Prevalidation of Epi2SensA, an assay using gene expression with the EpiDerm RhE model to predict skin sensitization in vitro

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Abstract ID # 928 /
Poster #P06-22

Abstract

The Epi²SensA is a similar method to the Epidermal Sensitization Assay (EpiSensA) in the 2024 version of the "OECD TG 492: In Vitro Skin Sensitisation" addressing the Adverse Outcome Pathway Key Event on Keratinocyte activation. Epi²SensA uses the EpiDerm™ reconstructed human epidermis (RhE) model from MatTek as the experimental system instead of the LabCyte RhE model in the validated reference method (VRM), EpiSensA. Thanks to the air liquid interface and metabolic activity of RhE models, the method showed better performances for pre/pro-haptens as well as for lipophilic substances compared to already 2D validated methods. The EpiDerm™ model is already validated in several OECD test guidelines (TG431, TG439, TG498) and is available in the majority of OECD member countries which is an important parameter for the implementation of this OECD test guideline. The Epi²SensA, like the EpiSensA, is based on gene expression quantification of four biomarkers related to the induction of skin sensitization: activating transcription factor 3 (ATF3) and interleukin 8 (IL-8) which reflect the inflammatory response of keratinocytes; glutamate-cysteine ligase, modifier subunit (GCLM) and DnaJ (Hsp40) homolog, subfamily B, member 4 (DNAJB4) which reflect the induction of cytoprotective gene pathways. The prediction model of the assays is based on the modulation of the expression of the four target genes quantified by quantitative real-time PCR analysis after topical exposure of test chemicals. The chemical is classified as skin sensitizer if the fold induction of the expression of at least one of the genes exceeded the respective cut-off value: 15-fold for ATF3, 2-fold for GCLM or DNAJB4, and 4-fold for IL-8.

The VRM protocol was optimized for the EpiDerm™ model with a set of chemicals including positive controls (Clotrimazole, 4-NBB), skin sensitizers (eugenol, 2-aminophenol, imidazolidinyl urea, methyl methacrylate) and non skin sensitizer chemicals (benzyl butyl phthalate, salicylic acid, and sodium dodecyl sulphate). The predictivity of the Epi²SensA has been further verified with the 20 chemicals of the performance standard. The me-too validation of the Epi2SensA is underway with 3 naïve laboratories, Burleson Research Technologies (BRT) in the US, Eurofins BioPharma Product Testing in Germany and the Food and Drug Safety Center Hatano Research Institute (FDSC) in Japan. Project 4.172 of the OECD work plan for the Me-Too validation of the EpiDerm™ model for the EpiSensA method was submitted by France and Japan.

Materials & Methods

The EpiDerm™ RhE model is reconstructed from normal, human-derived epidermal keratinocytes (NHEK) in a chemically defined medium. The NHEK are cultured on a Polytetrafluoroethylene (PTFE) membrane to form a multilayered highly differentiated model of the human epidermis.

Epi²SensA: Triplicate EpiDerm™ tissues are exposed to different concentrations of a test chemical. The volume of chemical applied was modified from 5 µL in the VRM protocol to 10 µL with the Epi²SensA to account for the increased surface area of EpiDerm™ which is twice that of Labcyte (Fig.1 and Table 2). After 1 hour, the surface of the RhE are rinsed and followed by a 5 hours post-incubation. Then, the gene expression of four markers of the keratinocyte response to the early phases of skin sensitization (induction of cytoprotective gene pathways and inflammatory responses) are evaluated by RT-PCR (Table 1). The fold induction of genes of interest in the treated tissues are calculated by comparing the relative gene expression with that in vehicle treated tissues. When the mean fold induction of at least one out of the four marker genes exceeds the respective cut-off value (ATF3: 15-fold; GCLM: 2-fold; DNAJB4: 2-fold; IL-8: 4-fold), the chemical is judged as a positive skin sensitizer.

Cell viability: The **LDH** (Lactate Dehydrogenase) cell viability assay is based on the measurement of LDH released from cells. LDH is a stable cytoplasmic enzyme which is rapidly released into the tissue culture medium upon damage to the plasma membrane. The quantity of LDH released by 100% killed control tissues is used to calculate cell viability of the tissues exposed to test samples. Killed controls are exposed to 10 µL of 10%Triton-X applied topically in the VRM and to 50 µL of 10%Triton-X added to the basolateral medium in the Epi²SensA . The **MTT** assay measures the colorimetric change of the MTT dye to an insoluble formazan product by mitochondrial enzymes of living cells. Cell viability is calculated by comparing the amount of formazan in the tissues exposed to test samples referenced to a control tissue treated with the vehicle only.

	volume	Surface of the RHE	Ratio volume /surface
EpiDerm™	10 µL	0.63 cm	15.9 µL/cm²
LabCyte	5 µL	0. 32 cm2	15.6 µL/cm²

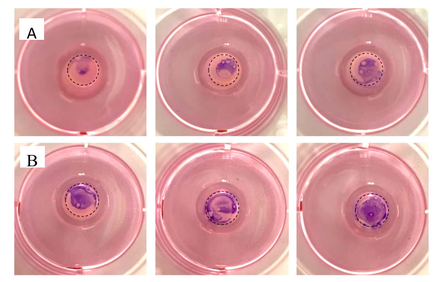

Fig.1: **Co**mparison between the two RHE models and impact of the volume exposure used. Visualization by addition of crystal violet dye in acetone/olive oil solvent (4:1) of the distribution on the RHE surface of a 5µL (A) or 10µL (B) application.

Results

The volume of test chemical applied was increased from 5uL to 10uL to take account of the surface area of the EpiDerm™ model (Fig.1). Using the 6h exposure length as proposed in the VRM, we experienced cytotoxicity limitation with some weak sensitizers such as eugenol, imidazolidinyl urea, and methyl methacrylate which require a higher concentration to induce a positive response in the EpiDerm™ model (Table 1). As a first step, we decided to reduce the duration of exposure from 6h in the VRM to 1h exposure stopped by rinsing and followed by a 5h post-incubation. In a second step, the LDH viability measurement protocol was optimized by conducting parallel cell viability measurements with LDH and MTT methods. To induce a maximum cell death in the Epi²SensA, killed control are produced by basolateral exposure to 10% Triton X-100 rather than topical exposure in the VRM (Fig.2). Thanks to these optimization, it was possible to identify weak skin sensitizers by testing higher concentrations while still meeting the acceptance criteria of 80% cell viability (Table 1). The predictivity of the Epi²SensA has been further verified with 18 chemicals of the performance standard that will be used in the Me-Too validation (Table 2).

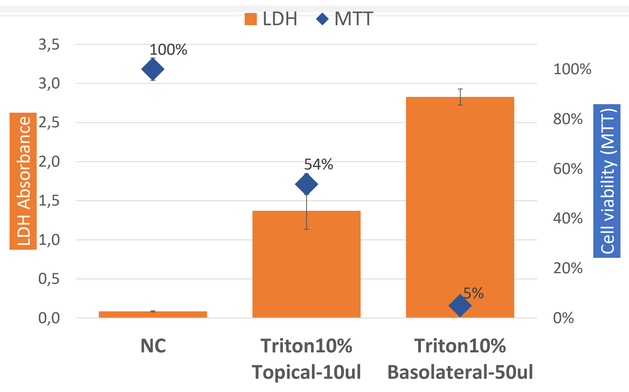

Fig.2: Comparison of amount of LDH measured in the medium with the cell viability measured by the MTT method. Only the basolateral exposure kills the EpiDerm® tissues. By comparison, with the topical exposure to 10% triton a residual cell viability of 54% was measured by MTT method.

Chemical name	CAS	Solvent	Potency (Ecetoc)	VRM SOP (6h)	Epi²SensA SOP (1+5h)
4NBB	100-11-8	AOO	Extreme	**S**	**S**
Clotrimazol	23593-75-1	AOO	Moderate	**S**	**S**
2-Aminophenol	95-55-6	AOO	Strong	**nt**	**S**
Eugenol	97-53-0	AOO	Weak	**NS**	**S**
Imidazolidinyl urea	39236-46-9	DW	Weak	**NS**	**S**
Methyl methacrylate	80-62-6	AOO	Weak	**NS**	**S**
Benzyl butyl phthalate	85-68-7	AOO	Negative	**NS**	**NS**
Salicylic Acid	69-72-7	AOO	Negative	***nt***	**NS**
SDS	151-21-3	DW	Negative	***nt***	**NS**

Table 1: Comparison between the VRM protocol and the optimized protocol for the EpiDerm™ model. The optimized protocol correctly classifies the 3 weak skin sensitizers that were not detected with the VRM protocol. **nt**: not tested, **NS** non sensitizer, **S**: sensitizer

Chemical name	CAS		Log Kow	Pre/pro	Solvent	GHS	Epi²SensA
4NBB	100-11-8		2.7		AOO	1A	**S**
Clotrimazole	23593-75-1		6.26		AOO	1A	**S**
DNCB	97-00-7	solid	2.27		AOO	1A	**S**
Tetrachlorosalicylanilide	1154-59-2	solid	5.87		AOO	1A	**S**
p-Phenylenediamine	106-50-3	solid	-0,39	X	AOO	1A	**S**
Lauryl Gallate	1166-52-5	solid	6.21	X	AOO	1A	**NS**
Metol	55-55-0	liquid	2.34		EtOH50%	1A	**S**
Isoeugenol	97-54-1	liquid	3.04	X	AOO	1A	**S**
Methyl heptine carbonate	111-12-6	liquid	3		AOO	1A	S
Glyoxal 40% solution in water	107-22-2	liquid	-1,66		DW	1A	S
Farnesol	4602-84-0	liquid	5.77		AOO	1B	**S**
Dibutyl aniline	613-29-6	liquid	5.12	X	AOO	1B	**NS**
Abiectic acid	514-10-3	solid	6.46	X	AOO	1B	**S**
α-Amyl cinnamaldehyde	122-40-7	liquid	4.35		AOO	1B	**S**
Imidazolidinyl urea	39236-46-9	solid	-0.86		DW	1B	**S**
Benzyl butyl phthalate	85-68-7	liquid	4.84		AOO	NS	**NS**
Diethyl phthalate	84-66-2	liquid	2,65		AOO	NS	**NS**
Lactic acid	50-21-5	Liquid	-0,65		DW	NS	**NS**

Table 2: The Epi2SensA protocol was used to assess the 20 reference substances that will be used in the Me-Too validation to determine the accuracy and reliability of the method. With this set of chemicals, the calculated sensitivity and predictivity of the Epi²SensA method were XX and XX% respectively.

Summary

- Pre-validation studies of the Epi²SensA method enabled the VRM protocol to be optimized for the EpiDerm™ model.
- The optimized protocol allowed correct classification of some weak sensitizers which previously were classified as false negatives in the VRM protocol.
- The transferability of the method was verified with a naive laboratory, in Italy (VitroScreen).
- Following this pre-validation, the Me-Too validation of the Epi2SensA method has been initiated with three laboratories on the American, European and Asian continents.