Research Article

Combined Use of *In Vitro* Phototoxic Assessments and Cassette Dosing Pharmacokinetic Study for Phototoxicity Characterization of Fluoroquinolones

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Received 28 April 2011; accepted 23 June 2011; published online 8 July 2011

Abstract. The present study aimed to develop an effective screening strategy to predict *in vivo* phototoxicity of multiple compounds by combined use of *in vitro* phototoxicity assessments and cassette dosing pharmacokinetic (PK) studies. Photochemical properties of six fluoroquinolones (FQs) were evaluated by UV spectral and reactive oxygen species (ROS) assays, and phototoxic potentials of FQs were also assessed using 3T3 neutral red uptake phototoxicity test (3T3 NRU PT) and intercalator-based photogenotoxicity (IBP) assay. Cassette dosing pharmacokinetics on FQs was conducted for calculating PK parameters and dermal distribution. All the FQs exhibited potent UV/VIS absorption and ROS generation under light exposure, suggesting potent photosensitivity of FQs. *In vitro* phototoxic risks of some FQs were also elucidated by 3T3 NRU PT and IBP assay. Decision matrix for phototoxicity prediction was built upon these *in vitro* data, taken together with outcomes from cassette dosing PK studies. According to the decision matrix, most FQs were deduced to be phototoxic, although gatifloxacin was found to be less phototoxic. These findings were in agreement with clinical observations. Combined use of *in vitro* photobiochemical and cassette dosing PK data will be useful for predicting *in vivo* phototoxic potentials of drug candidates with high productivity and reliability.

KEY WORDS: cassette dosing pharmacokinetic study; fluoroquinolones; phototoxicity; reactive oxygen species; 3T3 neutral red uptake phototoxicity test.

INTRODUCTION

Drug-induced phototoxicity is elicited after exposure of skin to photoreactive pharmaceutical substances and is trig-

gered by exposure to sunlight (1,2). The phototoxicity can be categorized as photoirritation, photogenotoxicity, or photoallergy, and some drugs can cause all three types of phototoxicity (3). A number of efforts have been made to develop effective screening systems to evaluate photosensitive/phototoxic potential through analytical and biological methods, with the aim of predicting adverse effects in early phases of drug discovery processes (1,4,5). Previously, our group proposed *in vitro* assay systems to assess the phototoxic risk of newly synthesized drug candidates, including a reactive oxygen species (ROS) assay (6), a derivatives of reactive oxygen metabolites assay (7), a capillary gel electrophoresis-based photocleavage assay (8), and an intercalator-based photogenotoxicity (IBP) assay (9). In addition to these *in vitro* phototoxic assessment tools, combined use of photochemical/photobiological and pharmacokinetic (PK) data has also been proposed as a new screening strategy for predicting *in vivo* phototoxic risk (10).

In drug discovery, *in vivo* PK study is essential to evaluate the absorption, distribution, metabolism, excretion, and PK profiles of drug candidates. However, single-compound discrete PK studies are time and resource consuming because large numbers of drug candidates have to be examined. Additionally, a large number of animals have to be killed to obtain sufficient data of drug candidates in the discrete PK approaches. To reduce the number of animals killed and improve the throughput of *in vivo* PK experiments, a cassette dosing approach has been suggested (11) and applied to drug discovery (12,13). In cassette dosing PK study, cocktail administration to a single animal enables evaluation of the PK profiles of multiple compounds at the same time;

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ABBREVIATIONS: 3T3 NRU PT 3T3, Neutral red uptake phototoxicity test; ACN, Acetonitrile; ANOVA, Analysis of variance; AUC_{0→∞}, Area under concentration *versus* time curve; AUMC_{0→∞}, Area under moment curve; C_{max}, Maximum concentration; CPFX, Ciprofloxacin; DMEM, Dulbecco's modified Eagle's medium; dsDNA, Double-stranded DNA; EBSS, Earle's balanced salt solution; EMEA, European Medicines Agency; FDA, Food and Drug Administration; FQ, Fluoroquinolone; GFLX, Gatifloxacin; IBP, Intercalator-based photogenotoxicity; k_{el}, Elimination rate constant; K_p, Value tissue to plasma concentration ratio; LFLX, Lomefloxacin; LVFX, Levofloxacin; MRT, Mean residence time; NBT, Nitroblue tetrazolium; NaPB, Sodium phosphate buffer; NFLX, Norfloxacin; OECD, Organisation for Economic Co-operation and Development; PBS, Phosphate-buffered saline; PIF, Photoirritation factor; PK, Pharmacokinetic; RNO, *p*-Nitrosodimethylaniline; ROS, Reactive oxygen species; SPFX, Sparfloxacin; t_{1/2}, Elimination half-life; T_{max}, Time to reach maximum level; TO, Thiazole orange; UPLC/ESI-MS, Ultra-performance liquid chromatography equipped with electrospray ionization mass spectrometry; UV, Ultraviolet; VIS, Visible light.

however, the highly productive PK approach has never been applied to evaluate *in vivo* phototoxic risk of compounds. The purpose of this study was to propose a high-throughput effective screening strategy to evaluate and compare *in vivo* phototoxic risk of multiple compounds by the combined use of photobiochemical and cassette dosing PK data.

In the present study, six fluoroquinolones (FQs), including norfloxacin (NFLX), ciprofloxacin (CPFX), levofloxacin (LVFX), gatifloxacin (GFLX), lomefloxacin (LFLX), and sparfloxacin (SPFX), were used as a model chemical series (Fig. 1). FQs, antibacterials, are well recognized to be a phototoxic chemical series (14), and the structure-phototoxicity relationships of FQs have been reported (15,16); substitution groups at the eight position play a key role on the phototoxic potential of FQs. Thus, the tested FQs were divided into three groups based on the substituent at the eight position of FQs in the present study, namely, free (NFLX and CPFX), non-halogenated (LVFX and GFLX), and halogenated (LFLX and SPFX) groups. To clarify the *in vivo* phototoxic risk of these FQ series, the photobiochemical properties and PK profiles of the FQs were examined. Photochemical properties of the FQs were evaluated with a focus on ultraviolet (UV) absorption for photoactivation and ROS generation for photoreactivity. Phototoxic potentials were assessed by 3T3 neutral red uptake phototoxicity test (3T3 NRU PT) for photoirritation and IBP assay for photogenotoxicity. Cassette dosing PK analyses of the FQs were also carried out, and PK parameters and tissue distribution of FQs with a focus on the skin were estimated.

MATERIALS AND METHODS

Chemicals

NFLX, CPFX, GFLX, LFLX, salmon sperm DNA, plasmid pBR322 DNA, imidazole, *p*-nitrosodimethylaniline

(RNO), nitroblue tetrazolium (NBT), thiazole orange (TO), Tween 20, disodium hydrogen phosphate 12 water, and sodium dihydrogen phosphate dihydrate were obtained from Wako Pure Chemical Industries (Osaka, Japan). LVFX, SPFX, nalidixic acid, trypsin/EDTA solution, and neutral red were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (ACN) and methanol were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). DMEM, new-born calf serum, PBS without Ca^{2+} and Mg^{2+} , EBSS without phenol red, penicillin/streptomycin solution were purchased from Invitrogen (Carlsbad, CA, USA).

UV Spectral Analysis

FQs were dissolved in 20-mM sodium phosphate buffer (NaPB; pH 7.4) at a final concentration of 20 μM . UV-VIS absorption spectra were recorded with a HITACHI U-2010 spectrophotometer (HITACHI, Tokyo, Japan) interfaced to a PC for data processing (software: Spectra Manager). A spectrofluorimeter quartz cell with 10-mm pathlength was employed.

Irradiation Conditions

FQs were stored in an Atlas Suntest CPS+ solar simulator (Atlas Material Technology LLC, Chicago, USA) equipped with a xenon arc lamp (1,500 W). A UV special filter was installed to adapt the spectrum of the artificial light source to natural daylight. The irradiation tests were carried out at 25°C with an irradiance of 250 W/m^2 (290–800 nm).

In a 3T3 NRU PT, UV BIO-SUN illuminator (Vilbert-Lourmat, Marne-la-vallee, France) was employed. The irradiation test was carried out with an irradiance of ca. 50 W/m^2 (320–800 nm).

FQs	R ⁸	R ¹	R ⁵	R ^{3'}	R ^{4'}	R ^{5'}
<u>Free group</u>						
NFLX	—H	—C ₂ H ₅	—H	—H	—H	—H
CPFX	—H		—H	—H	—H	—H
<u>Non-halogenated group</u>						
LVFX			—H	—H	—CH ₃	—H
GFLX	—OCH ₃		—H	—CH ₃	—H	—H
<u>Halogenated group</u>						
LFLX	—F	—C ₂ H ₅	—H	—CH ₃	—H	—H
SPFX	—F		—NH ₂	—CH ₃	—H	—CH ₃

Fig. 1. Structures of tested FQs

Determination of ROS

Both singlet oxygen and superoxide generated from irradiated chemicals were measured as we reported previously (17,18). Briefly, to monitor the generation of singlet oxygen, samples containing the compounds under examination, RNO (50 μ M) and imidazole (50 μ M) in 20 mM NaPB (pH 7.4), were irradiated with UVA/B and visible light, and then the UV absorption at 440 nm was measured using SAFIRE (TECAN, Männedorf, Switzerland). For the determination of superoxide, samples containing the compounds under examination and NBT (50 μ M) in 20 mM NaPB (pH 7.4) were irradiated with UVA/B and visible light, and the reduction of NBT was measured by the increase in the absorbance at 560 nm using SAFIRE.

3T3 Neutral Red Uptake Phototoxicity Test

Balb/c 3T3 cells were maintained in culture for 24 h for formation of monolayers. Two 96-well plates per test chemical were then pre-incubated with eight different concentrations of the chemical for 1 h. One plate was then exposed to a dose of 5 J/cm² UVA (+Irr experiment) whereas the other plate was kept in the dark (−Irr experiment). The treatment medium was then replaced with culture medium and after 24 h, cell viability was determined by neutral red uptake for 3 h. Cell viability obtained with each of the eight concentrations of the test chemical was compared with that of the untreated controls, and the percent inhibition was calculated. For prediction of phototoxic potential, the concentration responses obtained in the presence and in the absence of UV irradiation were compared, usually at the IC₅₀ level, *i.e.*, the concentration inhibiting cell viability by 50% of untreated controls. The photoirritancy factor (PIF) was determined:

$$\text{PIF} = \frac{\text{IC}_{50}(-\text{Irr})}{\text{IC}_{50}(+\text{Irr})}$$

IBP Assay

The photodynamic impairment of salmon sperm DNA by FQs was evaluated by IBP assay as reported previously (9). Briefly, in the irradiated group, each assay mixture (50 μ L) in the 96-well microplate, containing the FQ (200 μ M) and DNA (20 μ g/mL) in 20 mM NaPB (pH 7.4), was irradiated with UVA/B and visible light for 45 min, and then TO was added to each well at a final concentration of 1.3 μ M. As a control experiment, only 40 μ L of the FQ in 20 mM NaPB (pH 7.4) was exposed to UVA/B and visible light, since photodegradants sometimes affect assay system. Then, DNA and TO were added to the sample at the same final concentration of irradiated experiments. In both irradiation and control experiments, each assay mixture (100 μ L) was incubated at 37°C for 15 min to equilibrate intercalation of DNA with TO. To detect the intercalated TO, fluorescence (excitation, 509 nm and emission, 527 nm) was measured with SAFIRE.

In Vivo Preparations

Male Sprague–Dawley rats at 9 weeks of age (ca. 250–350 g, body weight) were purchased from SLC Inc. (Hama-

matsu, Japan) and housed in the laboratory with free access to food and water, and maintained on a 12-h dark/light cycle in a room with controlled temperature (24±1°C) and humidity (55±5%). All the procedures used in the present study were conducted according to the guidelines approved by the Institutional Animal Care and Ethical Committee of the University of Shizuoka.

All FQs were dissolved in 0.1-M acetic acid/sodium acetic acid buffer (pH 4.8) containing 0.05% Tween 20. Rats were fasted for approximately 18 h before drug administration and received the cocktail solution containing all six FQs orally (5 mg/kg each).

Plasma Concentration of FQs After Oral Co-administration

Rats were anesthetized using pentobarbital (50 mg/kg) and then a guide cannula (PUC-40, EICOM Corp., Kyoto, Japan) was inserted into the jugular vein before the day when FQs was co-administered orally. Blood samples (approximately 150 μ L) were collected from the cannulated jugular vein at the indicated times (0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 9, and 12 h) after oral co-administration of FQs. Plasma obtained by centrifugation (10,000×g, 10 min, 4°C) was deproteinized by addition of ACN. The mixture was mixed for a few seconds and centrifuged (2,000 rpm, 1 min, 4°C). The supernatants were filtered and 50% ACN solution including nalidixic acid (5 μ g/mL), an internal standard, was added to them (supernatant/nalidixic acid=9:1) for Ultra-performance liquid chromatography (UPLC) analysis.

Tissue Deposition of FQs After Oral Co-administration

At the indicated times (skin: 0.5, 1, 1.5, 2, 4, and 6 h; other tissues: 0.5 and 1 h) after oral co-administration of FQs, rats were killed by taking blood from the descending aorta under temporary anesthesia with diethyl ether, and the tissues were then perfused with cold saline from the aorta. The skin, liver, kidney, lung, cerebrum, cerebellum, white adipose, brown adipose, and eyes were dissected, and then fat and blood vessels were removed by trimming. The tissues were minced with scissors and homogenized in a Physcotron (Microtech Co., Ltd., Chiba, Japan) in 2 mL of ACN. The homogenates were transferred into stoppered test tubes. The tubes, which were used for homogenizing, were added to 2 mL of ACN for washing, and then the suspensions were also transferred into the stoppered test tubes. After shaking for 5 min and sonication for 10 min, the mixtures were centrifuged (3,500 rpm, 10 min). Extraction was repeated two times with ACN and the supernatants were pooled. The extraction was also repeated with 7 mL of Milli-Q and the supernatants were percolated through Waters Oasis HLB cartridges, which were preconditioned with 2 mL of methanol and 2 mL of Milli-Q. After the cartridges had been washed with 2 mL of Milli-Q (two times), the FQs were eluted with 4 mL of ACN. The collected eluents were pooled with ACN-extracts and the samples were evaporated to dryness under a gentle stream of nitrogen at 45°C. The residues were dissolved in deproteinized plasma solution including nalidixic acid (500 ng/mL) as an internal standard for UPLC analysis. The tissue to plasma concentration ratio (K_p value) was

calculated as the ratio of the tissue concentration of unchanged drug to the plasma concentration.

UPLC Analysis

The concentrations of FQs in rat tissues and plasma were determined with ultra-performance liquid chromatography equipped with electrospray ionization mass spectrometry (UPLC/ESI-MS) analysis. 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 Acquity UPLC™ BEH C₁₈ (particle size, 1.7 μm and column size, Φ 2.1×50 mm; Waters) was used, and column temperature was maintained at 40°C. The standards and samples were separated using a gradient mobile phase consisting of Milli-Q containing 0.1% formic acid (A) and methanol (B). The gradient condition of the mobile phase was 0–0.5 min, 80% A; 0.5–4 min, 80–25% A (gradient curve 8); 4–5 min, 5% A; and 5–6 min, 80% A, and the flow rate was set at 0.25 mL/min.

Pharmacokinetic Analysis

Pharmacokinetic characterization in the plasma was performed by non-compartmental analysis as implemented in WinNonlin Professional Version 5.2 (Pharsight Corporation, Mountain View, CA, USA) and that in skin was carried out by non-compartmental analysis. The elimination rate constant (k_{el}) was estimated by least square method from the terminal phase. The elimination half-life ($t_{1/2}$) was calculated using the following equation.

$$t_{1/2} = \frac{\ln 2}{k_{el}}$$

Area under concentration *versus* time curve ($AUC_{0 \rightarrow \infty}$), area under moment curve ($AUMC_{0 \rightarrow \infty}$), and mean residence time (MRT) were estimated using a trapezoid formula from 0 h to the last measurement time (T), after which the last observed concentration (C_T) and $t_{1/2}$ were used as follows:

$$AUC_{0 \rightarrow \infty} = \int_0^T C dt + \frac{C_T}{k_{el}}$$

$$AUMC_{0 \rightarrow \infty} = \int_0^T t \cdot C dt + \frac{T \cdot C_T}{k_{el}} + \frac{C_T}{k_{el}^2}$$

$$MRT = \frac{AUMC_{0 \rightarrow \infty}}{AUC_{0 \rightarrow \infty}}$$

where C is the observed plasma or skin concentration (plasma, ng/mL and skin, ng/g skin) and t equals the measurement time (h).

Data Analysis

For statistical comparisons, one-way analysis of variance (ANOVA) with pairwise comparison by Fisher's least signifi-

cant difference procedure was used. A P value of less than 0.05 was considered significant for all analyses.

Standard error of each PK parameter in skin was defined by the delta method using the root of the following equation (19,20):

$$V(\overline{AUC_{0 \rightarrow \infty}}) = \sum_{i=2}^m [(V(C_i)/n_i + V(C_{i-1})/n_{i-1}) \times ((t_i - t_{i-1})/2)^2] + (1/k_{el})^2 \cdot V(C_T)/n_T + (C_T/k_{el})^2 \cdot V(k_{el})/n_{\lambda}$$

$$V(\overline{AUMC_{0 \rightarrow \infty}}) = \sum_{i=2}^m [(V(C_i)/n_i + V(C_{i-1})/n_{i-1}) \times ((t_i - t_{i-1})/2)^2] + (1/k_{el}^2)^2 \cdot V(C_T)/n_T + (C_T \cdot 2/k_{el}^3)^2 \cdot V(k_{el})/n_{\lambda} + (1/k_{el})^2 \cdot V(C_T)/n_T + (T \cdot C_T/k_{el}^2)^2 \cdot V(k_{el})/n_{\lambda}$$

$$V(\overline{MRT}) = \frac{V(\overline{AUMC_{0 \rightarrow \infty}})}{(AUC_{0 \rightarrow \infty})^2} + \frac{V(\overline{AUC_{0 \rightarrow \infty}}) \cdot (AUC_{0 \rightarrow \infty})^2}{(AUC_{0 \rightarrow \infty})^4}$$

where m is the number of collection points, C_i is equal to the skin concentration of FQs, t_i equals the collection time, and n_i represents the number of each collection time.

RESULTS

Photochemical Characterization of FQs

In the early stage of phototoxic events, photosensitive compounds initially have to absorb UVA/B radiation and then are transferred from ground state to excited state. According to the first law of photochemistry, measuring the UV-absorbing property of a chemical would be indicative of photoactivation. Then, the excited compounds attack biomolecules including proteins, lipids and DNA via direct or indirect processes, possibly leading to photoallergy, photoirritancy, and photogenotoxicity (6). In this study, the UV spectral patterns of the FQs were recorded in 20 mM NaPB (Fig. 2a). On the basis of the obtained UV spectral patterns, all the FQs exhibited strong absorption in the UVA/B range and only SPFX had a different absorption spectral pattern in the UV and visible light region. Their lowest energy bands in the UVA had maxima at 336 (NFLX), 335 (CPFX), 333 (LVFX), 334 (GFLX), 327 (LFLX), and 367 nm (SPFX) (Table I). The spectrum of solar radiation that reaches the surface of the earth is composed of visible light (400–700 nm), UVA (320–400 nm), and a part of UVB (290–320 nm) (21). On the basis of the present results and a previous report, all the FQs could be excited after absorption of photon energy from sunlight, and potentially causing photochemical reactions, possibly leading to phototoxic responses.

In the indirect phototoxic process, ROS are known as principal intermediate species, including singlet oxygen via type II photochemical reaction and superoxide via type I photochemical reaction. The ROS can cause phototoxic reactions, such as photoirritation, photogenotoxicity, and photoallergy, via oxidative reactions with biomolecules. Thus,

Fig. 2. Photochemical properties of FQs. UV-absorption spectra of FQs (20 μ M) in 20 mM NaPB (pH 7.4) (a). Thin solid line, NFLX; thick solid line, CPFX; thin dashed line, LVFX; thick dashed line, GFLX; thin dotted line, LFLX; and thick dotted line, SPFX. Generation of singlet oxygen (b) and superoxide (c) from irradiated FQs (200 μ M). The FQs were dissolved in 20 mM NaPB (pH 7.4) and then exposed to UVA/B light (250 W/m²). Empty squares, NFLX; empty diamonds, CPFX; empty triangles, LVFX; empty circles, GFLX; four-pointed stars, LFLX; and inverted empty triangles, SPFX. Data represent mean $\pm$ SD for three experiments

monitoring ROS generation from UV-exposed compounds may be useful to clarify the phototoxic potential of chemicals. In the present study, to clarify and compare the phototoxic potential of the FQs, the ROS assay on the FQs (200 μ M) was carried out (Fig. 2b, c). All the FQs generated both singlet oxygen and superoxide, and, in particular, LFLX and LVFX have potent ability to generate these ROS compared with the other FQs. The singlet oxygen-forming ability was ranked as

follows: LFLX $\gg$ CPFX $>$ NFLX $>$ LVFX $>$ SPFX $>$ GFLX, and the descending order of superoxide-producing capacity was as follows: LVFX $\gg$ LFLX $>$ NFLX $>$ CPFX $>$ GFLX $>$ SPFX (Table I). On the basis of the data obtained, LVFX and LFLX would be expected to induce potent phototoxic reactions after exposure to UV, whereas GFLX and SPFX would be expected to be less phototoxic.

Detailed Phototoxic Reactions of FQs

According to the results from ROS assay, most FQs were deduced to have phototoxic potential, and their detailed phototoxic reactions needed to be evaluated. Previously, a number of effective *in vitro* methodologies were developed to characterize the detailed phototoxic potential of chemicals (1,4,22), and our group also proposed several *in vitro* photogenotoxic assessment tools (8,9). In the present study, the photoirritant and photogenotoxic risks of FQs were also evaluated by 3T3 NRU PT and IBP assay, respectively.

3T3 NRU PT has been established as an alternative *in vitro* methodology to various *in vivo* phototoxic evaluations (23). The assay is the only photosafety test which has been recommended by OECD guideline and has been validated to international standards (24). The test can assess the cytotoxic effects of UVA-irradiated compounds on Balb/c 3T3 mouse fibroblast cell line by using a concentration-dependent reduction of the uptake of neutral red. In this investigation, the viability curves of FQs with or without irradiation were determined up to 500 μ g/mL. Figure 3 shows representative cell viability curves of the 3T3 cells after exposure to GFLX and SPFX. At the tested concentration of GFLX, cytotoxicity was not observed without irradiation, and GFLX at the higher concentration ($>$ ca. 25 μ g/mL) had slight cytotoxicity after exposure to UVA light. The EC₅₀ values of the GFLX-treated group with/without UVA irradiation were estimated to be 122.0 and 42.77 μ g/mL, respectively, and these values provided a PIF value of 2.85 for GFLX. With respect to the SPFX-treated group, the higher concentration of SPFX exhibited cytotoxicity without UVA irradiation. On the other hand, cytotoxicity occurred at a lower SPFX concentration after exposure to UVA light, resulting in the enhancement of SPFX-induced cytotoxicity. The EC₅₀ values of the SPFX-treated group with/without UVA irradiation were 221.5 and 7.772 μ g/mL, and the PIF value of SPFX was calculated to be 28.5. The PIF values of the tested FQs are described in Table I. In the OECD guidelines, classification criteria based on PIF values are defined as three groups, including phototoxic molecules (PIF $>$ 5), mildly or probably phototoxic molecules (2 $<$ PIF $<$ 5) and non-phototoxic molecules (PIF $<$ 2) (24). On the basis of the classification criteria, most FQs tested, except GFLX, were found to be phototoxic, and GFLX was evaluated as a mildly/probably phototoxic compound. The *in vitro* phototoxic potential of FQs was deduced as follows: LVFX $>$ LFLX $>$ SPFX $>$ CPFX $>$ NFLX $>$ GFLX.

The IBP assay was developed as a high-throughput screening tool for evaluating the photogenotoxic potential of chemicals (9). The assay evaluates the photodynamic impairment of dsDNA by phototoxins on the basis of the differences of fluorescence emission from DNA-fluorescent intercalating dye complexes between control group and irradiated group. In the present study, the IBP assay was carried out on FQs to

Table I. Photochemical and Phototoxic Data on FQs

FQs	UV absorption ^a	ROS generation ^b		3T3 NRU PT (PIF)	Decrease in intercalated TO ($\Delta\%$ of vehicle)*
	$\lambda_{\max}$ (nm) [$\epsilon \times 10^4$ ($M^{-1} \text{ cm}^{-1}$)]	$^1\text{O}_2$ ($\Delta A_{440} \times 10^3$)	O_2^- ($\Delta A_{560} \times 10^3$)		
NFLX	324 [1.5], 336 [1.4]	310 $\pm$ 10	85 $\pm$ 6	>6.0	15*
CPFX	324 [1.5], 335 [1.5]	338 $\pm$ 13	63 $\pm$ 7	>10.0	15*
LVFX	333 [1.3]	214 $\pm$ 17	657 $\pm$ 29	73.1	13*
GFLX	334 [1.4]	39 $\pm$ 4	36 $\pm$ 3	2.9	10*
LFLX	327 [1.5]	668 $\pm$ 7	95 $\pm$ 13	>28.8	21*
SPFX	367 [0.8]	91 $\pm$ 7	23 $\pm$ 3	28.5	13*

* $P < 0.05$, significant difference between the control and irradiated data of each FQ

^a The wavelengths of $\lambda_{\max}$ were selected longer than the lower limit of UVB (290 nm)

^b Data represent mean $\pm$ SD for three experiments

clarify the photogenotoxic potential (Fig. 4). Compared with the control group of vehicle, the fluorescence emission in control groups of NFLX and CPFX slightly decreased, which suggested that these photolyses intercalate into DNA. A decrease of fluorescence emission was also observed in the irradiated groups of NFLX and CPFX, and was higher than that in control groups; the differencing fluorescence might be indicative of photodynamic DNA damage by NFLX and CPFX. In contrast, LVFX, GFLX, LFLX, and SPFX did not affect the intercalating behavior of TO in the control group; however, the fluorescence emission in the irradiated group was decreased owing to DNA damage by irradiated LVFX, GFLX, LFLX, and SPFX. According to the data obtained, all the FQs would have photogenotoxic potential. Previously, some compounds were shown to induce severe photodynamic impairment of DNA in the agarose gel electrophoresis-based photogenotoxicity assay, and their values of difference for intercalated levels of TO between irradiated and control groups were estimated at over 15% of that of vehicle (9). On the basis of the present results, NFLX, CPFX and LFLX were found to have strong photogenotoxic potential, and the descending order of decrease in intercalated TO level was shown to be as follows: LFLX > CPFX $\approx$ NFLX > LVFX $\approx$ SPFX > GFLX (Table I). According to the ROS and IBP data, there was an empirical correlation between the generation of singlet oxygen and the reduction of intercalated

TO level. Previously, DNA comet assay (25) and DNA photocleaving test (26) also demonstrated photogenotoxic potential of some FQs. In the DNA comet assay, the photogenotoxic potential of LFLX was found to be much higher than that of CPFX (25). In addition, three FQs such as LFLX, CPFX and NFLX exhibited potent DNA photocleaving activity in plasmid pBR322 DNA, and the photogenotoxic potential was ranked as follows: LFLX > CPFX, NFLX (26). Thus, these previous findings were partly consistent with the results from the IBP assay on FQs. Some FQs, including enoxacin, NFLX, CPFX, LFLX, and SPFX, induce DNA damage after exposure to UV, the major mechanisms of which may involve generation of singlet oxygen, radical chain reaction, and formation of thymine cyclobutane dimers as described in previous reports (27,28). Considering the present findings and previous reports, irradiated FQ-induced DNA damage may mainly occur through type II photochemical reaction.

PK Assessments of FQs

Determination of the specific skin distribution of FQs would enable estimation of *in vivo* phototoxic risk; thus, PK profiling on FQs after oral administration was also carried

Fig. 3. Phototoxicity of representative FQs in the 3T3 NRU PT. The 3T3 cells were treated with different concentrations of GFLX or SPFX and irradiated with UVA light (50 kJ/m²). Data represent mean $\pm$ SD for two to six experiments. Filled circles, GFLX without UVA; empty circles, GFLX with UVA; inverted filled triangles, SPFX without UVA; and empty squares, SPFX with UVA

Fig. 4. DNA damage by irradiated FQs. Each drug (200 μ M) was dissolved in 20 mM NaPB (pH 7.4) with/without DNA and then exposed to UVA/B light (250 W/m²). TO solutions with/without DNA were added to the assay mixture, and fluorescence emitted from the TO-DNA complexes was measured. Open bars, UV irradiated; and filled bars, control group. Data represent mean $\pm$ SD of four experiments. * $P < 0.05$, significantly different from each control group

out. In the present study, cassette dosing PK analysis was used to improve the throughput of experiments and reduce the number of animals killed. After single cocktail oral administration of FQs (5 mg/kg), the concentration-time curves in the plasma and skin were obtained by UPLC/ESI-MS analysis (Fig. 5) and the PK parameters were calculated from the data obtained (Table II). With regard to the plasma concentration of FQs, all the FQs reached the maximum concentration ($C_{\max}$) around 0.5 h after oral co-administration and the calculated $C_{\max}$ of plasma FQs was ranked as follows: LVFX>LFLX>GFLX>SPFX>CPFX>NFLX. As for the skin deposition of FQs, the times to reach maximum

level ($T_{\max}$) of FQs in the skin were ca. 0.5 h (NFLX) and ca. 1 h (other five FQs), and all $T_{\max}$ of skin FQs lagged behind each $T_{\max}$ of plasma FQs. The descending order of $C_{\max}$ of skin FQs was as follows: LFLX>GFLX>LVFX>SPFX>CPFX>NFLX. On the basis of the $C_{\max}$ of FQs, LFLX indicated the highest plasma and skin concentrations among the tested FQs at the same dose (5 mg/kg), and LFLX would be more likely to cause phototoxic reactions in the skin than the other FQs. In contrast, NFLX and CPFX might cause less phototoxic reactions in terms of their lower $C_{\max}$. In addition to the $C_{\max}$ of the plasma and skin, the remaining potency of drugs would also be a key factor affecting the duration of

Fig. 5. Plasma and skin concentration-time profiles after oral co-administration of the FQs (5 mg/kg). **a** NFLX, **b** CPFX, **c** LVFX, **d** GFLX, **e** LFLX, and **f** SPFX. Open symbols, plasma and filled symbols, skin. Data represent mean $\pm$ SE for six (plasma) and six to eight (skin) rats

Table II. Pharmacokinetic Parameters of FQs in the Plasma and Skin of Rats After Oral Co-administration

FQs	$C_{\max}$ (ng/mL or ng/g skin)	$T_{\max}$ (h)	$t_{1/2}$ (h)	MRT (h)
Plasma				
NFLX	257±27	0.38±0.06	0.98±0.09	1.51±0.11
CPFEX	351±31	0.43±0.05	0.69±0.08	1.11±0.09
LVFX	1,270±52	0.46±0.04	1.23±0.05	3.01±0.20
GFLX	1,060±62	0.63±0.06	1.42±0.08	2.16±0.13
LFLX	1,150±66	0.58±0.05	1.48±0.03	2.88±0.07
SPFX	717±40	0.58±0.08	2.05±0.21	4.40±0.48
Skin				
NFLX	100±12	0.5	0.71±0.27	2.82±0.03
CPFEX	187±14	1	0.59±0.14	2.58±0.01
LVFX	1,040±62	1	0.90±0.14	1.725±0.003
GFLX	1,110±72	1	1.34±0.38	1.995±0.003
LFLX	1,280±110	1	1.28±0.37	1.850±0.002
SPFX	658±33	1	1.99±0.86	3.638±0.006

Each value represents mean±SE for six (plasma) and six to eight (skin) rats

exposure risk of compounds to skin, a key trigger of phototoxicity; therefore, $t_{1/2}$ and MRT of the plasma and the skin were calculated. The $t_{1/2}$ of FQs was ranked as follows: SPFX>LFLX>GFLX>LVFX>NFLX>CPFEX (in the plasma) and SPFX>GFLX>LFLX>LVFX>NFLX>CPFEX (in the skin); the order of MRT was as follows: SPFX>LVFX>LFLX>GFLX>NFLX>CPFEX (in the plasma) and SPFX>NFLX>CPFEX>GFLX>LFLX>LVFX (in the skin). On the basis of these two parameters, SPFX had long-term exposure risk for the skin, and there is a possibility that the phototoxic events of SPFX persist over a longer time than the other FQs. Additionally, to clarify the selectivity of distribution property of the FQs, the tissue distribution for FQs at the $T_{\max}$ of skin concentration after oral co-administration was examined (Fig. 6) and the K_p values of the skin were estimated and ranked as follows: GFLX>LFLX>LVFX>SPFX>CPFEX>NFLX. On the basis of the present results, skin and brown adipose are considered to be major sites for the distribution of the FQs, except for liver and kidney, during the early period after oral co-administration, and GFLX and LFLX are considered to be highly distributed in the skin.

DISCUSSION

In the present investigation, the combined use of photochemical and cassette dosing PK data on model FQs was shown to be an effective and productive screening strategy for evaluating *in vivo* phototoxic potential of the FQs. From the present photochemical and phototoxic data, most tested FQs, except GFLX, were found to have potent *in vitro* phototoxic risk whereas the phototoxic potential of GFLX was not too strong. The cassette dosing approaches could provide PK parameters and skin distribution properties of multiple FQs with high throughput. Focusing on the skin deposition of the FQs, LVFX, GFLX, and LFLX were highly distributed in the skin and SPFX had moderate and long-term exposure risk to

skin. In contrast, the skin deposition properties for the free group, including NFLX and CPFEX, demonstrated extremely low values.

Previously, a cassette dosing PK study was proposed for evaluating PK profiles of multiple compounds at the same time (11). Generally, multiple compounds are prepared at relatively low concentrations in the same solution, and the cocktail solution is administered to animals. Then, the blood and/or tissue concentrations of chemicals are determined by sensitive analytical methodologies, and PK parameters of chemicals are estimated on the basis of the data obtained. This approach has been applied to various administration routes, such as intravenous, intravitreal, and oral routes of administration, to aid drug discovery (12,29,30). Unlike a discrete PK study, in the cocktail dosing PK study, PK interactions on absorption, distribution, metabolism, and elimination might occur among tested chemicals. However, the cassette dosing approach also has attractive advantages such as high throughput and reduction of labor, animals killed, and other research resources (30). They might outweigh the disadvantages of cassette dosing approach at least for screening purpose since a large number of new drug candidates have to be examined in an early phase of drug discovery.

FQs, as a model chemical series, are clinically used as antimicrobial agents for treating various infections (31), the mechanism of which is inhibition of DNA gyrase, an essential bacterial enzyme, resulting in the suppression of DNA synthesis in microbes (32,33). In spite of the broad spectrum of antimicrobial activity, FQ-induced phototoxicity was also reported as an adverse effect in clinical trials (34). On the basis of this previous report, the halogenated group of FQs was shown to have severe *in vivo* phototoxicity, and the free group of FQs clinically exhibits mild photosensitivity. Non-halogenated FQs have less *in vivo* phototoxic potential than other types of FQs. However, the skin photosensitivity of GFLX was not observed in a double-blind, placebo- and positive-controlled study (35). In addition to the previous clinical trials, structure–activity and structure–side-effect relationships for FQs were examined in *in vitro* and *in vivo* phototoxic studies (36,37), and, in particular, the substituent groups at the eight position of FQs were reported to play a key role in phototoxicity of FQs on the basis of their structure–phototoxicity relationships (15,16). A halogen atom at the eight position exhibited potent phototoxic potential whereas a methoxy group at the eight position had less phototoxic potential. Thus, the order for phototoxic potential of FQ subseries is demonstrated as follows: halogenated FQs>free FQs>non-halogenated FQs.

In the present study, various photobiochemical data on FQs were initially obtained using *in vitro* phototoxic assessment tools, and the PK profiles of FQs were also estimated using the data from cassette dosing approaches. To integrate these data for *in vivo* phototoxic evaluation of the FQs, a decision matrix was built upon several experimental outcomes (Table III). The decision matrix is a summarized schematic model of qualitative or quantitative values, and it helps us to systematically identify, analyze, and evaluate the complicated sets of information. There are two crucial factors in the decision matrix, such as *in vitro* photobiochemical and

Fig. 6. Tissue distribution of FQs at the T_{max} of the skin after oral co-administration of the FQs (5 mg/kg). The mean of K_p values (mL/g tissue) is indicated in/near each column. **a** NFLX, **b** CPF, **c** LVFX, **d** GFLX, **e** LFLX, and **f** SPFX. Each column represents mean $\pm$ SE for eight rats. * $P < 0.05$, significantly different from each skin concentration. [#] $P < 0.05$, significantly different from each K_p value of the skin

PK behaviors. When either of the two is at a low level, the tested chemical can be identified as mildly or less phototoxic. Of all PK parameters calculated, $t_{1/2}$ and MRT values in the skin should be of great importance since they were indicative of exposure period in the skin. On the basis of the phototoxic evaluation using the decision matrix, LFLX was deduced to have the most potent phototoxic risk because of its sensitive photoreactivity and high level in the skin, the mechanisms of which might include both photoirritation and photogenotoxicity mainly via type II photochemical reaction. LVFX would also have a potent phototoxic risk, especially photoirritancy, since both photoreacting and skin distribution properties were similar to those of LFLX except that LVFX had less

photogenotoxic risk than LFLX. Compared with the present results on LFLX and LVFX, those on SPFX were indicative of moderate *in vitro* phototoxic risk and skin deposition; therefore, SPFX would also have phototoxic risk. Notably, the $t_{1/2}$ and MRT values for SPFX demonstrated the long-term exposure risk in the skin, and *in vivo* phototoxic risk of SPFX might persist longer. The PK profiles of GFLX were quite similar to those of LFLX; however, weak phototoxic behavior in the *in vitro* screenings suggested that GFLX might have limited phototoxic potential. From these findings, halogenated FQs tend to show phototoxic risk, although phototoxicity of non-halogenated FQ seemed to be variable depending on its chemical structure and *in vitro* photo-

Table III. Decision Matrix

		NFLX	CPFX	LVFX	GFLX	LFLX	SPFX
ROS generation	$^1\text{O}_2$ ($\Delta A_{440} \times 10^3$)	310	338	214	39	668	91
	O_2^- ($\Delta A_{440} \times 10^3$)	85	63	657	36	95	23
Phototoxic reactions	3T3 NRU PT (PIF)	>6.0	>10.0	73.1	2.9	28.8	28.5
	Decrease of intercalated TO ($\Delta\%$ of vehicle)	15	15	13	10	21	13
PK profiles of skin	$C_{\max}$ (ng/g skin)	100	187	1,040	1,110	1,280	658
	K_p values (mL/g skin)	0.43	0.73	1.19	1.77	1.70	1.18
	$t_{1/2}$ (h)	0.71	0.59	0.90	1.34	1.28	1.99
	MRT (h)	2.82	2.58	1.72	1.99	1.85	3.64

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

reactivity. With respect to the free group of FQs, such as NFLX and CPFX, even if the free group had potent *in vitro* photoreactivity, it should have mild phototoxicity due to the lower migration to the skin than that of other groups at the present dosage (5 mg/kg). Overall, on the basis of the decision matrix, the order for the *in vivo* phototoxic risk of FQs was deduced as follows: LFLX>LVFX>SPFX>NFLX=CPFX>>GFLX. In previous clinical reports, most tested FQs, including NFLX, CPFX, LVFX, LFLX, and SPFX, were reported to be phototoxic in clinical trials and the order for phototoxic potential was summarized as follows: LFLX>SPFX>CPFX>NFLX=LVFX (34). As observed in the present study, cutaneous phototoxicity of GFLX was negligible in a clinical trial (35). The outcomes from the decision matrix approach were likely in agreement with the previous observations in clinical trials. From these findings, *in vivo* phototoxic risk of FQs could be estimated by combined use of *in vitro* photobiochemical and PK data.

For evaluating the phototoxic risk of new drug candidates, the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) established guidance on the photosafety testing of medical products, including phototoxicity (photoirritation), photogenotoxicity, photoallergy, and photocarcinogenicity tests (38–40). Both EMA guidance and FDA guidance have similarly described test chemicals, which absorb light ranging from 290 to 700 nm (UVA/B and VIS) and are applied directly to the skin and/or eyes or are distributed in these sites after systemic administration; they also recommend 3T3 NRU PT as an *in vitro* phototoxicity test. 3T3 NRU PT was indicated by OECD guideline 432 in 2004 (24), and the test has become a general *in vitro* methodology for evaluating phototoxicity. Although the 3T3 NRU test can strongly detect the phototoxic potential

of test chemicals, some positive chemicals in this test did not induce phototoxicity in *in vivo* phototoxicity tests in animals and/or human clinical photosafety studies (41). The overestimation of *in vitro* assessments such as 3T3 NRU PT might occasionally cause termination of development. Furthermore, the other contents of photosafety testing are markedly different between EMA guidance and FDA guidance, and, in particular, EMA guidance has no requirement of *in vivo* phototoxicity tests whether drug candidates have been determined as positive or negative in *in vitro* approaches. The assessment of photosafety for new drug candidates has not been completely elucidated yet, and more effective screening strategies for evaluating *in vivo* phototoxic risk are required. In the present study, the combined use of photobiochemical and PK data was proposed as one of the screening strategies for evaluating *in vivo* phototoxic risk of compounds, and the dermal PK data play a key role for better understanding of *in vivo* phototoxic potential.

In conclusion, *in vivo* phototoxicity of FQs could be evaluated by the combined use of photobiochemical and cassette dosing PK data. Additionally, the cassette dosing approaches would contribute to the improvement of throughput in PK analyses and save various resources. The present screening system would be an effective and high-throughput strategy for evaluating the *in vivo* phototoxic risk of new drug entities in an early stage of drug discovery.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid from the Food Safety Commission, Japan (no. 0807) and a Health Labour Sciences Research Grant from The Ministry of Health, Labour and Welfare, Japan.

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