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 (+IT) and the other one is kept in the dark (-IT).

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 $1x10^5$ 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 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_i}) 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

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

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.

- solubility of the test chemical in solvent;
- 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;
- 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.

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(19) This will be a hyperlink to the OECD web pages that contain the software.

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, 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 of solvent (negative) controls which have been taken through the whole test procedure (either +Irr or -Irr) but not treated with test chemical.

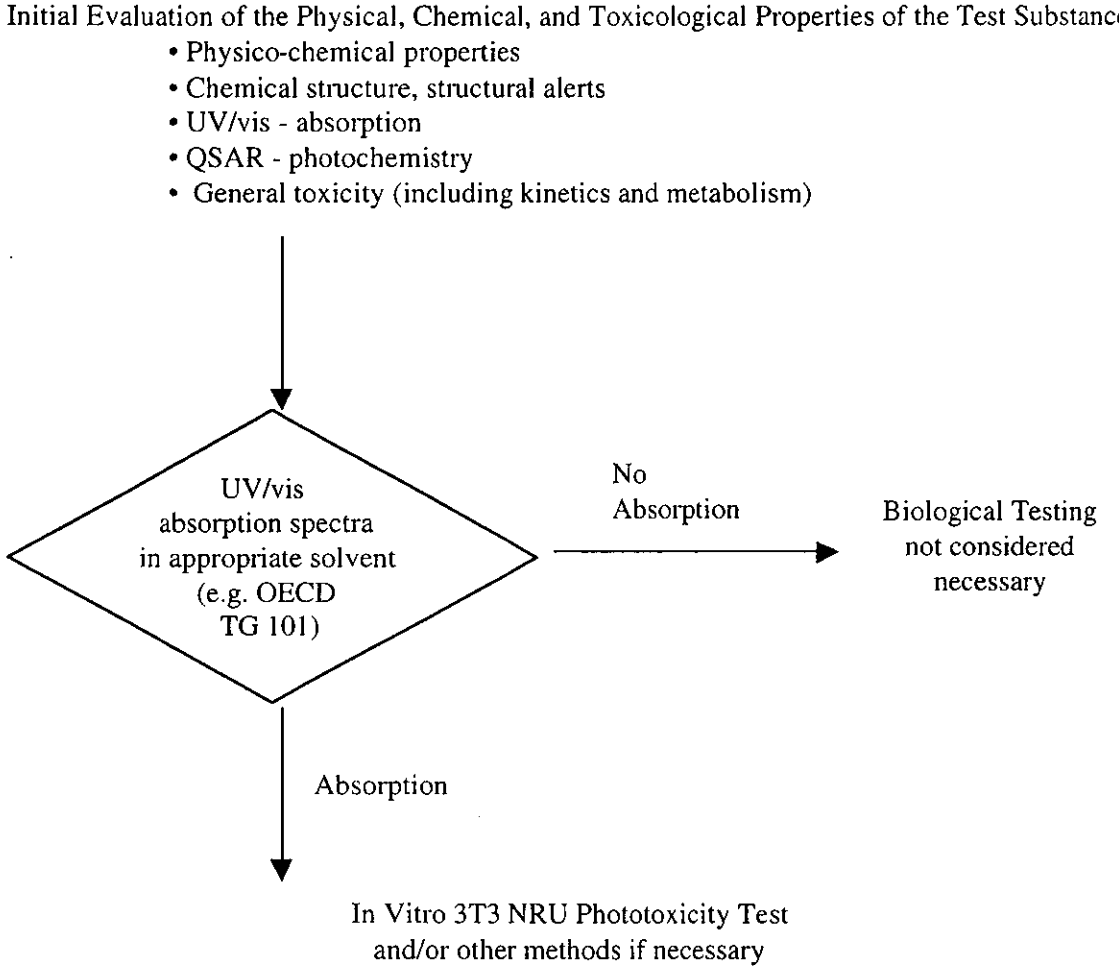
PIF (Photo-Irritation-Factor): factor generated by comparing two equally effective cytotoxic concentrations (IC_{50}) of the test chemical obtained in the absence (-Irr) and in the presence (+Irr) of a non-cytotoxic irradiation with UVA/vis light.

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)

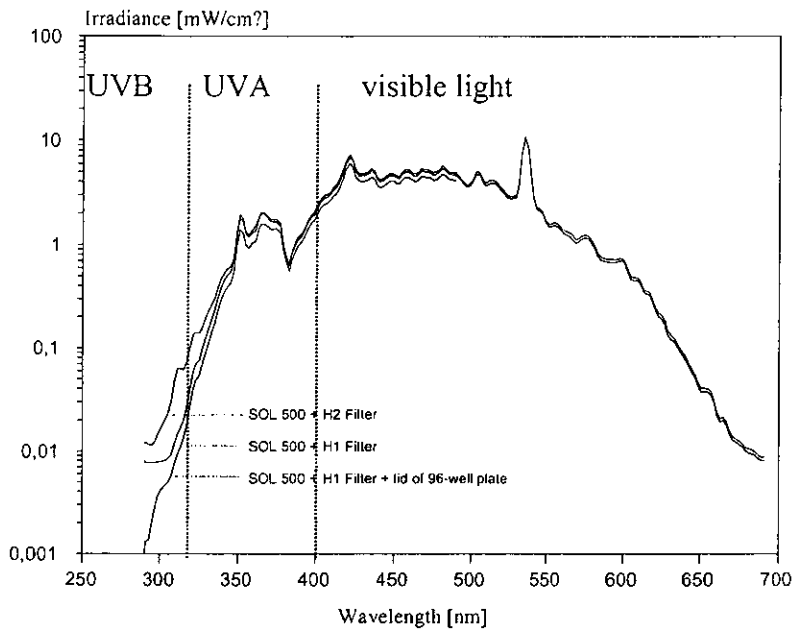
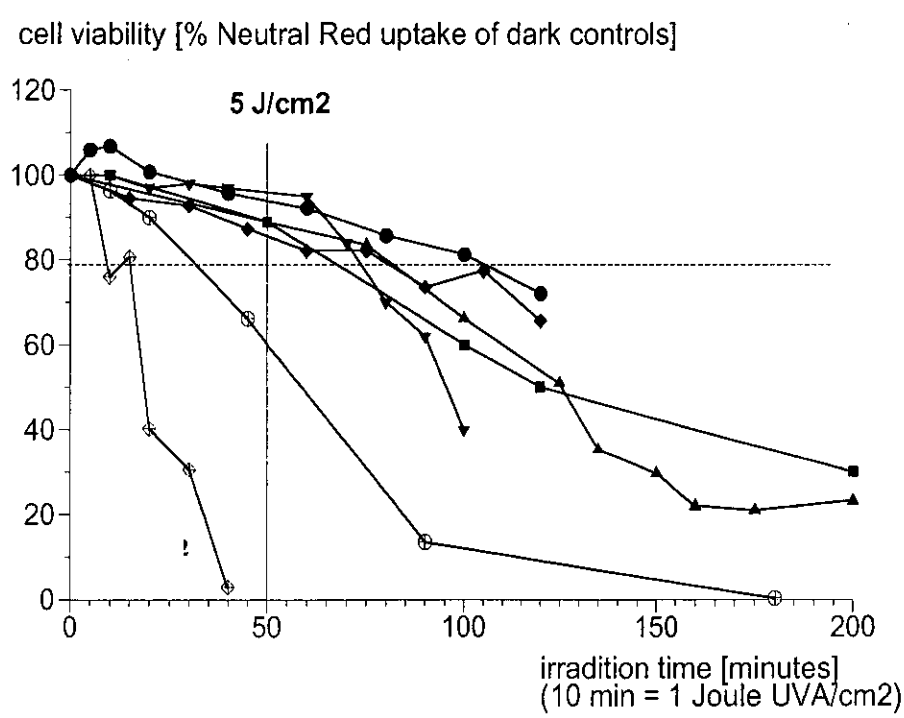


Figure 1 gives an example of an acceptable spectral power distribution of a filtered solar simulator. It is from the doped metal halide source used in the validation trial of the 3T3 NRU PT (1)(3)(12). The effect of two different filters and the additional filtering effect of the lid of a 96-well cell 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.

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/cm_ was derived (vertical dashed line). The horizontal dashed line shows in addition the maximum acceptable irradiation effect given in paragraph 29.

Comments on Draft TG 432

This new version is acceptable to us. Because this does not specifies the company that makes the UV lump and does not exclude the use of other cells than 3T3 cells. This lenders the flexibility to us.

I found there are several typing mistake and minor problems as follows.

1) paragraph 9, add at the end of the first sentence: , and therefore is recommended to obtain from well qualified cell depository.

1) paragraph 35, last of the sentence: (see paragraph 12) should be (see paragraph 17)
2) paragraph 35 line 1: Washing the cells with buffered solution should be soft not to detach the cells on the dishes. Carefully is rather ambiguos. Therefore, wash carefully should be wash softly .
3) paragraph 37: Exposure time to UV is fixed to 50 min . However, if the UV lump is not strong enough, there might be a case where addition exposure time is necessary to achieve 5J/cm2. Therefore, time should be about 50 min .
4) paragraph 40 line 1: type mistake, warmbuffering → warm buffering
5) paragraph 43: it says that Shake microtiter plate rapidly on --- . However, if the plate is shaken hard, extract may leak from the wells. Therefore, it should be Shake the microtiter plate gently on ---
6) paragraph 51, line 3 from DEc=--- equation: Sentence If C* cannot be determined because the response values of the +Irr curve are systematically higher or lower than REc the dose effect is set to 1. is strange because REc is the difference between Rc(-Irr) and Rc (+Irr). It might be If C* cannot be determined because the response values of the +Irr curve are systematically higher or lower than Rc(~~-Irr~~) the dose effect is set to 1.
7) next sentence of 6): The weighting factors i are given by --- should be The weighting factors Wi are given by ---
8) paragraph 51 last line: type mistake, (MPEc= or not, the chemical is classified as phototoxic). → (MPEc=0.15) or not, the chemical is classified as phototoxic.
9) paragraph 55 first line: First sentence needs modification because test chemical is classifies to be positive according to PIF and MPE. However, if only photosensitizing effects were observed only at the highest concentration, those values can not be calculated. Therefore, the sentence should be If photosensitizing effects were observed only at the highest concentration, --- .

ANNEX 1, Definitions, relative cell viability line 1: in relation of solvent (negative) controls → in relation to solvent (negative) controls

厚生科学研究班における光毒性試験代替法の評価

1，　評価委員会設立の経緯とその位置付け

安全性評価のための動物実験代替法の多くは適用できる化学物質の種類や評価できる毒性等に限界があります。また、in vivo 結果との対応が必ずしも十分でないことがあります。それらを十分に理解せずに代替法を利用すると誤評価してしまう可能性があります。それ故、適切なバリデーションおよび評価を行い代替法がどこまで従来の動物実験に代替し得るかを明確にしておくことが必須です。欧米では ECVAM や ICCVAM のような組織を設立し、バリデーションの支援や代替法を行政的に受け入れるための評価を行い、Episkin や Corrositex などの試験法を特定の行政目的のために受け入れてきました。一方、我が国にはそのような組織はなく、代替法の受け入れは十分ではありませんでした。

そこで、平成１３年度より始まった厚生科学研究「動物実験代替法の開発と利用に関する調査研究（主任研究者　大野泰雄)」では動物実験代替法として提案されている試験法を評価し、厚生労働省に報告する計画を立て、そのために代替法評価委員会と代替法評価会議を設置しました。

2，　評価委員会と評価会議の目的

安全性評価のための動物実験代替法として報告されている試験法を客観的、科学的に評価することにより、その利点と問題点、限界を明らかにし、試験法としての妥当性の範囲を明らかにし、認定することにより、動物実験代替法の使用を促進する。

3，　評価委員会と評価会議の業務と位置付け

評価委員会は代替法について具体的に調査し、評価するための機関であり、代替法の評価および評価の対象となる試験法の専門家から構成されています。一方、評価会議はより広い知識・経験・視野のもとで代替法を行政的な目的のための使用における妥当性について評価します。評価会議は臨床医師、統計の専門家、行政官、および厚生科学研究の班員、班友により構成されています。評価委員会は提出された代替法の申請書を評価し、評価文書を作成し、試験法が特定の目的のために妥当とされた場合には厚生科学研究班に設置された評価会議に上げ、更に評価されます。ここで申請された代替法が妥当とされた場合には公開のシンポジウムを開催し、広く意見を求め、その結果に基づいて最終評価を行います。

4，評価委員会の委員

委員長：

金子豊蔵（国立衛研　毒性部）

副委員長：

田中憲穂（食品薬品安全センター　秦野研究所）

委員：

板垣　宏（資生堂　ライフサイエンス研究センター安全性研究所）

今井弘一（大阪歯科大学　歯科理工学講座）

大野泰雄（国立衛研　薬理部）

大森　崇（国立衛研　審査センター）

岡本裕子（コーセー　基礎研究所）

小島肇夫（日本メナード化粧品　総合研究所）

畑尾正人（資生堂　基盤研究センター　薬剤開発研究所）

若栗　忍（食品薬品安全センター　秦野研究所）

5．評価会議の委員

委員長

大野泰雄（国立衛研　薬理部）

委員

金子豊蔵（国立衛研　毒性部）

田中憲穂（食品薬品安全センター　秦野研究所）

豊田英一（日本化粧品工業連合会・技術委員会）

西岡　清（東京医科歯科大学医学部）

林　憲一（厚生労働省医薬局審査管理課）

溝口　昌子（聖マリアンナ医科大学皮膚科学）

宮地　良樹（京都大学大学院医学研究科）

森本雍憲（城西大学薬学部）

吉村　功（東京理科大学工学研究科経営工学）

吉田武美（昭和大学薬学部）

６．評価する試験法について

評価する試験法の種類は年度毎に厚生科学研究班で決定します。平成１３年度は OECD のガイドライン案で示されている 3T3 細胞を用いた光毒性試験を文献的に評価しております。そこで、平成１４年度はそれ以外の光毒性試験代替法について評価を希望する試験法を公募します。

７．応募期限

平成１４年８月末日

８．評価スケジュール予定

９月：申請に必要な資料の確認

１０月：資料を評価委員に配布

１１月：評価委員会開催

１２月：評価文書の作成

１月：評価会議の開催

２月：公開シンポジウム開催

３月：評価文書作成

なお、施設間バリデーションに関するデータが無いものについては原則として妥当な試験法としては認められません。しかし、我が国における代替法開発の促進を考え、施設間バリデーションのデータが無い試験法についても評価委員会でそれを行う価値があると認めた場合には日本動物実験代替法学会バリデーション委員会に施設間バリデーションの実施を依頼し、その結果を合わせて評価し、評価会議にかける。この場合は評価会議の日程はバリデーションが終了してからとなります。

９．申請に際して提出していただく資料

１）代替しようとする試験法の名称

２）代替しようとする in vivo 試験法に関する資料（プロトコール、再現性、特異性、予測性についての記述を含む）

３）代替法の原理に関する資料

４）試験法の詳細なプロトコール

５）検討した被験物質のリストと化学物質としての特性に関する資料

６）検討した被験物質の in vivo 及び in vitro 試験結果の結果に関する資料

７）試験法の感度、特異性、予測性、精度（一致率）を記載した資料

8）試験法の特徴（適用範囲、false positive, false negative）

9）試験法のバリデーションとそのQCに関する資料

１０）　その他、データ解析上有用な資料（生データ等）

１１）　論文（または、学会発表資料&印刷中の論文原稿）

１２）　提案者の研究歴及び専門性を示す資料

１０．事務局

国立医薬品食品衛生研究所　安全性生物試験研究センター薬理部

大野泰雄

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厚生科学研究費補助金（医薬安全総合研究事業）
分担研究報告書

皮膚吸収試験代替法の開発と評価
（３次元ヒト皮膚培養モデルを用いた経皮吸収試験に関する研究）

分担研究者　大野泰雄　国立衛研　薬理部長
協力研究者　安藤正典、徳永裕司、国立衛研、環境衛生化学部

研究要旨

最近、皮膚ケラチノサイトを用いた皮膚３次元培養細胞モデルが我が国でも簡単に入手できるようになり、それらの製品の品質面あるいは堅牢性の面での評価も向上している。そこで、我々は、ヒトあるいは動物由来の皮膚の代替法として、角質層を有する皮膚３次元培養モデルを用い、各種化合物の in vitro 経皮吸収実験への応用を検討した。試験物質として、安息香酸ナトリウム（ＢＡ）、レゾルシン（ＲＮ）、サリチル酸（ＳＡ）、パラオキシ安息香酸メチル（ＭＰ）、パラオキシ安息香酸エチル（ＥＰ）および４－クロロ－ｍ－クレゾール（ＣＣ）を用い、皮膚３次元培養モデルとして、TESTSKIN および Vitrolife-Dermis の二種類の培養皮膚を用いた。ＳＡから得られた Flux が TESTSKIN あるいは Vitrolife-Dermis を用いた場合、ともに小さい値を示した。それ以外の試験物質では大きな値を示した。TESTSKIN の Flux は腹部剥離皮膚から得られた Flux の２．６～１１．４倍の値を、また、Vitrolife-Dermis の Flux は、腹部剥離皮膚から得られた Flux の１．５～７．７倍の値であった。ＳＡを除く５種類の試験物質の log k'と TESTSKIN、Vitrolife-Dermis あるいは腹部剥離皮膚から得られた透過速度との間には、類似したパターンを示すことが分かった。Vitrolife-Dermis の場合、スポンジ部分が付いた状態で測定を行っているが、透過律速は Vitrolife-Dermis が担っていることが分かった。界面活性剤の一種であるドデシル硫酸ナトリウム（ＳＤＳ）で処理した TESTSKIN の場合、ＢＡ、ＲＮ及びＳＡの透過速度を、それぞれ、０．５７，０．１５および０．５３倍に減少させた。塩化ベンザルコニウム（ＢＫ）で処理された TESTSKIN の場合、ＲＮあるいはＳＡの透過速度を０．４あるいは３．４倍に変化させた。Vitrolife-Dermis をＳＤＳで処理した場合、透過速度に変化がなかった。Vitrolife-Dermis をＢＫで処理した場合、ＭＰあるいはＥＰの透過速度を１．４倍に増加させ、ＳＡでは、０．７倍に減少させた。ＳＤＳあるいはＢＫで３０分間処理した後、３時間の透過実験を行った後の TESTSKIN 細胞の viability は２０～３０％であった。