New Photosafety Assessment Strategy Based on the Photochemical and Pharmacokinetic Properties of Both Parent Chemicals and Metabolites

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ABBREVIATIONS
3T3 NRU PT, 3T3 neutral red uptake phototoxicity test; CES, carboxylesterase; CYP, cytochrome P450; FA, fenofibric acid; FF, fenofibrate; ICH, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; MEC, molar extinction coefficients; MPE, mean photo effect; NADPH, nicotinamide
adenine dinucleotide phosphate; OECD, Organization for Economic Co-operation and Development; PK, pharmacokinetic; QN, quinine; RFA, reduced fenofibric acid; ROS, reactive oxygen species; SB, sulisobenzone; UPLC/ESI-MS, ultra-performance liquid chromatography equipped with electrospray ionization mass spectrometry; UV, ultraviolet; VIS, visible light
ABSTRACT

Photoreactivity and dermal/ocular deposition of compounds have been recognized as key considerations for evaluating the phototoxic risk of compounds. As some drugs are known to cause phototoxic reactions via generation of potent phototoxic metabolites, photosafety assessments on parent drugs alone may lead to false predictions about their photosafety. The objective of the present study was to establish a new photosafety assessment strategy for evaluating the in vivo phototoxic potential of both a parent substance and its metabolites. The in vivo phototoxic risk of fenofibrate (FF) and its metabolites, fenofibric acid (FA) and reduced fenofibric acid, were evaluated based on photochemical and pharmacokinetic analyses. FF and FA exhibited intensive ultraviolet absorption, with molar extinction coefficient values of 17,000 (290 nm) and 14,000 M⁻¹cm⁻¹ (295 nm), respectively. Superoxide generation from FA was significantly higher than from FF, and a marked increase in superoxide generation from FF was observed after incubation with rat hepatic S9 fractions, suggesting enhanced photoreactivity of FF after metabolism. FA showed high dermal/ocular deposition after oral administration (5 mg/kg, p.o.) although the concentration of FF was negligible, suggesting high exposure risk from FA. Based on these findings, FA was deduced to be a major contributor to phototoxicity induced by FF taken orally, and this prediction was in accordance with the results from in vitro/in vivo phototoxicity tests. This study suggested that this new screening strategy for parent substances and their metabolites gives reliable photosafety information on drug candidates and would be useful for drug development with wide safety margins.
INTRODUCTION

Drug-induced photosensitivity can be elicited by topical or systemic application of pharmaceutical substances in combination with subsequent exposure to sunlight or artificial light (Drucker and Rosen, 2011; Moore, 2002). As the photochemical reactions of drug molecules are a key trigger of phototoxic reactions, photochemical evaluations such as ultraviolet (UV) spectral analysis and reactive oxygen species (ROS) assay are carried out as photosafety assessments in pharmaceutical research to avoid adverse phototoxic events (Onoue et al., 2009; Seto et al., 2012). In addition to photochemical evaluation, pharmacokinetic (PK) evaluation with a focus on sunlight-exposed tissues (e.g. skin and eyes) can also be helpful for predicting in vivo phototoxicity, as phototoxic reactions mainly occur in the skin (Seto et al., 2009; Seto et al., 2011). Currently, regulatory agencies recommend PK characterization as well as photochemical characterization for the photosafety assessment on pharmaceuticals. For example, tissue distribution is recommended in the guidelines for photosafety assessment of pharmaceuticals published by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (ICH, 2013).

In many cases of drug-induced photosensitivity, phototoxic reactions are thought of as mainly being elicited by parent drugs, and the photosafety of drug metabolites is, in principle, outside the scope of regulatory oversight (ICH, 2013). However, the metabolites of phototoxic compounds can have phototoxicity that is as potent as that of their parent drugs, and some metabolites have even more potent phototoxic potential than their parent chemicals, including amiodarone, chlorpromazine, and fenofibrate. (Ferguson
et al., 1985; Ljunggren and Moller, 1977; Ljunggren, 1977; Miranda et al., 1994). In our previous investigation, in vivo phototoxic risk of chlorpromazine was predicted to be less phototoxic on the basis of the photochemical and PK characteristics of its parent compounds although, in fact, potent in vivo phototoxic reaction was observed in the rat skin after oral administration of chlorpromazine (Onoue et al., 2014). Chlorpromazine taken orally has been found to be extensively metabolized by cytochrome P450 (Wojcikowski et al., 2010), and its demethylated metabolites have been found to be more phototoxic than chlorpromazine (Ljunggren and Moller, 1977). In this context, false predictions might arise from the lack of photochemical and PK characterization on metabolites, and the photosafety assessments of metabolites as well as their parent chemicals should provide more reliable photosafety information on pharmaceuticals; however, the feasibility of such new screening strategies is unknown.

The present study aimed to establish a new photosafety assessment strategy with the combined use of photochemical and PK characterization on a parent drug and its metabolites, applying this new screening strategy to fenofibrate (FF) and its major metabolites, fenofibric acid (FA) and reduced fenofibric acid (RFA) (Fig. 1). FF, an anti-hyperlipoproteinemic agent, is clinically recognized as phototoxic (Leenutaphong and Manuskiatti, 1996; Machet et al., 1997; Roberts, 1989), and the phototoxic potential of FF has been investigated using several in vitro tools, demonstrating the potent in vitro phototoxicity of FF and FA (Miranda et al., 1994; Vargas et al., 1993). Thus, in the present study, FF and its metabolites were employed as model chemicals to confirm the feasibility of the new screening strategy. The photoreactivity of these compounds was
evaluated by UV spectral analyses and ROS determination before and after incubation with rat hepatic/intestinal S9 fractions. Dermal and ocular deposition of FF and its metabolites was characterized after the oral administration of FF to rats.
MATERIALS AND METHODS

Chemicals

FF was purchased from Wako Pure Chemical Industries (Osaka, Japan). FA and RFA were bought from AK Scientific Inc. (Union City, CA, USA) and Toronto Research Chemicals Inc. (Toronto, Ontario, Canada), respectively. Pooled IGS Sprague-Dawley rat liver S9 fraction, pooled IGS Sprague-Dawley rat intestinal S9 fraction, and nicotinamide adenine dinucleotide phosphate (NADPH)-regenerating system were obtained from Sekisui Medical (Tokyo, Japan). All other reagents were purchased from commercial sources. A quartz reaction container for irradiation of simulated sunlight to a 96-well plate was constructed by Ozawa Science (Aichi, Japan).

UV spectral analysis

UV spectral analysis was performed as previously described (Seto et al., 2013b) with minor modification. Briefly, FF (20 μM) was not dissolved in 20 mM sodium phosphate buffer (NaPB, pH7.4) due to its poor solubility, and thus, each compound was dissolved in ethanol at a final concentration of 20 μM. UV absorption spectra were recorded with a Hitachi U-2010 spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan) interfaced to a PC for data processing (software: Spectra Manager). A spectrofluorimeter quartz cell with 10 mm pathlength was employed. Molar extinction coefficient (MEC) values were calculated on the basis of maximum absorbance values in the wavelength range of 290–400 nm.
Irradiation conditions for determination of ROS

An Atlas Suntest CPS plus (Atlas Material Technology LLC, Chicago, IL, USA) equipped with a xenon arc lamp (1,500 W) and a cooling unit SR-P20FLE (Hitachi, Tokyo, Japan) was used for determination of ROS from irradiated chemicals. A UV special filter was installed to adapt the spectrum of the artificial light source to that of natural daylight; the Atlas Suntest CPS series has a high irradiance capability that meets CIE85/1989 daylight simulation requirements. The irradiation test was carried out at 25°C with an irradiance of ca. 2.0 mW/cm² as determined with a calibrated UVA detector Dr. Hönle #0037 (Dr. Hönle, München, Germany).

Determination of ROS from photo-irradiated compounds

Determination of singlet oxygen and superoxide generated from photo-irradiated compounds was conducted in accordance with established protocol (Seto et al., 2013a). Briefly, each tested compound was dissolved in dimethyl sulfoxide (DMSO) at 10 mM for stock solution. To monitor the generation of singlet oxygen, samples containing compounds (200 µM), p-nitrosodimethylaniline (50 µM) and imidazole (50 µM) in 20 mM NaPB (pH7.4) with 0.5% (v/v) Tween 20 were irradiated with simulated sunlight, and then the UV absorption at 440 nm was measured using SAFIRE (TECAN, Männedorf, Switzerland). For the determination of superoxide generation, samples containing the compounds (200 µM) and NBT (50 µM) in 20 mM NaPB (pH7.4) with 0.5% (v/v) Tween 20 were exposed to simulated sunlight, and the reduction of NBT was measured by the increase in the absorbance at 560 nm using SAFIRE. According to the results (mean of
triplicate determinations) from the micellar ROS (mROS) assay, photoreactivity for each tested chemical should be judged to be (i) positive with singlet oxygen ($\Delta A_{440\text{ nm}} \times 10^3$): 25 or more, and/or superoxide ($\Delta A_{560\text{ nm}} \times 10^3$): 20 or more; or (ii) negative with singlet oxygen ($\Delta A_{440\text{ nm}} \times 10^3$): less than 25, and superoxide ($\Delta A_{560\text{ nm}} \times 10^3$): less than 20. In the mROS assay, the final decision should be made as follows: (i) positive: above the threshold level for singlet oxygen or superoxide; or (ii) negative: below the threshold level for both singlet oxygen and superoxide (Onoue et al., 2013a).

**Determination of ROS from photo-irradiated compounds in enzyme-treated samples**

Rat hepatic/intestinal S9 fractions were pre-incubated for 2 min at 37°C (final concentration: 0.2 mg-protein/mL) in 0.3 mL of phosphate buffer (pH7.4) containing typical co-factors. FF was dissolved in DMSO at 10 mM as a stock solution. The reaction was initiated by the addition of FF at 100 $\mu$M, and the final concentration of DMSO was 1%. The reaction was terminated at 1 min by adding 0.2 mL of ice-cold ethanol. For comparison, FF (100 $\mu$M) was also incubated with heat-inactivated (ca. 80°C, 5 min) S9 fractions (denatured groups). The mixtures were evaluated by ROS assay (Onoue et al., 2013a). Briefly, to monitor the generation of singlet oxygen, enzyme-treated mixture, $p$-nitrosodimethylaniline (50 $\mu$M) and imidazole (50 $\mu$M) were dissolved in 20 mM NaPB (pH7.4). For the determination of superoxide generation, enzyme-treated mixture and NBT (50 $\mu$M) were dissolved in 20 mM NaPB (pH7.4). Both reaction mixtures theoretically contained 50 $\mu$M of FF. Then, these samples were
irradiated with simulated sunlight and measured in the same conditions with the ROS assay protocol.

Animals

Male Sprague-Dawley rats at 11–12 weeks of age (ca. 300–350 g, body weight) were purchased from SLC Inc. (Hamamatsu, Japan). For PK experiments, rats \( (n=39) \) were fasted for approximately 18 h before drug administration and orally received ethanolic solution of FF at a dose of 5 mg/kg. For in vivo phototoxicity test, rats \( (n=16) \) were anesthetized using pentobarbital (50 mg/kg i.p.), and then the hair on the abdomen was shaved at approximately 18 h before dermal application of drug solution. All the procedures used in the present study were conducted according to the guidelines approved by the Institutional Animal Care and Ethical Committee of University of Shizuoka.

PK study

Blood samples were taken in a volume of 200 μL from the tail vein in the indicated periods (0.5, 1, 2, 3, 4, 6, 8, 12, 24, and 48 h) after drug administration. The blood samples were centrifuged \( (10,000 \times g, 10 \text{ min, } 4^\circ C) \) to prepare plasma samples, and then the samples \( (100 \mu L) \) were deproteinized by addition of acetonitrile \( (250 \mu L) \). The supernatants were obtained by centrifugation \( (2,000 \text{ rpm, } 1 \text{ min, } 4^\circ C) \) and filtration \( (0.20 \mu m \text{ membrane filter; Millex}^\text{®}-\text{LG, Millipore, Billerica, MA, USA}) \) and kept frozen at \(-20^\circ C\) until they were analyzed.

At the indicated times \( (2, 4, 6, 8, 10, 12, 24, \text{ and } 48 \text{ h}) \) after oral administration of
FF, rats were humanely killed by taking blood from the descending aorta under anesthesia with pentobarbital Na (50 mg/kg), and the tissues were then perfused with cold saline from the aorta. The skin and eye were dissected, minced with scissors, and homogenized using Physcotron (Microtec, Chiba, Japan) in 4 mL of acetonitrile. After sonication for 10 min and shaking for 10 min, the samples were centrifuged (3,000 rpm, 10 min). Extraction was repeated twice with acetonitrile, and the supernatants were pooled. The collected eluents were pooled with acetonitrile extracts, and the samples were evaporated to dryness under a gentle stream of nitrogen at 45°C. The extracted and evaporated tissue samples were stored at 4°C until they were analyzed.

The ultra-performance liquid chromatography equipped with electrospray ionization mass spectrometry (UPLC/ESI-MS) system was employed for the determination of the drug concentration in plasma and tissue samples (Onoue et al., 2013b). The UPLC-ESI/MS system consisted of a Waters Acquity UPLC system (Waters, Milford, MA, USA), which included a binary solvent manager, a sample manager, a column compartment, and a micromass SQ detector connected with Waters Masslynx v 4.1. A Waters Acuity UPLC BEH C18 (particle size: 1.7 µm and column size: Φ2.1 × 50 mm; Waters) was used, and the column temperature was maintained at 40°C. The deproteinized plasma samples were mixed with 50% acetonitrile solution containing ketoprofen (1 µg/mL), an internal standard for UPLC/ESI-MS analysis (supernatant:ketoprofen=1:1), and for determination of tissue concentration, the extracted and evaporated samples were dissolved in 50% acetonitrile including ketoprofen (500 ng/mL). The standards and samples were separated using a gradient mobile phase consisting of purified water containing 0.1% formic acid (A)
and acetonitrile (B). The gradient conditions of the mobile phase were 0–1.0 min, 50% B; 1.0–5.0 min, 50–95% B (linear gradient curve); 5–5.5 min, 95% B; and 5.5–6 min, 50% B, and the flow rate was set at 0.25 mL/min. Analyses were carried out by monitoring specific m/z: 361.2 [M+H]+ for FF; 319.2 [M+H]+ for FA; 303 [M-OH]+ for RFA; and 255.2 [M+H]+ for ketoprofen (internal standard).

3T3 NRU PT

The Balb/c 3T3 mouse fibroblast cells (CloneA-31) were maintained in culture as previously reported (Spielmann et al., 1991). The 3T3 neutral red uptake phototoxicity test (3T3 NRU PT) and data analyses were carried out as described in the Organization for Economic Co-operation and Development (OECD) 432 guideline (OECD, 2004). Briefly, Balb/c 3T3 cells were maintained in culture for 24 h for the formation of monolayers. Two 96-well plates per test chemical were then pre-incubated with six different concentrations of the chemical dissolved in Earle’s Balanced Salt Solution (EBSS) for 1 h in duplicate. One plate was then exposed to a dose of 5 J/cm² UVA (+Irr experiment), whereas the other plate was kept in the dark by covering it with aluminum foil (-Irr experiment). UVA irradiation (ca. 30 min) was performed using a sol 500 Sun simulator (Dr. Hönle) equipped with a 500 W metal halide lamp and a H-1 filter to remove potentially cytotoxic UVB wavelengths. The treatment medium was then replaced with culture medium and, after 24 h, cell viability was determined by neutral red uptake for 3 h. After that, cells were lysed in eluate (ethanol:water:acetic acid=50:49:1), and the neutral red uptake was measured at the absorbance of 540 nm using the Benchmark™ Plus microplate...
spectrophotometer (BioRad, Hercules, CA, USA). Cell viability obtained with each of the six concentrations of the test chemical was compared with that of untreated controls, and mean photo effect (MPE) values were calculated by using Phototox Version 2.0 software (ZEBET, Berlin, Germany) on the basis of obtained cell viability curves in UVA-irradiated and non-irradiated groups of the test chemical for evaluating \textit{in vitro} phototoxicity.

\textbf{In vivo phototoxicity test}

Experiments were performed as described previously with minor changes in dermal administration (Seto et al., 2009). Each FF, FA, or control (quinine and sulisobenzone) was dissolved in DMSO at 100 mg/mL, and was applied to 2 application sites on rat skin at the abdomen (10 mg/site, \(n=4\)) using filter paper (2 cm \(\times\) 2 cm) under anesthesia with pentobarbital Na (50 mg/kg). At 4 h after dermal administration, the filter papers containing chemicals on the application sites were removed and wiped using cotton soaked with distilled water. Then, rats were irradiated individually using black light (FL15BL-B, National, Tokyo, Japan) as a UVA light source with an irradiance of ca. 2.7 mW/cm\(^2\) for ca. 3 h until the UV irradiance level reached 30 J/cm\(^2\). Because UVB light is highly cytotoxic, a UVA light source was employed for the \textit{in vivo} phototoxicity testing. During the UVA irradiation, rats were restrained on a sunbed under anesthesia with pentobarbital Na (50 mg/kg) to ensure uniform irradiation of their abdomen, and non-irradiated sites were wrapped in aluminum foil for protection from UV light. UV intensity was monitored using the calibrated UVA detector Dr. Hönle no. 0037 (Dr. Hönle).
was used as a measure of skin color. This instrument records 3-dimensional color reflectance, so-called $L^*a^*b^*$ system, as recommended by the Commission Internationale de l’Eclairage (CIE). The luminance ($L^*$) gives the relative brightness ranging from total black ($L^* = 0$) to total white ($L^* = 100$). The hue ($a^*$) axis represents the balance between red (positive values up to 100) and green (negative values up to −100), and the chroma ($b^*$) axis represents the balance between yellow (positive values up to 100) and blue (negative values up to −100). The differences in skin color ($\Delta E$) between before and after irradiation were described as follows (Pierard and Pierard-Franchimont, 1993; Westerhof et al., 1986):

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

Data analysis

The significance of differences was determined by Student’s $t$-test on the data from ROS generation after incubation with rat hepatic/intestinal S9 fractions. Other data were analyzed by one-way analysis of variance followed by Tukey’s multiple comparison tests. $P < 0.05$ was considered significant for all analyses. PK characterizations were performed by non-compartmental analysis as implemented in WinNonlin Professional Version 5.2 (Pharsight Corporation, Mountain View, CA, USA).
RESULTS

Photochemical characterization

Phototoxic reactions can be triggered by photochemical reactions of drug molecules following absorption of UV and visible light (VIS) (290–700 nm) (Moore, 1998; Onoue and Tsuda, 2006). Herein, the photochemical properties of FF and two major metabolites, FA and RFA, were analyzed with a focus on UV-absorbing properties and ROS-generating potentials. FF and FA exhibited intensive UV A/B absorption with maximal MEC values of 17,000 (290 nm) and 14,000 M\(^{-1}\)cm\(^{-1}\) (295 nm), and, on the other hand, only weak UVB absorption was observed in RFA with an MEC of 850 M\(^{-1}\)cm\(^{-1}\) at 290 nm (Fig. 2A). Previously, drug molecules with MEC of less than 1,000 M\(^{-1}\)cm\(^{-1}\) were demonstrated to be less phototoxic (Henry et al., 2009); thus, FF and FA can be identified to be photoexcitable.

To clarify the photoreactivity of FF and its metabolites, the generation of ROS from these compounds (200 \(\mu\)M) was determined under irradiation of simulated sunlight (Fig. 2B) since the good relationship between ROS data on chemicals at 200 \(\mu\)M and in vivo phototoxicity revealed the prediction capacity of the ROS assay (Onoue and Tsuda, 2006; Onoue et al., 2008a). FF and FA exhibited potent generation of singlet oxygen with values of 463 and 531 (\(\Delta A_{440\text{ nm}}\times10^3\)), and also generated superoxide with values of 171 and 332 (\(\Delta A_{560\text{ nm}}\times10^3\)), respectively. The levels of ROS generation from RFA were lower than those from FF and FA, and the values of singlet oxygen and superoxide generation were determined to be 61 (\(\Delta A_{440\text{ nm}}\times10^3\)) and 123 (\(\Delta A_{560\text{ nm}}\times10^3\)), respectively. From these findings, all compounds were judged to be photoreactive according to the ROS-data based
classification system, and the photoreactivity of the compounds was ranked as follows: FA > FF ≫ RFA.

ROS generation from enzyme-treated FF was determined after incubation with rat hepatic/intestinal S9 fractions to evaluate the possible photochemical transitions of FF after metabolism (Fig. 2C). Although no significant differences were observed in ROS generation from FF between pre-treatment of active and denatured rat intestinal S9 fractions, the generation of superoxide from FF was significantly increased by ca. 4.5-fold after pre-incubation with active rat hepatic S9 fractions compared with that from FF incubated with the denatured one ($P < 0.05$), suggesting enhanced photoreactivity of FF after metabolism in liver.

**PK characterization**

Phototoxic reactions mainly occur in skin and eyes; thus, dermal and ocular exposure to compounds can be a predictive factor for *in vivo* phototoxicity as well as systemic exposure (Boiy et al., 2008; Seto et al., 2009). PK characterizations of compounds were conducted with a focus on plasma, skin and eyes (Fig. 3 and Table 1). After oral administration of FF to rats, FF was negligible in all tissues (below the limit of detection: 10 ng/mL and 7.1 ng/g tissue), and on the other hand, its metabolites could be detected in plasma and tissues. Rapid and sustained increase of FA level was observed in all tissues up to 6.0–13 h, whereas RFA concentrations in plasma and skin were gradually elevated with $T_{\text{max}}$ of 12–16 h. The $C_{\text{max}}$ and AUC$_{0-\infty}$ values in plasma and skin of FA were ca. 3- to 4-fold higher than those of RFA, and only FA could be detected in rat eyes...
with the $C_{\text{max}}$ and $AUC_{0-\infty}$ values of 0.10 \(\mu\text{g/g tissue}\) and 2.6 h\(\cdot\mu\text{g/g tissue}\), respectively; therefore, FA would have higher exposure risk of UV-exposed tissues compared with RFA. On the other hand, RFA exhibited slower elimination from plasma and skin compared with FA, as evidenced by the ca. 1.5- to 2-fold-longer apparent half-lives of RFA compared with FA in plasma and skin, suggesting longer tissue retention of RFA. From the PK characteristics, dermal and ocular exposure was ranked as follows: FA $>$ RFA $>$ FF.

### Comparative in vitro/in vivo photosafety assessments

When both photochemical and PK data are taken into account, FA was more likely to be phototoxic than the others; then, the photosafety of FA was examined by both 3T3 NRU PT, a well-validated alternative method for photosafety assessment (Spielmann et al., 1998), and rat in vivo phototoxicity test, and FF was also tested for comparison. Quinine (QN) and sulisobenzone (SB) were employed as positive/negative controls in both in vitro/in vivo photosafety tests, respectively. In 3T3 NRU PT, cell viability curves were almost identical between UV-irradiated and non-irradiated groups treated with SB (Fig. 4A). In contrast, QN induced potent phototoxicity to 3T3 cells after UV irradiation. As observed for QN, FF and FA also exhibited enhanced cell death upon UV exposure, indicating potent phototoxicity to 3T3 cells (Fig. 4B). MPE values can distinguish phototoxic molecules (MPE $\geq$ 0.1) from non-phototoxic ones (MPE $<$ 0.1) (Holzhutter, 1997), and MPE values of FF and FA were calculated to be 0.40 and 0.54, respectively. Therefore, both FF and FA were identified to be phototoxic, and FA would be more phototoxic to 3T3 cells than FF because of its larger MPE value.
In vivo photosafety profiles were assessed based on the transitions in skin color (ΔE) after UV irradiation following dermal administration of FF, FA, QN, and SB (Fig. 4C). Dermal concentrations of FF and FA did not increase from 2 to 6 h after dermal application (data not shown), suggesting steady-state concentrations of the drugs in skin; then, the application period was confirmed at 4 h in the present study. In SB-treated groups, no significant differences were observed between UV-irradiated and non-irradiated rats. On the other hand, upon UV irradiation, QN induced significant increase of the ΔE value owing to the increase of the Δb* value, as previously observed (Nose and Tsurumi, 1993). UV-irradiated FF and FA also exhibited significantly higher ΔE values than each non-irradiated group (P<0.05), and these color changes were due to significant increases of Δb* values by 6.4 (for FF) and 5.9 (for FA). Furthermore, in FA-treated groups, the Δa* value was also significantly increased by 4.7 upon UV irradiation. Although ΔE values were not significantly different between UV-irradiated FF and UV-irradiated SB, the ΔE value of UV-exposed rats treated with FA was significantly higher than that of UV-exposed rats treated with SB (P<0.05). These results demonstrated more severe phototoxicity of FA than FF to rat skin.
DISCUSSION

For evaluating in vivo phototoxic risk of a chemical and its metabolites, development of the new photosafety screening system was attempted by photochemical and PK characterization, and the new screening strategy was applied to predict in vivo phototoxic risk of FF and its major metabolites, FA and RFA. According to the present outcomes, FA, a major metabolite of FF, can be the major contributor to FF-induced phototoxic skin responses because of its potent photoreactivity and high dermal/ocular exposure.

The primary trigger for phototoxic reactions can be photo-excitation of chemical molecules with UV/VIS irradiation (Moore, 1998), and then, excited molecules tend to undergo type I and/or II photochemical reactions with molecular oxygen and/or biomolecules. Generation of superoxide and singlet oxygen from photo-irradiated chemicals can be reliable indicators of type I and II photochemical reactivity, respectively (Onoue and Tsuda, 2006; Onoue et al., 2008a). From the ROS data, type I reactivity of FA was higher than that of FF, and, on the other hand, photoreactivity of RFA would be low among tested chemicals. In addition, enhanced superoxide generation could be observed after pre-incubation of FF with active rat hepatic S9 fraction compared with denatured group. Benzophenones including FF and FA can elicit lipid peroxidation, a major mechanism of photoirritation, via type I photochemical reactions after photoexcitation (Markovic et al., 1990). In this context, FA might be a major contributor to FF-induced phototoxicity after metabolism of FF. For photosafety assessments, exposure and retention to dermal/ocular tissues of compounds can also be a key consideration because phototoxic
reactions typically occur in skin and eyes (Boiy et al., 2008; Seto et al., 2009). Therefore, in the present study, the PK behavior of FF and its metabolites was assessed with a focus on plasma and skin/eyes in rats after oral administration of FF. Interestingly, FF was negligible in all samples, and only two metabolites could be detected in the present PK study. According to the present results, FA would have the highest dermal/ocular exposure risk, suggesting a major contributor of FF-induced phototoxicity. Although dermal exposure risk of RFA was lower than FA, RFA might have longer-term exposure risk to dermal compared with FA on the basis of its long elimination half-life. For comparison, the in vitro/in vivo phototoxicity tests also conducted, and FF and FA indicated potent phototoxicity was confirmed. According to the cell viability curves and ΔE values in UV-irradiated groups, the metabolic activity for conversion of FF to FA might not be high in 3T3 cells and rat skin; phototoxicity of FA would be more potent than that of FF on the basis of the data obtained.

In photosafety assessment, both photochemical and PK properties should be taken into consideration; thus, a summary table was built upon photochemical and PK data (Table 2), and values among the data are classified as high, moderate and low levels in accordance with our previous research (Seto et al., 2011). In the present photosafety prediction, high levels for both photochemical and PK data might indicate high phototoxic potential, whereas, low levels in either or both might be indicative of moderate or low phototoxic potential. FA was deduced to be a highly phototoxic metabolite because both photoreactivity and dermal/ocular exposure were high. FF and RFA were less phototoxic owing to limited dermal/ocular exposure of FF, moderate photoreactivity and limited ocular
exposure of RFA. In this context, the phototoxic risk of test compounds was deduced as follows: \( \text{FA} \gg \text{RFA} > \text{FF} \) (in the skin) and \( \text{FA} \gg \text{RFA=FF} \) (in the eyes). The deduced phototoxic potential of FF and FA was in agreement with the phototoxic outcomes from \textit{in vitro/in vivo} photosafety tests, suggesting the reliability of the present photosafety prediction on FF and FA. From these findings, phototoxic events related to FF would be attributed to FA, which might be the reason for the discrepancy between the observed phototoxicity after oral administration and the negative results in the photopatch test of FF (Leenutaphong and Manuskiati, 1996).

Many drugs seemed to exhibit different PK behavior between humans and non-human primates, which may partly be due to species differences of metabolic enzymes, such as CYPs, esterases and glucuronidases (Baillie and Rettie, 2011). FF is metabolized into FA by CES 1A1 in the liver after absorption, and a portion of it undergoes carbonyl reduction by CYP 3A4 to produce RFA, and then these metabolites and their glucuronides are excreted (Cornu-Chagnon et al., 1995; Fukami et al., 2010; Miller and Spence, 1998; Weil et al., 1988; 1990). Thus, outcomes from PK assessment could not be completely extrapolated to humans as long as inter-species differences existed in the enzymes related to FF metabolism. According to a previous report, no significant differences were reported between human and rat hepatic CES 1A1 activity, and furthermore, plasma PK behavior of FA in humans was in agreement with PK data in rats obtained in the present study (Lovin et al., 2003; Taketani et al., 2007). Thus, FA might also exhibit high dermal exposure in humans as observed in rats. On the other hand, in humans, there appeared to be inter-individual variability in PK, efficacy and safety profiles of orally administered FF
owing to the effect of food intake (Davidson et al., 2005). Yun et al. demonstrated that the oral administration of FF with a high fat meal can cause significant increases of $C_{\text{max}}$ and $\text{AUC}_{0-\infty}$ of FA compared with those under fasted conditions in humans (Yun et al., 2006); hence, the effect of food intake, especially high-fat meals, might have a major impact on the photosafety of oral FF therapy since dermal exposure of FA might be increased when FF is orally taken with high-fat meals.

To avoid undesired phototoxic events, early identification of a hazard for metabolite-mediated phototoxicity would be of great help in drug discovery. In general, to evaluate a hazard for metabolite-related toxicity without identification of metabolites, several methods have been developed based on a combination strategy of general toxicity tests and in vitro metabolism studies (Ames et al., 1973; Geissler and Faustman, 1988). As for phototoxicity, the phototoxic hazard of metabolites might be evaluated based on transitions of ROS generation from light-irradiated compounds after treatment with metabolizing enzymes, as observed in the present study; however, singlet oxygen generation from some irradiated samples was negligible in the ROS assay with S9 fractions, whereas potent singlet oxygen generation from irradiated FF and FA was observed in the mROS assay. Changes of photochemical reactions in the ROS assay were attributed to assay conditions, including concentration of chemicals and additives (Onoue et al., 2008b; Onoue et al., 2013c; Onoue et al., 2013a). Although further optimization of assay conditions is needed, the ROS assay employing drug-metabolizing enzymes might become a useful method for hazard identification of metabolite-mediated phototoxicity.

In conclusion, the established photosafety screening on FF with major metabolites
could provide reliable photosafety information on FF. Currently, the ICH S10 guideline for photosafety evaluation is published, that recommend photochemical testing, \textit{in vitro} phototoxicity assay, \textit{PK} study and dedicated clinical study. To avoid false prediction of drug photosafety in humans, these proposed assessments should be applied to both parent substances and their major metabolites in pharmaceutical research and development, possibly resulting in successful development of pharmaceutical products with wide safety margins.
DMD # 65060

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Kato, Suzuki, Ohtake, and Onoue.

Conducted experiments: Kato, Suzuki, and Ohtake.

Contributed new reagents or analytic tools: Kato, Seto, and Onoue.

Performed data analysis: Kato and Onoue.

Wrote or contributed to the writing of the manuscript: Kato, Seto, and Onoue.
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FOOTNOTES

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Figure legends

Fig. 1  Major metabolic pathways of FF in rats, monkeys and humans. FF is metabolically transformed into FA and RFA (Fukami et al., 2010; Miller and Spence, 1998).

Fig. 2  Photochemical properties of FF and metabolites. (A) UV-absorption spectra of compounds (20 μM) in ethanol. *Solid line*, FF; *dashed line*, RFA; and *dotted line*, FA. (B) Generation of ROS from each test compound (200 μM). *Filled columns*, generation of singlet oxygen; and *open columns*, generation of superoxide. *P*<0.05, vs. FF within singlet oxygen; †*P*<0.05, vs. FA within singlet oxygen; ‡*P*<0.05, vs. FF within superoxide; and ‡‡*P*<0.05, vs. FA within superoxide. Data represent the mean±SD (n=3). (C) Generation of ROS from enzyme-treated FF. §*P*<0.05, vs. superoxide in denatured rat hepatic S9 fractions. Data represent the mean±SD (n=3).

Fig. 3  Concentration-time profiles of FF, FA and RFA after oral administration of FF (5 mg/kg) in rats. (A) plasma, (B) skin, and (C) eyes. ◇, FF; ○, FA; and □, RFA. Data represent the mean±SEM (n=4–7).

Fig. 4  *In vitro/in vivo* phototoxicity tests on QN, SB, FF and FA. 3T3 NRU phototoxicity testing on (A) positive/negative controls, (B) FF and FA. △/▲, QN; ▽/▼, SB; ◇/◆, FF; and ○/●, FA. *Open symbols*, UV-irradiated groups; and *filled symbols*, non-irradiated groups. Data represent the mean of duplicate measurements. (C)
Colorimetric evaluation (ΔE) of phototoxic skin responses in rats. *Open columns*, UV-irradiated groups; and *filled columns*, non-irradiated groups. *P < 0.05 vs. the non-irradiated group of each compound; and #P < 0.05 vs. SB within UV-irradiated groups.

Data represent the mean±SEM (n=4).
Table 1  PK parameters in plasma, skin and eyes after oral administration of FF in rats

<table>
<thead>
<tr>
<th></th>
<th>t1/2 (h)</th>
<th>C_{max} (µg/mL) or C_{max} (µg/g tissue)</th>
<th>T_{max} (h)</th>
<th>AUC_{0-∞} (h・µg/mL) or AUC_{0-∞} (h・µg/g tissue)</th>
<th>MRT (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF</td>
<td>Plasma</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>Skin</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>Eye</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>FA</td>
<td>Plasma</td>
<td>6.4±0.40</td>
<td>6.3±1.1</td>
<td>9.7±1.1</td>
<td>97±14</td>
</tr>
<tr>
<td></td>
<td>Skin</td>
<td>11±1.5</td>
<td>0.34±0.017</td>
<td>13±3.9</td>
<td>8.5±1.3</td>
</tr>
<tr>
<td></td>
<td>Eye</td>
<td>22±6.9</td>
<td>0.10±0.0055</td>
<td>6.0±1.4</td>
<td>2.6±0.33</td>
</tr>
<tr>
<td>RFA</td>
<td>Plasma</td>
<td>12±1.0</td>
<td>1.6±0.10</td>
<td>12±0.0</td>
<td>33±3.2</td>
</tr>
<tr>
<td></td>
<td>Skin</td>
<td>17±3.3</td>
<td>0.080±0.0067</td>
<td>16±4.7</td>
<td>2.0±0.60</td>
</tr>
<tr>
<td></td>
<td>Eye</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

AUC_{0-∞}, area under the concentration vs. time curve from t=0 to t=∞ after administration; C_{max}, maximum concentration; t_{1/2}, terminal half-life; T_{max}, time to maximum concentration; and MRT, mean residence time. Each value represents the mean±SEM for 4–7 rats.

N.A., not available due to concentrations below the limit of detection.
Table 2  Decision matrix for evaluating *in vivo* phototoxicity risk of FF and its metabolites

<table>
<thead>
<tr>
<th></th>
<th>FF</th>
<th>FA</th>
<th>RFA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Photochemical properties</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV absorbance $\lambda_{\text{max}}$ (nm) [$\varepsilon$ (M$^{-1}$cm$^{-1}$)]</td>
<td>290 [17,000]</td>
<td>295 [14,000]</td>
<td>290 [850]</td>
</tr>
<tr>
<td>ROS data $^{1}\text{O}<em>2$ ($\Delta A</em>{440\text{nm}} \times 10^3$)</td>
<td>463</td>
<td>531</td>
<td>61</td>
</tr>
<tr>
<td>$\text{O}<em>2^-$ ($\Delta A</em>{560\text{nm}} \times 10^3$)</td>
<td>171</td>
<td>332</td>
<td>123</td>
</tr>
<tr>
<td><strong>Distribution to UV-exposed tissues</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin $t_{1/2}$ (h)</td>
<td>N.A.</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µg/g tissue)</td>
<td>N.A.</td>
<td>0.34</td>
<td>80</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (h$\cdot$µg/g tissue)</td>
<td>N.A.</td>
<td>8.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Eyes $t_{1/2}$ (h)</td>
<td>N.A.</td>
<td>22</td>
<td>N.A.</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µg/g tissue)</td>
<td>N.A.</td>
<td>0.10</td>
<td>N.A.</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (h$\cdot$µg/g tissue)</td>
<td>N.A.</td>
<td>2.6</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

Each crucial factor was divided into three levels. Black, gray and white cells represent high, moderate and low levels, respectively.

N.A., not available due to concentrations below the limit of detection.
Fig. 1

Fenofibrate (FF) → CES1A1 → Fenofibric acid (FA) → CYP3A4 → Reduced fenofibric acid (RFA)
**Fig. 2**

(A) Absorbance vs. Wavelength (nm)

(B) Graph showing singlet oxygen (decrease of A440 nm) and superoxide anion (increase of A560 nm)

(C) Comparison of singlet oxygen and superoxide anion in active and denatured states in rat hepatic and intestinal S9 fractions.
Fig. 3
Fig. 4

(A) Log [concentration (mg/mL)] vs. Cell viability (% of untreated control)

(B) Log [concentration (mg/mL)] vs. Cell viability (% of untreated control)

(C) Skin color changes (ΔE) with different concentrations of FF, FA, QN, and SB.