Report Approval

Dr. Fiona Jacobs **Study Director**	15SEP23

Methodology

The EpiDerm Phototoxicity Assay measures acute toxic response after exposure of skin to certain chemicals and subsequent exposure to light. The test is based upon a comparison of the cytotoxicity of a chemical when tested with and without additional exposure to a nontoxic dose of UVA + visible light. The test item was applied to the tissue surface in a 4-concentration dose range, in duplicate. Following a 24-hour incubation time, the dosed tissues were either exposed to UVA + visible light or remain in the dark. The tissues then underwent a gentle rinsing with PBS and an MTT assay was performed, and absorbance measurements taken at 570nm to determine the viability for each condition. A decrease of 30% viability between tissues that have been exposed to UVA + visible light and those that have been kept in the dark highlights a phototoxic effect.

Sponsor Information

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Test Facility Information

Test Facility

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Study Director Information

Study Director Name:

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Key Personnel

Test Facility Management:	Jan Ball
Study Scientist:	Michael Connolly
QA Manager:	Thomas Hand
Archivist:	Jan Ball

Study Dates

Experimental start date:	22AUG23
Experimental end date:	25AUG23
Study completion date:	Date of report signed by SD

Test Item Information

XCellR8 Code	SCB0001
Study Test Item code	TA1
Test Item Name	Mu-conotoxin TIIIA
Supplier	Sponsor
Batch/Lot Number	N/A
Expiry Date	01 December 2024
Physical State	Solid, White crystals, diluted in water. Dosed as a liquid
Storage Conditions	-17 to -25°C
Concentration tested	Prior to study start the test item was diluted to 500 ppm in water then frozen at -17 to -25°C until study start. The test item was diluted 100-fold (5 ppm, (0.0005%)) on the day of testing in water. This was the top concentration, the subsequent three concentrations were incrementally 3.16-fold smaller.
Administration method	50 µl Topical application on the tissue (vehicle water)

Vehicle Control

XCellR8, and Test Code	VC (Treated with Vehicle)
Name	H20 (50µL)

Nature and Purpose of the Study

This study was performed using the EpiDerm™ reconstructed tissue model EPI-200 (MatTek Corporation) based upon the EpiDerm Phototoxicity Protocol from MatTek. The phototoxic potential is determined based upon a reduction of tissue viability of >30% between the tissues exposed to UVA+ visible light and the identically treated tissues that have not been exposed to UVA+ visible light.

Experimental Design – Overview

Methods are adapted from the MatTek protocol MK-24-007-0069. Materials, suppliers, lot/batch numbers and expiry dates are recorded in study data.

Solubility checks and a preliminary test to check for test item interference with MTT were performed in advance of this study, in studies 23SCB01S50 and 23SCB01E50N. The concentrations used were based upon the top concentration set by the sponsor (5 ppm, 0.0005% in water). The four tested concentrations were 0.0005 %, 0.000158 %, 5.01×10^{-5} %, 1.58×10^{-5} % in water.

Upon tissue arrival, the tissues were equilibrated overnight in a humidified incubator set to 37°C, 5% CO_2. The following day, the tissues were dosed with the test items which have been dissolved in water or the VC (H_2O).

Following exposure to test or control items, plates were irradiated with 6 J/cm^2 broad spectrum visible light (290nm-700nm, 60min), while the negative irradiated plate was kept in the dark. After irradiation, the tissue surfaces are washed with a steady stream of PBS (20 washes) and incubated overnight (21 ± 3 hours) in a humidified incubator set to 37°C, 5% CO_2. Tissues viability was quantified by MTT, and optical densities measured at 570 nm without a reference filter.

Data was analysed in a bespoke Excel workbook (provided by Mattek). Data files of optical densities (ODs) generated by the microplate reader are copied from the reader software to the Windows Clipboard and then pasted into the Excel spreadsheet. Cell viability is calculated for each tissue as % of the corresponding vehicle control (water) either irradiated (+UVA) or non-irradiated (-UVA).

Schedule

- Day 1: Tissues received and conditioned overnight
- Day 2: Tissues dosed with 4 concentrations of test item dissolved in either water or sesame oil
- Day 3: Exposure to UVA+ light (or kept concealed from UVA+ light), rinsing. Incubate with MTT
- Day 4: End of MTT, measurements

Test System

Lot number 38763 of EPI-200 EpiDerm™ models were received from the MatTek Corporation and used for this study. The patented test system (EPI-200) is a ready to use 3D reconstructed human epidermis (RHE) model, that consists of normal human derived keratinocytes (NHEK) cultured on a porous membrane attached to a tissue culture insert. The keratinocytes are cultured at air-liquid interface allowing the Epiderm to have a keratinized top layer. The RHE model exhibits metabolically active human epidermal skin tissue structure that consist of a basal cell layer, spinous and a granular layer (8-12 cell layers) and cornified epidermis layers. The RHE model provided a NAM (new alternative method) for *in vitro* testing for dermal toxicologists and formulation scientist. The test system has ECVAM validation and OECD accepted test guidelines.

The lot number 38763 of EPI-200 has passed the quality acceptance criteria.

Test	Acceptance criteria	Result
Tissue viability	Optical Density between 1.0 - 3.0	1.519±0.150
Reproducibility	ET-50 between 4.77 – 8.72	4.86 hrs
Sterility	long term antibiotic and antimycotic free culture	No contamination
Pathogen test	no detection of HIV, hepatitis B, hepatitis C, bacteria, yeast, or other fungi	None detected

Results

Test items preparation and test concentrations

All test item solutions were prepared in water. The test item was tested at 0.0005 %, 0.000158 %, 5.01×10^{-5} %, 1.58×10^{-5} %.

Test Items

Study Acceptance criteria

- The absolute OD of the VC tissues in the MTT test: ≥0.8 ≤2.8
- The difference in viability between tissue duplicates that are treated identically must be ≤ 30%.

Criterion	Outcome
Is the OD of VC ≥0.8.	-UVA: 1.702, +UVA: 1.520. PASS
Does the tissue viability differ by >30% between two identically treated tissues?	No, biggest difference: 6.876. PASS

Prediction Model

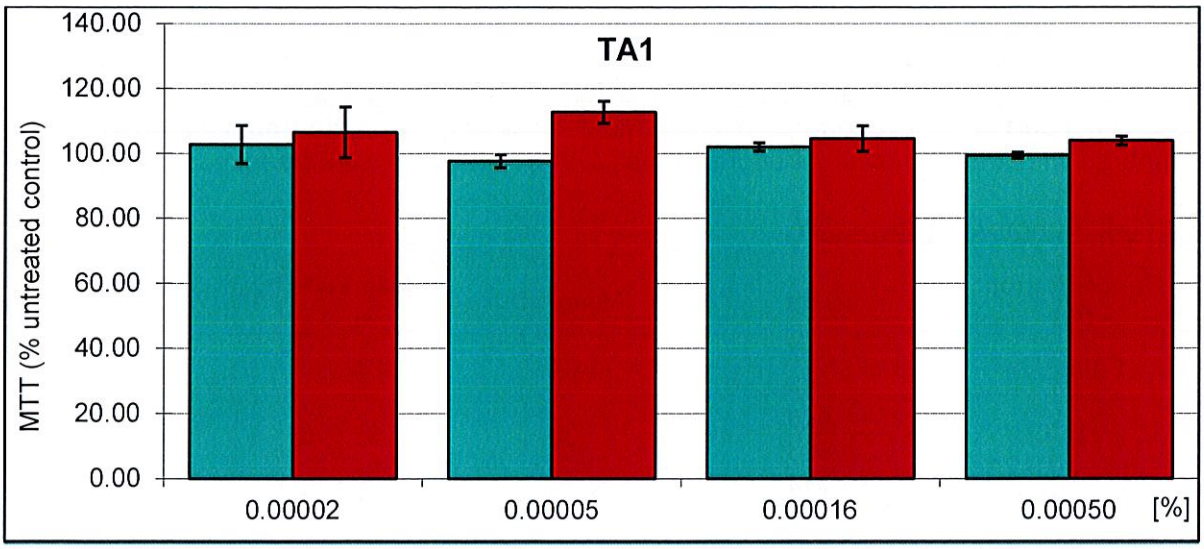
A test item is predicted to have a phototoxic potential if one or more test concentrations of the (+UVA) part of the experiment reveal a decrease in viability **exceeding 30%** when compared with identical concentrations of the (-UVA) part of the experiment.

TA1: (Mu-conotoxin TIIIA) - UVA

Concentration (%)	Mean	Mean OD	Mean Viability (%)	Difference tissue
$1.58x10^{-5}$	1.647	1.748	102.703	11.813
	1.848			
$5.01x10^{-5}$	1.626	1.661	97.605	4.085
	1.696			
0.000158	1.714	1.735	101.969	2.527
	1.757			
0.0005	1.707	1.691	99.383	1.881
	1.675			
VC	1.638	1.702	100.000	7.523
	1.766			

TA1: (Mu-conotoxin TIIIA) + UVA

Concentration (%)	Mean	Mean OD	Mean Viability (%)	Difference tissue
$1.58x10^{-5}$	1.500	1.619	106.498	15.66
	1.738			
$5.01x10^{-5}$	1.765	1.713	112.732	6.810
	1.662			
0.000158	1.529	1.589	104.540	7.929
	1.649			
0.0005	1.558	1.579	103.882	2.797
	1.600			
VC	1.552	1.520	100.000	4.244
	1.488			



The graph demonstrates phototoxicity potential of TA1 **(Mu-conotoxin TIIIA)** with and without UV dose of 6J/cm^2. No viability difference was noticed between +UVA and -UVA EpiDerm^{TM} (EPI-200) tissues treated identically with TA1 (green = -UVA, pink = +UVA) (Error bar = ± (Difference tissue/2)), therefore no phototoxicity potential was detected at these concentrations.

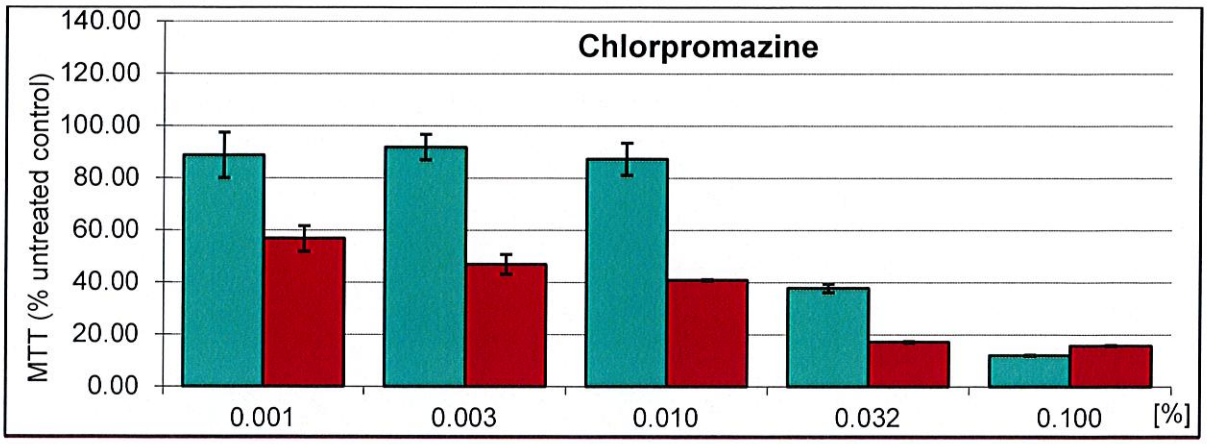
Conclusions/observations

The analysis of data revealed that exposure of the tissues to UVA+ visible light did not reduce the viability below 30% of the tissues exposed to the same concentration of test item that was not exposed to UVA+ visible light for the test item. Therefore, Mu-conotoxin TIIIA was not considered to have phototoxic potential.

Control compound

For the present study, it is not necessary to include a positive control into each phototoxicity test as this reduces the number of concentrations of the test chemical that can be assessed (*Phototoxicity Protocol for use with EpiDerm^{TM} Model (EPI-200) - ZEBET*). A full experiment with five concentrations of Chlorpromazine (dissolved in H_2O) ranging from 0.001% up to 0.1% was performed upon setting up the assay. A dose dependent reduction of cell viability occurred only in the UVA-irradiated tissues was observed between 0.001% and 0.01% confirming the proficiency of the test lab.

The graph below shows the percentage viability values for tissues dosed with the positive control item (chlorpromazine) at five different concentration and exposed to UVA+ visible light or kept in the dark. Percentage viability values were determined by comparison to the respective vehicle control (100%):



The graph demonstrates phototoxicity effect of chlorpromazine with and without UV dose of 6J/cm^2. The viability difference (between non-UV and UV irradiance) of identically treated EpiDerm™ (EPI-200) with chlorpromazine exceeds more than 30% at concentrations of 0.001%, 0.003%, and 0.010% w/v (in water). Thus, predicting it as a phototoxic compound (green = -UVA, pink = +UVA) (Error bar = ± (Difference tissue/2)).

Record Retention

All study records will be retained for a minimum of 5 years in the XCellR8 archive from issue of the final report. At the end of this period the Sponsor will be contacted to determine whether the records should be returned, retained or destroyed on their behalf and notified of the financial implication of each of these options.

One copy of the study plan and final report will be held indefinitely by XCellR8.

Records to be retained:

Original (ink-signed) study plan and any amendments, test and reference item formulation records (where relevant), manually recorded preliminary and main test data, authenticated hard copy output from the spectrophotometer, authenticated hard copy of data analysis results, original (ink-signed) copies of the final report and any subsequent report amendments(s) and re-issue(s).