OECD/OCDE **432**

Adopted :
13 April 2004

OECD GUIDELINE FOR TESTING OF CHEMICALS

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. It has been suggested that if the molar extinction/absorption coefficient is less than 10 litre x mol^{-1} x cm^{-1} the chemical is unlikely to be photoreactive. Such chemical 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)(9). 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, for example, 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 (10). NR is a weak cationic dye that readily penetrates cell membranes by non-diffusion, accumulating intracellularly in lysosomes. Alterations of the cell surface of the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged or dead cells, which is the basis of this test.

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 it is recommended that cells be obtained from a well qualified cell depository. 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 (11).

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 29 and 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, for example, 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, for example Earle's Balanced Salt Solution (EBSS), or other physiologically balanced buffer solutions, which must be free from protein components and light absorbing components (e.g., pH-indicator colours and vitamins) to avoid interference during irradiation. Since during irradiation cells are kept for about 50 minutes outside of the CO_2 incubator, care has to be taken to avoid alkalisation. If weak buffers like EBSS are used this can be achieved by incubating the cells at 7.5% CO_2. If the cells are incubated at 5% CO2 only, a stronger buffer should be selected.

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 precipitation 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, for example, 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)(12), 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 (13). 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, for example, 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 (14). Both, Xenon arcs and (doped) mercury-metal halide arcs are used as solar simulators (15). 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. Irrespective of measures taken to attenuate parts of the spectrum by filtering or by unavoidable filter effects of the equipment the spectrum recorded below these filters should not deviate from standardised outdoor daylight (14). 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)(16). 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 to be non-cytotoxic to Balb/c 3T3 cells and sufficiently potent to excite chemicals to elicit phototoxic reactions, (6)(17) for example 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 is 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:

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

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, for example 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 fulfil the requirement of adequate data quality.

Controls

28. *Radiation sensitivity of the cells, establishing of historical data:* Cells should be checked regularly (about every fifth passage) for sensitivity to the light source by assessing their viability following exposure to increasing doses of irradiation. Several doses of irradiation, including levels substantially greater than those used for the 3T3 NRU phototoxicity test should be used in this assessment. 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 and 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 $\geq$ 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)(16)(17):

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 $1xl0^5$ cells/mL in culture medium (= $1x10^4$ 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 12) 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 gently 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 paragraphs 12 and 17).

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 approximately 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, but not containing the test material. 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 pre-warmed buffer. 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 (16) 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 gently 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, and if possible the concentration of test chemical by which cell viability is reduced to 50% (IC_{50}). 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 (18). 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 (19). 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 R_C(-Irr) the dose effect is set to 1. The weighting factors w_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= 0.15) 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 (20).

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 phototoxicity. 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	water

Interpretation of data

55. If phototoxic effects are observed 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, for example, testing of the chemical in *in vitro* animal or human skin assays, 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 another model.

Test Report

57. The test report must include the following information:

Test substance:
- identification data, common generic names and IUPAC 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;
- solubility of the test chemical in solvent;

[1] Solvent used for measuring absorption

- percentage of solvent present in treatment medium.

Cells:
- type and source of cells;
- absence of mycoplasma;
- cell passage number, if known;
- Radiation sensitivity of cells, determined with the irradiation equipment used in the *in vitro* 3T3 NRU phototoxicity test.

Test conditions (1); *incubation before and after treatment:*
- type and composition of culture medium;
- incubation conditions (CO_2 concentration; temperature; humidity);
- duration of incubation (pre-treatment; post-treatment).

Test conditions (2); *treatment with the chemical:*
- rationale for selection of concentrations of the test chemical used in the presence and in the absence of irradiation;
- in case of limited solubility of the test chemical and absence of cytotoxicity: rationale for the highest concentration tested;
- type and composition of treatment medium (buffered salt solution);
- duration of the chemical treatment.

Test conditions (3); *irradiation:*
- rationale for selection of the light source used;
- manufacturer and type of light source and radiometer ;
- spectral irradiance characteristics of the light source;
- transmission and absorption characteristics of the filter(s) used;
- characteristics of the radiometer and details on its calibration;
- distance of the light source from the test system;
- UVA irradiance at this distance, expressed in mW/cm^2;
- duration of the UV/vis light exposure;
- UVA dose (irradiance x time), expressed in J/cm^2;
- temperature of cell cultures during irradiation and cell cultures concurrently kept in the dark.

Test conditions (4); *Neutral Red viability test:*
- composition of Neutral Red treatment medium;
- duration of Neutral Red incubation;
- incubation conditions (CO_2 concentration; temperature; humidity);
- Neutral Red extraction conditions (extractant; duration);
- wavelength used for spectrophotometric reading of Neutral Red optical density;
- second wavelength (reference), if used;
- content of spectrophotometer blank, if used.

Results:
- cell viability obtained at each concentration of the test chemical, expressed in percent viability of mean, concurrent solvent controls;
- concentration response curves (test chemical concentration vs. relative cell viability) obtained in concurrent +Irr and -Irr experiments;
- analysis of the concentration-response curves: if possible, computation/calculation of IC_{50}

(+Irr) and IC_{50} (-Irr);
- comparison of the two concentration response curves obtained in the presence and in the absence of irradiation, either by calculation of the Photo-Inhibition-Factor (PIF), or by calculation of the Mean-Photo-Effect (MPE);
- test acceptance criteria; concurrent solvent control ;
- absolute viability (optical density of Neutral Red extract) of irradiated and non-irradiated cells;
- historic negative and solvent control data; means and standard deviations;
- test acceptance criteria; concurrent positive control ;
- IC_{50}(+Irr) and IC_{50}(-Irr) and PIF/MPE of positive control chemical;
- historic positive control chemical data: IC_{50}(+Irr) and IC_{50}(-Irr) and PIF/MPE; means and standard deviations.

Discussion of the results.

Conclusions.

LITERATURE

(1) Lovell W.W. (1993). A scheme for *in vitro* screening of substances for photoallergenic potential. Toxic. In Vitro 7: 95-102.

(2) Santamaria, L. and Prino, G. (1972). List of the photodynamic substances. In "Research Progress in Organic, Biological and Medicinal Chemistry" Vol. 3 part 1. North Holland Publishing Co. Amsterdam. p XI-XXXV.

(3) Spielmann, H., Lovell, W.W., Hölzle, E., Johnson, B.E., Maurer, T., Miranda, M.A., Pape, W.J.W., Sapora, O., and Sladowski, D. (1994). *In vitro* phototoxicity testing: The report and recommendations of ECVAM Workshop 2. ATLA, 22, 314-348.

(4) Spikes, J.D. (1989). Photosensitization. In "The science of Photobiology" Edited by K.C. Smith. Plenum Press, New York. 2nd edition, p 79-110.

(5) OECD (1997) Environmental Health and Safety Publications, Series on Testing and Assessment No. 7 "Guidance Document On Direct Phototransformation Of Chemicals In Water" Environment Directorate, OECD, Paris.

(6) Spielmann, H., Balls, M., Döring, B., Holzhütter, H.G., Kalweit, S., Klecak, G., L'Eplattenier, H., Liebsch, M., Lovell, W.W., Maurer, T., Moldenhauer. F. Moore. L., Pape, W., Pfannbecker, U., Potthast, J., De Silva, O., Steiling, W., and Willshaw, A. (1994). EEC/COLIPA project on *in vitro* phototoxicity testing: First results obtained with a Balb/c 3T3 cell phototoxicity assay. Toxic. In Vitro 8, 793-796.

(7) Anon (1998). Statement on the scientific validity of the 3T3 NRU PT test (an *in vitro* test for phototoxicity), European Commission, Joint Research Centre: ECVAM and DGXI/E/2, 3 November 1997, ATLA, 26, 7-8.

(8) Spielmann, H., Balls, M., Dupuis, J., Pape, W.J.W., Pechovitch, G. De Silva, O., Holzhütter, H.G., Clothier, R., Desolle, P., Gerberick, F., Liebsch, M., Lovell, W.W., Maurer, T., Pfannenbecker, U., Potthast, J. M., Csato, M., Sladowski, D., Steiling, W., and Brantom, P. (1998). The international EU/COLIPA *In vitro* phototoxicity validation study: results of phase II (blind trial), part 1: the 3T3 NRU phototoxicity test. Toxic. In Vitro 12, 305-327.

(9) OECD (2002) Extended Expert Consultation Meeting on The In Vitro 3T3 NRU Phototoxicity Test Guideline Proposal, Berlin, 30th – 31st October 2001, Secretariat's Final Summary Report, 15th March 2002, OECD ENV/EHS, available upon request from the Secretariat.

(10) Borenfreund, E., and Puerner, J.A. (1985). Toxicity determination *in vitro* by morphological alterations and neutral red absorption. Toxicology Lett., 24, 119-124.

(11) Hay, R.J. (1988) The seed stock concept and quality control for cell lines. Analytical Biochemistry 171, 225-237.

(12) Lambert L.A, Wamer W.G., and Kornhauser A. (1996) Animal models for phototoxicity testing. In "Dermatotoxicology", edited by F.N. Marzulli and H.I. Maibach. Taylor & Francis, Washington DC. 5th Edition, p 515-530.

(13) Tyrrell R.M., Pidoux M (1987) Action spectra for human skin cells: estimates of the relative cytotoxicity of the middle ultraviolet, near ultraviolet and violet regions of sunlight on epidermal keratinocytes. Cancer Res., 47, 1825-1829.

(14) ISO 10977. (1993). Photography - Processed photographic colour films and paper prints - Methods for measuring image stability.

(15) Sunscreen Testing (UV.B) TECHNICALREPORT, CIE, International Commission on Illumnation, Publication No. 90, Vienna, 1993, ISBN 3 900 734 275.

(16) ZEBET/ECVAM/COLIPA - Standard Operating Procedure: *In Vitro* 3T3 NRU Phototoxicity Test. Final Version, 7 September, 1998. 18 pgs.

(17) Spielmann, H., Balls, M., Dupuis, J., Pape, W.J.W., De Silva, O., Holzhütter, H.G., Gerberick, F., Liebsch, M., Lovell, W.W., and Pfannenbecker, U. (1998) A study on UV filter chemicals from Annex VII of the European Union *Directive 76/768/EEC,* in the *in vitro* 3T3 NRU phototoxicity test. ATLA 26, 679-708.

(18) Holzhütter, H.G., and Quedenau, J. (1995) Mathematical modeling of cellular responses to external signals. J. Biol. Systems 3, 127-138.

(19) Holzhütter, H.G. (1997). A general measure of *in vitro* phototoxicity derived from pairs of dose-response curves and its use for predicting the *in vivo* phototoxicity of chemicals. ATLA, 25, 445-462.

(20) http://www.oecd.org/document/55/0,2340,en_2649_34377_2349687_1_1_1_1,00.html

ANNEX 1

DEFINITIONS

Irradiance: the intensity of ultraviolet (UV) or visible light incident on a surface, measured in W/m^2 or mW/cm^2.

Dose of light: the quantity (= intensity x time) of ultraviolet (UV) or visible radiation incident on a surface, expressed in Joules (= W x s) per surface area, for example, J/m^2 or J/cm^2.

UV light wavebands: the designations recommended by the CIE (Commission Internationale de L'Eclairage) are: UVA (315-400nm) UVB (280-315nm) and UVC (100-280nm). Other designations are also used; the division between UVB and UVA is often placed at 320nm, and the UVA may be divided into UV-A1 and UV-A2 with a division made at about 340nm.

Cell viability: parameter measuring total activity of a cell population (e.g., uptake of the vital dye Neutral Red into cellular lysosomes), which, depending on the endpoint measured and the test design used, correlates with the total number and/or vitality of the cells.

Relative cell viability: cell viability expressed in relation to solvent (negative) controls which have been taken through the whole test procedure (either +Irr or -Irr) but not treated with test chemical.

PIF (Photo-Irritation-Factor): factor generated by comparing two equally effective cytotoxic concentrations (IC_{50}) of the test chemical obtained in the absence (-Irr) and in the presence (+Irr) of a non-cytotoxic irradiation with UVA/vis light.

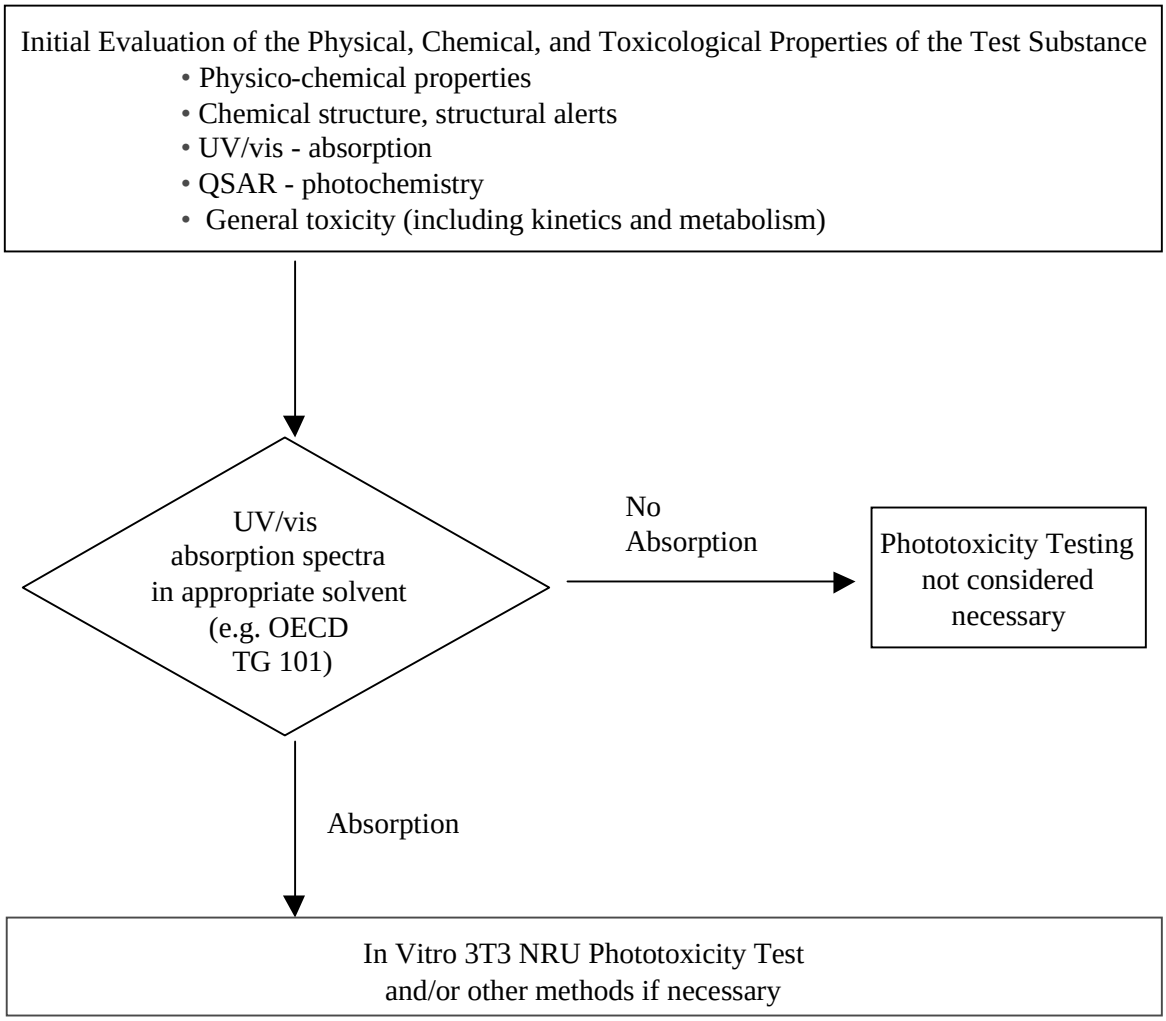
IC_{50}: the concentration of the test chemical by which the cell viability is reduced by 50%

MPE (Mean-Photo-Effect): measurement derived from mathematical analysis of the concentration response curves obtained in the absence (-Irr) and in the presence (+Irr) of a non-cytotoxic irradiation with UVA/vis light.

Phototoxicity: acute toxic response that is elicited after the first exposure of skin to certain chemicals and subsequent exposure to light, or that is induced similarly by skin irradiation after systemic administration of a chemical.

ANNEX 2

Role of the 3T3 NRU PT in a sequential approach to the phototoxicity testing of chemicals



ANNEX 3

Figure 1: Spectral power distribution of a filtered solar simulator

(see paragraph 22)

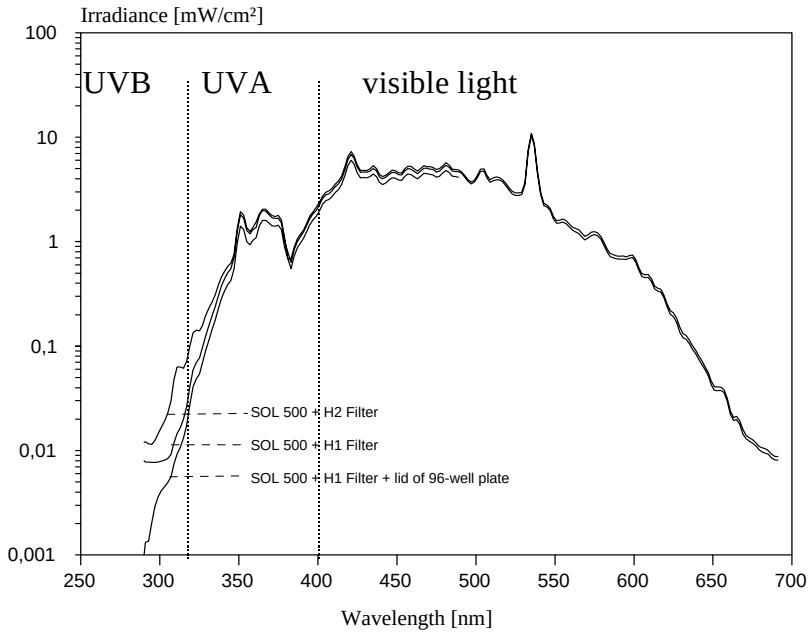
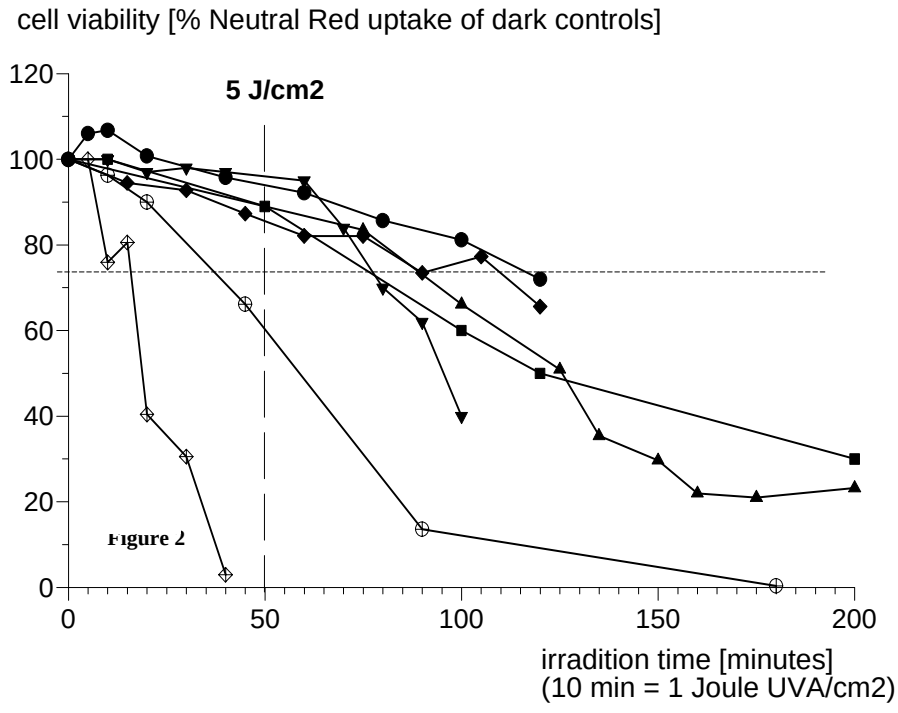


Figure 1 gives an example of an acceptable spectral power distribution of a filtered solar simulator. It is from the doped metal halide source used in the validation trial of the 3T3 NRU PT (6)(8)(17). The effect of two different filters and the additional filtering effect of the lid of a 96-well cell culture plate are shown. The H2 filter was only used with test systems that can tolerate a higher amount of UVB (skin model test and red blood cell photo-hemolysis test). In the 3T3 NRU-PT the H1 filter was used. The figure shows that additional filtering effect of the plate lid is mainly observed in the UVB range, still leaving enough UVB in the irradiation spectrum to excite chemicals typically absorbing in the UVB range, like Amiodarone (see Table 1).

Figure 2: Irradiation sensitivity of Balb/c 3T3 cells (as measured in the UVA range)

(see paragraphs 24, 28, 29)



Sensitivity of Balb/c 3T3 cells to irradiation with the solar simulator used in the validation trial of the 3T3NRU-Phototoxicity Test, as measured in the UVA range. Figure shows the results obtained in 7 different laboratories in the pre-validation study (1). While the two curves with open symbols were obtained with aged cells (high number of passages), that had to be replaced by new cell stocks the curves with bold symbols show cells with acceptable irradiation tolerance.
From these data the highest non-cytotoxic irradiation dose of 5 J/cm² was derived (vertical dashed line). The horizontal dashed line shows in addition the maximum acceptable irradiation effect given in paragraph 29.