An *In Vitro* Phototoxicity Assay Battery (Photohaemolysis and 3T3 NRU PT test) to Assess Phototoxic Potential of Fragrances

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Summary — The purpose of this study was to compare the *in vivo* and *in vitro* phototoxicity potentials of 13 fragrances. We used the 3T3 neutral red uptake phototoxicity (3T3 NRU PT) test and the photohaemolysis test as *in vitro* phototoxicity assays. In the 3T3 NRU PT test, all of the fragrances were non-phototoxic. Six fragrances were phototoxic in the photohaemolysis test. Three of the six photohaemolytic fragrances were phototoxic in the guinea-pig photoirritation test. These phototoxic fragrances did not cause cellular phototoxicity, but showed a photohaemolytic reaction. The photohaemolysis test was more sensitive than the 3T3 NRU PT test for screening for the phototoxicity of fragrances. The accuracy of this *in vitro* phototoxicity test battery was 82%. It is thought that the major phototoxic mechanism of fragrances is cell membrane damage. We suggest that a battery composed of the 3T3 NRU PT test and the photohaemolysis test is a simple and effective model for the *in vitro* phototoxicity assay of fragrances.

Key words: *fragrance,* in vitro *phototoxicity assay battery, photohaemolysis, 3T3 NRU PT test.*

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Introduction

The current toxicity assay for acute dermal phototoxicity is an animal test using guinea-pigs, rabbits, rats or mice. Although a standard protocol for phototoxicity testing in animals has been recommended (1), the acceptance of an animal test for an OECD guideline on phototoxicity testing has not yet occurred (2).

Phototoxicity can be described as an increase in the toxicity of a chemical on exposure to ultra-violet (UV) or visible radiation. Phototoxicity is caused by two oxygen-dependent reactions (3–6). Type I reactions are mediated via an electron or hydrogen transfer (free radical) process. Type II reactions are mediated by energy transfer from oxygen to produce an excited state singlet oxygen. Some compounds, such as psoralens, may react directly with biomolecules in their excited states (7). Some stable toxic photoproducts may be formed after absorption of light.

There are two main approaches to developing *in vitro* phototoxicity assays (5, 8). The 3T3 neutral red uptake phototoxicity (3T3 NRU PT) test is a method for evaluating damage to cell organelles or DNA (5, 9). The photohaemolysis test with red blood cells (RBCs) can detect impairment of the function and integrity of the cell membrane (5, 10–12). We studied *in vitro* phototoxicity assays, by using fragrances found in cosmetic products.

Materials and Methods

Test materials

Eighteen test materials (15 fragrances, 3 positive controls) were studied (Table 1).

UV radiation source and UV meter

PUVA800 lamps (model CUBE401, UVATEC. Inc., Sermon Oaks, CA, USA) were used as the UV radiation source. The UVB was filtered by glass, and the UV meter was the Waldman UV meter (Herbert Waldman GmbH & Co., Villingen-Schwenningen, Germany).

In vivo guinea-pig photoirritation test

This technique is modified from that of Lovell & Sanders (13). Healthy young adult male albino guinea-pigs, approximately 300–500g, were used. An aliquot of 25μl of the compound was applied to the shaved skin at a concentration of 20% in ethanol. After 30 minutes in the dark, the skin was exposed to the UVA light (15J/cm^2). The animals were observed at 24, 48 and 72 hours, and skin reactions were evaluated to give a Draize score.

Table 1: Test materials

Fragrances	Manufacturers	Specific ingredients
Ciel E-9942221/01	VMF	Galaxolide
Ciel E-9942221/02	VMF	Galaxolide
Ciel E-9942221/A	VMF	Galaxolide
Crypton fresh K-9838033	Kimex	
KX PC 3216	Kimex	
KX PC 3218	Kimex	
Bobby 123.869/P	Frimenich	
Roy 04	Kimex	
Enjoy 200	Kimex	
I#8	Pacific	Lemon oil, bergaptan-free bergamot oil
I#9	Pacific	Lemon oil, bergaptan-free bergamot oil
I-Herichrysum	Robertet	
Endless MP-2513	Soda Aromatics	
S.H. Green 35374	Hanbul	
Muscoflor 112856E	Frimenich	
Chlorpromazine HCl (positive control)	Sigma	
Bergamot oil (positive control)	Charabot	
Galaxolide (positive control)	IFF	

In vitro 3T3 neutral red uptake phototoxicity test

The 3T3 NRU PT test, as described in the OECD guideline, was used in this study (14–16). Balb/c 3T3 clone A31 fibroblasts were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum, 4mM glutamine, 100IU/ml penicillin, and 100μg/ml streptomycin. The individual wells of a 96-well tissue culture microplate were inoculated with 100μl of medium containing 1×10^4 cells. The plate was

Table 2: Results for the guinea-pig photoirritation test

Test materials	Number of guinea-pigs	Concentration (%)	Irritation index[a]		Results
			UV+	UV–	
Ciel E-9942221/01	5	20	0.75	0	+
Ciel E-9942221/02	5	20	0.75	0	+
Ciel E-9942221/A	5	20	0.38	0	+
Crypton Fresh K-9838033	5	20	0	0	–
KX PC 3216	5	20	0	0	–
KX PC 3218	5	20	0	0	–
Bobby 123.869/P	5	20	0	0	–
Roy 04	5	20	0	0	–
Enjoy 200	5	20	0	0	–
I#8	5	20	0	0	–
I#9	5	20	0	0	–
I-Herichrysum	5	20	0	0	–
Endless MP-2513	5	20	0	0	–
S.H. Green 35374	5	20	0	0	–
Muscoflor 112856E	5	20	0	0	–

[a]*Irritation index = (Σmaximum score of erythema – Σmaximum score of oedema)/number of animals.*

incubated for 24 hours to form a semi-confluent monolayer. The medium was then removed and washed with 100µl of Earle's balanced salt solution (EBSS, pH 7.2). Then 100µl of the test chemical solution with EBSS was added to each well. Cells with the substance were incubated in the dark for 60 minutes. The test plate was irradiated, while the control plate was stored in the dark. After UVA radiation (UVA 5J/cm^2), the solution was removed and the cells were washed with EBSS. Culture medium was added to the test and control plates, and the plates were incubated overnight. Cell viability was assayed by neutral red uptake. The IC50 (50% inhibition concentration of cell growth) was determined by using linear regression analysis. The cut-offs for photoirritation (photoirritation factor [PIF] = IC50 [–Irr]/IC50 [+Irr]) are PIF < 2: "no phototoxicity", 2 ≤ PIF < 5: "probable phototoxicity", PIF ≥ 5: "phototoxicity".

In vitro photohaemolysis test

A modification of the photohaemolysis technique described by Kahn & Fleichaker (17–20) was used in this study. The RBCs were obtained from healthy human volunteers, washed with phosphate-buffered saline (PBS; pH 7.4), then centrifuged for 15 minutes at 2500rpm. This procedure was repeated four times. The RBCs were suspended in PBS (1:200) and used within six hours.

Aliquots of 990µl of the RBC suspension were dispensed into 24-well microplates, 10µl of test chemical solution was added, and the microplates were incubated for ten minutes. The non-haemolytic maximal concentration in the non-irradiated condition was determined as the control concentration of the test material. The 24-well microplate was irradiated with UVA (15J/cm^2), while the non-irradiated control was kept in the dark at room temperature.

After irradiation, the microplates with samples were centrifuged for 15 minutes at 2500rpm. 100µl of supernatant was transferred to a 96-well microplate, and 50µl of Drabkin's reagent (Sigma) was added. Absorbance at 540nm was measured with a microplate reader, then:

$$\text{Photohaemolysis (\%)} = (A - B)/C \times 100$$

(where A is the optical density of the supernatant from the irradiated substance, B is the optical density of the supernatant from the non-irradiated test, and C is the optical density of 100% haemolysed RBCs). Mean haemolysis values of 10% or more were regarded as representing significant photohaemolysis.

Results

In the guinea-pig photoirritation test, Ciel E-9942221/01, Ciel E-9942221/02 and Ciel E-9942221/A were phototoxic (Table 2).

In the 3T3 NRU PT test, none of the fragrances were phototoxic (Table 3). Six of the 15 fragrances were phototoxic in the photohaemolysis test (Table 4). In the *in vitro* test battery, 13 of the 15 fragrances coincided with the results of *in vivo* tests. Three cases were classified as false-positive fragrances (Table 5). Ciel E-9942221/01, Ciel E-

Table 3: Results for the 3T3 NRU phototoxicity assay with 15 fragrances

Test materials	*In vivo* test	Mean IC50 (–Irr) (µg/ml)	Mean IC50 (+Irr) (µg/ml)	PIF	Results
Ciel E-9942221/01	+	161.75	178.27	0.908	–
Ciel E-9942221/02	+	159.00	250.30	0.635	–
Ciel E-9942221/A	+	528.13	662.59	0.846	–
Crypton fresh K-9838033	–	298.95	662.45	0.451	–
KX PC 3216	–	139.49	111.34	1.253	–
KX PC 3218	–	298.70	287.00	1.041	–
Bobby 123.869/P	–	663.74	531.43	1.249	–
Roy 04	–	970.11	1092.08	0.888	–
Enjoy 200	–	> 1000	> 1000	1.000	–
I#8	–	581.35	405.20	1.435	–
I#9	–	617.82	487.43	1.268	–
I-Herichrysum	–	> 1000	> 1000	1.000	–
Endless MP-2513	–	468.20	396.51	1.181	–
S.H. Green 35374	–	874.39	545.94	1.602	–
Muscoflor 112856E	–	759.68	633.92	1.198	–
Chlorpromazine (positive control)	+[a]	32.37	0.43	74.279	+

[a]*Chlorpromazine is a known phototoxic substance.*

Table 4: Results for the photohaemolysis assay with 15 fragrances

Test materials	*In vivo* test	Concentration (μg/ml)	Photohaemolysis (%)	Results
Ciel E-9942221/01	+	1000	14.08	+
Ciel E-9942221/02	+	1000	14.89	+
Ciel E-9942221/A	+	1000	25.42	+
Crypton Fresh K-9838033	–	1000	3.04	–
KX PC 3216	–	1000	–5.54	–
KX PC 3218	–	1000	3.90	–
Bobby 123.869/P	–	1000	8.57	–
Roy 04	–	1000	16.12	+
Enjoy 200	–	1000	0.78	–
I#8	–	1000	24.09	+
I#9	–	1000	21.55	+
I-Herichrysum	–	1000	–0.06	–
Endless MP-2513	–	1000	8.09	–
S.H. Green 35374	–	1000	3.02	–
Muscoflor 112856E	–	1000	8.35	–
Bergamot oil (positive control)	+[a]	63	32.52	+
Galaxolide (positive control)	+[a]	10000	84.79	+

[a]*These materials are known phototoxic substances.*

Table 5: Summarised data for the *in vitro* battery phototoxicity assay

		In vitro battery test		
Test materials	*In vivo* test	3T3 NRU PT	Photohaemolysis	Final results
Ciel E-9942221/01	+	–	+	+
Ciel E-9942221/02	+	–	+	+
Ciel E-9942221/A	+	–	+	+
Crypton fresh K-9838033	–	–	–	–
KX PC 3216	–	–	–	–
KX PC 3218	–	–	–	–
Bobby 123.869/P	–	–	–	–
Roy 04	–	–	+	+
Enjoy 200	–	–	–	–
I#8	–	–	+	+
I#9	–	–	+	+
I-Herichrysum	–	–	–	–
Endless MP-2513	–	–	–	–
S.H. Green 35374	–	–	–	–
Muscoflor 112856E	–	–	–	–
Chlorpromazine (positive control)	+[a]	+	NT	+
Bergamot oil (positive control)	+[a]	NT	+	+
Galaxolide (positive control)	+[a]	NT	+	+

[a]*These materials are known phototoxic substances; NT = not tested.*

9942221/02 and Ciel E-9942221/A contained phototoxic galaxolide. I#8 and I#9 contained bergaptan-free bergamot oil and lemon oil. Bergamot oil and lemon oil were reported to be phototoxic in humans, but bergaptan-free bergamot oil was not phototoxic in humans.

Discussion

The 3T3 NRU PT test is easy to perform and it appears very promising as a screening test. However, the data presented in Table 3 did show some differences with phototoxicity *in vivo*.

The photohaemolysis test is useful for screening chemicals and investigating phototoxic mechanism. Because chemicals that react with DNA cannot be detected in a photohaemolysis test, additional tests have to be performed to cover all mechanisms of phototoxicity (21, 22).

Conclusions

According to the results, the photohaemolysis test was more sensitive than the 3T3 NRU PT test for evaluating the phototoxic potential of fragrance. In this battery, no false negatives were observed. We suggest that a battery of the 3T3 NRU PT test and the photohaemolysis test is a simple and useful alternative to the guinea-pig photoirritation test.

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