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In Vitro Assays to Predict Phototoxicity of Chemicals: (II) Yeast Growth Inhibition Assay and Battery System with Photohemolysis Assay

Mariko Sugiyama, Hiroshi Itagaki and Shinobu Kato

Shiseido Safety & Analytical Research Center, 1050 Nippa-cho, Kohoku-ku, Yokohama, Japan, 223

Summary

The yeast growth inhibition assay, which is based on photodynamic reactions of compounds with cell organelles or DNA, was validated as possible alternative to animal testing to predict phototoxicity of chemicals after optimization of various factors. The data for 24 chemicals (8 fragrances, 5 UV absorbers, 4 drugs, 4 antimicrobials and 3 dyes) were compared with *in vivo* phototoxicity test results in guinea pigs. The sensitivity, specificity, positive predictive value, negative predictive value and equivalence of the *in vitro* test were 89%, 80%, 73%, 92% and 81%, respectively. Three chemicals were identified as false positive compounds, and one was identified as a false negative in the yeast growth inhibition assay. Although an excellent correlation was obtained between *in vivo* studies and yeast growth inhibition assay, a false negative result was still observed, so the combination of the photohemolysis test, which we have already reported, and the yeast test was used as a battery system. The battery stystem had a sensitivity of 100%, a specificity of 67%, a positive predictive value of 64%, a negative predictive value of 100% and an equivalence of 77%. Because of the sensitivity of 100%, this battery system, which is composed of two methods based on different mechanisms, is considered to be useful as a screening tool for predicting phototoxic potential of new chemicals, as an animal test alternative.

Key Words: *in vitro* test, yeast growth inhibition, phototoxicity, battery system.

Correspondence to: Mariko Sugiyama
Shiseido Safety & Analytical Research Center, 1050, Nippa-cho, Kohoku-ku, Yokohama, Japan, 223
Tel: (Japan) 045-542-1339
Fax: (Japan) 045-546-4456

Introduction

We have been investigating alternative methods to predict phototoxicity. In our previous report[1], the red blood cell (RBC) photohemolysis assay was shown to be a reliable tool because the results obtained correlated well with *in vivo* data. We concluded, however, that the photohemolysis technique alone is insufficient, because psoralens, which are a group of compounds well known to be phototoxic, did not cause photohemolysis, in accordance with the results of Freeman[2], Kahn and Fleischaker[3] and Johnson et al.[4]. This result suggested that phototoxicity of psoralens is not based on cellular membrane damage, so we looked for a suitable *in vitro* method based on intracellular changes in microorganisms.

In 1965, Daniels[5] demonstrated growth inhibition of yeast cells by suspected phototoxins upon exposure to UVA light. The yeast inhibition assay is an useful *in vitro* method in general, because it is simple, fairly rapid and inexpensive, and is especially sensitive to reactions involving DNA damage. The yeast

inhibition assay as an *in vitro* method for phototoxicity prediction has been reviewed previously[4,6]. Kavli and Volden[7] reported in detail the procedures and rationale of the *Candida* test for phototoxicity. Although the *Candida* phototoxicity test, introduced by Daniels, has been used by several investigators as a simple method, Kagan and Gabriel[8] warned against the use of a pathogenic microorganism for experiments and recommended the non-pathogenic yeast *Candida utilis* in place of *Candida albicans*. Weinberg and Springer[9] introduced a yeast inhibition test using non-pathogenic *Saccharomyces cerevisiae*.

Assays in yeast have been used to detect specific phototoxic substances such as bergapten or psoralens in plants, and to investigate the phototoxic potential of psoralen compounds[10–13]. Some of these previous reports suggested that this method might be useful for predicting phototoxicity with correlation analyses[9,15–17]. Although Tenenbaum et al.[15] and Bagley et al.[17] suggested that the use of yeast shows promise as a method to reduce or eliminate the use of animals for phototoxicity testing, their samples for studying the teast assay included only fragrance materials.

To develop a more reliable alternative method to predict phototoxicity, we selected the yeast inhibition assay, which reflects damage to DNA or cell organelles. In the present study, after optimizing the conditions including species of yeast, concentration of seeding, UV dose and incubation time, we used the yeast growth inhibition assay to assess the phototoxic potential of 24 chemicals. The results were compared to *in vivo* data, in order to evaluate the feasibility of using the yeast growth inhibition assay for predicting phototoxicity of chemicals. Furthermore, we attempted to combine the yeast growth inhibition assay with the RBC photohemolysis assay, the mechanism of which involves damage to the celular membrane, in a battery system to predict phototoxicity more accurately.

Materials and Methods

1. Test chemicals and in vivo test

Test chemicals

All test chemicals were obtained commercially and used without purification. Twenty-four chemicals (Table 1), consisting of 8 fragrance materials, 5 UV absorbers, 4 drugs, 4 antimicrobials and 3 dyes, including well-known phototoxic chemicals, were evaluated. All the test compounds show UV absorption. These chemicals (reagent grade) were from Nakalai Tesque, Inc. (Kyoto, Japan), Sigma Chemical Co. (St. Louis, U.S.A.), Aldrich Chemical Company, Inc. (Milwaukee, WIS, U.S.A.), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), and Eastman Kodak Company (N.Y., U.S.A.). UV absorbers were obtained from Givaudan-Roure Corp. (N.J., U.S.A.), Shonan Kagaku Kogyo Ltd. (Hiratuka, Japan) and Van Dyke & Company, Inc. (N.J., U.S.A.). Some of the fragrance materials were obtained from perfumeries.

In vivo test

Existing *in vivo* data reported by Sugiyama ea al.[1] were used for comparison with the *in vitro* yeast growth inhibition assay data, so that no further animal testing was performed solely for the purpose of the present study. The technique used in the *in vivo* study was basically the same as the phototoxicity test reported by Morikawa et al.[18]

2. Yeast growth inhibition

Media preparation

Forty grams of potato dextrose agar (Kyokuto Co. Ltd.) was dissolved in 1000 ml of distilled water. The medium was autoclaved for 20 min at 120°C, and 7 ml was dispensed into each well of 6-well microplates (Corning).

Table I. Test chemicals and *in vivo* data in guinea pigs.

Chemicals	Supplier	Concentration of chemical (%)	Phototoxic reaction Guinea pigs
Fragrance materials (8)			
Musk Ambrette	10	20.0	−
Musk Ketone	10	20.0	−
Musk Xylene	10	20.0	−
Phantolid	10	20.0	+
Galaxolide (50% in diethyl phthalate)	10	70.0	+
8-Methoxy Psoralen (8-MOP)	1	0.02	+
5-Methoxy Psoralen (5-MOP)	2	0.025	+
6-Methyl Coumarin (6-MC)	4	1.0	−
UV Absorbers (5)			
4-t-Butyl-4-methoxydibenzoylmethane (Parsol 1789)	7	10.0	−
2-Ethylhexyl-p-methoxycinnamate (Parsol MCX)	7	20.0	−
2-Hydroxy-4-methoxybenzophenone (ASL-24)	8	10.0	−
2-Hydroxy-4-methoxybenzophenone-5-sulfonic acid (ASL-24S)	8	5.0	−
2-Ethylhexyl-p-dimethylaminobenzoate (Escalol 507)	9	20.0	±
Drugs (4)			
Sulfanilamide	1	20.0	−
Indomethacin	2	1.0	−
Piroxicam	2	5.0	−
Chlorpromazine HCl (CPZ)	1	50.0	+
Antimicrobials (4)			
3,4,4'-Trichlorocarbanilide (TCC)	3	5.0	−
Bithionol	2	1.0	−
3,4',5-Tribromosalicylanilide (TBS)	5	1.0	−
3,3',4',5-Trichlorosalicylanilide (TCSA)	6	1.0	+
Dyes (3)			
Rose Bengal	1	1.0	−
Acridine	2	1.0	+
Anthracene	1	0.5	+

1: Nakalai Tesque, Inc.
2: Sigma Chemical Co.
3: Aldrich Chemical Company, Inc.
4: Wako Pure Chemical Industries, Ltd.
5: Tokyo Kasei Kogyo Co., Ltd.
6: Eastman Kodak Company.
7: Givaudan-Roure Corp.
8: Shonan Kagaku Kogyo, Ltd.
9: Van Dyke & Company, Inc.
10: Fragrance materials were obtained from perfumeries.

Yeast inoculum

Saccharomyces cerevisiae and *Candida albicans* were investigated as candidate yeast species. *Saccharomyces cerevisiae* was isolated from dry yeast (Oriental Yeast Co., Ltd.). Fifteen milligrams of dry yeast was suspended in 10 ml of sterilized distilled water and mixed with 1% potato dextrose agar (1:20). Four hundred microliters of the yeast-agar mixture (0.075 mg/ml) was overlaid on the prepared media in the 6-well microplate.

For *Candida albicans*, a preculture procedure was required. After 48 h of preculture, an appropriate amount of *Candida albicans* (ATCC No. C10231) was suspended in 5 ml of sterilized distilled water and adjusted to a

concentration of 1×7^7 cells/ml. Three hundred microliters of the suspension of *Candida albicans* was mixed with 6 ml of 1% potato dextrose agar, and 400 μl of this yeast-agar mixture (4.8×10^5 cells/ml) was overlaid on the prepared media in the 6-well microplate.

Application of samples

Twenty-microliter aliquots of test chemical solutions prepared by serial five-fold dilution were applied to 6-mm paper discs (Toyo Roshi Kaisha, Ltd.). Test compounds that were insoluble in water were dissolved in methanol, ethanol, acetone or dimethyl sulfoxide. None of these solvents themselves had any effect on yeast growth at their respective final concentrations. The disc was placed on the center of the seeded well using forceps.

UVA irradiation

The chemicals applied to a 6-well microplate were exposed to UVA at room temperature. As a radiation source, a transilluminator (Vilber Lourmat, France) was used with an emission spectrum from 320 nm to 400 nm with a peak at 365 nm for UVA (T-15L). The output of these lamps was set at 4.7 mW/cm^2 thrugh the 6-well microplate as measured with a UV radiometer (Topcon, Japan). The non-irradiated control was kept in the dark at room temperature.

Yeast Incubation

After irradiation, both irradiated and dark control plates were incubated at 25°C for 72 h.

Measurement of zones of inhibition

The zones of inhibition were measured using a metric ruler and were expressed in terms of the diameter of the inhibition circle minus the disc diameter.

Classification

In order to correlate the *in vitro* results with *in vivo* data, the yeast growth inhibition measured as the difference of the average zone between the irradiated group and the non-irradiated group was classified as follows: zone of growth inhibition<2 mm, −; zone of growth inhibition≥2 mm, +.

Statistical analysis

The *in vitro* results were compared using Student's t-test ($p<0.05$).

Results and Discussion

1. In vivo study

The guinea pig phototoxicity test results are shown in Table I. The numbers of chemicals classified into the positive, equivocal and negative reaction categories were 7, 1 and 16, respectively.

2. Yeast inhibition assay

1) Differences in responses between yeast species

Candida albicans and *Saccharomyces cerevisiae*, generally used for growth inhibition studies, were investigated. Although a previous report recommended use of *Candida utilis* instead of *Candida albicans*[8], we used the latter for growth inhibition studies because of it's availability. The concentrations of seeded yeast were 1.9×10^5 cells/well for *Can-*

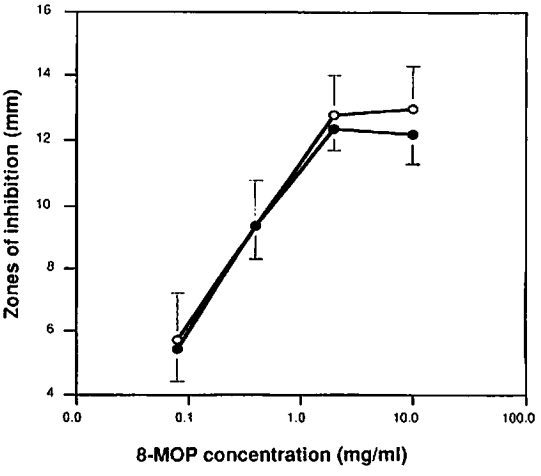

Fig. 1. Dose-response curves of the yeast inhibition zones for *Candida albicans* (○) and *Saccharomyces cerevisiae* (●). Data shown are mean±S.D. of four separate experiments. There were no statistically significant differences between *Candida albicans* and *Saccharomyces cerevisiae* at any concentration of 8-MOP used.

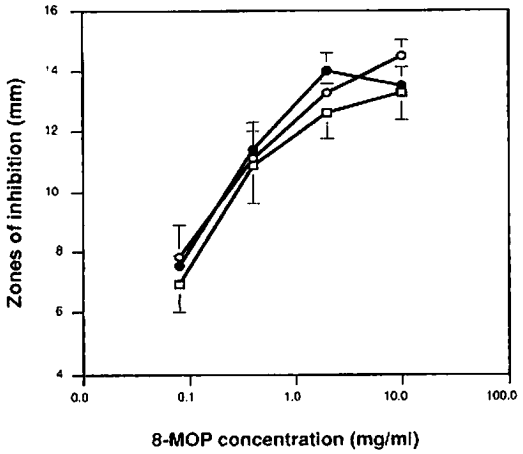

Fig. 2. Dose-response curves of the yeast inhibition zones for *Saccharomyces cerevisiae* at seedind concentration of 0.3 (○), 3.0 (●) and 30.0 (□) μg/well. Data shown are mean±S.D. of at least three separate experiments. There were no statistically significant differences between the three seeding concentration at any concentration of 8-MOP used.

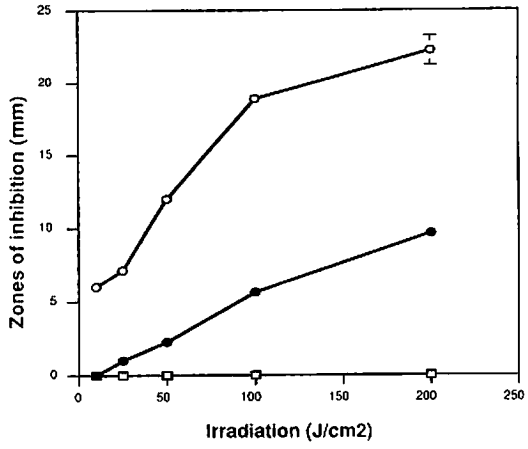

Fig. 3. Dose-response curves of the yeast inhibitation zones for *Saccharomyces cerevisiae* with 8-MOP (○), CPZ (●) and sulfanilamide (□) with UVA. Data shown are mean±S.D. of four separate experiments.

dida albicans and 30 μg/well for *Saccharomyces cerevisiae*. There were no marked differences between the sensitivity to growth inhibition of the two organisms at any concentration of 8-methoxy psoralen (8-MOP) with UVA irradiation (50 J/cm^2) (Fig. 1).

We selected *Saccharomyces cerevisiae*, because of its non-pathogenicity and because preculture was unnecessary.

2) Effect of inoculated concentration of yeast

Zones of growth inhibition were measured with dry yeast at 0.3–30.0 μg/well with UVA irradiation (50 J/cm^2) in the presence of 8-MOP (Fig. 2). The concentration of seeded yeast is an important factor for yeast inhibition assay, since too high a concentration may cause poorly difined zones of killing[5]. However, there were no marked differences in inhibition zones among these 3 inoculum sizes of dry yeast at any concentration of 8-MOP applied.

We selected 30 μg/well, because this concentration allowed easy measurement of inhibition zones.

3) Effect of UVA dose

UVA irradiation was conducted at doses of 10, 25, 50, 100 and 200 J/cm^2 in the presence of 2 mg/ml 8-MOP, chlorpromazine HCl (CPZ) or sulfanilamide to determine the optimum dose (Fig. 3), with *Saccharomyces cerevisiae* seeded at a concentration of 30 μg/well.

The inhibition zones with 8-MOP or CPZ increased with UVA dose in a dose-dependent manner, while sulfanilamide gave no reaction with any UVA dose applied. The growth of yeast was inhibited by UVA irradiation alone only at 200 J/cm^2. Other investigators have recommended UVA doses of 40–80 J/cm^2 for phototoxicity assays[9,15,16]. Based on this result and UV doses used by other researchers, we used 50 J/cm^2 as the irradiation dose since it gave clear zones of inhibition with CPZ.

4) Effect of incubation time

The zones of growth inhibition were measured at 24, 48, and 72 h and 7 d after UVA irradiation in the presence of 0.08, 0.4 and 2 mg/ml 8-MOP (Fig. 4). The concentration of seeded *Saccharomyces cerevisiae* was 30 μg/well and the UVA dose was 50 J/cm^2 throughout. The diameter of the zones of inhibition decreased with the growth of yeast until 72 h,

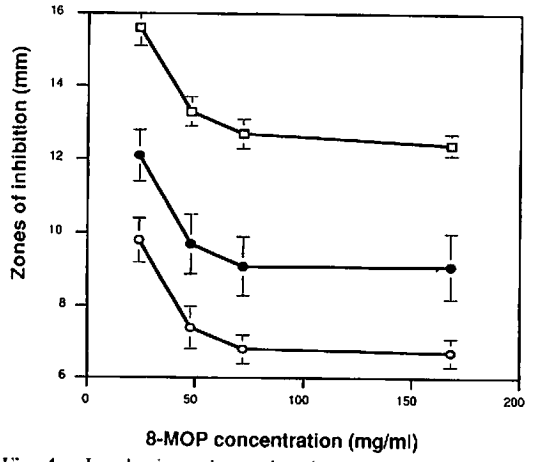

Fig. 4. Incubation time-related curves of the yeast inhibition zones for *Saccharomyces cerevisiae* with 8-MOP at 0.08 (○), 0.4 (●) and 2 mg/ml (□). Data shown are mean±S.D. of at least four separate experiments. Statistically significant differences were observed between 24 h and 48 h at all concentrations, and between 48 h and 72 h at a concentration of 2 mg/ml ($p<0.05$).

and showed little change thereafter. Therefore, we selected 72 h as the standard incubation time.

5) Yeast growth inhibition by 24 chemicals

We investigated yeast growth inhibition for 24 chemicals, under the conditions shown below.

Microorganism: *Saccharomyces cerevisiae* (isolated from dry yeast).

Concentration of inoculation: 30 μg/well.

UVA dose: 50 J/cm^2.

Incubation time: 72 h.

The results of yeast growth inhibition, classification and *in vivo* data are shown in Table II. For the purpose of comparison of phototoxic abilities predicted from the yeast growth inhibition assay and phototoxic reac-

Table II. Yeast growth inhibition by 24 chemicals.

Chemicals	(Solvent)	Growth inhibition zone (mm) (test concn; mg/ml)	Phtotoxic reaction *in vitro* Yeast assay	Phtotoxic reaction *in vivo* Guinea pigs
Fragrance materials (8)				
Musk Ambrette	(Acetone)	2.0 (20.0)	+	−
Musk Ketone	(Acetone)	0 (300.0)	−	−
Musk Xylene	(Acetone)	0 (300.0)	−	−
Phantolid	(Acetone)	1.4 (500.0)	−	+
Galaxolide	(Acetone)	2.1 (200.0)	+	+
8-MOP	(Ethanol)	14.1 (10.0)	+	+
5-MOP	(Ethanol)	10.0 (2.0)	+	+
6-MC	(Ethanol)	6.4 (100.0)	+	−
UV Absorbers (5)				
Parsol 1789	(Ethanol)	0 (100.0)	−	−
Parsol MCX	(Ethanol)	0 (not diluted)	−	−
ASL-24	(Ethanol)	0 (50.0)	−	−
ASL-24S	(Distilled water)	0 (50.0)	−	−
Escalol 507	(Ethanol)	3.0 (not diluted)	+	±
Drugs (4)				
Sulfanilamide	(Methanol)	0 (50.0)	−	−
Indomethacin	(Ethanol)	0 (10.0)	−	−
Piroxicam	(Acetone)	0 (20.0)	−	−
CPZ	(Distilled water)	2.4 (2.0)	+	+
Antimicrobials (4)				
TCC	(Acetone)	0 (20.0)	−	−
Bithionol	(Acetone)	0.3 (200.0)	−	−
TBS	(Acetone)	1.6 (2.0)	−	−
TCSA	(Acetone)	2.4 (10.0)	+	+
Dyes and dyestuffs (3)				
Rose Bengal	(Dimethyl sulfoxide)	11.5 (10.0)	+	−
Acridine	(Acetone)	6.8 (0.8)	+	+
Anthracene	(Acetone)	10.1 (0.08)	+	+

Table III. Comparison of phototoxic ability predicted from the yeast growth inhibition with the phototoxic reactions observed in guinea pigs.

in vivo / *in vitro*	+	±	−
+	Galaxolide 8-MOP 5-MOP CPZ TCSA Acridine Anthracene	Escalol 507	Musk Ambrette 6-MC Rose Bengal
−	Phantolid	None	Musk Ketone Musk Xylene Parsol 1789 Parsol MCX ASL-24 ASL-24S Sulfanilamide Indomethacin Piroxicam TCC Bithionol TBS

tions in guinea pigs, the results were arranged as shown in Table III. To evaluate the *in vitro* method, 5 parameters reported by Balls et al.[19] were used. The calculation method is shown in Table IV and Table V. The yeast assay had a sensitivity of 89%, a specificity of 80%, a positive predictive value of 73%, a negative predictive value of 92% and an equivalence of 81% (Table IV).

A fragrance material, Phantolid, which produced phototoxicity in guinea pigs, showed negative reaction in the yeast growth inhibition assay (classified as false negative). On the other hand, 3 other compounds, Musk Ambrette, Rose Bengal and 6-methyl coumarin (6-MC), showed positive reactions *in vitro*, while they were classified as negative in the *in vivo* test (false positive).

8-MOP and 5-methoxy psoralen (5-MOP) were positive in the present study in accordance with previous findings[5,9,15,17,20,21]. Phantolid, which did not give a positive

Table IV. Terms used to describe screening tests and screening programs.

Parameter	Meaning	Equation*	Value Yeast assay	RBC assay**	Battery system
Sensitivity	The ratio of the number of positive and equivocal measurements by the *in vitro* method to the total number of phototoxic and equivocal chemicals.	(a+b+d+e)/(a+b+d+e+g+h)	89%	67%	100%
Specificity	The ratio of the number of negative measurements by the *in vitro* method to the total number of non-phototoxic chemicals.	i/(c+f+i)	80%	73%	67%
Positive predictive Value	The ratio of the number of phototoxic and equivocal chemicals to the total number of positive and equivocal measurements *in vitro*.	(a+b+d+e)/(a+b+c+d+e+f)	73%	60%	64%
Negative predictive Value	The ratio of the number of non-phototoxic chemicals to the total number of negatives *in vitro*.	c/(g+h+i)	92%	79%	100%
Equivalence	The ratio of the number of chemicals classified into the same classification, positive, equivocal or negative, *in vitro* and *in vivo*, to the total number of chemicals.	$\frac{\{1.0(a+e+i)+0.5(b+d+f+h)\}\times 100}{a+b+c+d+e+f+g+h+i}$	81%	73%	77%

* a-i: The numbers of chemicals classified as shown in Table V.
**Data from Sugiyam et al.[1].

Table V. The relationship between *in vivo* and *in vitro* results.

		In vivo (guinea pigs) +	±	−
in vitro	+	a	b	c
	±	d	e	f
	−	g	h	i

a-i: The numbers of chemicals falling into the indicated classifications in the Table.

reaction in our *in vivo* study, was reported positive by some investigators: Weinberg and Springer[9] reported that Phantolid showed a positive reaction despite a small tail of absorption in the UVA spectral range (<1% of total absorbance). Phantolid showed slight only growth inhibition (<2 mm), so it was classified as negative, in agreement with the result reported previously by Tenenbaum et al.[14]. Sulfanilamide, which was negative in our present *in vivo* study, was reported as positive by Horio[22] in a different system, using *Trichophyton mentagrophytes* as the microorganism and colony-forming ability as the endpoint. Rose Bengal showed no phototoxicity in guinea pigs, although it was shown to induce phototoxicity in rabbits[18,23]. Although Daniels[5] reported that Rose Bengal did not cause yeast inhibition, we obtained a different result.

The results obtained using this *in vitro* growth inhibition method correlated reasonably well with those of the *in vivo* test, and the present procedure is both simple and reproducible. Moreover, the results suggest that the yeast growth inhibition assay may be useful as

Table VI. Phototoxic potential predicted by the battery system.

Chemicals	*in vitro* Photohemolysis	*in vitro* Yeast assay	***Battery system***	*in vivo* Guinea pigs
Fragrance materials (8)				
Musk Ambrette	+	+	+	−
Musk Ketone	−	−	−	−
Musk Xylene	−	−	−	−
Phantolid	+	−	+	+
Galaxolide	+	+	+	+
8-MOP	−	+	+	+
5-MOP	−	+	+	+
6-MC	−	+	+	−
UV Absorbers (5)				
Parsol 1789	−	−	−	−
Parsol MCX	−	−	−	−
ASL-24	−	−	−	−
ASL-24S	−	−	−	−
Escalol 507 (D)	−	+	+	±
Drugs (4)				
Sulfanilamide	−	−	−	−
Indomethacin	−	−	−	−
Piroxicam	−	−	−	−
Chlorpromazine HCl	+	+	+	+
Antimicrobials (4)				
TCC	−	−	−	−
Bithionol	+	−	+	−
TBS	+	−	+	−
TCSA	+	+	+	+
Dyes (3)				
Rose Bengal	+	+	+	−
Acridine	+	+	+	+
Anthracene	+	+	+	+

Table VII. Comparison of phototoxic ability predicted from the battery system with the phototoxic reactions observed in guinea-pigs.

in vitro \ *in vivo*	+	±	−
+	Galaxolide Phantolid 8-MOP 5-MOP TCSA CPZ Acridine Anthracene	Escalol 507	Musk Ambrette 6-MC Bithionol TBS Rose Bengal
±	None	None	None
−	None	None	Musk Ketone Musk Xylene ASL-24 ASL-24S Parsol 1789 Parsol MCX Sulfanilamide Indomethacin Piroxicam TCC

an alternative to animal experiments for predicting the phototoxicity of chemicals.

3. The battery system

We have previously reported the evaluation of the same 24 chemicals with the RBC photohemolysis assay[1]. In comparison of the 5 parameters between the two methods, all those in the yeast assay were higher than those in the RBC photohemolysis assay (Table IV). In the yeast growth inhibition assay, 8-MOP and 5-MOP exhibited photoreactions, while they were both negative in the photohemolysis assay.

Phantolid, Bithionol and Rose Bengal showed positive reactions in the photohemolysis assay, but were negative in the yeast assay. These results suggest that sensitivity to cellular membrane damage was higher in photohemolysis than in yeast assay, so the yeast assay alone seems to be insufficient to accurately assess phototoxicity, and we recommend the use of a combination of different techniques based on different mechanisms.

By combining a technique to predict damage to the cellular membrane (such as the photohemolysis test) with a technique to estimate the effects on cell organelles (such as the yeast growth inhibition assay), it should become possible to predict phototoxicity with considerable accuracy. A combination of the results of photohemolysis technique and the yeast assay were used as a battery system in which "positive" was defined as a positive result with either of these two methods (Table VI). For the purpose of comparison of phototoxic abilities predicted from the battery system and *in vivo* phototoxic reactions in guinea pigs, the results were arranged as shown in Table VII.

The sensitivity, specificity, positive predictive value, negative predictive value and equivalence of the battery system were 100%, 67%, 64%, 100% and 77%, respectively (Table IVa). Even though specificity, positive predictive value and equivalence were lower than those values in the yeast assay, no false negatives were observed in this battery system.

Five chemicals (Musk Ambrette, 6-MC, 3,4',5-tribromosalicylanilide[TBS], Bithionol and Rose Bengal), which were classified as negative in the *in vivo* test, showed positive reactions in the battery system (false positive). As reported previously, these five chemicals identified as false positive compounds in the battery system seem to induce photodynamic reactions. That is, both photohemolysis and yeast growth inhibition are phenomena based on photodynamic reactions of chemicals, but the test systems do not allow us to distinguish between phototoxicity and photoallergenicity.

In the present study, we did not consider factors of permeability and metabolism of chemicals in the skin. Some of the chemicals identified as false positive (Bithionol, Rose Bengal), do show positive reactions in other animal species, so the above factors may be important. Therefore, by introducing these factors into an *in vitro* test system, it may be

possible to predict phototoxicity more accurately.

When we evaluated the battery system in which the RBC photohemolysis assay and the yeast growth inhibition assay (methods based on different mechanisms) were combined for predicting phototoxicity more precisely, no false negative result was obtained. It is extremely important in risk assessment of chemicals that false negative results should be excluded. Therefore, we conclude that the present battery system is useful for predicting phototoxic potential of new chemicals as an alternative to animal experiments.

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