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Research Paper

Analytical Studies on the Prediction of Photosensitive/Phototoxic Potential of Pharmaceutical Substances

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Purpose. Phototoxic responses after administration of photosensitive pharmaceutics have been recognized as undesirable side effects, and predicting potential hazardous side effects is gaining importance as new drugs are introduced to the market. In this work, we characterize the photochemical/photobiological properties of model compounds to develop an effective screening method for the prediction of phototoxic/photosensitive potential.
Methods. Twenty-one known photosensitive/phototoxic compounds and five weak/nonphototoxic compounds were subjected to ultraviolet (UV) spectral analyses and photochemical evaluation including the determination of produced reactive oxygen species (ROS) and photostability study. The photooxidation of linoleic acid was also monitored in the presence of tested compounds, guided on the formation of thiobarbituric acid reactive substances.
Results. Most photosensitive/phototoxic drugs tested, even weak UV absorbers, at a concentration of 200 μM showed significant production of ROS under 18 h light exposure (30,000 lx). On the other hand, ROS generated from weak/nonphototoxic compounds, including strong UV absorber benzocaine, were low or negligible. Although exposure of quinine to light resulted in significant degradation (half-life, $t_{1/2}$ = 6.4 h), it was dramatically attenuated by the addition of ROS scavengers, especially sodium azide ($t_{1/2}$ = 122.6 h). Furthermore, concomitant exposure of photosensitive/phototoxic compounds (200 μM) and linoleic acid (1 mM) for 18 h led to the marked formation of lipoperoxide.
Conclusion. Results indicated that known photosensitive/phototoxic compounds tested have the ability to generate ROS under light exposure, and this photochemical reaction could be associated with their photoinstability and/or phototoxic responses. Based on these findings, determination of ROS, generated from photoirradiated compounds, may be an effective predictive model in recognizing their photosensitive/phototoxic potential.

KEY WORDS: lipid peroxidation; phototoxicity; reactive oxygen species; singlet oxygen; superoxide.

INTRODUCTION

Photosensitivity is a broad term used to describe unwanted phototoxic reactions of pharmaceutics, pigments, and food additives to nonionizing radiation (1). Phototoxic response to systemically or topically administered drugs requires exposure to the appropriate wavelength of light, especially UVA (320–400 nm) and UVB (290–320 nm) radiation, and the presence of drug in the skin. Several classes of drugs exhibit this type of side effect, including antibacterials (2,3), thiazide diuretics (4), nonsteroidal anti-inflammatory drugs (NSAIDs) (5), quinolones (6), and tricyclic antidepressants (7,8). Recently, the level of interest has markedly increased because of the awareness among the scientific community of the increase in the UV portion of the solar spectrum reaching the earth.

In phototoxic reactions, the drug absorbs energy from UVA/UVB light and releases it into the skin. Their ability to induce phototoxic response is believed to occur either by formation of photoadducts with biomolecules or through generation of reactive oxygen species (ROS), including singlet oxygen ($^1O_2$) and superoxide (O_2^-), which selectively modify various oxidative reaction mechanisms. Unsaturated membrane lipids in mammalian cells, including glycolipids, phospholipids, and cholesterol, are well-known targets of damaging and potentially lethal peroxidative modification. In addition to lipid peroxidation, it is well established that ROS react with nuclear DNA, resulting in the photodynamic breaking of the DNA strand (9). The DNA breakage was found to be facilitated by a noncovalent drug–DNA interaction, induced by both electron and energy transfer to DNA, as recently suggested for some NSAIDs (10). In

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ABBREVIATIONS: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; GSH, gluthatione; MDA, malondialdehyde; NBT, nitroblue tetrazolium; NSAIDs, nonsteroidal anti-inflammatory drugs; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substance; UV, ultraviolet.

this context, the primary event in any photosensitization process is the absorption of a photon, and the following free radical and singlet oxygen generation by photoexcited drug molecules may seem to be the principal intermediate species in the phototoxic response.

Screening for phototoxicity is necessary at the early phase of the drug discovery process and even before the introduction of drugs and chemicals into clinical therapy, which may help in preventing undesirable drug reactions in humans. Therefore, this investigation is aimed to design a model system for the assessment of photosensitive/phototoxic potential through analytical and biochemical methods. We evaluated the generation of singlet oxygen and superoxide, by colorimetric determination, upon exposure of some representative phototoxic/nonphototoxic compounds to light. We also investigated the photodynamic peroxidation of linoleic acid as a model of phototoxic injury and the photodegradation of quinine, a typical photosensitizer, for further clarification of the role of ROS in the photochemical/phototoxic response.

MATERIALS AND METHODS

Chemicals

All photosensitive and phototoxic compounds, including chlorpromazine, naproxen, ketoprofen, norfloxacin, nalidixic acid, indomethacin, ibuprofen, furosemide, benzoyl peroxide, amiodarone, quinine, oxytetracycline, diclofenac sodium, sulfamethoxazole, retinol, 8-methoxy psoralen, tamoxifen, omeprazole, carbamazepine, nitrendipine, 5-fluorouracil, benzocaine, phenytoin, aspirin, erythromycin, and sodium dodecyl sulfate (SDS), and several ROS scavengers, such as butylated hydroxyanisole (BHA), reduced gluthatione (GSH), sodium azide (NaN_3), and superoxide dismutase (SOD), were purchased from Sigma (St. Louis, MO, USA), Wako Pure Chemical Industries (Osaka, Japan), or Funakoshi (Tokyo, Japan). Linoleic acid, thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), *p*-nitrosodimethylaniline, imidazole, nitroblue tetrazolium (NBT), 1,1,3,3-tetraethoxypropane, and Tween 20 were obtained from Wako Pure Chemical Industries.

UV Spectral Analysis

All tested compounds were dissolved in 20 mM sodium phosphate buffer (NaPB, pH 7.4) at the final concentration of 10–50 μM. Ultraviolet–visible (UV–Vis) absorption spectra were recorded with a JASCO V-560 double-beam spectrophotometer (JASCO, Tokyo, Japan) interfaced to a PC for data processing (software: Spectra Manager). Spectrofluorimeter quartz cells with 10 mm pathlength were employed.

Irradiation Conditions

Each tested compound was stored in a light-irradiation tester Light-Tron Xenon (LTX-01; Nagano Science, Osaka, Japan) equipped with a xenon lamp. The spectral output of the lamps through the optical filter 310 and infrared cutting filter (Nagano Science) was 310–800, with a maximum at 470 nm. Illuminance was set at 30,000 lx, and the irradiation test was carried out at 25°C. Illuminance (30,000 lx) was checked on UVR-2 radiometer (Topcon, Tokyo, Japan) for each experimental procedure.

Determination of Reactive Oxygen Species

Singlet Oxygen

Singlet oxygen was determined following the procedure described by Kraljic and El Moshni (11), and measured in an aqueous solution by spectrophotometrically monitoring the bleaching of RNO at 440 nm using imidazole as a selective acceptor of singlet oxygen. Samples containing the compounds under examination (1–400 μM), *p*-nitrosodimethylaniline (50 μM) and imidazole (50 μM) in 20 mM NaPB (pH 7.4), were irradiated with UVA/UVB for different periods in 5-mL glass vial.

Superoxide Anion

Superoxide anion was determined according to procedure described by Pathak and Joshi (12). Samples containing the compounds under examination (1–400 μM) and nitroblue tetrazolium (NBT, 200 μM) in 20 mM NaPB were irradiated for indicated periods, and the reduction of NBT was measured by the increase in their absorbance at 560 nm. The same experiments were performed in the presence of SOD to clarify the involvement of superoxide in the NBT reduction.

Photostability Testing

Solutions of quinine (0.5 mg/mL) dissolved in 20 mM phosphate buffer (pH 7.4) in 5-mL glass vial were exposed to UVB–UVA irradiations. At selected times, the solutions were directly subjected to high-performance liquid chromatography (HPLC) analysis to monitor the photodegradation of quinine. The HPLC system consisted of Waters Alliance system (Waters, Milford, MA, USA), Inertsil ODS-3 (particle: size 3 μM, column size: ϕ4.6 $\times$ 250 mm; GL Sciences, Tokyo, Japan), and photodiode array detector (model 996; Waters) with the detection wavelength of 210 nm for quinine. The mobile phase consisted of 0.35% perchloric acid/acetonitrile 78:22 (v/v) with a flow rate of 1.0 mL/min.

Photosensitized Peroxidation of Linoleic Acid

Linoleic acid (1 mM), suspended in 20 mM NaPB (pH 7.4) containing 0.05% Tween 20, was irradiated in the presence of tested compound (200 μM), and lipid peroxidation was measured using a TBA assay as described previously (13). To the irradiated sample (500 μL), 0.67% TBA dissolved in 20 mM NaPB (pH 7.4, 1 mL) and 10 μL of 1.0% BHT solution in glacial acetic acid were added, and the mixture was heated at 95°C for 30 min. The mixture was extracted with 1.0 mL of 1-butanol, and absorbance of the extract was measured at 532 nm for the determination of TBA reactive substances (TBARS). A standard curve of 1,1,3,3-tetraethoxypropane was used to quantitate the amount of the produced malonaldehyde.

Data Analysis

For statistical comparisons, a one-way analysis of variance (ANOVA) with the pairwise comparison by Fisher's least significant difference procedure was used. A p value of less than 0.05 was considered significant for all analyses.

RESULTS

Photosensitizers and their UV Spectral Pattern

In this study, 21 photosensitizing drugs, including diuretic agents (4), NSAIDs (5,14), antipsychotic drugs (15), antimicrobials (16–19), antimalarials (20), cardiovascular drugs (21,22), anticonvulsants (23), proton pump inhibitors (24), psoralens (25), and anticancer drug (26), and five weak/nonphototoxic compounds (21,27,28) were selected as model compounds to examine and compare their photosensitive/phototoxic responses upon exposure to light. The UV absorption spectra of tested compounds were recorded in 20 mM NaPB, and the wavelength and absorbance of the long-wave peak were noted (Table I). According to Jagger's report (29), solar radiation reaches the surface of the earth after passage through the atmosphere, where the higher energy part is absorbed, mainly by nitrogen, oxygen, carbon dioxide, and ozone, resulting in the cutoff at a wavelength just below 300 nm. Here, the spectrum of solar radiation is composed of UVA, UVB, and visible light. Almost all tested photosensitizers showed the significant absorption of UVA/UVB, suggesting that they may absorb photon energy and may be excited under exposure to sunlight. In contrast, UVA/UVB absorption of weak or nonphototoxic compounds was extremely low, except for benzocaine.

The absorption spectra of quinine (an antimalarial drug), furosemide (a diuretic drug), tamoxifen (an antibreast cancer drug), and SDS (a control as nonphotosensitive compound) are shown in Fig. 1. These photosensitive drugs showed strong absorption in the UVA/UVB range, and the their lowest energy bands have maxima at 331 (quinine), 330.5 (furosemide), and 276 nm (tamoxifen). At this wavelength range, the spectral patterns of quinine and furosemide were similar and much higher than that of tamoxifen.

Table I. Photochemical Profile of Tested Compounds

Compounds	UVA/UVB absorption	Generation of ROS		Photochemical /toxicological reports
	λ_{max} (nm) (ε)[a]	$^1O_2$ (%)[b]	O_2^- (%)[c]	
Photosensitive/phototoxic compounds				
Chlorpromazine	307 (3831)	N.D.	21.2 ± 1.3	Photodermatitis,[d] Phototoxic (15)
Naproxen	318 (1460), 331 (1744)	18.4 ± 1.6	40.9 ± 2.6	Photodermatitis,[d] Phototoxic (5)
Ketoprofen	−[290 (4960)]	29.8 ± 1.2	12.7 ± 1.4	Photodermatitis,[d] Phototoxic (5)
Norfloxacin	324 (13,032)	21.5 ± 1.6	35.1 ± 1.0	Photodermatitis,[d] Phototoxic (16)
Nalidixic acid	336 (7831)	14.5 ± 1.0	31.3 ± 1.2	Photodermatitis,[d] Phototoxic (16)
Indomethacin	320 (6969)	N.D.	35.9 ± 3.1	Phototoxic (14)
Ibuprofen	−[290 (71)]	0.6 ± 0.3	60.3 ± 2.7	Phototoxic (5)
Furosemide	331 (4978)	8.0 ± 1.6	30.2 ± 2.6	Photodermatitis,[d] Phototoxic (4)
Benzoyl peroxide	−[290 (675)]	19.9 ± 1.2	5.6 ± 1.5	Phototoxic (19)
Amiodarone	303 (6209)	4.1 ± 0.6	19.9 ± 1.5	Photodermatitis,[d] Phototoxic (21)
Quinine	331 (4451)	43.5 ± 1.8	36.5 ± 2.5	Phototoxic (20)
Oxytetracycline	363 (13,518)	46.5 ± 2.4	96.1 ± 5.3	Phototoxic (17)
Diclofenac	−[290 (7753)]	16.9 ± 1.4	56.2 ± 1.0	Photodermatitis,[d] Phototoxic (5)
Sulfamethoxazole	−[290 (2620)]	N.D.	4.3 ± 0.5	Photodermatitis,[d] Phototoxic (18)
Retinol	370 (2342)	9.4 ± 0.5	10.5 ± 0.3	Phototoxic (21)
8-Methoxy psoralen	303 (11,818)	11.8 ± 0.3	28.3 ± 1.7	Photodermatitis,[d] Phototoxic (25)
Tamoxifen	−[290 (3619)]	N.D.	14.9 ± 1.2	Photosensitive[e]
Omeprazole	301 (15,158)	N.D.	31.8 ± 1.6	Photodermatitis,[d] Photosensitive (24)
Carbamazepine	−[290 (10,265)]	2.8 ± 0.4	7.0 ± 1.1	Photodermatitis,[d] Phototoxic (23)
Nitrendipine	358 (5689)	0.6 ± 0.2	9.5 ± 0.6	Photodermatitis,[d] Photosensitive (22)
5-Fluorouracil	−[290 (1837)]	N.D.	N.D.	Photodermatitis,[d] Photogenotoxic (26)
Weak/nonphototoxic compounds				
Benzocaine	−[290 (17,025)]	N.D.	0.6 ± 0.1	Weak or nonphototoxic (21)
Phenytoin	−[290 (6)]	N.D.	0.3 ± 0.1	Weak or nonphototoxic
Aspirin	−[290 (193)]	N.D.	N.D.	Nonphototoxic (27)
Erythromycin	−[290 (9)]	N.D.	N.D.	Nonphototoxic (27)
Sodium dodecyl sulfate	−[290 (312)]	N.D.	N.D.	Nonphototoxic (28)

[a] Measured in 20 mM NaPB (pH 7.4). If the peak and shoulder wavelengths are shorter than the lower limit of UVB (290 nm), the absorbance at 290 nm is noted in brackets.
[b] Percent of RNO bleaching by each compound (200 μM) under light exposure (30,000 lx) for 18 h.
[c] Percent of NBT reduction by each compound (200 μM) under light exposure (30,000 lx) for 18 h.
[d] Adverse drug reaction reporting, noted in the drug package insert.
[e] Noted in the interview form.

Photochemical Reactions of Photosensitizers

The generation of singlet oxygen was detected by spectrophotometric measurement of *p*-nitroso-dimethylaniline (RNO) bleaching, induced by imidazole as a singlet oxygen-specific substrate (11). In Fig. 2A, only four compounds (quinine, furosemide, tamoxifen, and SDS) are presented for the sake of clarity, and data show the kinetics of RNO bleaching after irradiation in the presence of photosensitizing drugs. Tamoxifen and SDS were not able to generate singlet oxygen to significant levels, and the order of singlet oxygen-forming ability was as follows: quinine > furosemide > tamoxifen ≒ SDS. Generation of singlet oxygen from irradiated quinine seems to be concentration-dependent, and quinine, kept in dark, did not show any RNO bleaching (Fig. 2B).

In addition to singlet oxygen, superoxide anion, generated from photoirradiated photosensitizers, was also measured by the reduction of NBT. The quinine solution at the several concentrations was exposed to UVA/UVB light for 18 h, and the absorbance of diformazan at 560 nm increased in a concentration-dependent manner (Fig. 3B). In contrast, the quinine-induced reduction of NBT was negligible without light exposure. To examine whether NBT was reduced by superoxide, SOD was added to the sample solution. SOD inhibited the reduction of NBT almost completely, indicating that NBT was mainly reduced by superoxide (data not shown). All tested compounds, except for SDS, produced superoxide as evidenced by NBT reduction (Fig. 3A), and the order of superoxide forming ability was as follows: quinine > furosemide > tamoxifen > SDS.

The capacity of the test compounds in an aqueous solution at the concentration of 200 μM to generate superoxide is shown in Table I. All known phototoxic/photosensitive compounds, except for 5-fluorouracil, indicated the ability to generate singlet oxygen, superoxide, or both, whereas weak/nonphototoxic compounds did not. Although there are so many UVA/UVB absorbers in the list of known phototoxic compounds, the ROS-forming abilities of these compounds did not correlate directly with the order of their UV absorption.

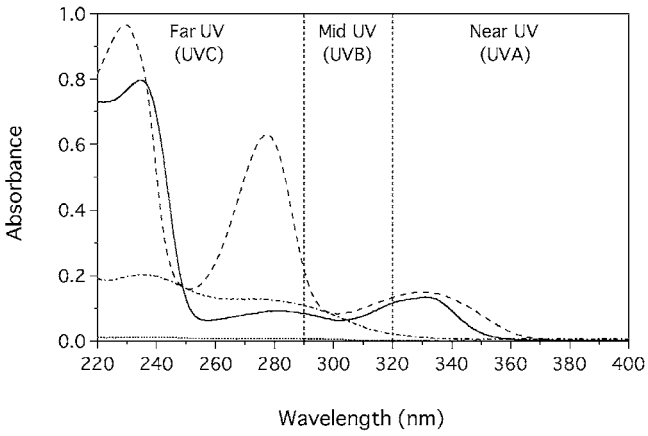

Fig. 1. Average intensity of sunlight at the earth's surface and UV absorption spectra of quinine, furosemide, tamoxifen, and SDS (30 μM) in 20 mM NaPB (pH 7.4). Quinine, solid line; furosemide, dashed line; tamoxifen, chain line; SDS, dotted line. Average intensity of sunlight (shaded) was reproduced from a previous report (29).

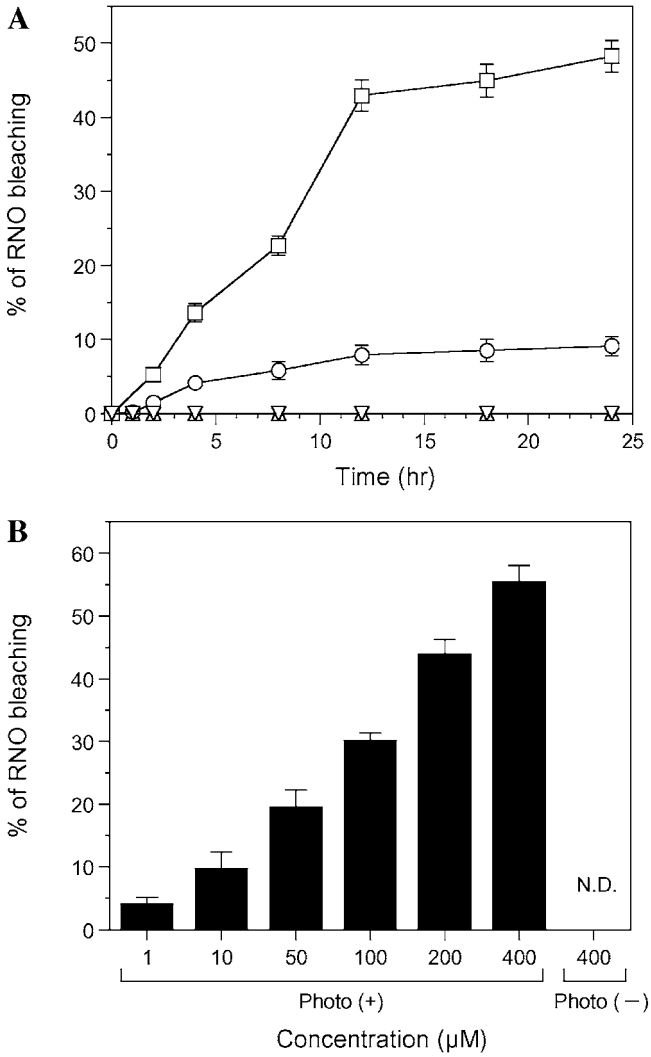

Fig. 2. Generation of singlet oxygen from photoirradiated photosensitizers. (A) Time course of singlet oxygen generation from photoirradiated compounds. Each tested compound (200 μM) was dissolved in 20 mM NaPB (pH 7.4), and exposed to UVA/UVB (30,000 lx) for indicated periods. □, quinine; ○, furosemide; △, tamoxifen; ▽, SDS (control). (B) Concentration-dependent generation of singlet oxygen from photoirradiated quinine. Quinine was dissolved in 20 mM NaPB (pH 7.4) at the indicated concentrations, and then exposed to UVA/UVB (30,000 lx) for 18 h. Data represent mean ± SD of four experiments.

ROS-Mediated Photodegradation of Quinine

Quinine (0.5 mg/mL) in 20 mM NaPB at pH 7.4 was exposed to UVA/UVB radiations from a solar simulator (xenon arc lamp, 30,000 lx) at 25°C, and a comparison of the resulting chromatograms shows the time-dependent photodegradation process (Fig. 4A). Although quinine, kept in the dark at 25°C, did not show any degradation in HPLC analysis, light exposure of quinine for 20 h resulted in complete decomposition, generating many unidentified photodegradants (Fig. 4B). A linear relationship was obtained according to the following equation: $\ln A = \ln A_0 - kt$ (apparent first-order kinetics; $r = 0.985$), where A is the remaining peak area, k is the slope, and t is time (h). The

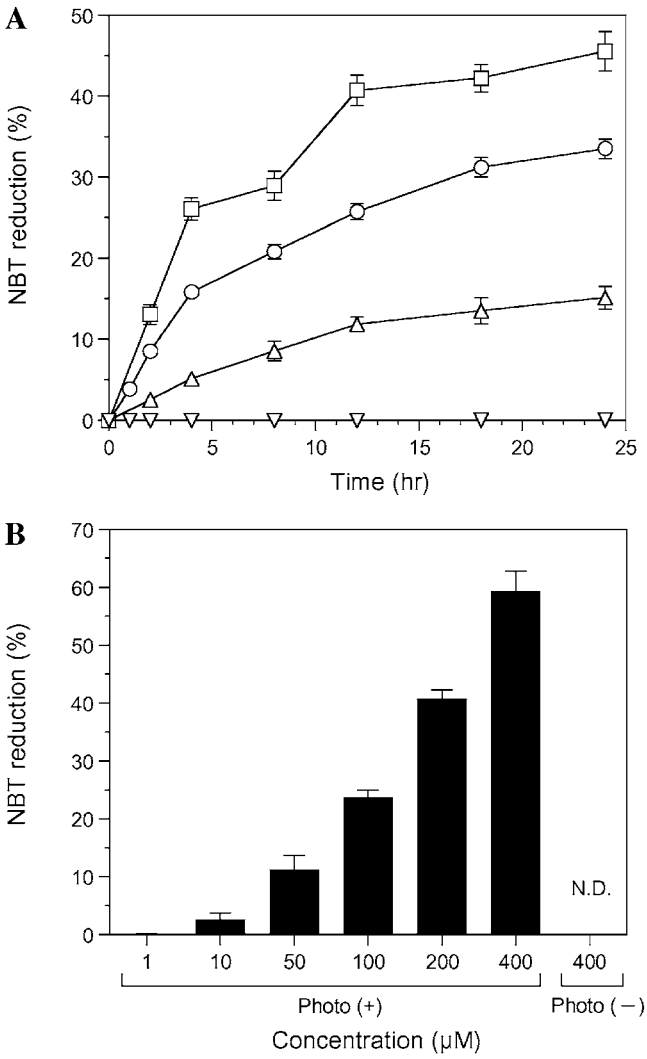

Fig. 3. Generation of superoxide from photoirradiated photosensitizers. (A) Time course of superoxide generation from photoirradiated compounds. Each tested compound (200 μM) was dissolved in 20 mM NaPB (pH 7.4), and exposed to UVA/UVB (30,000 lx) for indicated periods. □, quinine; ○, furosemide; △, tamoxifen; ▽, SDS (control). (B) Concentration-dependent generation of superoxide from photoirradiated quinine. Quinine was dissolved in 20 mM NaPB (pH 7.4) at the indicated concentrations, and then exposed to UVA/UVB (30,000 lx) for 18 h. Data represent mean ± SD of four experiments.

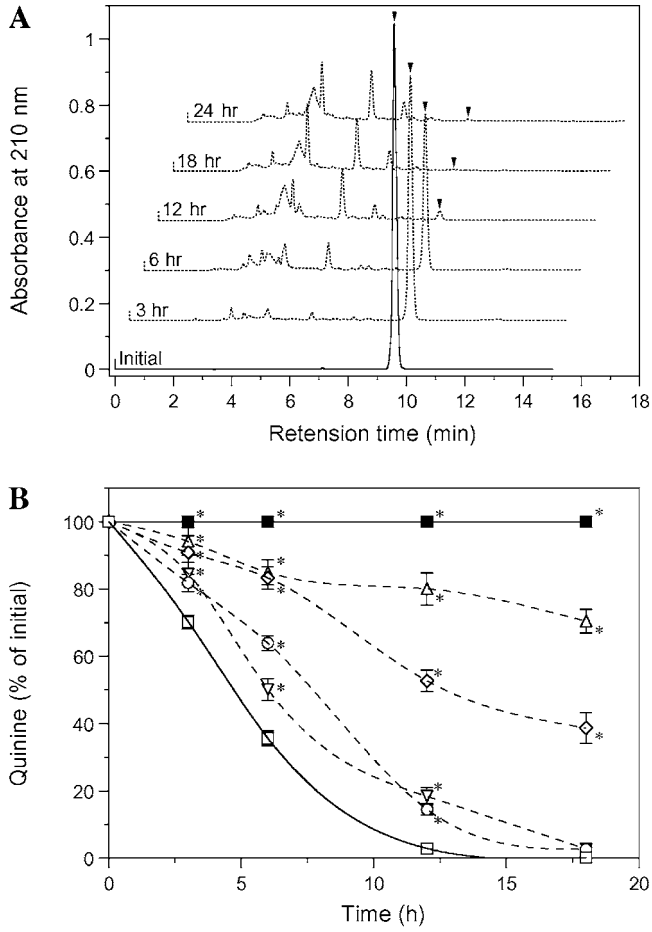

Fig. 4. Photodegradation of quinine and its attenuation by several scavengers. (A) HPLC chromatograms of quinine solution (0.25 mg/mL in 20 mM NaPB, pH 7.4) exposed to UVA/UVB radiation (30,000 lx) at 25°C for the indicated periods. Peak for intact quinine was arrowed. (B) Kinetic plot of light-induced degradation of quinine (0.25 mg/mL in 20 mM NaPB, pH 7.4) with or without radical scavengers. ■, Nonirradiated quinine; □, irradiated quinine; ◇, with BHA (100 μM); ○, with GSH (100 μM); △, with NaN_3 (100 μM); ▽, with SOD (100 U). Each point represents the means ± SD of four experiments. $^*P < 0.05$ with respect to photoirradiated quinine at the same time point.

degradation was evaluated on the basis of kinetic photodegradation constant k and half-life ($t_{1/2}$), with respect to the initial drug concentration, and the following data were obtained: slope (rate constant) = 0.358 h and $t_{1/2}$ = 6.43 h (Table II).

In an attempt to evaluate the possible role of ROS in the photodegradation of quinine, a series of experiments was performed in which various scavengers were included in quinine solution during irradiation. The scavengers used were as follows: NaN_3, a singlet oxygen scavenger; BHA and GSH, free radical scavengers; and SOD, a typical scavenger for superoxide (30). As shown in Fig. 4B, the addition of scavengers to the quinine solution produced a protective effect on photodegradation, and Table II summarizes the inhibitory effect of scavengers on the photodegradation of quinine. NaN_3 and BHA strongly inhibited the photodegradation of quinine, especially NaN_3, which could prolong half-life time by as much as 20-fold higher than quinine alone. In contrast, the protective effects of GSH and SOD were much

Table II. Rate Constants of Photodegradation for Quinine in Solution Forms with or without ROS Scavengers

Quinine solutions	Degradation constant, k (h^{-1})	Half-life time, $t_{1/2}$ (h)	Correlation, r
Quinine (0.25 mg/mL)	0.358	6.43	0.985
with BHA	0.055	41.47	0.990
with GSH	0.209	11.02	0.977
with NaN_3	0.019	122.63	0.987
with SOD	0.201	11.46	0.976

lower, and concomitant exposure of quinine and these scavengers to light showed the complete photodegradation at 24 h (data not shown). These results suggested that generation of ROS was tightly associated with photoinstability of quinine, and the main active species for its photodegradation may be singlet oxygen.

Photodynamic Lipid Peroxidation Induced by Irradiated Photosensitizers

Lipid peroxidation has been considered to be one of the major mechanisms in phototoxic skin responses induced by several NSAIDs (31). Thus, we attempted to investigate the ability of known phototoxic compounds to photosensitize peroxidation of linoleic acid. In this investigation, in order to evaluate and compare the lipid peroxide level, malondialdehyde (MDA), a secondary product of lipid peroxidation, was determined by TBA method (13). UV irradiation of a linoleic acid (1 mM), dissolved in 20 mM NaPB (pH 7.4) in the absence of photosensitizers formed a small amount of peroxidation products, but irradiation in the presence of photosensitive drugs (200 μM) produced a much greater amount of such products (Table III). Of all tested photosensitive/phototoxic compounds, only 5-fluorouracil did not show any significant lipid peroxidation under the experimental conditions. Weak or nonphototoxic compounds, except for benzocaine, did not enhance the production of MDA. These data were consistent with the results, indicating that they produce neither singlet oxygen nor superoxide under light exposure. Benzocaine, a weak photoreactive compound, showed photodynamic lipid peroxidation at a relatively low level as compared to known photosensitizers, possibly mediated by a small amount of superoxide generated. The results, taken together with other our photochemical experiments, may discriminate potential photosensitizers from nonphototoxic compounds, whereas the amount of TBARS, produced by photosensitizers, did not completely correlate with their ability to generate ROS.

Table III. Lipid Peroxidation Induced by Photoirradiated Compounds

Compounds tested (concentration)	TBARS (μM)	Significance
Linoleic acid (1 mM, not irradiated)	1.12 ± 0.07	**
Linoleic acid (1 mM, irradiated)	1.57 ± 0.13	
Linoleic acid (1 mM) with		
Photosensitive/phototoxic compounds (200 μM, photoirritants)		
Chlorpromazine	2.13 ± 0.15	**
Naproxen	4.96 ± 0.34	**
Ketoprofen	5.98 ± 0.49	**
Norfloxacin	2.15 ± 0.26	**
Nalidixic acid	5.82 ± 0.61	**
Indomethacin	1.94 ± 0.16	**
Ibuprofen	4.51 ± 0.38	**
Furosemide	2.62 ± 0.31	**
Benzoyl peroxide	6.19 ± 0.58	**
Amiodarone	3.55 ± 0.21	**
Quinine	2.97 ± 0.35	**
Oxytetracycline	2.51 ± 0.16	**
Diclofenac sodium	2.22 ± 0.27	**
Sulfamethoxazole	1.95 ± 0.24	**
Retinol	2.11 ± 0.30	**
8-Methoxy psoralen	5.14 ± 0.62	**
Tamoxifen	3.88 ± 0.34	**
Omeprazole	2.20 ± 0.08	**
Carbamazepine	2.58 ± 0.19	**
Nitrendipine	2.81 ± 0.45	**
5-Fluorouracil	1.55 ± 0.17	
Weak/nonphototoxic compounds (200 μM)		
Benzocaine	1.76 ± 0.16	*
Phenytoin	1.61 ± 0.13	
Aspirin	1.65 ± 0.19	
Erythromycin	1.68 ± 0.07	
Sodium dodecyl sulfate	1.51 ± 0.12	

Linoleic acid (10^{-3} M) and tested compounds were dissolved in 20 mM NaPB (pH 7.4) containing 0.05% Tween 20, and then exposed to light (30,000 lx) for 18 h. Lipid peroxidation was measured using a TBA assay, and a standard curve of 1,1,3,3-tetraethoxypropane was used to quantitate the amount of TBA reactive substance (TBARS) produced. **$p < 0.01$, *$p < 0.05$ with respect to photoirradiated linoleic acid.

DISCUSSION

The major findings of this study are that: (1) many phototoxic or photosensitive compounds have an ability to generate ROS under exposure to light, possibly leading to photodegradation and oxidative stress; and (2) the type and amount of ROS generated are different depending on the photosensitive compounds. The obtained results may also be very useful from the medical standpoint in the elucidation of the biological action of many pharmaceutical products *in vitro*. The prediction model proposed here may classify chemicals as phototoxic and/or photosensitive compounds on the basis of their photochemical properties.

Drug-induced phototoxic reactions can be categorized as either photoirritant, photogenotoxic, or photoallergic, and some drugs can cause all three types of reactions (32). Possible pathways of these photochemical/biological reactions are summarized in Fig. 5. The drug initially absorbs energy from UVA/UVB light, and then the photon energy excites an absorbing molecule into the singlet state. The energy is generally dissipated via radiative processes (fluorescence), or radiationless processes generating kinetic energy (heat), and then the molecule returns into the single ground state. It is also possible that the excited singlet molecule undergoes intersystem crossing into the more long-lived triplet state, and energy transfer from a donor to an acceptor molecule can occur when the two molecules enter into close contact during collision. Molecular oxygen, a triplet radical in its ground state, seems to be the predominant acceptor of excitation energy as its lowest excited level (singlet state) has a comparatively low value. Here, excitation of the drug by light may give rise to ROS such as singlet oxygen and superoxide, which may be one of causative agents for the three major drug-induced phototoxic responses, including photoirritation, photogenotoxicity, and photoallergy (32,33). First, photoirritation is frequently characterized as exaggerated sunburn that is sometimes mediated by oxidative stress in the cell membrane, and hyperpigmentation and desquamation may occur as a residual effect of a phototoxic reaction. Theoretically, if a high enough concentration of a phototoxic drug accumulates in the skin and the appropriate

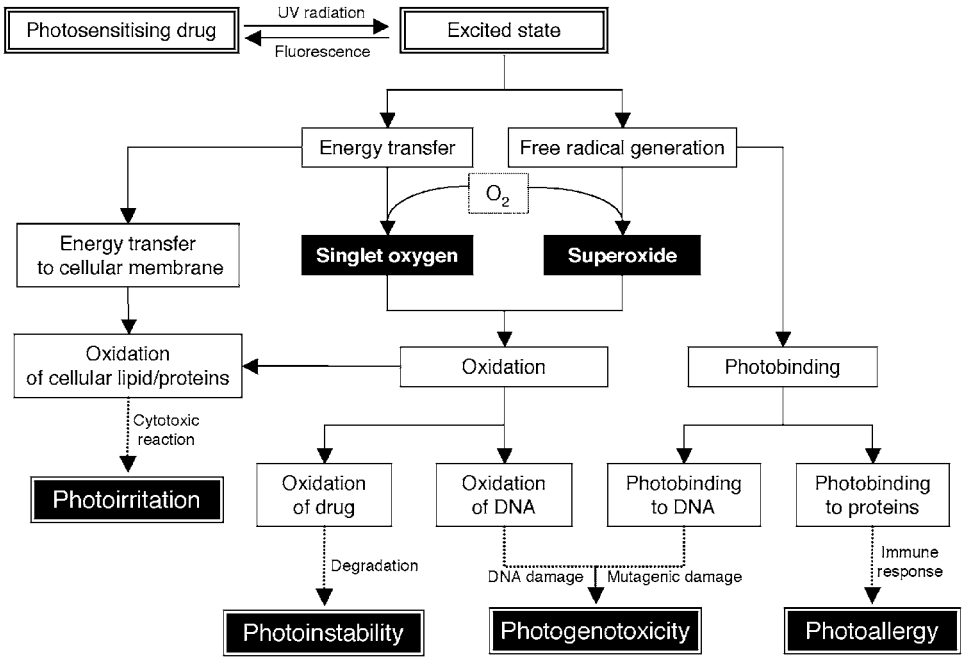

Fig. 5. Schematic representation of possible pathways for phototoxic responses induced by photosensitive drugs.

wavelength of light is present, any individual will develop a phototoxic reaction. In our investigation, lipid peroxidation was induced by some photosensitizers under photoirradiation, and this photochemical reaction certainly correlates with damage produced in the cell membrane, leading to the skin photoirritation. Second, photogenotoxiciy is attributable to the DNA damage and the formation of mutagenic photoadducts by a photosensitized interaction (26). With respect to DNA damage, there are several indirect mechanisms by which photoexcited molecules can damage DNA through the generation of ROS. Excited molecules can also directly transfer the excitation energy to DNA and thus give rise to pyrimidine dimer formation as observed upon direct DNA excitation. Third, photoallergy is an immune-mediated reaction in which light may cause a structural change in a drug so that it acts as a hapten, possibly by binding to proteins in the skin. As soon as an antigen, hapten–protein complex, is formed, Langerhans' cells residing in the epidermis can present the antigen to immunocompetent cells, thereby causing hypersensitivity (21). In addition to these phototoxic responses, photodegradation could occur through the generation of ROS, as evidenced by the fact that ROS scavengers, especially NaN_3, strongly inhibited the photodegradation of quinine in our study. There is also the probability that the photochemical reaction of compounds with ROS resulted in the yield of some toxic degradants. In this context, ROS assay could be predictive for the occurrence of photodegradation, as well as the potential of phototoxic responses.

To predict the potential of these phototoxic responses and photochemical reactions, an effective methodology to evaluate photochemical/biological properties was developed, with the aim of replacing the testing method performed in animals and humans (34). Several studies suggested a number of screening methods for recognizing photosensitizing drugs, including measurement of UV absorption, photohemolysis model (35), measurement of oxygen consumption in *Bacillus subtilis* (27), cutaneous phototoxic reaction model using human reconstituted epidermis Episkin (28), and 3T3 neutral red uptake (3T3 NRU) phototoxicity test (36). Although phototoxic responses were very complicated, most of the phototoxicity *in vitro* assays rely on a simple parameter of cytotoxicity (normally NRU or MTT tests). In particular, the 3T3 NRU phototoxicity test has been accepted in the EU/COLIPA validation program on "Photoirritation *in vitro*." However, this cell monolayer assay has the following main limitations: (1) low productivity due to the screening system, and (2) the assay is not sufficient for the discrimination of photoallergens and photoirritants. As an alternative method to 3T3 NRU phototoxicity test, the absorption spectrum of a compound was sometimes investigated, because no photochemical reaction can occur unless electromagnetic radiation is absorbed. Indeed, this could be an immediate and simple screening, but it might not always provide an effective indication for its capacity of participating in a photochemical process. In our investigation, the UVA/UVB absorptions of photoinactive chemicals, such as phenytoin, aspirin, erythromycin, and SDS, were weak or negligible; however, benzocaine showed a strong absorption peak within the sunlight region. Some photosensitive/phototoxic compounds showed low UVA/UVB absorption, whereas they were identified to be phototoxic according to clinical reports. Here, UVA/UVB absorption of chemicals may not always directly correlate with their phototoxic potential. These results suggest that some compounds would be falsely predicted as phototoxic or nonphototoxic based on UV spectral analysis only. In this context, the other or an additional screening method should be applied for the evaluation of photoreactivity so as to avoid giving false information.

Photooxidation of histidine was recently used as a test for photooxidizing potency; consequently, this test could be useful in hazard assessment practice (37). Because histidine reacts with singlet oxygen (11), the assay indicates the ability of photoirradiated compounds to generate singlet oxygen. With respect to ROS generated from photoirradiated com-

pounds, some photosensitizers, including chlorpromazine, indomethacin, sulfamethoxazole, tomoxifen, and omeprazole, failed to produce a detectable amount of singlet oxygen in our investigation; however, significant generation of superoxide was observed in these compounds. Interestingly, some photosensitizers also generated both species under light exposure. Single measurement of singlet oxygen or superoxide from photoirradiated compounds would provide false information, because two major photochemical pathways, such as the excited triplet state and free radical entities, may be involved in the phototoxic responses. This is a major reason why we proposed the measurement of both singlet oxygen and superoxide as a screening model for phototoxic/photosensitive evaluation. In our study, all known photosensitizers, except for 5-fluorouracil, showed ROS production under light exposure; however, they included weak UVA/UVB absorbers such as ibuprofen and benzoyl peroxide. This suggested that the present assay may be useful to evaluate the photosensitive and phototoxic potential of tested compounds with ease and high productivity.

Results from ROS and TBA assay indicated that phototoxic mechanisms of 5-fluorouracil may be different from those of other photosensitizers. Alternatively, the occurrence of phototoxicity in 5-fluorouracil may require concomitance of some biomolecules including DNA and RNA, in which phosphorylated 5-fluorouracil could be incorporated. Further investigation of the possible phototoxic cascades in 5-fluorouracil is required to understand the limitation of this assay, and the study may also enable the improvement of this assay or even the development of other effective predicting tools.

Based on the results obtained, we proposed a tiered strategy for phototoxicity/photosensitivity testing. In a first step, phototoxic potential is identified with ROS measurement following exposure to UVA/UVB light, using the RNO and NBT models for analysis of the concentration–response curves of compounds. For positive articles, the second step involves the photostability study and the 3T3 NRU assay. The test is suitable for compounds intended for topical and systemic administration, as well as for finished topical formulations.

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