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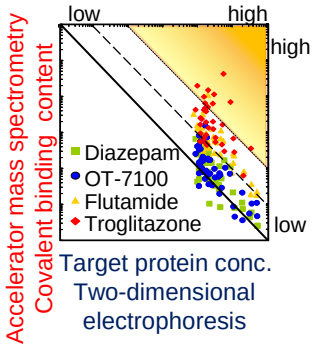
Graphic abstract



A zone analysis for imbalance of covalent bindings of substrates (bioactivated in chimeric mice with humanized liver) and target proteins could prove a useful tool for predicting hepatotoxic effects.

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ARTICLE

Zone analysis by two-dimensional electrophoresis with accelerator mass spectrometry of *in vivo* protein bindings of idiosyncratic hepatotoxicants troglitazone and flutamide bioactivated in chimeric mice with humanized liver

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Hiroshi Yamazaki,[a] Shunji Kuribayashi,[b] Tae Inoue, [c] Tomohiro Honda,[d] Chise Tateno,[c] Ken Oofusa,[e] Shinichi Ninomiya,[f] Toshihiko Ikeda[g] Takashi Izumi,[d] and Toru Horie,[h]

Analyses using electrophoresis with accelerator mass spectrometry revealed that *in vivo* bioactivated radiolabeled troglitazone and flutamide, both known to be hepatotoxic in humans, bound nonspecifically to a variety of microsomal and cytosolic proteins in livers from chimeric mice with humanized liver. Unlike those of radiolabeled diazepam (rarely hepatotoxic) and previously reported 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine (limited hepatotoxicity), some troglitazone and flutamide binding proteins were located in the top right area in a zone analysis, representing high covalent binding contents and high target protein concentrations. Among a variety of liver microsomal proteins bound, the binding target proteins of troglitazone and flutamide with the highest covalent binding contents (in terms of pmol equivalent/μg target protein) were 17 β-hydroxysteroid dehydrogenase and 3β-hydroxysteroid dehydrogenase, respectively. Troglitazone and flutamide were activated to reactive metabolites and apparently bound to different target proteins in livers from chimeric mice with humanized liver. The highest covalent binding contents for troglitazone was higher than that for flutamide under the present conditions. These results indicate that drug metabolism mediated by humanized livers (leading to binding *in vivo*) in combination with a zone analysis of covalent binding contents/target protein concentration data could be a good tool for evaluating the relationship between the nonspecific protein binding behavior of medicines and potential hepatotoxicity in humans. Thus, testing whether protein binding data of new medicines are unbalanced with respect to deviation from an inverse relationship or the presence of data points in the high covalent binding/high protein concentration zone might be an important concept in evaluating hepatotoxic potential.

Introduction

Drug-induced liver toxicity is the single most prominent adverse event causing non-approval of a drug or withdrawal of a drug from the market by regulatory agencies. In evaluating the potential for metabolic activation prior to market approval as well as during the post-marketing period, *in vitro* and *in vivo* studies play roles in the safe development and effective use of medicines. The US National Institutes of Health LiverTox database was developed to promote basic and clinical research on drug-induced liver toxicity.[1] It has been reported that drug-induced hepatotoxicity may be caused by active intermediates formed by animal and/or human P450 enzymes from the common analgesic acetaminophen[2] or from an idiosyncratic reaction to troglitazone, which was withdrawn from the market.[3] Species differences between experimental animals and humans in the roles of cytochrome P450 enzymes in drug metabolism are crucial factors in the evaluation of drug toxicity.[4] Chimeric mice with humanized liver or with human *P450* genes are used as animal models to investigate human metabolites during drug development.[5-7] There is an industrywide need for predictive

tools and an established knowledge base for quantifying the risks in humans.

The purpose of this study was to characterize *in vivo* idiosyncratic hepatotoxicants troglitazone[3] and flutamide[8] in comparison with diazepam (rarely hepatotoxic[1]) and previously reported 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine, a proximate metabolite of previous drug candidate OT-7100 (limited hepatotoxic effects in humans[9]), in terms of their nonspecific protein bindings to a variety of microsomal and cytosolic proteins in livers. We report herein a zone analysis for imbalance between potency and nonspecificity in protein binding using electrophoresis with accelerator mass spectrometry for metabolically activated idiosyncratic hepatotoxicants that bind nonspecifically to a variety of microsomal and cytosolic proteins in chimeric mice with humanized liver.

Results and discussion

Four ^{14}C-labeled substrates, namely diazepam (5 mg dose/kg body weight), 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine (3 mg dose/kg), flutamide (10 mg dose/kg), and troglitazone (150 mg dose/kg), were administered to chimeric mice. Liver microsomes and cytosol fractions were prepared 24 h, 12 h, 1 h, and 1 h after the treatments with ^{14}C-troglitazone, ^{14}C-flutamide, ^{14}C-5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine, and ^{14}C-diazepam, respectively. Many protein bands were present in the sodium dodecyl sulfate-polyacrylamide gel electrophoretogram after Coomassie blue staining; ten 1-mm-wide gel strips in the range 35–100 kDa in the liver microsome analysis were used to focus on diazepam and flutamide. After measurements of protein contents and radioactivities of the 10 analyte strips, covalent bindings per mg protein were calculated, as shown in Figure 1. Per milligram of total microsomal protein, approximately 1–6 pmole and 2–15 pmole equivalents of negative control diazepam and positive control flutamide, respectively, bound to a variety of liver microsomal proteins. As judged from the immunoblotting and molecular weights on the gel in combination with the radioactivity from the radiolabeled substrates, metabolically activated substrates bound to human P450 1A2 and 3A4 (strips 7 and 8, respectively), which were the primary enzymes involved in substrate activation[11,12], and to other many microsomal proteins in humanized livers.

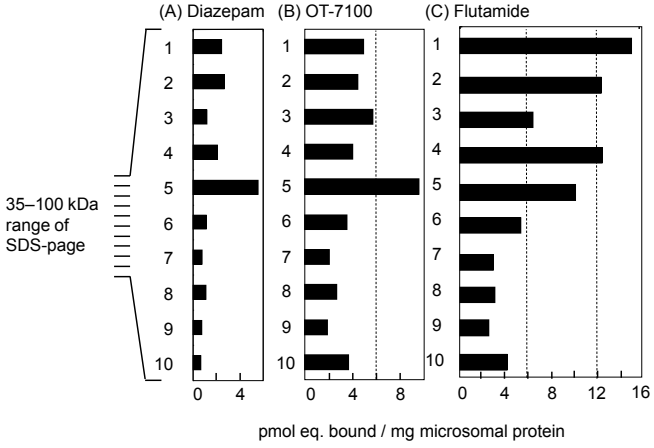


Figure 1. Electrophoresis for microsomal proteins, including P450 enzymes, bound to ^{14}C-labeled diazepam (A), ^{14}C-labeled 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine, a primary metabolite of OT-7100 (B), and ^{14}C-labeled flutamide (C) in chimeric mice with humanized liver. Gel slices 1 mm wide were cut out by hand between 35 and 100 KDa. Data for 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine were taken from Yamazaki et al.[9] for comparison

As can be seen in Figure 1, the overall protein binding levels reflect the hepatotoxicity of the three substrates, i.e., flutamide > OT-7100 > diazepam. Unfortunately, troglitazone could not be included in this part of the analysis because of sample limitations.

After administration of the four radiolabeled substrates to chimeric mice with humanized liver, covalent binding profiles of liver microsomal and cytosolic fractions were further investigated by two-dimensional electrophoresis. Protein samples (100 μg) were separated by isoelectric point (pI 3–10) and molecular weight (10–225 kDa). The target protein concentrations and covalent binding contents of the resulting analyte spots in the gels were determined. Two-dimensional electrophoresis with accelerator mass spectrometry analyses revealed that troglitazone, flutamide, 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine, and diazepam bonded covalently to a variety of liver microsomal (Figure 2A) and/or cytosolic (Figure 2B) proteins. The analyte spots with the 50 highest covalent binding contents were included in this analysis. The highest binding level of troglitazone was observed with 17 β-hydroxysteroid dehydrogenase (0.42 pmol equivalent/μg target protein) in microsomal proteins (target protein concentration 0.51 μg protein/mg microsomal protein, as shown in Figure 2A) and with glutathione *S*-transferase M2-2 (0.27 pmol equivalent/μg target protein) in cytosolic proteins (target protein concentration 0.14 μg protein/mg cytosolic protein, as shown in Figure 2B). These values for binding levels and target protein concentrations are considered to be high in the zone analysis.

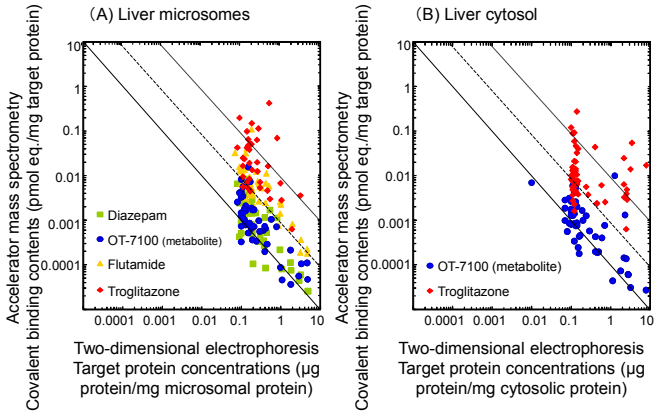


Figure 2. Covalent binding profiles of liver microsomal (A) and cytosolic (B) protein fractions separated by two-dimensional electrophoresis. The fractions were obtained from chimeric mice with at least 70% humanized liver after administration of radiolabeled substrates. Loaded protein samples (100 μg) underwent isoelectric focusing (pI 3–10) and were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10–225 kDa). *In vivo* protein bindings with metabolically activated ^{14}C-substrates were analyzed by accelerator mass spectrometry. Lines are drawn though convenient axis intersections to indicate an inverse relationship. Data for 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine, an OT-7100 metabolite, were taken from Yamazaki et al.[9] for comparison. Note that the data points in the top right zone (i.e., those with high covalent binding contents and high target protein concentrations) of

the left-hand graph (A) are predominantly those of troglitazone and flutamide, both of which are known to be hepatotoxic

Flutamide also bound covalently to a variety of liver microsomal proteins, the highest covalent binding target being 3β-hydroxysteroid dehydrogenase (0.11 pmol equivalent/μg target protein, target protein concentration 0.18 μg protein/mg microsomal protein, as shown in Figure 2A); however, the highest covalent binding contents for flutamide was lower than that for troglitazone. It is worth noting that the three target proteins quoted above with the highest covalent binding levels were different. The identities of the other analyte spot proteins are shown in Tables 1 and 2, including the names for reanalyzed binding proteins for 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine. The top right zone of Figure 2A contains activated drugs bound to abundant target protein molecules, indicating a nonspecific binding manner.

Table 1. Microsomal proteins bound with radiolabeled substrates in descending order of covalent binding content for radiolabeled diazepam.

Protein	Name
1	3-Ketoacyl-CoA-thiolase
2	NADPH-dependent carbonyl reductase
3	Plasma retinol-binding protein [precursor]
4	Branched-chain-amino-acid aminotransferase
5	3-Hydroxyanthranilic acid dioxygenase
6	Fatty acid desaturase 1
7	7-Dehydrocholesterol reductase
8	Selenium binding liver protein
9	Retinol dehydrogenase type III,
10	UDP-Glucuronosyltransferase
11	Endoplasmic reticulum protein
12	NADPH quinone oxide reductase 1
13	Acetaminophen binding protein
14	17β-Hydroxysteroid dehydrogenase
15	Steroid dehydrogenase homolog
16	Flavin-containing monooxygenase
17	Prolyl-4-hydroxylase
18	Retinol dehydrogenase type I
19	Superoxide dismutase
20	Sterol-4-α-carboxylate 3-dehydrogenase
21	Dihydrolipoamide acetyltransferase component
22	Putative UST1-like organic anion transporter
23	Tropomyosin 5
24	2,4-CoA reductase
25	Betaine−homocysteine S-methyltransferase
26	Carbonyl reductase, NADPH
27	Methionine adenosyltransferase
28	3β-Hydroxysteroid dehydrogenase type III
29	Acyl-CoA synthetase short-chain family member 2
30	Mitochondrial P450
31	Long-chain-fatty-acid−CoA ligase, liver isozyme
32	Glutathione peroxidase
33	Epoxide hydrolase
34	Keap-1
35	Oxidoreductase
36	NADH-cytochrome b5 reductase
37	Fructose-bisphosphate aldolase B
38	Urate oxidase
39	Catechol *O*-methyltransferase, membrane-bound form
40	NAD(P) transhydrogenase
41	Aldose reductase
42	Aldehyde dehydrogenase
43	NADPH-Cytochrome P450 reductase
44	Fatty aldehyde dehydrogenase
45	Glutathione *S*-transferase
46	Uricase
47	ATP-synthetase
48	Carbonic anhydrase
49	Protein disulfide isomerase
50	Serum albumin

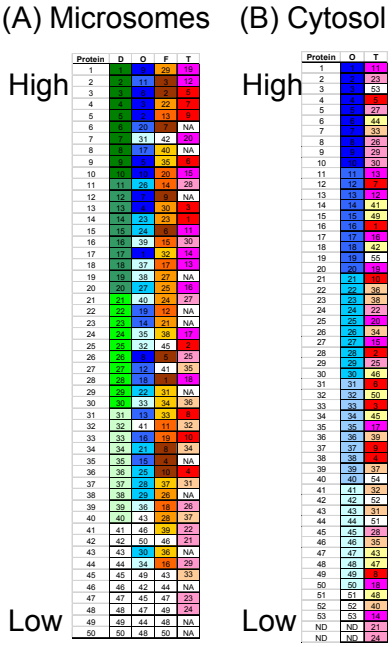
The peptides were analyzed by MASCOT database software (Matrix Science, Tokyo, Japan).

Table 2. Cytosolic proteins bound with radiolabeled substrates in descending order of covalent binding content for radiolabeled 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine.

Protein	Name
1	Catalase
2	Pyrophosphate phosphohydrolase
3	Protein DJ-1
4	UDP-*N*-Acetylglucosamine-peptide *N*-acetylglucosaminyl-transferase 110 kDa subunit
5	Golgi-associated protein, GCP360
6	Glutathione *S*-transferase Ya2
7	Thiopurine *S*-methyl transferase
8	Virus Tat binding protein, TBP-7
9	Virus Tat binding protein, TBP-1
10	Life tech mouse embryo 8 5dpc 10664019
11	Hypothetical protein clone pT-Adv JuaX22
12	Non-neural enolase
13	Phosphoglycerate kinase 1
14	Tumor protein p53
15	Transformed 3T3 cell double minute 2
16	Glutathione *S*-transferase, M2-2
17	Vesicle-associated calmodulin-binding protein
18	Solute carrier family 22 member-7, OAT2
19	Glutathione *S*-transferase, M2-3
20	Senescence marker protein-30
21	Heat shock protein 10
22	Glutathione *S*-transferase, M1-1
23	Mitochondrial P450
24	Phenylpyruvate tautomerase
25	Glutaredoxin
26	Kelch-like ECH-associated protein 1, KEAP 1
27	Proteasome activator subunit 1
28	NADPH quinone oxide reductase 1
29	Hydroxyprostaglandin dehydrogenase
30	Hemooxygenase-1
31	Phosphatidylethanolamine -binding protein
32	ES/130,180-kDa Ribosome receptor
33	pI 6.1 Esterase
34	Nuclear factor (erythroid-derived 2)-like 2, NRF 2
35	Oxalosuccinate decarboxylase
36	Ribonuclease UK114
37	Glutathione synthetase
38	*S*-Adenosylmethionine synthetase isoform type-1
39	Tropomyosin g
40	Tyrosine-ester sulfotransferase
41	Guanidinoacetate *N*-methyltransferase
42	Solute carrier family 10, NTCP
43	Glutathione peroxidase
44	Solute carrier family 22, member-1, OCT-1
45	Heat shock protein 90
46	Eukaryotic translation elongation factor 1 a1
47	Hydroxysteroid dehydrogenase
48	D-Dopachrome tautomerase
49	Triosephosphate isomerase
50	26S Proteasome regulatory subunit 6B
51	Glutathione *S*-transferase A1
52	Carbonic anhydrase
53	Guanine aminohydrolase
ND	Lamin A (Protein No.21 for troglitazone)
ND	Preadipocyte growth factor (protein No.24 for troglitazone)

The peptides were analyzed by MASCOT database software (Matrix Science, Tokyo, Japan). ND, not determined because of sample limitations

In Figure 3, fractioned microsomal and cytosolic proteins are listed in order of decreasing covalent binding contents with respect to diazepam (microsomal) and 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine (cytosolic). Dark colors indicate higher covalent binding contents for all four substrates. Fractioned proteins highly bound to the bioactivated substrates (darker colors in Figure 3) differed between idiosyncratic hepatotoxicants troglitazone[3] and flutamide.[8] In fact, the distribution of dark colors in Figure 3A becomes more imbalanced in the order troglitazone > flutamide > 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine > diazepam, the same order as for their hepatoxicities.



Protein	D	O	F	T
1	1	8	29	19
2	2	11	3	12
3	3	6	2	1
4	4	5	22	7
5	5	2	13	9
6	6	20	7	NA
7	7	31	42	20
8	8	17	40	NA
9	9	9	35	6
10	10	10	20	15
11	11	26	14	28
12	12	7	9	NA
13	13	4	30	4
14	14	23	23	5
15	15	24	6	11
16	16	39	15	30
17	17	1	32	14
18	18	37	17	13
19	19	38	27	NA
20	20	27	25	10
21	21	40	24	27
22	22	19	12	NA
23	23	14	21	NA
24	24	35	38	17
25	25	32	45	8
26	26	3	5	25
27	27	12	41	35
28	28	18	1	16
29	29	22	31	NA
30	30	33	34	36
31	31	16	33	3
32	32	41	11	32
33	33	15	19	18
34	34	21	8	34
35	35	13	4	NA
36	36	25	10	9
37	37	28	37	31
38	38	29	26	NA
39	39	36	18	26
40	40	43	28	37
41	41	46	39	22
42	42	50	46	21
43	43	30	36	NA
44	44	34	16	29
45	45	49	43	33
46	46	42	44	NA
47	47	45	47	23
48	48	47	48	24
49	49	44	48	NA
50	50	48	50	NA

Protein	O	T
1	1	11
2	2	23
3	3	53
4	4	6
5	5	27
6	6	44
7	7	33
8	8	26
9	9	29
10	10	30
11	11	13
12	12	7
13	13	12
14	14	41
15	15	49
16	16	1
17	17	16
18	18	42
19	19	55
20	20	19
21	21	10
22	22	36
23	23	38
24	24	22
25	25	20
26	26	34
27	27	15
28	28	2
29	29	25
30	30	46
31	31	8
32	32	50
33	33	3
34	34	45
35	35	17
36	36	39
37	37	9
38	38	4
39	39	37
40	40	54
41	41	32
42	42	52
43	43	31
44	44	51
45	45	28
46	46	35
47	47	43
48	48	47
49	49	5
50	50	18
51	51	48
52	52	40
53	53	14
ND	ND	21
ND	ND	24

Figure 3. Profiles of liver microsomal (A) and cytosolic (B) protein fractions separated by two-dimensional electrophoresis. The fractions were obtained from chimeric mice with at least 70% humanized liver after administration of radiolabeled substrates. Fractioned microsomal (A) and cytosolic (B) proteins are listed in order of decreasing covalent binding contents for radiolabeled diazepam and 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine, respectively. Colors in five dark to light shades indicate from high to low covalent binding contents for each substrate [so the color shades in the left-hand columns of (A) and (B) are highly ordered by definition]. D, diazepam; O, 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine, a primary metabolite of OT-7100; F, flutamide; and T, troglitazone. The numbers indicate individual target proteins, the names of which are shown in Tables 1 and 2. Binding data for 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine were taken from Yamazaki et al.[9] for comparison. For cytosolic proteins, diazepam and flutamide were not included because of sample limitations. NA, not available

The estimated covalent binding levels (pmol drug eq./μg target protein) apparently indicated an inverse relationship with the target protein concentrations in the case of the negative control, diazepam (a straight line, Figure 2A). The selective and balanced data pattern for diazepam may reflect the low hepatotoxicity of diazepam. A little imbalance between covalent binding content and target protein concentration was seen in the case of 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine, which might be involved as the primary metabolite in the limited hepatotoxic effects of OT-7100 in humans undergoing high-dose, long-term treatments.[10] Although data for several binding analyte spots for severely hepatotoxic flutamide overlapped those for diazepam and 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine, most were in the high covalent binding/high protein concentration zone (above the dashed line, Figure 2A). Furthermore, analyte spots for troglitazone deviated more from an inverse relationship than those of flutamide did. Both troglitazone and flutamide were activated to bind proteins with an imbalance between covalent binding content and target protein concentration, indicating that nonspecific binding to liver proteins can lead to hepatotoxicity. A zone analysis like that shown in Figure 2 in terms of intensity and imbalance of covalent binding contents and target protein concentrations could prove to be a useful tool for predicting hepatotoxic effects.

It can be speculated that *N*-oxidation of flutamide after hydrolysis[11] in humanized liver might lead to binding to P450 itself and to many other proteins in a nonselective and extensive manner. Troglitazone was withdrawn from the market and is reportedly activated to a quinone epoxide metabolite by human P450 3A4.[3,12] The drug–protein adducts of activated troglitazone are likely to nonselectively and extensively bind any abundant proteins in microsomal or cytosolic proteins shown in Figures 1 and 2. This would lead to a less inverse relationship on Figure 2 These lines of evidence suggest that substrates were bioactivated by human P450 enzymes in humanized liver *in vivo* and could bind to any proteins in both microsomes and cytosol, including the catalyst P450 itself, implying relatively low selectivity in target protein bindings. In the present study, two idiosyncratic hepatotoxic medicines, flutamide and troglitazone, were activated to reactive metabolites and apparently bound to different target proteins. It would be of great interest to test other nonhepatotoxic substrates and hepatotoxic medicines using this zone analysis approach and to accumulate target protein information in future studies.

Experimental

Chimeric mice with human liver cells (PXB-mice®, PhoenixBio Co., Hiroshima, Japan, n=3)[6] were orally treated with ^{14}C-troglitazone (150 mg/kg body weight, 1.75 MBq/mouse), ^{14}C-flutamide (the positive [hepatotoxic] control, 10 mg/kg, 0.866 MBq/mouse), and ^{14}C-diazepam (the negative [rarely hepatotoxic] control, 5.0 mg/kg, 0.370 MBq/mouse) and intravenously treated with ^{14}C-5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine (3.0 mg/kg, 0.645 MBq/mouse). In the chimeric mice, more than 70% of liver cells were estimated to have been replaced with human hepatocytes, as judged by measurements of human albumin concentrations in plasma. Liver microsome and cytosol fractions were prepared 24 h, 12 h, 1 h, and 1 h after the treatments with ^{14}C-troglitazone, ^{14}C-flutamide, ^{14}C-5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine[9], and ^{14}C-diazepam, respectively, based on their drug disposition. All animal experiments were performed in compliance with the relevant laws and institutional

guidelines. The institutional committees had approved the experiments.

In a one-dimensional analysis, sodium dodecyl sulfate–polyacrylamide gel electrophoretograms in 12% acrylamide gels with liver microsomes (10 μg) from chimeric mice were stained with 0.08% Coomassie Brilliant Blue R350 (GE Healthcare Bio-Science, Tokyo Japan).[9] The radioactivity content of each band on the gel was determined by a BAS-5000 Image Analysis System (Fujifilm, Tokyo, Japan). Ten 1-mm-wide gel slices from separate experiments were cut out by hand from the gel between 35 and 100 KDa after electrophoresis and were subjected to mass spectrometry to detect P450 enzymes.[9] After measurements of the protein contents and radioactivities of the analyte strips, covalent binding levels per μg microsomal protein were calculated.

For two-dimensional electrophoresis, microsomal or cytosolic protein samples (100 μg) were applied overnight to Immobiline Drystrip (GE Healthcare Bio-Science) by in-gel rehydration as described previously.[9] After the rehydrated gels were dried gently, isoelectric focusing was performed in a Pharmacia Hoefer Multiphor II electrophoresis chamber (GE Healthcare, Buckinghamshire, UK) as the first-dimension analysis, according to the manufacturer's instructions. As the second dimension, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in 9–18% acrylamide gradient gels using an IsoDalt electrophoresis chamber. The second-dimension gels were stained with SYPRO Ruby (Invitrogen, Carlsbad, CA, USA) for accurate quantification of spots using a fluorescent scanner following the manufacturer's protocols. The SYPRO Ruby-stained proteins were quantified using a Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA, USA) and Image Master Platinum image analysis software® (GE Healthcare) because this fluorescent stain has a wider linear-dynamic range than silver stain. In the two-dimensional electrophoresis procedure cytochrome P450 isoforms were not evaluated because membrane-bound proteins are generally difficult to resolve by the first pI-dependent separation step in two-dimensional electrophoresis.[9]

Identification of proteins was performed.[9] Because the fluorescent signals of stained gels were weakened during image analysis, the gels were re-stained with silver for accurate-picking of spots selected by the image analysis. Protein spots were excised from the dried silver-restained second-dimension gels and rehydrated for 20 min in 100 mM NH_4HCO_3. The gel spots were then destained for 20 min in a solution of 15 mM potassium ferricyanide and 50 mM thiosulfate, rinsed twice in water, and finally dehydrated in 100% acetonitrile until they turned opaque white. The spots were then dried in a vacuum centrifuge and subsequently rehydrated in a digestion solution consisting of 50 mM NH_4HCO_3, 5 mM $CaCl_2$, and 0.1 μg/μl modified sequence-grade trypsin (Promega, Madison, WI, USA). After overnight incubation at 37°C, the digestion was terminated in 5% trifluoroacetic acid for 20 min. Peptides were extracted three times (20 min each) with 5% trifluoroacetic acid in 50% acetonitrile, and the extracted peptides were pooled and dried in a vacuum centrifuge. The peptides were purified with ZipTip (Millipore, Billerica, MA, USA) using the manufacturer's protocols and analyzed using MASCOT database software (Matrix Science, Tokyo, Japan).

Accelerator mass spectrometry analyses were performed with an NEC 1.5SDH-1 0.6-MV Pelletron AMS system (National Electrostatics Corporation, Tokyo, Japan) to determine the $^{14}C/^{12}C$ content ratio in the microsomal and cytosolic protein samples after dilution, as described previously.[13] After measurements of protein contents and radioactivities of the analyte spots, the covalent binding levels were calculated.

Conclusions

In conclusion, the hepatotoxic substrates flutamide and troglitazone were activated by human livers to reactive intermediate(s) *in vivo* in humanized chimeric mice and could relatively nonspecifically bind to biomolecules. A zone analysis for intensity and imbalance in terms of covalent binding contents and target protein concentrations could provide important information on potential hepatotoxic effects. To test whether protein binding analyte spots of new medicines are unbalanced is an important concept, because if the protein binding data deviate greatly from an inverse relationship or if they are in the high covalent binding/high protein concentration zone, this might indicate a tendency to extensive nonspecific protein binding, and suggest the possibility of hepatotoxicity. Thus, the use of chimeric mice with humanized liver to realize *in vivo* covalent protein bindings of new drug substances in combination with the above-described zone analysis could prove to be a good predictor of drug toxicity *in vivo* in preclinical human studies.

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Notes and references

[a] Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan,
[b] Otsuka Pharmaceutical Factory, Inc., Naruto, Tokushima 772-8601, Japan
[c] PhoenixBio, Co., Higashi-Hiroshima, Hiroshima 739-0046, Japan
[d] Daiichi Sanyo, Co., Shinagawa-ku, Tokyo 140-8710, Japan
[e] Idea Consultants Inc., Suminoe-ku, Osaka 559-8519, Japan
[f] Sekisui Medical Co., Chuo-ku, Tokyo 103-0027, Japan
[g] Yokohama College of Pharmacy, Totsuka-ku, Yokohama 245-0066, Japan
[h] Drug Discovery and Development Institute, Tsukuba, Ibaragi 305-0036, Japan

1. Hoofnagle, J. H., Serrano, J., Knoben, J. E., and Navarro, V. J. LiverTox: a website on drug-induced liver injury. *Hepatology* 2013, *57*, 873-874.

2. James, L. P., Mayeux, P. R., and Hinson, J. A. Acetaminophen-induced hepatotoxicity. *Drug Metab. Dispos.* 2003, *31*, 1499-1506.

3. Kassahun, K., Pearson, P. G., Tang, W., McIntosh, I., Leung, K., Elmore, C., Dean, D., Wang, R., Doss, G., and Baillie, T. A. Studies on the metabolism of troglitazone to reactive intermediates in vitro and in

vivo. Evidence for novel biotransformation pathways involving quinone methide formation and thiazolidinedione ring scission. *Chem. Res. Toxicol.* 2001, *14*, 62-70.

4. Gonzalez, F. J., and Yu, A. M. Cytochrome P450 and xenobiotic receptor humanized mice. *Annu. Rev. Pharmacol. Toxicol.* 2006, *46*, 41-64.

5. Powley, M. W., Frederick, C. B., Sistare, F. D., and DeGeorge, J. J. Safety assessment of drug metabolites: implications of regulatory guidance and potential application of genetically engineered mouse models that express human P450s. *Chem Res Toxicol* 2009, *22*, 257-262.

6. Tateno, C., Yoshizane, Y., Saito, N., Kataoka, M., Utoh, R., Yamasaki, C., Tachibana, A., Soeno, Y., Asahina, K., Hino, H., Asahara, T., Yokoi, T., Furukawa, T., and Yoshizato, K. Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am. J. Pathol.* 2004, *165*, 901-912.

7. Katoh, M., Tateno, C., Yoshizato, K., and Yokoi, T. Chimeric mice with humanized liver. *Toxicology* 2008, *246*, 9-17.

8. Gomez, J. L., Dupont, A., Cusan, L., Tremblay, M., Suburu, R., Lemay, M., and Labrie, F. Incidence of liver toxicity associated with the use of flutamide in prostate cancer patients. *Am. J Med.* 1992, *92*, 465-470.

9. Yamazaki, H., Kuribayashi, S., Inoue, T., Tateno, C., Nishikura, Y., Oofusa, K., Harada, D., Naito, S., Horie, T., and Ohta, S. Approach for *in vivo* protein bindings of 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine bioactivated in chimeric mice with humanized liver by two-dimensional electrophoresis with accelerator mass spectrometry. *Chem. Res. Toxicol.* 2010, *23*, 152-158.

10. Kuribayashi, S., Goto, K., Naito, S., Kamataki, T., and Yamazaki, H. Human cytochrome P450 1A2 involvement in the formation of reactive metabolites from a species-specific hepatotoxic pyrazolopyrimidine derivative, 5-n-butyl-7-(3,4,5-trimethoxybenzoylamino)pyrazolo[1,5-*a*]pyrimidine. *Chem. Res. Toxicol.* 2009, *22*, 323-331.

11. Goda, R., Nagai, D., Akiyama, Y., Nishikawa, K., Ikemoto, I., Aizawa, Y., Nagata, K., and Yamazoe, Y. Detection of a new N-oxidized metabolite of flutamide, N-[4-nitro-3-(trifluoromethyl)phenyl]hydroxylamine, in human liver microsomes and urine of prostate cancer patients. *Drug Metab Dispos.* 2006, *34*, 828-835.

12. Yamamoto, Y., Yamazaki, H., Ikeda, T., Watanabe, T., Iwabuchi, H., Nakajima, M., and Yokoi, T. Formation of a novel quinone epoxide metabolite of troglitazone with cytotoxicity to HepG2 cells. *Drug Metab. Dispos.* 2002, *30*, 155-160.

13. Miyaoka, T., Isono, Y., Setani, K., Sakai, K., Yamada, I., Sato, Y., Gunji, S., and Matsui, T. Bioanalysis works in the IAA AMS facility: Comparison of AMS analytical method with LSC method in human mass balance study. *Nucl. Instrum. Meth. B* 2007, *259*, 779-785.

