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EEC/COLIPA in vitro Photoirritancy Program: Results of the First Stage of Validation[1]

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Phototoxicity is an acute reaction which can be caused by a single treatment with a chemical and UV or visible radiation. In vivo, the reaction can be evoked in all subjects provided that the concentration of chemical and dose of light are appropriate. The current toxicological assays 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 [1], acceptance of an animal test in an OECD guideline for phototoxicity testing could not be achieved. However, a sequential approach using in vitro testing prior to consideration of animal testing was recommended for phototoxicity testing. Consequently, COLIPA and DG XI of the EEC agreed to conduct a joint project on developing validated in vitro phototoxicity tests, which was coordinated by ZEBET.

During phase I of the study (1992/1993), a set of 20 test chemicals (12 phototoxins (PT), 4 non-PT and 4 UV-absorbing non-PT) was carefully selected by a COLIPA task force. Moreover, all laboratories had to perform an in vitro photo-

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toxicity assay based on the simple 3T3 cell neutral red uptake (NRU) cytotoxicity assay [2], which was modified for phototoxicity testing at the laboratory of Beiersdorf by exposing 3T3 cells both to test chemicals and UVA irradiation.

Since UVA is most relevant in phototoxic reactions, appropriate filters were used to avoid exposure to UVB, which has strong cytotoxic actions. Standardized exposure to UVA was an important technical aspect of the study, therefore, all laboratories had to use an identical light source and to apply an identical dose of 5 J/cm^2 UVA in all of the assays. In the present report, results from 3T3 cell NRU phototoxicity assay (3T3 NRU-PI assay) will be described and data from the other in vitro phototoxicity assays will briefly be summarized.

Materials and Methods

UVA Light Source, UVA Meter

In the 3T3 NRU-PI assay all laboratories used an identical UV light source, a doped mercury-metal halide lamp (SOL 500, Dr. Hönle, Martinsried, Germany), which simulates the spectral distribution of the natural sunlight. A spectrum almost devoid of UVB was achieved by filtering with a 50% transmission at a wavelength of 335 nm (filter: H1, Dr. Hönle). Emitted energy was measured with a calibrated UVA meter (Type No. 37, Dr. Hönle). Calibration was controlled with a second UVA meter which had to be kept in the dark. For the red blood cell (RBC) assay the SOL 500 was filtered with a H2 filter (Dr. Hönle, 50% transmission at 320 nm), cut-off wavelength of 305 nm), since RBC are more resistant to UVB. Phototoxicity testing with the dermal model Skin^2 was performed under the same UVA irradiation conditions as in the standard 3T3 NRU-PI assay.

Selection of Test Chemicals

Selection of test chemicals with high quality in vivo data is very difficult, since data from human patients are rare and quite often differ from animal data. Taking this into account, a COLIPA task force selected a list of 20 test chemicals which are supported by high quality human and animal data from the literature, which were assigned to 3 classes (table 1): class I – 12 phototoxins (PT); class II – 4 chemicals which are absorbing UVA light but are not PT, however some are photoallergens; class III – 4 chemicals which are neither UVA-absorbing nor PT.

According to recent studies [3] chemical No. 8 (piroxicam) should not be classified as a phototoxin, since products from UVA photolysis are inducing allergic reactions in patients either after repeated contact or by cross-reaction with contact sensitizers of similar chemical structure, as e.g., merthiolate [4]. Thus, piroxicam is assigned to class II rather than to class I.

Experimental Design of the 3T3 NRU-PI Assay

The NRU cytotoxicity assay with Balb/c 3T3 fibroblasts was adapted for phototoxicity testing in the following manner [5]: Balb/c 3T3 cells, clone 31 (ICN-Flow) were cultured in 96-well microtiter plates as described earlier [2]. After 24 h DMEM was removed, cells were washed twice in EBSS and eight concentrations of the test chemicals dissolved in EBSS were

added. Insoluble chemicals were dissolved in DMSO and added at a maximum of 1% DMSO in EBSS. After 24 h of incubation with test chemicals plates were exposed to UVA (1.67 mW/cm^2) for 50 min (= 5 J/cm^2). 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 h later according to Spielmann et al. [5]. To determine

Table 1. Phototoxicity of 20 chemicals tested with the 3T3 NRU phototoxicity assay

		In vivo human	In vivo animal	In vitro data			n	Result
				mean IC_{50}–UV µg/ml	mean IC_{50}+UV µg/ml	mean factor –UV/+UV		
Class I								
1	Promethazine	+	+/–	45.9	0.8	78.5	13	+
2	Chlorpromazine	++	++	24.6	0.6	46.6	13	+
3	6-Methylcoumarine	a	a	*	32.7		13	+
4	TCSA	a	+	19.8	0.4	55.6	12	+
5	Doxycycline	+	+	1,182	6.4	255	4	+
6	8-MOP	++	++	*	14.7		11	+
7	Tetracycline	+	+	1,916	16.8	374	9	+
9	Amiodarone	+	+	24.3	4.1	6	9	+
10	Bithionol	+	+	13.9	3.9	7	13	+
11	Neutral red	+		*	0.01		14	+
12	Rose bengal	+/–	–	4.2	0.2	70.2	13	+
Class II								
8	Piroxicam[1]	?	–	*	*		11	–
13	Cinnamic aldehyde	a	a	32.8	10.6	3.6	8	–
14	Chlorhexidine			61.5	74.4	1.5	11	–
15	Uvinul MS 40	+/–		15,958	11,577	1.4	11	–
16	PABA	a		10,463	9,780	1	7	–
Class III								
17	Penicillin G			53,914	49,755	1.1	8	–
18	*L*-Histidine[2]			*	*		12	–
19	Thiourea	a		17,651	16,944	1	13	–
20	Lauryl sulfate			35.6	24.2	1.5	14	–

No cytotoxicity could be determined up to the highest test concentration.
n = Number of determinations; means = arithmetic means of n determinations (± SD not shown); ++ = strong phototoxin; + = phototoxic; – = not phototoxic; +/– = inconclusive; a = (photo)allergen.
[1] Highest test concentration: 2,400 µg/ml.
[2] Highest test concentration: 46,400 µg/ml.

phototoxicity cytotoxic concentrations resulting in 50% reduction of viability (IC_{50}) were compared in the presence and absence of UVA irradiation, by calculation of the factor between the two IC_{50} values as described previously [5]:

$$\text{UV factor} = IC_{50}(-UV)/IC_{50}(+UV).$$

The cut-off value of the UV factor for discriminating between PTs and non-PTs was determined by discriminant analysis as outlined in the 'Results' section.

The Candida albicans *Phototoxicity Assay in Yeast*

In the simple yeast assay [6] *C. albicans* is seeded on agar plates and zones of growth inhibition around the applied test chemical in duplicate plates with and without UVA exposure are compared 24– 48 h after application. The assay is sensitive to phototoxins acting primarily on DNA [7].

Histidine Oxidation Test

Due to activation by UVA or UVB some chemicals will oxidize the amino acid histidine via singlet oxygen formation. Reduction of histidine can easily be monitored in a colorimetric assay [7].

Photohemolysis and Hemoglobin Oxidation with Red Blood Cells

The method is used to study the phototoxic potential of chemicals by their ability to damage the RBC membrane and/or to oxidize hemoglobin when exposed to UV and/or visible light. A new protocol [8] combines the widely used photohemolysis test [7] with the spectral changes characteristic of methemoglobin formation.

SOLATEX PI Assay

The principle of the commercial SOLATEX PI assay (In Vitro International, IVI, USA) is quantification of an enhanced response in a physicochemical model of dermal irritation upon exposure to UV radiation. Controls not exposed to UV radiation are providing the background irritation response. Two concentrations of a known photoirritant ('positive controls') are included to verify the test performance. The total response is measured spectrophotometrically at 400 nm. Materials inducing an increase >40% in OD after exposure to UV light compared to background, are classified as phototoxic.

Skin² PI Assay

The commercial Skin² dermal model ZK 1350 (Advanced Tissue Sciences, ATS, USA) is a three-dimensional human skin model consisting of dermal, epidermal and corneal layers. It is produced by seeding neonatal fibroblasts onto an inert nylon mesh and growing them into dermal tissue. Keratinocytes are seeded on top of the fibroblast layer and are differentiating into an epidermis including a multilayered stratum corneum. The tissue is cut into pieces of 9 mm^2 (ZK 1350) and sterilely sealed test kits are shipped worldwide by airfreight. In the Skin² PI assay, the cytotoxic effects of a chemical are compared to additional exposure to a nontoxic dose of UVA. Chemicals are applied topically and cytotoxicity is determined in the MTT assay in comparison to solvent controls. Cell viability is calculated for each piece of tissue as % of control and the viability of irradiated tissue and dark controls is compared. The cut-off value for classifying a chemical as phototoxic is a reduction in viability of more than 30% due to UVA exposure.

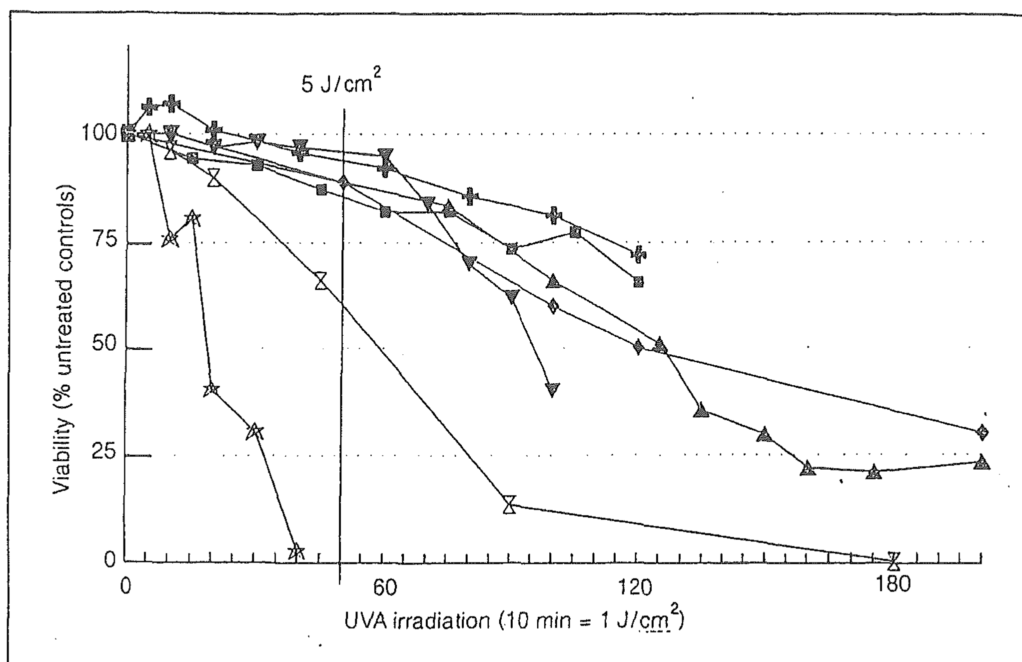

Fig. 1. UVA sensitivity of 3T3 mouse fibroblasts in 7 laboratories. 3T3 cells were exposed to UVA (1.67 mW/cm^2) in 96-well plates for up to 180 min in order to determine UVA sensitivity. Individual curves are representing data from 7 different laboratories. Curves with open symbols were obtained with older cells. The vertical line at 50 min exposure time indicates the standard UVA dose of 5 J/cm^2 which was used for phototoxicity testing in the 3T3 NRU assay.

Results

3T3 NRU Assay

UVA sensitivity of Balb/c 3T3 cells was tested in seven laboratories. Figure 1 shows that in 5 of the laboratories the viability of 3T3 cells with passage numbers between 70 and 80 was not affected within an UVA dose range of about 0–10 J/cm^2. However, in the same dose range in the 2 laboratories which were using 3T3 cells with passage numbers of 130–140, a significant reduction in viability was observed in the same dose range. Therefore, it was decided to use 3T3 cells with a low passage (<100) throughout the study. The result described in figure 1 reveals that for 3T3 cells the highest nontoxic UVA dose was 5 J/cm^2 (50 min exposure at an intensity of 1.67 mW/cm^2). This dose was chosen for further testing in order to detect even weak phototoxins.

A UV factor (IC_{50} -UV/IC_{50} +UV) could only be determined with 15 of the 20 test chemicals, since 5 chemicals (No. 3, 6, 8, 11 and 18) were not cytotoxic to

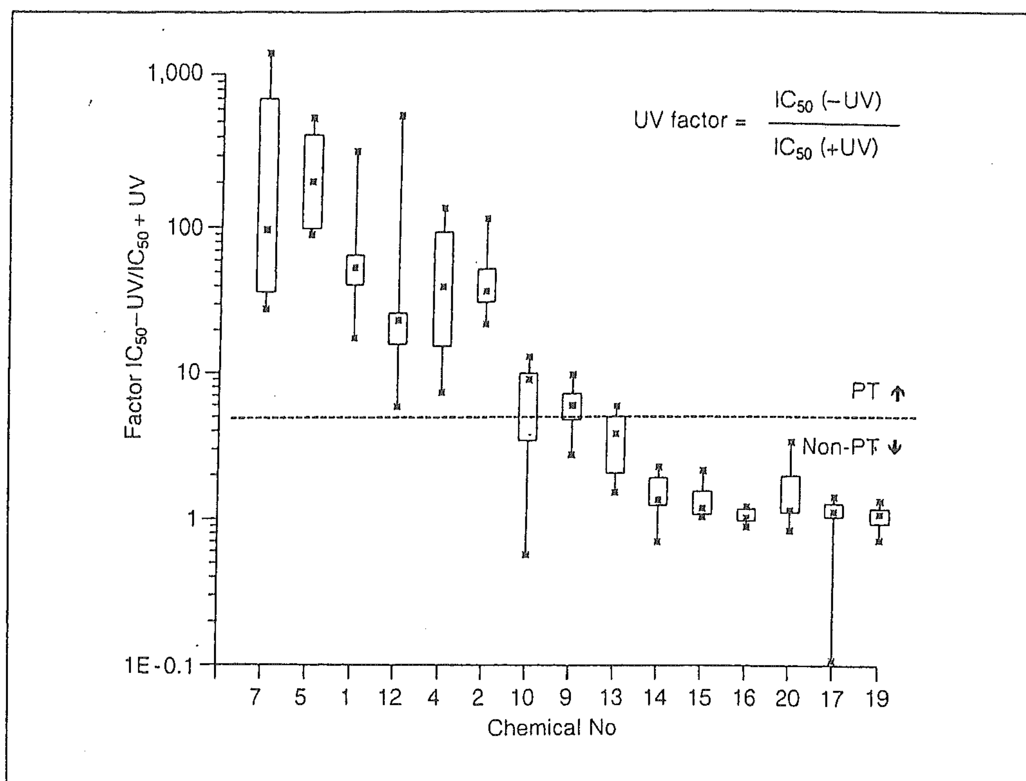

Fig. 2. 3T3 NRU phototoxicity assay: determination of UV photoactivation factors for 15 chemicals by discriminant analysis. UVA factors (IC_{50}–UV/IC_{50} +UV) were determined with 15 chemicals, which were cytotoxic both, with and without UVA exposure. The numbers of the chemicals correspond to tables 1 and 2. The box plots show medians, 95% confidence limits (boxes) and minima/maxima of factors determined in all of the laboratories. The dashed line indicates the cut-off value determined by discriminant analysis to discriminate between nonphototoxic and phototoxic chemicals.

3T3 cells in the dark even at the highest concentrations tested. Taking into account all 158 UV factors calculated with the 15 cytotoxic chemicals in all of the laboratories, discriminant analysis revealed a UV factor of 5.1 as cut-off value to discriminate between phototoxic and nonphototoxic test chemicals. Figure 2 gives the photoinactivation factors for the 15 chemicals as 'median box plots' of 158 determinations.

Table 1 proves that all of the 11 phototoxins and all of the 9 nonphototoxins were correctly identified in the 3T3 NRU-PI assay. UV factors ranging from 6 (No. 9) to 374 (No. 7) could only be determined with 8 of the 11 phototoxic chemicals (class I). Cytotoxicity could not be determined with chemicals 3, 6 and 11 of class I. However, a cytotoxic effect was measured in the NRU assay after UVA

Table 2. Summarized data of assays used in the EC/COLIPA validation study

	Mechanistic assays			Commercial assays		Growth inhibition assays			
	histidine photo-oxidation	RBC photo-hemolysis	RBC photo-Hb oxidation	SOLATEX PI	Skin[2] ZK 1300 ZK 1350	yeast growth inhibition	human lymphocytes MTT	human keratinocytes NRU	COMMON standard 3T3-NRU assay
Class I									
1 Promethazine	+	+	+	+	+	+	+	+	+
2 Chlorpromazine	(+)	+	+	+	+	(+)	+	+	+
3 6-MC	+	+	+	+	–	+	+	+	+
4 TCSA	+	+	+	+	+	+	–	+	+
5 Doxycycline	+	–	+	+	+	+	+	+	+
6 8-MOP	+	–	(+)	–	+	+	+	–	+
7 Tetracycline	+	–	+	+	+	–	–	+	+
9 Amiodarone	–	+	–	+	+	+	+	–	+
10 Bithionol	–	+	+	+/–	–	–	–	+	+
11 Neutral red	+	+	–	+	+	–	+	+	+
12 Rose bengal	++	+	(+)	+	+	+	n.t.	+	+
Class II									
8 Piroxicam*	–	–	–	–	–	–	–	–	–
13 Cinnamic aldehyde	(+)	–	–	–	–	–	–	–	–
14 Chlorhexidine	–	(+)	–	–	–	–	–	–	–
15 Uvinul MS 40	–	–	–	+	–	–	–	–	–
16 PABA	–	–	–	–	–	–	–	–	–
Class III									
17 Penicillin G	–	–	–	–	–	–	–	–	–
18 *L*-Histidine	–	–	–	–	–	–	–	–	–
19 Thiourea	–	–	–	n.q.	–	–	–	–	–
20 Lauryl sulfate	–	–	–	–	–	–	–	–	–

* For classification of piroxicam see 'selection of chemicals'.
n.q. = Test not qualified; n.t. = not tested.

exposure (table 1). The 3 PT were, therefore, correctly classified in the 3T3 NRU-PI assay without using the UV factor.

For the 5 UVA-absorbing chemicals which are not phototoxic in vivo (class II), UV factors between 1 (No. 16) and 3.6 (No. 13) were determined with the exception of piroxicam. UV factors between 1 and 1.5 were observed with the 3 cytotoxic non-UVA absorbing non-PTs (class III). With chemicals 8 (piroxicam) and 18 (*L*-histidine) cytotoxicity could not be determined in the absence or presence of UVA exposure, and they were correctly identified as 'non-PT'.

SOLATEX PI Assay

This new in vitro assay did not allow phototoxicity testing in a reproducible manner mainly due to problems of the software provided by the manufacturer. According to table 2 only 8 of the class I chemicals could be correctly identified in the SOLATEX PI assay, 1 'false-positive' result was obtained with the 4 non-PI chemicals of class II and 1 of the non-PI chemicals of class III could not be tested due to technical problems of the assay.

Skin² PI Assay

Table 2 demonstrates that 9 of the 11 PI chemicals of class I were correctly identified in the skin² ZK 1350 PI assay, No. 3 and No. 10 being the 'false negatives'. All of the negative chemicals of classes II and III were correctly identified as 'non-PI'.

Results Obtained with Additional Assays

Table 2 also gives the data from in vitro phototoxicity testing with assays, which are used to identify specific phototoxic mechanisms, e.g. histidine oxidation, RBC photohemolysis and RBC hemoglobin oxidation. Among different laboratories, the results were quite reproducible. Since these assays are related to specific mechanisms of phototoxicity, each of the tests gave a positive result only with a few of the phototoxic chemicals of class I. There was some overlap, since the phototoxicity of chemicals is quite often due to more than a single mechanism.

In contrast, data obtained with additional in vitro phototoxicity assays, e.g. the human lymphocyte and keratinocyte assays, were not reproducible, since the tests were still under development. As expected, the yeast assay, which is specific for UV-induced damage to DNA, was only positive with some of the PT chemicals of class I.

Discussion and Conclusions

The most important aspect of the EEC/COLIPA validation study of in vitro phototoxicity tests was the use of identical UV exposure conditions in all laboratories. Another important aspect of quality assurance was the determination of UVA sensitivity of the 3T3 cells before they were used in the study. In addition, it has to be emphasized that the quality of in vivo data of test chemicals was a critical aspect for validating in vitro phototoxicity assays, since human data are difficult to obtain and since data are suffering from differences in species specificity. This aspect is illustrated by the misleading animal data of piroxicam which had to be moved from class I to class II due to clinical data in humans.

The 3T3 NRU-PI assay, which was developed in the present study, showed the highest predictivity of all of the in vitro assays evaluated. The predictive value of the 3T3 NRU-PI assay was better than in an earlier study on 3T3 cell phototoxicity [9], in which 8-MOP and doxycycline were classified as 'false negatives' and PABA was classified as 'false positive'. Taking into account the many mechanisms of phototoxicity at the cellular level, it is surprising that a 100% in vitro/in vivo concordance was achieved with the simple 3T3 NRU-PI assay. This assay is, therefore, quite promising for further validation in phase II of the EEC/COLIPA study. Among the more 'general' in vitro phototoxicity tests, which can be used to identify the phototoxic potential of a chemical, the 3T3 cell NRU-PI assay, the RBC assay and the commercial Skin[2] and SOLATEX PI assays were performing much better in the present study than assays using yeast or human lymphocytes or keratinocytes (table 2). The 3T3 NRU-PI assay, the RBC assay and the two commercial in vitro assays will, therefore, also be candidates for further validation in a blind trial. Although the commercial assays are fairly expensive, they will play an important role in phase II of the validation study (blind trial), since they permit testing of solid and insoluble materials, which cannot be tested in the 3T3 cell NRU-PI assay.

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OECD guideline for testing of chemicals, Draft proposal for a new guideline : 432, *In Vitro* 3T3 NRU phototoxicity test (2002,3,15)

OECD GUIDELINE FOR TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE: 432

In Vitro 3T3 NRU phototoxicity test

INTRODUCTION

1. Phototoxicity is defined as a toxic response from a substance applied to the body which is either elicited or increased (apparent at lower dose levels) after subsequent exposure to light, or that is induced by skin irradiation after systemic administration of a substance.

2. The *in vitro* 3T3 NRU phototoxicity test is used to identify the phototoxic potential of a test substance induced by the excited chemical after exposure to light. The test evaluates photo-cytotoxicity by the relative reduction in viability of cells exposed to the chemical in the presence versus absence of light. Substances identified by this test are likely to be phototoxic *in vivo,* following systemic application and distribution to the skin, or after topical application.

3. Definitions used are provided in Annex 1.

INITIAL CONSIDERATION

4. Many types of chemicals have been reported to induce phototoxic effects (1)(2)(3)(4). Their common feature is their ability to absorb light energy within the sunlight range. According to the first law of photochemistry (Grotthaus-Draper Law), photoreaction requires sufficient absorption of light quanta. Thus, before biological testing is considered, a UV/vis absorption spectrum of the test chemical must be determined according to OECD Test Guideline 101. If the molar extinction/absorption coefficient is less than 10 litre x mol^{-1} x cm^{-1} the chemical has no photoreactive potential and may not need to be tested in the *in vitro* 3T3 NRU phototoxicity test or any other biological test for adverse photochemical effects (1)(5). See also Annex 2.

5. The reliability and relevance of the *in vitro* 3T3 NRU phototoxicity test was recently evaluated (6)(7)(8). The *in vitro* 3T3 NRU phototoxicity test was shown to be predictive of acute phototoxicity effects in animals and humans *in vivo*. The test is not designed to predict other adverse effects that may arise from combined action of a chemical and light, e.g., it does not address photogenotoxicity, photoallergy, or photocarcinogenicity, nor does it allow an assessment of phototoxic potency. In addition, the test has not been designed to address indirect mechanisms of phototoxicity, effects of metabolites of the test substance, or effects of mixtures.

6. Whereas the use of metabolising systems is a general requirement for all *in vitro* tests for the prediction of genotoxic and carcinogenic potential, up to now, in the case of phototoxicology, there are only rare examples where metabolic transformation is needed for the chemical to act as a phototoxin *in vivo* or *in vitro*. Thus, it is neither considered necessary nor scientifically justified for the present test to be performed with a metabolic activation system.

PRINCIPLE OF THE TEST METHOD

7. The *in vitro* 3T3 NRU phototoxicity test is based on a comparison of the cytotoxicity of a chemical when tested in the presence and in the absence of exposure to a non-cytotoxic dose of simulated solar light. Cytotoxicity in this test is expressed as a concentration-dependent reduction of the uptake of the vital dye Neutral Red when measured 24 hours after treatment with the test chemical and irradiation (9).

8. Balb/c 3T3 cells are maintained in culture for 24 h for formation of monolayers. Two 96-well plates per test chemical are pre-incubated with eight different concentrations of the test substance for 1 h. Thereafter one of the two plates is exposed to the highest non-cytotoxic irradiation dose whereas the other plate is kept in the dark. In both plates the treatment medium is then replaced by culture medium and after another 24 h of incubation cell viability is determined by Neutral Red uptake. Cell viability is expressed as percentage of untreated solvent controls and is calculated for each test concentration. To predict the phototoxic potential, the concentration responses obtained in the presence and in the absence of irradiation are compared, usually at the IC_{50} level, i.e., the concentration reducing cell viability to 50 % compared to the untreated controls.

DESCRIPTION OF THE TEST METHOD

Preparations

Cells

9. A permanent mouse fibroblast cell line, Balb/c 3T3, clone 31, either from the American Type Culture Collection (ATCC), Manassas, VA, USA, or from the European Collection of Cell Cultures (ECACC), Salisbury, Wiltshire, UK, was used in the validation study, and therefore is recommended. Other cells or cell lines may be used with the same test procedure if culture conditions are adapted to the specific needs of the cells, but equivalency must be demonstrated.

10. Cells should be checked regularly for the absence of mycoplasma contamination and only used if none is found (10).

11. It is important that UV sensitivity of the cells is checked regularly according to the quality control procedure described in this guideline. Because the UVA sensitivity of cells may increase with the number of passages, Balb/c 3T3 cells of the lowest obtainable passage number, preferably less than 100, should be used. (See paragraph 29and Annex 3).

Media and culture conditions

12. Appropriate culture media and incubation conditions should be used for routine cell passage and during the test procedure, e.g., for Balb/c 3T3 cells these are DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% new-born calf serum, 4 mM glutamine, penicillin (100 IU), and streptomycin (100 μg/mL), and humidified incubation at 37^0 C, 5-7.5% CO_2 depending on the buffer (see paragraph 17). It is particularly important that cell culture conditions assure a cell cycle time within the normal historical range of the cells or cell line used.

Preparation of cultures

13. Cells from frozen stock cultures are seeded in culture medium at an appropriate density and subcultured at least once before they are used in the *in vitro* 3T3 NRU phototoxicity test.

14. Cells used for the phototoxicity test are seeded in culture medium at the appropriate density so that cultures will not reach confluence by the end of the test, i.e., when cell viability is determined 48 h after seeding of the cells. For Balb/c 3T3 cells grown in 96-well plates, the recommended cell seeding density is 1×10^4 cells per well.

15. For each test chemical cells are seeded identically in two separate 96-well plates, which are then taken concurrently through the entire test procedure under identical culture conditions except for the time period where one of the plates is irradiated (+Irr) and the other one is kept in the dark (-Irr).

Preparation of test substance

16. Test substances must be prepared fresh, immediately prior to use unless data demonstrate their stability in storage. It is recommended that all chemical handling and the initial treatment of cells be performed under light conditions that would avoid photoactivation or degradation of the test substance prior to irradiation.

17. Test chemicals shall be dissolved in buffered salt solutions, e.g. Earle's Balanced Salt Solution (EBSS), or other physiologically balanced buffer solutions, which must be free from protein components, light absorbing components (e.g., pH-indicator colours and vitamins) to avoid interference during irradiation.

18. Test chemicals of limited solubility in water should be dissolved in an appropriate solvent. If a solvent is used it must be present at a constant volume in all cultures, i.e. in the negative (solvent) controls as well as in all concentrations of the test chemical, and be noncytotoxic at that concentration. Test chemical concentrations should be selected so as to avoid precipitate or cloudy solutions.

19. Dimethylsulphoxide (DMSO) and ethanol (ETOH) are the recommended solvents. Other solvents of low cytotoxicity may be appropriate. Prior to use, all solvents should be assessed for specific properties, e.g., reaction with the test chemical, quenching of the phototoxic effect, radical scavenging properties and/or chemical stability in the solvent.

20. Vortex mixing and/or sonication and/or warming to appropriate temperatures may be used to aid solubilisation unless this would affect the stability of the test chemical.

Irradiation Conditions

21. *Light source:* the choice of an appropriate light source and filters is a crucial factor in phototoxicity testing. Light of the UVA and visible regions is usually associated with phototoxic reactions *in vivo* (3)(11), whereas generally UVB is of less relevance but is highly cytotoxic; the cytotoxicity increases 1000-fold as the wavelength goes from 313 to 280 nm (12). Criteria for the choice of an appropriate light source must include the requirement that the light source emits wavelengths absorbed by the test chemical (absorption spectrum) and that the dose of light (achievable in a reasonable exposure time) should be sufficient for the detection of known photocytotoxic chemicals. Furthermore, the

wavelengths and doses employed should not be unduly deleterious to the test system, e.g., the emission of heat (infrared region).

22. Simulation of sunlight with solar simulators is considered the optimal artificial light source. The irradiation power distribution of the filtered solar simulator should be close to that of outdoor daylight given in (13). Both, Xenon arcs and (doped) mercury-metal halide arcs are used as solar simulators (14). The latter have the advantage of emitting less heat and being cheaper, but the match to sunlight is less perfect compared to that of xenon arcs. Because all solar simulators emit significant quantities of UVB they should be suitably filtered to attenuate the highly cytotoxic UVB wavelengths. Because cell culture plastic materials contain UV stabilisers the spectrum should be measured through the same type of 96-well plate lid as will be used in the assay. An example of the spectral irradiance distribution of the filtered solar simulator used in the validation study of the *in vitro* 3T3 NRU phototoxicity test is given in (8). See also Annex 3 Figure 1.

23. *Dosimetry:* 'The intensity of light (irradiance) should be regularly checked before each phototoxicity test using a suitable broadband UV-meter. The intensity should be measured through the same type of 96-well plate lid as will be used in the assay. The UV-meter must have been calibrated to the source. The performance of the UV-meter should be checked, and for this purpose the use of a second, reference UV-meter of the same type and identical calibration is recommended. Ideally, at greater intervals, a spectroradiometer should be used to measure the spectral irradiance of the filtered light source and to check the calibration of the broadband UV-meter.

24. A dose of 5 J/cm^2 (as measured in the UVA range) was determined in the validation study to be non-cytotoxic to Balb/c 3T3 cells and sufficiently potent to excite chemicals to elicit phototoxic reactions, e.g. to achieve 5 J/cm^2 within a time period of 50 min, irradiance was adjusted to 1.7 mW/cm^2. See Annex 3 Figure 2. If another cell line or a different light source are used, the irradiation dose may have to be calibrated so that a dose regimen can be selected that is not deleterious to the cells but sufficient to excite standard phototoxins. The time of light exposure is calculated in the following way:

$$t\,(\text{min}) = \frac{\text{irradiation dose }(\text{J/cm}^2)\times 1000}{\text{irradiance }(\text{mW/cm}^2)\times 60} \qquad (1\,\text{J} = 1\,\text{Wsec})$$

Test conditions

Test substance concentrations

25. The ranges of concentrations of a chemical tested in the presence (+Irr) and in the absence (-Irr) of light should be adequately determined in dose range-finding experiments. It may be useful to assess solubility initially and at 60 min (or whatever treatment time is to be used), as solubility can change during time or during the course of exposure. To avoid toxicity induced by improper culture conditions or by highly acidic or alkaline chemicals, the pH of the cell cultures with added test chemical should be in the range 6.5 - 7.8.

26. The highest concentration of the test substance should be within physiological test conditions, e.g. osmotic and pH stress should be avoided. Depending on the test chemical, it may be necessary to consider other physico-chemical properties as factors limiting the highest test concentration. For relatively insoluble

substances that are not toxic at concentrations up to the saturation point the highest achievable concentration should be tested. In general, precipitation of the test chemical at any of the test concentrations should be avoided. The maximum concentration of a test substance should not exceed 1000 µg/mL; osmolality should not exceed 10 mmolar. A geometric dilution series of 8 test substance concentrations with a constant dilution factor should be used (see paragraph 47).

27. If there is information (from a range finding experiment) that the test chemical is not cytotoxic up to the limit concentration in the dark experiment (-Irr), but is highly cytotoxic when irradiated (+Irr), the concentration ranges to be selected for the (+Irr) experiment may differ from those selected for the (–Irr) experiment to fulfill the requirement of adequate data quality.

Controls

28. *Radiation sensitivity of the cells, establishing of historical data:* Cells should be checked regularly for sensitivity to the light source by exposure to increasing doses of irradiation. These doses are easiest quantitated by measurements of UV parts of the light source. Cells are seeded at the density used in the *in vitro* 3T3 NRU phototoxicity test irradiated the next day. Cell viability is then determined one day later using Neutral Red uptake. It should be demonstrated that the resulting highest non-cytotoxic dose (e.g. in the validation study: 5 J/cm^2 [UVA]) was sufficient to classify the reference chemicals (Table 1) correctly.

29. *Radiation sensitivity, check of current test:* The test meets the quality criteria if the irradiated negative/solvent controls show a viability of more than 80% when compared with non-irradiated negative/solvent control.

30. *Viability of solvent controls:* The absolute optical density ($OD_{540\ NRU}$) of the Neutral Red extracted from the solvent controls indicates whether the $1x10^4$ cells seeded per well have grown with a normal doubling time during the two days of the assay. A test meets the acceptance criteria if the mean $OD_{540\ NRU}$ of the untreated controls is ≥ 0.4 (i.e. approximately twenty times the background solvent absorbance).

31. *Positive control:* A known phototoxic chemical shall be tested concurrently with each *in vitro* 3T3 NRU phototoxicity test. Chlorpromazine (CPZ) is recommended. For CPZ tested with the standard protocol in the *in vitro* 3T3 NRU phototoxicity test, the following test acceptance criteria were defined: CPZ irradiated (+Irr): IC_{50} = 0.1 to 2.0 µg/ml, CPZ non-irradiated (-Irr): IC_{50} = 7.0 to 90.0 µg/mL. The Photo Irritation Factor (PIF), should be > 6. The historical performance of the positive control should be monitored.

32. Other phototoxic chemicals, suitable for the chemical class or solubility characteristics of the chemical being evaluated, may be used as the concurrent positive controls in place of chlorpromazine.

Test procedure (6)(7)(8)(15)(16):

1st day:

33. Dispense 100 μL culture medium into the peripheral wells of a 96-well tissue culture microtiter plate (= blanks). In the remaining wells, dispense 100 μL of a cell suspension of 1×10^5 cells/mL in culture medium (= 1×10^4 cells/well). Two plates should be prepared for each series of individual test substance concentrations, and for the solvent and positive controls.

34. Incubate cells for 24 h (see paragraph 13) until they form a half confluent monolayer. This incubation period allows for cell recovery, adherence, and exponential growth.

2nd day:

35. After incubation, decant culture medium from the cells and wash carefully with 150 μL of the buffered solution used for incubation. Add 100 μL of the buffer containing the appropriate concentration of test chemical or solvent (solvent control). Apply 8 different concentrations of the test chemical. Incubate cells with the test substance in the dark for 60 minutes (see paragraph 12).

36. From the two plates prepared for each series of test substance concentrations and the controls, one is selected, generally at random, for the determination of cytotoxicity (-Irr) (i.e., the control plate), and one (the treatment plate) for the determination of photocytotoxicity (+Irr).

37. To perform the +Irr exposure, irradiate the cells at room temperature for 50 minutes through the lid of the 96-well plate with the highest dose of radiation that is non-cytotoxic (see also Annex 3). Keep non-irradiated plates (-Irr) at room temperature in a dark box for 50 min (= light exposure time).

38. Decant test solution and carefully wash twice with 150 μL of the buffered solution used for incubation. Replace the buffer with culture medium and incubate (see paragraph 12) overnight (18-22 h).

3rd day:

Microscopic evaluation

39. Cells should be examined for growth, morphology, and integrity of the monolayer using a phase contrast microscope. Changes in cell morphology and effects on cell growth should be recorded.

Neutral Red Uptake test

40. Wash the cells with 150 μL of the warmbuffering solution used for incubation. Remove the washing solution by gentle tapping. Add 100 μL of a 50 μg/mL Neutral Red (NR) (3-amino-7-dimethylamino-2-methylphenazine hydrochloride, CAS number 553-24-2; C.I. 50040) in medium without serum (15) and incubate as described in paragraph 12, for 3 h.

41. After incubation, remove the NR medium, and wash cells with 150 μL of the buffer. Decant and remove excess buffer by blotting or centrifugation.

42. Add exactly 150 µL NR desorb solution (freshly prepared 49 parts water + 50 parts ethanol + 1 part acetic acid).

43. Shake the microtiter plate rapidly on a microtiter plate shaker for 10 min until NR has been extracted from the cells and has formed a homogeneous solution.

44. Measure the optical density of the NR extract at 540 nm in a spectrophotometer, using blanks as a reference. Save data in an appropriate electronic file format for subsequent analysis.

DATA AND REPORTING:

Quality and quantity of data

45. The test data should allow a meaningful analysis of the concentration-response obtained in the presence and in the absence of irradiation. If cytotoxicity is found, both the concentration range and the intercept of individual concentrations shall be set in a way to allow the fit of a curve to the experimental data.

46. For both clearly positive and clearly negative results (see paragraph 53), the primary experiment, supported by one or more preliminary dose range-finding experiment(s), may be sufficient.

47. Equivocal, borderline, or unclear results should be clarified by further testing (see also paragraph 56). In such cases, modification of experimental conditions should be considered. Experimental conditions that might be modified include the concentration range or spacing, the pre-incubation time, and the irradiation-exposure time. A shorter exposure time may be appropriate for water-unstable chemicals.

Evaluation of results

48. To enable evaluation of the data, a Photo-Irritation-Factor (PIF) or Mean Photo Effect (MPE) may be calculated.

49. For the calculation of the measures of photocytotoxicity (see below) the set of discrete dose-response values has to be approximated by an appropriate continuous dose-response curve (model). Fitting of the curve to the data is commonly performed by a non-linear regression method (17). To assess the influence of data variability on the fitted curve a bootstrap procedure is recommended.

50. A Photo-Irritation-Factor (PIF) is calculated using the following formula:

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

If an IC_{50} in the presence or absence of light cannot be calculated, a PIF cannot be determined for the test material.

51. The mean photo effect (MPE) is based on comparison of the complete concentration response curves (18). It is defined as the weighted average across a representative set of photo effect values

$$MPE = \frac{\sum_{i=1}^{n} w_i \, PE_{c_i}}{\sum_{i=1}^{n} w_i}$$

The photo effect (PE_c) at any concentration (C) is defined as the product of the response effect (RE_c) and the dose effect (DE_c) i.e. $PE_c = RE_c \times DE_c$. The response effect (RE_c) is the difference between the responses observed in the absence and presence of light, i.e. $RE_c = R_c$ (-Irr) – R_c (+Irr). The dose-effect is given by

$$DE_c = \left| \frac{C/C^* - 1}{C/C^* + 1} \right|$$

where C* represents the equivalence concentration, i.e. the concentration at which the +Irr response equals the –Irr response at concentration C. If C* cannot be determined because the response values of the +Irr curve are systematically higher or lower than RE_c the dose effect is set to 1. The weighting factors i are given by the highest response value, i.e. w_i = MAX {Ri (+Irr), Ri (-Irr) }. The concentration grid Ci is chosen such that the same number of points falls into each of the concentration intervals defined by the concentration values used in the experiment. The calculation of MPE is restricted to the maximum concentration value at which at least one of the two curves still exhibits a response value of at least 10%. If this maximum concentration is higher than the highest concentration used in the +Irr experiment the residual part of the +Irr curve is set to the response value "0". Depending on whether the MPE value is larger than a properly chosen cut-off value (MPE_c= or not, the chemical is classified as phototoxic).

52. A software package for the calculation of the PIF and MPE is available from the Secretariat (19).

Interpretation of Results

53. Based on the validation study (8), a test substance with a PIF < 2 or an MPE < 0.1 predicts: "no phototoxicity". A PIF >2 and < 5 or an MPE > 0.1 and < 0.15 predicts: "probable phototoxicity" and a PIF > 5 or an MPE > 0.15 predicts: "phototoxicity".

54. For any laboratory initially establishing this assay, the reference materials listed in Table 1 should be tested prior to the testing of test substances for phototoxic assessment. PIF or MPE values should be close to the values mentioned in Table 1.

TABLE 1

Chemical and CAS No	PIF	MPE	Absorption Peak	Solvent[1]
Amiodarone HCL [19774-82-4]	>3.25	0.27-0.54	242 nm 300 nm (shoulder)	ethanol
Chloropromazine HCL [69-09-0]	>14.4	0.33-0.63	309 nm	ethanol
Norfloxacin [70458-96-7]	>71.6	0.34-0.90	316 nm	acetonitrile
Anthracene [120-12-7]	>18.5	0.19-0.81	356 nm	acetonitrile
Protoporphyrin IX, Disodium [50865-01-5]	>45.3	0.54-0.74	402 nm	ethanol
L – Histidine [7006-35-1]	no PIF	0.05-0.10	211 nm	water
Hexachlorophene [70-30-4]	1.1-1.7	0.00-0.05	299 nm 317 nm (shoulder)	ethanol
Sodium lauryl sulfate [151-21-3]	1.0-1.9	0.00-0.05	no absorption	--

Interpretation of data

55. If a positive result is obtained only at the highest test concentration, (especially for water soluble test chemicals) additional considerations may be necessary for assessment of hazard. These may include data on skin absorption, and accumulation of the chemical in the skin and / or data from other tests, e.g., testing of the chemical in *in vitro* animal or human skin, or skin models.

56. If no toxicity is demonstrated (+Irr and -Irr), and if poor solubility limited the concentrations that could be tested, then the compatibility of the test substance with the assay may be questioned and confirmatory testing should be considered (using, e.g., *in vitro* skin, *ex vivo* skin, or *in vivo* models).

Test Report

57. The test report must include the following information:

Test substance:
- identification data and CAS number, if known;
- physical nature and purity;
- physicochemical properties relevant to conduct of the study;
- UV/vis absorption spectrum;
- stability and photostability, if known.

Solvent:
- justification for choice of solvent;

[1] Solvent used for measuring absorption.