

B. 41. PHOTOTOXICITY - *IN VITRO* 3T3 NRU PHOTOTOXICITY TEST

1. METHOD

1.1 INTRODUCTION

Phototoxicity is defined as a 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.

Information derived from the *in vitro* 3T3 NRU phototoxicity test serves to identify the phototoxic potential of a test substance, i.e. the existence or absence of possible hazards likely to arise from a test substance in association with exposure to UV and visible light.

Since the toxicological endpoint of the *in vitro* test is determination of *photocytotoxicity*, induced by the combined action of a chemical and light, compounds that are phototoxic *in vivo* after systemic application and distribution to the skin, as well as compounds that act as photoirritants after topical application to the skin, can be identified by the test.

The *in vitro* 3T3 NRU phototoxicity test was developed and validated in a joint EU/COLIPA project from 1992-1997 (1)(2)(3), to establish a valid *in vitro* alternative to the various *in vivo* tests in use. In 1996 an OECD workshop recommended an *in vitro* tier testing approach for phototoxicity assessment (4).

Results from the *in vitro* 3T3 NRU phototoxicity test were compared with acute phototoxicity / photoirritation effects *in vivo* in animals and humans, and the test has been shown to give excellent predictivity for these effects. The test is not designed to predict other adverse effects that may arise from the combined action of a chemical and light, e.g. *photogenotoxicity*, *photoallergy*, and *photocarcinogenicity*, although many chemicals which show these specific properties will react positive in the *in vitro* 3T3 NRU phototoxicity test. In addition, the test is not designed to permit an assessment of *phototoxic potency*.

A sequential approach to phototoxicity testing of chemicals is set out in Annex 1.

1.2 DEFINITIONS

Irradiance: the intensity of ultraviolet (UV) or visible light incident on a surface, measured in W/m² or mW/cm².

Dose of light: the quantity (= intensity × time) of ultraviolet (UV) or visible radiation incident on a surface, expressed in Joules (= W × s) per surface area, e.g. J/m² or J/cm².

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

Prediction model: an algorithm used to transform the results of a toxicity test into a prediction of toxic potential. In the present test guideline, PIF and MPE can be used for transformation of the results of the *in vitro* 3T3 NRU phototoxicity test into a prediction of phototoxic potential.

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

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

Phototoxicity: an 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 the systemic administration of a chemical.

Photoirritation: a sub-species of the term 'phototoxicity', which is used to describe only those phototoxic reactions which are produced at the skin after exposure to chemicals (topically or orally). These phototoxic reactions lead always to non-specific cell damage (sunburn like reactions).

Photoallergy: an acquired immunological reactivity, which does not occur on first treatment with chemical and light, and needs an induction period of one or two weeks before skin reactivity can be demonstrated.

Photogenotoxicity: a genotoxic response observed with a genetic endpoint, which is elicited after the exposure of cells to a non-genotoxic dose of UV/visible light and a non-genotoxic chemical.

Photocarcinogenicity: carcinogenicity induced by repeated application of light and a chemical. The term 'photo co-carcinogenesis', is used if UV induced tumorigenesis is enhanced by a chemical.

1.3 REFERENCE SUBSTANCES

Besides the positive control chemical *Chlorpromazine*, which should be concurrently tested in each assay, for newly establishing the 3T3 NRU phototoxicity test it is recommended to use as reference chemicals a subset from the chemicals used in interlaboratory trials with the present test (1)(3)(13).

1.4 INITIAL CONSIDERATIONS

Many types of chemicals have been reported to induce phototoxic effects (5)(6)(7)(8). The only common feature is their ability to absorb light energy within the sunlight region. According to the first law of photochemistry (Grotthaus-Draper's Law) photoreaction requires sufficient absorption of light quanta. Thus, before biological testing according to the present test guideline is considered, a UV/vis absorption spectrum of the test chemical should be determined (e.g. according to OECD Test Guideline 101). If the molar extinction / absorption coefficient is less than $10 \text{ litre} \times \text{mol}^{-1} \times \text{cm}^{-1}$, the chemical has no photoreactive potential and does not need to be tested in the *in vitro* 3T3 NRU phototoxicity test or any other biological test for adverse photochemical effects (Annex 1).

1.5 PRINCIPLE OF THE TEST METHOD

Four mechanisms have been identified by which absorption of light by a (chemical) chromophore can result in a phototoxic response (7). All of them result in cell damage. Therefore, 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 UVA/vis light. Cytotoxicity in this test is expressed as a concentration dependent reduction of the uptake of the vital dye, Neutral Red (NR, (9)) 24 hours after treatment with the test chemical and irradiation.

Balb/c 3T3 cells are maintained in culture for 24 h for the formation of monolayers. Two 96-well plates per test chemical are then preincubated with eight different concentrations of the chemical for 1 h. Thereafter one of the two plates is exposed to a non-cytotoxic UVA/vis light dose of 5 J/cm² UVA (+UV experiment), whereas the other plate is kept in the dark (-UV experiment). 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 (NRU) for 3 h. Relative cell viability, expressed as percentage of untreated negative controls, is calculated for each of the eight test concentrations. To predict the phototoxic potential, the concentration responses obtained in the presence (+UV) and in the absence (-UV) of irradiation are compared, usually at the EC₅₀ level, i.e. at the concentration inhibiting cell viability by 50 % cf. untreated controls.

1.6 QUALITY CRITERIA

UVA sensitivity of the cells, historical data: Cells should be regularly checked for sensitivity to UVA. Cells are seeded at the density used in the *in vitro* 3T3 NRU phototoxicity test, irradiated the next day with UVA doses from 1-9 J/cm², and cell viability is determined one day later using the NRU assay. Cells meet the quality criteria, if their viability after irradiation with 5 J/cm² UVA is not less than 80% of the viability of dark controls. At the highest UVA dose of 9 J/cm², viability should not be less than 50% of that of dark controls. This check should be repeated about every 10th passage of the cells.

UVA sensitivity of the negative control cells, current test: The test meets the quality criteria if negative controls (cells in Earl's Balanced Salt Solution (EBSS) with or without 1% dimethylsulfoxide (DMSO) or 1% ethanol (EtOH)) in the +UVA experiment show a viability of not less than 80% of that of non-irradiated cells in the same solvent of the concurrent dark experiment (-UVA).

Viability of negative controls: The absolute optical density (OD_{540 NRU}) measured in the NR extract of the negative controls indicates whether the 1x10⁴ cells seeded per well have grown with normal doubling time during the two days of the assay. A test meets the acceptance criteria if the mean OD_{540 NRU} of untreated controls is ≥ 0.2

Positive control: A known phototoxic chemical shall be tested concurrently with each *in vitro* 3T3 NRU phototoxicity test. Chlorpromazine (CPZ) was used as positive control in the EU/COLIPA validation study and is therefore 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 (+UVA)*: EC₅₀ = 0.1 to 2.0 µg/ml, *CPZ non-irradiated (-UVA)*: EC₅₀ = 7.0 to 90.0 µg/ml. The Photo Irritation Factor (PIF), i.e. the shift of EC₅₀ should be at least 6.

Other known phototoxic chemicals, suitable for the chemical class or solubility characteristics of the test chemical being evaluated, may be used as the concurrent positive controls, in place of CPZ. In this case, based on historical data, the ranges of EC₅₀ values and PIF or MPE (Mean Photo Effect) should be adequately defined as acceptance criteria for the test.

1.7 DESCRIPTION OF THE TEST METHOD

1

.7.1 Preparations

1.7.1.1 Cells

A permanent mouse fibroblast cell line - Balb/c 3T3, clone 31 - either from ATCC or from ECACC was used in the validation study, and is therefore recommended. Other cells or cell lines may be successfully used with the same test protocol, if the culture conditions are adapted to the specific needs of the cells, but equivalency must be demonstrated.

Cells should be checked regularly for the absence of mycoplasma contamination and should only be used if the results of such checking was satisfactory.

Since the UVA sensitivity of cells may increase with the number of passages, Balb/c 3T3 cells of the lowest obtainable passage number should be used, preferably less than 100. It is important that UVA sensitivity of the Balb/c 3T3 cells is regularly checked according to the quality control procedure described in this Guideline.

1.7.1.2 *Media and culture conditions*

Appropriate culture media and incubation conditions should be used for routine cell passage and during the test procedure. For Balb/c 3T3 cells, these are DMEM supplemented with 10% new-born calf serum, 4 mM Glutamine, Penicillin and Streptomycin, and humidified incubation at 37°C / 7.5% CO₂. It is particularly important that cell culture conditions ensure a cell cycle time within the normal historical range of the cells or cell line used.

1.7.1.3 *Preparation of cultures*

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.

For the phototoxicity test cells are seeded in culture medium at a density such that cultures will not reach confluence by the end of the test, i.e. when cell viability is determined 48 h after the seeding of the cells. For Balb/c 3T3 cells grown in 96-well plates, 1×10⁴ cells per well is the recommended cell density.

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

1.7.1.4 *Metabolic activation*

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, no chemical is known for which 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.

1.7.1.5 *Test chemical / Preparation*

Test chemicals must be freshly prepared immediately prior to use, unless stability data demonstrate the acceptability of storage. Preparation under red light may be required when rapid photodegradation is likely to occur.

Test chemicals should be dissolved in buffered salt solutions, e.g. Earl's Balanced Salt Solution, (EBSS) or Phosphate Buffered Saline (PBS), which, to avoid interference during irradiation, must be free from protein components and light absorbing pH indicator colours.

Test chemicals of limited solubility in water should be dissolved in appropriate solvents at 100-fold the desired final concentration and then diluted 1:100 with the buffered salt solution. If a solvent is used it must be present at a constant volume of 1% (v/v) in all cultures, i.e. in the negative controls as well as in all concentrations of the test chemical.

Dimethylsulphoxide (DMSO) and ethanol (EtOH) are the recommended solvents. Other solvents of low cytotoxicity (e.g. acetone) may be appropriate, but they should carefully be assessed for specific properties, e.g. reaction with the test chemical, quenching of the phototoxic effect, radical catching properties.

Vortex mixing and / or sonication and / or warming to 37°C may be used, if necessary, to aid solubilization.

1.7.1.6 UV irradiation / Preparation

Light source: the choice of an appropriate light source and appropriate filtering is the most crucial factor in phototoxicity testing. UVA and visible regions are usually associated with photosensitization (7)(10), whereas UVB is of less relevance and is directly highly cytotoxic, increasing its cytotoxicity through 1000 fold from 313 to 280 nm (11). Criteria for the choice of an appropriate light source should include the essential requirement that the light source emits wavelengths absorbed by the test chemical and that the dose of light (achievable in a reasonable time) should be sufficient for the detection of known photosensitizers. Furthermore, the wavelengths and doses employed should not be unduly deleterious to the test system, which includes the emission of heat (infra red region).

The simulation of sunlight with solar simulators is considered the optimal light source. Both, Xenon arcs and (doped) mercury-metal halide arcs are used in solar simulators. The latter have the advantage of emitting less heat and of being cheaper, but the match to sunlight is not perfect. Since all solar simulators emit significant quantities of UVB, they should be suitably filtered to attenuate the highly cytotoxic UVB wavelengths.

For the *in vitro* 3T3 NRU phototoxicity test an irradiance spectrum practically devoid of UVB should be used (UVA:UVB ~ 1:20). 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 has been published (3).

Dosimetry: The intensity of light (irradiance) should be regularly checked before each phototoxicity test, by using a suitable broadband UV-meter. 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, but such instruments require skilled operation by appropriately trained persons.

A dose of 5 J/cm² (UVA) was determined in the validation study to be non-cytotoxic to Balb/c 3T3 cells and sufficiently potent to excite even weak phototoxic chemicals. To achieve 5 J/cm² within a time period of 50 min, irradiance has to be adjusted to 1.666 mW/cm². If another cell line or a different light source are used, the UVA dose may have to be slightly adapted, by using the criteria of being non-deleterious to the cells and sufficient to detect standard phototoxins. The time of light exposure is calculated in the following way:

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

1.7.2 Test Conditions

The maximum concentration of a test chemical should not exceed 100 µg/ml, since all phototoxic chemicals were detected at lower concentrations, whereas at higher concentrations the incidence of false positives (overpredictions) increases (13). The pH of the highest concentration of the test chemical should be satisfactory (pH range: 6.5 - 7.8).

The ranges of concentrations of a chemical tested in the presence (+UVA) and in the absence (-UVA) of light should be adequately determined in preceding range-finder experiments. Range and intercept of a concentration series shall be adjusted in such a way that concentration-response curves are sufficiently supported by experimental data. Geometric concentration series (with a constant dilution factor) should be used.

1.7.3 **Test Procedure¹**

1.7.3.1 *1st day*

Prepare a cell suspension of 1×10^5 cells/ml in culture medium and dispense 100 μ L culture medium only into the peripheral wells of a 96-well tissue culture microtiter plate (= blanks). In the remaining wells, dispense 100 μ L of a cell suspension of 1×10^5 cells/ml (= 1×10^4 cells/well). For each test chemical, prepare two plates: one for determination of cytotoxicity (-UVA), and the other for determination of phototoxicity (+UVA).

Incubate the cells for 24 h (7.5% CO₂, 37°C) until they form a half-confluent monolayer. This incubation period allows for cell recovery and adherence, and for exponential growth.

1.7.3.2 *2nd day*

After incubation, decant the culture medium from the cells and wash twice with 150 μ L EBSS/PBS per well. Add 100 μ L of EBSS/PBS containing the appropriate concentration of test chemical or just solvent (negative control). Apply 8 different concentrations of the test chemical. Incubate cells with the test chemical in the dark for 60 minutes (7.5% CO₂, 37°C).

To perform the (+UVA) part of the assay, irradiate the cells at room temperature for 50 minutes through the lid of the 96-well plate with 1.7 mW/cm² UVA (= 5 J/cm²). Ventilate with a fan to prevent H₂O condensation under the lid. Keep duplicate plates (-UVA) at room temperature in a dark box for 50 min (= UVA exposure time).

Decant test solution and wash twice with 150 μ L EBSS/PBS. Replace EBSS/PBS with culture medium and incubate (7.5% CO₂, 37 °C) overnight (18-22 h).

1.7.3.3 *3rd day*

Microscopic evaluation

Examine the cells under a phase-contrast microscope. Record changes in morphology of the cells due to cytotoxic effects of the test chemical. This check is recommended, to exclude experimental errors, but these records are not used for evaluation of cytotoxicity or phototoxicity

Neutral Red Uptake test

Wash the cells with 150 μ L prewarmed EBSS/PBS. Remove the washing solution by gentle tapping. Add 100 μ L NR medium and incubate at 37 °C, in a humidified atmosphere of 7.5% CO₂, for 3 h.

After incubation, remove the NR medium, and wash the cells with 150 μ L EBSS/PBS. Decant and blot EBSS/PBS totally. (*Optionally*: centrifuge reversed plate.)

Add exactly 150 μ L NR desorb solution (freshly prepared ethanol/acetic acid)

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

Measure the optical density of NR extract at 540 nm in a spectrophotometer, using blanks as a reference. Save the data in appropriate file format (e.g. ASCII) for subsequent analysis.

¹ Additional details can be found in reference (12)

2

DATA

2.1

QUALITY AND QUANTITY OF DATA

The data should permit a meaningful analysis of the concentration-response obtained in the presence and in the absence of UVA/vis irradiation. If cytotoxicity is found, both the concentration range and the intercept of individual concentrations should be set in such a way as to allow the fit of a curve to experimental data. Due to the fact that a test chemical might not be cytotoxic up to the defined limit concentration of 100 µg/ml in the dark experiment (-UVA), but highly cytotoxic when irradiated (+UVA), the concentration ranges to be tested in both parts of the experiment may need to differ by orders of magnitude to fulfil the requirement of adequate data quality. If no cytotoxicity is found in both parts of the experiment (-UVA and +UVA), testing with a great intercept between single doses up to the highest concentration is sufficient.

There is no requirement for verification of a clear positive result by performing a repeat experiment. In addition, clear negative results need not to be verified, provided the test chemical was tested at sufficiently high concentrations. In such cases, one main experiment, supported by one or more range-finding preliminary experiments, is sufficient.

Tests with borderline results near to the cut-off line of the prediction model should be repeated for verification.

If repeat testing is considered necessary, then variation of the experimental conditions may be important to achieve a clear result. A key variable in this test is preparation of solutions of the test chemical. Hence, variation of these conditions (co-solvent, trituration, sonication) may be most relevant in the repetition of a test. Alternatively, variation of the pre-irradiation incubation time may be considered. A shorter time can be relevant for water-unstable chemicals.

2.2

TREATMENT OF RESULTS

Where possible, the concentration of a test chemical reflecting a 50% inhibition of the cellular NRU (EC₅₀) is determined. This can be done by applying any appropriate non-linear regression procedure (preferably a Hill function or logistic regression) to the concentration-response data, or by using other fitting procedures (14). Before using an EC₅₀ for further calculations, the quality of the fit should be appropriately checked. Alternatively, graphical fitting methods can be used to calculate the EC₅₀. In this case, the use of probability paper is recommended (x-scale: log, y-scale: probit), as in many cases the concentration response function will become almost linear after this transformation.

2.3

EVALUATION OF RESULTS (PREDICTION MODELS)

2.3.1

Prediction model version 1: Photo-Irritation-Factor (PIF)

If both, in the presence (+UVA) and in the absence (-UVA) of light, complete concentration response curves are obtained, a Photo-Irritation-Factor (PIF) is calculated by means of the following formula:

$$(a) \quad PIF = \frac{EC_{50}(-UV)}{EC_{50}(+UV)}$$

A PIF < 5, predicts no phototoxic potential, whereas a PIF ≥ 5 predicts phototoxic potential.

If a chemical is only cytotoxic +UVA and is not cytotoxic when tested -UVA, the PIF cannot be calculated, although this is a result that indicates phototoxic potential. In such cases, a "> PIF" can be calculated if the (-UV) cytotoxicity test is performed up to the highest test concentration (C_{max}) and this value is used for calculation of the "> PIF":

$$(b) \quad > PIF = \frac{C_{max}(-UV)}{EC_{50}(+UV)}$$

If only a "> PIF" can be obtained, then any value >1 predicts phototoxic potential.

If both EC₅₀ (-UV) and EC₅₀ (+UV) cannot be calculated due to the fact that a chemical does not show any cytotoxicity up to the highest test concentration, this indicates no phototoxic potential. In such cases, a formal "PIF = *1" is used to characterise the result

$$(c) \quad \text{PIF} = *1 = \frac{C_{\max}(-UV)}{C_{\max}(+UV)}$$

If only a "PIF = *1" can be obtained, this predicts no phototoxic potential.

In cases (b) and (c), concentrations achieved in the *in vitro* 3T3 NRU phototoxicity test should be carefully taken into consideration when predicting phototoxic potential

2.3.2 Prediction model version 2: Mean-Photo-Effect (MPE)

Alternatively, a novel version of the model for predicting phototoxic potential can be applied, which has been developed by using data of the EU/COLIPA validation study (15) and tested under blind conditions in a subsequent study on the *in vitro* phototoxicity of UV filter chemicals (13). This model overcomes the limitation of the PIF model in cases where an EC₅₀ cannot be obtained. The model uses the "Mean Photo Effect" (MPE), a measure which is based on comparison of the complete concentration response curves. For application of the MPE model, a special computer software was developed at the Humboldt University (Berlin, D), which can be obtained free of charge.

2.4 INTERPRETATION OF RESULTS

A positive result in the *in vitro* 3T3 NRU phototoxicity test (PIF ≥ 5 or MPE ≥ 0.1) indicates that the test substance has phototoxic potential. If this result is obtained at concentrations below 10 µg/ml, the test chemical is also likely to act as phototoxin also under various exposure conditions *in vivo*. If a positive result is obtained only at the highest test concentration of 100 µg/mL, further considerations may be necessary for the assessment of hazard or phototoxic potency. These may include data on penetration, absorption and possible accumulation of the chemical in the skin, or testing of the chemical in a confirmatory alternative test, e.g. using a human *in vitro* skin model.

A negative result from the *in vitro* 3T3 NRU phototoxicity test (PIF < 5 or MPE < 0.1) indicates that the test substance was not phototoxic to the cultured mammalian cells under the conditions used. In cases where the chemical could be tested up to the highest concentration of 100 µg/ml, a negative result indicates that the chemical has no phototoxic potential, and phototoxicity *in vivo* may be considered unlikely. In cases where identical concentration-toxicity responses (EC₅₀+UV and EC₅₀-UV) were obtained at lower concentrations, the interpretation of data would be the same. In contrast, if no toxicity was demonstrated (+UV and -UV) and if aqueous solubility limited concentrations to values less than 100 µg/ml, then compatibility of the test substance with the assay may be questioned and confirmatory testing should be considered (e.g. using an *in vitro* skin model, or an *ex vivo* skin model or an *in vivo* test).

3 REPORTING

TEST REPORT

The test report must include the following information:

Test chemical:

- identification data and CAS no., if known
- physical nature and purity
- physicochemical properties relevant to conduct of the study
- stability and photostability, if known

Solvent:

- justification for choice of solvent
- solubility of the test chemical in this solvent
- percentage of solvent present in treatment medium (EBSS or PBS)

Cells:

- type and source of cells
- absence of mycoplasma
- number of cell passages, if known
- UVA sensitivity of cells, determined with the irradiation equipment used in the *in vitro* 3T3 NRU phototoxicity test

Test conditions (a); *incubation before and after treatment*:

- type and composition of culture medium
- incubation conditions (CO₂ concentration, temperature, humidity)
- duration of incubation (pre-treatment, post-treatment)

Test conditions (b); *treatment with the chemical*:

- rationale for selection of concentrations of the test chemical used both in the presence and in the absence of UV/vis 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 (c); *irradiation*:

- rationale for selection of the light source used
- spectral irradiance characteristics of the light source
- transmission / 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/cm²
- duration of the UV/vis light exposure
- UVA dose (irradiance × time), expressed in J/cm²
- temperature employed to cell cultures during irradiation and for cell cultures concurrently kept in the dark

Test conditions (d); *NRU test*

- composition of NR medium
- duration of NR incubation
- incubation conditions (CO₂ concentration, temperature, humidity)
- NR extraction conditions (extractant, duration)
- wavelength used for spectrophotometric reading of NR 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 mean viability of controls
- concentration - response curves (test chemical concentration vs. relative cell viability), obtained in concurrent +UVA and -UVA experiments
- data analysis of the concentration response curves: if possible, computation / calculation of EC₅₀ (+UVA) and EC₅₀ (-UVA)
- comparison of the two concentration-response curves obtained in the presence and in the absence of UVA/vis irradiation, either by calculation of the Photo Irritation Factor (PIF), or by calculation of the Mean Photo Effect (MPE)
- classification of phototoxic potential
- test acceptance criteria (a), *concurrent negative control*:
 - absolute viability (optical density of NR extract) of irradiated and non irradiated cells
 - historical data of negative control, mean and standard deviation
- test acceptance criteria (b), *concurrent positive control*:
 - EC50(+UVA) and EC50(-UVA) and PIF of positive control chemical
 - historical data of positive control chemical: EC50(+UVA) and EC50(-UVA) and PIF, mean and standard deviation

Discussion of the results

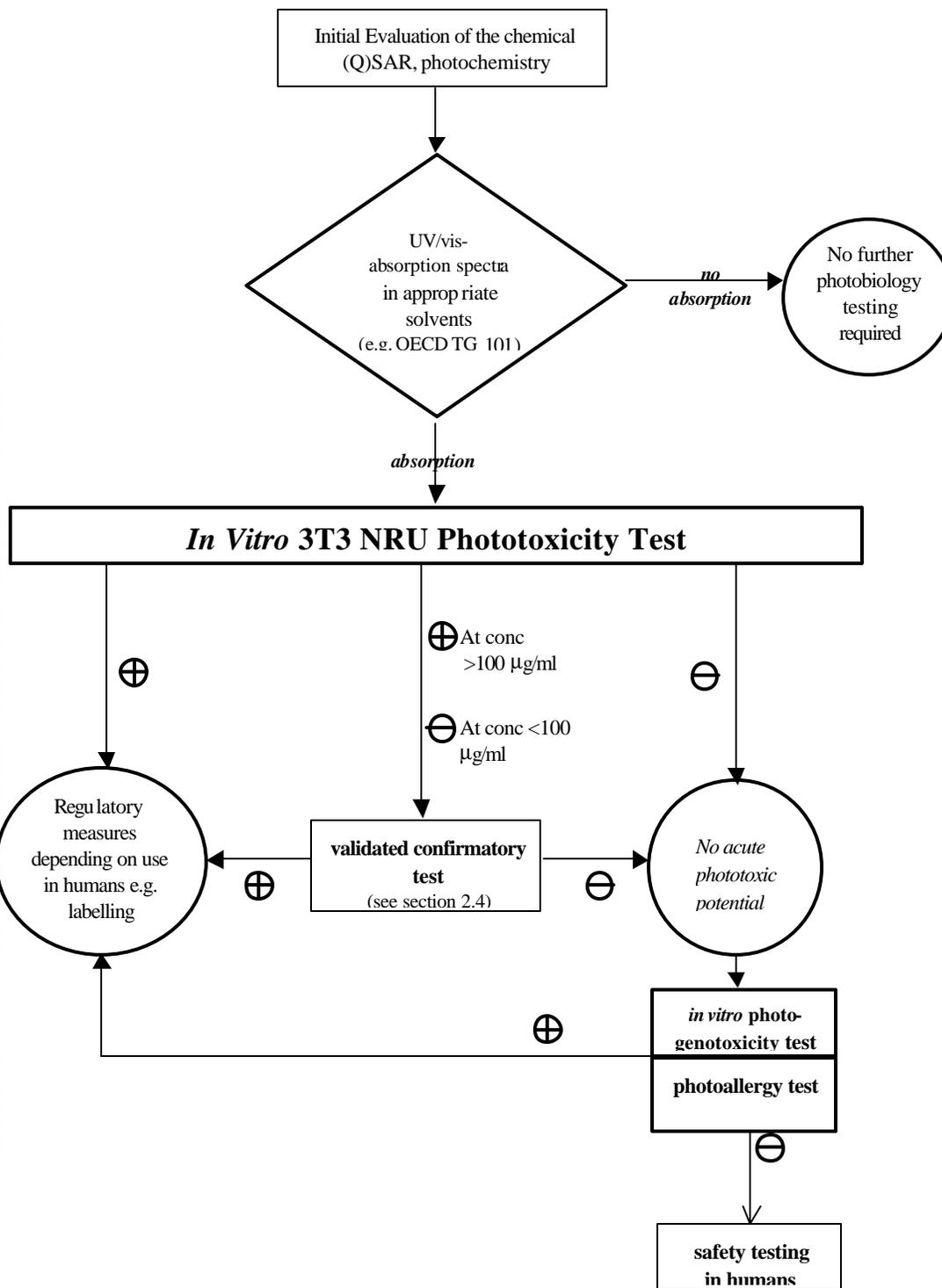
Conclusions

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ANNEX 1

Role of the 3T3 NRU PT in a Sequential Approach to Phototoxicity Testing of Chemicals



Please notice that only European legislation published in the paper editions of the Official Journal of the European Communities is deemed authentic. This text was prepared from the draft protocol sent for approval to the Member States. Minor editorial differences may exist between this version and the one published in the paper edition of the Official Journal. Care has been taken to ensure correctness of the text; nevertheless possibility of errors cannot be completely excluded. In case of doubt the reader is advised to consult the Official Journal.
 This method can be found in Dir. 2000/33/EC (O.J. L 136 2000) (English version corrected in Dir. 2000/33/EC, O.J. L 136, 2000).
 A complete list of Annex V Testina Methods and the corresponding OJ can be downloaded from a previous page in this site.