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RESEARCH PAPER

High-Throughput Screening System for Identifying Phototoxic Potential of Drug Candidates Based on Derivatives of Reactive Oxygen Metabolites

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ABSTRACT

Purpose The present study aimed to develop a high-throughput screening strategy for predicting the phototoxic potential of pharmaceutical substances, using a derivatives-of-reactive-oxygen-metabolites (D-ROM) assay.

Methods The assay conditions of the D-ROM assay were optimized with a focus on screening run time, sensitivity, solvent system, and reproducibility. The phototoxic potentials of 25 model compounds were assessed by the D-ROM assay, as well as by other screening systems for comparison, including the reactive oxygen species (ROS) assay, the DNA-photocleavage assay, and the 3T3 neutral red uptake phototoxicity test (3T3 NRU PT).

Results Some phototoxic drugs tended to yield D-ROM when exposed to simulated sunlight (250 W/m^2), whereas D-ROM generation was negligible for non-phototoxic chemicals. Compared with the ROS assay, the assay procedure for the D-ROM assay was highly simplified with a marked reduction in screening run time. Comparative experiments also demonstrated that D-ROM data were related to the outcomes of the DNA-photocleavage assay and the 3T3 NRU PT, with prediction accuracies of 76 and 72%, respectively.

Conclusion The D-ROM assay has potential for identifying the phototoxic potential of a large number of new drugs as a 1st screening system in the early stages of drug discovery.

KEY WORDS 3T3 neutral red uptake phototoxicity test · derivatives of reactive oxygen metabolites · photogenotoxicity · phototoxicity · reactive oxygen species

ABBREVIATIONS

3T3 NRU PT	3T3 neutral red uptake phototoxicity test
5-FU	5-fluorouracil
8-MOP	8-methoxypsoralen
AFM	atomic force microscopy
AGE	agarose gel electrophoresis
CD	circular dichroism
DEPPD	*N*,*N*-diethyl-*p*-phenylenediamine
DMEM	Dulbecco's Modified Eagle Medium
D-ROM	derivatives of reactive oxygen metabolites
EBSS	Earle's Balanced Salt Solution
EtBr	ethidium bromide
ECVAM	Europe Center for the Validation of Alternative Methods
OC	open circular
OECD	Organisation for Economic Co-operation and Development
PIF	photoirritation factor
ROS	reactive oxygen species
SC	supercoiled

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UV	ultraviolet
VIS light	visible light

INTRODUCTION

Drug-induced phototoxic skin responses are caused after the exposure of skin to photoreactive drugs, triggered by exposure to UVA (320–400 nm) and UVB (290–320 nm) radiation (1,2). There are at least three types of phototoxic skin reactions, including photoirritant, photogenotoxic, and photoallergic cascades, the mechanisms and pathologic features of which are quite different (3). Recently, the level of interest in phototoxicity has markedly increased owing to the awareness among the scientific community of the increased level of UV radiation from the sun reaching the earth. At early phases of the drug discovery process, the development of an efficacious phototoxicity testing system is essential for the avoidance of side effects. Therefore, a number of efforts have been made to provide a model system for the assessment of photosensitive/phototoxic potential through analytical and biochemical methods (1,4–11). Previously, our group proposed three screening systems to predict the phototoxic risk of newly synthesized drug candidates, which include the reactive oxygen species (ROS) assay for predicting phototoxic potential (12–14), the capillary gel electrophoresis-based photocleavage assay (15), and the DNA-binding assay (16) for photogenotoxic risk. In particular, ROS data on photo-irradiated chemicals could be effective for classifying such chemicals as phototoxic and/or photosensitive, since generation of reactive oxygens was found to be responsible for the induction of early photochemical and photobiological events (17).

A high-throughput ROS assay strategy, employing multiwell plates, might be useful as a first screening for phototoxic risk; however, there are some limitations of the ROS assay for screening purposes (18). The current ROS assay system is composed of two independent analytical processes to monitor type I and II photochemical reactions, and they require UV exposure with a high total irradiation energy, resulting in a long run time and data and operational complexity. Improvements to overcome these drawbacks would be of help to increase the productivity and usability of the ROS assay for phototoxicity assessment. Recently, attention has been drawn to the derivatives-of-reactive-oxygen-metabolites (D-ROM) test in the fields of clinical pharmacology and biochemistry (19–21). The D-ROM assay can detect peroxyl or alkoxyl radicals of a generic peroxide, which are indicative of oxidative stress conditions and generation of reactive oxygens (22). Accordingly, the D-ROM assay is currently recognized as an efficient and simplified analytical method for evaluating oxidative stress in the body (20). In addition to its clinical utility, the D-ROM assay might theoretically be applicable to phototoxicity screening by monitoring reactive oxygens-mediated photochemical events, although no efforts have been made to apply the D-ROM assay strategy to phototoxicity prediction.

The present investigation aimed to develop a novel prediction strategy for the phototoxic risk of drug candidates using a simplified D-ROM assay as an alternative to the ROS assay. The assay conditions of the D-ROM assay were optimized, focusing on irradiation time, sensitivity, solvent system, and robustness, and validation of the new assay system was also carried out. The new assay system was applied to 25 model compounds, including 20 phototoxic drugs and 5 non-phototoxic chemicals. To clarify the predictability of the D-ROM-based phototoxicity test, the phototoxic/photogenotoxic potentials of these chemicals were assessed by the ROS assay, the DNA-photocleaving assay, and the *in vitro* 3T3 neutral red uptake phototoxicity test (3T3 NRU PT).

MATERIALS AND METHODS

Chemicals

Naproxen, benzocaine, and sulisobenzone were purchased from Tokyo Chemical Industry (Tokyo, Japan), and 5-fluorouracil (5-FU), 8-methoxypsoralen (8-MOP), amiodarone, diclofenac, dimethyl sulfoxide, doxycycline, furosemide, imipramine, nalidixic acid, piroxicam, promethazine, quinine, and aspirin were purchased from Sigma (St. Louis, MO, USA). Chlorpromazine, *N*,*N*-diethyl-*p*-phenylenediamine (DEPPD), indomethacin, ketoprofen, nitrofurantoin, norfloxacin, erythromycin, ferrous sulfate, omeprazole, phenytoin, plasmid pBR322 DNA, *p*-nitrosodimethylaniline, imidazole, nitroblue tetrazolium, and quinidine were obtained from Wako Pure Chemical Industries (Osaka, Japan). Agarose L03 was purchased from Takara Bio (Shiga, Japan), and carbamazepine was bought from Acros Organics (Morris Plains, NJ, USA). Ethidium bromide (EtBr) was purchased from Nippon Gene (Toyama, Japan), and acetonitrile was purchased from Kanto Chemical (Tokyo, Japan). A quartz reaction container for high-throughput ROS assay was constructed by Ozawa Science (Aichi, Japan).

D-ROM Assay

The D-ROM assay is a spectrophotometric method that measures the alkoxy and peroxy radicals. D-ROM, generated from photosensitive chemicals under light exposure, were determined in accordance with the procedure of Hayashi *et al.* with some modifications (19). Briefly, assay mixtures containing the tested compounds (200 μM), DEPPD (600 μM), and ferrous sulfate (6.9 μM) in 0.1 M

acetic acid/sodium acetate buffer (pH 4.8) were prepared, and 245 μL of each assay mixture was stored in an Atlas Suntest CPS+solar simulator (Atlas Material Technology LLC, Chicago, USA) equipped with a xenon arc lamp (1,500 W). A UV special filter was installed to adapt the spectrum of the artificial light source to that of natural daylight. The irradiation test was carried out at 25°C with an irradiance of 250 W/m^2 (300–800 nm). Standard assays are typically performed in 96-well microtiter plates, employing a quartz reaction container as we proposed previously (14). After irradiation, the increase in absorbance at 505 nm was measured using a SAFIRE microplate spectrophotometer (TECAN, Männedorf, Switzerland).

ROS Assay

In our previous investigations, ROS assay was designed to detect both singlet oxygen and superoxide generated from photo-irradiated chemicals (13,14). In the ROS assay, each tested compound was stored in a light-irradiation tester Light-Tron Xenon (LTX-01, Nagano Science, Osaka, Japan) equipped with a xenon lamp (2,000 W). 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. The illuminance was set at 30,000 lux, and the irradiation test was carried out at 25°C. Singlet oxygen 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 sodium phosphate buffer (NaPB) (pH 7.4) were irradiated with UVA/B and Vis light (30,000 lux), and then the UV absorption at 440 nm was measured using a SAFIRE microplate spectrophotometer (TECAN). For the determination of superoxide, samples containing the compounds under examination and nitroblue tetrazolium (NBT, 50 μM) in 20 mM NaPB were irradiated with the UVA/B and Vis light (30,000 lux) for the indicated periods, and the reduction in NBT was measured by the increase in absorbance at 560 nm using a SAFIRE microplate spectrophotometer (TECAN).

DNA Photocleavage

In the DNA photocleavage assay, each assay mixture was stored in an Atlas Suntest CPS+solar simulator (Atlas Material Technology LLC) equipped with a xenon arc lamp (1,500 W) and a UV special filter. The irradiation test was carried out at 25°C with an irradiance of 250 W/m^2 (300–800 nm). The irradiated samples contained pBR322 DNA (final concentration, 10 μg/mL) dissolved in Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM boric acid, and 1 mM EDTA; pH 7.4) and the examined compounds at a final concentration of 200 μM. Irradiated plasmid pBR322 DNA was separated by electrophoresis (0.8% agarose gel in TAE buffer), stained with EtBr solution (0.5 μg/mL), and analyzed with image analyzing software Image J.

Atomic Force Microscopy (AFM)

AFM observation of plasmid DNA was carried out using an SPM-9600 scanning probe microscope (Shimadzu Co., Kyoto, Japan) in tapping mode with silicon cantilevers NCHR (NANOWORLD, Neuchâtel, Switzerland) whose spring constant and resonance frequency were 40 N/m and 300 kHz, respectively. The scan frequency was typically 1 Hz per line, and the modulation amplitude was a few nanometers. All samples were imaged in air at room temperature.

In Vitro 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, 08.06.2000, annexe II). Briefly, 96-well tissue culture plates were seeded with 1.0×10^4 cells/well 3T3 mouse fibroblast cells. The plates were 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 1,000 μg/mL. Chlorpromazine was used as a positive control. Duplicate plates were exposed for 20 min to UVA light (ca. 50 W/m^2) from UV BIO-SUN illuminator (Vilbert-Lourmat, Marne-la-Vallee, France) or were kept in the dark. After UVA exposure (total energy dose: 5 J/cm^2), the solutions were removed from all plates, and the cells were washed twice with EBSS and DMEM. The cells were then reincubated in culture medium overnight. Cell viability was assessed using the neutral red uptake (NRU) assay (23). 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) was calculated as an indicator of phototoxicity in accordance with a previous report (24). PIF is calculated by comparing two equally effective cytotoxic concentrations (EC_{50}) of irradiated and non-irradiated chemicals.

Data Analysis

For statistical comparisons, 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. To evaluate the robustness of the D-ROM assay, the Z'-factor, a statistical function, was calculated using the following equation: $Z' = 1 - (3\sigma_{c+} + 3\sigma_{c-})/|\mu_{c+} - \mu_{c-}|$ (25). The means of the positive and negative control signals are denoted as μ_{c+} and μ_{c-}, respectively. The SDs of the signals are denoted as σ_{c+} and σ_{c-}, respectively. The difference between the means, $|\mu_{c+} - \mu_{c-}|$, defines the assay dynamic range.

RESULTS AND DISCUSSION

Generation of D-ROM from UV-Excited Drugs

In phototoxic/photochemical events, the absorption of photon energy could be a key trigger for the photosensitization process, followed by the formation of reactive oxygens, such as superoxide and singlet oxygen through the type I and II reactions, respectively. Currently, these radical species are identified as the principal intermediate species in phototoxic responses (26). In the present investigation, the generation of D-ROM from irradiated drugs was monitored as an indicator for production of reactive oxygens. The generation of D-ROM could be detected using DEPPD, a chromogenic substrate for peroxyl radicals, followed by the formation of colored radical cations of the substrate (19). The exposure of quinine, a typical phototoxic drug, to UVA/B and Vis light (250 W/m^2) led to the marked production of D-ROM in a concentration-dependent manner (Fig. 1a). The generation of D-ROM was thought to be a photodynamic reaction, since quinine, protected from light, did not show potent D-ROM generation. Although it takes as long as 60 min to complete the ROS assay employing the same UV source as we proposed previously (14), the D-ROM assay could be completed within a few minutes, contributing to improved throughput of phototoxicity screening. According to the time evolution of the generation of radicals, taken together with the gradual increase in the basal level and discoloring of the reacted substrate, UV irradiation for 1 min was considered suitable for the D-ROM assay.

Some solvents act as modulators of radical species, so some differences in the generation of reactive oxygens might be observed depending on the solvent system used. For instance, dimethyl sulfoxide and isopropanol were found to act as quenchers of some reactive oxidants (27,28), whereas hexane and deuterium monoxide might stabilize some radical species with an extension of the half-life time (29,30). In our previous study, the use of acetonitrile-containing buffer was proposed for the ROS assay, because of its only slight influence on determination of singlet oxygen and superoxide (14). To clarify the effect of acetonitrile on the new screening system, D-ROM assays on quinine (200 μM) were also carried out in the presence of acetonitrile at various concentrations, ranging from 0 to 50% (Fig. 1b). The influence of acetonitrile at concentrations of less than 30% was negligible as evidenced by the constant outcomes for the D-ROM assay. However, acetonitrile at higher concentrations (>30%) attenuated D-ROM generation from quinine, which was possibly due to an altered photochemical property of quinine or measurement interference. Thus, D-ROM data might depend on the solvent system; therefore, the use of the same solvent system for both stock solution and assay mixture would ensure the most robust D-ROM assay for phototoxic prediction.

Validation of D-ROM Assay for Phototoxic Risk Assessment

To assess the robustness and reproducibility of the D-ROM assay, the Z'-factor was also calculated (25). The Z'-factor is

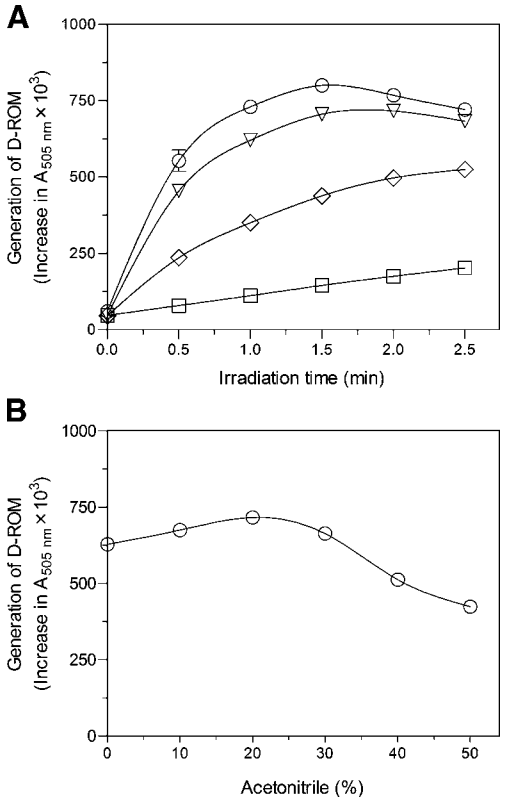

Fig. 1 Generation of D-ROM from photo-irradiated quinine. (A) Time course of D-ROM generation. Quinine was dissolved in 0.1 M acetic acid/sodium acetate buffer (pH4.8) and exposed to simulated sunlight for the indicated periods with an irradiance of 250 W/m^2. □, control (vehicle alone); ◊, quinine at 20 μM; ∇, 100 μM; and ○, 200 μM. Data represent mean ± SD of four determinations. (B) D-ROM generation from irradiated quinine in the presence of acetonitrile. Quinine (200 μM), dissolved in 0.1 M acetic acid/sodium acetate buffer (pH4.8) with various concentrations of acetonitrile, was exposed to simulated sunlight (250 W/m^2) for 1 min. Data represent mean ± SD of four determinations.

designed to reflect both assay signal noise ratio and the variation associated with the signal measurements. Hence, the Z'-factor is commonly utilized for quality assessment in assay development and optimization, as well as evaluation of the reproducibility of assays used for high-throughput screening campaigns (31). In an ideal assay, the Z'-factor is close to 1.0. In practical terms, a Z'-factor greater than 0.5 is indicative of an excellent assay, whereas assays with Z'-factor values less than 0.5 show a small separation band. Typical values from multiple measurements (20 times) of quinine (200 μM) and sulisobenzone (200 μM) are shown in Fig. 2. The Z'-factor for the D-ROM assay was calculated to be 0.75, demonstrating that the assay allows a large separation band between samples and blank signals and thereby confirming its suitability for high-throughput screening.

The overall precision of the method was evaluated by analyzing quinine standard solutions at 20 and 200 μM, and the intra-day precision (%RSD, $n=12$) and inter-day precision (days 1 and 3,%RSD, $n=24$) are shown in Table I. The intra-day%RSD values for the D-ROM assay were calculated to be 6.7 (20 μM) and 3.6 (200 μM), and the inter-day%RSD values were found to be 10.6 (20 μM) and 5.4 (200 μM). Thus, the precision of D-ROM assay at 20 μM was not high enough for high-throughput screening, although the %RSD value at 200 μM was below 6%. These data suggested that the proposed analysis has good intra- and inter-day precisions at higher analyte concentration (200 μM).

Phototoxic Risk Assessment on Model Compounds Using D-ROM Assay

On the basis of the optimized analytical method, the phototoxic risk of model compounds was evaluated (Table II). For comparison, the ROS assay was also carried out on these chemicals, in which both singlet oxygen and superoxide were monitored by independent colorimetrical determination. Of all chemicals tested, 8 phototoxic drugs exhibited significant D-ROM generation when exposed to simulated sunlight, which included chlorpromazine, ketoprofen, nalidixic acid, norfloxacin, omeprazole, promethazine, quinidine, and quinine. Quinidine is a stereoisomer of quinine, and there were no significant differences in photochemical behavior between quinine and quinidine ($p<0.05$), as evidenced by the results from ROS and D-ROM assays. Five non-phototoxic chemicals did not exhibit D-ROM production under light exposure, which was consistent with the results from the ROS assay. In addition to the non-phototoxic chemicals, some phototoxic drugs had no ability to generate D-ROM, whereas high amounts of singlet oxygen and/or superoxide were detected for these chemicals. Interestingly, significant generation of these reactive oxygens was observed for most phototoxic drugs examined, and only 5-FU was found to be less photoreactive among the phototoxic chemicals tested. We expected that D-ROM data could be highly related to the results from the ROS assay. However, there appeared to be a partial discrepancy between ROS and D-ROM data; in particular, the results for 8-MOP, diclofenac, doxycycline, furosemide, naproxen, nitrofurantoin, and piroxicam were quite different. Since the D-ROM assay could be indicative of typical reactive oxygen metabolites, such as alkoxy and peroxy radicals, it has been identified as one of the most reliable indicators of oxidative stress (20). However, the present findings suggested that other chemical pathways are involved in the metabolism of reactive oxygens and that the generated reactive oxygens might be captured via interaction with phototoxins, preventing the reaction with the chromogenic substrate. There also is the probability that non-oxidative photochemical intermediates may be formed under light exposure and that excited drug may

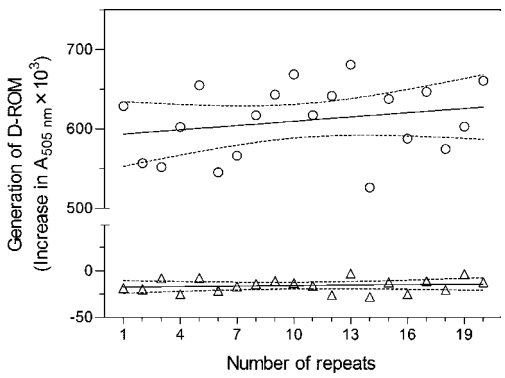

Fig. 2 Representative multiple measurement data used to calculate the Z'-factor for the D-ROM assay. Quinine as positive control (○) or sulisobenzone as negative control (Δ) at a concentration of 200 μM was dissolved in 0.1 M acetic acid/sodium acetate buffer (pH4.8) and exposed to simulated sunlight (250 W/m^2) for 1 min. Lines indicate mean ± 95% confidence interval.

Table I Intra-day and Inter-day (Day 1 and 3) Precision of D-ROM Assay

Quinine concentration (μM)	Generation of D-ROM (Increase in $A_{505\ nm} \times 10^3$)
Intra-day	
20	180 ± 12 (6.7)
200	620 ± 22 (3.6)
Inter-day	
20	168 ± 18 (10.6)
200	600 ± 33 (5.4)

Quinine (20 μM and 200 μM) was dissolved in 0.1 M acetic acid/sodium acetate buffer (pH 4.8) and exposed to UVA/B and Vis light (250 W/m^2) for 1 min. Data represent mean ± SD of three repeated experiments for intra-day ($n=12$) precision and six repeated experiments for inter-day precision ($n=24$). Values in parentheses are relative standard deviations.

Table II Photochemical and Photobiological Data on 20 Phototoxic and 5 Non-phototoxic Chemicals

Compounds (200μM)	D-ROM ($\Delta A_{505\ nm} \times 10^3$)	ROS data		DNA photocleavage (O.C.:% of total)	3T3 NRU PT (PIF)
		Singlet oxygen ($\Delta A_{440\ nm} \times 10^3$)	Superoxide ($\Delta A_{560\ nm} \times 10^3$)		
Photosensitizers					
5-Fluorouracil	19 ± 1	N.D.	2	5.2	1.0
8-Methoxy psoralen	6 ± 2	272	89	11.0	>74
Amiodarone	23 ± 4	365	N.D.	10.9	7.2
Carbamazepine	<1.0	N.D.	96	4.4	21.9
Chlorpromazine	121 ± 7	59	95	100.0	21.9
Diclofenac	<1.0	181	227	21.7	10.2
Doxycycline	<1.0	510	428	12.4	11.0
Furosemide	<1.0	519	135	10.3	1.0
Imipramine	<1.0	N.D.	100	8.1	1.0
Indomethacin	<1.0	13	121	6.2	1.0
Ketoprofen	151 ± 6	421	97	80.3	110
Nalidixic acid	349 ± 16	428	125	72.4	6.5
Naproxen	<1.0	306	131	34.2	3.7
Nitrofurantoin	<1.0	548	36	4.5	1.0
Norfloxacin	104 ± 13	411	126	23.3	>5.9
Omeprazole	77 ± 11	N.D.	156	52.0	2.7
Piroxicam	<1.0	542	84	5.9	1.0
Promethazine	280 ± 10	286	169	29.1	5.9
Quinidine	630 ± 29	673	115	88.7	29.9
Quinine	632 ± 40	686	124	85.5	15.7
Non-phototoxic chemicals					
Aspirin	7 ± 2	9	2	4.6	1.0
Benzocaine	<1.0	N.D.	25	4.1	1.0
Erythromycin	3 ± 2	N.D.	3	4.7	1.0
Phenytoin	<1.0	N.D.	15	3.5	1.0
Sulisobenzone	<1.0	N.D.	11	5.4	1.0

react with DEPPD directly. Thus, D-ROM assay has some possible limitations, since these photochemical reactions may yield either false positives or negatives.

On the basis of comparison with clinical information on drug-induced phototoxicity (2,32–34), the D-ROM assay might be less predictive of phototoxic risk than the ROS assay. However, the assay procedure for the D-ROM assay was highly simplified with two improvements. First, the D-ROM assay was designed for monitoring only one chromogenic substrate (DEPPD), which might offer reduced system complexity. In contrast, in the ROS assay that we proposed previously, determinations of both singlet oxygen and superoxide were necessary for reliable evaluation (12). Second, there was a marked reduction in screening run time compared with that in the ROS assay. Given these characteristics, especially highly improved throughput, the D-ROM assay might be useful for screening purposes in the drug discovery process.

Relatedness to DNA Photocleavage Assays

For further investigation of the relationship between D-ROM data and photogenotoxic potential, the pBR322 DNA-photocleaving activities of 25 model compounds were evaluated. Generally, DNA strand breaks can be readily observed by the structural conversion of supercoiled pBR322 DNA (SC) to the open circular (OC) form. In the present investigation, the conformation of photosensitized DNA cleavage products was analyzed by AFM (Fig. 3a). In AFM images of the irradiated pBR322 DNA without quinine, most DNA displayed the supercoiled form (Fig. 3a-I). In contrast, the majority of the irradiated DNA with quinine existed in the open circular form, reflecting a single-strand break in the DNA (Fig. 3a-II). The DNA-photocleaving activity of quinine (200 μM) was also analyzed by 0.8% agarose gel electrophoresis with EtBr staining (Fig. 3b). Irradiation of the plasmid DNA alone

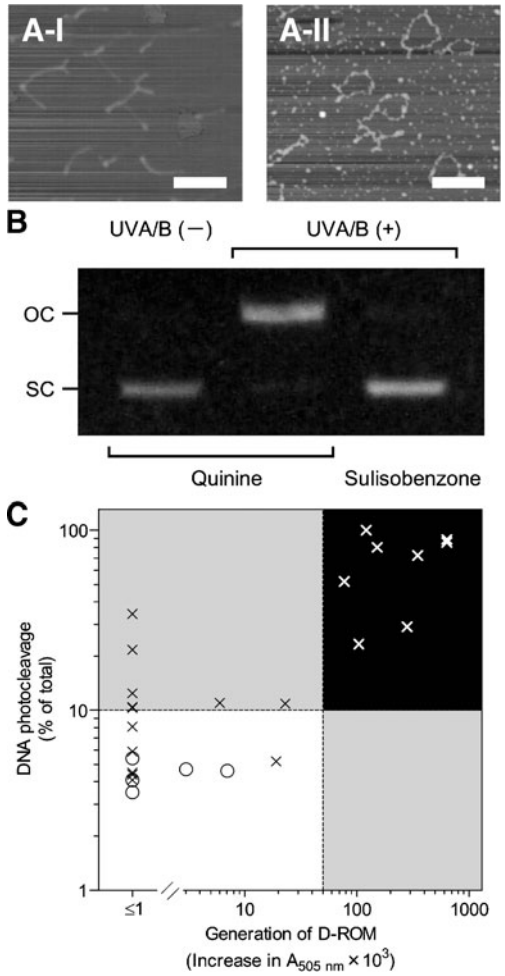

Fig. 3 DNA-photocleavage assay for predicting photogenotoxic potential. **a** AFM images from intact plasmid pBR322 DNA (A-I) and UV-exposed pBR322 DNA with quinine (200 μM; A-II). Scale bars represent 200 nm. **b** Photocleavage of plasmid pBR322 DNA by quinine. Supercoiled DNA was exposed to simulated sunlight (250 W/m^2) for 25 min with or without quinine or sulisobenzone (200 μM). Each pBR322 DNA sample was separated on 0.8% agarose gel and stained with ethidium bromide. OC, open circular form; and SC, supercoiled form. **c** A 2D plot of DNA-photocleavage *versus* D-ROM data for 25 compounds. ×, Phototoxic drugs; and ○, weak/non-phototoxic chemicals. According to tentative classification criteria, plot data were categorized into 3 regions; (1) shaded region, positive in both assays, (2) gray region, positive in only one assay, and (3) white region, negative in both assays.

with UVA/B and Vis light (250 W/m^2) for 25 min did not result in impairment of DNA (data not shown), and the addition of quinine (200 μM) to plasmid DNA also did not result in any structural conversion in the dark. However, significant DNA damage was caused by quinine after UV irradiation, although the DNA-photocleaving activity of sulisobenzone, a non-phototoxic chemical, was negligible under the same experimental condition. On the basis of its band intensity, there appeared to be a ca. 86% structural conversion of plasmid DNA after treatment with irradiated quinine.

The results of the AGE-based DNA photocleavage assay for other chemicals are summarized in Table II. Although non-phototoxic chemicals did not accelerate DNA photocleavage, photosensitizers tended to cause DNA damage upon light exposure. However, not all phototoxic drugs induced photodynamic impairment of DNA. In particular, no significant photocleaving activities were seen for 5-FU, carbamazepine, indomethacin, nitrofurantoin, and piroxicam. For further comparison, the results from DNA-photocleavage and D-ROM assays were analyzed on a 2D plot of DNA impairment *versus* D-ROM generation for various pharmaceutical substances (Fig. 3c). The plot data were categorized into 3 regions with tentative classification criteria: 10% DNA damage in DNA-photocleavage assay and 5×10^{-2} AU for D-ROM assay. Compounds in the shaded region were predicted as high risk in both assays. Chemicals in gray regions were predicted to have phototoxic risk by only one assay. Phototoxic/photogenotoxic risk was considered negligible for chemicals lying in the white region. Of all tested compounds, only 6 chemicals (24% of the total), including 8-MOP, amiodarone, diclofenac, doxycycline, furosemide and naproxen, can be found in the gray regions. From these findings, the D-ROM assay was shown to predict drug-induced DNA damage, reflecting photogenotoxic potential, with a prediction accuracy of 76%.

UV absorption usually generates singlet excited states. Although they are too short-lived to react chemically, more stable excited triplet states are sometimes formed by intersystem crossing, leading to direct and/or indirect DNA damage (2). In the photogenotoxic pathways, there are at least three direct mechanisms (35). First, photo-excited species sometimes induce direct DNA damage by covalent binding, resulting in formation of photoadducts. Second, an excited molecule can transfer the excitation energy to DNA, leading to pyrimidine dimer formation as observed upon direct DNA excitation. Last, photo-excited chromophore induces a one-electron or hydrogen abstraction, and the resulting DNA damage appears to consist mostly of oxidative guanine modification. In addition to these direct mechanisms of DNA damage by excited photoreactive chemicals, there are also at least two indirect mechanisms, which include (1) reactive oxygens-mediated DNA impairment and (2) generation of reactive decomposition products. Thus, possible mechanisms for photochemical genotoxicity can be quite complex and may involve a series of chemical reactions. All phototoxins might not always cause reactive oxygens-mediated photogenotoxic reactions, so that some phototoxins could not be captured by D-ROM assay. This might partially explain the data discrepancy between DNA-photocleavage and D-ROM assays.

Relatedness to 3T3 Neutral Red Uptake Phototoxicity Test

The 3T3 NRU PT was developed and validated under the auspices of ECVAM from 1992–1997 to establish a valid *in vitro* alternative to the various *in vivo* tests in use (36). The test is now accepted by the European Union commission and member states as being necessary for all compounds showing absorbance of UVA and Vis light (37). The 3T3 NRU PT assesses the cytotoxic effect of a test substance after exposure to a non-cytotoxic dose of UVA light compared with that in the absence of exposure, and the cytotoxicity is expressed as a concentration-dependent reduction in the uptake of Neutral Red. In this investigation, the viability curves of model compounds with or without irradiation were determined up to 1 mg/mL. Fig. 4a shows representative cell viability curves of the 3T3 cells after exposure to quinine and sulisobenzone. With respect to the quinine-treated group, upon irradiation, the cell viability was shifted to considerably lower concentrations. The EC_{50} values with and without UVA irradiation were 9.7 μg/mL and 150 μg/mL, respectively. These values produced a PIF of 15.7 for quinine. In contrast, the cell viability curve for 3T3 cells after exposure to irradiated sulisobenzone was almost identical to that without UVA exposure. Thus, the PIF value of sulisobenzone was calculated to be 1.0. Generally, PIF values are effective for distinguishing phototoxic molecules (PIF > 5) from non-phototoxic molecules, but are actually unable to correctly distinguish between mildly or probably phototoxic molecules (2 < PIF < 5) and non-phototoxic molecules (PIF < 2). On the basis of the classification criteria of PIF value, quinine was found to be phototoxic. In addition to quinine and sulisobenzone, the phototoxic potentials of other chemicals were also assessed by 3T3 NRU PT for further comparison (Table II). Of all phototoxic drugs, 14 phototoxic drugs (70% of all phototoxins) exhibited a potent phototoxic effect on cells as evidenced by a high PIF value (>2). However, some known phototoxic drugs, such as 5-FU, furosemide, imipramine, indomethacin, nitrofurantoin, and piroxicam, showed no significant transition in viability curves with or without UVA irradiation. Their PIF values (ca. 1.0) suggested a low phototoxic potential, and these findings were inconsistent with the results from DNA-photocleavage and D-ROM assays. On the basis of the present 3T3 NRU PT data and the adverse event information on the model chemicals listed in drug package inserts and several manuscripts (1,2,34,38), the prediction accuracy of 3T3 NRU PT was calculated to be 76%. The limited predictability of 3T3 NRU PT is not surprising since bioavailability and biokinetics can not be modeled in the assay, and it may result in the lack of *in vivo-in vitro* correlation (4).

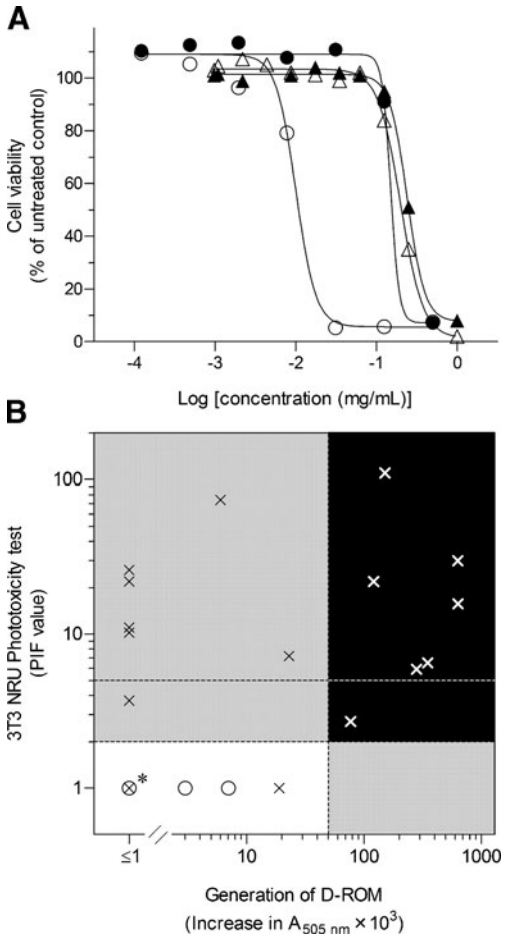

Fig. 4 *In vitro* phototoxicity assessment. **a** Phototoxicity of tested compounds in the 3T3 NRU PT. The 3T3 cells were treated with different concentrations of quinine or sulisobenzone, and irradiated with UVA light (50 kJ/m^2). Each value represents the mean of 6 replicates. ●, quinine without UVA (control); ○, quinine with UVA; ▲, sulisobenzone without UVA (control); and Δ, sulisobenzone with UVA. **b** A 2D plot of 3T3 NRU PT data *versus* D-ROM data for 25 compounds. ×, Phototoxic drugs; and ○, weak/non-phototoxic chemicals. According to tentative classification criteria, plot data were categorized into 3 regions; (1) shaded region, positive in both assays, (2) gray region, positive in only one assay, and (3) white region, negative in both assays. *, Overlapped symbols of 5 phototoxic and 3 non-phototoxic chemicals.

According to the 2D plot analysis of D-ROM and 3T3 NRU PT data (Fig. 4b), tested chemicals were categorized into three regions: 11 chemicals in the white region (both negative), 7 chemicals in the gray region (only one positive), and 7 chemicals in the shaded region (both positive). There is an empirical correlation of 72% between these two assays, and the exact reasons for the discrepancy are not fully understood. The 3T3 NRU PT can only use irradiation in the UVA, since UVB light is highly cytotoxic to the Balb/c 3T3 cells. Although UVB wavelengths are excluded in the 3T3 NRU PT, these wavelengths are an integral part of solar radiation responsible for photochemical reactions. Herein, the difference in irradiation condition might be a

part of the reason for data discrepancy between the 3T3 NRU PT and D-ROM assay. In addition, some phototoxins can induce phototoxic skin responses via reactive oxygens-independent pathways, and they may bind with lipids and proteins, as well as DNA (2). Photo-induced reactions with these biomolecules are important to the development of phototoxic and/or photoallergic responses (1). D-ROM assay can not completely predict the phototoxic risk as long as the photochemical reaction is reactive oxygens-independent. Further clarification will be helpful for understanding the limitations of the D-ROM assay and avoiding misleading data.

Previously, Lynch and co-workers demonstrated the link between phototoxicity and photoreactivity, and they proposed 3 key photochemical reactivity parameters, including the production of singlet oxygen, the production of superoxide, and the chemical photostability (11). Based on these findings, the photochemical reactivity assays may provide a good predictor of phototoxic liability *in vitro*. The extension of the photostability testing to assess potential phototoxic responses has already been included in the risk assessment strategy by the CPMP guidance on photosafety testing. In contrast, Henry and co-workers demonstrated that photostability testing alone was an inadequate predictor of possible photosafety liabilities, although the measurement of light absorption seemed to be a contributing part of an overall pre-clinical photosafety risk assessment process (4). Although the most reliable photoreactivity parameters are still under discussion, combination use of the efficacious photoreactive parameters may be useful for prediction of potential photosafety issues. Attempts to increase the predictability of D-ROM assay may need a further knowledge of the complex photochemical reaction pathways, and the combination use of D-ROM assay and other reliable predicting tools may provide a more reliable risk assessment strategy for the screening of the phototoxic potential of new drug entities in early development.

CONCLUSION

In the present investigation, an optimized D-ROM assay system was proposed for predicting the phototoxic potential of pharmaceutical substances. The photochemical and phototoxic behaviors of 25 model compounds were assessed by the ROS assay, the DNA-photocleavage test, and the 3T3 NRU PT, as well as the D-ROM assay, for comparison. The results from the D-ROM assay did not completely correlate with the ROS data; however, the D-ROM assay was partly indicative of photogenotoxic risk, as identified by the DNA photocleavage test, and phototoxic potential, as proposed by the 3T3 NRU PT, with prediction accuracies of 76 and 72%, respectively. These outcomes, taken together with the high-throughput contributing to highly reduced screening run time, suggest the usefulness of the D-ROM assay for identifying phototoxic potential and avoiding undesired side effects in the early stages of drug discovery.

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