Optimization of an *in vitro* pre-clinical screening tool for evaluation of anti-inflammatory activity of raw ingredients and finished products

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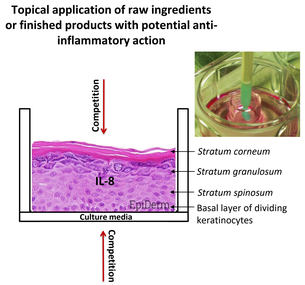
Abstract

In vitro testing methods based on three-dimensional (3D) reconstructed skin equivalents are reliable, rapid tools frequently used to screen actives and formulations for efficacy evaluation, including potential anti-inflammatory activity. Presented here are data generated using an established *in vitro* assay based on the EpiDerm™ Human Cell Construct (MatTek Corporation, Ashland, MA, USA) that was optimized to reduce the time of inflammatory cascade induction that may be more appropriate for the screening of certain actives or product lines. In the standard assay, the EpiDerm™ tissues are exposed topically for 6 hours to materials intended to counteract the inflammation induced by phorbol-12-myristate 13-acetate (PMA) added to the culture media. The tissue viability and interleukin (IL)-8 secretion in collected culture media are the assay endpoints evaluated at the completion of the exposure time. To accommodate the evaluation of products that need to be tested for short exposure times to the tissues depending on consumer uses or other considerations, the assay was optimized in two phases as further described. In the first phase, the kinetics of inflammatory events was investigated to determine if a shorter exposure of the tissues to PMA would induce significant expression of the IL-8 to allow reliable data interpretations. In these experiments, untreated EpiDerm™ tissues were first pre-incubated overnight in media without PMA; then 6 sets of duplicate tissues were transferred in media containing PMA for exposure times varying in hourly increments from 1 to 6 hours followed by 5 to 0 hours post-exposure to media without PMA, respectively. The culture media was collected at the completion of the exposure to PMA and then again every hour until the end of the post-exposure in media without PMA, for all groups; this allowed for the evaluation of IL-8 signal depletion in the absence of the induction agent. The results showed a time-dependent response of IL-8 expression by the tissues exposed to PMA and a time-dependent reduction of the cytokine signal in the post-exposure period. Of the treatment groups included in this phase, the one incubated in PMA-containing media for 3 hours followed by a 3 hours post-exposure without PMA showed a significant induction of the IL-8 to allow for reliable data analysis and it was selected for the second, confirmatory phase of the experiments. In this second phase, the untreated tissues were considered to be the assay negative control, while a commercially available Class 2 (high potency) anti-inflammatory cream was used as the assay positive control. These experiments confirmed the performance of the optimized assay based on the controls data. Our data demonstrate that a shorter 3 hours exposure to PMA induces a significant production of IL-8 as marker of inflammatory state to allow for data interpretation and ranking of products regarding their anti-inflammatory potential. This optimized approach not only shortens the assay length with similar results to the standard one but also accommodates testing of products that require a customized testing strategy that may be based on shorter exposure times to the inflammatory agent and/or prototypes investigated.

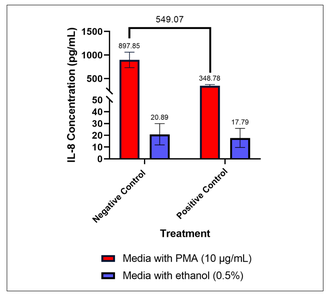
Materials & Methods



		Standard Protocol	Optimized Protocol
Pre-Incubation		At least 16 hours prior to treatment addition in Hydrocortisone-free culture media	
Tissue Number		6/ treatment group	2/treatment group
Controls	**Negative**	0.9% Saline	Untreated tissues
	Positive	Betamethasone Dipropionate Cream USP (augmented), 0.05%	
Dosing/ Incubation Conditions		Concurrent topical dosing of controls (100 µL) and incubation in culture media containing: 10 µg/mL PMA (3 tissues) and 0.5% ethanol (3 tissues), respectively.	Incubation in media containing 10 µg/mL PMA, then concurrent topical exposure to controls (100 µL) and incubation in media without PMA for set amount of time. (2 tissues per group). No ethanol control group tested.
Exposure time		6 hours	3-6 hours
Media Collection/ Treatment Group		Media collected after 6 hours	Group Identification: G3-3h, G3-4h, G3-5h, G3-6h; Hours 1–2: Incubation in media containing PMA; Hours 3–4: Concurrent topical exposure to controls and incubation in media; Hours 5–6: without PMA; Media Collection
Rinsing/MTT Reduction		Tissues were rinsed with CMF-DPBS* using a cut-tip wash bottle. Sterile, dry cotton swabs were used to remove residues of the positive control. The tissues were then placed into MTT for 3 hours. ** RT (room temperature) for the standard protocol and warmed at 37±1°C for the optimized protocol, respectively.*	
MTT Extraction		Isopropanol extraction of MTT for 2-3 hours with shaking at room temperature.	
MTT Endpoint		Spectrophotometric quantification using a 96-well plate-reader (OD_{570} nm)	
Cytokine Endpoint		The media samples collected were analyzed using Quantikine® ELISA Human IL-8 Immunoassay kit (values expressed as pg/mL).	

Standard Protocol

Fig. 1. Typical IL-8 results for negative and positive controls obtained using the standard protocol.



Question 1: Can this protocol be used to address action of products tested sequentially rather than concurrently (competitively) with the inflammation inducer (PMA)?

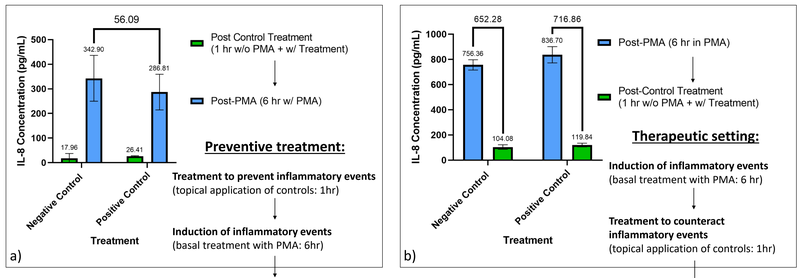
a) Preventive treatment: treatment with products of interest followed by assessment of their action to prevent inflammatory events (induced by PMA)

a) Therapeutic setting: induction of inflammatory events (by PMA) followed by treatment with products of interest

Optimized Protocol

Optimization – Phase I

Fig. 2. Evaluation of the standard protocol for sequential testing of controls and inflammation inducer (PMA) in a preventive treatment or therapeutic testing setting.



Answer 1: The therapeutic setting was further investigated to address the most common use of finished products with potential anti-inflammatory activity by consumers.

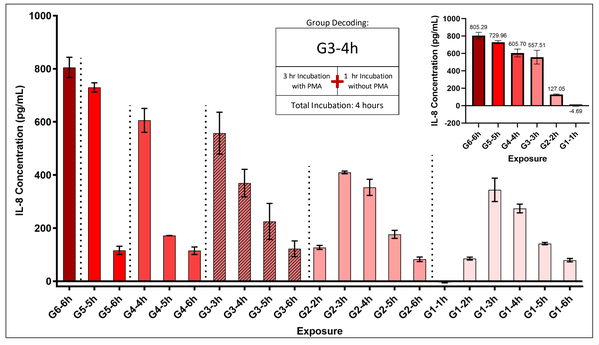
MTT Viability Results

Positive Control	74.1%-119.2%
Negative Control/ Untreated Tissues	100%

Question 2: What is the kinetics profile of IL-8 production after the incubation in the presence of PMA?

Optimization – Phase II

Fig. 3. IL-8 expression by EpiDerm™ tissues following PMA induction in a time-dependent manner within each exposure time group (1hr to 6hr). *Inset*: time-dependent increase in IL-8 production following exposure to PMA in hourly increments.

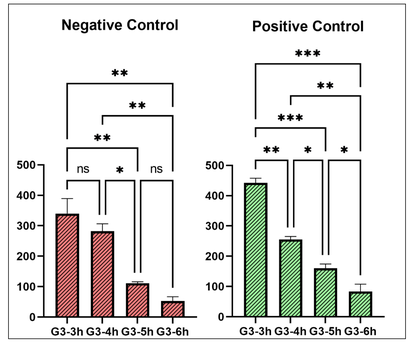


Question 3: Which exposure group provides the most reasonable time-dependent results for IL-8 production?

Answers 2-3: Group 3 (G3) showed a significant induction of IL-8 after 3 hours exposure to PMA and progressive, hourly time-dependent reduction of the cytokine expression in the post-exposure period.

Results

Fig. 4. Confirmation of the optimized protocol based on Group 3 results (**Fig. 3**) by using the assay controls (IL-8 results). Data analysis: one-way ANOVA Tukey multiple comparison approach.



Conclusions & Future Directions

- Reconstructed skin models (EpiDerm™) exposed topically to ingredients/formulations can be used to evaluate their anti-inflammatory activity based on the production of inflammatory cytokine IL-8 by while concurrently exposed basally to the inflammatory agent PMA (**Fig. 1** – standard protocol). IL-8 was selected based on its expression by the reconstructed tissues and its pro-inflammatory chemokine profile.
- Our preliminary results indicated that this testing method can be used in a therapeutic setting intended to assess the anti-inflammatory action of ingredients/formulations on the tissues similar to the most common use by consumers (**Fig. 2**).
- Based on intended consumer use, some products may be better evaluated using shorter exposure times to PMA. To address this need, we optimized the standard protocol by first conducting a kinetics experiment which confirmed that untreated tissues express IL-8 in response to PMA in a time-dependent manner (**Fig. 3 inset**). They also indicated that a 3 hr exposure of the tissues to PMA successfully induced the expression of IL-8 to levels that should allow for reliable subsequent data interpretations when the method would be used to evaluate prototypes.
- To confirm the preliminary optimization results, experiments using the positive control were conducted. They indicated that the positive control efficiently reduced the expression of IL-8 at all time points following the induction of inflammation for 3 hr (**Fig. 4**).
- The optimized protocol accommodates testing of products that require a customized testing strategy based on shorter exposure times to the inflammatory agent and the prototypes evaluated. Our next step will be to evaluate the optimized protocol by using products with available anti-inflammatory data to allow for paired clinical-*in vitro* data analysis.

References

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