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Title:

Characterization of fasiglifam-related liver toxicity in dogs

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Running title page

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Fasiglifam-related liver toxicity in dogs

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List of Nonstandard abbreviations:

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the plasma versus time concentration curve; BDC, bile duct-cannulated; Bsep, bile salt export pump; CL_b, biliary clearance; CL_{tot}, total plasma clearance; C_{max}, maximum concentration; DHB, 2,5-dihydroxybenzoic acid; DILI, drug-induced liver injury; $E_2$17βG, estradiol 17β-D-glucuronide; ER, biliary excretion ratio; GPR40, G protein-coupled receptor 40; HbA1c, glycated hemoglobin; HED, human equivalent dose; HEPES, 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; HPLC, high performance liquid chromatography; IC_{50}, half-maximal inhibitory concentration; ID, intraduodenal dose; LSC, liquid scintillation counter; MALDI TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Mrp2, multidrug resistance-associated protein 2; MS/MS, tandem mass spectrometry; NOAEL, no observable adverse effect level; Ntcp, sodium taurocholate cotransporting polypeptide; Oatp, organic anion transporting polypeptide; T2DM, type 2 diabetes mellitus; TCA, taurocholic acid; T_{max}, time to maximum concentration

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Abstract

Fasiglifam, a potent and highly selective agonist of the G protein-coupled receptor 40, was developed for the treatment of type 2 diabetes mellitus. However, phase III clinical programs were terminated owing to liver safety concerns. Fasiglifam-related liver toxicity was also observed in repeat-dose dog toxicology studies, characterized by the granulomatous inflammation with crystal formation in the liver and/or bile ducts. These histopathological changes were not observed in the rat toxicology studies. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) analysis of dog liver sections obtained from a repeat-dose toxicology study indicated that the crystalline material in the affected dog liver contained fasiglifam and fasiglifam glucuronide (fasiglifam-G). Nonclinical mechanistic studies indicated that after 14 days of repeated oral dosing with [^{14}C]fasiglifam at 200 mg/kg/day to dogs, the concentrations of fasiglifam and fasiglifam-G in the bile exceeded the solubility limit of these compounds in the bile (approximately 3000 μg/mL). After single oral 2- and 200-mg/kg doses administered to rats and dogs, fasiglifam and fasiglifam-G concentrations in dog bile were 5- to 10-fold higher than those in rat bile for the same dose of fasiglifam, while the bile flow rate adjusted by body weight was 4- to 8-fold lower in dogs than in rats. High fasiglifam and fasiglifam-G concentrations in dog bile together with lower bile flow rate could cause crystal formation in dog bile, resulting in secondary granulomatous inflammation in the dog liver.

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Introduction

Fasiglifam (TAK-875), a potent and highly selective agonist of the G protein-coupled receptor 40 (GPR40), was developed for the treatment of type 2 diabetes mellitus (T2DM). GPR40 is highly expressed in pancreatic beta cells, and its activation induces insulin secretion. The insulinotropic effect of fasiglifam is strictly glucose-dependent (Tsujihata et al., 2011). Fasiglifam only enhanced insulin secretion in islet cells in the presence of high ambient glucose, which is essential for a desired safety profile for the treatment of T2DM in terms of hypoglycemia risk. In clinical studies, fasiglifam reduced blood glucose and glycated hemoglobin (HbA1c) levels with a low risk of hypoglycemia in T2DM patients (Kaku, 2013). However, the clinical development of fasiglifam was terminated in phase III clinical trials because of liver safety concerns (Kaku et al., 2015; Kaku et al., 2016). From the fasiglifam clinical data focusing on liver safety findings, one definite Hy's Law case [alanine aminotransferase (ALT) or aspartate aminotransferase (AST) > 3 x ULN (upper limit of normal) and total bilirubin > 2 x ULN] and two 'near' Hy's Law cases were identified in fasiglifam-treated patients (Marcinak et al., 2018). Fasiglifam-related liver toxicity was also observed in repeat-dose dog toxicity studies. Fasiglifam treatment in these studies resulted in liver toxicity characterized by elevation of plasma AST, ALT, alkaline phosphatase (ALP), and/or bilirubin. Associated histopathological changes were characterized as portal/periportal granulomatous inflammation with crystal formation (Supplemental Figures 1 and 2). This toxicity was observed in a 4-week study at a dose of 1000 mg/kg/day. The toxicity measures were greater than those observed for a dose of 150 mg/kg/day in a 13-week study and for a dose of 80 mg/kg/day in 39-week study, indicating that the toxicity was both dose-

and duration-dependent (Supplemental Table 1). Although elevations of serum bilirubin and ALT were observed in repeat-dose rat studies (Wolenski et al., 2017), no histopathological changes indicative of liver injury were observed in any rat studies even at the lethal dose of 2000 mg/kg/day.

Our previous study indicated that disposition and metabolism of fasiglifam were similar between human and animals (Kogame et al., 2018). Fecal excretion was the primary elimination route for unchanged fasiglifam in all species. In addition, no human-specific metabolites were noted, and all metabolites found in human were also observed in the nonclinical animal toxicology studies, suggesting that the fasiglifam-related liver toxicity observed in humans and dogs might not have been caused by the production of specific metabolites. Moreover, since glucuronidation is the major fasiglifam metabolic elimination pathway, the glucuronide of fasiglifam, fasiglifam-G, could be considered as a liver toxicity candidate metabolite in addition to unchanged fasiglifam.

The dog liver toxicity was observed before the phase III study. Therefore, we conducted the present study to support the clinical development of fasiglifam. We identified the composition of foreign body materials observed in dog liver obtained from the 39-week repeat-dose studies by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) analysis. Furthermore, we used several *in vitro* and *in vivo* approaches to characterize the mechanism of fasiglifam-related dog liver toxicity. Nonclinical mechanistic studies included (1) metabolic profiles of [^{14}C]fasiglifam in the plasma and liver samples; (2) potential covalent binding of fasiglifam-G to plasma and liver proteins; (3) [^{14}C]fasiglifam accumulation in the plasma and liver of dogs; (4) disposition and metabolism of

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[^{14}C]fasiglifam in rats and dogs; (5) hepatic transporter studies of fasiglifam and fasiglifam-G; (6) concentrations of total radioactivity, fasiglifam, and fasiglifam-G in bile after single or repeated dosing of [^{14}C]fasiglifam; (7) bile flow rate and bile pH in rats and dogs in the presence of [^{14}C]fasiglifam; and (8) *in vitro* solubility study of fasiglifam and fasiglifam-G. Based on these studies, we predicted fasiglifam and fasiglifam-G concentrations in human bile of T2DM patients receiving 50 mg dose to justify the phase III studies. The potential risk of hepatotoxicity in humans was assessed as discussed herein.

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Materials and Methods

Chemicals and biologicals

Fasiglifam ([(3*S*)-6-({2′,6′-dimethyl-4′-[3-(methylsulfonyl)propoxy]biphenyl-3-yl}methoxy)-2,3-dihydro-1-benzofuran-3-yl]acetic acid hemihydrate) and 2′,6′-dimethyl-4′-[3-(methylsulfonyl)propoxy]biphenyl-3-carboxylic acid (M-I) were synthesized by Takeda Pharmaceutical Company Limited (Kanagawa, Japan). Further, [(3*S*)-6-({2′,6′-dimethyl-4′-[3-(methylsulfonyl)propoxy]biphenyl-3-yl}[^{14}C]methoxy)-2,3-dihydro-1-benzofuran-3-yl]acetic acid hemihydrate ([^{14}C]fasiglifam) and glucuronic acid conjugate of [^{14}C]fasiglifam ([^{14}C]fasiglifam-G) were synthesized by DAIICHI PURE CHEMICALS CO., LTD. (Tokyo, Japan) and Quotient Bioresearch (Pharmaron UK Limited, Cardiff, UK), respectively. Dog bile was purchased from Kitayama Labes Co, Ltd. (Yamaguchi, Japan). Human bile was purchased from KAC Co., Ltd. (Kyoto, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan). [^{3}H]Estradiol 17β-D-glucuronide ([^{3}H]$E_2$17βG) and [^{3}H]taurocholic acid ([^{3}H]TCA) were purchased from PerkinElmer, Inc. (Waltham, MA). [^{3}H]Pravastatin sodium salt was obtained from American Radiolabeled Chemicals (St. Louis, MO). Unlabeled pravastatin sodium salt and cyclosporin A were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Membrane vesicles were purchased from GenoMembrane Co., Ltd. (Kanagawa, Japan). The products were prepared from purified plasma membrane isolated from Sf9 cells infected with baculovirus expressing rat and dog multidrug resistance-associated protein 2 (Mrp2) or bile salt export

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pump (Bsep). Rat and dog cryopreserved hepatocytes were purchased from Life Technologies Corporation (Carlsbad, CA).

Animals

Eight-week-old male Sprague–Dawley rats weighing 251–282 g were purchased from Charles River Laboratories Japan, Inc., (Ibaraki, Japan). Male beagle dogs (7–11 months old, 8.41–11.2 kg) were purchased from Kitayama Labes Co., Ltd. They were fed laboratory chow (CR-LPF; Oriental Yeast Co., Ltd., Tokyo, Japan, for rats; CD-5M; CLEA Japan, Inc., Tokyo, Japan; or Labo D Stock; Nosan Corporation, Yokohama, Japan, for dogs), had free access to water, and were housed in temperature- and humidity-controlled rooms (18–28°C, 38–82%), with 12-h light/dark cycles, for more than 1 week before use. All experiments involving animals were reviewed and approved by the Animal Care and Use Committee of Takeda Pharmaceutical Company Limited.

MALDI TOF MS

The composition of the foreign body material in the liver in the dog toxicity studies was determined by conducting direct mass spectrometric measurement of fresh frozen sections of liver tissue containing the foreign body materials obtained from a dog showing hepatotoxicity in the 39-week oral toxicity study of fasiglifam at 150 mg/kg/day with a 13-week recovery period. Sections were cut at a thickness of 10 µm with a cryostat microtome (CM1950; Leica Microsystems, Germany). The sections were placed onto

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conductive slide glasses (Indium Tin-Oxide-coated slide glass) and were stored at -80°C until MALDI-TOF MS analysis. The tissue slices were equilibrated to room temperature for at least 20 min before analysis. The glass slides were placed onto a special AXIMA MS plate for tissue analysis and then immobilized using double-sided conductive tapes. Furthermore, 200 nL of peptide solution (10 nmol/mL angiotensin II in 0.1% trifluoroacetic acid and 10 nmol/mL ACTH 18-39 in 0.1% trifluoroacetic acid) were spotted for external calibration by manually pipetting near the tissue slices, and then 200 nL of matrix solution (20 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 50% acetonitrile/0.1% trifluoroacetic acid for positive mode or 4 mg/mL 9-aminoacridine in 70% methanol for negative mode) were manually spotted at the same position.

The tiny foreign body materials in the liver tissue slices were directly measured by microdispensing a trace amount of the matrix solutions onto the lesion sites containing the foreign body materials in the tissue slices. First, the location of the foreign body materials was observed by microscopy, and then a landmark was prepared in the proximity of that location on the tissue slices by using a needle or a pen. The sample plates, on which glass slides were immobilized with double-sided conductive tape, were set on the stage part of a chemical printer, and then an image of the sample plate was acquired using a scanner attached to a chemical printer instrument. The print positions to microdispense the matrix solution onto tissue sections were determined based on the landmark on the obtained image. Three hundred picoliters of 20 mg/mL DHB in 50% acetonitrile/0.1% trifluoroacetic acid or 4 mg/mL 9-aminoacridine solution in 70% methanol were microdispensed onto the tissue sections at a total volume of 18 nL on a single spot.

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Matrix solution was microdispensed onto spots on each tissue slice (s) (s1, s2, and s4 in Figure 1) as the matrix toward the foreign body materials. After matrix deposition, each matrix deposit on the tissue slices was observed by microscopy. The matrix deposit, including foreign body materials, was measured by analyzing the foreign bodies (these spots are indicated by red letters-numbers in Figure 1). The printed positions corresponding to the normal region are shown by black letters-numbers in Figure 1.

Direct measurement was performed on the tissue slices by using MALDI-TOF MS. The text file of the print positional information was exported from the chemical printer, and then direct analysis of the printed positions was performed based on the X and Y coordinates from the text file. The peptide solution, which was manually pipetted onto the glass slides, was used as an external calibration. Furthermore, tandem mass spectrometry (MS/MS) experiments were performed for the significant ions observed in the foreign body materials. The parameters for collision-induced dissociation were optimized to effectively produce fragment ions.

Rat studies

The metabolite profiling studies of fasiglifam were investigated in intact and bile duct-cannulated (BDC) male rats. After a single oral administration of [^{14}C]fasiglifam (final specific activity: 4.19 MBq/mg) at 2 and 200 mg/kg to the intact rats, blood and liver were collected at 1 (T_{max} of fasiglifam in rats (Negoro et al., 2010)) and 8 h after administration. The blood was collected from the abdominal aorta under anesthesia by using diethyl ether. The blood was immediately cooled under ice-chilled conditions, and the plasma was

obtained by centrifugation at 8000 × *g* at 4°C. Subsequently, the total radioactivity in the plasma was measured. The residual plasma samples were used for the metabolite profiling study.

For the BDC rat study, the rats were cannulated under anesthesia with isoflurane. After the efflux of bile was confirmed, the [^{14}C]fasiglifam formulation was administered into the duodenum of the rats at 2 and 200 mg/kg (final specific activity: 0.763 MBq/mg for 2 mg/kg and 0.00741 MBq/mg for 200 mg/kg). After administration, the rats were housed in Bollman cages. Bile was collected at 1-h intervals for 8 h, and then 8–24 h under dry ice-chilled conditions. The total radioactivity in the bile was measured. The residual bile samples in each period were mixed together with the obtained weight ratio and used for the metabolite profiling study. The pH of the bile was measured using pH test paper (MACHEREY-NAGEL; Düren, Germany).

Dog studies

The blood, liver, and bile were collected at 2 (T_{max} of fasiglifam in dogs (Negoro et al., 2010)) and 8 h after single oral doses of 2 and 200 mg/kg (final specific activity: 4.01 MBq/mg for 2 mg/kg and 4.19 MBq/mg for 200 mg/kg) or 14-day repeated oral doses (final specific activity: 0.215 kBq/mg) of [^{14}C]fasiglifam at 200 mg/kg to intact dogs. The dogs were anesthetized with thiopental or sodium pentobarbital at an intravenous dose of 25 mg/kg for the collection of the liver and bile. During the repeated dosing study, the blood was collected at 5, 15, and 30 min and 1, 2, 4, 8, and 24 h after the 1st, 7th, 13th, and 14th administration. The blood was collected from the cephalic vein. Blood samples were collected from the

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cephalic vein, immediately cooled under ice-chilled conditions, and plasma was obtained by centrifugation at approximately 10000 × *g* at 4°C. Subsequently, total radioactivity in the plasma was measured. The gallbladder and liver were excised and immediately stored under ice-chilled conditions. Bile was collected almost entirely from the gallbladder by using syringes. The residual plasma, liver, and bile samples at 2 and 8 h in the single dose study and after 14th daily dose were used for metabolite profiling. The residual plasma samples in the repeated dose study were used for the determination of fasiglifam concentration by using high performance liquid chromatography (HPLC).

Time course of radioactivity in the bile after single oral administration of [^{14}C]fasiglifam at doses of 2 and 200 mg/kg (final specific activity: 0.747–0.772 MBq/mg for 2 mg/kg and 0.00741 MBq/mg for 200 mg/kg) was investigated in BDC dogs. The dogs were sedated with xylazine at a subcutaneous dose of about 3 mg/kg, and anesthesia was maintained with isoflurane. Just before the operation, the dogs were given cefazolin as an intravenous dose of about 25 mg/kg and meloxicam at a subcutaneous dose of about 0.2 mg/kg. During the operation, dextrose/acetate Ringer's solution was intravenously infused via the cephalic vein at a rate of about 100 mL/h/body. A cannula was inserted into the bile duct toward the liver, and another cannula was inserted into the bile duct toward the duodenum. The ends of the cannulas were interconnected on the dorsal region with a joint tube until dosing. After stable bile flow into the cannula was confirmed, [^{14}C]fasiglifam was orally administered. Bile was collected at 1-h intervals for 8 h, and then at 8–24 h under dry ice-chilled conditions. The total radioactivity in the bile was measured. The residual bile samples in each period were mixed together with the obtained weight ratio and used for the metabolite

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profiling study. The pH of the bile was measured using a pH test paper (MACHEREY-NAGEL).

Metabolite profiling studies

The metabolite profiles in the plasma, bile, and liver obtained from single administration studies were elucidated as reported previously by using an on-line radioisotope (RI) detection method (Kogame et al., 2018). In brief, HPLC analyses were performed on a Shimadzu Prominence or LC-VP HPLC system (Shimadzu Corporation, Kyoto, Japan). The separation was performed on a reversed-phase HPLC column (Inertsil ODS-3; 4.6 mm I.D. × 250 mm, 5 μm; GL Sciences Inc., Tokyo, Japan) at a flow rate of 1 mL/min. The column temperature was maintained at 40°C. The HPLC eluate of the animal samples was mixed with Ultima-Flo^{TM} AP (PerkinElmer, Inc.) at 1:3, and the radiolabeled components were quantified after background subtraction. The mobile phases were 0.01 mol/L ammonium formate/acetonitrile/formic acid (900:100:1, v/v/v; solvent A) and 0.01 mol/L ammonium formate/acetonitrile/formic acid (100:900:1, v/v/v; solvent B)., The samples were eluted using the following gradient: 40–50% B, 0−45 min; 50–100% B, 45–45.1 min; 100% B, 45.1−50 min; 100–40% B, 50–50.1 min; 40% B, 50.1−60 min.

The metabolite profiles in the liver obtained from repeat dosing were elucidated using the offline HPLC method employing an ultra-low background liquid scintillation counter (Onishi et al., 2013). Fractions were collected every 1 min, and the radioactivity was measured using an ultra-low background liquid scintillation counter (1220 Quantulus; PerkinElmer, Inc.). The radioactivity in each elute was detected as described in the Measurement of radioactivity section.

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Hepatobiliary transporter studies

Uptake of [^{14}C]fasiglifam into hepatocytes

Hepatic uptake of [^{14}C]fasiglifam was investigated using cryopreserved hepatocytes from rat and dog (two donors for each species). Hepatocytes preserved in the gas phase of liquid nitrogen were thawed at 37°C, and then suspended in recovery medium (High Viability CryoHepatocyte Recovery Medium, BD Biosciences, Franklin Lakes, NJ). The suspensions were mixed gently 3 times and centrifuged at approximately 100 × *g* for 10 min at 23°C. The supernatants were removed, and the residual hepatocytes were suspended in the plating medium (CryoHepatocyte Plating Medium, BD Biosciences). The cells were resuspended in the incubation medium (118 mmol/L NaCl, 5.0 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 25 mmol/L $NaHCO_3$, 2.5 mmol/L $CaCl_2$, 13 mmol/L 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 10.0 mmol/L glucose) to yield a cell density of 1.6×10^6 viable cells/mL for the uptake study. The number of viable cells was determined using trypan blue staining. Before the uptake studies, the cell suspension- and test solution-containing test substrates were prewarmed in an incubator at 37°C for 5 min. The uptake studies were initiated by the addition to the cell suspension of an equal volume of buffer containing [^{14}C]fasiglifam (0.3 μmol/L) or [^{3}H]pravastatin (0.3 μmol/L), a substrate of organic anion transporting polypeptides (Oatps), in the presence or absence of 10 μmol/L cyclosporin A, a potent inhibitor of Oatps and sodium taurocholate cotransporting polypeptide (Ntcp; final density of the hepatocytes: 0.8×10^6 cell/mL as viable cells). After incubation at 37°C or 4°C for designated times, reaction solutions were gently added to the cell separation

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tubes. The tubes were immediately centrifuged at 10000 × *g* for 15 s at room temperature. The reaction was terminated by transferring the cells to a KOH layer, and then an aliquot of the remaining reaction solution was measured for radioactivity using a liquid scintillation counter (LSC). The uptake clearances of pravastatin were estimated from the uptake volumes at 37°C for 20 and 90 s. The uptake clearances of fasiglifam were estimated from the uptake volumes of fasiglifam at 37°C for 30 s and at 4°C for 20 and 60 s, because the uptake clearance of fasiglifam was too high to evaluate at 37°C between 2 time points. All incubations were made in duplicate.

Inhibitory effects of fasiglifam and fasiglifam-G on hepatobiliary transporters

Transport studies were performed using the rapid filtration technique (van Staden et al., 2012). Briefly, 40 μL of transport medium (50 mmol/L MOPS-Tris, 70 mmol/L KCl, 7.5 mmol/L $MgCl_2$, pH 7.0 for Mrp2, or 10 mmol/L HEPES-Tris, 100 mmol/L KNO_3, 10 mmol/L $Mg(NO_3)_2$, 50 mmol/L sucrose, pH 7.4 for Bsep) containing radiolabeled compounds ([^{3}H]$E_2$17βG for Mrp2 and [^{3}H]TCA for Bsep) was preincubated at 37°C for 5 min. The transport reaction was initiated by the addition of 10 μL of membrane vesicle suspension (50 μg of protein) into the preincubated transport medium. The reaction mixture contained 5 mmol/L of ATP or AMP. The transport reaction was terminated by addition of 1 mL of ice-cold buffer (40 mmol/L MOPS-Tris, 70 mmol/L KCl, pH 7.0 for Mrp2, or 10 mmol/L HEPES-Tris, 100 mmol/L KNO_3, 50 mmol/L sucrose, pH 7.4 for Bsep). The stopped reaction mixture was filtered through a glass fiber filter (Merck Millipore, Darmstadt, Germany) and then washed twice with 10 mL of ice-cold buffer. The

radioactivity retained on the filter was determined using LSC.

Because the initial linear uptake was confirmed for TCA into Bsep-expressing vesicles and $E_2$17βG into Mrp2-expressing vesicles at 1 and 2 min, respectively, the uptake study for [^{3}H]TCA (0.2 μmol/L) and [^{3}H]$E_2$17βG (0.02 μmol/L) was conducted at 1 and 2 min, respectively, to estimate IC_{50} values of fasiglifam and fasiglifam-G. The uptake clearances of [^{3}H]TCA or [^{3}H]$E_2$17βG into the membrane vesicles were investigated in the presence of 1, 3, 10, 30, and 100 μmol/L of fasiglifam or fasiglifam-G. All incubations were made in triplicate. The IC_{50} values were obtained from curve fitting of the concentration-inhibition curves by nonlinear regression analysis by using SAS System Version 8.02 (SAS Institute Inc., Cary, NC).

Measurement of radioactivity

Radioactivity in the plasma, bile, *in vitro* biological samples, and organic solvent extracts was measured directly using LSC (LSC-5100; Aloka Co., Ltd., Tokyo, Japan, or Tri-Carb 2100TR, 2700TR, 2900TR, 3100TR, or 3110TR; PerkinElmer Inc. or LS 6000IC or 6500; Beckman Coulter, Inc., Brea, CA) with Hionic-Fluor™ (PerkinElmer Inc.) and liquid scintillator A (toluene-based scintillator; Wako Pure Chemical Industries Ltd., Osaka, Japan). Radioactivity in the liver homogenate was measured using the combustion method by employing a sample oxidizer (Model 307 or A030701; PerkinElmer Inc.). Resultant [^{14}C]CO_2 was trapped in carbon dioxide absorbent (Carbo-Sorb® E, PerkinElmer Inc.), Permafluor® E^+ (PerkinElmer Inc.), or liquid scintillator B (toluene-based scintillator; Wako Pure Chemical Industries Ltd.) was then added, and radioactivity was assayed using LSC. Metabolite profiles in the dog liver samples

obtained after repeat dosing were generated using HPLC with fraction collection, followed by measuring the radioactivity employing ultra-low background LSC (1220 Quantulus; PerkinElmer Inc.) by using Ultima Gold uLLT (PerkinElmer Inc.) as a liquid scintillator. In the on-line detection during the HPLC, radioactivity was measured using an online RI detector (model 505TR or 625TR; PerkinElmer Inc.), with Ultima-Flo^{TM} AP (PerkinElmer Inc.) was used as the liquid scintillator. The lower limit of quantitation was defined as twice and three times the background value for off-line and on-line detection, respectively.

Quantification of fasiglifam in plasma

The concentrations of fasiglifam in plasma in the dog multiple dosing study were determined using HPLC. Forty-microliter aliquots of the plasma samples were mixed with 960 μL of the 5% ammonia solution. The mixed samples were loaded onto SPE cartridges (Oasis MAX extraction cartridge, 1 cc/30 mg; Waters Corporation, Milford, MA), which were successively washed with 1 mL of the 5% ammonia solution and 1 mL of methanol. The analytes were eluted to glass test tubes with 1 mL of the 1% trifluoroacetic acid methanol solution. The eluates were mixed with 100 μL of the propylene glycol solution. These mixtures were evaporated under a nitrogen stream at 40°C. The residues were dissolved in 300 μL of the mobile phase by vortex-mixing for about 30 s, sonicating for about 1 min, and vortex-mixing for about 30 s. The mixtures were centrifuged at approximately 1,800 × *g* at room temperature for 3 min. The supernatants were injected into the Shimadzu LC-VP HPLC system (Shimadzu Corporation) equipped with an XBridge C_{18} column (4.6 mm ID × 150 mm, 5 μm; Waters Corporation) maintained at 40°C. The mobile phase

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consisted of 10 mmol/L ammonium formate (pH 3)/acetonitrile (9:11, v/v). A flow rate of 1 mL/min was maintained for the entire run. The eluted fasiglifam was monitored with absorbance at 235 nm. The lower limit of quantitation for fasiglifam was 0.2 μg/mL.

Solubility of fasiglifam and fasiglifam-G in bile

The solubility of fasiglifam and fasiglifam-G in dog and human bile was assessed by measuring the recovery of radioactivity in the supernatant of the bile after centrifugation of the bile samples spiked with [^{14}C]fasiglifam and [^{14}C]fasiglifam-G at concentrations of 1, 3, and 10 mg/mL. Dog bile was obtained from 8 male and female beagle dogs with an age range of 10 to 12 months. Human bile was obtained from 9 men and women with an age range of 55 to 82 years. The spiked samples were set in a water bath at 37°C for 60 min and centrifuged at approximately 1850 × *g* at 25°C for 10 min, and then the radioactivity in the supernatant was measured as the recovery of radioactivity.

Prediction methods for fasiglifam in bile

The maximum concentration of fasiglifam in bile (C_{max_bile}) was estimated using the maximum plasma concentration (C_{max_plasma}) of fasiglifam, biliary clearance (CL_b), and the bile flow rate (Eq.1):

$$C_{max\_bile} = \frac{CL_b \times C_{max\_plasma}}{\text{Bile flow rate}} \qquad \text{(Eq. 1)}$$

Crystal formation is likely to be driven by C_{max_bile} of fasiglifam and fasiglifam-G. The steady-state C_{max_plasma} of fasiglifam can be used to predict the corresponding value at steady-state in bile (Eq.1). Biliary

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clearance (CL_b) of fasiglifam may be determined using the biliary excretion ratio (ER) and the total plasma clearance (CL_{tot}) values of fasiglifam ($CL_b = ER \times CL_{tot}$).

Data analysis

Data are expressed as mean values or mean values ± S.D. of the results from two to four animals. Values for C_{max_plasma} and time to C_{max_plasma} (T_{max}) were directly noted from the data. The terminal half-life ($t_{1/2}$) and area under the plasma versus time concentration curve (AUC) were calculated from the measured values by using the non-compartmental model in WinNonlin (Ver. 4.1, Pharsight Corporation, Mountain View, CA).

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Results

MALDI TOF MS

Direct mass spectrometric measurements were conducted for lesion sites containing the foreign body material in the liver sections from a dog in a 39-week oral gavage toxicity study of fasiglifam. The ions that corresponded to fasiglifam and fasiglifam-G were detected in the lesions containing the foreign body material (Supplemental Figure 3), whereas the corresponding peaks were not observed in normal regions (Supplemental Figure 4). The MS/MS experiment was performed at *m/z* 563.2 and *m/z* 739.2 as precursor ions. Therefore, the ion at *m/z* 563.2 was identified to be potassiated fasiglifam by comparing with the MS/MS spectrum of the reference standard of fasiglifam (Supplemental Figure 5). The ion at *m/z* 739.2 was estimated to be fasiglifam-G because a typical neutral loss of 176 Da was observed in the MS/MS spectrum (Supplemental Figure 6). The predominant product ion in this MS/MS experiment also corresponded to the mass value of potassiated fasiglifam (*m/z* 563.2). The relative intensities of the observed ions corresponding to both fasiglifam and fasiglifam-G were much higher in the spots in the lesions containing the foreign body materials than in those in the normal regions (Supplemental Table 2).

Evaluation of the metabolic profiles of [^{14}C]fasiglifam in the plasma and liver samples

The concentrations of radioactivity in the plasma and liver were determined in rats and dogs treated with [^{14}C]fasiglifam at single oral doses of 2 and 200 mg/kg. Following a single oral administration of the same

dose, the concentrations of total radioactivity in the rat liver were 2- to 6-fold higher than those in the dog liver (Table 1). Metabolic profiling studies indicated that fasiglifam was the major component in the plasma (>85% of radioactivity) and liver (>57% of radioactivity) in rats and dogs. The concentrations of fasiglifam in the rat liver were 2- to 10-fold higher than those in the dog liver, while the concentrations of fasiglifam-G in the liver tended to be higher in dogs than in rats at the same oral dose (Figure. 2). The concentrations of fasiglifam-G were significantly lower than those of fasiglifam in the livers of rats and dogs.

Evaluation of the potential covalent binding of fasiglifam-G to plasma and liver proteins

The potential covalent binding of fasiglifam-G (an acyl glucuronide) to the plasma and liver proteins was evaluated in rats and dogs treated with [^{14}C]fasiglifam. Following a single oral and/or intravenous administration of [^{14}C]fasiglifam to intact rats and dogs, the mean total recovery of radioactivity ranged from 95.9% to 99.3% (Kogame et al., 2018). These findings indicate that the administered [^{14}C]fasiglifam was completely excreted in both species. No acyl migration of [^{14}C]fasiglifam-G was detected in the plasma and liver of rats and dogs after the oral administration of [^{14}C]fasiglifam, and the total radioactivity in the plasma and liver of rats and dogs was extracted completely at an efficiency ranging from 91% to 109%. In addition, potential covalent binding of fasiglifam and its metabolites to the microsome samples was evaluated. *In vitro* cytochrome P450 (CYP) and uridine diphosphate glucuronosyltransferase (UGT) reaction phenotyping studies generated oxidative metabolites (M-I, T-1676427 and unidentified

metabolites) and fasiglifam-G, respectively (Kogame et. al., 2018). The recovery of the radioactivity through the pretreatment ranged 98.4 to 104.9%.

Evaluation of [^{14}C]fasiglifam accumulation in the plasma and liver of dogs

Fourteen-day repeat-dose studies were conducted in male dogs at an oral [^{14}C]fasiglifam dose of 200 mg/kg/day. The concentration-time profiles of total radioactivity and fasiglifam after the 14th administration were similar to those after the 1st, 7th, and 13th administration of [^{14}C]fasiglifam (Supplemental Figure 7), suggesting that neither total radioactivity nor fasiglifam accumulated in the plasma during the 14-day dosing period.

In the liver, the concentrations of total radioactivity, fasiglifam, and fasiglifam-G at 2 and 8 h postdose of the 14th administration were similar or slightly higher than those after the first administration (Supplemental Table 3), indicating that there was no appreciable accumulation of total radioactivity, fasiglifam, or fasiglifam-G in the liver after 14 days of repeated dosing.

Evaluation of the disposition and metabolism of [^{14}C]fasiglifam in rats and dogs

Disposition and metabolism of [^{14}C]fasiglifam was evaluated in bile duct-cannulated male rats and in dogs following a single intraduodenal dose (ID) in rats and after oral administration to dogs. At a dose of 2 mg/kg, the mean total recovery of radioactivity at 24 h postdose was 84.5% and 82.4% in rats and dogs, respectively; 81.4% and 78.2% of the dosed radioactivity was excreted into the bile of rats and dogs,

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respectively. These results showed that [^{14}C]fasiglifam was well absorbed in rats and dogs at a dose of 2 mg/kg, and that the absorbed fasiglifam and related compounds were mainly excreted into the bile. At a dose of 200 mg/kg, the mean total recovery of radioactivity at 24 h postdose was 84.4% and 54.7% in rats and dogs, with 54.1% and 42.0% of the dosed radioactivity being excreted into the bile, respectively for both. Data regarding excretion of total radioactivity are presented in Supplemental Table 4. A previous study showed that metabolism plays a greater role in the elimination of fasiglifam than biliary and urinary excretion of fasiglifam in both the species, and glucuronidation of fasiglifam was the predominant metabolic pathway in both the species (Kogame et al., 2018). The proposed metabolic pathways of fasiglifam are presented in Supplemental Figure 8. Hence, fasiglifam-G was the main component in the rat and dog bile (ranging from 41.6% to 78.8% to the total radioactivity), and metabolic profiles of bile samples were qualitatively similar between rats and dogs (Table 2). The biliary excretion of unchanged parent drug in dogs was similar to slightly higher than that in rats (the adjusted biliary excretion ratio of fasiglifam were 13.8% and 7.0% of the dose in dogs and rats, respectively, at 2 mg/kg), whereas the amount of fasiglifam-Tau formed in dogs was at least 3-fold lower than that in rats.

Hepatic transporter studies of fasiglifam and fasiglifam-G

The uptake of [^{14}C]fasiglifam into hepatocytes was investigated in rats and dogs, and the results are summarized in Table 3. The hepatic uptake of [^{3}H]pravastatin in hepatocytes was also investigated as a positive control for evaluating Oatp function. In the presence of cyclosporin A, the hepatic uptake clearance

of pravastatin was significantly reduced in rat and dog hepatocytes. These findings are consistent with reported data (Shitara et al., 2004; Wilby et al., 2011), suggesting that rat and dog hepatocytes used in the present study retained Oatp function. The hepatic uptake clearance values of [^{14}C]fasiglifam were comparable between rats and dogs, indicating that no species difference existed in the hepatic uptake clearance of [^{14}C]fasiglifam. The uptake clearance values of [^{14}C]fasiglifam in rats and dogs were ≥43-fold higher than those of [^{3}H]pravastatin, a substrate for Oatps. In addition, cyclosporin A, an inhibitor of Oatps and Ntcp, had no or little inhibition (<20%) on the hepatic uptake clearance of [^{14}C]fasiglifam in rats and dogs. These findings suggest that passive diffusion plays a more important role than transporter(s) in the hepatic uptake of [^{14}C]fasiglifam in rats and dogs. The inhibitory effects of fasiglifam and fasiglifam-G on hepatobiliary transporters were evaluated, and the results are summarized in Table 4. No obvious species differences in potential inhibition were observed.

Determination of the concentrations of total radioactivity, fasiglifam, and fasiglifam-G in bile after single or repeated dosing of [^{14}C]fasiglifam

Concentrations of total radioactivity in the bile

All bile samples described in this study were collected from the common bile duct of rats and dogs and/or from the gallbladder of dogs. Time-course studies showed that the concentrations of total radioactivity in dog bile were up to 7-fold higher than those in rat bile after receiving a single oral (dogs) or ID (rats) dose

of 2 or 200 mg/kg (Figure 3). The concentrations of total radioactivity in the bile were also determined in dogs after 14 days of repeated dosing at an oral [^{14}C]fasiglifam dose of 200 mg/kg/day. After the 14th administration, the concentrations of total radioactivity in dog bile at 2 and 8 h postdose were 12143, and 18357 μg equiv./mL, respectively, which were approximately similar or slightly higher than those in dogs after a single oral dose of 200 mg/kg (6668 and 14089 μg equiv./mL at 2 and 8 h, respectively). The results also indicated that the concentrations of total radioactivity in dog bile were 54- to 173-fold higher than those in the liver after 14 days of repeated dosing at an oral dose of 200 mg/kg/day (224 and 106 μg equiv./mL at 2 and 8 h, respectively).

Concentrations of fasiglifam and fasiglifam-G in the bile

The concentrations of fasiglifam and its metabolites in the pooled rat and dog bile over 24 h after a single ID or oral dose of 2 or 200 mg/kg were determined. Within the same dose group, the concentrations of fasiglifam and fasiglifam-G in the dog bile were 5- to 10-fold higher than those in the rat bile (Table 5). The concentrations of M-I and fasiglifam-Tau in dog bile were similar or lower than those in rat bile. The concentrations of fasiglifam and fasiglifam-G in dog bile were also determined after 14 days of repeated dosing of [^{14}C]fasiglifam at 200 mg/kg/day. Following repeated dosing, the average concentrations of fasiglifam and fasiglifam-G in dog bile at 2 and 8 h postdose ranged from 5280 to 11087 μg/mL (individual values in 4 dogs ranged from 3509 to 12956 μg/mL; Table 6). Note that since the measurements in Table 5 are concentrations over 24 hours, the values in Table 5 cannot be compared with the individual time

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measurements in Table 6. No precipitate/crystalline material was observed in the bile collected from the gallbladder during the study. This study also indicated that the concentrations of fasiglifam and fasiglifam-G in the bile slightly increased (≤2-fold) with repeated dosing compared to those in dogs after a single oral dose of 200 mg/kg.

Determination of bile flow rate and pH in rats and dogs in the presence of [^{14}C]fasiglifam

Bile flow rates and pH values in rats and dogs were determined following a single ID (rats) or oral (dogs) administration of [^{14}C]fasiglifam at 0 (vehicle control), 2, or 200 mg/kg. Data from the literature (Davies and Morris, 1993) and the present study indicate that the mean normal bile flow rate adjusted by body weight in dogs is about 4- to 8-fold slower than that in rats. At 2 mg/kg, the bile flow rates in rats and dogs were similar to those observed in the vehicle control groups (Table 7). At 200 mg/kg, the bile flow rates in rats and dogs were slightly higher than those observed in the vehicle control groups (Table 7). No significant change in the pH values of bile in rats and dogs was observed after a single ID (rats) and oral (dogs) dose of [^{14}C]fasiglifam at 2 or 200 mg/kg (Table 7). However, following 14 days of repeated dosing at an oral dose of 200 mg/kg/day, the pH of the dog bile increased to a range of 6.8 to 7.6.

The *in vitro* solubility of fasiglifam and fasiglifam-G

The *in vitro* solubility of fasiglifam and fasiglifam-G in dog and human bile was determined using individual bile samples collected from the respective species spiked with different concentrations of

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[^{14}C]fasiglifam or [^{14}C]fasiglifam-G. Results of these studies showed that the solubility limit for both fasiglifam and fasiglifam-G was approximately 3000 μg/mL in dog bile, and approximately 1000 μg/mL in human bile (Table 8). No apparent effect of either total bile acid concentrations or bile pH was noted on solubility.

Predicted maximum concentrations of fasiglifam in dog bile

Using Eq. 1 we would have predicted a maximum biliary concentration of 2590 μg/mL. This was calculated using the maximum plasma concentration of 315 μg/mL reported in Table 1, the total dog clearance of fasiglifam of 29.8 ml/hr/kg reported by Kogame et al. (2018), the extraction ratio of 0.138 determined in Table 2, and a bile flow rate of 0.5 ml/hr/kg as given in Table 7, which is also the value reported by Davies and Morris (1993).

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Discussion

Fasiglifam treatment in 4- to 39-week repeated-dose toxicology studies in dogs resulted in liver toxicity characterized by the elevation of AST, ALT, ALP, and/or bilirubin. Associated histopathological changes were characterized as portal/periportal granulomatous inflammation with crystal formation. This toxicity was both dose- and duration-dependent. In the 39-week dog study, the liver toxicity observed at 150 mg/kg/day had either completely resolved or showed signs of recovery following a 13-week recovery period. The observed fasiglifam-related dog liver toxicity was characterized by conducting nonclinical mechanistic studies *in vitro* and *in vivo*. For comparison, the mechanistic studies were also conducted in rats. In the repeated-dose toxicology studies in rats, the histopathological changes observed in dogs were not found in rats at doses up to 2000 mg/kg/day. For the *in vivo* mechanistic studies, oral doses of 2 and 200 mg/kg/day were used because 2 mg/kg/day was considered non-toxic (human equivalent dose [HED] is approximately 67 mg/day based on body surface area), whereas doses of ≥150 mg/kg/day (HED for 150 mg/kg/day in dog is 5000 mg/day) resulted in liver toxicity after repeated dosing for ≥13 weeks in dogs.

MALDI TOF MS analysis indicated that the crystalline material in the liver sections of affected dogs contained fasiglifam and fasiglifam-G, indicating that the dog liver toxicity could be characterized in terms of pharmacokinetics and physicochemistry of fasiglifam and its related compounds.

No dog-specific metabolites were found in the plasma, liver, or bile; further, no evidence of covalent binding of fasiglifam-G (an acyl glucuronide) to the plasma or liver proteins was noted in the dogs. Moreover, no significant accumulation of total radioactivity, fasiglifam, and/or fasiglifam-G was noted in

the dog plasma or liver following 14 days of repeated oral dosing of [^{14}C]fasiglifam at 200 mg/kg/day. Although the concentrations of fasiglifam in the rat liver were 2- to 10-fold higher than those in the dog liver at the same dose level (Figure 2), no histopathological liver injury was observed in any rat toxicology studies, suggesting that fasiglifam is not directly toxic to hepatocytes.

Transporter studies showed that passive diffusion played a more important role than active transport in the hepatic uptake of fasiglifam in rats and dogs. No species difference was noted between rats and dogs in the clearance of fasiglifam hepatic uptake and fasiglifam-G hepatobiliary efflux, or potential inhibition of hepatobiliary transporters by fasiglifam and fasiglifam-G. Therefore, the involvement of hepatic transporters could not solely account for the differences in the liver findings in the rat and dog toxicity studies.

Following a single ID or oral dose of [^{14}C]fasiglifam at 2 or 200 mg/kg to rats and dogs, within the same dose group, the concentrations of total radioactivity, fasiglifam, and fasiglifam-G in dog bile were 5- to 10-fold higher than those in rat bile (Table 5), whereas their excretion (% of administered dose) was comparable between the two species (Table 2). The differences in concentrations are attributed to the difference in bile flow rate between the two species. In the vehicle group, bile flow rate in dogs (12–13 mL/day/kg) was considerably slower than that in rats (44–49 mL/day/kg), which was consistent with published data (Davies and Morris, 1993). We note that the maximum plasma concentration reported here (Table 1) is consistent with that in the 39-week toxicity study in dogs where a maximum concentration was 78 μg/mL for 40 mg/kg/day.

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The bile concentration of fasiglifam was 7269 μg/mL at 8 h after 14 days of repeated dosing of [^{14}C]fasiglifam at 200 mg/kg/day as shown in Table 6. This value is just under 3 times the calculated maximum biliary concentration in dogs of 2590 μg/mL using Eq. 1. We attempted to predict the maximum bile concentration in dogs using Eq. 1, to see if it would be possible to predict the maximum bile concentration in humans. Assuming that the extraction ratio is similar between dogs and humans (0.138), using the total human clearance of fasiglifam of 14.6 mL/hr/kg (Kogame et al., 2018), the maximum plasma concentration of 5.3 μg/mL observed in T2DM patients for a 50 mg multiple daily dose study (Leifke et al., 2012), and a human bile flow rate of 0.24 mL/hr/kg (Davies and Morris, 1993), a maximum bile concentration of 44.5 μg/mL would be predicted. We suggest that the solubility limit would be 20-fold higher than the predicted fasiglifam concentration, and that even if our calculations underpredict by 1/3 of the dog data, or even if the underprediction is 7-fold off in a worst case scenario, we would not expect biliary concentrations of fasiglifam to exceed the solubility limit for this compound. The *in vitro* solubility limit of fasiglifam in dog bile was approximately 3000 μg/mL. However, no crystalline material was visually observed in the dog bile collected from the gallbladder at the end of the 14-day dosing period (data on file), possibly because it is difficult to find microscopic nature of the crystals and/or the duration of the study was insufficient for the crystal growing.

The C_{max_bile} of fasiglifam-G could not be estimated due to the low or undetectable levels of the metabolite in plasma. From Tables 2 and 5 it can be seen in both rats and dogs that average fasiglifam-G bile concentrations over 24 hours range from 3.7- to 7.2-fold higher than fasiglifam bile concentrations. The same

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pattern is observed for the dog concentrations at 2 and 8 hr in Table 6; however, the ratios are only 1.3 to 1.6 greater. If fasiglifam-G bile concentrations in humans follow the same relative pattern with fasiglifam as observed in rats and dogs, again we would not expect fasiglifam-G concentrations to exceed solubility limits in the previous human studies.

Histopathological examination of the liver from affected dogs in repeat-dose toxicity studies with fasiglifam showed that crystal formation occurred primarily in and around the portal triads of the dog liver, and granulomatous inflammation was a secondary reaction to the presence of crystals in these areas. Toxicity of this nature is dose-dependent and explains the dose- and duration-dependent nature of the dog liver toxicity. In addition, assuming linear pharmacokinetics, we predicted the maximum concentrations of fasiglifam and fasiglifam-G in dog bile at the no observable adverse effect level (NOAEL) of 40 mg/kg/day. The predicted bile concentrations of these compounds at the NOAEL in the dog study did not exceed the solubility limit in the dog bile, indicating that crystal formation might not occur in the biliary tree of dogs at NOAEL.

As shown above, the predicted concentration in human bile indicated that the risk of crystal formation in human bile was minimal at the maximum recommended human dose (50 mg). The safety margin based on ratio of dog AUC at NOAEL (854000 ng•h/mL at 40 mg/kg, Wolenski et al., 2017) to human AUC at the clinically efficacious dose (61463.4 ng•h/mL at 50 mg, Mayer et al., 2014) was estimated to be more than 10-fold. However, the fasiglifam clinical development program was terminated owing to liver safety concerns. These facts indicate that the mechanism of the liver toxicity might be different between humans

and dogs. Although the fundamental pathogenesis of fasiglifam-induced liver injury in humans is still not well understood, nonclinical *in vivo* and *in vitro* studies have proposed theories for the pathogenesis caused by a fasiglifam-specific factor (Li et al., 2015; Wolenski et al., 2017), in which the inhibitory effect of fasiglifam on several hepatobiliary transporters might provide a possible mechanism of fasiglifam-induced liver injury in human. Hepatobiliary transporters are known to play an important role in bile acid homeostasis (Kock and Brouwer, 2012; Chiang, 2013). Therefore, the inhibition of the transporters is considered to lead to abnormal intracellular concentration of bile acid and subsequently liver injury, since bile acids have detergent-like property (Pauli-Magnus et al., 2005; Marion et al., 2007). However, in many cases, drug-induced liver injury (DILI) cannot be predicted by nonclinical animal models (Chan and Benet, 2018), and since severe DILI has a very low incidence (Guidance for Industry, 2009, Bjornsson et al., 2013; Fontana, 2014), in addition to drug-specific risk factors, patient-specific risk factors such as, age, genetic polymorphisms, alcohol consumption, and/or medical history might also contribute to the susceptibility of DILI. Unfortunately, the patient-specific risk factors in fasiglifam clinical studies are not available.

In summary, high concentrations of fasiglifam and fasiglifam-G that exceeded the solubility limits of these compounds in dog bile were most likely the main causes of dog liver toxicity observed in repeat-dose toxicology studies. The formation of crystals and toxicity development require high biliary concentrations and sufficient study duration. The present study showed the importance of pharmacokinetic studies in drug development in terms of clarifying the mechanism of toxicity and safety assessment, although the mechanism of fasiglifam-related liver toxicity in clinical is still unclear.

Authorship Contributions

Participated in research design: Kogame, Moriya, Pan, Fukui, Tagawa, Benet

Conducted experiments: Kogame, Moriya, Mori, Ebihara

Performed data analysis: Kogame, Moriya, Mori, Ebihara, Fukui, Tagawa

Wrote or contributed to the writing of the manuscript: Kogame, Moriya, Mori, Pan, Morohashi, Ebihara, Fukui, Tagawa, Benet

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Figure Legends

Figure 1. Optical images of the matrix-deposited region on fresh frozen liver tissues.

Letters in black: spots on the normal region

Letters in red: spots on the foreign body material

Figure 2. Concentrations of fasiglifam and fasiglifam-G in the rat and dog liver after a single oral dose of [^{14}C]fasiglifam. Data in (A) and (B) represent the concentrations at 2 mg/kg in rats and dogs, respectively. Data in (C) and (D) represent the concentrations at 200 mg/kg in rats and dogs, respectively. Each value represents the data from one animal.

Figure 3. Time course of the concentrations of radioactivity in bile after the administration of a single dose of [^{14}C]fasiglifam at 2 mg/kg (A) and 200 mg/kg (B). Each value represents of an average of two animals. The individual measurements are presented in Supplemental Table 6

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Tables

Table 1 Concentrations of radioactivity in the plasma and liver after a single oral administration of [^{14}C]fasiglifam in rats and dogs

Species	Dose (mg/kg)	Time (h)	Concentration of radioactivity (μg equiv./mL or g)	
			Plasma	Liver
Rat	2	1	4.98	18.1
		8	3.32	13.8
	200	1	237	275
		8	352	341
Dog	2	2	5.04	10.6
		8	1.84	5.08
	200	2	315	179
		8	39.0	55.3

Data were obtained from one animal for each collection time.

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Table 2 Mean levels (percentages in parentheses) of fasiglifam and its metabolites in the bile of rats and dogs during 0 to 24 h following a single dose of [^{14}C]fasiglifam

Compound	% of administered dose			
	Dog		Rat	
	2 mg/kg	200 mg/kg	2 mg/kg	200 mg/kg
Total radioactivity	78.2 (100.0)	42.0 (100.0)	81.4 (100.0)	54.1 (100.0)
Fasiglifam	10.8 (13.8)	4.6 (11.0)	5.7 (7.0)	5.3 (9.8)
M-I	LOQ (0.0)	LOQ (0.0)	LOQ (0.0)	1.3 (2.4)
Fasiglifam-G	39.4 (50.4)	33.1 (78.8)	38.8 (47.7)	22.5 (41.6)
Fasiglifam-Tau	4.5 (5.8)	0.2 (0.5)	15.2 (18.7)	12.2 (22.6)
Others	23.5 (30.0)	4.2 (9.7)	21.9 (26.6)	13.0 (23.6)

Each value represents an average of two animals. Figures in parentheses represent proportions to the total radioactivity (%). The individual measurements are presented in Supplemental Table 5.

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Table 3 Uptake of [^{14}C]fasiglifam and [^{3}H]pravastatin into rat and dog hepatocytes and the inhibitory effect of cyclosporin A

Compound	Concentration	Uptake clearance (μL/min/mg protein)			
		Rat		Dog	
		Rs704	RS740	DB252	DB268
Pravastatin	0.3 μmol/L	15.8	19.9	4.8	2.7
Pravastatin + CyA	0.3 μmol/L + 10 μmol/L	2.8	3.2	0.7	0.9
Fasiglifam	0.3 μmol/L	689.6	858.6	586.8	522.6
Fasiglifam + CyA	0.3 μmol/L + 10 μmol/L	632.8	715.2	556.2	421.8

CyA: cyclosporin A

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Table 4 IC_{50} values of fasiglifam and fasiglifam-G on hepatobiliary transporters

Species	IC_{50} (μmol/L)			
	Mrp2		Bsep	
	Fasiglifam	Fasiglifam-G	Fasiglifam	Fasiglifam-G
Rat	-	2.9	20.6	82.5
Dog	-	5.1	16.1	18.2

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Table 5 Concentrations of fasiglifam and its metabolites in pooled bile over 24 h after a single dose of [^{14}C]fasiglifam

Dose (mg/kg)	Compound	Concentration (μg equiv./mL) (n = 2)		Fold difference
		Rat	Dog	Dog/Rat
2	Fasiglifam	1.80	18.2	10.1
	Fasiglifam-G	12.4	66.6	5.4
	Fasiglifam-Tau	4.83	7.65	1.6
	M-I	LOQ	LOQ	NA
200	Fasiglifam	101	470	4.7
	Fasiglifam-G	433	3341	7.7
	Fasiglifam-Tau	231	16.0	<1
	M-I	23.9	LOQ	<1

[^{14}C]fasiglifam was administered intraduodenally to rats and orally to dogs. The individual measurements are presented in Supplemental Table 7.

LOQ, below the lower limit of quantitation; NA, not available.

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Table 6 Concentrations of fasiglifam and fasiglifam-G in dog bile after a single or repeating dose of [^{14}C]fasiglifam administered at 200 mg/kg/day

Time after dosage (h)		Concentration (μg equiv./g)	
		Fasiglifam	Fasiglifam-G
1st dose	2	2407	3934
	8	5002	7862
14th dose	2	5280	6863
	8	7269	11087

Data were obtained from one animal for each collection time at the first dose. Each value at the 14th dose represents the mean of two animals (the individual measurements are presented in Supplemental Table 8).

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Table 7 Range of bile flow rates and pH values (triplicate samples) after a single dose of [^{14}C]fasiglifam

Species	Bile flow rate (mL/day/kg) (n = 2)			pH (n = 1)	
	Vehicle	2 mg/kg	200 mg/kg	2 mg/kg	200 mg/kg
Rat	44–49	61–68	92–115	7.4–7.7	7.4–8.0
Dog	12–13	12	20–24	6.5–6.8	6.8

[^{14}C]fasiglifam was administered intraduodenally to rats and orally to dogs.

The pH of the bile was obtained from 0–24 h postdose in rats and at 2 and 8 h postdose in dogs.

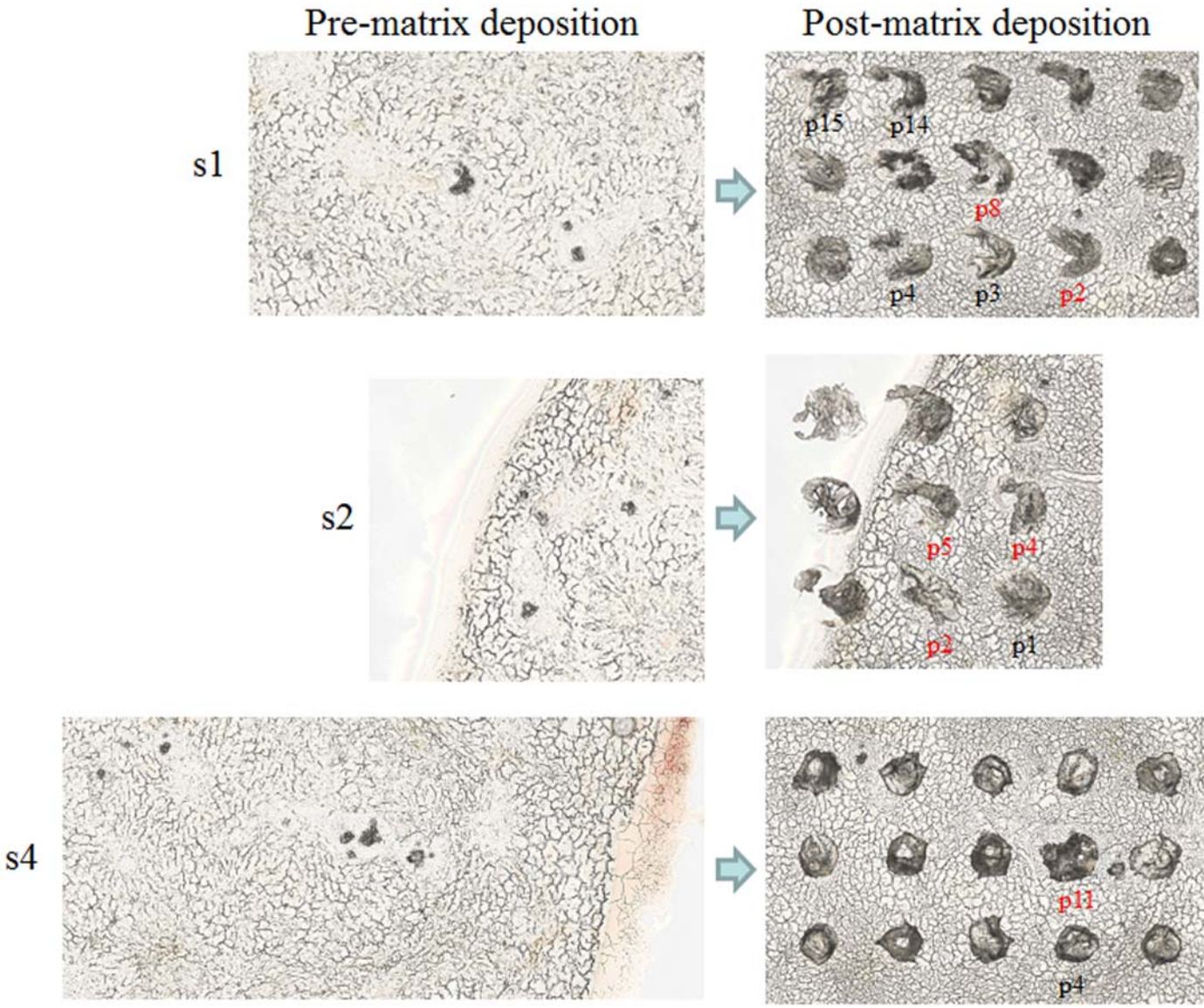
Published bile flow rate is 90 mL/day/kg in rats and 12 mL/day/kg in dogs (Davies and Morris, 1993).

Table 8 The *in vitro* solubility of [^{14}C]fasiglifam and [^{14}C]fasiglifam-G, pH, and total bile acid concentrations in dog bile

	Dog (n=8)		Human (n=9)	
	[^{14}C]Fasiglifam	[^{14}C]Fasiglifam-G	[^{14}C]Fasiglifam	[^{14}C]Fasiglifam-G
Recovery* at 1000 μg/mL (%)	91.6-96.7	98.5-102	92.9-99.9	91.7-98.0
Recovery* at 3000 μg/mL (%)	80.5-90.6	92.0-103	63.9-96.4	60.3-96.1
Recovery* at 10000 μg/mL (%)	63.3-77.5	55.0-102	20.6-78.1	24.3-80.2
pH	6.8-7.8		6.4-7.6	
Total bile acid concentration (mmol/L)	119-187		11.6-139	

*Recovery of the spiked radioactive material.

Values represent the range for eight dogs.



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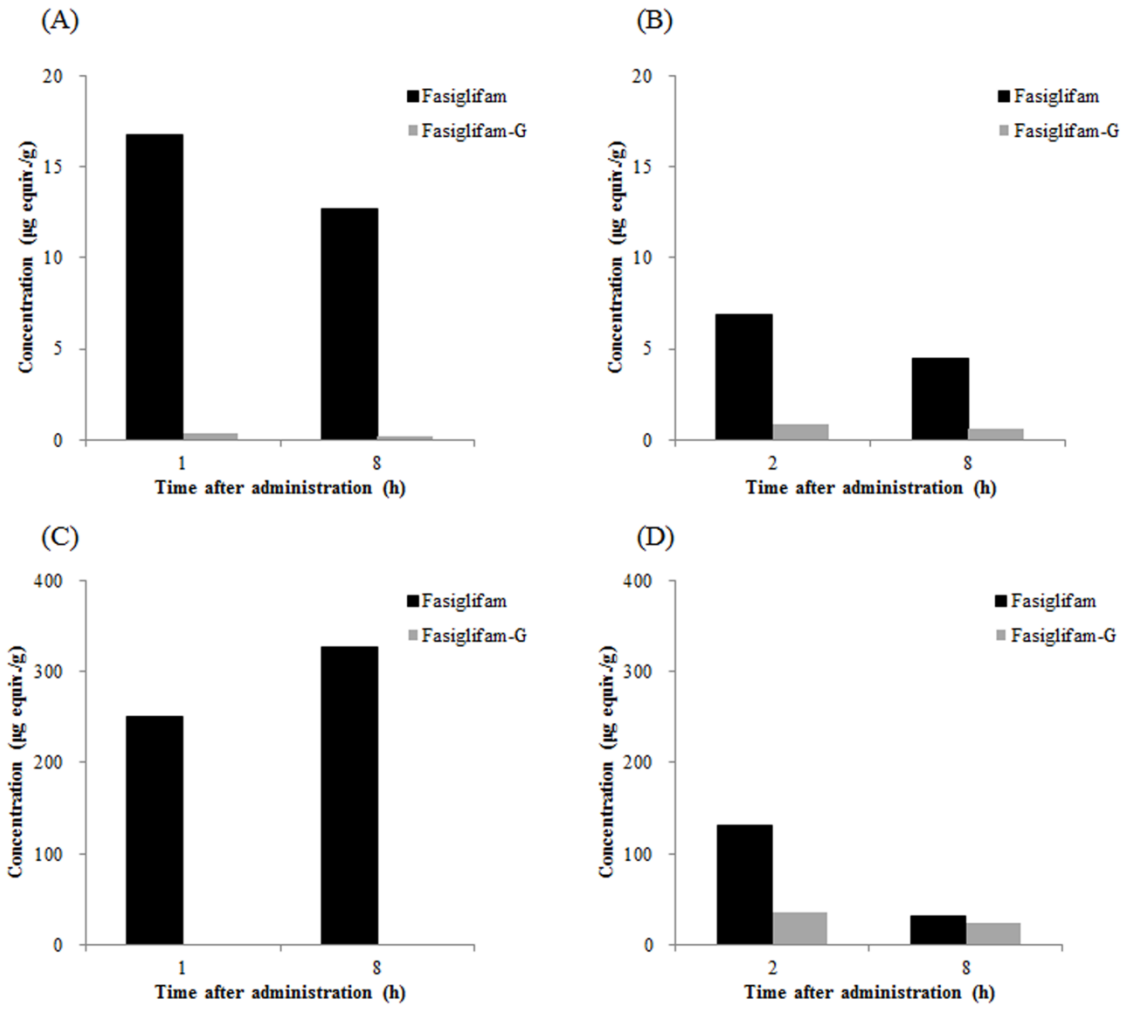

Figure 2

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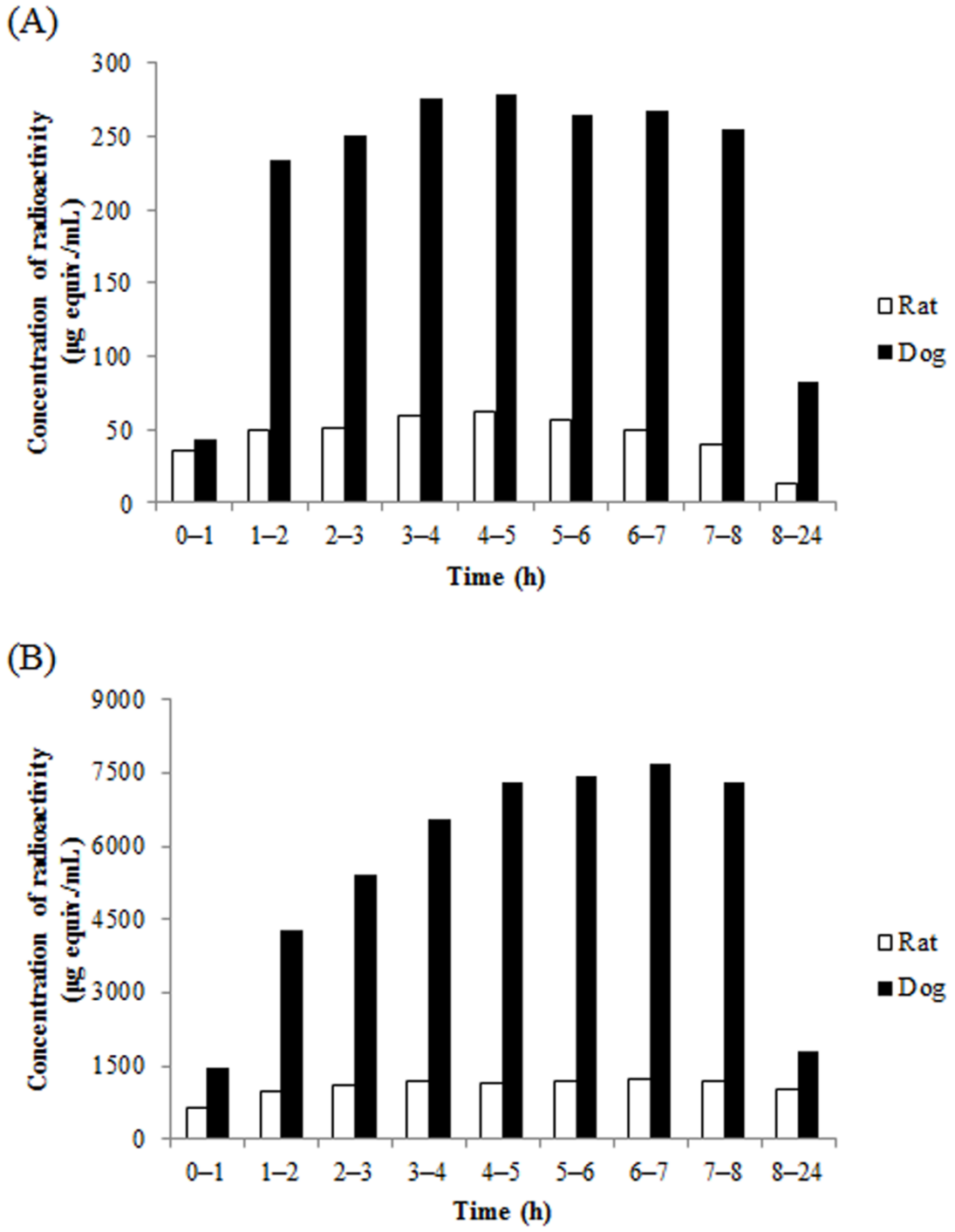

Figure 3