Application of Induced Pluripotent Stem Cell-Derived Keratinocytes for Toxicology Endpoints: Assay Development

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Introduction
The Seventh Amendment to the Cosmetics Directive (76/768/EEC) has driven in vitro toxicology investment and development to a point where there are now many in vitro assays that can be used for toxicology endpoints. Regulatory necessity further pushes demand and through international testing agencies as well as private industry, new technologies and assays are continually being developed.

The AOP-based Toxicology Outcomes Pathway (AOP) paradigm created a framework that highlighted the specific key biological events that lead to an in vivo outcome making it possible to replace an in vivo assay with a battery of in vitro tests instead. Reengineering somatic cells to become induced pluripotent stem cells (iPSCs) opened the door to the prospect of having human cells of any lineage for therapy, drug discovery and efficacy testing.

Capitalising on successes from previous international assay development projects, six projects from the 7th European RTD Innovation Programme came together under SCER&Tox. The goal was moving towards replacement of dose dependent toxicity testing in vivo by expanding knowledge and technologies in stem cells, organotypic cell systems, epigenetics, biomarkers, in vitro to in vivo extrapolation and in silico methods. The SCER consortium (FP7 – EU grant number 266753 & part of SEURAT-1) developed technical and biological resources to investigate in vivo toxicology endpoints using human induced pluripotent stem cell (iPSC) based in-vitro assays. A subsequent range of concentrations that exclude highly cytotoxic concentrations of trans-Cinnamaldehyde used as positive control. Initial concentration range tested was 0.05-1.6 µM (Figure 4).

Methods

Cells
3. 3D viability and morphology was performed to determine appropriate assay parameters starting with seeding density and cell type.

Flow Cytometry
1. Cell culture and viability was monitored over 72 hours post-exposure using 0.5% Trypan Blue

Experimental Design
1. Per experiment, 1 well of cells was cultured and counted to approx. 80% confluence in cells counted in the appropriate plexiglass plates. Each plate was pre-seeded to the appropriate plexiglass plates.

Conditions Optimised
1. Cell seeding density
2. Exposure time (48 hours versus 72 hours)
3. Cytotoxicity method
4. Positive Control concentration (trans-Cinnamaldehyde [SigmaAldrich])

Results

Conformation of Keratinocyte Phenotype
1. Prior to use, cells were checked for Pan-CK keratinocyte markers, lack of stem cell markers (SSEA-3 & TRA-1-60) and lack of a foreskin marker as characterisation of the cell line (Figure 2).

Optimisation of Seeding Density
1. Cells initially seeded at concentrations of 10,000 to 59,000 per well (n=2) and cultured for 72 hours to investigate maximum time cells could be cultured for under each condition (Figure 3, top chart)
2. Optimal seeding density of 30,000 cells/well initially selected by confocal on Day 6
3. 30,000 cells/well selected as no improvement in cytotoxicity measure read-out of higher seeding density used (Figure 3, bottom chart)

Positive Control
1. Trans-Cinnamaldehyde used as positive control. Initial concentration range tested was 0.05-1.6 µM
2. A subsequent range of concentrations that exclude highly cytotoxic effects (IC50) was then selected, 0.1-1.6 µM

Use of iPS Cells for In vitro Skin Sensitisation Assay
To evaluate the potential of an iPS-derived keratinocyte cell based assay for the assessment of skin sensitisation in vitro, cells were exposed to the three sensitisers: SA, EGD and 2,4-DNCB. Analysis was by cytotoxicity and qPCR.
1. As expected, cytotoxicity was not observed (data not shown)
2. qPCR results for NQO1 and HMOX1 showed no effect with SA, a dose response with 2,4-DNCB and an inverted dose response with EGD (Figure 6)
3. Cytotoxicity and qPCR data were comparable to published data using the KeratinoSens™ (Givaudan Scherina AG, Switzerland) assay.
4. Increases in pro-inflammatory cytokines IL-6 not detected (data not shown)
5. Increases in anti-inflammatory cytokines IL-10 and IL-18 not detected (data not shown)
6. No clear increases in HMOX1 protein observed for any chemical tested

Conclusions
30,000 iPS cells were seeded for 72 hours exposure to trans-Cinnamaldehyde in the KeratinoSens™ (Givaudan Scherina AG, Switzerland) assay. The KeratinoSens™ was used as an in vitro model as part of the integrated testing strategy (ITS) for skin sensitisation prediction.

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References

Figure 1. Key events in the skin sensitisation AOP from chemical structure to population response.

Figure 2. Flow cytometric characterisation of iPSC-derived keratinocytes. Left panel: Double positive culture using Alamar Blue as vital dye. Right panel: Single positive culture for PS (negative for PS markers (APC)) show presence of Pan-CK keratinocyte markers and lack of PS markers on cells confirming keratinocyte lineage.

Figure 3. Column graphs showing MTT assay results for tested seeding densities of 10k to 59k showing no significant differences. Top chart shows results for each of the 10k to 59k. Bottom chart shows results from 30k to 59k. Cytotoxicity pattern was the same for each whether 48 hour or 72 hour exposure optimal for skin sensitisation assessment (by qPCR)

Figure 4. AlamarBlue® assay readout is Optical Density (OD) at 570 nm. Error bars show standard deviation from mean (30 wells used for each).

Figure 5. qPCR data for genes downstream of NRF2 following exposure to trans-Cinnamaldehyde. Top panel: Data following 48 hour exposure. Only NQO1 has increased over the Untreated Control (UTC). Bottom panel: Data following 72 hour exposure. Increases in both NQO1 and HMOX1 observed.