Covalent Binding and Tissue Distribution/Retention Assessment of Drugs Associated with

Idiosyncratic Drug Toxicity

Hideo Takakusa, Hiroshi Masumoto, Hideo Yukinaga, Chie Makino, Shintaro Nakayama, Osamu

Okazaki, Kenichi Sudo

Drug Metabolism & Pharmacokinetics Research Laboratories, R&D Division, Daiichi Sankyo Co.,

Ltd. 1-2-58, Hiromachi, Shinagawa-ku, Tokyo, 140-8710, Japan.
Running title: Covalent binding and tissue retention of drugs

Corresponding author: Hideo Takakusa, Ph.D.

Drug Metabolism & Pharmacokinetics Research Laboratories, R&D Division, Shinagawa R&D Center, Daiichi Sankyo Co., Ltd.

1-2-58, Hiromachi, Shinagawa-ku, Tokyo, 140-8710, Japan.

Telephone: +81-3-3492-3131

Fax: +81-3-5436-8567

Email: takakusa.hideo.yb@daiichisankyo.co.jp

Number of text pages: 28 (including references and legends)

Number of tables: 4

Number of figures: 5

Number of References: 31

Number of words in the Abstract: 250

Number of words in the Introduction: 590

Number of words in Discussion: 1686

Non-standard abbreviations

AE, adverse event; ARG, autoradiography; GSH, glutathione; HLM, human liver microsome; IDT,
idiosyncratic drug toxicity; NCE, new chemical entity; RLM, rat liver microsome; RM, reactive metabolite.
ABSTRACT

Bioactivation of a drug to a reactive metabolite and its covalent binding to cellular macromolecules is believed to be involved in clinical adverse events, including IDTs. For the interpretation of the covalent binding data in terms of risk assessment, the in vitro and in vivo covalent binding data of a variety of drugs associated with IDTs or not were determined. Most of the ‘problematic’ drugs, including ‘withdrawn’ and ‘warning’ drugs, exhibit higher HLM in vitro covalent binding yields than the ‘safe’ drugs. Although some of the ‘problematic’ drugs that are known to undergo bioactivation other than CYP-mediated oxidation exhibited only trace levels of HLM covalent binding like ‘safe’ drugs, a rat in vivo covalent binding study could assess the bioactivation of such drugs. Furthermore, the tissue distribution/retention of the drugs was also examined by rat autoradiography (ARG). The residual radioactivity in the liver observed at 72 or 168 h post-dose was found to be well correlated with the rat in vivo covalent binding to liver proteins and thus the in vivo covalent binding yields of the drugs could be extrapolated from the retention profiles observed by means of ARG. Long-term retention of radioactivity in the bone marrow was observed with some drugs associated with severe agranulocytosis, suggesting a spatial relationship between the toxicity profile and drug distribution/retention. Taken together, the covalent binding and tissue distribution/retention data of the various marketed drugs obtained in the present study should be quite informative for the interpretation of data in terms of risk assessment.
Drug-induced toxicity is a major impediment of drug therapy and drug development. Although toxic drugs should be screened out during preclinical safety studies, in some cases, rare but serious toxicities, so-called idiosyncratic drug toxicities (IDTs) (Kaplowitz, 2005; Li, 2002; Seguin and Uetrecht, 2003), become apparent after the launch of new chemical entities (NCEs). Many drugs, including troglitazone, tienilic acid, clozapine, flutamide and ticlopidine have been withdrawn from the market or given a ‘black box’ warning as a result of adverse events (AEs) that had not been predicted. 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 (Evans et al., 2004; Walgren et al., 2005; Zhou et al., 2005). Therefore, it is important to assess the potential of NCEs to generate reactive metabolites and form drug-protein covalent adducts in the preclinical stages. Several in vitro techniques to evaluate reactive metabolite formation have been recently developed, including the qualitative LC-MS/MS detection of the reactive metabolites trapped with nucleophiles such as glutathione (GSH) and cyanide (Baillie and Davis, 1993). Since these techniques can provide us with structural information regarding the reactive metabolites trapped with the nucleophiles, they are helpful in understanding the mechanism of metabolic bioactivation and in modifying the structure to minimize metabolic bioactivation. The quantitative assessment of reactive metabolite formation using radio-labeled GSH and fluorophore-tagged GSH has also been reported and utilized (Gan et al., 2005; Masubuchi et al., 2007).

Radio-labeled tracers of NCEs enable us to conduct the study of covalent binding, so that the
amount of radioactivity covalently bound to proteins can be determined. Covalent binding studies are considered to be the most general and definitive assessments of bioactivation because they can provide direct evidence of the covalent binding of NCEs to proteins. In order to interpret the covalent binding data in terms of risk assessment, it is important to collect sufficient background data on a variety of marketed drugs by a standard method. Evans et al. in Merck Research Laboratories have provided protocols for in vitro covalent binding studies in a liver microsomal system and in an in vivo study using rats, as well as a decision tree for assessing the suitability of NCEs for development based on bioactivation considerations (Evans et al., 2004). Liver microsomes are employed to assess the intrinsic covalent binding yields catalyzed primarily by oxidative enzymes. Rat in vivo studies can be used to assess the metabolic bioactivation that occurs in systems other than liver microsomal systems. Based on these protocols, the covalent binding data of some drugs known to undergo bioactivation, such as acetaminophen, furosemide and tienilic acid, were collected by Masubuchi et al. (Masubuchi et al., 2007). For further consideration of the relationship between the incidence of drug-induced toxicities and the amount of covalent binding to liver proteins, we have collected the covalent binding data in human and rat liver microsomal systems and rats in vivo of sixteen 14C-labeled ‘problematic’ drugs associated with drug-induced toxicities, including IDTs, as well as ‘safe’ drugs.

Whole-body quantitative autoradiography (ARG) allows us to acquire the tissue distribution/retention properties of 14C-labeled-NCEs. If tissue retention properties are correlated
with covalent binding yields, ARG can be useful for risk assessment. In this study, the tissue
distribution/retention of a number of drugs has been also investigated by a rat ARG in order to
investigate the relationship between covalent binding and tissue retention. We also focused on the
relationship between the toxicity profile and drug distribution based on the ARG data.
Methods

Materials

$^{14}$C-labeled amodiaquin, benzbromarone, clozapine, flutamide, imipramine, nevirapine, sulfamethoxazole, tacrine, varsartan, zafirlukast and zomepirac were obtained from BlyChem Ltd. (Billingham, England). $^{14}$C-labeled aminopyrine, caffeine, erythromycin, phenacetin, procainamide and valproic acid were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). $^{14}$C-labeled phenytoin was purchased from PerkinElmer (Boston, MA, USA). $^{14}$C-labeled warfarin was purchased from GE Healthcare (Buckinghamshire, UK). $^{14}$C-labeled amlodipine was purchased from Moravek Biochemicals (Brea, CA, USA). Unlabeled amlodipine, amodiaquin, benzbromarone, clozapine, erythromycin, flutamide, imipramine, phenytoin, sulfamethoxazole, tacrine, warfarin and zomepirac were obtained from SIGMA (St. Louis, MO, USA). Unlabeled aminopyrine and valproic acid were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Unlabeled caffeine, nevirapine, phenacetin and zafirlukast were obtained from Fluka (Buchs, Switzerland), Toronto Research Chemicals Inc. (North York, ON, Canada), ULTRA Scientific (North Kingstown, RI, USA), Cayman Chemical (Ann Arbor, MI, USA), respectively. Unlabeled procainamide and varsartan were synthesized in-house. Pooled human (n = 50, mix gender) and male Sprague-Dawley rat liver microsomes were purchased from XenoTech (Lenexa, KS, USA). NADP and glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Oriental Yeast Co. Ltd, (Tokyo, Japan) and glucose-6-phosphate (G6P) was obtained from SIGMA. All other reagents and solvents were of the
highest grade commercially available.

**Animals**

Male Crj: CD (SD) IGS rats (4-8 weeks) were obtained from Charles River Laboratories Japan, Inc (Kanagawa, Japan). The rats were acclimated for one week to a 12 h light/dark cycle in a humidity and temperature-controlled environment, and allowed access to food and water until experimental use, whereupon food was withdrawn for 16-18 h before administration of the compounds. The rats were cared for and treated in accordance with the National Institute of Health Guidelines for Laboratory Animal Welfare. The protocols were approved by our Institutional Animal Care and Use Committee.

**In vitro covalent binding study**

The experimental procedure was based on that previously reported (Evans *et al.*, 2004; Masubuchi *et al.*, 2007). Briefly, the complete incubation of the $^{14}$C-labeled test compounds with HLMs or RLMs containing the following: 10 μM $^{14}$C-labeled test compound (substrate), 2 mg/mL HLMs/RLMs, 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. A 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. Incubation samples without β-NADP$^+$ were used as (-) NADP control systems, respectively.
As the substrates were dissolved in acetonitrile, the final incubation mixture contained 1% v/v acetonitrile. After 1 h incubation, the reaction was terminated by the addition of 0.5 mL ice-cold acetonitrile. After vortexing and centrifugation, the 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 aliquots were taken for the protein assay with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and determination of the radioactive content by liquid scintillation counting with Hionic-fluor scintillation fluid cocktail (PerkinElmer, Wallesley, MA, USA). The amount of the test compound-related material as radioactivity covalently bound to the microsomal protein was determined and the covalent binding [pmol/mg protein] was calculated. As a background, the radioactivity of the samples without incubation was also determined.

**In vivo covalent binding study**

The experimental procedure was based on that previously reported (Evans et al., 2004; Masubuchi et al., 2007). Briefly, 14C-labeled and unlabeled compounds were dissolved or suspended in 0.5% methylcellulose (400cP) to prepare a solution at a concentration of 2 mg/mL as free form for oral administration to fasted rats. Following a single oral administration of each test compound at a dose of 20 mg/kg to fasting male rats, the rats were exsanguinated at 2, 6 and 24 h (N = 3 animals per time points), their plasma and liver samples were collected and stored frozen until analysis. The
obtained liver samples were weighed and homogenated with aqueous 1.15% \( v/v \) KCl. In the same way as in the \textit{in vitro} covalent binding study, the liver homogenate and plasma samples were washed with the organic solvents, followed by a protein assay and the determination of the radioactivity covalently bound to the protein.

\textbf{Rat whole-body autoradiography}

For oral administration to fasted rats, \( ^{14}\text{C} \)-labeled and unlabeled compounds were dissolved or suspended in 0.5 % \( w/v \) aqueous methylcellulose (400cP) to prepare a solution at a concentration of 0.5 mg/mL as a free form. Following a single oral administration of each test compound at a dose of 3 mg/kg (4 MBq/kg) to fasting male rats, the rats were killed by ether inhalation, deep frozen and embedded in carboxy-methylcellulose at -70°C. Sagittal sections (30 \( \mu \text{m} \)) were cut using a cryomicrotome (CM3600, Leica, Nussloch, Germany) and lyophilized. Selected sections were exposed to Imaging Plates (TYPB-BAS SR2040, Fuji Photo Film Ltd, Tokyo, Japan) with plastic standards for quantification in cassettes. After exposure, the Imaging Plates were scanned using an imaging analyzer (FUJIX BAS2500, Fuji Photo Film Ltd) to obtain the autoradiograms. The radioactivity concentrations in the tissues of interest were calculated from the standard curves derived from the plastic standards.
Results

In vitro covalent binding study

The in vitro covalent binding yields of 16 problematic and 4 safe drugs were determined by incubation for 1 h with HLM and RLM. The structures, the proposed reactive metabolite species and the reported toxicities caused by the reactive metabolites of the drugs tested are shown in Table 1. The covalent binding yields of radioactivity from the 14C-labeled drugs tested incubated with HLM are shown in Table 2. In the complete incubations with HLM, benzbromarone exhibited the highest level of covalent binding, 389.9 ± 18.9 [pmol/mg protein], and amodiaquin, flutamide, tacrine and imipramine exhibited relatively high levels of covalent binding, 208.1 ± 13.4, 178.0 ± 10.9, 137.0 ± 7.5 and 133.8 ± 7.0 [pmol/mg protein], respectively. The NADPH-dependent covalent binding values of benzbromarone, flutamide and tacrine were also at a high level (> 100 [pmol/mg protein]), indicating that their covalent binding to liver microsomal proteins occurred via NADPH-dependent metabolism. On the other hand, amodiaquin and imipramine exhibited relatively high levels of covalent binding in the (-)NADP incubation with HLM, 258.2 ± 14.7 and 91.0 ± 15.7 [pmol/mg protein], showing that their covalent binding contained NADPH-independent processes. Covalent binding studies of the drugs tested using RLM showed results similar to HLM (Table 3). In the complete incubations with RLM, benzbromarone exhibited the highest level of covalent binding, 313.0 ± 79.4 [pmol/mg protein], and imipramine, amodiaquin, clozapine and flutamide exhibited relatively high levels of covalent binding, 234.7 ± 11.2, 125.6 ± 11.4, 117.6 ± 4.3 and 106.5 ± 0.1
Amodiaquin and imipramine also exhibited high levels of covalent binding in the (−)NADP incubation with RLM, 94.3 ± 2.7 and 130.6 ± 21.0 [pmol/mg protein]. The safe drugs amlodipine, caffeine, varsartan and warfarin gave levels of covalent binding lower than 50 [pmol/mg protein] in both HLM and RLM.

In order to observe any species differences in covalent binding, the covalent binding yields of the drugs tested in the complete incubation with HLM were plotted versus that with RLM, as shown in Figure 1. The log-linear regression line was calculated by the least-squares method. The correlation coefficient (R) between HLM and RLM the in vitro covalent binding of the drugs tested was calculated to be 0.84. In the case of tacrine, the covalent binding in HLM was approximately 7-fold higher than that in RLM. In the cases of aminopyrine, clozapine, nevirapine, phenytoin, procainamide, sulfamethoxazole and warfarin, on the contrary, the covalent binding in RLM was 2 to 5-fold higher than that in HLM.

**In vivo covalent binding study**

The potential of the in vivo metabolic bioactivation of the drugs was assessed in rats. After a single oral administration of 20 mg/kg dosed of 14C-labeled drugs to rats (n = 3), radioactivity irreversibly bound to proteins in the liver and plasma homogenates were detected using a liquid scintillation counter. The relatively high dose (20 mg/kg) was chosen in order to highlight the potential of metabolic bioactivation and to balance maximizing analytical sensitivity with
standardizing protocol. The maximum levels of radioactivity covalently bound to rat liver and plasma proteins are shown in Table 4. Aminopyrine, imipramine, clozapine, valproic acid and amodiaquin exhibited relatively high levels of covalent binding to liver proteins, with levels of more than 100 [pmol/mg protein]. Aminopyrine, imipramine and valproic acid also gave high levels (> 100 [pmol/mg protein]) of covalent binding to plasma proteins, whereas most of the other drugs showed only trace levels (< 10 [pmol/mg protein]). Thus, the levels of covalent binding to liver and plasma protein did not correspond exactly.

In order to investigate the in vitro-in vivo correlation of the covalent binding, the rat in vivo covalent binding to liver proteins was plotted to the in vitro RLM covalent binding, as shown in Figure 2. The correlation coefficient (R) between RLM in vitro and rat in vivo covalent binding yields of the drugs tested was calculated to be 0.47 from a log-linear regression analysis, thus a quite weak correlation between them was observed. Valproic acid, aminopyrine and zomepirac exhibited significantly high levels of rat in vivo covalent binding relative to those of RLM in vitro covalent binding, with levels of approximately 30, 6 and 5-fold, respectively. Conversely, benzbromarone showed a markedly low level of rat in vivo covalent binding relative to those of RLM in vitro covalent binding.

Rat quantitative whole-body autoradiography

The distribution and retention of total radioactivity was investigated by means of rat quantitative
whole-body autoradiography. Eleven drugs, including amodiaquin, benz bromarone, caffeine, clozapine, flutamide, nevirapine, imipramine, sulfamethoxazole, tacrine, zafirlukast and zomepirac, were tested. Autoradiograms were taken at 3, 6, 24, 72 and 168 h after a single oral administration of $^{14}$C-labeled compounds (3 mg/kg) to fasted male rats. Because washout of the free and reversibly bound fraction of radioactivity is critical for detecting the covalently bound fraction of radioactivity in this experiment, we chose the relatively low dose (3 mg/kg). Typical autoradiograms at 72 and 168 h post-dose are shown in Figure 3. The temporal changes of the concentration (% to the maximum concentration) in some tissues are shown in Figure 4. In most of the tissues, the maximum levels of radioactivity were observed at 3 or 6 h after administration and then the levels of radioactivity were decreased. Amodiaquin, clozapine, flutamide and tacrine gave residual radioactivity, 2-6% of the maximum radioactivity concentration, in the liver up to 168 h after administration. Amodiaquin and clozapine, which are associated with agranulocytosis, also showed the retention of radioactivity in the bone marrow and spleen up to 168 h after administration. In the cases of benz bromarone, nevirapine, zafirlukast and zomepirac, slight residual radioactivity, 1-3% of the maximum radioactivity concentration, was detected in the liver at 72 h after administration and was eliminated at 168 h. Imipramine exhibited the retention of radioactivity in almost all the tissues observed up to 168 h after administration. In the case of sulfamethoxazole, the radioactivity was almost completely eliminated from all tissues at 72 h after administration.

Next, we investigated the relationship between the retention profiles of radioactivity in the liver
and the covalent binding of radioactivity from the drugs to liver proteins. The plots of the residual radioactivities in liver (% to the maximum concentration) of the eleven drugs observed by means of a rat ARG versus the covalent binding yields to liver proteins obtained by rat in vivo covalent binding studies are shown in Figure 5. The retention of the radioactivity in the liver was found to be well correlated with the rat in vivo covalent binding to liver proteins. The correlation coefficients between the residual radioactivities at 24, 72 and 168 h after administration and the covalent binding yields were 0.82, 0.91 and 0.91, respectively.
Discussion

Since covalent binding studies are presently considered to provide the most definitive data for the risk assessment of IDT, the covalent binding data of NCEs usually become available at a late preclinical stage and influence their fates as candidates for further development. In order to make decisions based on the covalent binding data, it is important to accumulate the reference data of a variety of marketed drugs by a standard method. Most of the drugs used in the present study are known to form various reactive metabolites (Kalgutkar et al., 2005), as shown in Table 1. This includes several types of reactive metabolites, such as quinone imine derived from amodiaquin (Christie et al., 1989); ortho-quinone from benzbromarone (MacDonald and Rettie, 2007); quinone methide from tacrine (Madden et al., 1993); nitrenium ion from clozapine (Pirmohamed et al., 1995); iminium ion from nevirapine (Kalgutkar et al., 2005) and zafirlukast (Kassahun et al., 2005); nitroso species from erythromycin (Larrey et al., 1983), phenacetin (Koymans et al., 1990), procainamide (Uetrecht, 1985) and sulfamethoxazole (Naisbit et al., 2002); arene oxide from imipramine (Masubuchi et al., 1996); acyl glucuronide from valproic acid (Baillie, 1988) and zomepirac (Wang et al., 2001); and radical species from aminopyrine (Uetrecht et al., 1995), flutamide (Kang et al., 2007) and phenytoin (Cuttle et al., 2000). Quinone imine formation from amodiaquin was reported to be caused by autoxidation (Maggs et al., 1987), and thus the high levels of covalent binding without NADPH observed in this study seem to be reasonable. In the case of imipramine, as far as we know, no NADPH-independent bioactivation pathway has been reported and the reason for the high levels of
covalent binding without NADPH remains unclear. Presumably, some unknown NADPH-independent pathway might contribute to the covalent binding of imipramine. The formation of reactive metabolites is thought to cause a variety of AEs (Kaplowitz, 2005; Zhou et al., 2005). Amodiaquin, benzbromarone, erythromycin, flutamide, nevirapine, tacrine, valproic acid, zafirlukast and zomepirac cause hepatotoxicity. As a result of their hepatotoxicity, amodiaquin and zomepirac were withdrawn from the market, and benzbromarone, flutamide, nevirapine and valproic acid received black box warnings. Aminopyrine, amodiaquin and clozapine are known to cause severe and life-threatening agranulocytosis, and thus aminopyrine was withdrawn from the market and clozapine received a warning. Imipramine, phenacetin, phenytoin, procainamide and sulfamethoxazole are associated with hypersensitivity reactions. Thus, the drugs in the present study are useful in investigating the relationship between the covalent binding profile and the occurrence of drug-induced toxicities.

The HLM in vitro covalent binding yields of these drugs indicated that the levels of covalent binding of most of the ‘problematic’ drugs were higher than those of the ‘safe’ drugs. Evans et al. proposed a rationale for compound evaluation; both in vitro HLM and rat in vivo covalent binding yields of < 50 pmol/mg protein are desirable (Evans et al., 2004). Considering the HLM in vitro covalent binding data in light of these criteria, the covalent binding yields of the safe drugs were lower than 50 pmol/mg protein and the drugs exhibiting higher levels of covalent binding than 50 pmol/mg protein were all problematic. However, the drugs that exhibited levels of in vitro HLM covalent
binding lower than 50 pmol/mg protein include some ‘problematic’ drugs containing the ‘withdrawn’
drugs such as aminopyrine and zomepirac, and ‘black box warning’ drugs such as clozapine, valproic
acid and nevirapine. Therefore, it appears to be difficult to completely predict the risk of toxicity
only by judgments based on the *in vitro* HLM covalent binding yields. The rat *in vivo* covalent
binding yields of the drugs were different from those of the HLM *in vitro* covalent binding yields.
Meanwhile the ‘safe’ drugs exhibited a level of covalent binding lower than 50 pmol/mg protein and
the drugs exhibiting levels of covalent binding higher than 50 pmol/mg protein were all problematic
drugs. In particular, aminopyrine, clozapine, nevirapine and valproic acid showed high levels (> 50
pmol/mg protein) of rat *in vivo* covalent binding relative to those of HLM *in vitro* covalent binding (<
50 pmol/mg protein). Therefore, most (7 out of 8) of the ‘withdrawn’ or ‘black box warning’ drugs
exhibited levels of covalent binding higher than 50 pmol/mg protein in the *in vitro* HLM assay and/or
rat *in vivo* assay. As shown in Figure 2, the plots of the *in vitro-in vivo* correlation revealed large
differences between the *in vitro* and *in vivo* covalent bindings of aminopyrine and valproic acid.
From these observations, it is suggested that aminopyrine and valproic acid have a major bioactivation
pathway other than CYP-mediated oxidation in liver microsomal systems. In fact, it has been
reported that aminopyrine could be metabolized to a reactive species by myeloperoxidase (MPO) in
activated neutrophils (Uetrecht *et al.*, 1995). The bioactivation pathways of valproic acid are known
to be glucuronidation of the acyl group and mitochondrial β-oxidation (Baillie, 1988; Bailey *et al.*,
1996). Since it has been proved that a rat *in vivo* covalent binding study can find drugs with a risk of
bioactivation other than CYP-mediated oxidation, such as aminopyrine and valproic acid, this type of study will be essential for risk assessment, in addition to an HLM in vitro study. As well as HLM in vitro and rat in vivo covalent binding studies, a covalent binding study using hepatocytes would be feasible and effective, since it can serve to reveal the metabolic bioactivation processes that depend on the presence of a full component of cellular enzyme systems.

In rat in vivo covalent binding study, some drugs exhibited higher levels of covalent binding to plasma proteins than those to liver proteins. The level of covalent binding to plasma proteins is considered to be dependent on the distribution, reactivity and lifetime of reactive metabolites in plasma. As in the case with aminopyrine, the drugs bioactivated by reactive oxygen species generated from leukocytes in blood seem to exhibit the high levels of covalent binding to plasma proteins. Also, if a reactive metabolite generated in liver is relatively stable such as the acyl glucuronide derived from valproic acid, it can be translocated from liver to blood and can bind to plasma proteins.

Rat ARG visually gives us information regarding the tissue distribution and retention properties of the drugs. Because the residual radioactivity observed in the liver at 72 or 168 h post-dose was found to be well correlated with the rat in vivo covalent binding to liver proteins, as shown in Figure 5, the major fraction of the long term-retained radioactivity was thought to be covalently bound to liver proteins. Thus, it seems possible that the in vivo covalent binding yields of the drugs should be extrapolated from their retention profiles observed by means of ARG. From the obtained data, drugs
exhibiting residual radioactivity of more than 5% to the maximum radioactivity in liver at 72 h post-dose or that more than 2% at 168 h post-dose will show high levels (> 50 pmol/mg protein) of covalent binding yields to the liver with a high probability. Exceptionally, caffeine exhibited residual radioactivity in several tissues, including the liver, although it does not undergo bioactivation and form covalent binding. The reason for this remains unclear, but presumably caffeine is metabolized to nucleobases such as xanthines and is assimilated into endogenous molecules in tissues.

Regarding tissues other than the liver, long-term retention of radioactivity in the bone marrow was observed with some drugs associated with severe agranulocytosis, such as amodiaquin and clozapine, implying that the covalent binding of their reactive species to the proteins in bone marrow might cause agranulocytosis. The mechanism of drug-induced agranulocytosis has not been fully defined. However, it has been proposed that the covalent binding of reactive metabolites to neutrophil proteins could lead to agranulocytosis either by direct toxicity or through an immune-mediated reaction (Gardner et al., 1998; Pumford et al., 1997). Alternatively, it has been also proposed that the stromal cells in the bone marrow, which are required for the growth and maturation of neutrophil precursors, rather than neutrophils or their precursors, are the targets of toxicity (Guest and Uetrecht, 1999). A long-lived drug covalently bound to proteins can be a hapten and lead to an immune response, when stimulated with 'danger signals' caused by cellular damage or stress. From the point of view of the hapten and danger hypotheses (Uetrecht, 2008), the long-term retention of drug-protein adducts is considered to be a risk factor for IDT. It appears to be important
and useful as a risk assessment for drug-induced agranulocytosis to examine the retention of radioactivity in bone marrow by means of ARG.

In conclusion, we have determined the HLM in vitro and rat in vivo covalent binding yields of a variety of drugs by a standard method. Most of ‘problematic’ drugs, including ‘withdrawn’ and ‘warning’ drugs, exhibit higher HLM in vitro covalent binding yields than ‘safe’ drugs, and some ‘problematic’ drugs that undergo bioactivation other than CYP-mediated oxidation exhibited only trace levels of HLM covalent binding like ‘safe’ drugs. Since rat in vivo covalent binding studies can assess the bioactivation of such drugs, they are essential for risk assessment, in addition to an HLM in vitro study. Although the criteria (50 pmol/mg protein) from Merck have some false negatives, they are likely to be useful because most (7 out of 8) of the ‘withdrawn’ or ‘black box warning’ drugs exhibited levels of covalent binding higher than 50 pmol/mg protein in the in vitro HLM assay and/or rat in vivo assay. Accumulation of safe drug data is still important to improve the reliability of the risk border for covalent binding studies. Furthermore, by means of ARG, we have also shown the strong correlation between the covalent binding yields of the drugs and the long-term retention of radioactivity in autoradiograms, as well as the spatial relationship between the toxicity profile and drug distribution/retention. ARG enables us to estimate the covalent binding yields in various tissues at a time without extensive washing procedures, suggesting that ARG would be useful for IDT risk assessment. Taken together, the covalent binding and tissue distribution/retention data of the various marketed drugs obtained in this study are quite informative for the interpretation of data in terms of
risk assessment.
References


perspective on minimizing the potential for drug bioactivation in drug discovery and development.

*Chem Res Toxicol 17*: 3-16.


of a cytochrome P450 2D enzyme in rat liver microsomes: in relation to covalent binding of its

(2002) Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major

Pirmohamed M, Williams D, Madden S, Templeton E and Park BK (1995) Metabolism and

Pharmacol Toxicol* 37: 91-117.


Uetrecht JP (1985) Reactivity and possible significance of hydroxylamine and nitroso metabolites of


Footnotes

Address correspondence to:

Hideo Takakusa, Ph.D.

Drug Metabolism & Pharmacokinetics Research Laboratory, R&D Division, Shinagawa R&D Center,

Daiichi Sankyo Co., Ltd.

1-2-58, Hiromachi, Shinagawa-ku, Tokyo, 140-8710, Japan.

E-mail: takakusa.hideo.yb@daiichisankyo.co.jp
Legends for figures

Figure 1. Correlation between HLM and RLM in vitro covalent binding of the drugs tested.

Figure 2. Plots of rat in vivo covalent binding to liver proteins versus RLM in vitro covalent binding of the drugs tested.

Figure 3. Typical whole-body autoradiograms (left axis aspect) 72 and 168 h after a single oral administration of 14C-labeled drugs at a dose of 3 mg/kg to rats. (A) Amodiaquin, (B) clozapine, and (C) flutamide.

Figure 4. Temporal changes of the concentration (% to the maximum concentration) in some tissues. (A) Amodiaquin, (B) benzbromarone, (C) caffeine, (D) clozapine, (E) imipramine, (F) flutamide, (G) nevirapine, (H) sulfamethoxazole, (I) tacrine, (J) zafirlukast and (K) zomepirac.

Figure 5. Plots of residual radioactivity ratios of the eleven drugs observed in the liver in rat quantitative whole-body ARG versus rat in vivo covalent binding to liver proteins.
Table 1. The structures, proposed reactive metabolites and toxicities of the drugs tested.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Structure</th>
<th>Proposed reactive metabolite</th>
<th>Possible relevant toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminopyrine</td>
<td><img src="" alt="Structure" /></td>
<td>Radical species</td>
<td>Aguranulocytosis (Withdrawn)</td>
</tr>
<tr>
<td>Amodiaquin</td>
<td><img src="" alt="Structure" /></td>
<td>Quinone imine</td>
<td>Aguranulocytosis (Withdrawn)</td>
</tr>
<tr>
<td>Benz bromarone</td>
<td><img src="" alt="Structure" /></td>
<td>Quinone</td>
<td>Hepatotoxicity (Black box warning)</td>
</tr>
<tr>
<td>Clozapine</td>
<td><img src="" alt="Structure" /></td>
<td>Nitrenium ion</td>
<td>Aguranulocytosis (Black box warning)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td><img src="" alt="Structure" /></td>
<td>Nitroso</td>
<td>Hepatotoxicity, Hypersensitivity</td>
</tr>
<tr>
<td>Flutamide</td>
<td><img src="" alt="Structure" /></td>
<td>Radical species</td>
<td>Hepatotoxicity (Black box warning)</td>
</tr>
<tr>
<td>Imipramine</td>
<td><img src="" alt="Structure" /></td>
<td>Arene oxide</td>
<td>Hypersensitivity</td>
</tr>
<tr>
<td>Nevirapine</td>
<td><img src="" alt="Structure" /></td>
<td>Iminium ion</td>
<td>Hepatotoxicity (Black box warning)</td>
</tr>
<tr>
<td>Phenacetin</td>
<td><img src="" alt="Structure" /></td>
<td>Nitroso, Quinone imine</td>
<td>Hypersensitivity</td>
</tr>
</tbody>
</table>
Phenytoin  
Radical species, Quinone  
Hypersensitivity

Procainamide  
Nitroso  
Hypersensitivity, Agranulocytosis

Sulfamethoxazole  
Nitroso  
Hypersensitivity

Tacrine  
Quinone methide  
Hepatotoxicity

Valproic acid  
Acyl glucuronide, \(\alpha, \beta\)-unsaturated carbonyl  
Hepatotoxicity (Black box warning)

Zafirlukast  
\(\alpha, \beta\)-unsaturated iminium ion  
Hepatotoxicity

Zomepirac  
Acyl glucuronide  
Hepatotoxicity (Black box warning)

Amlodipine  
—  
—

Caffeine  
—  
—

Varsartan  
—  
—

Warfarin  
—  
—
Table 2. Covalent binding yields of the drugs tested incubated with HLMs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Covalent Binding (Mean ± S.D.) [pmol/mg protein]</th>
<th>Covalent Binding (Mean ± S.D.) [pmol/mg protein]</th>
<th>Covalent Binding (Mean ± S.D.) [pmol/mg protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complete (-) NADP NADPH-dependent</td>
<td>Complete (-) NADP NADPH-dependent</td>
<td>Complete (-) NADP NADPH-dependent</td>
</tr>
<tr>
<td>Aminopyrine*</td>
<td>30.9 ± 3.3 5.3 ± 3.1 25.7</td>
<td>208.1 ± 13.4 258.2 ± 14.7 -50.0</td>
<td>389.9 ± 18.9 4.8 ± 0.5 385.1</td>
</tr>
<tr>
<td>Amodiaquin*</td>
<td>44.7 ± 2.6 19.4 ± 5.7 25.3</td>
<td>57.1 ± 6.7 4.5 ± 0.2 52.6</td>
<td>178.0 ± 10.9 12.2 ± 0.3 165.8</td>
</tr>
<tr>
<td>Benzbromarone*</td>
<td>133.8 ± 7.0 91.0 ± 15.7 42.8</td>
<td>23.6 ± 3.6 14.1 ± 3.0 9.4</td>
<td>19.1 ± 1.3 20.2 ± 2.0 -1.2</td>
</tr>
<tr>
<td>Clozapine*</td>
<td>137.0 ± 7.5 2.7 ± 0.3 134.3</td>
<td>36.4 ± 2.0 1.7 ± 0.1 34.7</td>
<td>6.3 ± 3.3 2.3 ± 0.2 4.0</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>4.4 ± 0.4 2.6 ± 0.3 1.7</td>
<td>4.4 ± 0.4 2.6 ± 0.3 1.7</td>
<td>6.4 ± 0.5 2.9 ± 0.9 3.4</td>
</tr>
<tr>
<td>Flutamide*</td>
<td>3.2 ± 0.7 2.8 ± 2.0 0.5</td>
<td>9.1 ± 0.3 2.6 ± 0.3 6.5</td>
<td>9.9 ± 1.6 2.3 ± 1.2 7.6</td>
</tr>
<tr>
<td>Flutamide*</td>
<td>1.4 ± 0.6 0.6 ± 0.2 0.7</td>
<td>15.9 ± 2.6 7.0 ± 3.4 8.9</td>
<td>6.4 ± 0.5 2.9 ± 0.9 3.4</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>1.4 ± 0.6 0.6 ± 0.2 0.7</td>
<td>15.9 ± 2.6 7.0 ± 3.4 8.9</td>
<td>6.4 ± 0.5 2.9 ± 0.9 3.4</td>
</tr>
<tr>
<td>Tacrine</td>
<td>137.0 ± 7.5 2.7 ± 0.3 134.3</td>
<td>36.4 ± 2.0 1.7 ± 0.1 34.7</td>
<td>6.3 ± 3.3 2.3 ± 0.2 4.0</td>
</tr>
<tr>
<td>Warfarin</td>
<td>9.1 ± 0.3 2.6 ± 0.3 6.5</td>
<td>9.9 ± 1.6 2.3 ± 1.2 7.6</td>
<td>1.4 ± 0.6 0.6 ± 0.2 0.7</td>
</tr>
</tbody>
</table>

* “Withdrawn” or “black box warning” drugs.
Table 3. Covalent binding yields of the drugs tested incubated with RLMs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Covalent Binding (Mean ± S.D.) [pmol/mg protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complete</td>
</tr>
<tr>
<td>Aminopyrine*</td>
<td>90.2 ± 5.4</td>
</tr>
<tr>
<td>Amodiaquin*</td>
<td>125.6 ± 11.4</td>
</tr>
<tr>
<td>Benzbromarone*</td>
<td>313.0 ± 79.4</td>
</tr>
<tr>
<td>Clozapine*</td>
<td>117.6 ± 4.3</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>34.9 ± 4.7</td>
</tr>
<tr>
<td>Flutamide*</td>
<td>106.5 ± 0.1</td>
</tr>
<tr>
<td>Imipramine</td>
<td>234.7 ± 11.2</td>
</tr>
<tr>
<td>Nevirapine*</td>
<td>49.5 ± 1.2</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>18.4 ± 1.7</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>17.2 ± 4.7</td>
</tr>
<tr>
<td>Procainamide</td>
<td>26.8 ± 3.8</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td>Tacrine</td>
<td>18.9 ± 1.1</td>
</tr>
<tr>
<td>Valproic acid*</td>
<td>4.3 ± 0.7</td>
</tr>
<tr>
<td>Zafirlukast</td>
<td>45.6 ± 4.0</td>
</tr>
<tr>
<td>Zomepirac*</td>
<td>5.7 ± 0.4</td>
</tr>
<tr>
<td>Amlodipine</td>
<td>7.5 ± 0.2</td>
</tr>
<tr>
<td>Caffeine</td>
<td>7.4 ± 1.8</td>
</tr>
<tr>
<td>Varsartan</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Warfarin</td>
<td>41.1 ± 7.8</td>
</tr>
</tbody>
</table>

* “Withdrawn” or “black box warning” drugs.
Table 4. Covalent binding of radioactivity to liver and plasma proteins after a single oral administration (20 mg/kg) to rats.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Covalent Binding (Mean ± S.D.) [pmol/mg protein]</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Plasma</td>
<td></td>
</tr>
<tr>
<td>Aminopyrine*</td>
<td>555.7 ± 58.1</td>
<td>793.9 ± 40.6</td>
<td></td>
</tr>
<tr>
<td>Amodiaquin*</td>
<td>126.3 ± 11.0</td>
<td>4.1 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Benzbromarone*</td>
<td>13.6 ± 1.1</td>
<td>3.1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Clozapine*</td>
<td>156.6 ± 9.6</td>
<td>12.9 ± 6.6</td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>54.5 ± 21.6</td>
<td>99.0 ± 47.2</td>
<td></td>
</tr>
<tr>
<td>Flutamide*</td>
<td>59.8 ± 5.0</td>
<td>9.1 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>Imipramine</td>
<td>326.8 ± 86.0</td>
<td>526.5 ± 78.0</td>
<td></td>
</tr>
<tr>
<td>Nevirapine*</td>
<td>79.5 ± 11.4</td>
<td>1.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Phenacetin</td>
<td>16.8 ± 4.3</td>
<td>1.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Phenytoin</td>
<td>34.2 ± 7.6</td>
<td>3.1 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Procainamide</td>
<td>26.5 ± 8.3</td>
<td>1.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>13.2 ± 0.2</td>
<td>1.0 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Tacrine</td>
<td>46.3 ± 3.3</td>
<td>2.6 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Valproic acid*</td>
<td>135.7 ± 21.5</td>
<td>183.5 ± 53.5</td>
<td></td>
</tr>
<tr>
<td>Zafirlukast</td>
<td>14.2 ± 7.2</td>
<td>0.8 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Zomepirac*</td>
<td>28.1 ± 9.1</td>
<td>6.5 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>Amlodipine</td>
<td>0.1 ± 0.1</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td>Caffeine</td>
<td>21.0 ± 3.6</td>
<td>6.5 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Varsartan</td>
<td>6.3 ± 1.0</td>
<td>5.5 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>Warfarin</td>
<td>17.1 ± 4.5</td>
<td>10.6 ± 5.5</td>
<td></td>
</tr>
</tbody>
</table>

* “Withdrawn” or “black box warning” drugs.
Figure 1
\[ y = 5.0981x^{0.5593} \]

\[ R = 0.47 \]

Figure 2

In vitro RLM covalent binding \([\text{pmol/mg protein}]\)

Rat \textit{in vivo} covalent binding to liver protein \([\text{pmol/mg protein}]\)

- Aminopyrine
- Valproic acid
- Benz bromarone
- Amlodipine

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 3

(A) 72 h

(B) 72 h

(C) 72 h

Liver

Bone marrow

Liver

Bone marrow

Liver

Liver

72 h

168 h

168 h

168 h
Figure 4
Figure 4
Figure 5

Residual radioactivity in liver autoradiogram [ratio to the maximum concentration, %]

- 24 h post-dose
- 72 h post-dose
- 168 h post-dose

Rat in vivo covalent binding to liver [pmol/mg protein]

- $y = 0.104x + 8.544$  
  $R = 0.82$
- $y = 0.078x + 1.173$  
  $R = 0.91$
- $y = 0.039x - 0.104$  
  $R = 0.91$