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Short communication

High-throughput screening strategy for photogenotoxic potential of pharmaceutical substances using fluorescent intercalating dye

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ABSTRACT

The aim of the present study was to provide an intercalator-based photogenotoxicity (IBP) assay as a high-throughput *in vitro* screening system for predicting the photogenotoxic potential of pharmaceutical substances. The conditions of the high-throughput IBP assay using thiazole orange (TO), a fluorescent intercalating dye, were optimized and validated by a fluorescence titration experiment and reproducibility/robustness test. The IBP assay was applied to 27 phototoxic and 5 weak/non-phototoxic commercially available compounds, and other phototoxicity screenings were also carried out for comparison; these included the reactive oxygen species (ROS) assay for overall phototoxic potential and the DNA-photocleavage assay for photogenotoxic risk. According to the results from the comparative experiments, a decreased level of intercalated TO in the IBP assay could theoretically be related to the DNA-photocleaving behaviors of phototoxic drugs, but not to their ROS-generating abilities. The IBP assay could predict the photodynamic DNA impairment caused by irradiated drugs with a prediction accuracy of 78%. These findings suggest that the IBP assay could be a fast and reliable tool for predicting the photogenotoxic potential of a large number of drug candidates at early stages of drug discovery.

1. Introduction

Drug-induced phototoxic skin responses are elicited after the exposure of skin to topical or systemic administration of pharmaceutical substances, triggered by exposure to sunlight [1]. There are at least three types of drug-induced phototoxic reactions, including photoirritation, photoallergy, and photogenotoxicity, the mechanisms and pathologic features of which are quite different [2]. To avoid side effects at an early phase of the drug discovery process, our group previously proposed a reactive oxygen species (ROS) assay for evaluating the phototoxic potential of pharmaceutical substances [3]. However, the ROS assay might lack specificity for three types of phototoxic reactions, since phototoxic compounds do not always exhibit all phototoxic reactions [4–6]. Therefore, other efficacious screening strategies should be developed to predict each type of phototoxic response in more detail.

Over the past few years, the development of effective methodologies to evaluate photogenotoxicity has been attempted, and a number of screening methods for recognizing photogenotoxic drugs have been suggested [7,8]. Our group also proposed novel *in vitro* tools for assessing photogenotoxic risk using capillary gel electrophoresis (CGE) analysis [9] and DNA-binding assay as a second screening tool following ROS assay [10]. Although these assays proposed possible mechanisms of drug-induced photogenotoxicity, some technical problems were encountered, most notably limited throughput in early stages of drug discovery. Improvement of the photogenotoxicity assay is necessary in terms of enhancing reliability and productivity, which could possibly lead to the development of a novel high-throughput screening system. Previously, ethidium bromide (EtBr), an intercalating dye, was used as a fluorescent probe to demonstrate the metal-catalyzed impairment of DNA in the base-pair region [11]. Theoretically, the photogenotoxic potential of photosensitizers is also predictable by intercalator-based screening, since oxidative DNA damage is observed upstream of drug-induced photogenotoxicity [2]. An intercalator-based screening system for oxidative DNA damage using multiwell plates might be efficacious for screening a number of new drugs with high reproducibility, although the assay has never been applied to photogenotoxicity prediction.

The present study aimed to provide an intercalator-based photogenotoxicity (IBP) assay as a more effective and streamlined *in vitro* assessment tool for predicting drug-induced photogenotoxicity. For evaluating the drug-induced impairment of DNA, thiazole orange (TO), a highly sensitive intercalating dye, was used as a fluorescent probe in the IBP assay. TO itself is virtually non-fluorescent, however, the intercalation of DNA with TO was found to result in a 3000-fold enhancement of fluorescence [12,13]. The assay systems were optimized and validated using ketoprofen as a model com-

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pound with focus on irradiation conditions and concentration of assay mixture. The IBP assay system was applied to 27 photosensitizers and 5 non-phototoxic compounds, and the data obtained were also compared with the results from a ROS assay and an agarose gel electrophoresis (AGE)-based DNA-photocleavage assay to clarify the predictability of the IBP assay.

2. Materials and methods

2.1. Chemicals

All phototoxic and weak/non-phototoxic compounds were purchased from Sigma (St. Louis, MO, USA), Wako Pure Chemical Industries (Osaka, Japan), or Tokyo Chemical Industry (Tokyo, Japan). Salmon sperm DNA, plasmid pBR322 DNA, *p*-nitrosodimethylaniline (RNO), imidazole, nitroblue tetrazolium (NBT), disodium hydrogenphosphate, sodium dihydrogenphosphate dihydrate, and thiazole orange (TO) were bought from Wako Pure Chemical Industries. Ethidium bromide (EtBr) and agarose L03 were purchased from Nippon Gene (Toyama, Japan) and Takara Bio (Shiga, Japan), respectively.

2.2. Fluorescence titration

A fluorescence titration experiment was carried out to optimize the TO concentration for the IBP assay. In a 96-well microplate (AGC Techno Glass, Chiba, Japan), 10 μL of DNA solution (100 μg/mL), dissolved in 20 mM sodium phosphate buffer (NaPB, pH 7.4), was mixed with 20 μL of 20 mM NaPB (pH 7.4) and 70 μL of TO at various final concentrations, ranging from 0.01 to 3 μM. The assay mixtures were incubated at 37 °C for 15 min. After equilibration, the fluorescence (excitation, 509 nm; and emission, 527 nm) of each mixture (100 μL) in the 96-well microplate was measured with SAFIRE (TECAN, Männedorf, Switzerland).

2.3. Irradiation conditions

Each tested compound was stored in an Atlas Suntest CPS+ solar simulator (Atlas Material Technology LLC, Chicago, USA) equipped with a xenon arc lamp (1500 W). A UV special filter and a 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 250 W/m^2.

2.4. Intercalator-based photogenotoxicity (IBP) assay

The photodynamic impairment of salmon sperm DNA by phototoxic chemicals was evaluated by IBP assay. Each assay mixture (50 μL) in the 96-well microplate, containing the tested compound (200 μM) and DNA (20 μg/mL) in 20 mM NaPB (pH 7.4), was irradiated with UVA/B for 45 min, and then TO was added to each well at a final concentration of 1.3 μM. As a control experiment, only 40 μL of the tested compound in 20 mM NaPB (pH 7.4) was exposed to UVA/B, and then DNA (10 μg/mL) and TO (1.3 μM) were added to the sample. In both irradiation and control experiments, each assay mixture (100 μL) was incubated at 37 °C for 15 min to equilibrate intercalation of DNA with TO. To detect the intercalated TO, fluorescence (excitation: 509 nm and emission: 527 nm) was measured with SAFIRE.

2.5. Determination of reactive oxygen species (ROS)

Both singlet oxygen and superoxide generated from irradiated chemicals were measured as we reported previously [14,15]. Briefly, to monitor the generation 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, and then the UV absorption at 440 nm was measured by SAFIRE. For the determination of superoxide, samples containing the compounds under examination and nitroblue tetrazolium (NBT, 50 μM) in 20 mM NaPB (pH 7.4) were irradiated with UVA/B, and the reduction of NBT was measured by the increase in the absorbance at 560 nm using SAFIRE.

2.6. DNA-photocleavage assay

The irradiated samples contained pBR322 DNA (final concentration, 10 μg/mL) dissolved in Tris–acetic acid–EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) and the tested compounds at a final concentration of 200 μM. Samples were exposed to UVA/B (375 kJ/m^2). Irradiated plasmid pBR322 DNA was separated by electrophoresis (0.8% agarose gel in TAE buffer), visualized with EtBr staining, and analyzed with image analyzing software Image J.

2.7. 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 discussion

3.1. Development of IBP assay

Some fluorescent intercalating dyes, such as EtBr and TO, form an intense fluorescent complex with DNA [11,13]. Interestingly, the fluorescent yield is reduced upon DNA denaturation by oxidative stress and becomes very weak when intramolecular hydrogen bonds in single strands are further destabilized [16]. Thus, the intercalating behavior of DNA-specific fluorescent probes should reflect oxidative-stress-induced alterations in the DNA base-pair region. In the present study, to evaluate DNA damage due to photoirradiated compounds, an IBP assay was developed with the use of fluorescent intercalating dyes. TO was chosen for the IBP assay because of a 150-fold higher sensitivity than EtBr. Upon a fluorescence titration experiment, the binding constant of TO with DNA was calculated to be as low as 360 nM. To evaluate DNA impairment with a high sensitivity, the concentration of TO in the IBP assay was set at 1.3 μM, equivalent to the concentration at 80% of maximum binding (data not shown). In addition to the TO concentration, other conditions for the IBP assay, such as UVA/B irradiation time and concentration of compounds, were also optimized using ketoprofen, a typical phototoxic drug. After concomitant exposure of ketoprofen and DNA to UVA/B for the indicated periods, TO was added to the assay mixture to monitor the intercalating behavior (Fig. 1A). Although no significant changes in the intercalating behavior of TO were observed as long as samples were protected from light, the UVA/B exposure of ketoprofen and DNA resulted in a marked reduction in TO-intercalating capacity. The reduction in intercalated TO is indicative of oxidative impairment of DNA, and the photodynamic DNA damage appeared to be saturated at 45 min. According to the concentration response curve (Fig. 1B), only a slight reduction in intercalated TO was observed at 300 μM ketoprofen without UV exposure, whereas irradiated ketoprofen at just over 30 μM caused significant DNA damage.

3.2. Assay precision and robustness

The overall precision of the method was evaluated by analyzing the photogenotoxic potential of ketoprofen standard solution

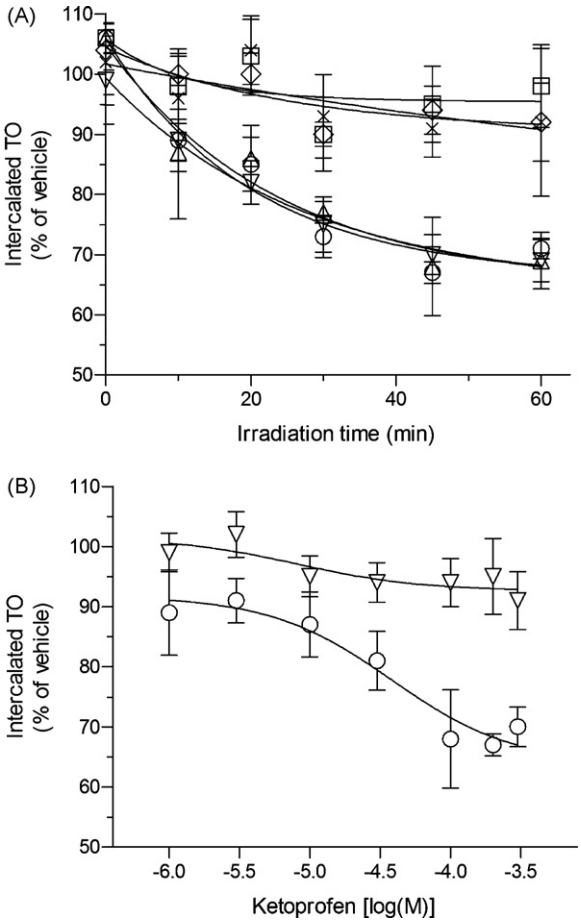

Fig. 1. DNA damage induced by ketoprofen. (A) Time-dependent damage of salmon sperm DNA. Irradiated group: △, 100 μM; ○, 200 μM; and ▽, 300 μM; and control group: ◊, 100 μM; □, 200 μM; and ×, 300 μM. (B) Ketoprofen-induced DNA damage occurred in a dose-dependent manner. Ketoprofen was irradiated with UVA/B in the presence or absence of salmon sperm DNA. ○, Irradiated data; ▽, control data. Data represent mean ± SD of four experiments.

at 100, 200, and 300 μM under 45-min UV exposure. The intra-day precision (%RSD, $n = 3$) and inter-day precision (days 1 and 2%RSD, $n = 6$) are shown in Table 1. The intra-day %RSD values for irradiated and control experiments ranged from 2.98 to 6.86 and 5.12 to 5.51, respectively, and the inter-day %RSD values varied from 5.91 to 6.65 (irradiated group) and 4.97 to 5.27 (control group). On the basis of the data for ketoprofen at 100–300 μM, the IBP assay had potent intra- and inter-day precision, and the concentration of

Table 1
Intra-day and inter-day precision for DNA damage by irradiated ketoprofen.

Concentration (μM)	Intercalated TO [% of vehicle, mean ± SD (%RSD)]	
	Irradiated	Control
Intra-day		
100	72.9 ± 5.00 (6.86)	96.0 ± 4.92 (5.12)
200	76.4 ± 2.27 (2.98)	93.5 ± 4.87 (5.21)
300	76.2 ± 3.93 (5.16)	92.2 ± 5.08 (5.51)
Inter-day		
100	71.6 ± 4.67 (6.53)	95.4 ± 4.74 (4.97)
200	74.0 ± 4.37 (5.91)	93.9 ± 4.91 (5.22)
300	75.3 ± 5.01 (6.65)	93.5 ± 4.93 (5.27)

Ketoprofen (200 μM) and salmon sperm DNA (20 μg/mL) were co-exposed to UVA/B light (675 kJ/m^2) or protected from light. TO (1.3 μM) solution was added to the assay mixture, and fluorescence emitted from the intercalated TO was measured. Data represent mean ± SD of three experiments for intra-day precision and six experiments for inter-day precision.

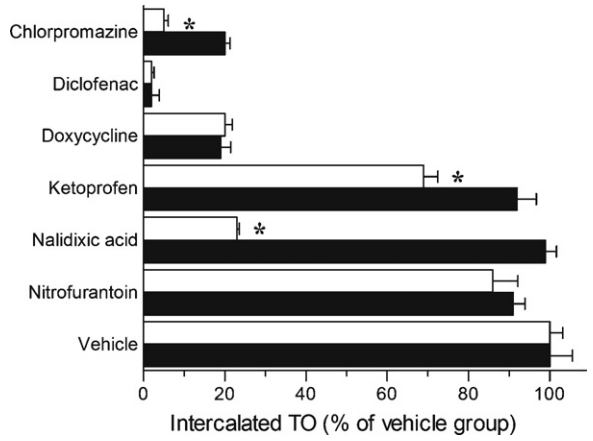

Fig. 2. DNA damage by irradiated phototoxic drugs. Each drug (200 μM) and salmon sperm DNA (20 μg/mL) were co-exposed to UV or protected from light. TO solution at a final concentration of 1.3 μM was added to the assay mixture, and fluorescence emitted from the intercalated TO was measured. Open bar, UV-irradiated; and filled bar, control group. Data represent mean ± SD of four experiments. *$P < 0.05$, significantly different from control.

tested chemicals for the IBP assay was set at 200 μM. Further evaluation on robustness of the IBP assay was also made with emphasis on the influences of co-existing solvents in the assay mixtures and equilibration temperature. By the use of stock solution of tested chemicals, small quantity of organic solvent could be involved in the assay mixture, and this might lead to variable outcomes. However, there were no significant differences among the results from IBP assay of ketoprofen (200 μM) with or without organic solvents (1%) such as dimethyl sulfoxide (DMSO), acetonitrile, methanol, and ethanol (data not shown). In addition, transition of equilibration temperature (25–40 °C) did not affect the IBP data (data not shown).

3.3. Application of IBP assay to known phototoxic/non-phototoxic drugs

The IBP assay was carried out on 27 phototoxic and 5 weak/non-phototoxic commercially available drugs to clarify the predictability and usefulness of the IBP assay. According to the results from the IBP assay on several phototoxic drugs (Fig. 2), ketoprofen, nalidixic acid, and nitrofurantoin did not affect the intercalating behavior of TO without UV exposure. The UV irradiation of ketoprofen and nalidixic acid in the presence of DNA resulted in 23 and 76% reductions in the level of intercalated TO, respectively, although nitrofurantoin was found to be less photogenotoxic as evidenced by the lack of a significant difference in the intercalation of TO between control and irradiated groups. These data were consistent with previous observations on CGE analyses, which showed that both ketoprofen and nalidixic acid could cause photocleavage of plasmid pBR322 DNA, but nitrofurantoin did not [9]. The IBP assay could be applied to most test compounds; however, some drugs, including chlorpromazine, diclofenac, and doxycycline, affected the intercalating behavior of TO without light exposure, leading to a narrow screening window. The decrease in intercalated TO levels might be attributable to drug-induced quenching of fluorescence from DNA-TO complexes and/or strong intercalation between DNA and the drugs. On the basis of the decrease in intercalated TO levels, chlorpromazine might have photogenotoxic potential; however, doxycycline did not seem to be photogenotoxic. It would be very challenging to evaluate the photogenotoxic risk of diclofenac precisely, because of the very limited screening range. Thus, the IBP assay might not be suitable for some chemicals that lack an adequate assay range.

Table 2
Photochemical and photogenotoxic data on phototoxic compounds.

Compounds	ROS generation[a]		DNA photocleavage (OC form, %)	Decrease in intercalated TO (% of vehicle)
	$^1O_2$ ($\Delta A_{440} \times 10^3$)	O_2^- ($\Delta A_{560} \times 10^3$)		
Phototoxic				
5-FU	5 ± 3	N.D.	5.2	N.D.
8-MOP	31 ± 7	51 ± 3	11.0	1
Acetazolamide	19 ± 1	N.D.	2.4	4
Amitriptyline	4 ± 2	8 ± 10	9.9	1
Carbamazepin	7 ± 1	N.D.	4.4	6
Chlorpromazine	N.D.	113 ± 1	100	15*
Clomipramine	60 ± 4	11 ± 1	91.5	24*
Diazepam	12 ± 4	N.D.	4.3	2
Diclofenac	181 ± 7	370 ± 14	21.7	N.D.
Doxycycline	131 ± 8	344 ± 6	12.4	N.D.
Flutamide	25 ± 9	N.D.	10.6	11*
Furosemide	139 ± 5	111 ± 16	10.3	4
Imipramine	21 ± 1	16 ± 1	8.1	15*
Indomethacin	8 ± 4	186 ± 16	N.D.	N.D.
Ketoprofen	348 ± 12	45 ± 5	73.9	23*
Methotrexate	206 ± 17	425 ± 27	8.2	53*
Nalidixic acid	132 ± 2	231 ± 2	72.4	76*
Naproxen	172 ± 7	207 ± 2	34.2	53*
Nitrazepam	174 ± 9	49 ± 5	34.2	61*
Nitrofurantoin	72 ± 7	15 ± 1	4.5	5
Omeprazole	25 ± 7	150 ± 1	52.0	15*
Oxytetracycline	257 ± 21	275 ± 18	6.5	N.D.
Piroxicam	83 ± 7	96 ± 9	5.9	5
Promethazine	52 ± 4	34 ± 2	29.1	18*
Quinine	376 ± 11	240 ± 6	85.5	38*
Tetracycline	203 ± 29	169 ± 13	10.4	6
Tolbutamide	1 ± 6	N.D.	3.3	8
Weak/non-phototoxic				
Aspirin	9 ± 2	N.D.	4.6	2
Benzocaine	N.D.	4 ± 0	N.D.	1
Erythromycin	N.D.	5 ± 0	4.7	3
Phenytoin	11 ± 8	8 ± 1	3.5	5
Sulisobenzone	N.D.	N.D.	5.4	5

N.D.: not detected.
[a] Data represent mean ± SD for three experiments.
* $P < 0.05$, significantly different from control.

The results from the IBP assay on other drugs are summarized in Table 2, and the levels of photodynamic DNA damage by tested chemicals were estimated by decreases in the level of TO intercalation. Of all the drugs tested, 15 phototoxic compounds and all the weak/non-phototoxic chemicals were found to be less photogenotoxic. However, 12 phototoxic drugs exhibited photodynamic DNA damage, which was indicative of photogenotoxic risk.

3.4. Experimental comparative study of IBP and ROS assays

Generally, photosensitizers generate ROS, such as superoxide and singlet oxygen, after exposure to UVA/B, and the ROS act as principal toxic mediators and attack biomolecules including phospholipids, proteins, and DNA [2]. Therefore, the availability of ROS data would be efficacious to realize the potential of all phototoxic reactions including photoirritation, photoallergy, and photogenotoxicity [3,8]. In the present investigation, the ROS assay was also carried out on 27 phototoxic and 5 weak/non-phototoxic drugs (Table 2). Nine phototoxic compounds, including 8-MOP, diclofenac, doxycycline, furosemide, indomethacin, nitrofurantoin, oxytetracycline, piroxicam, and tetracycline, exhibited significant ROS generation, whereas they were predicted to be non-photogenotoxic in the IBP assay. We found no clear relationship between ROS and IBP data directly. Theoretically, the ROS assay could be used to evaluate all types of phototoxic risk, although the IBP assay could be indicative of photogenotoxic potential specifically. This might partially explain the discrepancy between ROS and IBP data.

3.5. Experimental comparative study of IBP and DNA-photocleavage assays

The photogenotoxic potential of 32 model compounds was evaluated from pBR322 DNA-photocleaving activities for comparison, as well as from the newly developed IBP assay. Generally, DNA strand breaks can be readily observed by following the conversion of supercoiled pBR322 DNA (SC) to the open circular (OC) form. The DNA-photocleaving activity of ketoprofen (200 μM) was analyzed by AGE (Fig. 3A). The addition of ketoprofen to plasmid DNA did not result in DNA photocleavage in the dark; however, pBR322 DNA damage was clearly induced by ketoprofen after UVA/B irradiation. On the basis of its band intensity, there appeared to be a ca. 74% structural conversion of plasmid DNA from the SC to the OC form.

DNA-photocleaving activities of other chemicals were determined and are summarized in Table 2. No significant structural conversion of plasmid DNA was observed in negative control groups. Interestingly, not all phototoxic drugs induced photodynamic impairment of DNA. These findings were partly consistent with the results from the IBP assay, although the order of DNA damage severity observed in the AGE analysis did not completely correspond to that in the IBP assay. For further comparison, the results from the DNA-photocleavage and IPB assays were ana-

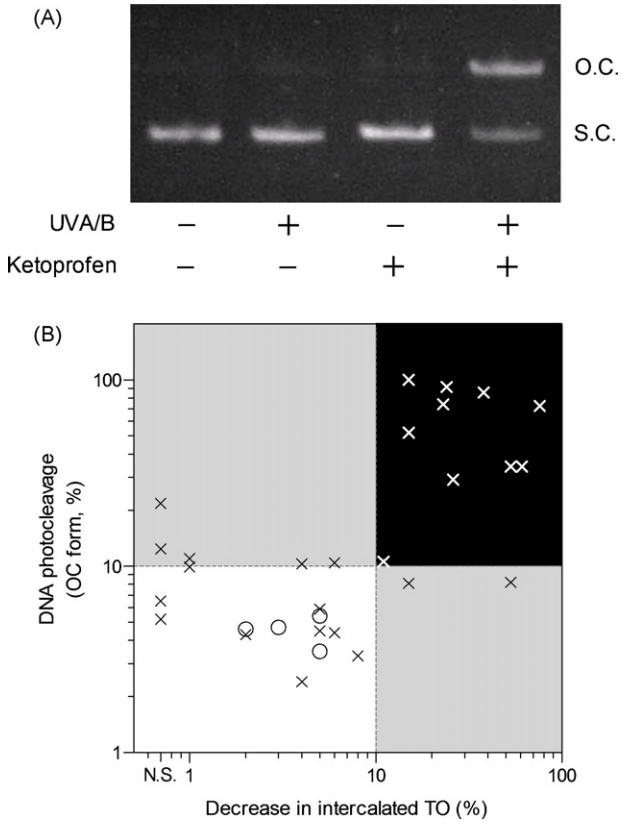

Fig. 3. Photodynamic impairment of DNA induced by irradiated photosensitizers. (A) Ketoprofen-induced photocleavage of plasmid pBR322 DNA. Supercoiled DNA was exposed to UV with or without ketoprofen. EtBr-stained 0.8% agarose gels are shown. OC, open circular form, and SC, supercoiled form. (B) A 2D plot of DNA-photocleavage versus IBP data for 32 compounds. ×, phototoxic drugs; and ○, weak/non-phototoxic drugs. According to tentative classification criteria, plot data were categorized into three regions: (1) shaded region, positive in both assays, (2) gray region, positive in only one assay, and (3) white region, negative in both assays.

lyzed on a 2D plot of generated OC form versus reduced TO intercalation for various pharmaceutical substances (Fig. 3B). With tentative classification criteria (10% DNA damage) to discriminate photogenotoxins from non-photogenotoxic chemicals, plot data were categorized into three regions. Thus, compounds in the shaded region were predicted to be photogenotoxic in both assays, and those predicted to be photogenotoxic by only one assay are plotted in the gray regions. The chemicals for which the photogenotoxic risk might be negligible are in the subthreshold white region. Of all tested compounds, only seven chemicals (22% of the total) were present in the gray regions, that is, they showed a discrepancy between the data from DNA-photocleavage and IBP assays. DNA-photocleavage assay could indicate the impairment of DNA directly, although IBP assay was indicative of decrease in TO-intercalating capacity, reflecting the oxidative DNA damage. The different measuring systems might lead to the data discrepancy between themselves. The 2D-plot analysis suggested that the IBP assay could predict drug-induced DNA damage, reflecting the photogenotoxic potential, with a prediction accuracy of 78%.

Comparative studies of the IBP and DNA-photocleavage assays demonstrated possible limitations of the IBP assay. There is the possibility that the emission of fluorescence from intercalated TO is sometimes quenched by tested compounds or their photodegradants, leading to a limited screening window and misleading results. In the present study, only diclofenac exhibited an extremely limited screening window, suggesting that the photogenotoxic risk of diclofenac might be unpredictable using the IBP assay. For the avoidance of misleading information, the level of intercalated TO in control groups should be compared with that in vehicle groups, which would enable the detection of compounds unsuitable for the IBP assay. However, the IBP assay exhibited some advantages compared with the DNA-photocleavage assay and other photogenotoxic assessment tools such as the CGE analysis [9] and the DNA-binding assay [10]. First, the use of multiwell plates enables the IBP assay to be used to evaluate large numbers of compounds at the same time and to simplify the methodology in preparation and data processing. Second, there is a marked reduction in screening run time compared with that in DNA-photocleavage assay and CGE analysis, because of no electrophoretic process. Last, the DNA-binding assay is not indicative of photogenotoxic risk without ROS data, although the IBP assay does not need them for the risk assessment.

These findings, taken together with those from previous study on ROS assay strategies, indicate that the IBP assay can be employed for detecting the photogenotoxic potential of phototoxic compounds as a 2nd screening tool following the ROS assay, and that this strategy gives more precise and specific prediction of drug-induced photogenotoxicity. The combination of these simplified assay systems would be suitable for evaluating a large number of pharmaceutical candidates and especially effective in early stages of drug discovery.

4. Conclusion

The IBP assay was newly developed for predicting the photogenotoxic potential of pharmaceutical substances. The new assay strategy was found to be more convenient than the prediction tools that we had proposed previously, including the AGE- or CGE-based DNA-photocleavage assay and the DNA-binding assay, with simplified procedures and improved throughput. In this study, we evaluated the photochemical and phototoxic behaviors of 32 model compounds using the ROS assay, the DNA-photocleavage assay, and the IBP assay. Although the results from the IBP assay did not completely correlate with ROS data, the IBP assay exhibited a 78% prediction precision for the oxidative impairment of DNA caused by irradiated drugs. These findings suggest the usefulness of the IBP assay for identifying photogenotoxic risk and avoiding undesired side effects in the early stages of pharmaceutical development. The new assay can be used for screening purposes, and further accumulation of data will allow us to estimate practical classification criteria to identify photogenotoxic chemicals more precisely.

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