Navigating the Claim Substantiation Maze in Cosmetic Industry

The Perspective of the Contract Laboratory Specialized in Animal-Free Testing

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Key words: *in vitro* methods; cosmetics; pigmentation; skin tone modulators; anti-inflammatory

Skin (Photo-)ageing and Solutions from Cosmetic Industry

The skin is the largest organ that provides not only the unique, wide palette of color tones distinctive to human race, but also the critical protection needed against potentially harmful chemical, biological and environmental agents. Besides the natural process of ageing affecting the entire body as a complex biological system, skin additionally undergoes photo-ageing due to its continuous exposure to ultraviolet (UV) radiation. Signs of the biological ageing affecting the skin usually encompass changes in the texture (fine lines, deep wrinkles), firmness of cutaneous tissue (collagen destruction, tissue atrophy), vascular disorders (spider veins) and pigmentation heterogeneities (age spots, uneven skin tone) (Flament *et al.,* 2013). While the natural ageing process depends on time and genetics and is unavoidable, the photo-ageing can be prevented by reducing the exposure to the damaging effects of the UV (from sun or artificial sources) that aggravate the other signs of natural ageing.

Short and long term protection of the skin against the action of potentially harmful agents can be achieved by repeated applications of cosmetic products that provide mostly superficial effects intended primarily to cleanse and beautify the skin (Saraf and Kaur, 2010). Weather from natural sources or synthetic, the ingredients included in the composition of finished cosmetic products are selected for their demonstrated capacity to nourish the health, texture and integrity of the skin and to confer photo-protection (Draelos, 2010). In response to the increasing demand from consumers, cosmetic industry is continuously searching for novel, multifunctional, safe and efficacious ingredients formulated into products claiming benefits appealing to the consumer and preferably tested without the need of animals (Secchi *et al.,* 2016).

Both European and U.S. authorities require claims substantiation although the processes differ in several practical aspects. In the European Union, the cosmetic standards are set by the European Commission which then enforces their use by regulatory authorities of member countries that require manufacturers to register cosmetic products and provide proof of claims *before* the products reach the consumers (OJ EU L342, 2009). In the U.S., the Federal Drug Administration (FDA) and Federal Trade Commission have jurisdiction over cosmetic claims and individual states also have regulatory authority. *Pre-market clearance of safety/efficacy is not required* (except for color additives), however manufacturers are expected to supply scientific support of the claims when requested by authorities. The safety and/or efficacy claims accompanying the cosmetic products may be based on the collective weight of evidence supplied by peer-reviewed scientific literature, patents, expert opinions, suppliers' data, as well as by reliable, reproducible and statistically relevant scientific results of pre-clinical or clinical studies. Highlighting the biological effect of a unique ingredient or desirable benefit often provides the much needed fine-tuned market margin that successfully promotes new products to consumers.

The Challenge to Design Efficacious Cosmetic Products Addressing Inflammatory and Pigmentary Events in the Skin Following UV Exposure

Aesthetic alterations of the skin are easily noticed due to their colored appearance. One of the main contributory factors to changes in the physiological status and appearance of the skin is the UV irradiation. UV promotes the synthesis of cytokines, vasoactive and neuroactive mediators in the cell populations resident in the skin that subsequently results in an inflammatory response. The first visible effect is erythema (Schröder, 1995; Slominski and Wortsman, 2000; Clydesdale *et al.,* 2001; Skobowiat *et al.,* 2011; Slominski *et al.,* 2012) typified by hyperaemic redness. The second visual effect of inflammation is induced by the down-

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Safranal
Damascenone Total
Alpha Damascone
Iris OA
Beta Galbutenone
Karanaorg
Orspirane
Beta Damascone
Tetrafloran
Tridecenor
Safranal Ultra pure
Floral Dec
Sandafleur
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regulation of molecular factors responsible for the structural integrity of the *stratum corneum,* the superficial layer of the skin. The disturbance of the *stratum corneum* results in flakiness commonly referred to as scale, which can be further aggravated by moisture loss due to impaired barrier function and hyper-proliferation of the epidermis (common in inflammatory conditions). Finally, the third aesthetic effect of inflammation is clinically presented as pigmentary alterations. Age spots present on skin areas commonly exposed to UV (face, dorsum of the hand, etc.) represent a concern often addressed by the use of cosmetic products. The molecular pathways activated by skin's exposure to UV lead to an exaggerated response of the over-stimulated melanocytes that synthesize increased amounts of the pigment melanin as a defense mechanism (D'Orazio *et al.,* 2013).

The molecular events following complex UV-induced inflammatory cascades (Costin and Hearing, 2007; Birlea *et al.,* 2016) result in a diverse array of skin ailments (hyper-pigmentation, compromised epidermal barrier, impaired moisture retention, erythema, scale, wrinkles, ageing, photo-damage) (Amaro-Ortiz *et al.,* 2014) that have a considerable impact on cutaneous aesthetics and are of significant concern to the affected individuals. The complexity of the molecular pathways affected also presents a challenge to the cosmetic chemist when attempting to formulate products that could best address these effects (Ganceviciene *et al.,* 2012). For example, sunscreens became an invaluable first line tool in skin care for the prevention of inflammation caused by UV and of down-stream effects represented by sunburn and photo-ageing. Other products are designed to conceal age spots, prevent appearance and/or reduce the signs of ageing, and to improve the overall perceived look of the skin as a measure of its health status.

Compounds that prevent and mitigate inflammation and its effects have become of increasing interest in cosmetic industry when designing products intended to maintain the normal structural and physiological properties of the skin. To improve the skin tone and texture while reducing the appearance of ageing, ingredients with anti-inflammatory action and skin tone/color modulatory capacity are now commonly incorporated into skin care products (Baumann *et al.,* 2009; Burger *et al.,* 2016). Botanicals gained much interest in recent years due to a perceived efficacy in part generated by their natural origin and a low incidence of reported adverse effects. Delivery technologies advanced equally fast: for example, the advent of nanotechnology has provided a new avenue for the bioavailability of critical ingredients such as anti-inflammatory compounds or others that pose penetration challenges. The anti-inflammatory and skin lightening activity of promising candidates needs to be demonstrated by laboratory methods that can support scientifically their effects on cutaneous physiology, barrier function and aesthetics and thus provide an overall sense of skin well-being to the end user.

Confronted with the complexity of skin structure, physiology and ailing conditions related to ageing and exposure to harmful toxicants, manufacturers of personal care products need to utilize lab testing methods to support the claims of increasingly milder and efficacious products **(Figure 1).** In the following sections we will discuss the most advanced pre-clinical testing technologies and strategies used to assess the safety and efficacy of cosmetic products without the need for animals, which is nowadays a claim in itself made by manufacturers and used as part of the labels (U.S. FDA, 2000) **(Figure 1).**

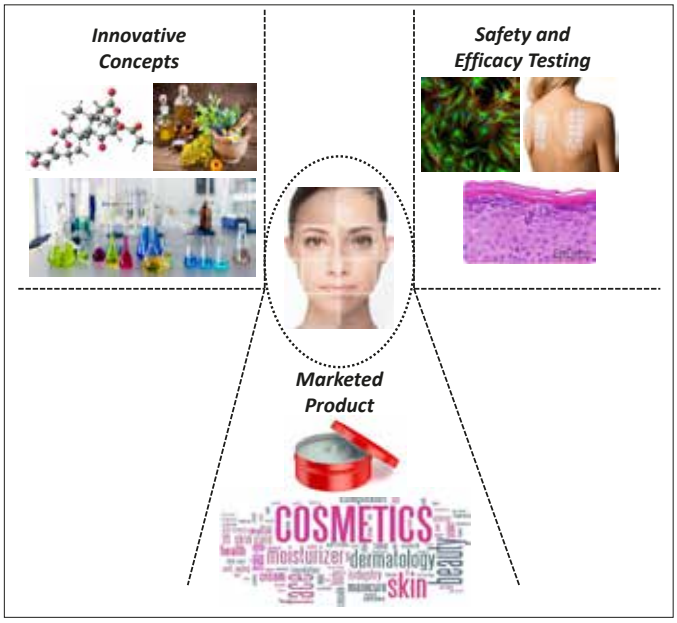

Fig. 1. Translating innovative concepts into marketed products: science meeting the marketing and the consumer. *Innovative ideas converted into active ingredients/finished formulas are subsequently subjected to safety and efficacy testing using pre-clinical, in vitro cell- or reconstructed tissues-based assays and finally advanced to clinical testing. Evidence collected through testing contributes to the claims listed on the labels of the finished products.*
The reconstructed skin tissue model EpiDerm™ - courtesy of the manufacturer, MatTek Corporation, Ashland, MA, USA.

The Assessment of Safety and Efficacy of Raw Ingredients and Cosmetic Products Using Alternative Methods to Animal Testing

The field of *in vitro* toxicology and testing is a fascinating area of science focused on perfecting non-animal techniques which are reliable, scientifically relevant, fast, cost-effective and capable to reduce downstream need for human volunteers in clinical trials (Ashawat *et al.,* 2009; Datta and Paramesh, 2010). The strategies based on non-animal methods provide a unique testing platform conveniently adaptable to certain chemistries or line of skin care products. Pre-clinical *in vitro* technologies come to meet not only the consumers' assurance need for safe and efficacious products use, but also to support the companies' mission to avoid animal testing and to promote the 3Rs (Replacement, Reduction and Refinement) as a framework for humane animal research (Flecknell, 2002). This is of particular importance for cosmetic industry as

animal testing for cosmetic ingredients and finished products is currently banned in the European Union (OJ EU L66, 2003). The law further prohibits marketing of any cosmetic products in the EU that involves new animal testing and calls for scientifically sound, proven methods using biological and computational models be used as replacement for the traditional animal tests (OJ EU L66, 2003).

Assessment of raw ingredients' and finished products' anti-inflammatory potential

To keep up with the fast paced industry focused on the discovery/design of novel ingredients for cosmetic and personal care formulations, the pre-clinical testing strategies used to investigate anti-inflammatory potential became increasingly complex and sophisticated over the years. For example, the *in chemico* method developed in the '60s (Mizushima and Kobayashi, 1968) is a rapid screening tool that measures by absorbance the denaturation of egg albumin upon contact with agents with anti-inflammatory action (Banerjee *et al.,* 2014). Other *in vitro* methods are based on the human myelomonocytic cell line THP-1 (Singh *et al.,* 2005) or human peripheral blood mononuclear cells (PBMCs) (Winkler *et al.,* 2006). The cells are pre-incubated with anti-inflammatory candidates prior to stimulation with lipopolysaccharide (THP-1 cells) or phytohemagglutinin/concanavalin A (PBMCs). Measurement of tumor necrosis factor (TNF)-α or neopterin production/tryptophan degradation have been established as reliable endpoints in these cell-based assays (THP-1cells and PBMCs, respectively).

In general, compounds with demonstrated anti-inflammatory action in cell-based assays are further studied in relevant animal models and finally tested on human volunteers. For example, anti-inflammatory activity of compounds administered orally can be assessed by measuring the percentage inhibition of edema induced by injection of carrageenan in the plantar tissues of the hind paw of Wistar rats (Winter *et al.,* 1962; Zhang *et al.,* 2013; Leslie, 2014).

To overcome significant challenges posed by the cell-based test systems (limited spatial interactions and solubility issues of complex cosmetic finished products in the cell culture media) (Wilmer *et al.,* 1994) and ethical concerns with animal testing, *in vitro* assay based on human skin reconstructed tissue models have been designed (de Yzaguirre *et al.,* 2014). These assays show promise addressing industry's need for testing strategies to qualify prototypes for subsequent clinical studies investigating anti-inflammatory action and are scientifically relevant due to the human-derived cell lines/tissues used.

We designed a novel *in vitro* assay based on the reconstructed tissue model EpiDerm™ (MatTek Corporation, Ashland, MA, USA) derived from human skin keratinocytes. The tissues were exposed topically for 6 hours to materials investigated for their

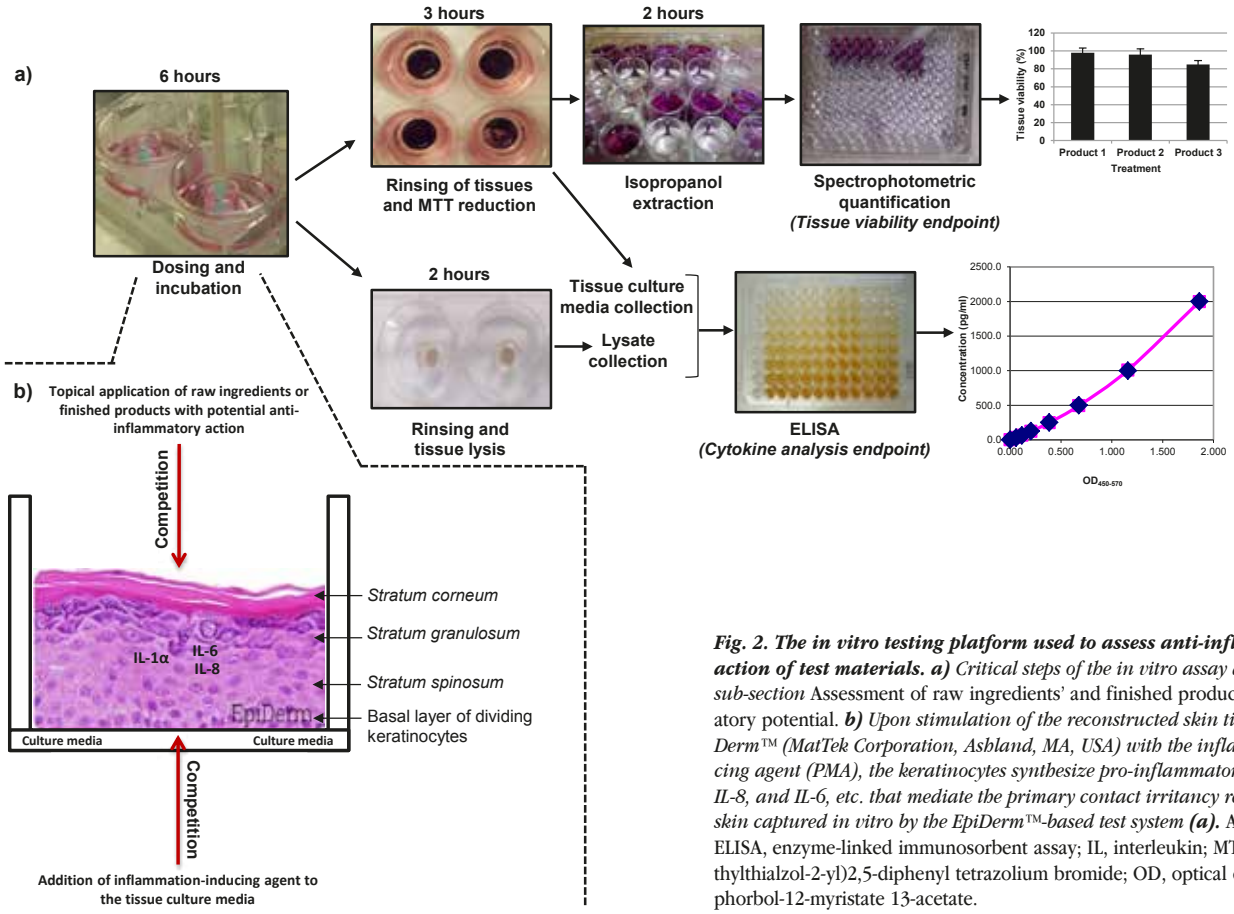

Fig. 2. The in vitro testing platform used to assess anti-inflammatory action of test materials. a) *Critical steps of the in vitro assay described in sub-section* Assessment of raw ingredients' and finished products' anti-inflammatory potential. ***b)*** *Upon stimulation of the reconstructed skin tissue model EpiDerm™ (MatTek Corporation, Ashland, MA, USA) with the inflammation-inducing agent (PMA), the keratinocytes synthesize pro-inflammatory cytokines IL-1α, IL-8, and IL-6, etc. that mediate the primary contact irritancy reactions in the skin captured in vitro by the EpiDerm™-based test system* ***(a).*** Abbreviations: ELISA, enzyme-linked immunosorbent assay; IL, interleukin; MTT, 3-(4,5-dimethylthialzol-2-yl)2,5-diphenyl tetrazolium bromide; OD, optical density; PMA, phorbol-12-myristate 13-acetate.

capacity to counteract the inflammation induced by phorbol-12-myristate 13-acetate (PMA) added to the culture media (Pidathala *et al.,* 2015) **(Figure 2a, 2b).** Upon the initiation of inflammatory pathways by PMA, the keratinocytes synthesize pro-inflammatory cytokines interleukin (IL)-1α, IL-8, and IL-6, etc. that mediate the primary contact irritancy reactions in the skin (Schröder, 1995) and that can be captured *in vitro* by the EpiDerm™-based test system **(Figure 2a).** Two different ingredients with known anti-inflammatory activity formulated as creams were evaluated: over-the-counter (OTC)-Class 7 low potency, and prescription (Rx)-Class 2 high potency, formulated for augmented penetration. The low potency active was also tested as an alcohol-based spray containing the same concentration of the active present in the cream version. To avoid over-prediction of the skin irritation (Vavilikolanu *et al.,* 2009), the alcohol-based formulation was applied to the tissues at a reduced dosing volume of 30 µl, while the creams were applied as 100 µl doses. The tissue viability (%) was assessed by 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 **(Figure 2b).** The cytokines analyzed were IL-1α and IL-8.

Our data showed that the analysis of the compartmentalized cytokine synthesis (IL-1α analyzed in the lysed tissues) and secretion (IL-8 analyzed in the culture media) represent reliable indicators of anti-inflammatory actions of the materials tested and intended for human skin application. Both cytokine indicators showed that the Rx cream formulated for augmented penetration was the most effective of the creams in reducing the cytokines' levels, thus supporting the Class 2 high potency for clinical use **(Figure 3).** Furthermore, the OTC-Class 7 active formulated as a spray had a stronger anti-inflammatory action compared to its cream counterpart despite the reduced dosing volume, and similar to the Rx-Class 2 cream. Our data support the use of the Rx-Class 2 cream and the OTC-Class 7 spray as reference materials for screening formulations investigated for anti-inflammatory action.

Our experiments (Pidathala *et al.,* 2015) provide a proof of concept for the testing platform based on reconstructed skin equivalents treated with pharmaceutical compounds with known patient use performance as reference materials. This is a reliable, rapid tool to screen actives and formulations with anti-inflammatory action and also to identify triggers of excessive inflammation. Our future plans will focus on screening a diverse range of actives and formulations relevant for personal care and cosmetic industry in order to expand the applicability domain of the *in vitro* assay and will also consider widening the cytokine profiling.

Assessment of raw ingredients' and finished products' potential to modulate skin color

Chronic exposure to UV has been demonstrated to induce skin ageing and photo-ageing, and the molecular mechanisms responsible for these complex biological events have been investigated thoroughly (reviewed in Kim and Park, 2016). In recent years, another significant contributor to skin ageing was determined as an independent pathogenic factor: tobacco smoke (Kennedy *et al.,* 2003; Schröder *et al.,* 1995). Furthermore, the study by Vierkötter *et al.,* 2010 found a significant association between traffic-related airborne particles and signs of extrinsic skin ageing (*i.e.,* pigment spots on face and nasolabial fold). The strongest effect was determined to be induced by soot, which carried a high concentration of surface-bound polycyclic aromatic hydrocarbons (PAHs) that are ligands for aryl hydrocarbon receptors and have been recently shown to induce melanocyte proliferation and skin tanning in mice (Krutmann *et al.,* 2008). PAHs are frequently bound to the surface of combustion-derived particulate matter (PM) and this mechanism may therefore

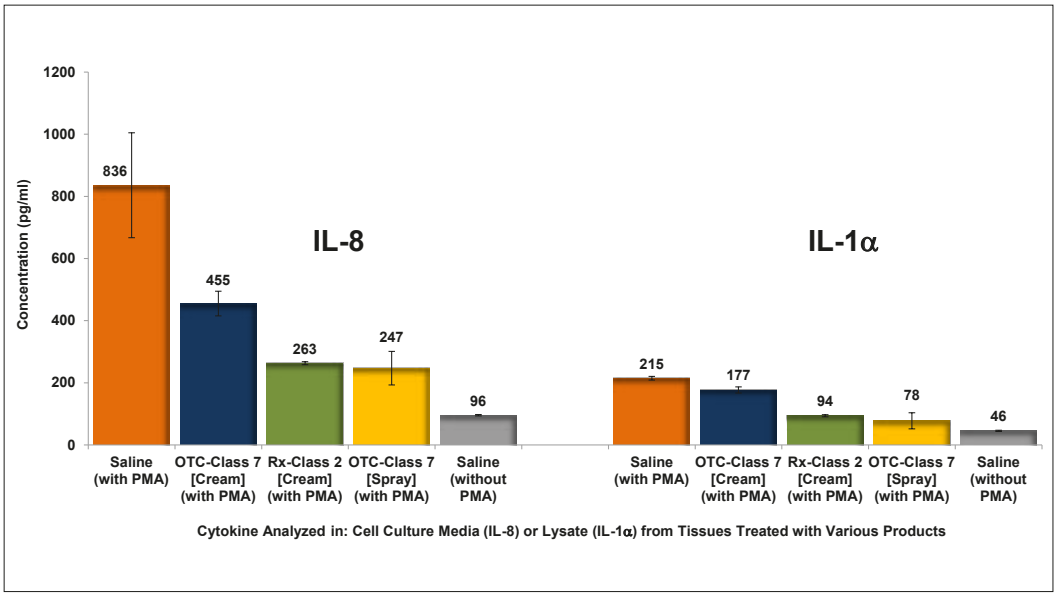

Fig. 3. Induction of cytokine expression (IL-1α, IL-8) by PMA in the reconstructed skin tissue model EpiDerm™ (MatTek Corporation, Ashland, MA, USA). *The tissues were treated with: the assay negative control (0.9% Saline); OTC-Class 7 cream; OTC-Class 7 spray and Rx-Class 2 cream. The induction of cytokines in the negative control-treated tissues and cultured in PMA-containing media was significant compared to the tissues cultured in media without PMA: 836 pg/ml vs 96 pg/ml for IL-8 and 215 pg/ml vs. 46 pg/ml for IL-1α, respectively. These results demonstrate the induction of the inflammatory events by PMA in the reconstructed tissues used in the assay.* Abbreviations: IL, interleukin; OTC, over-the-counter; PMA, phorbol-12-myristate 13-acetate; Rx, prescription (drug).

provide a scientific rationale for the reported association between pigment spots and exposure to traffic-related PM (Vierkötter *et al.,* 2010).

A multitude of methods of diverse complexity and endpoints have been perfected over the years to investigate the efficiency of ingredients or complex formulations to modulate skin color and to address the pigmentary conditions related to (photo)-ageing (Costin, 2016; Gunia-Krzyazak, 2016). While screening of large numbers of compounds is reasonably achieved for safety and efficacy purposes using *in silico* and subsequently cell-based assays (Land *et al.,* 2006), mechanistic and confirmation assays are best achieved using *in vitro* pre-clinical test systems based on human reconstructed pigmented models (Costin, 2016).

We optimized a 2-phase, tiered testing platform initiated with a 1-week dose-range finding pre-screening assay followed by a 1-week screening assay using the reconstructed human skin model complete with melanocytes of Asian phototype (MelanoDerm™) from MatTek Corporation (Ashland, MA, USA) (Costin and Raabe, 2013). Our testing platform is designed to rapidly evaluate candidate actives that modulate melanin production in human skin and to address the needs of a fast paced product release-oriented cosmetics industry. Macroscopic and microscopic pictures taken on Day 0 (baseline, untreated tissues) and Day 7 were used as supportive evidence of the impact of compounds on melanin production and on melanocytes' physiology **(Figure 4a).** The dose-range finding pre-screening assay used the tissue viability (%) endpoint (by MTT) to determine the toxicity profile of candidate materials before advancing to the screening assay **(Figure 4b).** The results obtained for various concentrations of the assay positive control (1% Kojic acid) demonstrated the sensitivity of the test system evaluated by its response with lowest viability (29%) pertaining to higher concentration (5%) of the assay positive control. Kojic acid is known to inhibit tyrosinase, the key enzyme of the melanogenic pathway, by chelating copper at the active site of the enzyme (Cabanes *et al.,* 1994). The melanin production endpoint was assessed during the screening assay and evaluated the tissues treated with compounds of interest in comparison to the negative control (sterile, deionized water)- or positive control (1% Kojic acid)-treated tissues **(Figure 4c).** The melanin production in Kojic acid-treated tissues was similar to the concentration of the pigment at Day 0 in untreated tissues, indicating a significant effect on the overall melanin concentration compared to the negative control-treated tissues. We also tested *N*-butyldeoxynojirimycin (NB-DNJ), which is an inhibitor of the endoplasmic reticulum N-glycan processing enzymes α-glucosidases I and II. NB-DNJ was shown to affect the glycan maturation of tyrosinase (Petrescu *et al.,* 1997). The study used mouse melanoma cells treated with 0.5 mM NB-DNJ and showed that the treatment significantly reduced the enzymatic activity of tyrosinase, subsequently reflected in a lower melanin production in the NB-DNJ-treated cells. Our data showed that the same concentration of the agent applied onto the tissues at a 4-fold higher dosing volume compared to the assay controls (100 µl vs. 25 µl) was not efficient enough to reduce the melanin production as in the cell-based assay reported previously. These data indicate that a dose-range finding assay is necessary when transitioning from cell-based assays to reconstructed tissue-based testing models. Preliminary investigations should take into account a multitude of factors that may contribute to a final efficacious action to reduce pigment production such as the penetration rate of the actives, bioavailability, differentiation of the tissues while in culture, etc.

In conclusion, our optimized 2-phase testing platform using the MelanoDerm™ reconstructed pigmented skin model represents a tool suitable for rapid pre-clinical evaluation of the efficacy of ingredients/final formulations to modulate pigmentation in

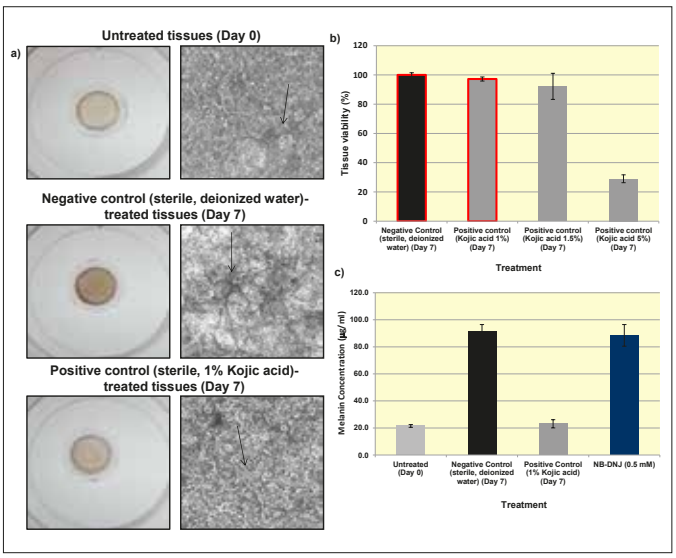

Fig. 4. Typical results obtained with the in vitro testing platform used to assess the potential of materials to modulate skin color. a) *Macroscopic (left column) and microscopic pictures (right column, magnification 15X) of MelanoDerm™ tissues (Asian phototype) untreated (Day 0) and treated for 7 days with the assay negative (sterile, deionized water) or positive control (1% Kojic acid), respectively. The macroscopic pictures were taken with a CANON camera, Powershot, SX130IS, 12x optical zoom, manual setting, while the microscopic pictures were taken with an Infinity 2 camera connected to an inverted Nikon Eclipse TE 2000 U microscope and captured using the Infinity Analyzer Software. Arrows on the microscopic pictures point towards melanocytes displaying long dendrites and low melanin production (untreated tissues, Day 0), long pigmented dendrites (tissues treated with the assay negative control, Day 7) or dendrites lacking pigment (tissues treated with the assay positive control, Day 7).* ***b)*** *Typical tissue viability (%) results obtained for the tissues treated with the assay negative control (sterile, deionized water) defined as 100% (baseline) vs. various concentrations of the assay positive control (Kojic acid). The standard concentration of the assay positive control is 1% (w/v) that did not induce a significant reduction in tissue viability (2.8%) compared to the 5% concentration (71% reduction in tissue viability). The results indicate that the test system is sensitive to various concentrations of the assay positive control reflected in the reduction of tissue viability. Bars with red outline indicate the typical assay controls.* ***c)*** *Typical melanin concentration results obtained for the tissues treated with the assay controls; also tested was the glycosylation inhibitor NB-DNJ (0.5 mM). NB-DNJ did not induce significant toxicity to the tissues (data not shown) in the testing method used.*
Abbreviation: NB-DNJ, N-butyldeoxynojirimycin.

human skin. Use of this strategy generates valuable *in vitro* data that can provide guidance prior to the commencement of costly clinical trials. The assay could also provide mechanistic insights when complemented with histological evaluations or other endpoints. Last but not least, the testing platform can accommodate chronic/repeated exposures which may be of interest when investigating *in vitro* the effects of PAHs, for example. The advances in non-animal testing technologies support the need for fast, scientifically relevant and sensitive methods such as those described herein and optimized to assess the efficacy (and safety) of actives designed to ameliorate inflammatory or pigmentary events occurring in human skin.

Conclusions – Future of Cosmetics and the Advances in Testing Strategies

Manufacturers of actives or formulations with potential anti-inflammatory and skin tone modulatory action are in need of rapid, reliable and relevant *in vitro* assays to be used as screening tools before initiation of clinical testing. A key driver for the technological advancements and innovations in the field of *in vitro* toxicology testing was the oposition to animal testing of cosmetic products and ingredients, as well as an outright ban across the EU which has a ripple effect on global manufacturers. The assays we presented herein come to fill that gap and provide data to support the use of reconstructed skin models and cytokine or other specialized (*e.g.,* melanin) endpoints to interpret the efficacy of various classes of products to protect or improve the health of the skin. These efforts are concentrated on implementation of new test methods into practical testing for screening purposes and also on supporting industry's efforts to make novel, safe and efficacious cosmetic products available to the end-user.

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