Phototoxicity Evaluation of Pharmaceutical Substances with a Reactive Oxygen Species Assay Using Ultraviolet A

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With ultraviolet and visible light exposure, some pharmaceutical substances applied systemically or topically may cause phototoxic skin irritation. The major factor in phototoxicity is the generation of reactive oxygen species (ROS) such as singlet oxygen and superoxide anion that cause oxidative damage to DNA, lipids and proteins. Thus, measuring the generation of ROS can predict the phototoxic potential of a given substance indirectly. For this reason, a standard ROS assay (ROS assay) was developed and validated and provides an alternative method for phototoxicity evaluation. However, negative substances are over-predicted by the assay. Except for ultraviolet A (UVA), other UV ranges are not a major factor in causing phototoxicity and may lead to incorrect labeling of some non-phototoxic substances as being phototoxic in the ROS assay when using a solar simulator. A UVA stimulator is also widely used to evaluate phototoxicity in various test substances. Consequently, we identified the applicability of a UVA simulator to the ROS assay for photoreactivity. In this study, we tested 60 pharmaceutical substances including 50 phototoxins and 10 non-phototoxins to predict their phototoxic potential via the ROS assay with a UVA simulator. Following the ROS protocol, all test substances were dissolved in dimethyl sulfoxide or sodium phosphate buffer. The final concentration of the test solutions in the reaction mixture was 20 to 200 µM. The exposure was with 2.0~2.2 mW/cm² irradiance and optimization for a relevant dose of UVA was performed. The generation of ROS was compared before and after UVA exposure and was measured by a microplate spectrophotometer. Sensitivity and specificity values were 85.7% and 100.0% respectively, and the accuracy was 88.1%. From this analysis, the ROS assay with a UVA simulator is suitable for testing the photoreactivity and estimating the phototoxic potential of various test pharmaceutical substances.

Key words: Alternative testing method, Photosafety screening, Phototoxicity, Photoreactivity, Reactive oxygen species, Ultraviolet A

INTRODUCTION

Phototoxicity is an acute light-induced skin irritation when...
ment of the molar extinction coefficient (MEC), a standard ROS assay, a 3T3 neutral red uptake phototoxicity assay and a reconstructed human skin model assay.

The ROS assay was developed to screen photoreactivity of drugs through generation of the superoxide anion (type 1 reaction) and singlet oxygen (type 2 reaction) and their generation is an early stage chemical reaction as part of the phototoxicity mechanism (6). The ROS assay protocol was established and the validation studies were conducted under the direction of the Japanese Center for the Validation of Alternative Methods (JaCVAM) (9-11). For the assay, high sensitivity and reproducibility were demonstrated and as part of the test, two solar simulators Suntest CPS series and SXL-2500V2 were evaluated. This assay, however, has low specificity, showing high false positive results. For this reason, we only focused on the effect of UVA (315–400 nm), which is much important than other ultraviolet in phototoxicity. In addition, a UVA simulator is commonly used for phototoxicity evaluations. In this study, we evaluated the performance of the ROS assay with UVA (UVA ROS assay) instead of sunlight (290–700 nm) using 50 phototoxins and 10 non-phototoxins.

MATERIALS AND METHODS

Chemicals and materials. Sixty test substances, including reference chemicals and phototoxic/non-phototoxic drugs, were selected for evaluation from the ROS assay protocol, a validation report of the ROS assay, package inserts and previous studies (6,9-16). 4-Aminobenzoic acid, 6-methylcoumarin, 8-methoxy psoralen, acridine, amiodarone, amiodipine, amoxapine, aspirin, atorvastatin, benzocaine, bezafibrate, bithionol, chlorothiazide, chlorpromazine HCl, ciprofloxacin, dapsone, demeclocycline, diclofenac, doxycycline, erythromycin, fenofibrate, flutamide, fluvastatin, furosemide, gliclazide, glufosinulvin, hydrochlorothiazide, ibuprofen, ketoprofen, levofloxacin, losartan, lovastatin, metotrexate, naldixic acid, naproxen, nifedipine, nitrofurantoin, norfloxacain, octyl salicylate, ofloxacin, omeprazole, oxytetracyclline HCl, penicillin G, perphenazine, phenytoin, piroxicam, pro-methazine HCl, quinidine, quinine HCl, tetracycline, tiaprofenic acid, dimethyl sulfoxide (DMSO), sodium phosphate monobasic, sodium phosphate dibasic, p-nitrosodimethylaniline (RNO), imidazole and nitroblue tetrazolium chloride (NBT) were purchased (10). All test substances and reagents were protected from light.

UV spectral analysis. UV spectral analysis was conducted as described in a previous study (6). The test substances were dissolved in 20 mM sodium phosphate buffer (NaPB, pH 7.4) at 20 µM (final concentration). The UV/VIS absorption spectra were analyzed with a microplate spectrophotometer (Mecasys, Daejeon, Korea), and a spectrophotometer cuvette with a 10-mm pathlength was used. The MEC values were calculated using the highest absorption peaks from 290 to 700 nm.

Conditions of irradiation. A UVA simulator equipped with 40 W lamps was used (Vilbert-Lourmat, Marne-la-vallee, France). The UVA irradiation test was conducted at 25°C with 2.0 mW/cm² irradiance measured by a UVA detector (UVP, Cambridge, UK).

Reactive oxygen species (ROS) assay. ROS assay was performed as previously described in Onoue et al. (9,11) and the ROS assay protocol (10). Stock solutions of all test substances were prepared at 10 mM in DMSO or 20 mM sodium phosphate buffer (NaPB, pH 7.4) and used within the same day for accurate data. To detect the generation of singlet oxygen and superoxide anion, all prepared test substance stock solutions were mixed in reagents, containing 400 µM NBT in 20 mM NaPB (pH 7.4) for singlet oxygen (SO) and 200 µM RNO and 50 µM imidazole in 20 mM NaPB (pH 7.4) for superoxide anion (SA) detection, and the final concentration of test substances in the reaction mixture was at 200 µM. When precipitation was observed using a microscope (×100 magnification) in the reaction mixture, appropriate final concentrations (20, 50, 100 µM) were used. Two hundred microliters of each reaction mixture were put into three wells of a 96-well plate. Before UVA exposure, absorbance was measured at 440 nm for SO and 560 nm for SA by a microplate spectrophotometer and then a quartz reaction container was installed in the plate. The plate was irradiated with a UVA simulator. After irradiation, the absorbance at 440 nm and 560 nm for the plate was measured. Following the ROS assay protocol, ROS generation of SO and SA was calculated by mean absorbance before and after irradiation.

Data judgment. Photoreactivity of the test substances was judged according to the following criteria (10,11). A test substance was classified as a photoreactive substance when an SO value 25 or more and/or an SA value 20 or more was measured; in turn, it was judged to be a non-photoreactive substance when values of less than 25 for SO and
RESULTS

Optimization of the irradiance dose. To use a UVA simulator, we performed a preliminary study in order to find the appropriate UVA dose with the reference chemicals listed in the ROS assay protocol (10). We exposed at 2.0 mW/cm² irradiance, which is in the irradiance range of Atlas Suntest CPS/CPS+ in the ROS assay (10). We irradiated at up to 18 J/cm² at intervals of 3 J/cm² and recorded the phototoxic information. The results produced at 9 or more J/cm² were matched with their phototoxic potential (Table 1). Also, values of positive and negative substances met the acceptance criteria (17,18). Considering the results obtained with the irradiation times, we selected the UVA dose of 9 J/cm² for the main study.

Results of ROS assay using UVA simulator. Using the selected UVA dose, we identified the performance of the ROS assay with 60 test substances including 50 phototoxins and 10 non-phototoxins (Table 2). Firstly, we measured an MEC for all the test substances if they were photoreactive. Four substances, ibuprofen, erythromycin, penicillin G and phenytoin, had an MEC of less than 1,000. Next, we tested their solubility and identified precipitation, coloration or any other interference at 200 µM in the reaction mixture. Amiodarone, demeclocycline, fenofibrate, piroxicam and rosiglitazone showed precipitation in the reaction mixture for SO and/or SA and an appropriate concentration was further explored for them. Except for amiodarone, the substances with the solubility issues were dissolvable at least at 20 µM. As such, the evaluable test substances were determined be 59 of the original 60. The test results indicated that the UVA ROS assay correctly classified 42 of 49 phototoxins and 10 of 10 non-phototoxins. Amoxapine, atorvastatin, flutamide, griseofulvin, hydrochlorothiazide, nifdefipine and nitrinderpine were falsely judged as being non-phototoxins. The predictive capacity showed an 85.7% sensitivity, 100.0% specificity and 88.1% accuracy (Table 3).

DISCUSSION

To use a UVA simulator instead of validated solar simulators, we established the irradiation condition of the ROS assay with UVA. We chose a UVA irradiance of 2.0 mW/cm², which has been used in Atlas Suntest CPS series and has shown the lowest variation in a previous study (6). We found an appropriate UVA intensity that was compatible with phototoxic information of reference chemicals (Table 1). Also, the selected intensity sufficiently generated ROS in the range of 5~20 J/cm², which is widely used in in vitro and in vivo phototoxic assays (1).

Solubility evaluation of test substance solutions proceeded right before the assay, in order to prevent interference, such as precipitation and coloration that might affect

Table 1. Selection of UVA irradiation conditions using reference chemicals

| No. | Substance name          | CAS no. | Phototoxic information* | Concentration (µM) | ROS assay (J/cm²)** |
|-----|-------------------------|---------|-------------------------|-------------------|---------------------|
|     |                         |         | 3T3 NRU | Animal | Human | 3  | 6  | 9  | 12 | 15 | 18 |
| 1   | Quinine HCl (PC)        | 6119-47-7 | P   | P   | P   | 200 | 200 | +  | +  | +  | +  |
| 2   | Sulisobenzone (NC)      | 4065-45-6 | N   | N   | N   | 200 | 200 | −  | −  | −  | −  |
| 3   | 4-Aminobenzoic acid     | 150-13-0 | N   | N   | N   | 200 | 200 | −  | −  | −  | −  |
| 4   | 8-Methoxy psoralen      | 298-81-7 | P   | P   | P   | 200 | 200 | −  | +  | +  | +  |
| 5   | Acridine                | 260-94-6 | P   | P   | P   | 200 | 200 | +  | +  | +  | +  |
| 6   | Benzocaine              | 94-09-7  | N   | N   | N   | 200 | 200 | −  | −  | −  | −  |
| 7   | Chlorpromazine          | 69-09-0  | P   | P   | P   | 200 | 200 | −  | +  | +  | +  |
| 8   | Diclofenac              | 15307-79-6 | P | 200 | 200 | +  | +  | +  | +  |
| 9   | Doxycycline             | 10592-13-9 | P | 200 | 200 | +  | +  | +  | +  |
| 10  | Erythromycin            | 114-07-8 | N   | N   | N   | 200 | 200 | −  | −  | −  | −  |
| 11  | Fenofobrate             | 49562-28-9 | P | 20  | 20  | −  | +  | +  | +  |
| 12  | Furosemide              | 54-31-9  | P/N | P   | 200 | 200 | −  | +  | +  | +  |
| 13  | L-Histidine             | 71-00-1  | P   | N   | P   | 200 | 200 | −  | −  | −  | −  |
| 14  | Ketoprofen              | 22071-15-4 | P | 200 | 200 | +  | +  | +  | +  |
| 15  | Nalidixic acid          | 389-08-2 | P   | P   | P   | 200 | 200 | +  | +  | +  | +  |
| 16  | Norfloxacin             | 70458-96-7 | P | 200 | 200 | +  | +  | +  | +  |
| 17  | Omeprazole              | 73590-58-6 | P | 200 | 200 | −  | +  | +  | +  |
| 18  | Promethazine HCl        | 58-33-3  | P   | P   | P   | 200 | 200 | +  | +  | +  | +  |

*Phototoxic information was from JaCVAM (17) and Onoue et al. (11); P, phototoxic; N, non-phototoxic.
**+, Positive result; −, Negative result.
Table 2. Results of the UVA ROS assay

| No. | Substance name          | CAS no. | UV absorption* \(\lambda_{max}\) (nm) | MEC \(M^{-1} cm^{-1}\) | Concentration \((\mu M)\) | UVA ROS assay** | Phototoxic information |
|-----|------------------------|---------|-------------------------------------|----------------------|------------------------|------------------|------------------------|
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The ROS Assay Using a UVA Simulator

The test results. To overcome a solubility problem, the ROS assay was modified to use the micelle system using Tween 20 (18). Thus, low solubility substances could be evaluated for their phototoxic potential by adapting the micellar system to the UV A ROS assay.

The study results showed that the 7 phototoxic substances, amoxapine, atorvastatin, flutamide, griseofulvin, hydrochlorothiazide, nifedipine and nitrendipine, were classified as non-phototoxins (Table 2). These substances showed a UV A absorption and MEC of over 1,000 but did not generate ROS, either singlet oxygen or superoxide anion species. Moreover, irradiation of UV A at up to 18 J/cm$^2$ did not generate ROS (data not shown). However, these substances were correctly classified in previous studies, which used a solar simulator (6,18). Different results between a UV A simulator and a solar simulator could be related with UVB wavelength. UVB may be an essential factor to generate ROS of these chemicals, even they absorbed UV A wavelength. Therefore, misclassified chemicals may not generate ROS.

On the other hand, the final concentration of a test chemical, 200 µM, could be limited to generate ROS in the UV A ROS assay system. If using more higher concentration than the final concentration, these chemicals would be generated ROS and met the criteria of photoreactivity. L-Histidine, penicillin G and phenytoin that were false positive substances in the ROS assay were correctly classified as non-phototoxins (Table 2) (6,9,11). Surprisingly, bezafibrate, gliclazide, ibuprofen and losartan generated superoxide anion and were thus classified as phototoxins even though they had low MEC values in the UV/VIS range (290–700 nm). These results were consistent with those of the previous study, and MEC values cannot be always used to evaluate-phototoxic potential of chemicals (6). We conducted the UVA ROS assay with 60 test substances to identify their phototoxic potential. The results showed that this assay could adequately evaluate phototoxicity of the test substances analyzed. In addition, the UVA ROS assay has higher specificity and lower sensitivity than the ROS assay, and the performance of the UVA ROS assay is comparable with that of the ROS assay (Table 3). Our findings suggest that the UVA ROS assay could be used as a method for phototoxicity evaluation of pharmaceutical substances.

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