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EEC/COLIPA PROJECT ON *IN VITRO* PHOTOTOXICITY TESTING: FIRST RESULTS OBTAINED WITH A BALB/C 3T3 CELL PHOTOTOXICITY ASSAY

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Abstract—In a joint validation project eight laboratories from the European Cosmetic Industry Association (COLIPA) as well as FRAME (England) and ZEBET (Germany) are trying to develop validated *in vitro* methods to be incorporated into new international guidelines for acute phototoxicity testing. The first stage of the study involved selection of the most promising *in vitro* phototoxicity tests for further validation. 20 chemicals with known phototoxic properties (12 phototoxins, four UV-absorbing non-phototoxins and four non-UV absorbing non-phototoxins) were tested under identical conditions of UV exposure conditions (sun simulator, UVA 5 J/cm^2) in a standardized cytotoxicity assay with Balb/c 3T3 fibroblasts (endpoint: neutral red uptake, NRU). 19 of the 20 chemicals were correctly classified by the 3T3 NRU phototoxicity test, and therefore, this simple assay for phototoxicity seems very promising and should be validated further.

INTRODUCTION

The current toxicological test systems for 'acute dermal phototoxicity' are animal tests using guinea pigs, rabbits, rats or mice. Although a standard protocol for phototoxicity testing in animals has recently been recommended by Nilsson *et al.* (1993), agreement on a regulatory guideline has not been achieved, mainly because of disagreements on animal species and detailed procedures. However, a sequential approach, proceeding by way of *in vitro* testing before consideration of any requirement for animal testing was accepted. Therefore, COLIPA (European Cosmetic Industry Association) and DGXI of the EEC agreed to conduct a joint validation project on *in vitro* phototoxicity tests. The project is aimed at providing both the cosmetics industry and regulators with well validated *in vitro* methods for phototoxicity testing.

During the first stage of the project (1992) a set of 20 chemicals [12 phototoxins (PTs), four non-UV absorbing non-PTs and four UV-absorbing non-PTs) was selected by COLIPA members to compare *in vitro* phototoxicity tests that are established in industry, and, for the purpose of standardization, an interlaboratory trial was performed with an *in vitro* phototoxicity assay using cultured mammalian cells. A fibroblast cell line, Balb/c 3T3 cells, was chosen because it was considered stable, readily available and readily cultured. A toxicity protocol (Spielmann *et al.*, 1991) was modified for phototoxicity by using Earle's balanced salt solution (EBSS) as the medium during irradiation and by introducing a pre-irradiation incubation with the test substance. The light source emitted UVA and visible light; UVB was attenuated both to allow a large dose of UVA to be used and to avoid problems arising from variation of UVB spectral emission. The same type of light source and the same model of calibrated UVA meter were used throughout the study in all laboratories. The present study reports the first results obtained with the 3T3 cell NRU cytotoxicity assay in *in vitro* phototoxicity testing.

MATERIALS AND METHODS

UVA light source and UVA meter. The UV light source used in all laboratories was obtained from the same manufacturer. A doped mercury-metal halide lamp (SOL 500, Dr Hönle, Martinsried, Germany), which simulates the spectral distribution of the natural sunlight, was chosen. A spectrum practically

Abbreviations: DMEM = Dulbecco's modified Eagle's medium; DMSO = dimethyl sulfoxide; EBSS = Earle's balanced salt solution; 8-MOP = 8-methoxypsoralen; NRU = neutral red uptake; PABA = *p*-aminobenzoic acid; PT = phototoxin.

devoid of UBV was achieved by fitting a filter with a 50% transmission at a wavelength of 335 nm, to the SOL 500. Emitted energy was measured with a simple calibrated UVA meter (type no. 37, Dr Hönle), and calibration was controlled with a reference UVA meter of the same type which was not handled and kept in the dark.

Selection of test chemicals. On the basis of information, from the literature, on phototoxicity in animals and humans, 20 chemicals (Table 1) were selected for validation: 12 phototoxins (PTs), four chemicals that absorb UV light but are not PTs (although some may be known allergens) and four chemicals that neither absorb UV nor are PTs.

Experimental design. The neutral red uptake (NRU) growth inhibition assay using Balb/c 3T3 fibroblasts to determine cytotoxicity (Borefreund and Puerner, 1985) was adapted for phototoxicity testing in the following manner: Balb/c 3T3 cells, clone 31 (ICN-Flow) were cultured in 96-well microtitre plates as described earlier (Spielmann *et al.*, 1991). After 24 hr the medium [Dulbecco's modified Eagle's medium (DMEM)] was removed, the cells were washed twice in EBSS and eight concentrations of the test chemicals dissolved in EBSS were added. Test chemicals that were insoluble in EBSS were dissolved in dimethyl sulfoxide (DMSO) before use and added at a maximum of 1% DMSO in EBSS. After 1 hr of preincubation with the test chemicals the plates were exposed to UVA (1.6 mW/cm^2) for 50 min (= 5 J/cm^2). During this period a second set of plates with the same chemicals was kept in the dark. After light exposure EBSS was replaced by DMEM (without any test chemicals) and NRU was determined 24 hr later according to the method of Spielmann *et al.* (1991).

Evaluation of in vitro data and statistical analysis. The following criteria were chosen for data analysis of the Balb/c 3T3 phototoxicity test: the cytotoxic concentrations resulting in a 50% reduction of viability (IC_{50}) with and without UVA radiation, were compared by calculation of the factor between both IC_{50} values: factor = $IC_{50}(-UV)/IC_{50}(+UV)$. The cut-off value of the factor to discriminate between PTs and non-PTs was calculated using a discriminant analysis of all factors determined in all laboratories on all of the test chemicals.

RESULTS AND DISCUSSION

UVA sensitivity of 3T3 fibroblasts

UVA sensitivity of Balb/c 3T3 cells was tested in seven laboratories. Figure 1 shows that the viability of 3T3 cells was not affected within a UVA dose range of about 0 to 10 J/cm^2 in five laboratories. However, in the same dose range in two laboratories, 3T3 cells showed a significant reduction in viability. Since the two laboratories had used 3T3 cells with passage numbers of 130–140 and the other laboratories had used younger and less UV-sensitive 3T3 cells from passages 70–80, it was decided to use 3T3 cells with a low passage (< 100) from the same

Table 1. Phototoxicity of 20 chemicals in the 3T3 cell NRU cytotoxicity assay

Chemical			Phototoxicity *in vitro**		3T3 NRU cytotoxicity†				
No.	Name	CAS No.	Human	Animal	Mean IC_{50} − UV (μg/ml)	Mean IC_{50} + UV (μg/ml)	Mean factor −UV/+UV	n	Result
			Class I UV-absorbing, phototoxic						
1	Promemthazine	58-33-3	+	+/−	45.9	0.8	78.5	13	+
2	Chlorpromazine	69-09-0	+ +	+ +	24.6	0.6	46.6	13	+
3	6-Methylcoumarin	92-48-8	A	A	§	32.7		13	+
4	TCSA	1154-59-2	A	+	19.8	0.4	55.6	12	+
5	Doxycycline	100 929-47-3	+	+	1182	6.4	255	4	+
6	8-MOP	298-81-7	+ +	+ +	§	14.7		11	+
7	Tetracycline	64-75-5	+	+	1916	16.9	374	9	+
8	Piroxicam‡	36322-904	(+)	−	§	§		11	−
9	Amiodarone	1951-25-3	+	+	24.3	4.1	6	9	+
10	Bithionol	97-18-7	+	+	13.9	3.9	7	13	+
11	Neutral red	553-24-2	+		§	0.01		14	+
12	Rose bengal	632-69-9	+/−	−	4.2	0.2	70.2	13	+
			Class II UV-absorbing non-phototoxic						
13	Cinnamic aldehyde	104-55-2	A	A	32.8	10.6	3.6	8	−
14	Chlorhexidine	3697-42-5			61.5	74.4	1.5	11	−
15	Uvinul MS 40	4065-45-6	+/−		15 958	11 577	1.4	11	−
16	PABA	150-13-0	A		10 463	9780	1	7	−
			Class III non-UV-absorbing non-phototoxic						
17	Penicillin G	69-57-8			53 914	49 755	1.1	8	−
18	L-Histidine‡	71-00-1			§	§		12	−
19	Thiourea	62-56-6	A		17 651	16 944	1	13	−
20	Lauryl sulfate	151-21-3			35.6	24.2	1.5	14	−

TCSA = 3,3′,4′,5-tetrachlorosalicylonilid 8-MOP = 8-methoxypsoralen PABA = *p*-aminobenzoic acid.
*+ = phototoxic; − = non-phototoxic; +/− = inconclusive; A = photoallergen.
†Means are arithmetic means for the number of calculations (n) shown (standard deviations are not shown).
‡Piroxicam, highest test concentration 2.4 mg/ml; L-histidine, highest test concentration 46.4 mg/ml; other chemicals were tested at
§No IC_{50} could be determined.

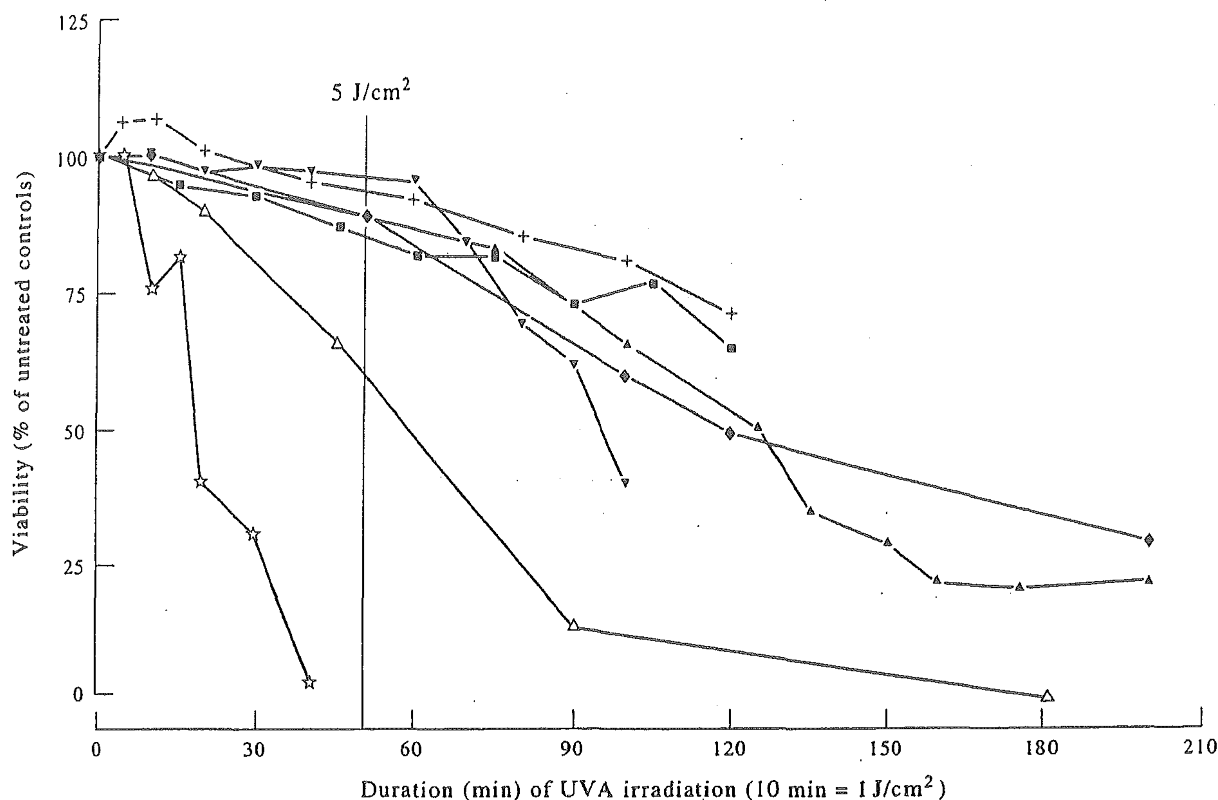

Fig. 1. UVA sensitivity of Balb/c 3T3 fibroblasts. The cells were exposed to UVA (1.6 mW/cm^2) in 96-well plates. The vertical line at 50 min exposure time indicates the standard UVA dose of 5 J/cm^2 which was used for phototoxicity testing of chemicals in the Balb/c 3T3 neutral red uptake assay. The individual curves represent data from seven different laboratories.

supplier (Flow) in all experiments. To detect even weak phototoxins, the highest non-toxic UVA dose, 5 J/cm^2 (50 min exposure at an intensity of 1.6 mW/cm^2), was chosen for further testing (Fig. 1).

Phototoxicity of test chemicals in the 3T3 NRU cytotoxicity assay

Table 1 shows that 11 of 12 known PTs and all eight non-PTs were correctly identified with the assay. A photoinactivation factor $[IC_{50}(-UV)/IC_{50}(+UV)]$ could only be determined for 15 of the 20 test chemicals since five chemicals (nos 3, 6, 8, 11 and 18) were not cytotoxic to 3T3 cells in the dark (−UV) even at the highest concentrations tested. Therefore, only for eight of the 12 phototoxic chemicals (Class I) could the UVA-dependent increase in toxicity be expressed by a photoinactivation factor with mean factors ranging from 6 (no. 9) to 374 (no. 7). For the four UV-absorbing chemicals that are not phototoxic *in vivo* (Class II), −UV/+UV factors between 1 (no. 16) and 3.6 (no. 13) were determined, and for the three cytotoxic non-UV absorbing non-PTs (Class III) −UV/+UV factors ranged from 1 to 1.5. Taking into account all 158 −UV/+UV factors calculated on the 15 cytotoxic chemicals, a discriminant analysis revealed a cut-off value of factor 5.1 to discriminate between phototoxic and non-phototoxic test chemical.

Although cytotoxicity could not be measured with chemicals nos 3, 6 and 11 of Class I without UV a phototoxic effect $[IC_{50}(+UV)]$ was observed after UV exposure (Table 1). These three PTs were, therefore, correctly classified in the 3T3 NRU assay, although a −UV/+UV factor could not be calculated. For chemical no. 8 (piroxicam) and no. 18 (L-histidine), however, no cytotoxic effects could be determined either with or without exposure to UVA. L-Histidine was tested up to a concentration of 46.6 mg/ml and is, therefore, correctly identified as non-phototoxic, whereas piroxicam was not detected as phototoxic (false negative). This may be due to the fact that piroxicam is probably not phototoxic itself but that its photoproducts are (Serrano *et al.*, 1992). The few descriptions of piroxicam's phototoxic properties in humans so far have not been confirmed in animal tests. When using UVA + UVB piroxicam was detected in another 3T3 assay (Duffy *et al.*, 1987). Thus, in the present 3T3 cell phototoxicity test 19 of the 20 test chemicals could be classified correctly.

Taking into account the many mechanisms of phototoxicity at the cellular level, for example action on the cell membrane, nucleus and macromolecules (for review see Johnson *et al.*, 1986), it is surprising that the *in vitro/in vivo* correlation was better for this simple assay than for any of the other *in vitro* phototoxicity tests that have been evaluated in the EEC/COLIPA project. These tests have included the photohaemolysis test (Pape *et al.*, 1993), the histidine oxidation test and the *Candida albicans* test (Johnson *et al.*, 1986) and some commercially available tests

(SOLATKX PI[tm], Skin[2tm], TESTSKIN[tm]). Furthermore, the predictive value of the present 3T3 cell assay was better than that of the 3T3 cell phototoxicity assay reported by Duffy *et al.* (1987), in which 8-MOP and doxicycline were classified as false negatives and tetracycline and PABA were false positive. Therefore, the improved version of the 3T3 cell phototoxicity assay described in the present study seems promising and should be validated further under blind conditions.

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Toxicology in Vitro

The International EU/COLIPA *In Vitro* Phototoxicity Validation Study: Results of Phase II (Blind Trial). Part 1: The 3T3 NRU Phototoxicity Test

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Abstract—To date, no standardized international guideline for the testing of chemicals for phototoxic potential has been accepted for regulatory purposes. In 1991, the European Commission (EC), represented initially by the Directorate General XI and later by ECVAM (the European Centre for the Validation of Alternative Methods) and COLIPA (the European Cosmetic, Toiletry and Perfumery Association), agreed to establish a joint EU/COLIPA programme on the development and validation of *in vitro* phototoxicity tests. The first phase (phase I, 1992–93) was designed as a prevalidation study, to identify *in vitro* test procedures and test protocols for a formal validation trial under blind conditions. In the second phase (phase II, 1994–95), the formal validation study, the most promising *in vitro* phototoxicity tests were validated with 30 carefully selected test chemicals in 11 laboratories in a blind trial. The 3T3 mouse fibroblast neutral red uptake phototoxicity test (3T3 NRU PT) was performed as a core test in nine laboratories, since it provided the best results in phase I of the study. The purpose of phase II was to confirm the reliability and relevance of the *in vitro* tests for predicting phototoxic effects and for identifying phototoxic chemicals. In phase II the phototoxic potential of test chemicals in the 3T3 NRU PT test was either assessed by determining the phototoxicity factor (PIF) by using a cut-off value of 5 as in phase I of the study, or by determining the mean photo effect (MPE) by using a cut-off value of 0.1, as recently proposed by Holzhütter (1997). Results obtained with both approaches in the 3T3 NRU PT test in phase II were reproducible in the nine laboratories, and the correlation between *in vitro* and *in vivo* data was very high. Therefore, ECVAM and COLIPA conclude from this formal validation trial under blind conditions that the 3T3 NRU PT test is a scientifically validated *in vitro* test which is ready to be considered for regulatory purposes for assessing the phototoxic potential of chemicals. A draft OECD Guideline for "*In Vitro* Phototoxicity Testing", incorporating the standard protocol of the 3T3 NRU PT test, will be submitted to the OECD test guidelines programme in due course.
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Abbreviations: CV = classification variability; DMEM = Dulbecco's modified Eagle's medium; DMSO = dimethyl sulfoxide; EBSS = Earle's balanced salt solution; MPE = mean photo effect; MT = management team; NRU = neutral red uptake; OECD = Organisation for Economic Cooperation and Development; PIF = phototoxicity factor; RBC = red blood cells; SOP = standard operating procedure; TF = task force.

Keywords: *in vitro* toxicology; phototoxicity; validation study; phototoxic potential; photoirritation; cosmetic ingredients.

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:ment Team members.

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INTRODUCTION

Background and goals of the study

Photosensitization is defined as a process in which reactions to normally ineffective radiation doses are induced in a system by the introduction of a specific, radiation-absorbing substance, the photosensitizer, which causes another substance, the substrate, to be changed by radiation. When used to describe the reaction of skin to an exogenous chemical and UV or visible radiation, the term includes phototoxic and photoallergic reactions, as well as photomutagenicity and photocarcinogenicity (Spielmann *et al.*, 1994c).

Phototoxicity (=*chemical phototoxicity*) is the term used for an acute reaction which can be induced by a single treatment with a chemical and UV or visible radiation. *In vivo*, the reaction can be evoked in all subjects, provided that concentration of chemical and dose of light are appropriate. The term *photoirritation* is used to describe phototoxic reactions in skin produced by substances applied topically to the skin or via the systemic route and exposure to UV or visible light. *Photoallergy* is an acquired immunological reactivity. The skin reaction does not occur on first treatment with a chemical or light. Rather, an induction period is required before skin reactivity can occur.

The current toxicity assays for "*acute dermal phototoxicity*" are animal tests using guinea pigs, rabbits, rats or mice. Although standard protocols for phototoxicity testing in animals have recently been published (Nilsson *et al.*, 1993; OECD, 1991), no animal phototoxicity test has yet been accepted by the OECD. Instead, OECD experts recommended a sequential approach for phototoxicity testing, involving the use of *in vitro* assays prior to testing in animals (OECD, 1995). In 1991, DG XI of the EU and COLIPA agreed to conduct a prevalidation study on *in vitro* phototoxicity tests. It was the goal of the *EU/COLIPA validation project on in vitro phototoxicity tests* to determine whether currently available *in vitro* methods were capable of predicting the phototoxic potential to humans of chemicals applied via the systemic route or topically to the skin.

Among the assays currently developed for *in vitro* phototoxicity testing, two main types can be distinguished, namely cellular assays for screening purposes and mechanistic assays to identify specific mechanisms of phototoxicity (Spielmann *et al.*, 1994c). The basic mechanism in phototoxicity can be described as an increase in toxicity of a chemical induced by exposure to UV or visible radiation. Therefore, the phototoxic potential of a chemical can be measured as an increase in cytotoxicity after exposure to UV or visible light. A large variety of test systems have been used to screen for phototoxic potential, including mammalian and non-mammalian permanent cell lines and primary cell cultures (Pape *et al.*, 1994; Spielmann *et al.*, 1994c).The EU/COLIPA *in vitro* phototoxicity testing programme relies on both cellular and mechanistic assays. The 3T3 NRU PT, an *in vitro* test for chemical phototoxicity, was the most promising *in vitro* test to identify chemicals with phototoxic potential in both phase I and phase II of the EU/COLIPA validation study. Therefore, the 3T3 NRU PT will be recommended as the first test in the EU/COLIPA testing strategy for phototoxic potential. To prove that this core test has been properly validated and is ready to be accepted for regulatory purposes, the formal validation of the 3T3 NRU PT is reported here in detail, as *Part 1* of two reports on the results of phase II of the EU/COLIPA *in vitro* phototoxicity validation study, the blind trial. The results obtained with other *in vitro* phototoxicity tests in phase II of study will be given in *Part 2* of the report. How to use *in vitro* assays within a testing strategy for regulatory purposes is an important question, but is beyond the scope of a formal validation study and will, therefore, not be discussed here.

Management and funding

To co-ordinate the EU/COLIPA validation project, a management team (MT) of six scientists was appointed, three representing the EU (Michael Balls, Georges Pechovitch, Horst Spielmann) and three from COLIPA (Jack Dupuis, Wolfgang Pape, Odile de Silva). In phase II of the study, the MT set up a management structure which basically followed the recommendations of the ECVAM *Workshop on Practical Aspects of the Validation of Toxicity Test Procedures* (Balls *et al.*, 1995). A Chemicals Task Force (TF) was established, lead laboratories were appointed for each of the tests to be validated, and Standard Operating Procedures (SOPs), including statistically-based prediction models, were prepared and approved by the MT for each *in vitro* assay. Finally, the distribution and coding of chemicals, as well as the biostatistical analysis of the data to be produced, were contracted out to independent institutions. The biostatistical analysis was performed according to the guidelines of the ECVAM Task Force (TF) on Biostatistics (Holzhütter *et al.*, 1996). COLIPA and ZEBET provided *in vivo* and *in vitro* phototoxicity data for selecting test chemicals and for establishing the phototoxicity data base. In collaboration with the lead laboratories, ZEBET helped to establish SOPs for each of the *in vitro* tests to be validated, and ZEBET acted as lead laboratory for the primary core test of phase II, the 3T3 NRU PT test.

Selection of *in vitro* tests and chemicals in phase I

A COLIPA *Task Force (TF) on In Vitro Phototoxicity* carefully selected 20 chemicals (11 phototoxins, four non-phototoxic and five UV-absorbing non-phototoxic) according to historical

in vivo animal data and human clinical data. It was agreed to compare the performances of *in vitro* phototoxicity assays established in laboratories of the European cosmetics industry. To standardize the quality of the work, all the participating laboratories agreed to perform an *in vitro* phototoxicity assay based on the 3T3 mouse fibroblast neutral red uptake (NRU) cytotoxicity test (Borenfreund and Puerner, 1985), which was modified for phototoxicity testing by exposing 3T3 cells to test chemicals in the presence and absence of UVA. In addition, the following established *in vitro* assays were evaluated in one or more laboratories during phase I of the study (Spielmann *et al.*, 1995), the prevalidation stage: photohaemolysis and haemoglobin oxidation in red blood cells (RBC), histidine oxidation, a *Candida albicans* assay, a human keratinocyte assay, and two new commercial assays, the physicochemical SOLATEX PT™ assay, and the Skin² PT™ assay, with reconstructed human skin (Edwards *et al.*, 1994; Liebsch *et al.*, 1995).

Standardization of exposure to UVA was an important technical aspect of phase I of the study. Therefore, all laboratories agreed to use an identical light source in all of the assays.

Results of phase I

The results of phase I of the study have been published (Spielmann *et al.*, 1994b,c and 1995). The 20 test chemicals covered a representative spectrum of phototoxic and non-phototoxic chemicals. To facilitate testing in simple tissue culture systems, most of the chemicals were water soluble.

Evaluation of the outcome of phase I showed that all of the test chemicals could correctly be identified in the 3T3 NRU PT test, in which a phototoxicity factor (PIF) was used to discriminate between phototoxic and non-phototoxic chemicals.

Goals and structure of phase II

Taking this encouraging result into account, the MT and the TF decided to use the 3T3 NRU PT test as a core assay in nine laboratories during phase II of the study, that is, for formal validation in a blind trial. In addition to the 3T3 NRU PT test, the following *in vitro* tests from phase I were included in phase II, although most of them were still at the stage of test development or prevalidation (Curren *et al.*, 1995):

— the RBC PT test, (three labs)
— the SOLATEX PT test (two labs)
— the histidine oxidation test (two labs)
— a protein binding test (two labs)
— a human keratinocyte test (one lab)
— the skin² ZK1350 PT test (one lab), and
— a complement PT test (one lab).

Phase II was conducted according to the recommendations of European experts on validation (Balls *et al.*, 1995), as a blind trial in 11 laboratories in Europe and the USA. The test chemicals were mainly from those selected by a panel of experts at an ECVAM *Workshop on In Vitro Phototoxicity Testing*, who took into account high-quality human data from clinical trials (Spielmann *et al.*, 1994c).

The MT of the study has decided to publish the results of phase II in two parts. In *Part 1*, the present report, the results of the formal validation of the 3T3 NRU PT test will be described in detail. In *Part 2*, the prevalidation data obtained from the remaining seven *in vitro* phototoxicity tests will be presented and analysed.

The participating laboratories and the names of all those who actively contributed to the study are listed in Table 1.

MATERIALS AND METHODS

Selection of chemicals for the blind trial

In 1993, an ECVAM *Workshop on In Vitro Phototoxicity Testing* (Spielmann *et al.*, 1994c) selected a list of reference chemicals for phototoxicity validation studies, which was entirely based on human data. At the ECVAM workshop, data were presented from a clinical trial in which a standardized *photopatchtest* was used to evaluate the acute skin phototoxic potential and photoallergy potential of drugs and chemicals to the human skin (Hölzle *et al.*, 1991). The list of chemicals recommended for *in vitro* phototoxicity testing by the workshop covers the most important groups of phototoxic chemicals.

In Phase I of the study, chemicals had been selected not only to cover those of interest to the cosmetic industry, but also to provide an approximately equal proportion of phototoxic and non-phototoxic chemicals, and, finally, to be applicable to cell culture tests. Therefore, predominantly water soluble materials were tested. In phase II of the study, insoluble materials were deliberately included, in order to provide information on the limits of the 3T3 NRU PT test.

For Phase II of the study, the Chemicals TF selected 32 test chemicals for the blind trial, all taken from a list of chemicals recommended by the ECVAM *Workshop on In Vitro Phototoxicity Testing*. The Chemicals TF thereafter carefully reviewed the existing literature and compiled an *in vivo* and *in vitro* phototoxicity database (Table 2a,b), which includes human and animal *in vivo* data, as well as results from *in vitro* phototoxicity tests and information on photoallergy. The Chemicals TF classified the test chemicals under the heading "evaluation of phototoxic potential" as either "phototoxins" or "photoallergens". It should be noted that the amount and quality of the available information are not the same for each of the chemicals. Moreover, owing to the lack of sufficient acute phototoxicity data, three of the chemicals had to be

Table 1. Institutions and scientists participating in Phase II of the international EU/COLIPA *in vitro* phototoxicity validation study

Participating institutions	Names of contributing scientists
ZEBET at the BgVV Berlin, Germany	Horst Spielmann Manfred Liebsch Beate Döring Susanne Boy Dieter Traue
COLIPA, The European Cosmetic, Toiletry and Perfumery Association	Jack Dupuis
European Commission-ECVAM Ispra, Italy	Michael Balls Paolo Catalani Angelo Collotta
European Commission-DG XI Brussels, Belgium	Georges Pechovitch
Beiersdorf AG Hamburg, Germany	Wolfgang J. W. Pape Uwe Pfannenbecker
BIBRA International Carshalton, UK	Paul G. Brantom
CIBA-GEIGY Ltd Basel, Switzerland	Thomas Maurer
Henkel KGaA Düsseldorf, Germany	Winfried Steiling
Hoffmann-La Roche AG Basel, Switzerland	Jörg M. Potthast Miklos Csato
Humboldt-Universität zu Berlin (Charité) Berlin, Germany	Hermann-Georg Holzhütter Eckard Misdom
L'Oreal Aulnay-Sous-Bois, France	Odile de Silva Pierre Desolle
The Procter & Gamble Company Cincinnati, USA	G. Frank Gerberick Lynn W. Cruse
Unilever Environmental Safety Laboratory Sharnbrook, UK	Will W. Lovell Penny Jones
FRAME Alternatives Laboratory University of Nottingham, UK	Richard Clothier Helen Cox Louise Hughes Angela Wilshaw
Warsaw Medical School Warsaw, Poland	Dariusz Sladowski

Names of the nine laboratories which performed the 3T3 NRU PT test are marked in bold letters.

classified "weak (±)" in the "phototoxin" column and "positive" in the "photoallergen" column.

From the final list of 32 test chemicals, BIBRA selected, coded and distributed 30 materials (Table 2) for use in the blind trial, so that the participating laboratories would not know how many phototoxic and how many non-phototoxic materials were supplied to them. Three of the chemicals (4/5, 19/20, 27/28) were tested both as salts and as free acids or bases. Thus, in essence, 27 different chemicals from the original list of 32 were tested in phase II.

Irradiation

To maximize the interlaboratory reproducibility of the results, interlaboratory differences in irradiation characteristics had to be reduced to a minimum. Therefore, for the 3T3 NRU PT assay, all except one of the laboratories used an identical UV light source in phase I and phase II of the study, a doped mercury metal halide lamp (SOL 500; Dr Hönle, Martinsried, Germany), which simulates the spectral distribution of natural sunlight (Fig. 1). The US laboratory, which did not take part in phase I, used a slightly different type of sun simulator (SOL 3; Dr Hönle) with an identical spectral output in the range of 290–550 nm and a higher output than the SOL 500 in the visible wavelength range of 550–690 nm. Despite this difference in the UV sources used, a spectrum almost devoid of UVB (< 320 nm) was achieved by filtering with 50% transmission at a wavelength of 335 nm (filter: H1; Dr Hönle). Irradiation was adjusted to 1.7 mW/cm^2 (about 60 cm distance) with a calibrated UVA-meter (Type No. 37; Dr Hönle). Measurements were made through the polystyrene lids of the 96-well plates used in the 3T3 NRU PT test. Calibration was controlled with a second calibrated reference UVA meter of the same type.

Design of the 3T3 NRU PT test

The NRU cytotoxicity assay with Balb/c 3T3 fibroblasts (Borenfreund and Puerner, 1985) was adapted for phototoxicity testing in the following

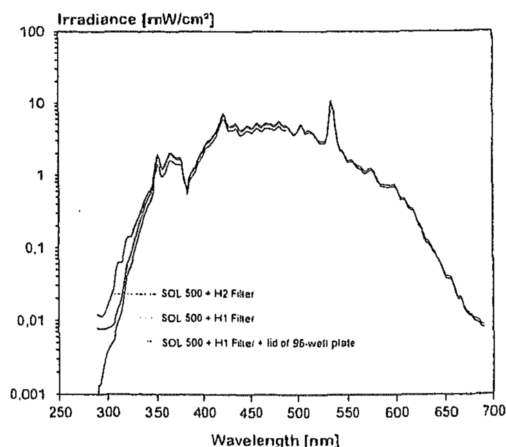

Fig. 1. Filtered spectral output of the sun simulator SOL 500. The upper curve shows the spectral output (irradiance in mW/cm^2) of the SOL 500 when filtered with a H2 filter (Dr Hönle). The middle curve shows the spectral output when filtered with the H1-filter. The lower curve shows the H1 filtered output plus additional filter effect of the polystyrene lid of the 96-well microtitre plate which covered the cells during light exposure in the 3T3 NRU PT test.

Table 2a. 30 Selected test chemicals used in Phase II of the EU/COLIPA study assessment of *in vivo* and *in vitro* databases and solubility

Ch. no.	Chemical	Phototoxicity data: *in vivo* animals	*in vivo* humans	*in vitro* (at least in one test)	Evaluation of phototoxic potential: photo-toxin	photo-allergen	Solubility class*: H_2O	ETOH
1	2-Hydroxy-4-methoxybenzophenone	no data	(±)	–	?	?	7	2
2	5-Methoxypsoralene (5-MOP)	+	+	+	+	–	7	3
3	6-Methylcoumarin	weak +	+	+	+	+	7	?
4	*Acridine–hydrochloride*	+	+	+	+	–	3	?
5	*Acridine–free base*	+	+	+	+	–	?/5	2
6	Amiodarone	+	+	+	+	–	6	4
7	Anthracene	+	+	+	+	–	7	4
8	Bergamot oil	+	+	+	+	–	?	2
9	Bithionol	s + / t–	+	+	+	+	7	5
10	Chlorhexidine dihydrochloride	no data	–	–	–	(+)?	4/6	6
11	Chlorpromazine	+	+	+	+	+	1	2
12	Demeclocycline	+	+	+	+	–	3/4	5
13	Fenofibrate	no data	(+)	+	+	?	7	5
14	Furosemide	no data	+	no data	+	–	5/7	4
15	Hexachlorophene	–	(±)?	–(UVB: +)	–	?	6/7	2
16	Ketoprofen	–	(±)	+	(±)	+	7	2
17	Sodium lauryl sulfate	no data	no data	–	–	–	3	?
18	Musk ambrette	–	(±)	+	(±)	+	7	?
19	*Nalidixic acid–sodium salt*	+	+	+	+	(+)?	?	6
20	*Nalidixic acid–free acid*	+	+	+	+	(+)?	6	5/6
21	Neutral red	+	+	+	+	–	3	3
22	Norfloxacin	+	+	(+)	+	–	4/5	5
23	Ofloxacin	+	+	+	+	–	?/5	?
24	*p*-Aminobenzoic acid (PABA)	–	(±)?	–	–	+	5	2
25	Penicillin G sodium salt	no data	no data	–	–	–	2/3	5
26	Promethazine	+	+	+	+	+?	1	2
27	*Protoporphyrin IX–free acid*	+	no data	+	+	–	?	?
28	*Protoporphyrin IX–disodium salt*	+	no data	+	+	–	?	?
29	Rose bengal	–	+	+	+	–	6	3
30	Tiaprofenic acid	+	+	+	+	–	5	3

*Solubility classes.
s = systemic application t = topical application ETOH = ethanol.
(+) = weak positive (±) = positive, but insufficient data ? = unclear.

Table 2b.

Term	Range of solubility	Solubility class
very soluble	> 1 000 mg/ml	1
freely soluble	> 100 mg/ml–1000 mg/ml	2
soluble	> 30 mg/ml–100 mg/ml	3
sparingly soluble	> 10 mg/ml–30 mg/ml	4
slightly soluble	> 1 mg/ml–1 mg/ml	5
very slightly soluble	> 0.1 mg/ml–1 mg/ml	6
practically insoluble	0.1 mg/ml and lower	7

manner (INVITTOX PROTOCOL No. 78, Spielmann *et al.*, 1994a,b): Balb/c 3T3 cells, clone 31 (ICN-Flow) were cultured in 96-well microtitre plates. After 24 hr, the Dulbecco's modified Eagle's medium (DMEM) was removed, cells were washed twice in Earle's balanced salt solution (EBSS), and eight concentrations of the test chemicals, dissolved in EBSS, were added. Insoluble chemicals were dis-

solved in a solvent compatible with the 3T3 cells [preferably dimethyl sulfoxide (DMSO) or ethanol] and added at a maximum of 1% (v/v) to the EBSS. After 60 min of incubation with test chemicals, the cells in the microtitre plates were exposed to the sun simulator (UVA irradiance: 1.7 mW/cm^2) for 50 min (UVA dose = 5 J/cm^2). Concurrently, a second set of plates with the same test chemicals were kept in the dark. After exposure to UV light, EBSS was replaced by DMEM (without test chemical), and NRU was determined 24 hr later in a plate-reader at 540 nm (Spielmann *et al.*, 1994a,b).

The complete methodology, including assay acceptance criteria, routine culture of the 3T3 cells and the prediction model, was fixed in an SOP. The SOP was drafted by the lead laboratory (ZEBET) from a protocol originally developed at Beiersdorf, then amended and approved by all participating laboratories. According to the SOP, each of the 30 test chemicals had to be tested twice, independently on separate occasions. Prediction of phototoxic potential was achieved by applying the prediction models described below The IC_{50} is defined as the concentration of test material which causes a 50% reduction of NRU compared with that of untreated control cultures.

Prediction model

Original version based on the Photoinhibition Factor (PIF)

The prediction model described in the SOP used in this validation study was derived from analysis of interlaboratory data obtained in the preceding EU/COLIPA prevalidation study (Spielmann *et al.*, 1994a,b). It is based on a comparison of two equally effective cytotoxic chemical concentrations (IC_{50} values) obtained in concurrently performed experiments in the presence (+UV) and absence (−UV) of UVA irradiation. The IC_{50} values obtained in the light and dark experiments were compared by calculating a PIF (photoinhibition factor):

$$\mathrm{PIF} = \frac{IC_{50}(-UV)}{IC_{50}(+UV)}$$

Discriminant analysis of the results obtained in the prevalidation study revealed a cut-off value of PIF = 5 for predicting phototoxic potential (e.g. Liebsch *et al.*, 1994).

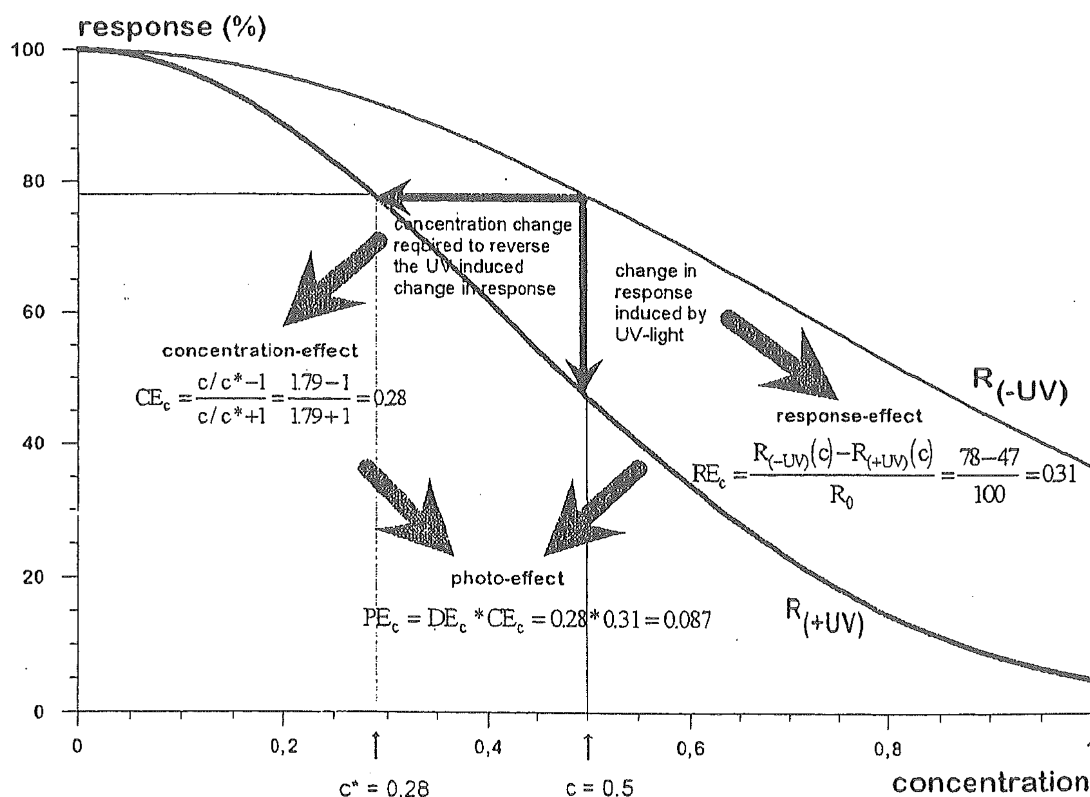

Fig. 2. Definition of the measures, concentration effect, response effect and photo effect used for determination of the mean photo effect (MPE). The MPE prediction model is based on a comparison of the +UV and −UV concentration–response curves on a grid of concentrations c_i (1 = 1,..., N) chosen from the common concentration range of the dark and light experiments. The photo effect PE_i at concentration d_i is computed as product of the concentration effect CE_i and the response effect RE_i. The mean photo effect (MPE) is obtained by averaging across all PE_i values.

Since the PIF could be only calculated in cases where both IC_{50} values existed, that is, where the concentration–response curves in the presence and the absence of UV light dropped below 50% NRU of the controls, the prediction model defined in the SOP contained two additional classification rules:

1. If a chemical is only cytotoxic +UV and is not cytotoxic when tested −UV, the factor shown above cannot be calculated, although this is a clear result **indicating phototoxic potential**. In this case, a >PIF comparable between different laboratories can be calculated, if the cytotoxicity assay is performed in fixed-concentration steps of 10, and the highest testable concentration (−UV) is used for calculation of the >PIF:

$$> \mathrm{PIF} = \frac{C_{max}(-UV)}{IC_{50}(+UV)}$$

2. If both, IC_{50} (−UV) and IC_{50} (+UV) cannot be calculated due to the fact that a chemical does not show cytotoxicity up to the highest testable concentration, this **indicates no phototoxic potential**.

For biometrical analysis, the verbal classification rules of the SOP were applied, as follows:

1. If only a ">PIF" could be obtained, then any value > 1 predicts a phototoxic potential.
2. If no cytotoxicity was obtained, in both the dark and light experiments, a formal "PIF = *1" was assigned to the data.

In summary, the prediction model according to the SOP comprised three classification rules:

(1) If IC_{50} (−UVA) and IC_{50} (+UVA) were determined:
PIF ⩾ 5 = phototoxic
PIF < 5 = non-phototoxic

(2) If C_{max} (−UVA) and IC_{50} (+UVA) were determined:
">PIF">1 = phototoxic
">PIF" ⩽ 1 = non-phototoxic *(did not occur)*

(3) If C_{max} (−UVA) and C_{max} (+UVA) were determined:
PIF=*1 = non-phototoxic

Refined version of the prediction model based on the Mean Photo Effect (MPE)

A major limitation of the prediction model described above is the fact that the PIF is based on the comparison of two equi-effective concentrations (IC_{50}) in the dark and light experiments, which cannot be determined in every case. For example, for case (2), above, the absolute numerical values achieved as ">PIF" depend on the determination of the highest testable concentration of the test chemical. To overcome this limitation, a novel measure for the phototoxic potential of chemicals, the mean photo effect (MPE), has recently been proposed (Holzhütter, 1997). It is based on a comparison of the +UV and −UV concentration–response curves on a grid of concentrations c_i ($I=1, \ldots, N$) chosen from the common concentration range of the dark and light experiments. The photo effect (PE_i) at concentration c_i is computed as a product of the concentration effect (CE_i) and the response effect (RE_i) (c.f. Fig. 2). The MPE is obtained by averaging across all PE_i values. Analogous to PIF, the MPE can be used in a prediction model for the phototoxic potential of chemicals by comparing it with a critical cut-off value, MPE_c. The cut-off value $MPE_c=0.1$ was derived from a first application of the MPE-based prediction model to data obtained in phase II of the EU/COLIPA study in a test carried out by the FRAME/University of Nottingham laboratory according to the same test design, but with primary human keratinocytes instead of 3T3 cells (Holzhütter, 1997).

Both the MPE prediction model and the PIF prediction model are based on a comparison of two concentration–response curves obtained with a chemical concurrently with and without UVA irradiation. The two models have the same basic structure, but use different parameters for comparing differences in the same set of +UVA and −UVA concentration–response curves of a chemical. Thus, the application of the MPE prediction model to the data obtained in the present study is regarded as mathematical refinement and not a *post hoc* change to the basic model itself (Archer *et al.*, 1997). In addition, the refined MPE prediction model is currently being applied in a further ECVAM special study on *in vitro* phototoxicity, with a new set of chemicals.

Biometrical analysis

Quality check and processing of the raw data

Raw data from the 3T3 NRU PT test were submitted by participating laboratories on standard MS EXCEL spreadsheets to the independent biostatistician, where they were carefully registered and checked for consistency. This check included:

— *completeness of the data*
— *correct numerical format of all sheet entries*
— *identification of apparently wrong concentration units*
— *identification of deviations from the SOP*

The participating laboratories reported all problems detected during this quality check to the

biostatistician via the MT. This indirect dialogue between the biostatistician and the experimental toxicologists resulted in an improvement of the data sheets for statistical analyses.

Analysis of concentration–response curves

The prediction model of the 3T3 NRU PT test is based on comparison of the cytotoxicities (IC_{50}) of a chemical in the presence and absence of UV light. For the curve fit, a novel mathematical concentration–response model, FITGRAPH, was used (Holzhütter and Quedenau, 1995), which can adequately fit even complex (e.g. non-monotonic or biphasic) concentration–response relations. Goodness of fit was assessed by applying the SIGNS-test, Wilcoxon's Rank-Sum-test, RUNS-test, χ^2-test and, finally, the Z-test of Zwanzig, which determines whether the sum of deviation squares of residuals is significantly smaller than the variance of the data. The last-named test was found to be the most sensitive one in discriminating between "good" and "bad" data fits. A detailed description of these tests and a comparison of their performance is given in Buckwitz and Holzhütter (1990) and Holzhütter *et al.* (1996).

The variability of the IC_{50} and MPE values estimated from a single pair of concentration–response curves (intra-assay-error) was assessed by computer-aided Monte-Carlo simulations (for details, see Holzhütter *et al.*, 1996). This was necessary, because these values do not represent the parameters of the model, so the conventional technique for deriving approximate values for variances of the model parameters could not be applied. As the total number of single response measurements (3–6) at a given concentration was too small to obtain a reliable estimate of data variance, the bootstrap resampling method (Efron and Tibshirani, 1993) was employed, which is known to work well with small sample sizes. An example of this application of the bootstrap resampling method to data for chemical hexachlorophene (no. 15), obtained in one single run of the 3T3 NRU PT test, is shown in Fig. 3. The bundle of curves obtained after 10 resampling trials with the +UV and −UV concentration–response values is shown.

Comparison of in vitro/in vivo classification of phototoxic potential

The *in vivo* phototoxic potentials of the 30 test chemicals are reported in terms of an ordinal classification (Table 2). The predictive capacity of the 3T3 NRU PT test was assessed by means of 2 × 2 contingency tables, to compare the *in vivo* classifi-

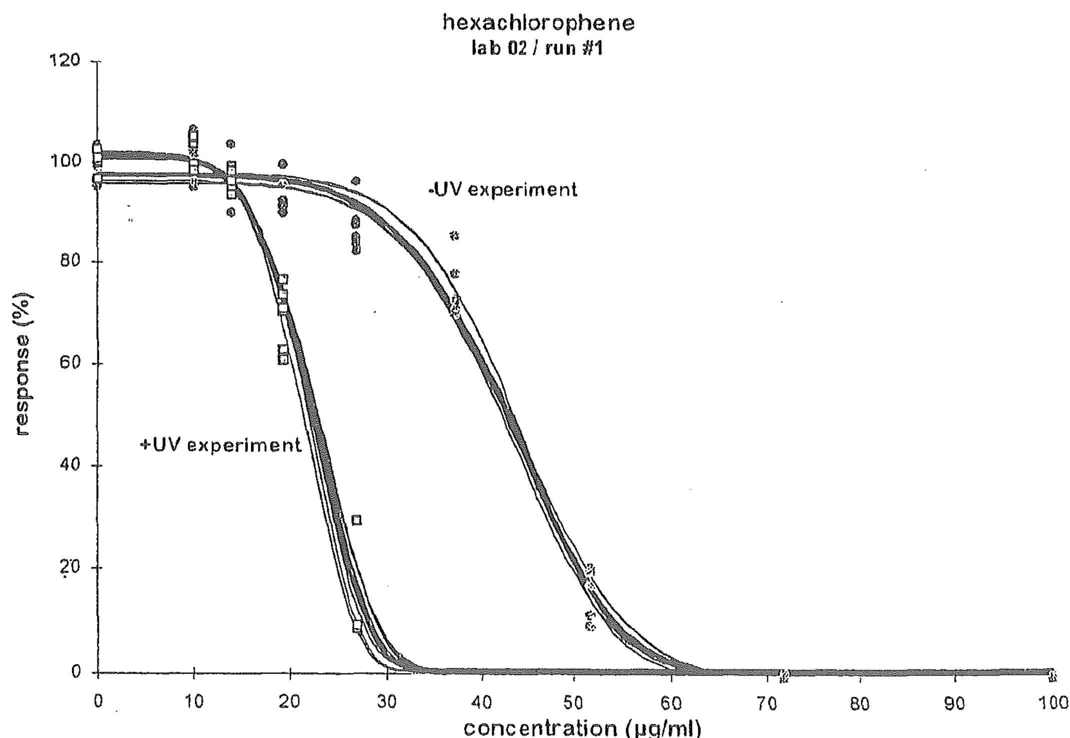

Fig. 3. Assessment of variability: curve fit including bootstrap resampling. Averaging across the two bundles of curves obtained in a single run of the 3T3 NRU PT test in one laboratory reveals a PIF = 1.93 ± 0.007 and a MPE = 0.067 ± 0.005. Thus, according to both prediction models (see Materials and Methods), hexachlorophene would be classified as non-phototoxic, based on the difference between the +UV and the −UV curves produced in this experiment. (Note: classifications given in Table 3 and Table 5 are based on mean values obtained in two independent experiments).

cations of phototoxicity of the test chemicals with the *in vitro* predictions obtained in the *in vitro* test. This procedure is recommended as a standard way of assessing data from validation studies (Balls *et al.*, 1990; Holzhütter *et al.*, 1996). The following performance measures were calculated from the contingency tables: *sensitivity, specificity, positive predictivity, negative predictivity, accuracy, and prevalence.* In addition, to assess the degree of *in vitro/in vivo* association, a χ^2-test (corrected according to Yates for sample-sizes of N = 20–60) was performed. For N = 30 chemicals and an error probability of $\alpha = 0.05$, there is significant degree of association between the two classifications, if the value of χ^2 is larger than the critical value, 3.8.

Assessment of intralaboratory variability of in vitro classifications

Each chemical was tested only twice in independent experiments in each laboratory, so the analysis of variance (ANOVA) was not very reliable. Instead, the reproducibility of the two independent runs was tested by computing a classification variability (CV) for each chemical.

Assessment of interlaboratory variability of in vitro classifications

In both versions of the prediction model, PIF and MPE, chemicals are assigned to two classes of phototoxicity: either "phototoxic" or "non-phototoxic". Therefore, variability of the experimental data and hence of the endpoint values derived from these data may only cause concern if it has an impact on the classifications derived. The classifications of phototoxicity were obtained by comparing the mean values of PIF and MPE with the corresponding cut-off values. In order to see how robust these classifications were against variability of the underlying data, a CV was computed for each chemical. The CV gives the percentage of cases in which the classification based on the single-experiment values of PIF or MPE deviates from the classification based on mean values of PIF or MPE:

$$CV = \frac{100}{N} \sum_{i} \sum_{jlab} \left| C^{lab} - C_{ij}^{lab} \right|$$

Here, C^{lab} is the classification of photoirrititation assessed by the laboratory (lab) on the basis of the mean value of PIF or MPE, respectively, and C_{ij}^{lab} denotes the classification which would be assigned to the chemical if this decision was based on a single value of PIF or MPE obtained from combining randomly the i-th bootstrap UV(+) concentration–response curve with the j-th (bootstrap) UV(−) concentration–response curve for the same experiment. The denominator (N) is the product of all bootstrap combinations and of all laboratories involved.

RESULTS

PIF values: correlation with phototoxicity *in vivo*

The PIF values (including type "<", and type "*1" results) obtained in nine laboratories in two determinations with each of the test chemicals are shown in Table 3. One of the laboratories provided data produced according to the SOP for only 16 test chemicals. According to the classification scheme whereby PIF >5="phototoxic" in the NRU PT test, the discordance rate column (DR) of Table 3 gives a summary of the false positive and negative classifications in comparison with the *in vivo* classification of the test chemicals given in Table 2. The first analysis of the data demonstrates that most of the test chemicals were correctly identified by the majority of laboratories, except for chemical 14, even before the appropriate use of solvents and of concentration ranges had been analysed (see Tables 7 and 8). Three chemicals were classified falsely as "non-phototoxic" by more than two laboratories: *furosemide* (no. 14) with six discordances, *bergamot oil* (no. 8) with four discordances, and *amiodarone* (no. 6) with three discordances. Chlorhexidine dihydrochloride was classified falsely as "phototoxic" by three laboratories, although it is classified "non-phototoxic" *in vivo* according to Table 2.

One chemical was excluded from the calculation of *in vivo/in vivo* correlations, since the quality of the *in vivo* data was insufficient according to Table 2, namely *2-hydroxy-4-methoxybenzophenone* (no. 1).

A 2×2 contingency table summarizing the classification results from Table 3 is given as Table 4. The accuracy of the prediction of *in vivo* phototoxicity from the PIF values in the 3T3 NRU PT test amounts to 88%, with a specificity of 82% and a sensitivity of 90%. The test is characterized by a very high positive predictivity of 96% and by a negative predictivity of 64%, which may be due to the imbalance in the testing set, with four non-phototoxic chemicals *v.* 25 phototoxic ones. This assumption is supported by results from a recent special study on the phototoxic potential of UV-filter chemicals (data not yet published), in which a more balanced testing set was used (10 phototoxic and 10 non-phototoxic test chemicals), a negative predictive value of 95% was obtained for PIF in the 3T3 NRU PT test.

MPE values: correlation with phototoxicity in vivo

The same set of phototoxicity data obtained in the 3T3 NRU PT test were analysed by using the MPE as the measure for phototoxic potential, and the MPE values are shown in Table 5. Again, for Laboratory 1, data from only 16 chemicals could be used to calculate MPE values. Table 5 shows that, for the other eight laboratories, MPE values could be determined for all the chemicals tested. This is a

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Table 3. 3T3 NRU PT test: PIF values and classification of phototoxic potential

Chemical	Ch. no.	*In vivo* classification	PIF									DR	CV (%)
			LAB 1	LAB 2	LAB 3	LAB 4	LAB 6	LAB 8	LAB 9	LAB 10	LAB 11		
2-Hydroxy-4-methoxybenzophenone	1	*unclear*	≠	* 1	* 1	0.9	* 1	> 2.7	* 1	1.1	* 1	*unclear*	0.0
Sodium lauryl sulfate	17	non-ph.irrit.	1.2	1.6	1.0	1.2	1.3	1.0	1.2	1.5	1.1	0/9	0.0
Hexachlorophene	15	non-ph.irrit.	1.4	> 1.5	1.4	1.8	2.6	1.2	1.3	1.1	1.2	1/9	0.0
p-Aminobenzoic acid (PABA)	24	non-ph.irrit./ *ph.allerg.*	0.9	5.3	1.0	* 1	1.0	* 1	1.2	> 2.6	* 1	2/9	12.5
Penicilin G	25	non-ph.irrit.	2.0	* 1	> 1.0	1.2	* 1	1.0	* 1	14.7	* 1	2/9	1.3
Chlorhexidine dihydrochloride	10	non-ph.irrit.	1.3	> 1.2	> 1.2	* 1	> 3.1	1.2	1.6	1.2	* 1	3/9	16.3
Furosemide	14	ph.irrit.	not tested	1.8	1.1	* 1	> 1.3	* 1	1.1	* 1	> 2.8	6/8	8.8
Bergamot oil	8	ph.irrit.	9.5	> 2.6	10.2	4.0	> 2.5	2.1	2.2	103.0	* 1	4/9	17.5
Amiodarone	6	ph.irrit.	5.2	> 2.7	3.3	7.7	1.8	14.9	9.2	1.6	> 2.4	3/9	6.3
Nalidixic acid-free acid	20	ph.irrit.	≠	> 154.7	> 4.1	* 1	* 1	> 3.2	> 1,4	> 2.1	> 16.4	2/8	18.8
Nalidixic acid-sodium salt	19	ph.irrit.	> 5.6	> 25.3	37.9	> 15.8	3.6	2228.4	18.6	2.9	> 4.8	2/9	3.8
Ofloxacin	23	ph.irrit.	≠	> 6.4	> 4.6	> 7.7	15.5	> 6.7	> 1.1	* 1	> 16.4	1/8	6.3
Anthracene	7	ph.irrit.	≠	* 1	> 1.4	> 395.2	> 165.5	> 46497.0	> 893.3	> 1388.0	> 37.0	1/8	6.3
Fenofibrate	13	ph.irrit.	≠	* 1	49.4	46.1	> 16.3	585.9	> 99.6	128.2	> 7.4	1/8	17.5
Bithionol	9	ph.irrit.	10.1	59.8	9.2	7.2	> 12.2	13.2	7.9	16.7	6.1	0/9	17.5
Chlorpromazine	11	ph.irrit.	21.8	42.7	26.7	60.9	20.2	35.3	308.2	18.9	28.4	0/9	13.8
Promethazine	26	ph.irrit.	17.3	86.2	53.1	44.8	13.6	24.0	47.5	20.5	496.6	0/9	16.3
Rose bengal	29	ph.irrit.	23.3	25.5	77.5	11.2	1490.2	106.7	60.7	36.5	> 115.9	0/9	17.5

Protoporphyrin IX - free acid	27	ph.irrit.	242.5	> 951.5	349.4	> 163.3	> 19.7	233.6	50844.1	2321.2	26567.0	0/9	6.3
6-Methylcoumarin	3	ph.irrit.	≠	308.6	168.2	> 23.1	> 7.0	26.7	50.3	30.5	> 75.5	0/8	11.3
Protoporphyrin IX–disodium salt	28	ph.irrit.	32827.4	> 275.2	361.6	> 35.5	> 232.6	4486.8	16191.3	996.0	> 26.2	0/9	2.5
Tiaprofenic acid	30	ph.irrit.	1543.2	41216.1	2013.2	> 713.4	> 65.0	> 707.6	4630.3	> 321.5	2129.7	0/9	7.5
Demeclocycline	12	ph.irrit.	111.4	> 311.5	735.6	> 216.9	> 89.9	> 237.6	390.5	81.8	> 950.4	0/9	3.8
Acridine–hydrochloride	4	ph.irrit.	≠	> 236.5	2485.9	559.4	> 1264.6	> 5595.2	518.5	684.4	> 881.1	0/8	2.5
Acridine–free base	5	ph.irrit.	≠	> 275.7	> 775.7	90.7	> 9515.8	9447.6	1562.3	348.9	> 553.0	0/8	5.0
Norfloxacin	22	ph.irrit.	≠	45.7	> 8.6	> 2.8	> 1.6	> 28.9	127.7	> 2.8	> 13.9	0/8	10.0
Neutral red	21	ph.irrit.	≠	14890.0	> 13479.1	> 4344.8	336.3	> 20736500.0	> 1317.6	> 847.0	> 9317.5	0/8	2.5
5-Methoxypsoralene (5-MOP)	2	ph.irrit.	≠	> 50.6	> 65.2	> 8.8	> 1.0	> 1498.1	> 28.8	> 3.4	> 105.3	0/8	5.0
Musk ambrette	18	ph.irrit./ *ph.allerg.*	≠	* 1	> 8.0	> 42.3	15.5	10.9	> 24.7	5.4	> 4.9	1/8	12.5
Ketoprofen	16	ph.irrit./ *ph.allerg.*	≠	1622.5	2304.4	> 408.9	> 1.5	> 123.3	1057.1	> 1040.7	> 1395.1	0/8	10.0

Ch. no. = chemical number according to Table 2.
≠ = testing not performed according to SOP.
DR = discordance rate = misclassifications per total classifications.
CV = classification variability = for details see Materials and Methods.

Table 4. 3T3-NRU test: comparison of *in vitro* and *in vivo* classifications of phototoxicity based on PIF

		In vivo classification			
		phototoxic	non-phototoxic	unclear	total
In vitro classification	phototoxic	182	8	1	191
	non-phototoxic	21	37	7	65
	total	203	45	8	256
		table statistics for the 2 × 2 table in the box			
	sensitivity:	90%		*prevalence:*	4.51
	specificity:	82%			
	positive predicitivity:	96%			
	negative predicitivity:	64%			
	accuracy:	**88%**			
	χ^2:	**102.24**	(≫3.8)		

major advantage of the new measure in comparison with the PIF system.

When Holzhütter's prediction model was used to classify chemicals by using a cut-off value of MPE > 0.1 = "phototoxic" in the 3T3-NRU PT test, the discordance rate (DR) column of Table 5 gives a summary of the false positive and negative classifications compared to the *in vivo* classification given in Table 2. These data demonstrate that about 60% of the test chemicals were correctly classified by all nine laboratories even before the appropriate use of solvents and concentration ranges had been analysed (see Tables 7 and 8). Again, most of the remaining test chemicals were correctly identified by the majority of laboratories, except for chemical 14. Taking into account the striking consensus among laboratories to classify chemical 14 as "non-phototoxic" in the 3T3 NRU PT test, when using either PIF or MPE, the *in vivo* classification of this chemical needs careful reconsideration.

One of the "non-phototoxic" chemicals was classified as "phototoxic" by more than two laboratories when the MPE approach was used: *p-aminobenzoic acid* (no. 24), with four discordances. Because of the insufficient quality of the *in vivo* data, *2-hydroxy-4-methoxybenzophenone* (no. 1) was again excluded from calculation of the *in vivo v. in vivo* correlations.

A 2 × 2 contingency table summarizing the classification results from Table 5 is given as Table 6. The accuracy of the *in vivo* predictions from MPE values of the 3T3 NRU PT test amounts to 92%, with a specificity of 84% and a sensitivity of 93%. The MPE approach confirmed the very high positive predictivity of 96% and a negative predictivity of 73%. In the recent study on the phototoxic potential of UV-filter chemicals (data not yet published), in which a more balanced testing set was used, as mentioned above, a negative predictive value of 100% was obtained for MPE in the 3T3 NRU PT test. The highly significant result of the χ^2 test confirms that the classification of test chemicals into the two *in vivo* phototoxicity groups—phototoxic *v.* non-phototoxic—from *in vitro* testing in the 3T3 NRU PT test, is highly significant. The accuracy of the predictions from the MPE is identical when the three photoallergens (chemicals no. 14, no. 16 and no. 24) are excluded from the calculation.

Intralaboratory and interlaboratory variability of data

Each chemical was tested in two independent runs in each of the laboratories. Intra-assay reproducibility is shown in Fig. 4 for chemicals 1–16. The graphs demonstrate that from the original concentration response plots, and even without determining PIF or MPE values, the phototoxic potential of the test chemicals can easily be assessed in the following manner: chemicals no. 1, no. 8, no. 10, no. 14 and no. 15 are "non-phototoxic", and all of the others are "phototoxic".

The variability of data between laboratories and the concentration ranges used for testing is illustrated for PIF and MPE in Fig. 5 for three chemicals (no. 7, no. 12 and no. 18). It is obvious from Fig. 5 that there was a variability of data at lower concentrations and that the phototoxic potential of the test chemicals was correctly identified when either PIF or MPE were used by all laboratories for the tetracycline demeclocycline (no. 12). A similar pattern was obtained with the other two chemicals in all of the laboratories except laboratories 1 and 2. Taking into account the use of solvents as given in Table 7, the negative results obtained in laboratory 2 with both anthracene (no. 7) and musk ambrette (no. 18), which are practically insoluble in water (solubility class 7), is due to the use of EBSS rather than more appropriate solvents in this particular laboratory. The data from laboratory 1

Table 5. 3T3 NRU PT test: MPE values and classification of phototoxic potential (cut-off: 0.1)

Chemical	Ch. no.	*In vivo* classification	MPE LAB 1	LAB 2	LAB 3	LAB 4	LAB 6	LAB 8	LAB 9	LAB 10	LAB 11	DR	CV (%)
2-Hydroxy-4-methoxybenzophenone	1	*unclear*	≠	0.055	0.017	−0.010	0.049	0.195	0.055	0.021	0.092	*unclear*	20.0
Sodium lauryl sulfate	17	non-phototoxic	0.027	0.013	0.003	0.017	0.033	−0.002	−0.010	0.013	0.002	0/9	0.0
Hexachlorophene	15	non-phototoxic	0.077	0.036	0.023	0.060	0.233	0.030	0.015	0.005	0.008	1/9	12.5
p-Aminobenzoic acid (PABA)	24	non-phototoxic	0.001	0.178	0.007	0.032	−0.065	0.117	0.010	0.742	0.276	4/9	15
Penicillin G sodium	25	non-phototoxic	0.108	−0.054	0.068	−0.002	0.000	0.054	0.015	0.386	0.065	2/9	20.0
Chlorhexidine dihydrochloride	10	non-phototoxic	0.043	0.040	−0.005	0.022	−0.094	0.022	0.029	−0.011	0.004	0/9	2.5
Furosemide	14	phototoxic	≠	0.139	0.003	0.027	0.340	0.030	0.010	−0.004	0.215	5/8	7.5
Bergamot oil	8	phototoxic	0.387	0.325	0.454	0.459	0.239	0.123	0.442	0.794	0.181	0/9	8.8
Amiodarone	6	phototoxic	0.321	0.738	0.146	0.294	0.127	0.583	0.540	0.089	0.362	1/9	8.8
Nalidixic acid–free acid	20	phototoxic	≠	0.510	0.576	0.261	0.020	0.556	0.281	0.341	0.334	1/8	3.8
Nalidixic acid–fsodium salt	19	phototoxic	0.312	0.417	0.717	0.790	0.102	0.539	0.842	0.168	0.633	0/9	6.8
Ofloxacin	23	phototoxic	≠	0.949	0.861	0.750	0.742	0.780	0.186	0.282	0.792	0/8	6.3
Anthracene	7	phototoxic	≠	0.025	0.098	0.687	0.704	0.865	0.566	0.851	0.803	2/8	8.8
Fenofibrate	13	phototoxic	≠	0.064	0.604	0.407	0.196	0.598	0.486	0.799	0.496	1/8	3.8
Bithionol	9	phototoxic	0.438	0.676	0.046	0.519	0.599	0.612	0.314	0.253	0.479	1/9	6.3
Chlorpromazine	11	phototoxic	0.386	0.707	0.806	0.883	0.529	0.808	0.279	0.382	0.275	0/9	2.5
Promethazine	26	phototoxic	0.652	0.639	0.907	0.706	0.711	0.289	0.322	0.632	0.763	0/9	3.8

Rose bengal	29	phototoxic	0.631	0.698	0.477	0.622	0.508	0.531	0.518	0.823	0.332	0/9	1.3
Protoporphyrin IX–free acid	27	phototoxic	0.647	0.966	0.940	0.599	0.700	0.916	0.884	0.721	0.659	0/9	0.0
6-Methylcoumarin	3	phototoxic	$\neq$	0.836	0.581	0.797	0.302	0.458	0.413	0.796	0.742	0/8	0.0
Proptoporphyrin IX–disodium salt	28	phototoxic	0.697	0.627	0.990	0.677	0.473	0.280	0.976	0.828	0.747	0/9	3.8
Tiaprofenic acid	30	phototoxic	0.681	0.973	0.679	0.813	0.701	0.803	0.916	0.938	0.813	0/9	0.0
Demeclocycline	12	phototoxic	0.327	0.704	0.539	0.335	0.877	0.707	0.590	0.357	0.821	0/9	1.3
Acridine–hydrochloride	4	phototoxic	$\neq$	0.603	0.847	0.630	0.708	0.704	0.395	0.887	0.556	0/8	0.0
Acridine–free base	5	phototoxic	$\neq$	0.641	0.815	0.5940	0.511	0.806	0.665	0.801	0.538	0/8	1.3
Norfloxacin	22	phototoxic	$\neq$	0.782	0.845	0.449	0.335	0.614	0.901	0.559	0.779	0/8	0.0
Neutral red	21	phototoxic	$\neq$	0.938	0.772	0.602	0.656	0.991	0.566	0.876	0.672	0/8	0.0
5-Methoxypsoralene (5-MOP)	2	phototoxic	$\neq$	0.744	0.653	0.672	0.049	0.516	0.669	0.660	0.746	1/8	0.0
Musk ambrette	18	*ph.allerg.*	$\neq$	0.091	0.834	0.692	0.692	0.609	0.669	0.084	0.629	2/8	8.8
Ketoprofen	16	*ph.allerg.*	$\neq$	0.494	0.874	0.633	0.156	0.515	0.582	0.941	0.508	0/8	1.3

Ch no. = Chemical number according to Table 2.
$\neq$ = testing not performed according to SOP.
DR = discordance rate = misclassifications per total classifications.
CV = classification variability = for details see Materials and Methods.