OPTIMIZED *IN VITRO* PIGMENTATION SCREENING ASSAY USING A RECONSTRUCTED THREE DIMENSIONAL HUMAN SKIN MODEL

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Abnormal pigmentation is related to a variety of cosmetic and clinical conditions including melasma, lentigo, age spots, vitiligo, etc. Numerous actives designed to modulate skin tone have been utilized as cosmetics and pharmaceuticals to address these conditions. We report on an optimized short-term (1-week) protocol using the Asian MelanoDerm™ model (MatTek Corporation) and designed to rapidly evaluate candidate actives that modulate melanin production in human skin. Initial assay development activities identified the shortest in-life assay phase sufficiently sensitive to identify melanogenesis modulators. Based upon the initial results, an optimized protocol was subsequently evaluated for reproducibility in four independent trials.

Key words: melanin, pigmentation, *in vitro* assay, three dimensional human skin model.

INTRODUCTION

Abnormal pigmentation is related to a variety of cosmetic and clinical conditions including melasma, lentigo, age spots, inflammatory or trauma-induced hyperpigmentation, vitiligo, etc. (1, 2). The striking contrast in skin tones created by pigmentary disorders and conditions has a significant psychological and social impact on affected individuals. Thus, the goal of cosmetic or clinical treatment is to balance the skin tone without causing undesirable pigmentation changes or skin irritation to surrounding normally-pigmented skin. A large number of actives designed to cause darkening or lightening of the skin have been developed and utilized in cosmetics and pharmaceuticals for external use to address these conditions (3, 4). These products are also able to cosmetically alter one's natural skin color, or to provide natural protection from ultraviolet (UV) irradiation (by increasing the melanin content of the skin). Due to the ever-increasing need for

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novel, safe and efficacious melanogenesis modulators, the manufacturers of such classes of products are actively and continuously looking for alternatives to expensive and time consuming clinical and whole animal testing approaches.

To aid in the development and testing of these products, several commercially-available reconstructed human skin models complete with melanocytes are available and their use for screening ingredients targeting modulation of melanogenesis is growing (5-7). They allow for investigation of melanogenic pathways modulated by compounds topically applied to the skin, or via the systemic exposure route; the latter is evaluated by dosing actives in the culture medium under the tissue constructs. *In vitro* studies using three-dimensional (3-D) skin models containing melanocytes can be designed to investigate the mechanisms of action of various melanogenic factors that influence skin pigmentation, to discover new modulators of melanogenesis and assess their safety and efficacy, or to evaluate the photoprotective properties of melanin.

Here we report on an optimized short-term (1-week) protocol using the MelanoDerm™ model produced by MatTek Corporation (Ashland, MA, USA) and designed to rapidly evaluate candidate actives to address the needs of a fast paced product release-oriented cosmetics industry. To address the specific needs for screening and evaluating skin lightening products by the Asian cosmetic market, we selected the Asian MelanoDerm™ model. Initial assay development activities were targeted towards selecting the ideal culture medium, tissue culture maintenance and handling methods, and identifying the shortest in-life assay phase sufficiently sensitive to identify ingredients with melanogenesis modulating activity. Based upon the results of the initial developmental activities, an optimized protocol was proposed and subsequently evaluated for reproducibility in four independent trials.

MATERIALS AND METHODS

MELANODERM™ – THE RECONSTRUCTED PIGMENTED HUMAN SKIN EPIDERMIS MODEL

The Asian MelanoDerm™ (MEL-300-A) model used in this study was obtained from MatTek Corporation. The MelanoDerm™ tissues are viable reconstituted 3-D human skin equivalents consisting of normal, human epidermal keratinocytes (NHEK) and melanocytes (NHM) derived from neonatal foreskin tissue of Asian donors. According to the manufacturer, the cells used to obtain the 3-D model are co-cultured on a collagen-coated membrane to form a multilayered, highly differentiated model of the human epidermis. The apical surfaces of the reconstructed tissues are exposed to air whereas the bottom surfaces remain in contact with the culture medium. The NHM localized in the basal cell layer of

MelanoDerm™ tissues are dendritic and spontaneously produce melanin granules which progressively populate the layers of the tissue leading to varying levels of pigmentation. Although the normal NHM:NHEK ratio within native human epidermis is 1:30 (8), the use of a 1:10 ratio in the MelanoDerm™ models amplifies differences between tissues exposed to various chemicals or final formulations and untreated or control(s)-treated tissues, thus allowing for shorter duration experiments.

The MelanoDerm™ kit contains 24 units of 9-mm diameter tissues shipped embedded in agarose. The manufacturer's histology analysis shows that the reconstructed tissues are comprised of 8 to 12 cellular layers (with representative basal, spinous, and granular cell layers) and a *stratum corneum* of 10 to 15 layers. This stratified feature is ideal for testing finished formulations (creams, lotions, etc.) since one can apply them topically onto the tissue surface without dilution, thus avoiding solubility issues typically associated with aqueous dilution-based assays. Also, these reconstructed tissues allow for modeling the penetration of actives through the *stratum corneum*.

TISSUE CULTURE MEDIUM

In our experiments, the MelanoDerm™ tissues were cultured in two different maintenance medium formulations. The tissue manufacturer specifies that the Long Life Maintenance Medium (LLMM) (EPI-100-LLMM) may be used for incubations of 1 to 2 weeks, while the New Maintenance Medium (NMM)-113 (EPI-100-NMM-113) may be used for longer cultures (3 weeks). Based upon these media and culture conditions, the manufacturer claims that tissues cultured in EPI-100-LLMM will be darker than the tissues cultured in EPI-100-NMM-113. The EPI-100-LLMM is a 2:1 formulation of Dulbecco's Modified Eagle's Medium (DMEM) and MCDB medium, and it contains two additives known to stimulate melanogenesis: beta-fibroblast growth factor (β-FGF), and alpha-melanocyte stimulating hormone (α-MSH). The EPI-100-NMM-113 is a DMEM-based medium that contains β-FGF, α-MSH, and keratinocyte growth factor (KGF), which is not included in the composition of EPI-100-LLMM. The two medium formulations also contain epidermal growth factor, insulin, hydrocortisone, other proprietary stimulators of epidermal differentiation, gentamicin (5 µg/ml), amphotericin B (0.25 µg/ml), and lipid precursors used to enhance epidermal barrier formation. Neither medium formulation contains serum.

MELANIN ASSAY

On the day of the melanin extraction assay, the excised MelanoDerm™ tissues were thawed at room temperature. The melanin was extracted from the tissues using Solvable (PerkinElmer, Waltham, MA, USA), an aqueous-based

reagent used to solubilize tissues. Two hundred fifty µl of Solvable were added to each microfuge tube containing the harvested tissues. A 1 mg/ml melanin standard stock solution was prepared by dissolving synthetic melanin (Sigma, St. Louis, MO, USA) in Solvable. A series of melanin standards was prepared from the 1 mg/ml stock ranging from 0.0041 to 0.33 mg/ml. The standard series was prepared by adding 0.6 ml of the 1 mg/ml melanin standard stock solution to 1.2 ml Solvable, and then making a series of five more dilutions (dilution factor of 3). Solvable was used as the 0.0 mg/ml standard. The tissue samples and the melanin standard series were incubated for at least 16 hours at 60±2ºC in a dry bath.

Upon completion of the melanin extraction, the extracted tissue samples and the melanin standards were cooled and centrifuged at 13,000 rpm for 5 minutes at room temperature. Two hundred µl of samples or standards were transferred to the appropriate wells of a 96-well plate (BD Biosciences, Sparks, MD, USA), and 200 µl of Solvable were added to the wells designated as blanks. The standards and blanks were transferred into duplicate wells, while the extracted tissue samples were transferred for reading in single wells. The absorbance at 490 nm (OD_{490}) of each well was measured with a Molecular Devices Vmax plate reader (with the Automix function selected) (Molecular Devices, Downingtown, PA, USA). Melanin concentration was expressed as µg/ml of dissolved tissue.

TISSUE VIABILITY ASSAY

The typical assay used to assess viability of 3-D cultures is based on the reduction of the yellow tetrazolium salt 3-(4,5-dimethylthialzol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) to the purple formazan dye by mitochondrial succinate dehydrogenase in viable cells. If a topically-applied material induces cytotoxicity, it will result in a corresponding decrease in mitochondrial activity, as measured by a decrease in the amount of MTT reduction.

A 1.0 mg/ml solution of MTT in warm MTT Addition Medium was prepared no more than 2 hours before use. After the appropriate exposure times, the MelanoDerm™ tissues designated for the MTT endpoint were extensively rinsed with Ca^{++} and Mg^{++} Free Dulbecco's Phosphate Buffered Saline (CMF-DPBS) (Gibco, Grand Island, NY, USA) and the wash medium was decanted. Three-tenths ml of MTT reagent were added to designated wells in a pre-labeled 24-well plate. The MelanoDerm™ tissues were transferred to the appropriate wells after rinsing. The plates were incubated for approximately three hours at standard culture conditions.

After the incubation period with MTT solution, the MelanoDerm™ tissues were blotted on sterile, absorbent paper, cleared of excess liquid, and transferred to a pre-labeled 24-well plate containing 2.0 ml of isopropanol in each designated well. Then the plates were shaken for at least two hours at room temperature.

At the end of the extraction period, the liquid within the tissue inserts was decanted into the well from which the tissue insert was taken. The extract solution was mixed and 200 µl were transferred to two wells of a 96-well plate designated for each sample. Two x 200 µl of isopropanol were placed in the two wells designated as the blanks. The absorbance at 550 nm (OD_{550}) of each well was measured with a Molecular Devices Vmax plate reader.

For the tissues processed at Day 0 (untreated – see section EVALUATION OF THE OPTIMIZED PROTOCOL), the mean OD_{550} value of the blank wells was calculated. The corrected OD_{550} value of each untreated tissue was determined by subtracting the mean OD_{550} value of the blank wells from the OD_{550} values of each untreated tissue. The individual % viability values were tabulated for each individual tissue by dividing the individual corrected OD_{550} value by the mean of all OD_{550} values calculated for the untreated tissues. Mean (and standard deviation) viability values were calculated for the untreated tissues. An overall mean % viability was calculated and plotted on a bar graph (with ± 1 standard deviation error bar).

For the tissues treated with the negative and positive control(s) and processed at Day 7, the mean OD_{550} value of the blank wells was calculated. The mean corrected OD_{550} value of the negative control was determined by subtracting the mean OD_{550} value of the blank wells from their mean OD_{550} values. The corrected OD_{550} values of the individual positive control-treated tissues were determined by subtracting from each mean OD_{550} value for the blank wells. The following percents of control calculations were made for the positive control-treated tissues:

$$\% \text{ of Control} = \frac{\text{corrected } OD_{550} \text{ of Positive Control}}{\text{corrected mean } OD_{550} \text{ Negative Control}} \text{ X } 100$$

The individual % of Control values were averaged to calculate the mean % of Control per positive control. An overall average of the MTT viability was calculated and plotted on a bar graph (with ± 1 standard deviation error bar).

RESULTS

INITIAL ASSAY DEVELOPMENT

A paramount goal of the initial assay development activities was to determine the optimal culture media, culture conditions, and treatment times to maximize melanin production under controlled conditions while enhancing the ability to detect inhibition of melanogenesis. To minimize the risks for biological contamination and infections, specific culture techniques and rinsing steps were utilized as will be further detailed.

Immediately upon receipt, the culture medium and MelanoDerm™ tissues were stored at 2-8ºC. On the evening of the day of receipt, the culture medium (either EPI-100-LLMM or EPI-100-NMM-113) was warmed to approximately 37ºC in a water bath. Nine-tenths (0.9) ml of each of the culture media were aliquoted into the appropriate wells of 6-well plates (BD Biosciences). MelanoDerm™ tissues were transferred aseptically from the 24-well shipping plates into the 6-well plates containing 0.9 ml of culture medium. The MelanoDerm™ tissues were incubated at 37±1ºC in a humidified atmosphere of 5±1% CO_2 in air (standard culture conditions) overnight (at least 16 hours), to acclimate the tissues.

At least 16 hours after initiating the cultures, the tissues were transferred to new 6-well plates containing 5.0 ml of either fresh, pre-warmed EPI-100-LLMM or EPI-100-LLMM-113. Tissue stands (MEL-STND) were used to hold the tissues at air-liquid interface thus offering the convenience of avoiding daily replacement of medium. The MelanoDerm™ tissues from the same lot number were cultured in EPI-100-LLMM for either 1 or 2 weeks (trials T1 and T2, respectively), or in EPI-100-NMM-113 for 3 weeks (20 days) (trial T3) (Table 1). The number of tissue replicates used for the various treatment groups ranged from two to four, and are presented in Table 1. To minimize the impact of potential biocontamination events, the 6-well plates and the tissue stands were replaced occasionally. In this study, the tissues were refed with fresh medium every 48±2 hours, typically on the days of treatment addition.

To compare the impact of the various culture conditions on melanogenesis, tissues were treated throughout the trials with 25 µl of the negative control (sterile deionized water, Quality Biological, Inc., Gaithersburg, MD, USA) and positive control (1% aqueous solution of Kojic acid) (Sigma) on the first day, and every 48±2 hours thereafter. The 1% Kojic acid solution was prepared in sterile deionized water, filtered, and used in the assays within 2 hours. The negative control is not expected to impact either tissue viability or melanogenesis, while the positive control is known to inhibit tyrosinase by chelating copper at the active site of the enzyme (9).

At the end of each 48-hour treatment cycle, the MelanoDerm™ tissues were rinsed with sterile CMF-DPBS to remove any residual control material. An autoclaved squirt bottle was used to dispense the CMF-DPBS onto the tissues under sterile conditions. The outsides of the tissue inserts were then blotted dry on sterile paper towel and sterile cotton tip applicators were used as needed to remove any residual CMF-DPBS from the tissue surfaces after rinsing. The tissues were then treated with the appropriate control and incubated at standard culture conditions for another 48-hour treatment cycle. At the end of the 1-, 2-, or 3-week timeframe specified for each treatment group, the MelanoDerm™ tissues were removed from the inserts using sterile scalpels (Miltex Inc., York, PA, USA), placed into individual labeled 1.5 ml microfuge tubes (Fisher Scientific, Pittsburgh, PA, USA), and stored at ≤-60ºC for subsequent melanin analysis.

Table 1

Technical aspects – trials T1-T7

Trial #	Culture medium	Weeks in culture	Tissue manipulation			Tissue replicates		Notes
			Re-feeding schedule; Culture medium (ml)	Use of culture stands	Tissue rinsing procedure	NC	PC	
INITIAL ASSAY DEVELOPMENT								
1 (T1)	EPI-100-LLMM	1	Every 48±2 hours; 5 ml; plates changed occasionally	Yes	Tissues rinsed with CMF-DPBS dispensed from an autoclaved squirt bottle, and blotted dry on sterile absorbent paper; sterile cotton tip applicators used to remove any residual CMF-DPBS used for rinsing	3	3	***Recommended study design for further evaluation***
2 (T2)		2				3	4	-
3 (T3)	EPI-100-NMM	3				2	4	The tissues became infected at Day 20; therefore the assay was terminated a day prior to the intended end date.
EVALUATION OF THE OPTIMIZED PROTOCOL								
4-7 (T4-T7)	EPI-100-LLMM	1	Every 24±2 hours; 0.9 ml; plates changed daily	No	Tissues rinsed three times with ~ 500 µl CMF-DPBS; aspiration of rinses performed with single use sterile aspirators	3	3	UT replicates: **T4**: 2; **T5**: 3; **T6**: 1; **T7**: 3

Abbreviations: CMF-DPBS, Ca^{++} and Mg^{++} Free Dulbecco's Phosphate Buffered Saline; LLMM, Long Life Maintenance Medium; NC, negative control; NMM, New Maintenance Medium; PC, positive control; UT, untreated tissue.

We assessed the production of melanin as a measure of the enzymatic activity of tyrosinase, the rate limiting enzyme of the melanogenic pathway. The results from the initial developmental trials (trials T1-T3) are presented in Fig. 1. The melanin concentration of the negative control-treated tissues cultured in EPI-100-LLMM for 1 week (59.5 µg/ml) was virtually the same as that for the tissues cultured for 2 weeks (59.3 µg/ml), suggesting that optimal melanin production occurs during the first week of culture in EPI-100-LLMM. In contrast, the melanin concentration of the negative control-treated tissues cultured in EPI-100-NMM-113 for 3 weeks was almost 2-fold lower (36.5 µg/ml), suggesting that the tissues produce less melanin in this culture medium and/or possibly at a slower rate. These results confirm the manufacturer's claims that the tissues cultured in EPI-100-LLMM will be darker than those cultured in EPI-100-NMM-113.

An analysis of the relative melanin concentration extracted from the tissues treated with 1% Kojic acid showed that the melanin concentration was reduced by at least 1.5-fold in all three trials (T1-T3) (Fig. 1). Any difference in melanin concentration from trial to trial is likely the result of variability in the tissue responses. Since the ratio between the melanin concentration of the negative control-treated tissues and the positive control-treated tissues was greater than 1.5 in all trials, we concluded that notable assay sensitivity for screening melanogenesis inhibitors can be achieved using a short-term 1-week assay.

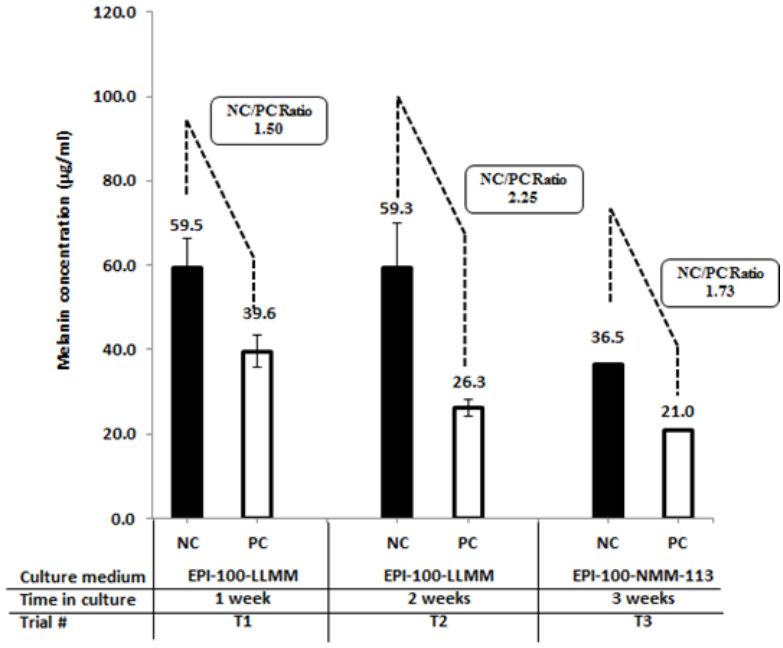

Fig. 1 – Analysis of melanin concentration (µg/ml) in MelanoDerm™ tissues treated with the negative control (NC) (black bars) and positive control (PC) (white bars) in trials T1-T3. LLMM, Long Life Maintenance Medium; NMM, New Maintenance Medium.

EVALUATION OF THE OPTIMIZED PROTOCOL

The results from the initial assay development activities were used to further refine and optimize the melanogenesis modulator screening protocol based on the setup used in trial T1 considered to be the shortest in-life assay sufficiently sensitive to identify ingredients with melanin modulating activity. Several changes were introduced into the study design and are presented in Table 1. These modifications were warranted to improve the execution of the experiments, to reduce the risks for culture infections, and to overcome several technical challenges such as proficiency in rinsing the tissues. The same cell culture initiation procedures, including the 16-hours pre-incubation at standard culture conditions, employed in the initial development trials were utilized in the evaluation of the optimized protocol (trials T4-T7). Four trials were performed and each used a different lot of tissues that were cultured only in EPI-100-LLMM for 1-week timeframe.

At least 16 hours after initiating the cultures, the tissues were transferred to new 6-well plates containing 0.9 ml of fresh, pre-warmed EPI-100-LLMM. Three MelanoDerm™ tissues were treated topically on the first day, and every 48 hours (within a timeframe of 48±2 hours from previous treatment) with 25 µl of either the positive or negative control. Medium was refreshed daily (within 24±2 hours after the previous re-feeding). On the days of treatment, the MelanoDerm™ tissues were first gently rinsed three times using approximately 500 µl of CMF-DPBS to remove any residual control, and the rinses were carefully aspirated using aseptic techniques. The tissues were treated with the negative or positive control and then transferred to a new 6-well plate containing 0.9 ml of fresh, pre-warmed EPI-100-LLMM. The tissues were then incubated at standard culture conditions for the appropriate exposure times.

An additional set of untreated (UT) MelanoDerm™ tissues was introduced to provide a baseline for the melanin assay. At least 16 hours after initiating the cultures (*i.e.*, Day 0), the untreated MelanoDerm™ tissues were rinsed with CMF-DPBS, blotted dry on sterile absorbent paper, and cleared of excess liquid. They were then removed from the inserts using sterile scalpels, placed into labeled 1.5 ml individual microfuge tubes, and stored at ≤-60ºC for subsequent melanin analysis. This procedure was also used for the tissues completing the 1-week timeframe of the assay (*i.e.*, Day 7).

The results from the optimized protocol evaluation (trials T4-T7) are presented in Fig. 2a. The melanin production in the negative control-treated tissues ranged from a minimum of 27.2 µg/ml (trial T5) to a maximum of 97.0 µg/ml (trial T6). Melanin production in the 1% Kojic acid-treated tissues was reduced by at least 1.5-fold relative to the negative control tissues (Fig. 2a) in all four trials (T4-T7). Based upon a non-statistical assay performance evaluation, we suggest that the melanin production of tissues treated with the positive control should be approximately half that of the tissues treated with the negative control in order to achieve sufficient assay sensitivity. Additional trials should be performed to

establish a robust and reliable database of responses to both controls. Untreated MelanoDerm™ tissues, whose melanin content was assessed at Day 0 as a baseline measure of pigmentation, were included in the evaluation of the optimized protocol. The start concentration of melanin varied from lot to lot, from a low level (~19.0 μg/ml – in trials T4 and T5) to higher levels (24.8 μg/ml and 53.6 μg/ml – in trials T7 and T6, respectively).

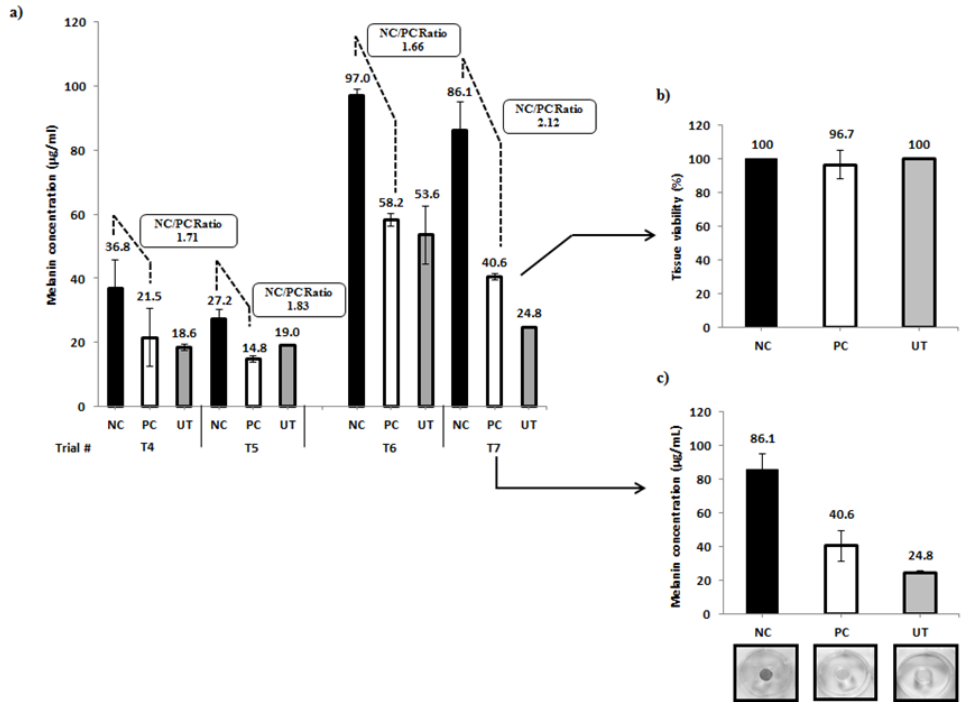

Fig. 2 – a) Analysis of melanin concentration (μg/ml) in untreated MelanoDerm™ tissues (UT) (grey bars), and in MelanoDerm™ tissues treated with the negative control (NC) (black bars) and positive control (PC) (white bars) and cultured in LLMM for 1-week assay (trials T4-T7); UT were processed at Day 0, while the NC- and PC-treated tissues were processed at Day 7; b) Analysis of the viability of UT, NC- or PC-treated tissues by MTT assay (for trial T7); UT were processed at Day 0, while the NC- and PC-treated tissues were processed at Day 7; UT (Day 0) and NC (Day 7) values defined as 100% (baseline); c) Representative macroscopic photos of tissues included in trial T7 - correlation with melanin concentration results; pictures were taken with a Canon PowerShot SX130IS (12.1 Mega Pixels) camera before the tissues were processed for the endpoints considered in each trial.

DISCUSSION

In our studies we used 2-4 tissue replicates (Table 1), however we consider that for the melanin endpoint, the use of triplicate MelanoDerm™ tissues for each treatment group is sufficient to provide reproducible and reliable results. It is also

recommended to include at least duplicate untreated tissues for the melanin endpoint at Day 0 in order to assess the baseline melanin production of the test system.

In addition to the melanin endpoint, several other endpoints should be considered depending on the specific goals of each project. A tissue viability endpoint should always be conducted in parallel to the melanogenesis endpoint as many materials may reduce melanin production due to their cytotoxicity, thus leading to incorrect data interpretation. In these instances, test material doses or exposures may need to be modified to ensure the viability of the tissues. The relative viability of the MelanoDerm™ tissues treated with 1% Kojic acid should be in general at least 80% (Fig. 2b), a cut-off that provides reasonable evidence that the inhibition of melanogenesis is not due to overt cytotoxic effects.

Another endpoint to consider is the photographic representation of changes in melanin content. In general, the tissues' degree of pigmentation can be visually assessed and it correlates with objective melanin concentration analyses (Fig. 2c), thus providing supplementary substantiation of the test system's performance. Macroscopic and microscopic photographs may provide insights on the influence of controls and test materials on melanin production as assessed by observation of cellular morphology.

Certain caution should be taken into consideration for data interpretation. The MelanoDerm™ model seems to reach a certain melanin content which is often times reduced compared to that of a whole population of melanocytes cultured in flasks. Therefore, the maximum melanin concentration obtained in this assay results in relatively low values, typically in the lowest range of the standard curve. Consequently, the users of this testing platform should consider using a melanin determination assay capable to detect a range of 5 µg/ml to 100 µg/ml of human melanin, such as an ELISA kit. Overall, discrimination between melanin concentration values among candidate skin lighteners should be approached with caution and take into consideration that the differences may be minor and data interpretation should be part of an entire profile of a candidate material of interest (animal, *in vitro*, and clinical safety and efficacy data).

For data interpretation, it is advisable for each testing laboratory to generate a reliable and robust negative and positive control database, capturing at least melanin concentration and viability of tissues of the same lot or belonging to different lots. Furthermore, the ratio between the tissue's responses to the negative and positive controls should be considered as a critical parameter both for evaluating the tissue quality and responses of each lot, and for interpretation of responses to test material exposure. While protocol designs can be flexible and tailored to meet various testing needs (*i.e.*, safety, efficacy), especially to meet the wide-ranging needs of the R&D efforts, the performance of such assays should be strictly monitored by inclusion of standard controls, the performance of which is evaluated based on established control databases.

CONCLUSIONS

Physiologically relevant *in vitro* models such as the MelanoDerm™ model are used to assess pigmentation, epidermal histology, melanin synthesis, melanosome translocation and distribution to keratinocytes in controlled *in vitro* laboratory environments (10-14). While melanogenic cell lines are simpler and less expensive systems for routine screening of melanogenic modifiers and potential bioactives, they do not model the barrier function of the *stratum corneum* or the migrational interplay between epidermal keratinocytes and melanocytes. Accordingly, promising actives should be tested in 3-D models, before proceeding to clinical testing. When using this testing platform it is worth considering that certain resident skin cells such as Langerhans' cells and dermal fibroblasts are not included in the MelanoDerm™ model. Their absence does not account for their contribution to the intracellular interactions that occur normally in the skin and that are responsible for the melanin production (15, 16). Furthermore, the development of a thicker *stratum corneum*, which can lead to reductions/changes in the rate of percutaneous penetration of the active ingredients/final formulations, could make dose-related data analyses and interpretation rather difficult.

Longer-term assays using 3-D tissues are inherently exposed to increased risks for infections particularly when final products not prepared under sterile conditions are tested. Another important point to consider is the potential for increased culture time-related accumulation of melanin degradation products that can adversely influence tissue viability. Finally, a melanin "saturation" effect may occur where the tissues reach a maximum melanin content above which its production is potentially slowed or inhibited by a feed-back mechanism, thus possibly affecting assay sensitivity. Therefore, short-term assays avoid uncontrollable tissue growth and performance characteristics, and melanin accumulation, and are preferable due to their reduced risks for infections. They are also more cost-effective and amenable to industry's need for quick and reliable data to support their time-sensitive decision process.

In conclusion, our optimized, short-term (1-week) assay using the MelanoDerm™ model represents a relevant testing platform suitable for rapid pre-clinical evaluation of the efficacy of skin lightening products to modulate pigmentation in human skin.

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