Predictability of Idiosyncratic Drug Toxicity Risk for Carboxylic Acid-Containing Drugs Based on the Chemical Stability of Acyl Glucuronide

Ryoko Sawamura, Noriko Okudaira, Kengo Watanabe, Takahiro Murai, Yoshimasa Kobayashi, Masaya Tachibana, Takashi Ohnuki, Kayoko Masuda, Hidehito Honma, Atsushi Kurihara, and Osamu Okazaki

Drug Metabolism and Pharmacokinetics Research Laboratories, Daiichi Sankyo Co., Ltd., Tokyo, Japan

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 AGs is supposed to relate to their reactivity. In this study, the half-lives of 21 AGs of carboxylic drugs in 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 The carboxylic drugs were classified into three safety categories of “safe,” “warning,” and “withdrawn” in terms of their IDT risk.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in HSA solution or in human fresh plasma with regard to the separation of the safe drugs from the warning drugs or the withdrawn drugs. In KPB, whereas the half-lives in the safe category were 7.2 h or longer, those in the withdrawn category were 1.7 h or shorter. The classification value of the half-life in KPB, which separated the safe drugs from the withdrawn drugs was calculated to be 3.6 h by regression analysis. In conclusion, this is the first report that clearly shows the relationship between the IDT risk and chemical stability of AGs in several in vitro systems. The KPB system was considered to be the best for evaluating the stability of AGs, and the classification value of the half-life in KPB serves 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 a 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 the 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. 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). Furthermore, 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 idiosyncrasy of drug toxicity (IDT) (Uetrecht, 2001; Zhou et al., 2007). Many carboxylic acid-containing drugs, such as nonsteroidal anti-inflammatory drugs, 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. (1993) showed good correlation between the extent of covalent binding to HSA and the apparent first-order degradation rate of AGs of six drugs, and the half-life of AG has been regarded as corresponding to its chemical reactivity. After that, several groups determined half-lives of AGs in 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 AGs 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. However, to date, there have been 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. Furthermore, a proper incubation material to determine the half-life has not been suggested. It is considered that the

ABBREVIATIONS: AG, acyl glucuronides; HSA, human serum albumin; IDT, idiosyncratic drug toxicity; KPB, potassium phosphate; IS, internal standard; NCE, new chemical entity.
classification value of the half-life in the proper material that 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. Therefore, in the present study, the half-lives of 21 AGs of carboxylic drugs (Fig. 1) were determined in potassium phosphate buffer (KPB), HSA solution,

A AGs of the safe drugs

Flufenamic acid AG

Gemfibrozil AG

Meclofenamate AG

Levofloxacin AG

Montelukast AG

Telmisartan AG

Repaglinide AG

FIG. 1. Structures of the 21 AGs used in this analysis.
and human fresh plasma. Half-lives of AGs obtained 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 of the AGs except levofloxacin AG, HSA (fatty acid-free), and dimethyl sulfoxide (ACS reagent) were purchased from Sigma-Aldrich (St. Louis, MO). Sitaflaxacin used as an IS for levofloxacin AG was synthesized in-house. KPB (0.5 M, pH 7.4) was obtained from BD Gentest (Woburn, MA). 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 ethics committee. AGs of gemfibrozil, ibufenac, levofloxacin, meclofenamate, mefenamic acid, and repaglinide were prepared in-house by bioconversion or by chemoenzymatic 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 buffered to pH 7.4 with 1 M KPB (pH 7.0), 0.1 M KPB (pH 7.4), and 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 concentration 10 \( \mu \)M) were added to 0.1 M KPB (pH 7.4) and 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 were performed. The sample aliquots of 50 \( \mu \)L 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 a 2-fold volume of 5%
formic acid in acetonitrile including IS on ice. The sample aliquots of levo-
floxacin AG (100 μl) were taken at several time points from the start of the
incubation, followed by the immediate addition of a 4-fold volume of 50 mM
acetate buffer (pH 5.0) including IS on ice. The samples were stored at −20°C
until analysis.

**Analytical Method.** The samples were filtered with Captiva. A 2-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 Corporation, Milford, MA) interfaced with an Alliance 2795 (Waters
Corporation). Carboxylic acid, its AG, and the isomers of AG were detected by
multiple reaction monitoring in the positive ion mode except for gemfibrozil
AG. The incubation sample of AG was analyzed to obtain the multiple reaction
monitoring 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 performed 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). The concentration of levofloxacin AG was determined by a high-
performance liquid chromatography method with fluorescence detection, using
an L-7480 fluorescence detector interfaced with and L-7100 pump (Hitachi,
Ltd., Tokyo, Japan). The high-performance liquid chromatography separation
was performed on a Symmetry Shield RP18 column (3.5 μm, 4.6 × 150 mm;
Waters Corporation). 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 and maintained at 80:20 for 6.9
min. Analyses were run at a flow rate of 1 ml/min at 40°C. The 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 concentrations 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
0 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 to 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 of the tested drugs were
classified into “safe,” “warning,” and “withdrawn” (Table 1). The first safety
category of safe includes drugs with no warnings in RxList or in Japanese drug
labeling. This category includes seven drugs: flufenamic acid, gemfibrozil,
levofloxacin, meclofenamate, montelukast, repaglinide, and telmisartan. The
**TABLE 1**

<table>
<thead>
<tr>
<th>Drug IDT Risk</th>
<th>Toxicity</th>
<th>Maximum Daily Dose</th>
<th>mg</th>
</tr>
</thead>
<tbody>
<tr>
<td><em><strong>Safe</strong></em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td>750</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>1200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>750</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meclofenamate</td>
<td>400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Montelukast</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repaglinide</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Telmisartan</td>
<td>80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Warning**
- Ibuprofen, Anaphylaxis, DILI, SJS
- Furosemide, Neutropenia, thrombocytopenia
- Indomethacin, Anaphylaxis, SJS
- Mefenamic acid, Anaphylaxis, SJS
- Naproxen, Anaphylaxis, SJS
- Probenecid, Anaphylaxis
- Tolmetin, Anaphylaxis, DILI, SJS
- Ibuprofen, Anaphylaxis, DILI

**Withdrawn**
- Ibufenac, Anaphylaxis, DILI
- Fenclofenac, Anaphylaxis
- Ibuprofen, Anaphylaxis
- Zomepirac, Anaphylaxis, DILI

Half-lives of AGs in KPB, HSA solution, and human plasma

<table>
<thead>
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<th>Toxicity</th>
<th>Maximum Daily Dose</th>
<th>mg</th>
</tr>
</thead>
<tbody>
<tr>
<td><em><strong>Safe</strong></em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td>7.2 ± 0.6</td>
<td>7.7 ± 1.1</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>71.4 ± 7.1</td>
<td>7.6 ± 0.8</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>16.1 ± 2.3</td>
<td>16.3 ± 3.8</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>Meclofenamate</td>
<td>28.1 ± 1.6</td>
<td>16.6 ± 0.9</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>Montelukast</td>
<td>37.5 ± 5.2</td>
<td>21.8 ± 1.7</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>11.5 ± 1.0</td>
<td>7.0 ± 0.2</td>
<td>7.0 ± 0.1</td>
</tr>
<tr>
<td>Telmisartan</td>
<td>45.6 ± 5.5</td>
<td>111.8 ± 14.0</td>
<td>11.7 ± 12</td>
</tr>
<tr>
<td>Ibufenac</td>
<td>0.7 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>Furosemide</td>
<td>3.2 ± 0.0</td>
<td>7.2 ± 0.3</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>Probenecid</td>
<td>2.7 ± 0.1</td>
<td>1.3 ± 0.0</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Tolmetin</td>
<td>1.7 ± 0.1</td>
<td>0.6 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Mefenamic acid</td>
<td>17.0 ± 0.5</td>
<td>51.3 ± 6.2</td>
<td>5.8 ± 1.1</td>
</tr>
<tr>
<td>Naproxen</td>
<td>1.1 ± 0.0</td>
<td>1.0 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Probencid</td>
<td>2.2 ± 0.1</td>
<td>0.3 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.3 ± 0.0</td>
<td>0.8 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Probenecid</td>
<td>0.4 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Benoxaprofen</td>
<td>0.7 ± 0.0</td>
<td>0.8 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>Fenclofenac</td>
<td>1.4 ± 0.1</td>
<td>1.8 ± 0.0</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>1.7 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Zomepirac</td>
<td>0.8 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>Ibufenac</td>
<td>0.4 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
</tbody>
</table>

**Results**

**Correlation between the Risk of IDT and the Half-Lives of AGs in KPB.** In KPB, although 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 that 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, although 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,
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 the 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. 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 $\beta_2$ to $\beta_1$ 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 $\beta_0$ to $\beta_1$ 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 those of the warning drugs.

Discussion

Drugs were classified in terms of IDT according to the definition in the review of Uetrecht (2009) that IDT is an adverse drug reaction that does not occur in most patients within the normal therapeutic dose range and does not involve the therapeutic effects of the drug. One of the mechanisms of IDT is that a drug or reactive metabolite acting as a hapten modifies several proteins and finally the immune response is induced (Uetrecht, 2009). IDT includes several symptoms, such as Stevens-Johnson syndrome, anaphylaxis, drug-induced liver injury, neutropenia, agranulocytosis, and thrombocytopenia. The mechanisms of all IDTs are not the same, but they are likely to be immune-mediated (Tesfa et al., 2009; Uetrecht, 2009). The main IDTs of the carboxylic acid-containing drugs in the warning or withdrawn category were anaphylaxis, drug-induced liver injury, neutropenia, Stevens-Johnson syndrome, and thrombocytopenia (Table 1). In this study, we systematically investigated the relationship between the IDT risk and the half-life of AGs 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 was to determine 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 that has some influence on the

### TABLE 4

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

<table>
<thead>
<tr>
<th>Test System</th>
<th>Whole-Model Test: Logit $r^2$</th>
<th>$\hat{p}_0$</th>
<th>$\hat{p}_1$, (Half-Life)</th>
<th>$\hat{p}_1$, (Maximum Daily Dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KPB</td>
<td>1</td>
<td>$-9.8$</td>
<td>80.9</td>
<td>$-11.4$</td>
</tr>
<tr>
<td>HSA solution</td>
<td>1</td>
<td>$-12.8$</td>
<td>80.7</td>
<td>$-10.7$</td>
</tr>
<tr>
<td>Human plasma</td>
<td>1</td>
<td>$54.3$</td>
<td>146.5</td>
<td>$-20.9$</td>
</tr>
</tbody>
</table>

FIG. 2. The half-lives of AGs in KPB (A), 4% HSA solution (B), and human plasma (C). Numbers associated with symbols correspond to names of AG 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, mefenamic 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.

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.

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</tr>
</tbody>
</table>
IDT risks as reported previously (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 for which the AG half-life is longer than the classification value has a more than 50% probability of being in the safe category. The classification value in human fresh plasma could not be calculated because of 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, whereas those in HSA solution were less than the classification value except for mefenamic acid and furosemide. Mefenamic acid is not only glucuronidated but also oxidized by P450 at the 3-methyl position. The 3-hydroxymethylmefenamic acid 1-O-AG is formed after the oxidation. The 3-carboxymefenamic acid 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-hydroxymethylmefenamic acid, and 3-carboxymefenamic acid (McGurk et al., 1996). The exposure to all three AGs may cause the IDT of mefenamic acid although each of them is chemically stable. Therefore, the KPB system was considered to show a better separation than the HSA solution system.

It has been reported that KPB promotes isomerization of AGs, whereas HSA solution and human fresh plasma catalyze more hydrolysis of AGs than KPB (Spahn-Langguth and Benet, 1992; Georges et al., 2000). 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 AGs. 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 are false-negative drugs, such as mefenamic acid, in the warning category. Therefore, it is important that we continue to pay attention to the IDT risk of AGs and perform clinical studies and postmarketing surveys carefully even if the reactivity of the 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 half-life. It is reported that the occurrence of IDT is related to clinical doses (Nakayama et al., 2009; Usui et al., 2009) and that 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. However, in the result, the warning drugs were not distinguished from the withdrawn drugs because the maximum daily doses of the withdrawn drugs were similar to those of the warning drugs. The absolute ratio of $\beta_2$ to $\beta_1$, 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 of the safe drugs were longer than those from all of 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 the factors that contributes to IDT risk with regard to AGs.

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 the 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 a benzoic acid substitution at the $\alpha$-carbon should be considered as a 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 also 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 because there was no safe drug among the drugs for which the AG half-life was shorter than the classification value. Moreover, calculating the half-life is a reproducible method because the half-lives, which were determined in this study, were similar to those in another report (Boelsterli, 2002). The assessment for IDT risk can 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 biocconversion, the organic synthetic method, or an enzymatic method before selecting a NCE. However, in consideration of a risk that clinical study would be forced to be discontinued or a NCE would be forced to be withdrawn from the market because of a crucial IDT, we think that it is extremely important to perform this assessment after the identification of the metabolic profile and synthesis of AG in early drug development.

In conclusion, the present study is the first report that clearly showed the relationship between the IDT risk and chemical stability in several in vitro systems. The KPB 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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