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Short communication

High-throughput reactive oxygen species (ROS) assay: An enabling technology for screening the phototoxic potential of pharmaceutical substances

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Abstract

Recently, attention has been drawn to drug-induced phototoxic skin responses, and avoidance of this undesired side effect is necessary for pharmaceutical development. We previously proposed that determination of reactive oxygen species (ROS) generated from photoirradiated compounds would be effective for the prediction of the phototoxic potential. In this investigation, a high-throughput ROS assay system was developed using a multiwell plate and quartz reaction container. The experimental conditions of irradiance uniformity, UV intensity, exposure time, temperature and solvent systems were found to affect the generation of ROS, and thus the conditions of the ROS assay were optimized. The intra- and inter-day R.S.D. values for the determination of ROS from quinine (200 μM) irradiated at 250 W/m^2 for 1 h was found to be less than 3.3 and 4.5%, respectively. The results from the ROS assay of 39 compounds allowed us to estimate classification criteria to identify the ability of phototoxic/photochemical responses. The developed assay system will be an effective tool for predicting the phototoxic potential of pharmaceutical candidates in early stage of pharmaceutical development.

Keywords: Phototoxicity; Reactive oxygen species; Singlet oxygen; Superoxide; UV

1. Introduction

Photosensitivity is a broad term used to describe unwanted phototoxic skin reactions of pharmaceutics, pigments, and food additives to nonionizing radiation [1]. Drug-induced phototoxic reactions can be categorized as photoirritation, photogenotoxicity, or photoallergy, and some drugs can cause all three types of reactions [2]. To avoid these undesirable side effects, screening of drug-induced phototoxicity is necessary at the early phase of the drug discovery process. Our group previously proposed a model system for the assessment of the photosensitive/phototoxic potential of pharmaceutical substances on the basis of their photochemical behaviors; generation of reactive oxygen species, including superoxide (Type I reaction) and singlet oxygen (Type II reaction), upon exposure of compounds to simulated sunlight [3,4].

In the early stage of pharmaceutical development, a high-throughput and high-performance ROS assay is necessary, since enormous numbers of synthetic compounds have to be evaluated for their phototoxic potential. Therefore, the main purpose of this study was to develop a simple multiwell plate-based ROS assay system for the prediction of phototoxic potential. For the multiwell plate-based ROS assay, we designed a quartz reaction container whose advantages include the reduction of sample volume, the improvement of assay productivity, and highly uniform irradiation. In the present study, we optimized the conditions of the ROS assay and investigated the influence of experimental conditions such as irradiation uniformity, UV intensity,

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exposure time, temperature and solvent systems on the generation of ROS. These experiments validated the utility of the system, and the multiwell plate-based ROS assay was found to be robust and adaptable to high-throughput screening. In addition to assay development, the ROS assay was carried out for 32 photosensitizers and 6 non-phototoxic substances in order to provide possible criteria for discrimination of pharmaceutical candidates having phototoxic potential.

2. Experimental

2.1. Materials

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, nitrofurantoin, 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). *p*-Nitrosodimethylaniline, imidazole, and nitroblue tetrazolium were obtained from Wako Pure Chemical Industries. The quartz reaction container designed for high-throughput ROS assay was constructed by Ozawa Sciences (Nagoya, Japan).

2.2. Irradiation conditions

Each tested compound was stored in an Atlas Suntest CPS+ solar simulator (Atlas Material Technology LLC, Chicago, IL) or 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 14–250 W/m^2. Irradiance was checked on a UVR-2 radiometer (Topcon, Tokyo, Japan) for each experimental procedure.

2.3. Determination of reactive oxygen species

2.3.1. Singlet oxygen

Singlet oxygen was determined following the Kraljic and El Moshni procedure [5], and it was 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, *p*-nitrosodimethylaniline (50 μM) and imidazole (50 μM), in 20 mM NaPB (pH 7.4) were irradiated with UVA/B (250 Wh/m^2), and then UV absorption at 440 nm was measured by a SpectraMax plus 384 microplate spectrophotometer (Molecular Devices, Kobe, Japan).

2.3.2. Superoxide anion

Superoxide anions were determined according to the procedure of Pathak and Joshi [6]. Samples containing the compounds under examination and nitroblue tetrazolium (NBT, 50 μM) in 20 mM NaPB were irradiated for the indicated periods, and the reduction of NBT was measured by the increase of their absorbance at 560 nm using a SpectraMax plus 384 microplate spectrophotometer (Molecular Devices).

2.4. UV spectral analysis

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

2.5. 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.

3. Results and discussion

3.1. Quartz reaction container and light source

Multiwell plates have far-ranging use in high-throughput methodologies including biological assays [7], purification [8], and even salt screening [9], since the use of multiwell plates provides an assay system for rapid and parallel measurements of a large amount of samples. In this investigation, a quartz reaction container was designed for a high-throughput ROS

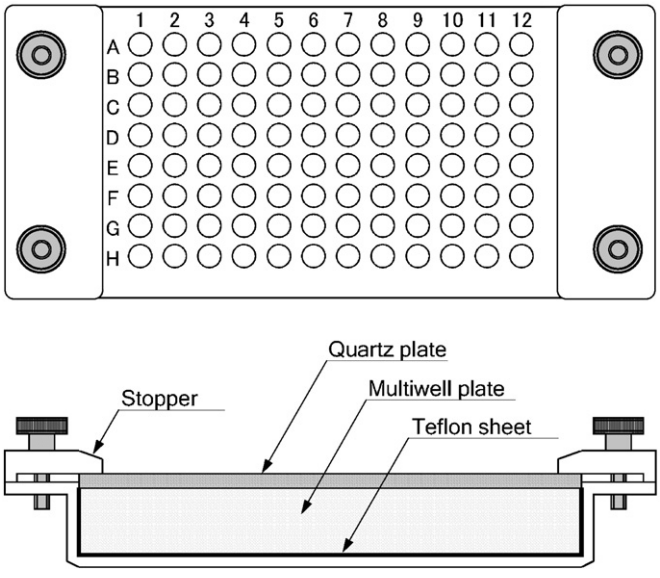

Fig. 1. Quartz reaction container designed for ROS assay.

assay (Fig. 1) that consisted of a quartz plate, a multiwell plate, and a steel multiwell retainer. UV radiation is usually divided into several ranges based on its physiologic effects: (1) UVA (near UV): 320–400 nm (UVA I: 340–400 nm and UVA II: 320–340 nm), (2) UVB (middle UV): 290–320 nm, and (3) UVC (far UV): 180–290 nm [10,11]. UVA radiation is easily transmitted through air and glass, and there is penetration through the epidermis and the anterior ocular media. UVB and UVC radiation is also transmitted through air and through quartz but partially absorbed by ordinary glass. The sun emits ultraviolet radiation in the UVA, UVB, and UVC bands, but because of absorption by the atmosphere's ozone layer, the main ultraviolet radiation that reaches the Earth's surface is UVA [12]. In this investigation, both a UV special filter and a window glass filter were utilized to produce simulated sunlight composed of UVA and partial UVB. For the effective exposure of the tested samples to simulated sunlight, a quartz plate was used as a sample cover.

3.2. Generation of ROS from quinine exposed to UVA/B

The generation of singlet oxygen was detected by spectrophotometric measurement of *p*-nitrosodimethylaniline (RNO) bleaching, followed by decrease of the absorbance of RNO at 440 nm [5]. In addition to singlet oxygen, the generation of superoxide from photoirradiated compounds could also be determined by the reduction of nitro-substituted aromatics such as NBT [13]. Quinine solution at the several concentrations (50–200 μM) was exposed to UVA/B light for the indicated periods, and both generation of singlet oxygen and superoxide from irradiated quinine seemed to be concentration- and time-dependent (Fig. 2). However, samples did not show any RNO bleaching or NBT reduction without UVA/B irradiation (data not shown).

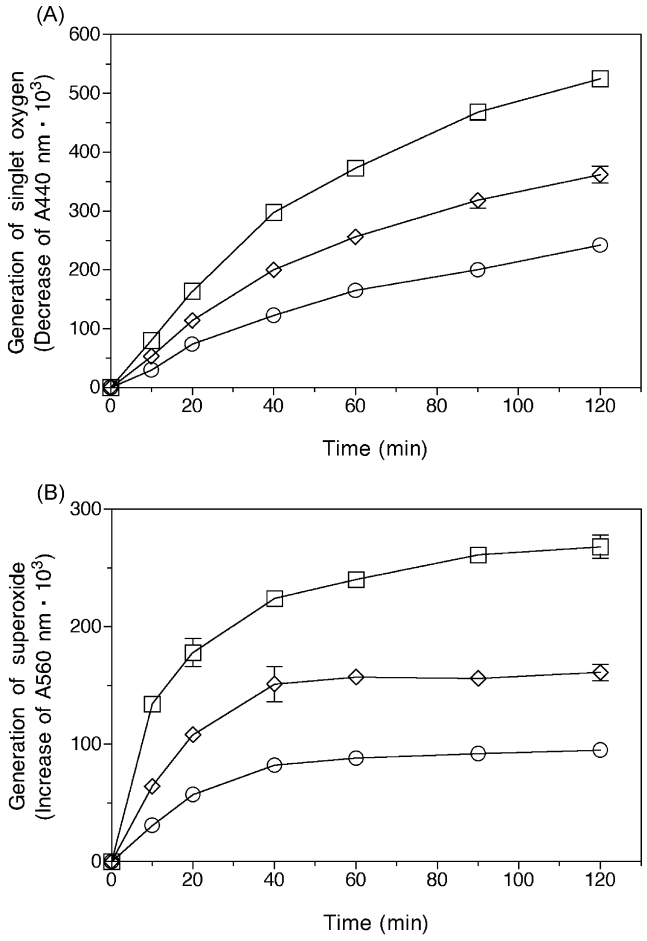

Fig. 2. Generation of reactive oxygen species from photoirradiated quinine. Time course of singlet oxygen (A) and superoxide (B) generation from quinine in 20 mM NaPB (pH 7.4) exposed to UVA/B (250 W/m^2) for indicated periods. (◯) 50 μM; (◊) 100 μM; (□) 200 μM. Data represent mean ± S.D. of four experiments.

3.3. Several factors influencing the determination of ROS from photoirradiated quinine

3.3.1. Irradiance uniformity on the multiwell plate

Quinine solution at the concentration of 200 μM was added to all wells in the 96-well plate and exposed to UVA/B at 250 W/m^2 for 1 h. Then, generation of singlet oxygen was determined by RNO bleaching measured by the decrease of absorption at 440 nm. Although ROS data on all wells seemed to be similar as evidenced by the fact that the CV was found to be 0.6%, the ROS generation on the outer lane (A1–H1, A12–H12, A2–A11, and H2–11) tended to be 1.2–1.9% less than the inner zone (B2–G11; data not shown). It is unclear why this discrepancy occurred; however it might be attributed to differences in irra-

Table 1
Effect of solvent system on ROS generation from photoirradiated quinine

Solvent system	Generation of reactive oxygen species (mean ± S.D.)	
	Singlet oxygen (decrease of A440 nm × 10^3)	Superoxide (increase of A560 nm × 10^3)
20 mM NaPB (pH 7.4)	382 ± 5	250 ± 10
With		
MeOH (1%)	367 ± 6**	206 ± 5**
EtOH (1%)	372 ± 6*	230 ± 9*
IPA (1%)	378 ± 4	257 ± 9
MeCN (1%)	389 ± 7	223 ± 4**
DMSO (1%)	447 ± 7**	337 ± 17**

Quinine (200 μM) was dissolved in 20 mM NaPB (pH 7.4) with or without the indicated solvents and exposed to UVA/B (250 W/m^2) for 1 h. Significantly different from control (20 mM NaPB alone), *$P < 0.05$, and **$P < 0.01$.

Table 2
ROS generation from quinine exposed to UVA/B at various levels of irradiation

Irradiation condition	Generation of reactive oxygen species (mean ± S.D.)	
	Singlet oxygen (decrease of A440 nm × 10^3)	Superoxide (increase of A560 nm × 10^3)
14 W/m^2 for 18 h	686 ± 7**	124 ± 9**
250 W/m^2 for 1 h	376 ± 11	240 ± 6
500 W/m^2 for 0 min	344 ± 19*	245 ± 35
750 W/m^2 for 20 min	438 ± 25**	203 ± 17**

Quinine (200 μM) was dissolved in 20 mM NaPB (pH 7.4) containing 1% MeCN and exposed to UVA/B (250 Wh/m^2). Significantly different from control sample irradiated at 250 W/m^2 for 1 h, *$P < 0.05$ and ** $P < 0.01$.

diance among wells. For a robust ROS assay, it would therefore be better to only use the inner zone.

3.3.2. Coexisting solvents in the assay reaction mixture

Since it is well established that some solvents act as modulators of radical species, e.g. dimethyl sulfoxide (DMSO) and isopropanol (IPA) as quenchers of some reactive oxidants [14,15] and hexane and D_2O as stabilizers of some radical species that extend the half-life time of oxygen radicals [16,17], the effects of coexisting solvents on ROS generation from quinine were investigated (Table 1). Among the organic solvents tested, DMSO tended to enhance the generation of singlet oxygen and superoxide by 20 and 35%, respectively; however, methanol (MeOH) and ethanol (EtOH) exhibited 3–18% inhibition of ROS generation, especially that of superoxide. Acetonitrile (MeCN) showed 11% attenuation of the generation of superoxide but not singlet oxygen. Influences of IPA on the ROS assay were negligible in this assay condition. Thus, there appeared to be some differences in the generation of ROS depending on the solvent system; therefore, the use of same solvent system for both stock solution and assay mixture would allow the most robust ROS assay.

3.3.3. Irradiation condition

In order to evaluate the influence of irradiation intensity on ROS generation, ROS assays on quinine were carried out with deliberate variation of irradiation (Table 2). UVA/B irradiance intensity ranged from 14 to 750 W/m^2, and the irradiation time was adjusted to allow the same total energy (250 Wh/m^2). As shown in Table 2, the results of the ROS assay under various conditions were quite different. Quinine at 14 W/m^2 exhibited the highest generation of singlet oxygen, whereas it showed 2-fold less superoxide generation when compared to quinine at 250 W/m^2. ROS data on quinine at 250 and 500 W/m^2 seemed to be relatively similar; however, quinine at 500 W/m^2 as well as 750 W/m^2 showed relatively high variation with a %R.S.D. of 5.4% for singlet oxygen and 14.3% for superoxide. On the basis of these findings, taken together with the test duration (1 h),

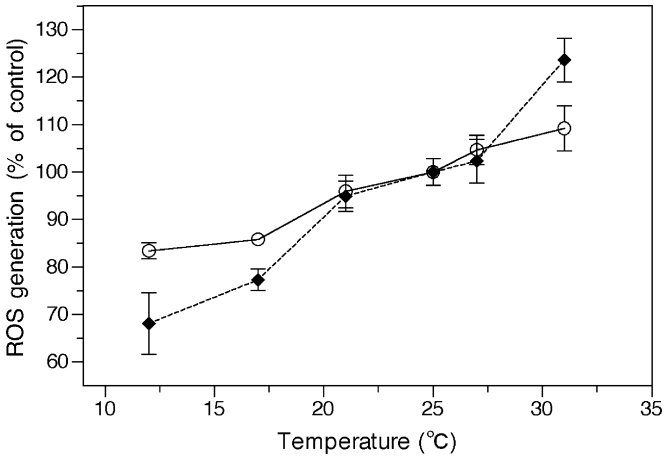

Fig. 3. Effect of assay temperature on ROS generation. Quinine (200 μM) was dissolved in 20 mM NaPB (pH 7.4) and exposed to UVA/B (250 W/m^2) for 1 h. (◯) Singlet oxygen and (◆) superoxide. Percent expression was calculated as the percentage of ROS generation compared to the control data at 25 °C. Data represent mean ± S.D. of four experiments.

Table 3
Intra-day and inter-day (days 1 and 3) precision for ROS of irradiated quinine at three concentrations

Concentration (μM)	Generation of reactive oxygen species, mean ± S.D. (%R.S.D.)	
	Singlet oxygen (decrease of A440 nm × 10^3)	Superoxide (increase of A560 nm × 10^3)
Intra-day		
50	165 ± 3 (1.8)	88 ± 7 (8.0)
100	254 ± 6 (2.4)	154 ± 5 (3.4)
200	373 ± 7 (1.9)	241 ± 8 (3.3)
Inter-day		
50	159 ± 6 (3.8)	93 ± 6 (6.5)
100	262 ± 11 (4.2)	156 ± 5 (3.2)
200	378 ± 9 (2.4)	246 ± 11 (4.5)

Quinine (200 μM) was dissolved in 20 mM NaPB (pH 7.4) containing 1% MeCN and exposed to UVA/B (250 W/m^2) for 1 h. Data represent mean ± S.D. of five experiments for intra-day precision and 10 experiments for inter-day precision.

irradiation at 250 W/m^2 was deduced to be suitable for the ROS assay.

3.3.4. Assay temperature

In this work, temperature dependencies of ROS generation from irradiated quinine (200 μM) were measured in the temperature range of 12–32 °C. As shown in Fig. 3, variations in assay temperature altered the photochemical properties of quinine, including both singlet oxygen and superoxide generating abilities. Quinine at temperatures ranging from 23 to 27 °C exhibited similar ROS data (data not shown). According to the data, lowering the temperature from 32 to 12 °C resulted in a significant reduction of superoxide, whereas the decrease was much more moderate for singlet oxygen. Overall, there was definite dependency of ROS generation on assay temperature. This is consistent with previous observations showing that photochemical and phototoxic events occurred more rapidly at the higher temperature [18,19]. These observations suggested that strict control of temperature in the ROS assay would be required for high reproducibility.

3.4. Precision

The overall precision of the method was evaluated by analyzing five samples of quinine standard solutions at 50 (25%), 100 (50%), and 200 μM (100%), representing low, middle and

Table 4
ROS generation from phototoxic/non-phototoxic substances

Compounds	UVA/B absorption[a], λ_{max} (nm)/ε (M^{-1} cm^{-1})	Generation of reactive oxygen species[b]	
		Singlet oxygen (ΔA440 × 10^3)	Superoxide (ΔA560 × 10^3)
Phototoxic compounds			
5-Fluorouracil	−[290 (1.8 × 10^3)]	N.D.	3 ± 0
8-Methoxy psoralen	303 (1.2 × 10^4)	31 ± 7	51 ± 3
Amlodipine	365 (3.7 × 10^3)	5 ± 2	53 ± 3
Amoxapine	297 (1.0 × 10^4)	N.D.	28 ± 4
Benzoyl peroxide	−[290 (6.8 × 10^2)]	88 ± 4	N.D.
Bufexamac	−[290 (1.3 × 10^2)]	16 ± 6	21 ± 1
Carbamazepine	−[290 (1.0 × 10^4)]	N.D.	16 ± 2
Chlorothiazide	312 (9.5 × 10^3)	32 ± 5	32 ± 2
Chlorpromazine	307 (3.8 × 10^3)	N.D.	113 ± 1
Diclofenac	−[290 (7.8 × 10^3)]	181 ± 7	370 ± 14
Doxycycline	352 (8.4 × 10^3)	131 ± 8	344 ± 6
Furosemide	331 (5.0 × 10^3)	139 ± 5	111 ± 16
Haloperidol	−[290 (1.8 × 10^2)]	19 ± 7	36 ± 1
Ibuprofen	−[290 (70)]	N.D.	165 ± 5
Imipramine	−[290 (2.9 × 10^3)]	21 ± 1	16 ± 1
Indomethacin	320 (7.0 × 10^3)	8 ± 4	186 ± 16
Ketoprofen	−[290 (5.0 × 10^3)]	284 ± 9	108 ± 2
Nalidixic acid	336 (7.8 × 10^3)	132 ± 2	231 ± 2
Naproxen	318/331 (1.4/1.7 × 10^3)	172 ± 7	207 ± 2
Nifedipine	344 (5.2 × 10^3)	32 ± 1	1 ± 0
Nimodipine	362 (1.2 × 10^3)	N.D.	154 ± 6
Nitrendipine	358 (5.7 × 10^3)	55 ± 10	52 ± 1
Nitroflantoin	381 (1.8 × 10^4)	72 ± 7	15 ± 1
Norfloxacin	324 (1.3 × 10^4)	147 ± 5	106 ± 4
Omeprazole	301 (1.5 × 10^4)	N.D.	129 ± 3
Oxytetracycline	363 (1.4 × 10^4)	201 ± 11	370 ± 7
Piroxicam	354 (1.6 × 10^4)	83 ± 7	96 ± 9
Promethazine	299 (3.5 × 10^3)	52 ± 4	34 ± 2
Quinine	331 (4.5 × 10^3)	376 ± 11	240 ± 6
Retinol	370 (2.3 × 10^3)	79 ± 3	32 ± 3
Sulfamethoxazole	−[290 (2.6 × 10^3)]	66 ± 1	4 ± 1
Tamoxifen	−[290 (3.6 × 10^3)]	119 ± 7	239 ± 5
Tryptophan	−[290 (4.1 × 10^3)]	45 ± 4	46 ± 0
Weak/non-phototoxic compounds			
Aspirin	−[290 (1.9 × 10^2)]	9 ± 2	N.D.
Benzocaine	−[290 (1.7 × 10^4)]	N.D.	4 ± 0
Erythromycin	−[290 (9)]	N.D.	5 ± 0
Phenytoin	−[290 (6)]	11 ± 8	8 ± 1
SDS	−[290 (3.1 × 10^2)]	N.D.	N.D.
Sulisobenzone	320 (6.6 × 10^3)	N.D.	N.D.

[a] Measured in 20 mM phosphate buffer (pH 7.4). If the peak/shoulder wavelengths were shorter than the lower limit of UVB (290 nm), the absorbance at 290 nm was noted in parenthesis.
[b] Irradiated at 250 W/m^2 for 1 h. Data represent mean ± S.D. for four independent experiments.

high concentrations. The intra-day precision (%R.S.D., $n = 5$) and inter-day precision (days 1 and 3 %R.S.D., $n = 10$) are shown in Table 3. The intra-day %R.S.D. values for the detection of singlet oxygen and superoxide ranged from 1.8 to 2.4 and 3.3 to 8.0, respectively. The inter-day %R.S.D. values varied from 2.4 to 4.2 (singlet oxygen) and 3.2 to 6.5 (superoxide). The precision of the singlet oxygen assay was better than that of superoxide as indicated by the lower R.S.D.s. Thus, in all cases, the %R.S.D. obtained was below 10%, showing that the proposed analytical method has good intra- and inter-day precision.

3.5. Application of the ROS assay for phototoxic assessment

In this investigation, the high-throughput ROS assay developed here was carried out for 33 photosensitizing substances and 6 non-phototoxic compounds, and the capacity of the test compounds at the concentration of 200 μM to generate ROS is shown in Table 4. All known phototoxic/photosensitive compounds, except for 5-fluorouracil, demonstrated 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 from a medical standpoint for the elucidation of the photochemical properties of many pharmaceutical products *in vitro*. With respect to classification, plot analysis of ROS data provided criteria (2.0×10^{-2} for singlet oxygen and 2.5×10^{-2} for superoxide) to discriminate the photosensitizers from non-phototoxic substances (Fig. 4). Compounds in the shaded region appear to have a low potential for phototoxic skin responses.

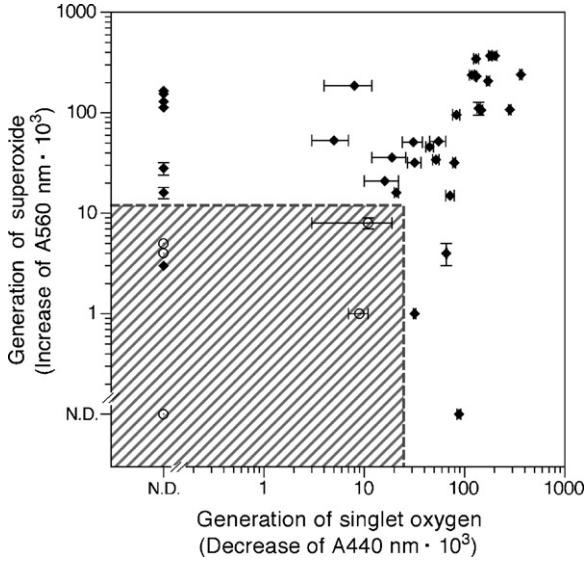

Fig. 4. A plot of singlet oxygen data vs. superoxide data for 39 compounds. Each tested compound (200 μM) was dissolved in 20 mM NaPB (pH 7.4) and exposed to UVA/B (250 W/m^2) for 1 h. (◯) Non-phototoxic compounds and (♦) phototoxic compounds. Data represent mean ± S.D. of four experiments. The shaded region is indicative of low phototoxic potential.

To predict the potential of these phototoxic responses and photochemical reactions, the development of effective methodologies to evaluate photochemical/biological properties have so far been attempted with the aim of replacing animal test [20]. These studies suggested various screening methods for recognizing phototoxins, including the photohemolysis model [21], measurement of oxygen consumption in *Bacillus subtilis* [22], and the 3T3 neutral red uptake (3T3 NRU) phototoxicity test [23]. These assays using biochemical systems take some time due to their complexity, so the absorption spectrum of a compound was sometimes investigated as an immediate and simple screening according to the first law of photochemistry (alternatively referred to as the Grotthus–Draper law), which states that no photochemical reaction can occur unless electromagnetic radiation is absorbed [24]. In our investigation, the UVA/B absorptions of non-phototoxic chemicals such as phenytoin, aspirin, erythromycin and SDS were found to be weak or negligible; however, benzocaine and sulisobenzone exhibited a strong absorption peak within the sunlight region. Interestingly, the photosensitive/phototoxic compounds; bufexamac, haloperidol and ibuprofen showed low UVA/B absorption, although they were identified to be phototoxic according to clinical reports. These findings indicate that UVA/B absorption of chemicals is not always directly indicative of their phototoxic potential. These results also suggest that some compounds might be falsely predicted to be phototoxic or non-phototoxic based on UV spectral analysis. In this context, other or additional screening methods should be applied for evaluation of phototoxic potential in order not to provide false information. Based on the findings obtained in this investigation, the high-throughput ROS assay system could be of use as a first screening to predict phototoxic risk of chemicals in the early stage of pharmaceutical development.

4. Conclusion

In the present study, a high-throughput ROS assay combining the use of a multiwell plate and quartz reaction container was developed and validated. Although some experimental conditions, especially light intensity and temperature, had significant influences on photochemical properties of the photosensitizer, the repeated analysis was indicative of the robustness and precision of the assay. The results of the ROS assay for 33 photosensitizers and 6 negative controls allowed us to estimate classification criteria that could be useful for recognizing the phototoxic potential of drug candidates.

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