OECD GUIDELINE FOR TESTING OF CHEMICALS

In Vitro 3T3 NRU phototoxicity test

INTRODUCTION

1. Phototoxicity is defined as a toxic response elicited by topically or systemically administered photoreactive chemicals after the exposure of the body to environmental light.

2. The *in vitro* 3T3 Neutral Red Uptake (NRU) phototoxicity test is used to identify the phototoxic potential of a test chemical activated by exposure to light. The test evaluates photo-cytotoxicity by the relative reduction in viability of cells exposed to the test chemical in the presence versus absence of light. Chemicals identified as positive in this test may be phototoxic *in vivo*, following topical application or systemic application and distribution to the skin.

3. Definitions used in this Test Guideline 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. Photoreactions require sufficient absorption of light quanta. Thus, before testing is considered, a UV/vis absorption spectrum of the test chemical may be determined according to OECD Test Guideline 101. It has been suggested that if the molar extinction/absorption coefficient (MEC) is less than 1000 L mol^{-1} cm^{-1}, the chemical is unlikely to be photoreactive (5). Such chemicals may not need to be tested in the *in vitro* 3T3 NRU phototoxicity test or any other biological test for adverse photochemical effects (1)(6). In general, this principle applies to all test chemicals, however, depending on the intended use of the chemical or potential exposure conditions, more specific guidelines may apply (such as ICH S10 for pharmaceuticals) (5). See also Annex 2.

5. The reliability and relevance of the *in vitro* 3T3 NRU phototoxicity test was evaluated (7)(8)(9)(10). 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, *per se*. Furthermore, the test has not been designed to address indirect mechanisms of phototoxicity, effects of metabolites of the test chemical, or effects of mixtures. However, in some cases, a negative in vitro 3T3 NRU phototoxicity test may obviate the need for other testing, e.g. photogenotoxicity (see Note 2(5))(11)(12).

6. The *in vitro* 3T3 NRU phototoxicity test does not need to be performed with a metabolic activation system, because there are only rare examples where metabolic transformation is needed for the test chemical to act as a phototoxin, *in vivo*, or *in vitro* (12).

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 (NR) when measured 18-24 hours after treatment with the test chemical and irradiation (13). NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. NR is not charged at close-to-neutral pH of the cytoplasm but becomes positively charged and trapped in low pH of lysosomal lumen. The low pH of lysosomal lumen is actively maintained, requires ATP, and is dependent on integrity of lysosomal membrane. Phototoxins can induce cell damage through formation of Reactive Oxygen Species (ROS) and other mechanisms that lead to increased permeability of the lysosomal membrane, reduction in the pH gradient, and other changes that gradually become irreversible (214)(15). 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 and damaged or dead cells.

8. BALB/c 3T3 cells are maintained in culture for 18-24 h for formation of monolayers. Two 96-well plates per test chemical are pre-incubated with eight different concentrations of the test chemical for 1 h. Thereafter one of the two plates is exposed to an irradiation dose whereas the other plate is kept in the dark. In both plates, the treatment buffer is then replaced with culture medium and overnight incubation (18-24 h) cell viability is determined by NRU. Cell viability is expressed as percentage of test chemical-treated NRU values compared with 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, including the concentration reducing cell viability to 50 % compared to the solvent controls (i.e., IC_{50}).

DESCRIPTION OF THE TEST METHOD

Preparations

Cells

9. A immortalised mouse fibroblast cell line, BALB/c 3T3, clone A31, obtained from 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. It is recommended that cells be obtained from a well-qualified cell depository (22). 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 (i.e., appropriate responses to reference chemicals), in accordance with the principles of Guidance Document No. 34 (21).

10. Cells should be checked for the absence of mycoplasma contamination by the supplier and upon arrival in the laboratory (see (16) for recommendations) and only used if none is found (17).

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 with a total passage number preferably less than 100 should be

used in the assay (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, 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 important that cell culture conditions assure a cell division 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 chemical

16. Test chemicals must be prepared fresh on the day of testing 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 chemical prior to irradiation.

17. Test chemicals shall be dissolved in buffered salt solutions, e.g. Earle´s or Hanks' Balanced Salt Solution (EBSS or HBSS), 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 are used (e.g., DPBS or EBSS) this can be achieved by incubating the cells at 7.5% CO_2. If the cells are incubated at 5% CO_2 only, a stronger buffer (e.g., HEPES) should be selected.

18. Test chemicals of limited solubility in water should be dissolved in an appropriate intermediate solvent. If an intermediate solvent is used it must be present at a constant volume in all cultures (i.e., in the solvent controls, as well as in all concentrations of the test chemical) and be non-cytotoxic at that concentration.

19. Test chemical concentrations should be selected so as to avoid precipitation or cloudy solutions. 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, sonication, and/or warming to appropriate temperatures may be used to aid solubilisation unless this compromises the stability of the test chemical.

21. UV light absorption is a requirement of chemicals acting as phototoxicants and chemicals that do not have a MEC greater than 1000 L mol^{-1} cm^{-1} at any wavelength between 290 and 700 nm are not considered sufficiently photoreactive to result in phototoxicity (5). Thus, UV absorption of MEC $\geq$ 1000 L mol^{-1} cm^{-1} between 290 and 700 nm should be considered a threshold requirement for testing phototoxicity.

Irradiation Conditions

22. *Light source* (i.e., solar stimulator): The choice of an appropriate solar stimulator 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)(18), 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 (19). Acceptable solar simulators emit the entire solar spectrum (290 nm through 700 nm) and adjustment of the spectrum can be performed using filters to attenuate UVB while allowing UVA and visible light (see Annex 3). Furthermore, the wavelengths, doses employed, and light source equipment used (e.g., open or closed system) should not be unduly deleterious to the test system (e.g., emission of heat/ wavelengths in the infrared region).

23. 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 (20). Both, xenon arcs and (doped) mercury-metal halide arcs are used as solar simulators (21). 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. All solar simulators emit significant quantities of UVB and should be suitably filtered to attenuate the highly cytotoxic UVB wavelengths (Annex 1). 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 (20). External light standard D65, the internationally recognized emission standard for outdoor daylight, is provided in ISO DIS 18909:2006. 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 (9)(22). See also Annex 3 Figure 1.

24. *Dosimetry:* The intensity of light (irradiance) should be regularly checked before each phototoxicity test using a suitable broadband UVA-meter (Annex 1). Irradiance should be measured through the same type of 96-well plate lid as will be used in the assay. The UVA-meter must have been calibrated to the source. At greater intervals, an externally calibrated reference UV-vis spectroradiometer should be used to measure spectral irradiance of the filtered light source on-site and to adjust the calibration of the broadband UVA-meter if needed. Alternatively, regular calibration of the UVA-meter could be performance at a central calibration laboratory provided that this facility is equipped with an identical light source/filter combination.

25. 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 (7)(23). 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. Alternate exposure times and/or irradiance values may be used to achieve 5 J/cm2 using the formula:

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

26. Similarly, if another cell line or a different light source is used, the irradiation should be calibrated so that a dose regimen can be selected that is not deleterious to the cells but sufficient to excite standard phototoxins (e.g., proficiency chemicals described in Table 1) (27).

Test conditions

Test chemical concentrations

27. 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 to 8.

28. The highest concentration of the test chemical 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 chemicals that are not toxic at concentrations up to the saturation point, the highest achievable concentration should be tested. For non-cytotoxic chemicals (no IC_{50} value up to precipitation), it might be useful to demonstrate the solubility limit under assay conditions. In this case, including two or three concentrations in the main experiment that will likely show precipitation may be useful. The maximum concentration of a test chemical should not exceed 1000 µg/mL; osmolality should not exceed 10 mM. In many cases, the maximum concentration can be reduced to 100 µg/mL, since compounds without any significant cytotoxicity (under irradiation) up to this limit can be considered as being devoid of relevant phototoxicity (5). Higher maximum concentration without irradiation might still be considered to establish IC_{50} values for PIF calculation. A geometric dilution series of 8 test concentrations with a constant dilution factor should be used (see paragraph 47).

29. 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

30. *Radiation sensitivity of the cells, establishing of historical data:* A working bank of cells may be checked at least once for sensitivity to the light source by assessing their viability following exposure to increasing doses of irradiation. It is recommended to check the sensitivity from time to time with increasing number of the cell passage. Several doses of irradiation, including levels greater than those used

for the 3T3 NRU phototoxicity test should be used in this assessment. These doses are quantitated easier by measurements of UV parts of the light source. Cells are seeded at the same density used in the *in vitro* 3T3 NRU phototoxicity test and irradiated the next day (see **Test procedure** section). Cell viability is then determined on the third day 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 proficiency chemicals (Table 1) correctly.

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

32. *Viability of solvent controls:* The absolute optical density ($OD_{540\pm10\ NRU}$) 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 $OD_{540\pm10\ NRU}$ of the solvent controls is ≥ 0.4 (i.e., approximately twenty times the background solvent absorbance).

33. Attention should be paid to crystallisation of the Neutral Red (NR) solution during the incubation with the cells, since crystals may lead to high variability. A shift in the pH of the neutral red solution may trigger formation of NR crystals. Addition of pH stabilisers (e.g., HEPES) to the cell culture medium may prevent crystallization (28). It is recommended to pre-qualify the stock Neutral Red before use in the experiments since the quality from various suppliers may differ. Filtration or centrifugation of the solution of Neutral Red in the cell culture media is highly recommended.

34. *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. Each laboratory performing this assay should establish their own historical databases including Mean Photo Effect (MPE) to monitor the performance over time (Table 1).

35. 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 (Table 1).

Test procedure (7)(8)(9)(22)(23):

1st day:

36. 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 chemical concentrations, and for the solvent and positive controls.

37. Incubate cells for 18-24 h (see paragraph 12) until they form a half confluent monolayer. This incubation period allows for cell recovery, adherence, and exponential growth.

2nd day:

38. After incubation, decant culture medium from the cells and wash gently with 150 µL of the buffer solution used for incubation (see paragraph 17). 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 to both plates. Incubate cells with the test chemical in the dark for 60 minutes.

39. From the two plates prepared for each series of 8 test chemical concentrations and the controls, one plate is selected for the determination of cytotoxicity (-Irr) (i.e., the control plate), and one (the treatment plate) for the determination of photocytotoxicity (+Irr).

40. 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 (i.e., 5 J/cm^2; see also Annex 3). Keep non-irradiated plates (-Irr) at room temperature in a dark box for approximately 50 min (= light exposure time).

41. Decant test solution and carefully wash twice with 150 µL of the buffer solution used for incubation, but not containing the test material. Replace the buffer with culture medium and incubate overnight (18-24 h; see paragraph 12).

3rd day:

Microscopic evaluation

42. 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

43. Wash the cells with 150 µL of the pre-warmed (37^{o}C) buffer solution. Remove the buffer solution. 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 (22) and incubate (as described in paragraph 12) for 3 h.

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

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

46. 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.

47. Measure the optical density of the NR extract at 540±10 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

48. Appropriate concentrations which capture the dose-responses in the presence and absence of irradiation should be selected to allow meaningful analysis of the data, and if possible a determination of the concentration of test chemical by which cell viability is reduced to 50% (IC_{50}). If cytotoxicity is observed, the ranges of concentrations tested may be updated to capture the range of dose responses (e.g., those concentrations which result in viabilities above and below 50%).

49. 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.

50. 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

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

52. 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 (24). To assess the influence of data variability on the fitted curve a bootstrap procedure is recommended.

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

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

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

55. The Mean Photo Effect (MPE) is based on comparison of the complete concentration response curves (25). 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,. $RE_c = R_c(\text{-Irr}) - R_c(\text{+Irr})$. 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 wi 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.

56. A software package for the calculation of the PIF and MPE is available from the OECD Secretariat (26).

Interpretation of Results

57. Based on the validation study (8), a test chemical 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".

58. For any laboratory initially establishing this assay, the reference materials listed in Table 1 should be tested to establish proficiency prior to the routine testing of test chemicals for phototoxicity. PIF or MPE values should be close to the values mentioned in Table 1 (9).

TABLE 1*. Proficiency chemicals.

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

*Values from Spielmann et al. 1998 (9).

Interpretation of data

59. 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, e.g., testing of the chemical in *in vitro* animal or human skin assays, or skin models.

60. 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 chemical with the assay may be questioned and confirmatory testing should be considered using another model.

Test Report

61. The test report should include the following information:

Test chemical:
- 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;
- percentage of solvent present in treatment medium.

Cells:
- type and source of cells;

- Certificate of Origin from the supplier of the cells
- absence of mycoplasma and other contamination;
- cell passage number,
- Radiation sensitivity of cells from a particular passage range, 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), and/or by

calculation of the Mean-Photo-Effect (MPE) depending on the dose-response curve;
- 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) ICH S10 Photosafety Evaluation of Pharmaceuticals. Guidance for Industry. January 2015. https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm065007.htm

(6) 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

(7) 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.

(8) 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.

(9) 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.

(10) 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

(11) Lynch AM, Guzzie PJ, Bauer D, Gocke E, Itoh S, Jacobs A, Frul CA, Schepky A, Tanaka N, Kasper P. (2011) Consideration on photochemical genotoxicity. II: report of the 2009 International Workshop on Genotoxicity Testing Working Group. Mutat Res 723: 91-100.

(12) Ceridono M, Tellner Par, Bauer D, Barroso J, Alépée N, Corvi R, De Smedt A, Fellows MD, Gibbs NK, Heisler E, Jacobs A, Jirova D, Jones D, Kanádarová H, Kasper P, Akunda JK, Krul C, Learn D, Liebsch M, Lynch AM, Muster W, Nakamura K, Nash JF, Pfannenbecker U, Phillips G, Robles C, Rogiers V, Van De Water F, Liminga UW, Vohr HW, Wattrelos O, Woods J, Zuang V, Kreysa J, Wilcox P. (2012) The 3T3 neutral red update photoxiticity test: practical experience and implications for phototoxicity Testing – The report of an ECVAM-EFPIA workshop. Reg Tox Pharm. 63: 480-488.

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

(14) Brunk UT, Svensson I (1999) Oxidative stress, growth factor starvation and Fas activation may all cause apoptosis through lysosomal leak. Redox Report; 4(1-2): 3-11.

(15) Johansson A-C, Appelqvist H, Nilsson C, Kågedal K, Roberg K, Öllinger K. (2010) Regulation of apoptosis-associated lysosomal membrane permeabilization. Apoptosis.;15(5):527-540. doi:10.1007/s10495-009-0452-5)

(16) OECD (2018). Guidance Document on Good In Vitro Methods Practices (GIVIMP). OECD Series on Testing and Assessment No. 286. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2018)1&doclanguage=en

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

(18) 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.

(19) 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.

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

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

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

(23) 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.

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

(25) 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.

(26) http://www.oecd.org/env/ehs/testing/section4software.htm

(27) INVITTOX Protocol 78. 3T3 Neutral Red Uptake (NRU) Phototoxicity Assay. ECVAM DB-ALM; 2008. *http://ecvam-dbalm.jrc.ec.europa.eu/*

(28) Baker C. S., 1998. Crystallization of neutral red vital stain from minimum essential medium due to pH instability. In Vitro Cell Dev Biol Anim. 1998 Sep;34(8):607-8

ANNEX 1

DEFINITIONS

Mixture: A mixture or a solution composed of two or more chemicals in which they do not react (4).

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, e.g., 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.

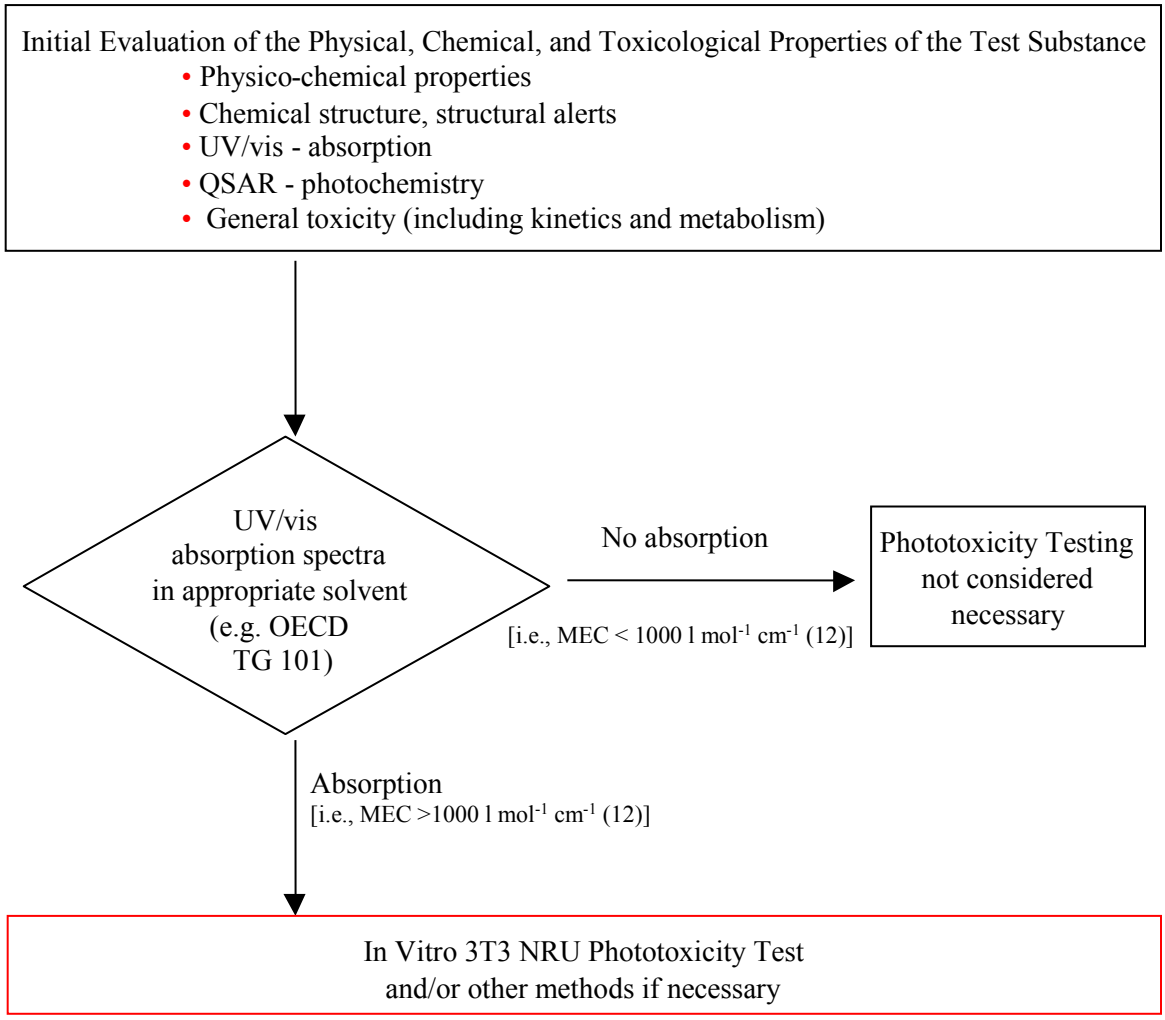
IC_{50}: the concentration of the test chemical by which the cell viability is reduced by 50%

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.

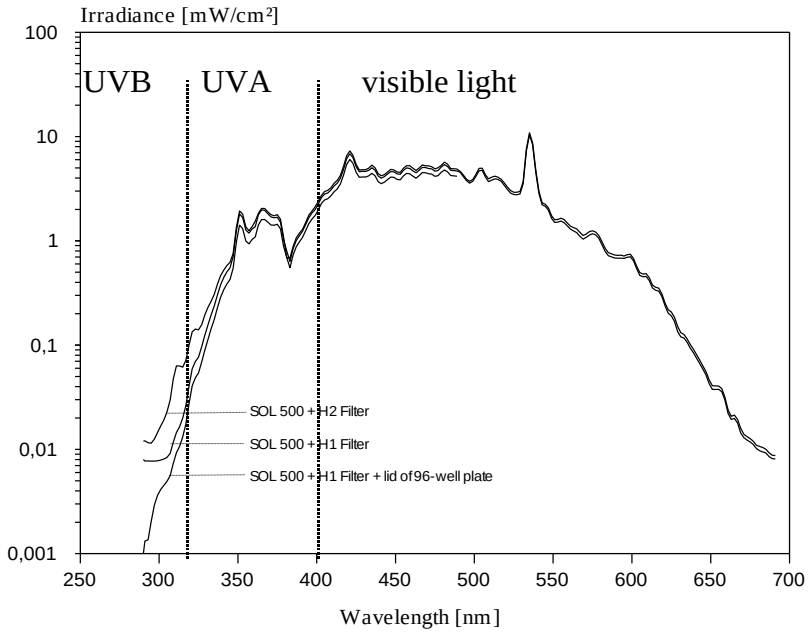
ANNEX 2

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

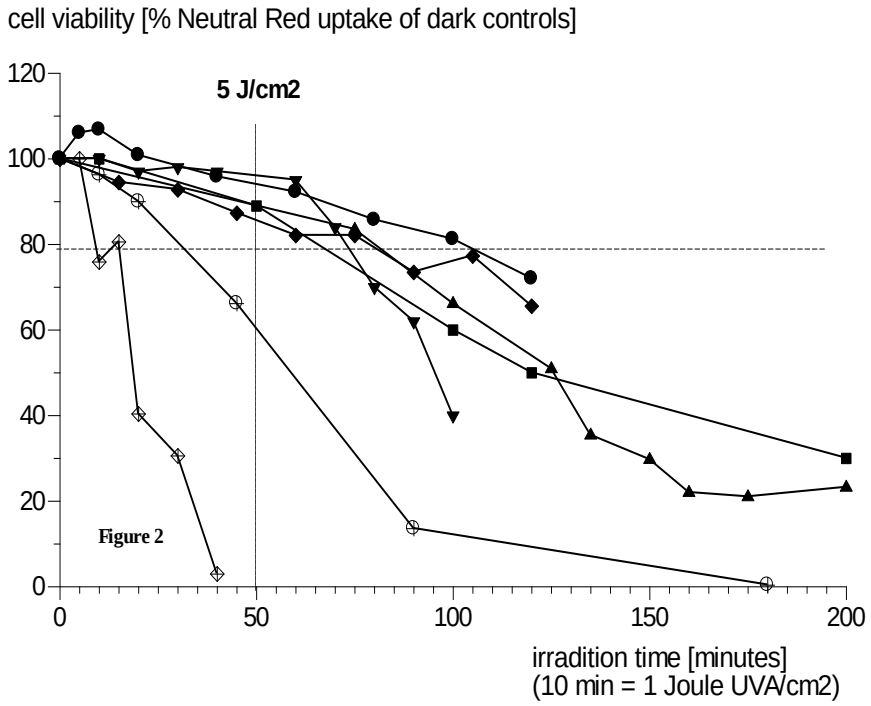


(see paragraph 22)

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 cells from a high passage number that were replaced with 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.