

MB RESEARCH LABORATORIES

STANDARD PROTOCOL

702-02

1.0 **TITLE OF STUDY:** 3T3 Neutral Red Uptake Phototoxicity Test

2.0 **OBJECTIVE:** The cytotoxicity and phototoxicity of the test compound to 3T3 cells (in the presence or absence of UVA light) is assessed by Neutral Red Uptake. The 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU PT), based on the OECD test guidelines, is designed to detect the phototoxicity induced by the combined action of a test article and light by using an *in vitro* cytotoxicity assay with the Balb/c 3T3 mouse fibroblast cell line. The test identifies aqueous-soluble compounds (or formulations) that have the potential to exhibit *in vivo* phototoxicity after systemic application.

3.0 **TEST ARTICLE:**

3.1: **Source:** All test articles will be supplied by the sponsor. The test article identification and analysis of the purity, strength, pH, stability, solubility, uniformity and safety is the responsibility of the sponsor. The test and control articles and the vehicle will be considered 100% active/pure for the purpose of dosage calculations, unless directed otherwise by Sponsor.

3.1.1: **Absorbance Spectrum:** The sponsor will supply MB Research with the UV-VIS absorbance spectrum of the test article prior to study initiation.

3.2: **Label:** Each test article will be identified by source, name and/or code number, date of receipt at MB Research, and MB Project Number.

3.3: **Storage:** The test article will be stored at room temperature and humidity unless otherwise specified by the sponsor.

3.4: **Hazards:** Based on the information provided by the sponsor, appropriate routine safety precautions will be exercised in the handling of the test article.

3.5: **Vehicle and Controls** will be considered 100% active/pure for the purpose of dosage calculations.

3.5.1: **Vehicle:** The vehicle will be sterile HBSS. If necessary, a test article can be prepared in a solvent (e.g. DMSO, or EtOH) at 100-fold the desired final concentration and will be specified in section 13.3.3 of this protocol.

3.5.2: **Positive Control:** Chlorpromazine (CPZ), source and other I.D. or composition data will be recorded in the raw data and included in the final report.

4.0 **TEST SYSTEM AND JUSTIFICATION:** Balb/c 3T3 cells (*American Type Culture Collection (ATCC), Rockville, MD, Clone A31*) are recommended by the test guidelines.

5.0 **EXPERIMENTAL DESIGN:**

5.1: **Basis of the Method:** The basis of this test is a comparison of the cytotoxicity of a test article when tested in the presence and in the absence of a non-cytotoxic dose of UVA light. Cytotoxicity is measured as an inhibition of the capacity of the cell cultures to take up a vital dye, Neutral Red, one day after treatment, according to Borenfreund & Pruener (1985)¹.

5.2: Experimental Description

Endpoint and Detection : Cell viability, determined as inhibition of the capacity of the cell cultures to take up vital dye, Neutral Red

Test Parameter : EC₅₀, EC_{50UV}

Test System : Balb/c 3T3 mouse fibroblast cell line (ATCC CCL-163, clone A31)

Balb/c 3T3 cells are seeded in microplates and maintained in culture for 24 hrs for formation of monolayers. Ideally, the central 60 wells of the microplate are used. Two 96-well plates per test article are then preincubated with up to eight different concentrations of the test article for 1 hour. One plate is then exposed to a dose of 5 J/cm² UVA (+UV experiment), whereas the other plate is kept in the dark (-UV experiment). The treatment medium is then replaced with culture medium and, after 24 hrs, cell viability is determined by Neutral Red Uptake for 3 hrs (See Annex A).

Cell viability obtained with each of the concentrations of the test article is compared with that of vehicle controls and the percent inhibition (of viability) is calculated. For prediction of phototoxic potential, the concentration responses obtained in the presence and absence of UVA irradiation are compared, usually at the EC₅₀ level, i.e. the concentration inhibiting cell viability by 50% of vehicle controls.

5.3: Neutral Red (NR) Medium

Prepare NR solution to yield a 50 ug/ml working solution. The NR medium should be allowed to stand at room temperature, or incubated overnight at 37°C, and centrifuged at approximately 600X g for 10 minutes (to remove NR crystals) before adding to the cells. Alternatively, 0.2 – 10 um filtering can be used.

5.4: Neutral Red Extractant

- 1) 1.0% Glacial Acetic Acid Solution, 50% Ethanol, 49% H₂O
- or
- 2) 95% Ethanol, 5% H₂O

Best prepared immediately prior to use.

5.5: Preparation of Test Articles

Exposure of the cells with test articles and subsequent irradiation is performed in buffered salt solution, since these treatment media are free of proteins and pH indicators. Test articles are dissolved in either HBSS or PBS. The highest final concentration of the test article shall not exceed 1000 ug/ml (Spielmann et al., 1998)².

The solubility of the test article is assessed prior to the assay to establish the optimum solvent system, i.e. whether the stock solution should be made in HBSS, PBS or in organic solvent. For pretesting of solubility, use of the hierarchical procedure shown in Annex B is recommended.

- Test articles that are soluble in water up to 1000 ug/ml should be dissolved in sterile prewarmed (37°C) HBSS or PBS.
- Test articles of limited solubility in water (< 1000 ug/ml) should be dissolved in Dimethylsulfoxide (DMSO) at 100-fold the desired final concentration. Ethanol (EtOH) may be considered as the third optional solvent. The solvent shall be present at a constant volume of 1% (v/v) in the vehicle controls and in all eight test concentrations, i.e. the test article is dissolved in a solvent (vehicle) consisting of 1 part DMSO or EtOH added to 99 parts of sterile prewarmed (37°C) HBSS or PBS.

Measure the pH of the highest concentration of the test article. Since strong acids and bases may influence the buffer capacity of HBSS or PBS, they should be neutralized with 0.1N NaOH or 0.1N HCl. In this case, prepare highest concentration of the test article in ~ 80% of final HBSS/PBS volume, measure pH, neutralize, and add HBSS or PBS to final volume. Vortex mixing and/or sonication and/or warming to 37°C may be used, if necessary, to aid solubilization. The concentrations used for relatively insoluble test articles should range from the soluble to the precipitating dose. If a precipitate is present, centrifuge the concentration at 100X g – 200X g for 5 min and use the supernatant for dosing.

The test article must be freshly prepared immediately prior to use. Preparation under red light may be necessary, if rapid photodegradation is known or likely to occur.

5.6: Range-Finder and Main Experiment

5.6.1: Irradiation Procedures

The –UVA plate will be kept in the dark at RT. The +UVA plate will be irradiated with approximately 1.7 mW/cm² (= 5 J/cm²) of UVA from a SOL 500 Solar Simulator. The UVA dose will be monitored with a calibrated UVA radiometer. Temperature will be recorded using a calibrated digital thermometer.

5.6.2: Range Finder Experiment

Test eight concentrations of the test article, +UVA and –UVA, by diluting the stock Test Article solution with a constant factor (e.g. $^2\sqrt{10} = 3.16$, see ANNEX C) with HBSS/PBS, covering a large range starting at 1000µg/ml, or 0.1%, e.g.:

1000⇒316⇒100 ⇒31.6⇒10⇒3.16⇒1.0⇒0.316 µg/ml

5.6.3: Main Experiment

Depending on the slope of the concentration-response curve estimated from the range finder, the dilution factor in the concentration series of the main experiment should be smaller (e.g. $^6\sqrt{10} = 1.47$, see ANNEX C) centered on the two EC₅₀'s, the EC_{50+UVA} and the EC_{50-UVA (dark)}. Each relevant concentration range will be targeted to include at least three cytotoxic concentrations between 10% and 90%. If the chosen concentration range does not accomplish this, the test will be considered a screen and the main experiment will be repeated.

6.0: TEST PROCEDURE

6.1: 1st day After growing up the cells from frozen stock

Prepare a cell suspension of 1x10⁵/ml in culture medium (DMEM with 10% NBC Serum and Gentamicin 50 µg/ml). Using a multi-channel pipette, dispense 100 µl culture medium only (no cells) into the peripheral wells of a 96-well tissue culture microtiter plate (= blanks, See Annex D). In the remaining central 60 wells, dispense 100 µl of a cell suspension of 1x10⁵ cells/ml (= 1x10⁴ cells/well). Per one test article, prepare two plates each: one for determination of cytotoxicity (–UVA), and the other for determination of phototoxicity (+UVA).

Incubate cells for approximately 24 hr (5.0% ± 1.0% CO₂, 37°C) until they form a half-confluent monolayer. This incubation period allows for cell recovery and adherence.

6.2: 2nd day

After incubation, decant culture medium from the cells and wash carefully with 150 ul HBSS/PBS. Decant and blot excess rinse buffer if necessary. Add 100 ul of HBSS/PBS containing the appropriate concentration of test article. Incubate cells for 1 hr (5.0% \pm 1.0% CO₂, 37°C).

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

After UVA irradiation, decant test solution and wash once with 150 ul HBSS/PBS. Replace HBSS/PBS with culture medium and incubate at 37°C overnight (18-24 hrs).

6.3: 3rd day

6.3.1: Microscopic Evaluation

Examine cells under a phase contrast microscope. Record changes in morphology of the cells due to cytotoxic effects of the test article. This check is performed to exclude experimental errors. Records are not used for evaluation of cytotoxicity or phototoxicity.

6.3.2: Measurement of Neutral Red Uptake (NRU)

The uptake of the vital dye Neutral Red into the lysosomes/endosomes and vacuoles of living cells is used as a quantitative indication of cell number and viability.

1. Remove culture medium and add 100 ul Neutral Red (NR) medium. Incubate at 37°C, in a humidified atmosphere of 5.0% CO₂, for three hrs.
2. After incubation, remove the NR medium and wash cells with 150 ul HBSS/PBS.
3. Decant and blot HBSS/PBS totally. (Optionally: centrifuge reversed plate.)
4. Add exactly 150 ul NR Extractant (95% ethanol or ethanol/acetic acid) solution.
5. Shake microtiter plate, either rapidly on a microtiter plate shaker for 10 min. or slowly for up to 2 hrs, on a platform shaker until NR has been extracted from the cells and formed a homogeneous solution.
6. Measure the absorption of the resulting colored solution at 540 nm (without a reference filter) in a microtiter plate reader, using the mean of the outer wells (blanks) as a reference (see Annex D). Immediately print readout or save file in Standard ASCII format.

7.0: QUALITY CHECK OF ASSAY (I): POSITIVE CONTROL

Chlorpromazine (CPZ) tested in a full-scale phototoxicity test on two plates, according to Section 3.6 "Test Procedure", concurrently with the test articles serves as positive reference. Determine NRU in the absence (-UVA) and presence (+UVA) of irradiation (5 J /cm²):

A test meets acceptance criteria if, for CPZ:

- the EC₅₀ +UVA is within the range of: 0.1-2.0 ug/ml
- the EC₅₀ -UVA is within the range of: 7.0-90.0 ug/ml
- the Photo-Irritancy Factor (PIF) between the two EC₅₀ values is at least: 6

8.0: QUALITY CHECK OF ASSAY (II): VEHICLE (NEGATIVE) CONTROL

The optical density (OD₅₄₀) obtained in the vehicle control indicates whether the 1 X 10⁴ (10,000) cells seeded per well have proliferated normally time during the two days of the assay. A test meets acceptance criteria if the mean OD₅₄₀ of vehicle controls is greater than 0.3 AU (0.3 is acceptable, but OECD Test Guideline 432 recommends at least 0.4, if achievable).

To check for systematic errors, Vehicle Controls (VC) are placed at both the left side (row 2) and the right side (row 11) of the center 60 wells of the 96-well plate (see ANNEX D). A test meets acceptance criteria if the mean of the six left (VC_L) wells and the mean of the six right (VC_R) wells vehicle control do not differ by more than 15% from the mean of all twelve VC wells (Global Mean = VC₁₂). Thus, VC must be ≤ 0.15. See formula below:

$$\frac{VC_{12} - VC_R}{VC_{12}} \quad \text{and} \quad \frac{VC_{12} - VC_L}{VC_{12}} \quad \text{must} \leq 0.15$$

9.0: ANALYSIS OF DATA

9.1: To apply the rules for predicting phototoxic potential (see "**Prediction Model**" section, Annex E) it is necessary to analyze the concentration-cytotoxicity response curves concurrently obtained in the presence (+UVA) and absence (-UVA) of irradiation.

9.1.1: Photo-Irritancy Factor (PIF)/Mean Photo Effect (MPE) Prediction Model: The default analysis for the 3T3 NRU Phototoxicity Study will use the PIF model (see Annex E). To apply the PIF, any appropriate procedure can be used to calculate the EC₅₀ (EC₅₀ = the concentration inhibiting all viability by 50%). A compound is a phototoxin if PIF is ≥ 5. See example below:

$$PIF = \frac{EC_{50} (-UV)}{EC_{50} (+UV)} \geq 5$$

If EC₅₀ in the presence or absence of light cannot be calculated, phototoxicity can be determined by the MPE using the 3T3 NRU Phototox Prediction Software developed by ZEBET (see Annex E).

9.1.2: Options to Calculate EC₅₀:

- 9.1.2.1: Apply any appropriate non-linear regression procedure (preferably a Hill function or logarithmic regression) to the concentration-response data. Before using the EC₅₀ for further calculations, the quality of the fit should be appropriately checked.
- 9.1.2.2: Plot viability data between EC₁₆ and EC₈₄ versus the test concentrations and apply a logarithmic regression to allow interpolation of the EC₅₀. An r² or correlation coefficient of >0.90 should be achieved to confirm "quality of fit".
- 9.1.2.3: Graphical Probit Method: Apply a simple graphical fitting method. In this case it is recommended to use 3-cycle log-probit paper with "x=log" and "y=probit" scales as in most cases the concentration response function will become almost linear after this transformation. The quality of the fit should be appropriately checked.

9.1.2.4: Logarithmic Extrapolation Method: $x = \log(\text{concentration})$ is plotted vs. $y = \% \text{ viability}$. Use a logarithmic regression (such as in MS Excel) to obtain an equation that allows interpolation of the EC_{50} . The points chosen for the regression are the TA concentrations yielding values just below and above 50% viability.

10.0 REVISION OF THE PROTOCOL:

Any amendment to or deviation from this protocol will be fully documented in the study file, including the reason for the change, the authority for said change and the date thereof.

11.0 RECORDS TO BE MAINTAINED:

11.1: Collection of Data: All data generated during the conduct of this study will be recorded in ink on worksheets. All entries will be dated, initialed and verified by another person.

11.2: Reports

11.2.1: Draft Report: A draft report will be submitted to the sponsor prior to submission of the final report.

11.2.2: Final Report: The final report will include, but not be limited to:

- Test article information, i.e., manufacturer, source, purity, stability, lot/batch number, etc. when provided to testing facility by the sponsor.
- UV-VIS absorbance spectrum from 220 to 790 nm, to be supplied by the Sponsor.
- Identification and composition of positive and vehicle (negative) control articles.
- Identification and composition of solvent for vehicle and justification for choice of solvent when applicable.
- Description of all test procedures including irradiation (UV source, emission spectra, and filters used, radiometer used, UV-dose (J/cm^2), irradiance of the UV-source (W/cm^2), duration of exposure, distance from UV source to test site)
- Individual and test group data presented in tabular form
- Statistical treatment of the results, when applicable, will be limited to tabulated mean values and standard deviations
- Conclusions

11.3: Retention of Data:

11.3.1: Raw Data will be filed at MB Research by project number.

11.3.2: Final Report will be filed at MB Research by sponsor name and MB project number.

11.3.3: Test Article: Any remaining test article will be returned to the sponsor upon submission of the study report.

11.3.4: Test Article Mixtures: These are not routinely retained. However, upon written request from the sponsor, an aliquot of the test article mixture will be forwarded to the sponsor.

12.0 GOOD LABORATORY PRACTICES:

This study will be conducted in accordance with the Good Laboratory Practices of the EPA, 40 CFR 160 and 792, FDA 21 CFR Part 58, and as specified in The Testing of Chemicals, published by the Organization for Economic Cooperation & Development (OECD), 1997.

- 12.1: Protocol: MB Research will have on file a copy of this protocol, signed and dated by both the responsible MB Study Director and the sponsor's authorized representative.
- 12.2: Quality Assurance: The Quality Assurance Unit will inspect at least one critical phase of this study, audit the raw data and audit the report in accordance with the Standard Operating Procedures of MB and the applicable regulatory requirements.

13.0 SPONSOR REQUEST:

13.1: The sponsor requests that this protocol be implemented:

As written (or) Amended per attached description of amendments

13.2: Will report be submitted to a regulatory agency? No Yes _____(agency)

13.3: Test Article will be identified in the report and supporting documentation exactly as indicated below:

13.3.1: Identity: The test article is identified as follows (use additional copies of this page for multiple TA's):

I.D.: _____

Description: _____

pH (when applicable): _____ Lot/Batch #: _____

13.3.2: UV/visible Absorbance Spectrum supplied to MB? (Refer to section 3.1.1) Yes No

13.3.3: Solvent necessary to prepare at 100X stock concentration (if insoluble in HBSS): _____

13.3.4: Range Finder: Conduct Prescreen from ___ to ___ % Do Not Conduct

13.3.5: Characterization of the test article is required in support of data submissions and should include identity, strength, purity, composition, stability and uniformity. This data must be reviewed by the Study Director prior to study initiation and included in the final report. (EPA 40 CFR 160.105 and 792.105; FDA 21 CFR 58.105, OECD 6.2). This information is:

provided (or) not available

13.3.6: Material Safety Data Sheet Supplied: Yes No

13.3.7: DOT Hazardous Material: No Yes (indicate DOT shipping Name) _____

EPA Hazardous Waste: No Yes (indicate EPA Waste Number) _____

13.3.8: Shipping Instructions for Return of Residual Test Article: (Call or refer to Study Initiation Information for costs)

UPS / Ambient temperature Express carrier / Ambient temperature
 Overnight carrier / Dry Ice Overnight carrier / Ice packs

13.4: Authorization Statement: This protocol is authorized for implementation at MB Research. This study is necessary to estimate the toxic effects of the test compound. To the best of my knowledge and information, this test is not an unnecessary duplication of any previous studies.

13.4.1: Confidentiality: Study results and reports will be released only to the below named sponsor representative unless other sponsor representatives are identified below.

BY: _____
(signature) (date)

(type/print name)

(title)

FOR: _____
(company)

(address)

(city) (st) (zip)

(phone) (fax)

Optional: Other Sponsor representative: _____

14.0 MB RESEARCH ACKNOWLEDGMENT: Request for implementation of this protocol and receipt of the test article is acknowledged by MB Research.

14.1 Test Article Identity: _____

14.1.1: Date Received: _____

14.1.2: Physical Description: _____

14.1.3: Test Article Characterization:

14.1.3.1: Not supplied by Sponsor, or

14.1.3.2: Received and Reviewed by Study Director

14.2: MB Project Number assigned to this study: _____

14.3: Proposed Study Dates:

14.3.1: Experimental Start Date: _____

14.3.2: Experimental Term Date: _____

14.3.3: Study Completion Date (Submission of Report): The target date for report submission is approximately 4 weeks following Experimental Term Date.

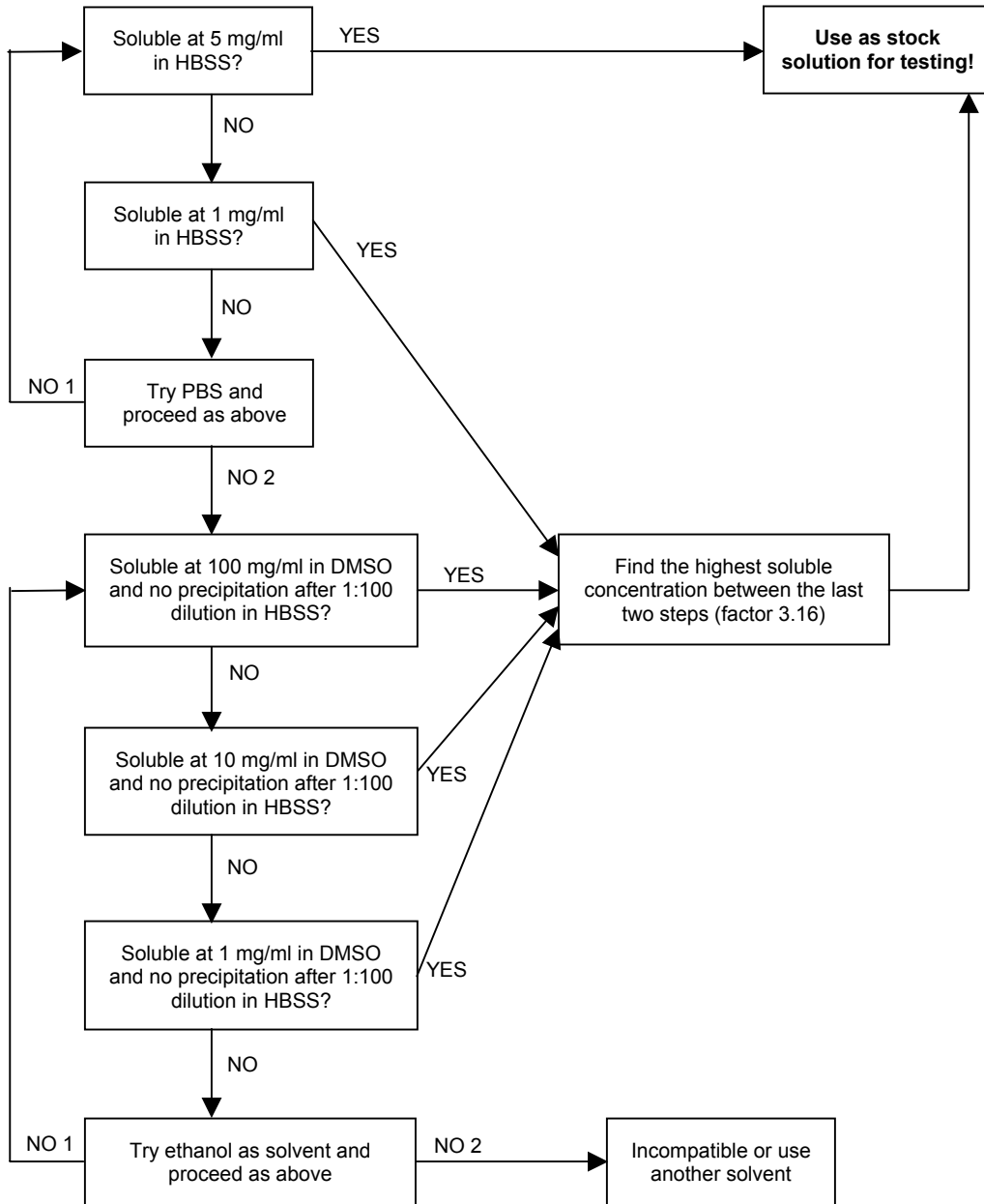
14.4: Approval: This protocol is approved for implementation at MB Research by the below named MB Study Director.

BY: _____ (date)
Study Director
Testing Facility MB Research Laboratories
1765 Wentz Road, P. O. Box 178
Spinnerstown, PA 18968

ANNEX A NRU PT TEST: FLOW CHART

Time (h)	PROCEDURE		
0.00 h	Seed 96-well plates: 1×10^4 cells / 100 ul DMEM culture medium / well <i>Incubate (37°C / 5.0% CO₂ / 24 h)</i>		
24.00 h	Remove culture medium. Wash once with HBSS.		
24.00 h	Treat with 8 conc. Of 100 ul test article solved in HBSS (vehicle zero control = HBSS) <i>Incubate (37°C / 5.0% CO₂ / 1 h)</i>		
25.00 h	<table border="1" style="width: 100%;"> <tr> <td style="background-color: yellow; text-align: center;"> Phototoxicity: Expose to UVA 1.67 mW/cm² for 50 min (= 5 J/cm²) at room temp. </td> <td style="background-color: blue; color: white; text-align: center;"> Cytotoxicity: Keep duplicate plate for 50 min. in the dark at room temperature </td> </tr> </table>	Phototoxicity: Expose to UVA 1.67 mW/cm ² for 50 min (= 5 J/cm ²) at room temp.	Cytotoxicity: Keep duplicate plate for 50 min. in the dark at room temperature
Phototoxicity: Expose to UVA 1.67 mW/cm ² for 50 min (= 5 J/cm ²) at room temp.	Cytotoxicity: Keep duplicate plate for 50 min. in the dark at room temperature		
25.50 h	Remove treatment medium, wash with HBSS. Replace HBSS by culture medium <i>Incubate (37°C / 5.0% CO₂ / overnight)</i>		
48.00 h	Microscopical control of morphological alterations Remove culture medium. Add 100 ul Neutral Red medium. <i>Incubate (37°C / 5.0% CO₂ / 3 h)</i>		
51.00 h	Discard NR Medium Wash once with 150 ul HBSS Add 150 ul fixative (Ethanol/Acetic Acid Solution)		
51.40 h	Shake plate for 10 min.		
51.50 h	Detect NR Absorption at 540 nm (i.e. cell viability)		

ANNEX B STRATEGY FOR THE USE OF SOLVENTS



ANNEX C DECIMAL GEOMETRIC CONCENTRATION SERIES

In general, dose-response relationships are nonlinear, but can be linearized to some extent by logarithmic transformation of the x-axis. Usually this has to be done when EC₅₀ values are calculated either by regression analysis or by graphical estimation. If dose series (in cell culture: concentration series!) are done with arithmetic steps, transformation of the x-axis will result in an unequal distribution of measuring points. Therefore, the use of geometric concentration series (= constant dilution factor) is recommended. The simplest geometric series are dual geometric series, e.g. factor 2. These series have the disadvantage of permanently changing chains within the series (2, 4, 8, 16, 32, 64, 128, 256, etc.). The decimal geometric series, first described by Hackenberg & Bartling (1959)³ for the use in toxicological and pharmacological studies, has the advantage that independent experiments with wide and narrow dose factors can be easily compared, and under certain circumstances can even be merged together:

EXAMPLE:

10						31.6						100
10				21.5				46.4				100
10		14.7		21.5		31.6		46.4		68.1		100
10	12.1	14.7	17.8	21.5	26.1	31.6	38.3	46.4	56.2	68.1	82.5	100

The dose factor of **3.16** ($= \sqrt[2]{10}$) divides a decade into 2 equal chains, the dose factor of **2.15** ($= \sqrt[3]{10}$) divides a decade into 3 equal chains, the dose factor of **1.47** ($= \sqrt[6]{10}$) divides a decade into 6 equal chains, and the dose factor of **1.21** ($= \sqrt[12]{10}$) divides the decade into 12 equal chains.

Therefore, for reasons of an easier biometrical evaluation of the data it is recommended to use decimal geometric concentration series rather than dual geometric series.

The technical production of decimal geometric concentration series is very easy. An example is given for 1.47:

Dilute one volume of highest dose by adding 0.47 volumes of diluent. After equilibration, dilute one volume of this solution by adding 0.47 volumes of diluent...(and so on). Thus, assuming an initial concentration of 1000 µg/ml, and using the dose factor of 1.47 ($= \sqrt[6]{10}$), the resulting concentrations would be 1000, 681, 464, 316, 215, 147, 100 and 68.1 µg/ml.

ANNEX D 96-WELL PLATE CONFIGURATION

Note: The plate configuration shown below is a recommendation. In case the software NRU-PIT2 is used for analyzing the data the configuration has to be exactly as shown below. Alternatively, an MS Excel worksheet template is configured to analyze and graph the data based on the template below.

Note: Since evaporation may take place in the peripheral wells, it is recommended to use these wells for blanks only, which correct possible adsorption of Neutral Red to the plastic.

	1	2	3	4	5	6	7	8	9	10	11	12
A	b	b	b	b	b	b	b	b	b	b	b	b
B	b	VC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC	b
C	b	VC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC	b
D	b	VC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC	b
E	b	VC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC	b
F	b	VC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC	b
G	b	VC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC	b
H	b	b	b	b	b	b	b	b	b	b	b	b

- VC = VEHICLE CONTROL
(mean viability set to 100%)
- C1 - C8 = TEST ARTICLE at eight concentrations
(C1 = lowest, C8 = highest)
- b = BLANKS
(containing no cells, but treated with NR medium and with NR Desorb solution)

ANNEX E PREDICTION MODEL

The mathematical rule for the prediction of *in vivo* toxicity potential from *in vitro* test data is called the Prediction Model (PM, for details see Archer *et al.*, 1997)⁴. At present, test prediction of phototoxic potential is based on comparison of the two concentration-response curves concurrently obtained in the presence (+UVA) and absence (-UVA) of UVA irradiation. This can be achieved either by comparison of two equally effective inhibition concentrations by comparison of the +UVA and -UVA concentration response curves on a grid of concentrations from the concentration range shared by both curves (refined PM, see MEAN PHOTO EFFECT Section).

ORIGINAL VERSION BASED ON THE PHOTO-IRRITANCY FACTOR (PIF)

The PM was developed from interlaboratory data obtained in a EU/COLIPA prevalidation study (Spielmann *et al.*, 1994a,b)⁵ and applied in a formal validation study (Spielmann *et al.*, 1998)⁶. It is based on comparison of two equally effective cytotoxic Test Article concentrations (EC₅₀ values) obtained in concurrently performed experiments in the presence (+UVA) and absence (-UVA) of UVA irradiation, which are used to calculate a photo-irritancy factor (PIF):

Discriminant analysis of the PIFs obtained in the prevalidation study revealed a cut-off value of PIF = 5 for phototoxic potential (e.g. Liebsch *et al.*, 1994)⁷. The resulting classification rule is:

$$(1) \quad \text{PIF} = \frac{\text{EC}_{50} (- \text{UV})}{\text{EC}_{50} (+ \text{UV})}$$

If **PIF < 5**: no phototoxic potential predicted. If **PIF ≥ 5**: phototoxic potential predicted

The PIF can be calculated only if the concentration-response curves obtained in the presence and the absence of UVA-light drop down below 50% of the controls, because only in these cases two EC₅₀ values (-UVA and +UVA) can be determined. Therefore, the prediction model takes into account two additional classification rules:

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

$$(2) \quad > \text{PIF} = \frac{\text{C}_{\text{max}} (- \text{UV})}{\text{EC}_{50} (+ \text{UV})}$$

Since the "> PIF" is not an exact numerical value, no biostatistical procedure could be applied to determine the optimum cut-off. Consequently, the classification rule has to be:

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

If both EC₅₀ (-UVA) and EC₅₀ (+UVA) cannot be calculated because a test article does not show any cytotoxicity up to the highest test concentration, this indicates no phototoxic potential. In this cases a formal "PIF = *1" is used to characterize the result:

$$(3) \quad \text{PIF} = *1 = \frac{\text{C}_{\text{max}} (- \text{UV})}{\text{EC}_{50} (+ \text{UV})}$$

A "PIF = *1" predicts no phototoxic potential

Note: In cases (2) and (3) the absolute concentrations achieved should be taken into consideration.

ANNEX E PREDICTION MODEL (cont'd)**REFINED VERSION OF THE PREDICTION MODEL BASED ON THE MEAN PHOTO EFFECT (MPE)**

A major limitation of the PIF prediction model is the fact that the PIF is based on the comparison of two equally effective concentrations (EC_{50}) in the dark and light experiments, which cannot be determined in every case.

To overcome this limitation, a novel measure for the phototoxic potential of test articles, the mean photo effect (MPE), has recently been proposed (Holzhütter, 1997)⁸. It is based on a comparison of the +UVA and -UVA concentration response curves on a grid of concentrations i ($i=1, \dots, N$) chosen from the common concentration range of the (-UVA) and (+UVA) experiments. The photo effect (PE_{*i*}) at concentration (c_i) is computed as a product of the concentration effect (CE_{*i*}) and the response effect (RE_{*i*}). The mean photo effect (MPE) is obtained by averaging across all PE_{*i*} values.

To apply the refined Mean Photo Effect (MPE) prediction model, special software has to be used. This software uses the algorithms published by Holzhütter (1997)⁸. The software is presently available for MS Windows.

Data files of optical densities (OD_{540}) generated by the microplate reader are printed out and data is recorded and entered into MS Excel or equivalent program (which fits the concentration response curves with the procedure FitGraph, specially developed for the proper handling of non-monotonous curves (Holzhütter & Quedenau 1995)⁹).

The program compares corresponding (+UVA) and (-UVA) couples of experiments, predicts the phototoxic potential (both according to the PIF model and the MPE model) and calculates a toxicity probability which takes into account whether predictions based on results near the classification cut-off are influenced by the intra-assay and inter-assay variability.

Analogous to PIF, the MPE can be used in a prediction model for the phototoxic potential of test articles by comparing it with a critical cut-off value, MPE_c. The cut-off value MPE_c = 0.1 was derived from a first application of the MPE-based prediction model to data obtained in phase II of the EU/COLIPA study in a test carried out by the FRAME/University of Nottingham laboratory according to the same test design, but with primary human keratinocytes instead of 3T3 cells (Holzhütter, 1997)⁸.

MPE	Prediction of <i>in vivo</i> phototoxic potential
< 0.1	Non-phototoxic
≥ 0.1	Phototoxic

Note: In contrast to the PIF, the MPE cannot be easily calculated by everyone without using special software. A special program, "3T3 NRU Phototox, v. 2.0", was obtained from ZEBET¹⁰.)

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