomepirac	Anaphylaxis , DILI	600

DILI - Drug-induced liver injury

SJS - Stevens-Johnson syndrome

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Table 2. The half-lives of AGs in KPB, HSA solution and human plasma

No.	AG	Half-life (h) (Mean ± S.D.)		
		KPB	HSA	Human plasma
1	Flufenamic acid AG	7.2 ± 0.6	7.7 ± 1.1	4.4 ± 0.6
2	Gemfibrozil AG	71.4 ± 7.1	7.6 ± 0.8	1.7 ± 0.1
3	Levofloxacin AG	16.1 ± 2.3	16.3 ± 3.8	3.1 ± 0.2
4	Meclofenamate AG	28.1 ± 1.6	16.6 ± 0.9	3.0 ± 0.2
5	Montelukast AG	37.5 ± 5.9	21.8 ± 1.0	1.7 ± 0.3
6	Repaglinide AG	11.5 ± 1.0	7.0 ± 0.2	7.0 ± 0.1
7	Telmisartan AG	45.6 ± 5.5	111.8 ± 14.0	11.7 ± 1.2
8	Diclofenac AG	0.7 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
9	Furosemide AG	3.2 ± 0.0	7.2 ± 0.3	3.7 ± 0.2
10	Ibuprofen AG	2.7 ± 0.1	1.3 ± 0.0	0.8 ± 0.1
11	Indomethacin AG	1.7 ± 0.1	0.6 ± 0.0	0.2 ± 0.0
12	Mefenamic acid AG	17.0 ± 0.5	51.3 ± 6.2	5.8 ± 1.1
13	*R*-Naproxen AG	1.1 ± 0.0	1.0 ± 0.1	0.3 ± 0.1
14	*S*-Naproxen AG	2.2 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
15	Probenecid AG	0.3 ± 0.0	0.8 ± 0.0	0.2 ± 0.0
16	Tolmetin AG	0.4 ± 0.0	0.5 ± 0.0	0.2 ± 0.0
17	*R*-Benoxaprofen AG	0.7 ± 0.0	0.8 ± 0.0	0.1 ± 0.0
18	*S*-Benoxaprofen AG	1.4 ± 0.1	1.8 ± 0.0	0.7 ± 0.1
19	Fenclofenac AG	1.7 ± 0.1	0.9 ± 0.2	0.8 ± 0.1
20	Ibufenac AG	0.8 ± 0.0	0.6 ± 0.0	0.3 ± 0.0
21	Zomepirac AG	0.4 ± 0.0	0.2 ± 0.0	0.1 ± 0.0

Table 3. Ordinal logistic regression analysis of half-life in KPB, HSA solution and human plasma

Test system	Whole-model test	Analysis of maximum likelihood estimation					
	Logit r^2	β_0			β_1 (half-life)		
		Estimate	S.E.	p value	Estimate	S.E.	p value
KPB	1	-48.5	0.0	<0.0001	87.6	0.0	<0.0001
HSA solution	1	-54.0	0.0	<0.0001	97.3	0.0	<0.0001
Human plasma	N.C.	N.C.	-	-	N.C.	-	-

N.C. - Not calculated due to convergence failure

Table 4. Ordinal logistic regression analysis of half-life and maximum daily dose in KPB, HSA solution and human plasma

Test system	Whole-model test	Analysis of maximum likelihood estimation								
	Logit r^2	β_0			β_1 (half-life)			β_2 (maximum daily dose)		
		Estimate	S.E.	p value	Estimate	S.E.	p value	Estimate	S.E.	p value
KPB	1	-9.8	0.0	<0.0001	80.9	0.0	<0.0001	-11.4	0.0	<0.0001
HSA solution	1	-12.8	592300	1	80.7	0.0	<0.0001	-10.7	0.0	<0.0001
Human plasma	1	54.3	566000	1	146.5	0.0	<0.0001	-20.9	0.0	<0.0001

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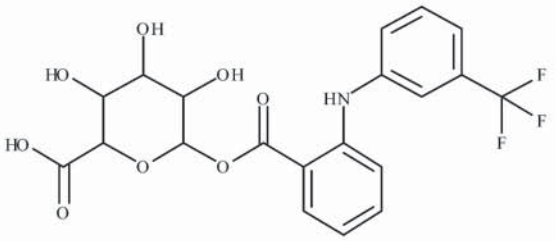
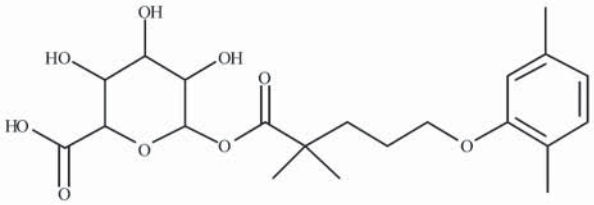
Fig. 1

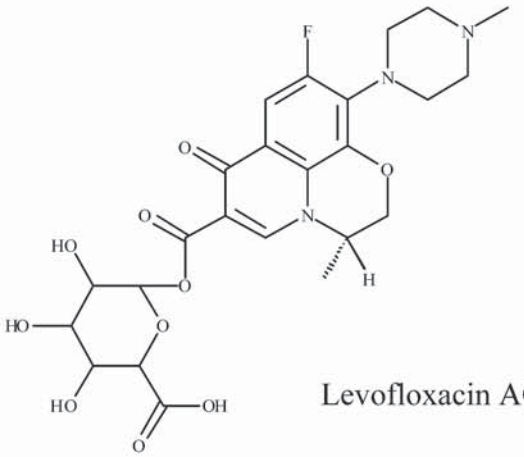
AGs of the 'safe' drugs



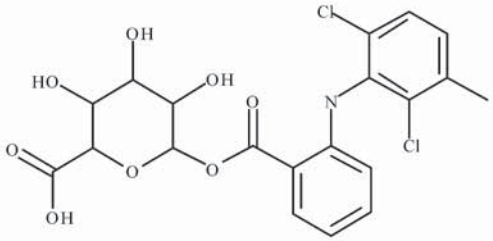
Flufenamic acid AG



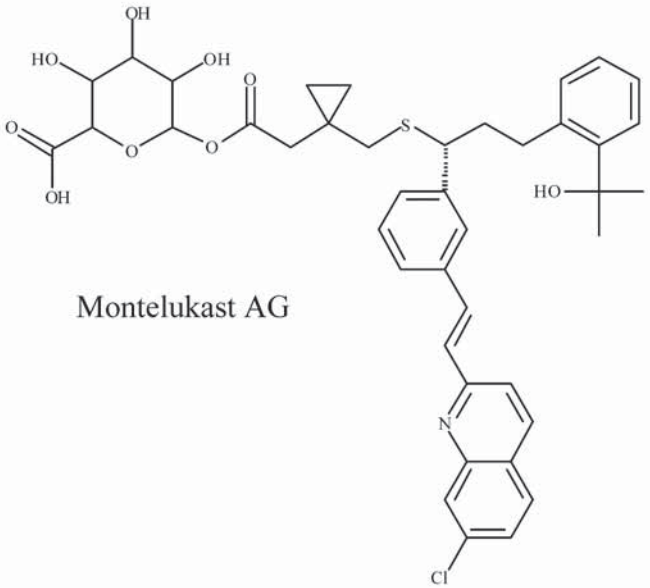
Gemfibrozil AG



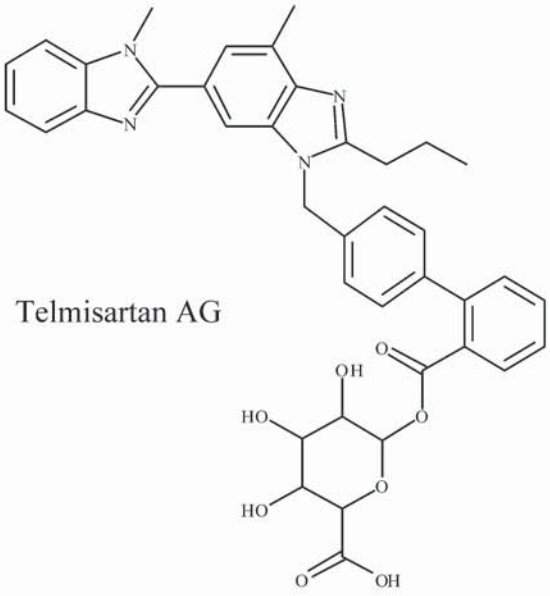
Levofloxacin AG



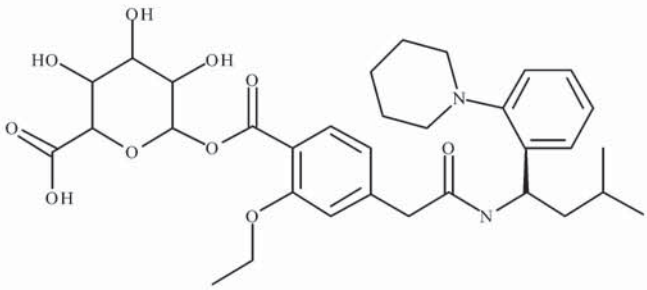
Meclofenamate AG



Montelukast AG



Telmisartan AG



Repaglinide AG

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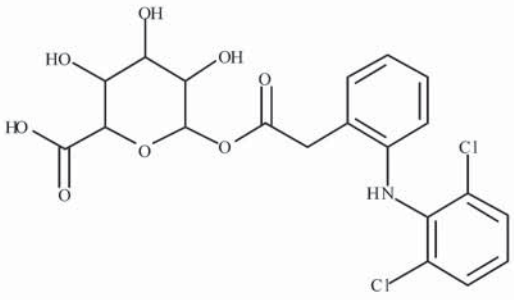
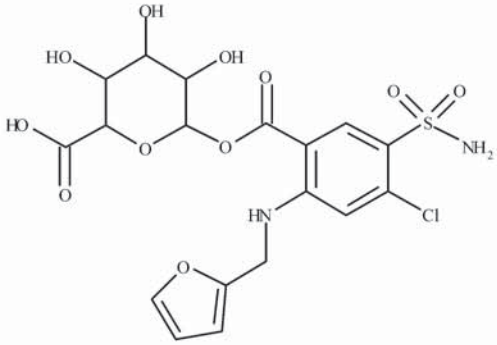
Fig. 1 (contd)

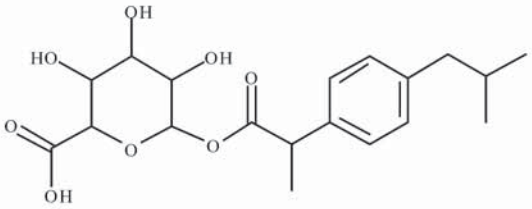
AGs of the 'warning' drugs



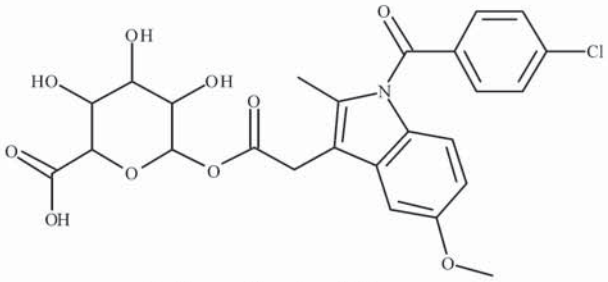
Diclofenac AG



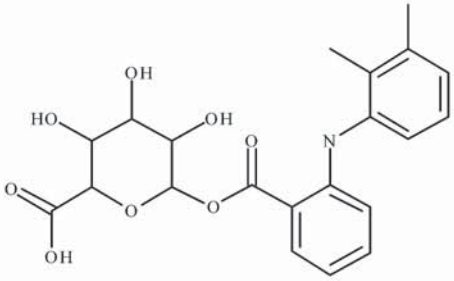
Furosemide AG



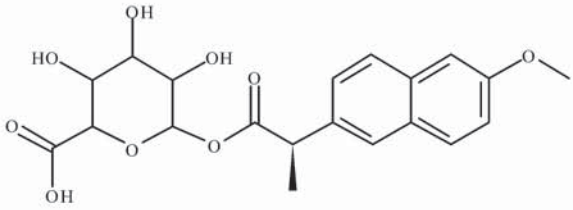
Ibuprofen AG



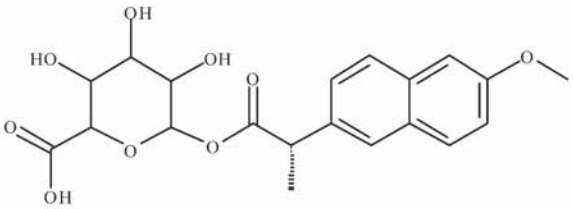
Indomethacin AG



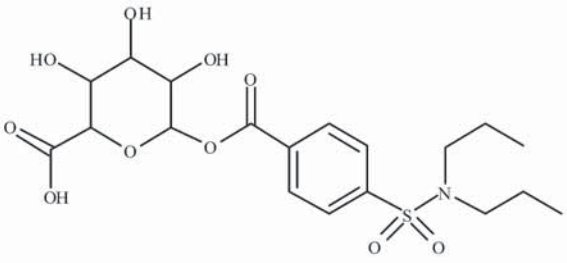
Mefenamic acid AG



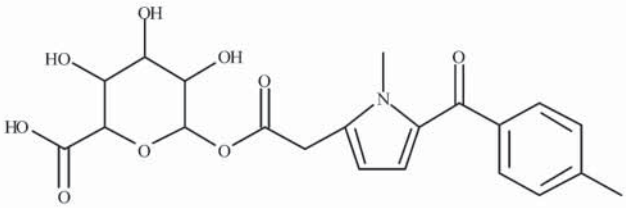
R-Naproxen AG



S-Naproxen AG



Probenecid AG



Tolmetin AG

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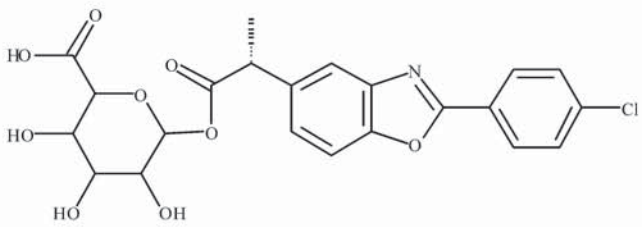
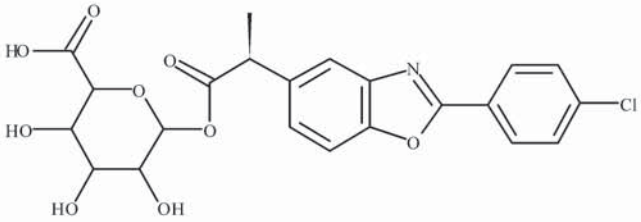
Fig. 1 (contin)

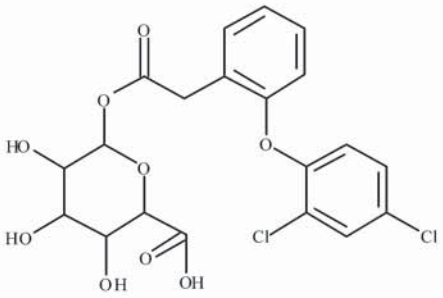
AGs of the 'withdrawn' drugs



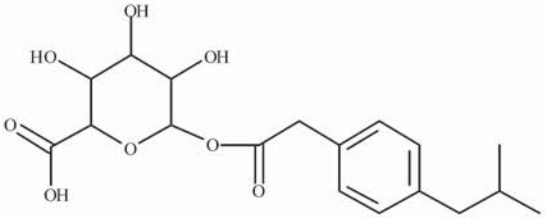
R-Benoxaprofen AG



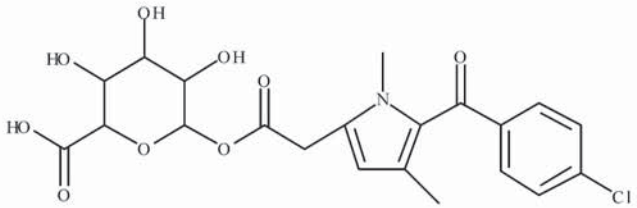
S-Benoxaprofen AG



Fenclofenac AG



Ibufenac AG



Zomepirac AG

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Fig. 2