Skin Tone Modulation: *In Vitro* Pre-clinical and Clinical Efficacy Testing Strategies and Innovative Solutions in Cosmetics

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29 June 2017
OUTLINE

- Integration of the *in vitro* pre-clinical safety and efficacy testing methods in the skin care products manufacturing framework

- Pre-clinical and clinical testing platforms used for the assessment of skin tone modulation potential of prototypes
  - Melanin biosynthesis pathway
  - Key steps in the processing of melanogenic proteins and points of impact from modulators of the pathway

- Pre-clinical optimized melanin modulator screening assay
  - Key elements
  - Assay optimization
  - Endpoints: tissue viability, melanin production, macro- and microscopic pictures

- Conclusions
Integration of the *in vitro* pre-clinical safety and efficacy testing methods in the skin care products manufacturing framework.

**Proof of concept**

**Safety Testing**

**Efficacy Testing**

**Product: concept** → **Raw ingredient/ Final formulation** → **Product: production and launching**

**Efficacy testing** ← --- ← **Safety testing**

**Pre-clinical testing (in vitro)**

**Skin tone modulation**

**Skin irritation**

**Skin sensitization**

**Co-cultured cell-based models**

**Ex-vivo models**

**Reconstructed tissue models**

**Clinical testing**

**Cell-based models**

Modified in part from: Gertrude-Emilia Costin and Kimberly G. Norman. Application of In Vitro Methods in Preclinical Safety Assessment of Skin Care Products, Springer-Verlag Berlin Heidelberg 2015 M.A. Farage et al. (eds.), Textbook of Aging Skin, DOI 10.1007/978-3-642-27814-3_130-1; and Gertrude-Emilia Costin, Decoding and modulating the color of human skin. Cosmetic Chemist 2016. Available at: http://d19cgyi5s8w5eh.cloudfront.net/e/ml/VPwvPaYmSi-Uohf6UcNzhA?e=roger_mcmullen%40fd.edu&a=Hn7-iRf_ToiDAzh-g4Tduw&f=8&t=1
Pre-clinical and clinical testing platforms used for the assessment of skin tone modulation potential of prototypes

In vitro

In silico

Going “skin deep”

Native skin (explant)

Pigmented reconstructed skin model

Melanocytes/Keratinocytes Co-cultures

Melanocytes

Melanosome

Proof of concept

Safety Testing

Efficacy Testing

Clinical

“Journey” to the surface

Journey to the surface

Tissue

Cell(s)

Organelle

Modified in part from: Costin GE. Decoding and modulating the color of human skin. November Newsletter of the Cosmetic Chemist. 2016. Available at: http://d19cgyi5s8w5eh.cloudfront.net/eml/VPwvPaYmSi-Uohf6UcNzhA?e=roger_mcmullen%40edu.edu&a=Hn7-iRf_ToiDAzh-g4Tduw&f=8t=1
Melanin biosynthesis pathway

Tyrosine → DOPA → DOPAquinone → Cysteine

DOPAchrome tautomerase / DCT/TRP2 → CycloDOPA

DHICA → DOPAchrome → DHI

Indole-5,6-quinone-carboxylic acid → Indole-5,6-quinone

DHICA oxidase / TYRP1/TRP1 → DHICA polymerase / Pmel17/silver protein

DHI oxidase / Tyrosinase (TYR)

CysteinylDOPA

Alanyl-hydroxybenzothiazine

Very slow

Slow

Very fast

Fast

DHICA-Melanin

DHI-Melanin

PheoMelanin

a) Histology assays
   • Fontana-Mason staining for melanin
   • DOPA staining (electron microscopy) for tyrosinase activity

b) Enzymatic assays
   • DOPA oxidase assay – spectrophotometric analysis or on-gel assessment
   • Tyrosine hydroxylation assay – radioactive ($^3$H)
   • Melanin formation assay – radioactive ($^{14}$C)

c) Chemical assays
   • HPLC assays for determination of melanin type and concentration: eu- vs pheo-melanin (PTCA, AHP)
   • Determination of melanin concentration: chemical extraction with NaOH

d) Genotoxicity assays (induced by UV)
   • CPDs
   • 6-4 PPs

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Key steps in the processing of melanogenic proteins and points of impact from modulators of the pathway

1. Transcription
2. Maturation
3. Transport to melanosomes and participation in melanin biosynthesis
4. Melanosomes
5. Degradation proteolysis

Modified in part from: Costin GE. Decoding and modulating the color of human skin. November Newsletter of the Cosmetic Chemist. 2016. Available at: http://d19cgyv5w5eh.cloudfront.net/eml/VPwvPaYmSi-Uoh6UcNzhA?e=roger_mcmullen%40fdu.edu&a=Hn7-iRf_ToiDAzh-g4Tduw&f=&t=1

Petrescu S.M. et al. Inhibition of N-glycan processing in B16 melanoma cells results in inactivation of tyrosinase but does not prevent its transport to the melanosome. J. Biol. Chem. 272, 15796-15803 (1997)
Pre-clinical optimized melanin modulator screening assay key elements

• **Test system:** Reconstructed human epidermis (RhE) models containing melanocytes of different phototypes: Asian, African-American, Caucasian, etc.

• **Providers:** Cell Systems (Toisdorf, Germany) - epics-M EpiSkin (Lyon, France) - Reconstructed Human Pigmented Epidermis (RHPE) MatTek Corporation (Ashland, MA, USA) - MelanoDerm™ of Asian phototype TegoScience (Seoul, Korea) - NeoDerm-ME

• **Assay endpoints:** tissue viability (%) – MTT (dose range finding: 10μl and 25μl, respectively) melanin concentration (overnight extraction in Solvable at 62°C) histology (Fontana-Mason; Hematoxylin-Eosin) micro- and macroscopic pictures

• **Assay controls:** negative (NC) (sterile, deionized water) positive (PC) (1% Kojic Acid)

• **Applicability:** support of Product Development, New Technology, Innovation projects screening of actives/finished formulations with potential to modulate melanin production and impact skin tone

• **Limitation:** small differences in melanin production between tissues treated with various actives/final formulations (depending on the phototype used)
Melanin modulator screening assay: assay optimization

*Tissues manipulation - Assay optimized to:*

- **Avoid infections**
  - Change of plates
  - Media change
  - Rinsing procedure
  - Dedicated incubator/hood

- **Accommodate the dosing of a variety of test materials that pose challenges (creams, lotions, gels, etc.)**
  - Use of sterile dosing devices
  - Use of sterile pins/bulb rods

Melanin modulator screening assay: assay optimization

*Exposure times, culture media, tissue viability and melanin production*

![Graph showing melanin concentration and NC/PC ratio over time.]

**Typical protocol**

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Time in culture</th>
<th>Trial #</th>
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</thead>
<tbody>
<tr>
<td>EPI-100-LLMM</td>
<td>1-week</td>
<td>T1</td>
</tr>
<tr>
<td>EPI-100-LLMM</td>
<td>2-weeks</td>
<td>T2</td>
</tr>
<tr>
<td>EPI-100-NMM-113</td>
<td>3-weeks</td>
<td>T3</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Tuesday</th>
<th>Wednesday</th>
<th>Thursday</th>
<th>Friday</th>
<th>Saturday</th>
<th>Sunday</th>
<th>Monday</th>
<th>Tuesday</th>
<th>Wednesday</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Overnight incubation</strong></td>
<td><strong>Treatment 1</strong></td>
<td><strong>Treatment 2</strong></td>
<td><strong>Treatment 3</strong></td>
<td><strong>Treatment 4</strong></td>
<td>Endpoints</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Melanin modulator screening assay: endpoints macroscopic pictures

Untreated Tissues

Asian phototype

Negative Control (di water)

Day 0

Day 7

Positive Control (1% Kojic Acid)

African-American phototype
Melanin modulator screening assay: endpoints microscopic pictures

Asian phototype

Untreated Tissues

Negative Control (di water)

Positive Control (1% Kojic Acid)

African-American phototype

Day 0

7-days assay

14-days assay
Melanin modulator screening assay: endpoints tissue viability

**Dose range finding assay**

- 10µl and 25µl dose: ≥75%
- 25µl dose: ≤75%
- 10µl dose: ≤75%

**Definitive assay**

- 25µl dose to be used
- 10µl dose to be used
- Material likely cytotoxic

**Treatment**

<table>
<thead>
<tr>
<th>Tissue viability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dose range finding assay</strong></td>
</tr>
<tr>
<td>10µl and 25µl dose: ≥75%</td>
</tr>
<tr>
<td>25µl dose: ≤75%</td>
</tr>
<tr>
<td>10µl dose: ≤75%</td>
</tr>
</tbody>
</table>
Factors to consider when transitioning from cell-based to tissue-based assays (and in preparation for clinical studies)

- Differentiation of the tissues while in culture
- Penetration rate of the actives
- Bioavailability (encapsulation – pH sensitive liposomes)

**Petrescu S.M. et al.** Inhibition of N-glycan processing in B16 melanoma cells results in inactivation of tyrosinase but does not prevent its transport to the melanosome. J. Biol. Chem. 272, 15796-15803 (1997)

Yellow MTT $\rightarrow$ purple formazan

Melanin modulator screening assay: overcoming challenges

- **Hydroquinone**
- **Vitamin A**
- **KOH**
- **Corn oil**
- **Vitamin E**
- **Eugenol**
- **Ascorbic Acid**
- **Heptanal**

**Alternatives**
- Killed control tissues
- ATP assay

**Points to consider – optimization**

- **Challenge**: The ATP assay uses a lysis buffer $\rightarrow$ extraction of melanin $\rightarrow$ variability
- **Possible solution**: drying of tissues before extraction in Solvable

- **Challenge**: accuracy of the combined approach
- **Possible solution**: direct comparison with a standard melanin assay (without prior ATP extraction) is needed for validation of the combined approach
CONCLUSIONS

The optimized, specialized efficacy testing platform using reconstructed tissue models completed with melanocytes can be used to:
The optimized, specialized efficacy testing platform using reconstructed tissue models completed with melanocytes can be used to:

- Rapidly obtain pre-clinical evaluation of actives for their ability to modulate melanin production
- Analyze melanin production in reconstructed tissue models of various phototypes
- Deliver reliable, fast, and relevant results regarding the safety and efficacy of promising prototypes
- Be adapted to specific testing needs and mechanistic approaches
- Accommodate chronic/repeated exposures, which may be of interest to various industries
- Address the increasing market demand for innovative, safe and efficacious skin lightening products capable of modulating pigmentation in human skin

CONCLUSIONS
Acknowledgments

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Brandan Nokes

Industry Collaborators

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Skin Tone Modulation

Manpreet Randhawa PhD
06/29/2017
Agenda

• Background on skin tone

• Why do we need different technologies to address skin tone issues?

• Types of claims and their substantiation

• Case study

• Conclusions
Skin Tone Issues Are Dictated By External And Internal Aggressors

- Aging
- Sun
- Genetic predisposition
- Pollution
- Hormones
- Ethnicity
- Cosmetics
Skin Tone Is A Result Of Different Skin Factors

- Redness
- Yellowness
- Hydration
- Pigmentation
- Texture
Pigmentation Is The Main Deciding Factor For Skin Tone Issues

FROM RACES

<table>
<thead>
<tr>
<th></th>
<th>African</th>
<th>Oriental</th>
<th>Caucasian</th>
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<tbody>
<tr>
<td>Melanin composition</td>
<td>Pheomelanin Eumelanin</td>
<td>Pheomelanin Eumelanin</td>
<td>Pheomelanin Eumelanin</td>
</tr>
<tr>
<td>Melanin grains in epidermis</td>
<td>Complexed</td>
<td>Complexed</td>
<td>Complexed</td>
</tr>
<tr>
<td>Melanin morphology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melanin grain Size (nm)</td>
<td>1*0.5</td>
<td>0.6*0.3</td>
<td>0.5*0.3</td>
</tr>
</tbody>
</table>

TO FITZPATRICK SKIN TYPES

Skin reaction to sun

https://www.skinbarnyc.com
Pigmentary Disorders Are Regulated Differently

**Melasma**
- The melanocytes are markedly increased in number and show pendulous change.

**Freckles**
- The melanocytes do not increase in number but are hyperactive.

**Age Spots**
- Markedly increase in the number of melanocytes along with hyperactivity. Cell turn over rate is slow.

**Vitiligo**
- No melanocytes accompanied by inflammation infiltration.
Different Technologies Are Needed To Address The Pigmentation Issues

**Pigmentation targets**

- Stratum corneum
- Keratinocytes
- Melanosome Transfer
- Tyrosinase Activity
- Melanocyte Activity

**Technologies**

- Light reflectors
- Sunscreens
- Retinol, Exfoliators (glycolic acid)
- Soy
- Licorice extract Kojic acid
- Ascorbic acid
- Hydroquinone
- Hexyl Resorcinol
- Tranexamic Acid
- Resveratrol

*Blue* — melanin in basal layer,  
*Red* — melanin in the middle layer  
*Green* — melanin in upper layers.
Evolution Of Claims From Skin Whitening To Ideal Fairness

Pigmentation dependent MOA and clinical benefits

Pigmentation & Aging dependent MOA and clinical benefits

- Even skin tone
- Minimal hyper-pigmentation spots
- Reflecting a healthy clarty skin from within

Healthy Skin Tone

Ideal Fairness
Healthy glow & clarity from within

- Smooth skin texture with healthy skin cell turn over
- No rough, no dull caused by accumulated melanin pigments or dead skin cells
- Reflecting healthy radiant from within

Healthy Skin Texture

Healthy Skin HYDRATION

- Hydration is key to light transmission
- Well hydrated skin allows optimized light penetration & scattering, providing translucent skin looking
- Well hydrated skin allows well delivery of actives

Haght textures leads to light being poorly transmitted in skin, resulting dull & dark looking
FDA Statement For Cosmetics Beauty Claims

The Federal Food, Drug & Cosmetic Act (FD&C Act) defines cosmetics as "articles intended to be rubbed, poured, sprinkled, or sprayed on, introduced into, or otherwise applied to the human body...for cleansing, beautifying, promoting attractiveness, or altering the appearance." Included in this definition are products such as skin moisturizers, perfumes, lipsticks, fingernail polishes, eye and facial makeup preparations, shampoos, permanent waves, hair colors, toothpastes, and deodorants, as well as any material intended for use as a component of a cosmetic product.
Types Of Claims

• Clinical claims: Clinical testing
  • Speed
  • Efficacy

• Consumer claims: Consumer testing

• Instrumental claims: Chromameter and mexameter

• Invitro claims: Cell based assays, 3-D models
Skin Benefits Are Captured Under Different Claim Categories

• Instant claims
  • Instant glow

• 1 week claims
  • Radiance
  • Luminosity
  • Translucent
  • Transparent

• 2 week claims: Even tone claims

• 4 week claims: Dark spots claims including age spots and post acne marks are faded
Neutrogena® Rapid Tone Repair
Night Moisturizer

Works quickly for visible results in just one week.

An exclusive combination of Vitamin C and Accelerated Retinol SA boosts surface skin cell renewal to brighten skin’s tone and help reverse the look of even the most stubborn dark spots revealing noticeably more even, younger looking skin.

You will see increased clarity and more radiant skin in just one week, and see diminished signs of dark spots, discoloration and blotchiness over time.

Clinically proven to help:
- Reduce the look of dark spots and discoloration
- Brighten and even skin’s tone
- Improve skin’s clarity and radiance
- Smooth fine lines & texture
Olay Natural White All-in-One
Fairness Night Cream

Benefits:
- * Reduces appearance of dark spots
- * Increases skin cell renewal to lighten for radiant looking skin
- * Provides anti-oxidant protection
- * Keeps skin well moisturized

WHITE PERFECT DAY CREAM
BRIGHTENS SKIN FOR A
MORE TRANSPARENT,
HEALTHY GLOW

INTENSE WHITENING +
FULL PROTECTION
1) Instant glow
2) Fairer skin
3) Even tone
4) Anti-dark spots
5) Anti-shine effects
6) Anti-pollution
7) Anti-UVA
8) Anti-UVB
9) Anti-oxydation
10) Anti-oiliness effect

Benefits: Reduces spots by controlling melanin production and visibly brightens skin tone for a more transparent skin
Elements to be considered for claims

- Questions to be asked from the study
  - Clinical
  - Consumer
  - Invitro study

- Robustness of the study
  - Controls (comparators), placebo controlled
  - Recruitment criteria
  - Global vs. skin type claims
  - Statically significance
  - Reproducibility
  - Comparison to baseline or comparator product
  - Seasonality

- Sunscreens have been shown to impact pigmentation. Study With/Without a sun filter

*M Randhawa et al., 2016*
Case study

4-Hexyl-1,3-phenylenediol (Hexinol™)

• Invitro studies
  • Cell culture model
  • 3-D model

• Clinical study

• Products with 4-Hexyl-1,3-phenylenediol
Cell Culture (2D)

4-Hexyl-1,3-phenylenediol inhibits Tyrosinase activity and melanogenesis

![Graphs showing the inhibition of Tyrosinase activity and melanogenesis](chart)

Pigmented Epidermal Equivalents (3D)

4-Hexyl-1,3-phenylenediol reduces pigmentation

<table>
<thead>
<tr>
<th>Untreated control</th>
<th>Vehicle control</th>
<th>0.2 mg/ml</th>
<th>1 mg/ml</th>
<th>2.5 mg/ml</th>
</tr>
</thead>
</table>

*YK Won et al., 2014*
Clinical study

Protocol

- Study design: 12-week, double blind, vehicle-controlled, randomized
- Time period: November to January
- Skin type: 65 female subjects, aged 30–40, Fitzpatrick type III and IV, and regular users of moisturizing products
- Inclusion criteria: Subjects must have clinically determined mild to moderate dyschromia (solar lentigines) on both sides of the face, with at least five pigmented spots per cheek.

Clinical evaluations

- Clinical evaluations were performed by two dermatologists at baseline and at weeks 2, 4, 8 and 12.
- Clinical assessments were scored on a 0 (none) to 9 (severe) scale and included the following benefit areas:
  - overall skin lightening; appearance of spot on the cheeks; overall contrast between spots and surrounding skin; overall pigmentation size; overall skin tone evenness;

4-Hexyl-1,3-phenylenediol formula significantly reduces skin hyperpigmentation

\[ YK \text{ Won et al., 2014} \]
4-Hexyl-1,3-phenylenediol Reduces Hyperpigmentation In Clinical Study

YK Won et al., 2014
Hexinol™ products impacting skin tone

Ingredients
Hyaluronic acid
Hexinol

In a clinical study, women saw noticeable improvements in the 5 main signs of aging in as little as 4 weeks:
• Discolorations appeared visibly improved
• Helped restore elasticity and firmness to sagging skin
• Hydrated thirsty, dry skin
• Improved overall radiance of skin
• Smoothed out the appearance of fine lines and wrinkles

By combining the fairness efficacy with hydration efficacy the product will be provide translucency, watery shine as well as fairness
Conclusions

Skin tone is a result of multitude of factors

Pigmentation is a complex mechanism and regulated by different pathways

Skin tone claims can be addressed by different model system

Claim substantiation depends on the robustness of the study