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Short communication

Reactive oxygen species assay-based risk assessment of drug-induced phototoxicity: Classification criteria and application to drug candidates

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ABSTRACT

We have previously demonstrated that the phototoxic potential of chemicals could be partly predicted by the determination of reactive oxygen species (ROS) from photo-irradiated compounds. In this study, ROS assay strategy was applied to 39 marketed drugs and 210 drug candidates in order to establish provisional classification criteria for risk assessment of drug-induced phototoxicity. The photosensitizing properties of 39 model compounds consisting of phototoxic and non-phototoxic chemicals, as well as ca. 210 drug candidates including 11 chemical series were evaluated using ROS assay and the 3T3 neutral red uptake phototoxicity test (NRU PT). With respect to marketed drugs, most phototoxic drugs tended to cause type I and/or II photochemical reactions, resulting in generation of singlet oxygen and superoxide. There seemed to be a clear difference between phototoxic drugs and non-phototoxic compounds in their abilities to induce photochemical reactions. A plot analysis of ROS data on the marked drugs provided classification criteria to discriminate the photosensitizers from non-phototoxic substances. Of all drug candidates tested, 35.2% compounds were identified as phototoxic or likely phototoxic on the basis of the 3T3 NRU PT, and all ROS data for these phototoxic compounds were found to be over the threshold value. Furthermore, 46.3% of non-phototoxic drug candidates were found to be in the subthreshold region. These results verify the usefulness of the ROS assay for understanding the phototoxicity risk of pharmaceutical substances, and the ROS assay can be used for screening purposes in the drug discovery stage.

1. Introduction

Drug-induced photo-irritation can be defined as an inflammatory reaction of the skin after topical or systemic administration of pharmaceutical substances [1]. In many cases of drug-induced phototoxicity, skin reactions can be triggered by doses of sunlight regarded as harmless and most often in the ultraviolet A (UVA) range (320–400 nm). Several classes of drugs including antibacterials [2,3], thiazide diuretics [4], non-steroidal anti-inflammatory drugs (NSAIDs) [5], quinolones [6], and tricyclic antidepressants [7,8], even though non-toxic by themselves, may become reactive under exposure to environmental light, leading to undesired side effects [1].

The primary event in any photosensitization process is the absorption of photons of the appropriate wavelength, which allows chromophore to reach an excited state. The excitation energy is often transferred to oxygen molecules, followed by generation of reactive oxygen species (ROS): superoxide through type I reaction and singlet oxygen through type II reaction by photo-excited drug molecules. These appear to be the principal intermediate species in the phototoxic response [9,10]. In cells, this cascade gives rise to local oxidative stress and damage to genomic DNA, proteins, and lipids within cell membranes [11]. From the standpoint of risk assessment, we previously proposed that determination of ROS from pharmaceutical substances irradiated with UVA and ultra-

Abbreviations: EBSS, Earle's balanced salt solution; MPE, mean photo effect; NSAIDs, non-steroidal anti-inflammatory drugs; OECD, Organisation for Economic Co-operation and Development; PIF, photo-irritancy factor; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; 3T3 NRU PT, 3T3 neutral red uptake phototoxicity test; UV, ultraviolet.

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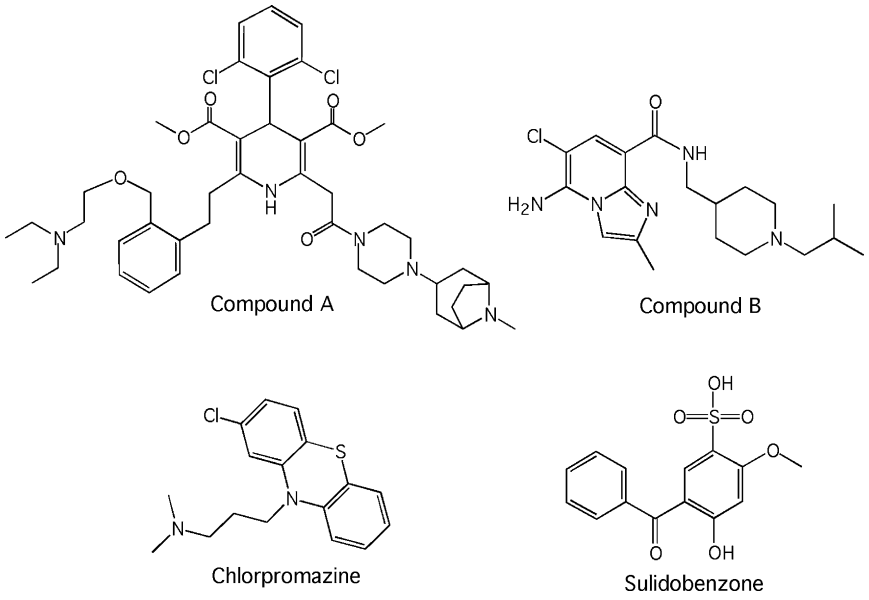

Fig. 1. Structures of chemicals tested; compound (A) a dihydropyridine derivative, compound (B) an imidazopyridine derivative, chlorpromazine and sulisobenzone.

violet B (UVB) would be of help in recognizing their phototoxic potential [10,12–14].

The utility of the ROS assay for understanding the phototoxic potential was already partially confirmed [10], and the present study is aimed to provide a provisional threshold for classification of substances as phototoxic or non-phototoxic, as well as verifying the predictability of the ROS assay. In this study, photochemical and photobiological behaviors of many marketed drugs and newly synthesized drug candidates were evaluated by analytical and biochemical methodologies, including UV spectral analysis, ROS assay, and 3T3 neutral red uptake phototoxicity test (NRU PT). Particularly, photochemical and photobiological properties of dihydropyridine derivative (compound A), imidazopyridine derivative (compound B) and chlorpromazine were investigated since these chemical series and drug were often identified to be highly phototoxic [15–17]. In addition to the phototoxic chemicals, sulisobenzone, a benzophenone derivative, was used as negative control because of its low phototoxic potential [18]. The photosensitizing abilities of 39 model compounds consisting of 33 phototoxic and 6 non-phototoxic chemicals were assessed with the use of the ROS assay, and then we established provisional classification criteria to identify the phototoxic risk. Based on the criteria obtained, the phototoxic potential of 210 drug candidates including 11 chemical series were evaluated, and the relationship between ROS and 3T3 NRU PT data is discussed.

2. Materials and methods

2.1. Chemicals

All photosensitive/phototoxic compounds including 5-fluorouracil, 8-methoxy psoralen, amlodipine, amoxapine, benzoyl peroxide, bufexamac, carbamazepine, chlorothiazide, chlorpromazine, diclofenac, doxycycline, furosemide, haloperidol, ibuprofen, imipramine, indomethacin, ketoprofen, nalidixic acid, naproxen, nifedipine, nimodipine, nitrendipine, nitroflantoin, norfloxacin, omeprazole, oxytetracycline, piroxicam, promethazine, quinine, retinol, sulfamethoxazole, tamoxifen, tryptophan, aspirin, benzocaine, erythromycin, phenytoin, sodium dodecyl sulfate (SDS), and sulisobenzone were purchased from Sigma (St. Louis, MO), Wako Pure Chemical Industries (Osaka, Japan), or Funakoshi (Tokyo, Japan). Imidazole, *p*-nitrosodimethylaniline, and nitroblue tetrazolium were obtained from Wako Pure Chemical Industries. All drug candidates used in this investigation, including (4*R*)-(−)-dimethyl4-(2,6-dichlorophenyl)-2-(2-{[2-(diethylamino)ethoxy]methyl}phenyl)-6-{2-[4-(8-methyl-8-azabicyclo[3.2.1.]oct-3-yl)-1-piperazinyl]-2-oxoethyl}-1,4-dihydro-3,5-pyridinedicarboxylate mono-benzoate (compound A) and 5-amino-6-chloro-*N*-[(1-isobutylpiperidin-4-yl)methyl]2-methylimidazo[1,2-*a*]pyridine-8-carboxamide (compound B) shown in Fig. 1, were chemically synthesized in the Nagoya Laboratories of Pfizer Global Research and Development (Aichi, Japan).

2.2. Irradiation conditions

Each tested compound was stored in a Light-Tron Xenon (LTX-01, Nagano Science, Osaka, Japan) equipped with a xenon arc lamp. UV special filter and window glass filter were installed to adapt the spectrum of the artificial light source to natural daylight. The irradiation test was carried out at 25 °C with an irradiance of 1.8 mW/cm^2.

2.3. UV spectral analysis

All tested compounds 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 JASCO V-560 double-beam spectrophotometer (JASCO, Tokyo, Japan) interfaced to a computer for data processing (software: Spectra Manager). Spectrofluorimeter quartz cells with a 10-mm path length were employed.

2.4. Determination of reactive oxygen species

Singlet oxygen was determined following the Kraljic and El Mohsni procedure [19], and it was measured in an aqueous solution by spectrophotometrically monitoring the bleaching of *p*-nitrosodimethylaniline (RNO) at 440 nm using imidazole as a selective acceptor of singlet oxygen. Samples containing the compounds under examination, *p*-nitrosodimethylaniline (50 μM) and imidazole (50 μM), in 20 mM NaPB (pH 7.4) were irradiated with UVA/B (30,000 lx), and then UV absorption at 440 nm was mea-

Table 1
UV and ROS data for tested compounds

Compounds	UVA/B absorption[a] λ_{max} (nm)/ε (M^{-1} cm^{-1})	Generation of reactive oxygen species[b]	
		Singlet oxygen (decrease of $A_{440\,nm} \times 10^3$)	Superoxide (increase of $A_{560\,nm} \times 10^3$)
Compound A	377 (4.2×10^3)	58 ± 4	185 ± 15
Compound B	349 (1.3×10^4)	424 ± 17	63 ± 3
Chlorpromazine	307 (3.8×10^3)	59 ± 14	95 ± 3
Sulisobenzone	320 (6.6×10^3)	Not detected	Not detected

[a] Measured in 20 mM phosphate buffer (pH 7.4).
[b] Irradiated with UVA/B at 30,000 lx for 18 h. Data represent mean ± S.D. for four independent experiments.

sured by a SpectraMax plus 384 microplate spectrophotometer (Molecular Devices, Kobe, Japan). Superoxide anion was determined according to the Pathak and Joshi procedure [20]. Samples containing the compounds under examination and nitroblue tetrazolium (NBT, 50 μM) in 20 mM NaPB were irradiated with UVA/B (30,000 lx) for the indicated periods, and the reduction of NBT was measured by increased absorbance at 560 nm using a SpectraMax plus 384 microplate spectrophotometer.

2.5. 3T3 NRU phototoxicity test (3T3 NRU PT)

The *in vitro* 3T3 NRU PT was carried out as described in the Organisation for Economic Co-operation and Development (OECD) 432 guideline and the European Community Official Journal (L 136/9, 8 June 2000, annexe II). Briefly, 96-well tissue culture plates were seeded with 1.0×10^4 cells/well 3T3 mouse fibroblast cells. The plates were then incubated at 37 °C in a humidified 5% CO_2 incubator for 24 h. Cells were exposed to dilutions of the test compounds in Earle's balanced salt solution (EBSS) for 60 min. Compounds were tested at various concentrations ranging from 0.061 to 1000 μg/mL. Chlorpromazine was used as a positive control. Duplicate plates were exposed for 50 min to UVA–vis light at 1.7 mW/cm^2 from a doped mercury metal halide lamp (solar simulator SOL500, Dr. Honle UV technology, Munich, Germany) equipped with the UV special filter for giving artificial sunlight, or they were kept in the dark. After a 50-min exposure, the solutions were removed from all plates, and the cells washed twice with EBSS and DMEM, respectively. The cells were then reincubated in culture medium overnight. Cell viability was assessed using the neutral red uptake (NRU) assay [21]. The NRU assay consisted of a 3-h incubation with neutral red (50 μg/mL in DMEM) followed by extraction with a mixture (150 μL) of acetic acid, ethanol and water (1:50:49). The absorbance was measured at 540 nm. The photo-irritancy factor (PIF) and mean photo effect (MPE), indicators of phototoxicity, were calculated according to a previous report [22]. PIF is generated by comparing two equally effective cytotoxic concentrations (EC_{50}) of irradiated and non-irradiated chemical, and MPE is derived from mathematical analysis of the complete shape of two concentration response curves obtained in the absence and presence of a non-cytotoxic irradiation with UV.

2.6. Data analysis

For statistical comparisons, a one-way analysis of variance (ANOVA) with 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.

3. Results and discussions

3.1. UV spectral analysis

The first law of photochemistry, alternatively referred to as the Grotthus–Draper law, states that no photochemical reaction can occur unless electromagnetic radiation is absorbed [23]. According to this photochemical principle, the absorption spectrum of a compound can be used as an immediate and simple prediction of photoreactivity. In this study, UV spectral patterns of marketed drugs and drug candidates were recorded in 20 mM NaPB, and UV spectra of chlorpromazine, a phototoxic drug, and sulisobenzone, a non-phototoxic substance, and two drug candidates: compound A, a dihydropyridine derivative, and compound B, an imidazopyridine derivative, are shown in Fig. 2.

Solar radiation reaches the surface of the earth after passage through the atmosphere where the higher energy portion is absorbed, resulting in filtering of the UVC region [24]. The spectrum of solar radiation is therefore composed of UVA, UVB and visible light (Fig. 2). According to the UV spectral patterns obtained, all tested compounds showed strong absorption in the UVA/B or visible range. Their UV spectral patterns partly overlapped the sunlight spectrum, and the order of UV absorption intensity in the sunlight region was as follows: compound B > sulisobenzone > compound Achlorpromazine. In addition to compounds A and B, almost all tested drug candidates exhibited significant absorption of UVA/B (data not shown). Thus, most compounds tested here were found to absorb sunlight, suggesting that they may absorb photon energy and be excited under exposure to sunlight.

3.2. ROS generation from chemicals irradiated with UVA/B

It is well established that ROS such as singlet oxygen and superoxide act as major toxic mediators in the upstream of drug-induced phototoxic cascades [1] and are responsible for oxidative damage against various biomolecules including phospholipids, proteins, and DNA. Based on these observations, we previously proposed that the ROS assay system, which monitors ROS generation from test compounds irradiated with UVA/B, is indicative of the phototoxicity of drug candidate [10]. In this study, ROS assays on compounds A

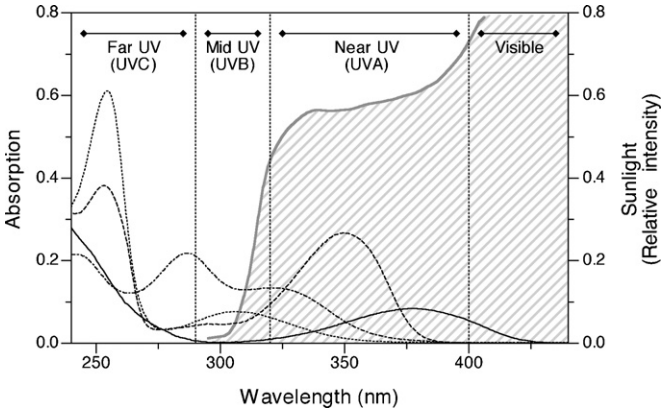

Fig. 2. UV-absorption spectra of compounds A and B, chlorpromazine and sulisobenzone (20 μM) in 20 mM NaPB (pH 7.4). Solid line, compound A; dashed line, compound B; dotted line, chlorpromazine; and chain line, sulisobenzone.

and B, chlorpromazine, and sulisobenzone (200 μM) were carried out to evaluate the phototoxic potential.

The generation of singlet oxygen was detected by spectrophotometric measurement of RNO bleaching, followed by decreased absorbance of RNO at 440 nm [19]. Although singlet oxygen does not react chemically with RNO, the RNO bleaching is a consequence of singlet oxygen capture by the imidazole ring, resulting in the formation of a *trans*-annular peroxide intermediate capable of inducing the bleaching of RNO as follows:

$$\text{Singlet oxygen} + \text{imidazole} \rightarrow [\text{peroxide intermediate}]$$

$$\rightarrow \text{oxidized imidazole}$$

$$[\text{Peroxide intermediate}] + \text{RNO} \rightarrow \text{RNO bleaching} + \text{products}$$

The generation of superoxide could be determined by the reduction of NBT [25] as indicated below: NBT can be reduced by superoxide anion via a one-electron transfer reaction, yielding partially reduced ($2e^-$) monoformazan (NBT^+) as a stable intermediate. Thus, superoxide can reduce NBT to NBT^+, whose formation can be monitored spectrophotometrically at 560 nm.

$$\text{Superoxide} + \text{NBT} \rightarrow O_2 + NBT^+$$

Table 1 summarizes the results from ROS assays on tested compounds. Compounds A and B, and chlorpromazine could generate both singlet oxygen and superoxide to significant levels under light exposure, whereas in the dark, they did not show any ROS generation. Interestingly, sulisobenzone, a strong UVA/B absorber, did not show generation of superoxide, as well as singlet oxygen. The order of ROS-forming ability was as follows: compound B > compound A > chlorpromazine ≫ sulisobenzone. According to the results of the ROS assay, the phototoxic potentials of compounds A and B, and chlorpromazine, could be deduced. UV-absorbing property was not directly related to ROS data since sulisobenzone was found to be less photoreactive. According to Jablonski diagram [26,27], once a molecule has absorbed energy in the form of electromagnetic radiation, there are a number of routes by when it can return to ground state. The excited molecules often emit photons (fluorescence, phosphorescence, or delayed fluorescence) to fall back into lower energy states, and three nonradiative deactivation processes are also important: internal conversion, intersystem crossing and vibrational relaxation. The relaxation system of excited chemicals would be different depending on the chemical structure and molecular state, and it affected the photochemical/photobiological behavior of chemicals. On the basis of our findings, irradiated sulisobenzone might be deactivated immediately through emission of photon energy or nonradiative transitions, so that it could not be phototoxic and had no ability to generate ROS under light exposure.

3.3. Phototoxicity in 3T3 mouse fibroblasts

The 3T3 NRU PT is designed to detect phototoxicity induced by the combined action of a chemical and UVA/B light by using an *in vitro* cytotoxicity assay in the Balb/c 3T3 mouse fibroblast cell line. The 3T3 NRU PT was carried out to further clarify the phototoxic potential of the tested compounds (Fig. 3). Fig. 3A shows representative cell viability curves of 3T3 cells after exposure to compound A. In the presence of irradiation, cell viability was shifted to considerably lower concentrations, and the PIF and MPE values of compound A were calculated to 8.1 and 0.25, respectively (Table 2). Generally, PIF values are effective for discriminating phototoxic molecules (PIF > 5) from non-phototoxic molecules, but are actually unable to correctly discriminate mildly or probably phototoxic molecules (2 < PIF < 5) from non-phototoxic molecules (PIF < 2). According to the MPE model, a test substance is considered to have phototoxic potential if the MPE is over 0.15; also a test substance can be considered as "probable phototoxic" (0.1 < MPE < 0.15) or "non-phototoxic" (MPE < 0.1). Based on the classification criteria

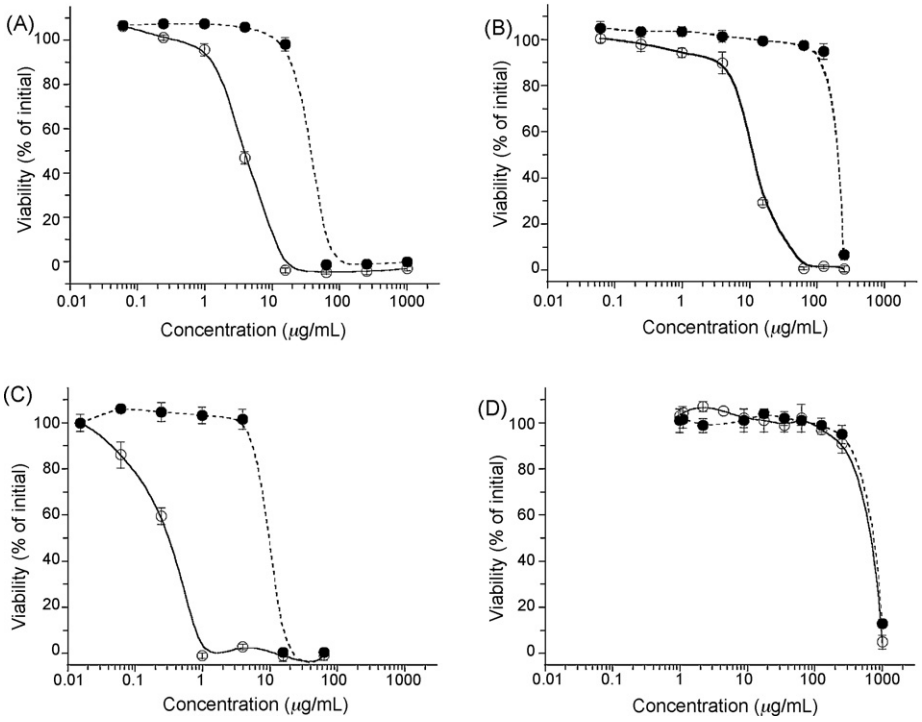

Fig. 3. Phototoxicity of tested compounds in the 3T3 NRU PT. The 3T3 cells were treated with different concentrations of tested compounds: (A) compound A, (B) compound B, (C) chlorpromazine and (D) sulisobenzone, and irradiated with UVA/B light (50 kJ/m^2). Each value represents the mean ± S.D. of six replicates. (●) Compound kept in dark and (○) compound with UVA/B irradiation.

Table 2
Phototoxic behavior of tested compounds on Balb/c mouse 3T3 fibroblast cells

Compounds	3T3 NRU PT data	
	Photo-irritation factor (PIF)	Mean photo effect (MPE)
Compound A	8.1	0.25
Compound B	17.9	0.37
Chlorpromazine	26.0	0.45
Sulisobenzone	1.0	0.04

of PIF and MPE values, compound A was found to be phototoxic. Compound B and chlorpromazine also exhibited a strong phototoxic effect on cells as evidenced by the higher PIF (17.9 and 26.0) and MPE (0.37 and 0.45) values. On the contrary, sulisobenzone showed no significant transition in viability curves with or without UV irradiation, and their PIF and MPE values suggest a low phototoxic potential. These findings were consistent with the results of the ROS assay, and experimental evidence obtained in the present study suggests that compounds A and B cause phototoxic responses probably through oxidative damage.

3.4. Relationship between ROS generation and phototoxicity

To assess the ability of 33 photosensitizing drugs, 5-fluorouracil, 8-methoxy psoralen, amlodipine, amoxapine, benzoyl peroxide, bufexamac, carbamazepine, chlorothiazide, chlorpromazine, diclofenac, doxycycline, furosemide, haloperidol, ibuprofen, imipramine, indomethacin, ketoprofen, nalidixic acid, naproxen, nifedipine, nimodipine, nitrendipine, nitroflantoin, norfloxacin, omeprazole, oxytetracycline, piroxicam, promethazine, quinine, retinol, sulfamethoxazole, tamoxifen and tryptophan; and 6 non-phototoxic compounds: aspirin, benzocaine, erythromycin, phenytoin, SDS and sulisobenzone (200 μM) to cause photochemical responses, the ROS assay was carried out. The capacity of the tested compounds (200 μM) to generate ROS is shown in Fig. 4A. All known phototoxic/photosensitive compounds, except for 5-fluorouracil, exhibited the ability to generate singlet oxygen, superoxide, or both, whereas weak/non-phototoxic compounds, even strong UVA absorbers such as benzocaine and sulisobenzone, did not. There seemed to be clear differences between photosensitizers and non-phototoxic compounds in their abilities to induce photochemical reactions, and the results obtained may be useful for the elucidation of the photochemical properties of many pharmaceutical products in a cell-free system. Plot analysis of the ROS data provided classification criteria (5.0×10^{-2} for both singlet oxygen and superoxide) to discriminate the photosensitizers from non-phototoxic substances. Thus, compounds in the shaded region appear to have a low potential for phototoxic skin responses. Interestingly, 5-fluorouracil, a phototoxic drug, lay in the subthreshold region, and 5-fluorouracil was also evaluated to be not phototoxic by 3T3 NRU PT (PIF < 2, MPE < 0.1). In our previous work, the results of biochemical experiments indicated that the phototoxic mechanisms of 5-fluorouracil might be different from those of other photosensitizers [10]. The phototoxicity of 5-fluorouracil might require the concomitant activity of biomolecules including DNA and RNA in which phosphorylated 5-fluorouracil could be incorporated. The exact reason why 5-fluorouracil did not show photochemical reactions and cytotoxicity in these screening systems still remains unclear, however further clarification will be helpful for understanding the limitations of the ROS assay and avoiding misleading data. Generation of radical species would not be always involved in phototoxic processes, since some phototoxic chemicals could bind with DNA and proteins under light exposure, resulting in induction of photogenotoxicity and photoallergy, respectively. In addition, some photodegradants after UV-irradiation of pharmaceutical substances might exhibit toxic responses directly. Thus, there is the probability that ROS assay provides false negative results in some chemicals.

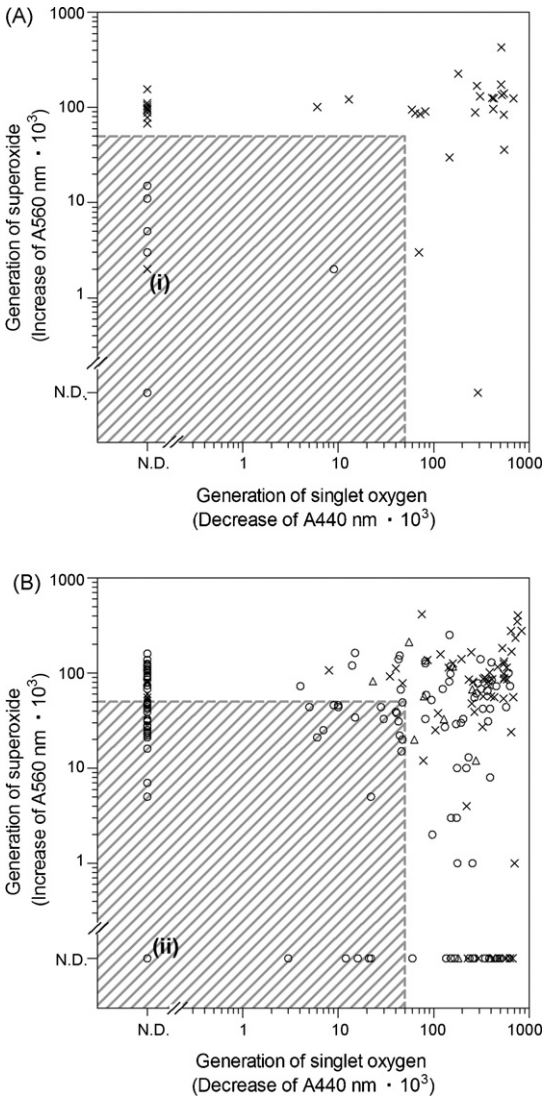

Fig. 4. Plot of singlet oxygen data vs. superoxide data for pharmaceutical substances. (A) ROS data for marketed drugs. (○) Non-phototoxic drugs and (×) phototoxic drugs. (B) ROS data for 210 drug candidates. According to results from 3T3 NRU PT data, each drug candidate was evaluated as (○) non-phototoxic compounds; (△) probably phototoxic compounds; and (×), phototoxic drugs. For determination of ROS generation, each tested compound (200 μM) was dissolved in 20 mM NaPB (pH 7.4) and exposed to UVA/B (1.8 mW/cm^2) for 18 h. Data represent mean of four experiments. The shaded region is indicative of low phototoxic potential. (i) ROS data for 5-fluorouracil and (ii) ROS generation was not detected (N.D.) in 12 drug candidates.

In addition to the model compounds, the phototoxic potential of 210 drug candidates consisting of 11 chemical series, azaindoles, benzimidazoles, cyanobenzenes, dihydropyridines, furopyridines, imidazopyridines, pyridines, pyrazoles, pyridines, pyrimidines and quinolines, were also assessed with the use of the ROS assay and 3T3 NRU PT. According to results from 3T3 NRU PT, 60 compounds (28.6% of total) and 14 compounds (6.7% of total) were identified to be phototoxic and probable phototoxic, respectively. It should be noted that all the phototoxic and probable phototoxic compounds showed a significant amount of ROS generation that exceeded the threshold level (Fig. 4B). In addition, 136 compounds (64.8% of total) were found to be non-phototoxic on the basis of 3T3 NRU PT data,

and 46.3% of non-phototoxic compounds showed no significant ROS generation, lying in the subthreshold region. Thus, the provisional classification criteria based on the ROS assay provided no false negatives as compared to the 3T3 NRU PT, and they could be used as first screening to identify the phototoxic potential of drug candidates.

There are at least three types of drug-induced phototoxic skin reactions consisting of the photo-irritant, photogenotoxic and photoallergic cascades, the mechanisms and pathologic features of which are quite different [28]. 3T3 NRU PT was designed to evaluate the photo-irritant potential of drug substances. On the contrary, the ROS assay can detect type I and/or II photochemical reactions induced by irradiated compounds, which were observed in the upstream of drug-induced phototoxic cascades. In this context, the ROS assay might capture photogenotoxic and photoallergic drugs, as well as the photo-irritant chemicals that are identified by 3T3 NRU PT. Furthermore, photolabile substances would also be recognized as phototoxic since they sometimes show significant ROS generation during the photodegradation process. These factors could contribute to the discrepancy observed between the 3T3 NRU PT and ROS assays.

Recently, new pharmaceutical or cosmetic compounds must have their phototoxic potential tested when they absorb wavelengths in the range of sunlight composed of UVA and partial UVB [11]. There is thus an obvious need for a phototoxic screening strategy based on complementary *in vitro* tests [29]. Based on the findings obtained in this study, the ROS assay strategy could be of use as a first screening to classify chemicals in the early stage of pharmaceutical development.

4. Conclusion

In this study, we evaluated the photochemical and phototoxic behaviors of 39 model compounds and 210 drug candidates consisted of 11 chemical series. The major findings of this study are that (1) ROS assay showed a correlation with the phototoxic risk as determined by the 3T3 NRU PT and (2) ROS determination of 39 model compounds provided provisional threshold values to identify the phototoxic risk. By using the classification criteria, ROS assay could be indicative of the phototoxic potential of drug candidates. These findings support the usefulness of the ROS assay for identifying the phototoxic risk and avoiding undesired side effects in the early stage of pharmaceutical development. However, ROS assay might not always discriminate phototoxic chemicals exactly since phototoxic skin responses could also be caused by various factors other than ROS generation. In conclusion, the ROS assay can be used for screening purposes, and further accumulation of ROS data will allow us to estimate new classification criteria to discriminate phototoxic compounds more precisely.

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