Original Contribution

Journal of Cosmetic Dermatology, **0**, 1–8

In vitro and clinical evaluation of SIG1273: a cosmetic functional ingredient with a broad spectrum of anti-aging and antioxidant activities

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Summary

Background Isoprenylcysteine (IPC) small molecules were identified as a new class of anti-inflammatory compounds over 20 years ago. Since then, they have been developed as novel cosmetic functional ingredients (CFI) and topical drug candidates. SIG1273 is a second generation CFI that has previously been shown to provide a broad spectrum of benefits for the skin through its anti-inflammatory and antimicrobial properties.

Objective To determine whether SIG1273 possesses anti-aging properties *in vitro* and evaluate the tolerability and activity of SIG1273 when applied topically to human subjects.

Methods To model photoaging *in vitro*, human dermal fibroblasts (HDFs) were exposed in culture to UVA to induce collagenase (MMP-1) production. An *in vitro* wound-healing model was based on the activation of HDF migration into cell-free tissue culture surface. Hydrogen peroxide-induced oxidative stress was performed using HDFs to measure intracellular ROS activity. Radical scavenging capacity was determined using a colorimetric antioxidant assay kit (ABTS method). Lastly, a 4-week, 29-subject study was performed in which SIG1273 was applied topically as a cream to assess its tolerance and activity in reducing the appearance of aging.

Results *In vitro* studies demonstrate SIG1273 inhibits UVA-induced MMP-1 production, hydrogen peroxide-induced oxidative stress and promotes wound healing. Moreover, SIG1273 was shown to be a radical scavenging antioxidant. Clinical assessment of SIG1273 cream (0.25%) showed it was well tolerated with significant improvement in the appearance of fine lines, coarse wrinkles, radiance/luminosity, pore size, texture/smoothness, hydration and increased firmness.

Conclusions SIG1273 represents a novel CFI with antioxidant, anti-aging, and anti-inflammatory properties that when applied topically is well tolerated and provides benefits to individuals with aging skin.

Keywords: anti-aging, anti-inflammatory, antioxidant, cosmeceutical

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Accepted for publication November 26, 2015

Introduction

Isoprenylcysteine (IPC) small molecules have been identified as a novel class of topical anti-inflammatory

and antimicrobial cosmetic functional ingredients (CFI).[1–3] IPCs have been shown to inhibit signaling activation in the membrane, by competing with isoprenoid groups for prenyl-binding sites on membrane-associated proteins.[4–7] In addition, IPCs modulate signal transduction by inhibiting heterotrimeric G-protein formation and/or presumably by blocking downstream G-protein–effector interactions[8,9] and more recently by binding and activating PPARγ.[10] *In vitro* studies have shown IPC compounds to be effective down modulators of inflammatory responses in platelets, macrophages, and neutrophils.[11–13] Treatment of endothelial cells with IPC derivatives inhibits pro-inflammatory TNF-α stimulation of vascular cell adhesion molecule-1 (VCAM-1) by modulating Rac1 activity[14,15] as well as suppressing purinergic receptor-mediated IL-8, monocyte chemotactic protein-1, growth-regulated oncogene α release.[16] Moreover, topically applied IPC analogs demonstrate *in vivo* anti-inflammatory activity, whose activity is restricted to the site of application,[1] including first in class cosmetic functional ingredient, N-acetyl-S-farnesyl-L-cysteine, which is currently present in several cosmetic products as well as SIG990, a topical drug candidate for rosacea.[17]

SIG1273 (tetramethylhexadecenyl succinyl cysteine) is a second generation IPC CFI with anti-inflammatory activity and was the first reported IPC to also have antimicrobial properties.[2,3] SIG1273 has a broad range of anti-inflammatory activities, abrogating toll-like receptor 2 (TLR2), toll-like receptor 4 (TLR4), and T-cell receptor (TCR) signaling.[3] Moreover, SIG1273 was shown to inhibit ultraviolet B (UVB), phorbol 12-myristate 13-acetate (TPA) and *Propionibacterium acnes* induced pro-inflammatory cytokine release and in a clinical study was shown to provide a benefit to individuals with acne prone skin by reducing *P. acnes* counts and reducing inflammation.[2]

In this study, we report *in vitro* and clinical data demonstrating SIG1273 possesses anti-aging properties against these hallmarks of skin aging. We show SIG1273 is active against damaging ROS acting both as a radical scavenging antioxidant and protecting dermal fibroblasts from hydrogen peroxide-induced oxidative stress. In addition, SIG1273 reduces ultraviolet A (UVA)-induced MMP-1 production, which plays a critical role in the photoaging process[18] and promotes HDF migration *in vitro*, predictive of wound-healing enhancement. Lastly, clinical assessment indicates SIG1273, formulated in a topical cream is well tolerated and provides several benefits to human facial skin including significant improvement in the appearance of fine lines, coarse wrinkles, radiance/luminosity, pore size, and hydration/firmness.

Materials and methods

Reagents

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Organic solvents and hydrogen peroxide were purchased from Fisher Scientific (Hampton, NH, USA). SIG1273 was synthesized according to methods as described in US patent application US 12/616 781. All chemicals were analyzed by LC/MS (Agilent 1100), ^{1}H, and ^{13}C NMR (500 and 125 MHz, Bruker) for structural identity and confirmed to be >95% purity by analytical HPLC (Agilent 1200, Santa Clara, CA, USA).

Cells

Primary human dermal neonatal fibroblasts (HDFs) were purchased from Life Technologies (Gibco, Carlsbad, CA, USA). Cells underwent two passages, using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS).

Antioxidant assay

Radical scavenging antioxidant assay was estimated using the colorimetric antioxidant assay kit obtained from Cayman Chemical Company (Ann Arbor, MI, USA). ABTS (2, 2′-azino-bis-[3-ethylbenzthiazoline sulfonate]) was used as the chromogen, which changes into a colored monocation radical form ($ABTS^{\bullet+}$) by metmyoglobin in the presence of hydrogen peroxide, and monitored by measuring absorption at 750 nm using a plate reader (Envision-PerkinElmer, Waltham, MA, USA). Antioxidant inhibition was calculated based on the discoloration of ABTS by serial concentrations of test compounds added simultaneously with myoglobin and hydrogen peroxide.

Cell-based antioxidant assay

Human dermal fibroblast cells were cultured in DMEM with 10% FBS and seeded into black wall 96-well plates and incubated for 24 h before treatment. Cells were pretreated for 3 h with and without test compounds (0.01–3 μM final concentrations) and labeled with 50 μM of dichloro-dihydro-fluorescein diacetate (DCFH-DA). Total fluorescence (excitation = 485 nm; emission = 535 nm) was measured after cells were co-

incubated for 20 min with 0.1 mM H_2O_2 and the test compounds. Cell protection against oxidative stress was calculated using the following formula:

$$\begin{aligned}\text{Cell protection (\%)} = [&(\text{test compound (\%)} \\ &- \text{hydrogen peroxide only (\%)})/ \\ &(\text{test compound (\%)} \\ &- \text{untreated control (\%)})] \times 100.\end{aligned}$$

Ultraviolet A-induced collagenase assay

Human dermal fibroblast cells were seeded into 24-well plates and incubated for 48 h before treatments. After 24-h incubation, media were replaced with FBS-depleted DMEM medium. Later, medium was replaced with fresh medium containing test compounds (0.03–30 μM final concentrations) and cells were incubated for 6 h, then irradiated with 12.5 J/cm^2 UVA (350–12 nm; Daavlin Co., Bryan, OH, USA) and later incubated for additional 24 h. Media supernatants were harvested for collagenase level measurements.

ELISAs

The levels of human collagenase (pro-MMP1) were measured from HDFs media supernatants by sandwich ELISA using appropriate standards and following manufacture's protocols (R&D Systems Inc., Minneapolis, MN, USA).

In vitro HDF migration assay

The effect of SIG1273 on dermal wound closure was investigated using the CytoSelect™ Wound Healing Assay Kit (Cell Biolabs Inc., San Diego, CA, USA). HDF cells were seeded in full supplemented medium into 24-well plates containing wound field inserts and incubated for 48 h. After reaching confluence, inserts were removed from wells, washed with 1 × DPBS and later treated with test compounds for 8 h. Cells were fixed and stained according to manufacturer's instructions. Representative images of the wound field were photographed using a bright field microscope (Olympus BX41; Olympus Corp., Melville, NY, USA) equipped with a digital CCD camera (Lumenera Corp., Ottawa, ON, Canada) and analyzed using NIH-ImageJ software.[19] Cell migration activity of compounds was compared to untreated controls by counting the number of cells that migrated inside the open field after compound treatments.

Clinical study

The human use study was conducted at Reliance Clinical Testing Services, Inc. (RCTS) (Irving, TX, USA) in accordance with the intent and purpose of Good Clinical Practice regulations described in Title 21 of the U.S. Code of Federal Regulations (CFR), the Declaration of Helsinki, International Conference on Harmonization (ICH) harmonized tripartite guidelines for Good Clinical Practice and/or RCTS Standard Operating Procedures. This was a 31-subject, 4-week study. SIG1273 cream was applied once daily to one side, while the other side of the face was utilized to assess a different formulation and CFI and thus is not included in this report. All subjects gave informed consent and received a topical cream containing 0.25% SIG1273 for application. There were two study visits: at baseline and at week 4. Measurements, observations, and results were only taken from the same SIG1273 treated side of the face. Data obtained for clinical grading were statistically compared to baseline scores using a Wilcoxon signed-rank test. Cutometer measurements were statistically compared to baseline scores using a paired difference *t*-test. Changes from baseline were considered significant at the $P < 0.05$ level. All photographs were taken using the Canfield VISIA CR system which is a self-contained photographic device designed to take photographs under standard, reproducible settings. The device is software driven, and the settings are programmed into the device. The flashes are set for consistent output which ensures reproducible photographs between sessions. The test formulation was applied once daily on designated side of face, and subjects were told to use no other skincare products on their faces during study participation, except for water washable eye makeup and lipstick.

Statistical analysis

Statistical significance was determined by ANOVA followed by a Dunnett multiple comparisons test using *P*-values less than 0.05 as a significant difference. Dose–response curves were generated by fitting data with the Hill, three-parameter equation using the Sigma Plot software, from which the IC_{50} and maximum inhibition were determined.

Results and discussion

SIG1273 protects against reactive oxygen species (ROS)

The production of reactive radicals is significantly increased during cell metabolism and dermal tissue

aging mechanisms.[20] Antioxidant molecules present in the environment and in our bodies function to counteract ROS. We thus sought to determine SIG1273's antioxidant properties by testing its activity as a free radical scavenger and efficacy in inhibiting the induction of intracellular ROS in response to H_2O_2 exposure.

SIG1273's capacity to scavenge reactive radicals was quantitated using a colorimetric antioxidant assay kit (see Materials and Methods). Results demonstrate SIG1273 inhibits hydrogen peroxide-induced free radical reactive oxygen species (IC_{50} = 68 ± 8 μM) with similar activity to vitamin E (α-tocopherol; IC_{50} = 25 ± 0 μM) but significantly greater potency than lipoic acid (IC_{50} = 682 ± 169 μM); two antioxidants commonly used in skin-care products (Fig. 1).[21]

The accumulation of intracellular ROS over time has been implicated in cellular aging mechanisms correlated to sun damage, pollution, and other environmental factors inducers on skin.[22] Intracellular free radicals and other reactive species are constantly generated by cellular metabolism and exacerbated by environmentally produced oxidative damage to lipids, nucleic acids, and proteins.[23] The onset of oxidative stress can be delayed by the introduction of functional antioxidant molecules that can penetrate dermal cells and scavenge these species before their interaction with biomolecules. Therefore, we used a HDF cell-based assay for measuring intracellular reactive oxygen species activity employing the cell-permeable fluorogenic probe 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is diffused into cells pre-treated with potential antioxidants, and the fluorescence intensity is proportional to intracellular ROS levels.[24] We sought to determine SIG1273's intracellular antioxidant capacity of reactive radical scavenging using this method (see Materials and Methods). Results demonstrate SIG1273 inhibits intracellular oxidative stress (37 ± 2%) with similar activity to vitamin E (α-tocopherol; 42 ± 3%) and greater potency than lipoic acid (26 ± 4%) (Table 1). Given the similarity in their lipid tail structures, SIG1273 likely functions similar to vitamin E as a chain-breaking antioxidant preventing the propagation of free radical reactions.[25] In addition, SIG1273 contains a cysteine residue which also has been reported to have antioxidant properties.[26]

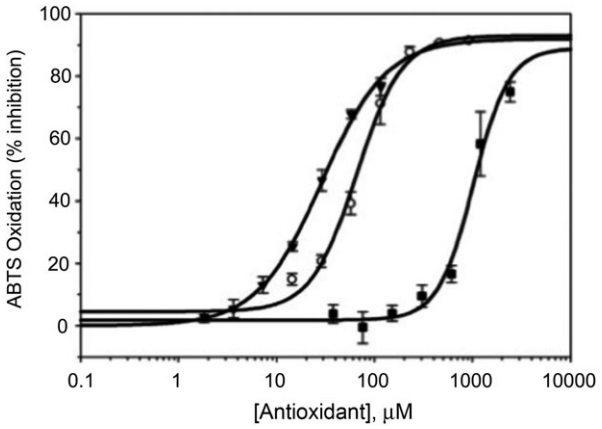

Figure 1 SIG1273 is a direct antioxidant ingredient. Radical scavenging ABTS colorimetric assay: SIG1273 (○), vitamin E (▼) and lipoic acid (■). The data represent the mean ± SD of three independent experiments. The IC_{50} value was determined via the four parameter logistic curve fit using SigmaPlot graphical software (San Jose, CA).

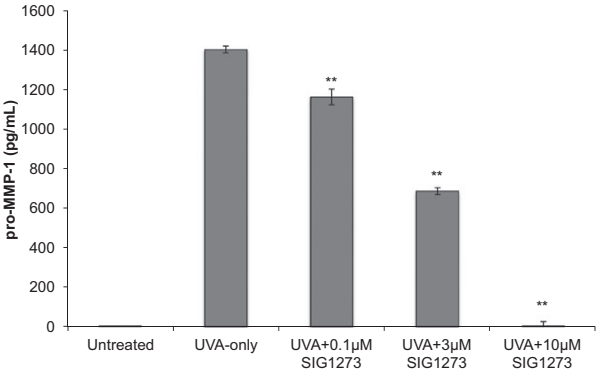

Figure 2 SIG1273 inhibits UVA-induced MMP-1 release from HDFs. Media supernatants were collected 24 h after UVA irradiation, and the content of pro-MMP-1 was measured by ELISA. The data represent the mean ± SD of two independent experiments. $^{**}P < 0.01$ indicates a statistically significant difference compared to UVA-only irradiated cells.

SIG1273 inhibits UVA-induced human fibroblasts collagenase production

UV-induced secretion of matrix metalloproteinases (MMPs) is thought to be a major participant in human cutaneous photoaging.[27] MMP-1, that is, interstitial col-

Table 1 Antioxidant effects of SIG1273 on hydrogen peroxide-treated human dermal fibroblasts (HDFs)*

Treatment	Maximum DCFH-DA fluorescence inhibition (%)
SIG1273	37 ± 2
Lipoic acid	26 ± 4
Vitamin E	42 ± 3

*HDFs were treated with antioxidants for 3 h, and intracellular ROS was probed by DCFH-DA fluorescence after H_2O_2 (0.1 mM) incubation. The data represent the mean ± SD of two independent experiments.

lagenase, induced by ultraviolet A (UVA) irradiation[28] breaks down the extracellular matrix (type I, II, and III collagen) contributing to wrinkle formation. To determine SIG1273's antiwrinkle potential, we tested for its ability to mitigate UVA-induced pro-MMP-1 release from cultured HDFs. SIG1273 dose dependently inhibits pro-MMP-1 release with an IC_{50} = 3 μM (Fig. 2), similar to previous studies with a known MMP-1 release inhibitor, epigallocatechin-3-gallate (EGCG).[29]

SIG1273 promotes human fibroblasts migration

Skin wound-healing capacity is compromised with age, resulting in decreased cell migration, proliferation, and inflammatory responses.[30,31] As one ages, skin elasticity and collagen are reduced hindering the skin's wound-healing ability as well.[30] To evaluate the potential skin repair activity of SIG1273, we utilized the *in vitro* "scratch-open" field assay measuring the stimulation of HDF migration into a cell-free gap on the tissue culture dish.[32] Results show SIG1273 significantly increases the number of cells migrating into the gap with a maximum 49% increase in migratory response at 0.1 μM treatments compared to untreated cells after 8 h (Fig. 3). In addition, positive controls TGF-β1, previously shown to be effective in this assay,[33] were also tested and provided a 36% increase in migratory response activity (Fig. 3). These results are consistent with similar histopathological *in vivo* data observed after topical application of vitamin K1,[34] which also possesses a long chain isoprenoid. This suggests that the cell migration acceleration activity observed by SIG1273 could be due to its phytol moiety. Altogether, the increased activation of HDF migration by SIG1273 suggests it could enhance cutaneous repair.

SIG1273 cream clinical assessment

The data reported here and previously[2,3] demonstrating SIG1273's *in vitro* antioxidant and anti-inflammatory activity suggests this CFI could reduce the signs of aging in human facial skin. We therefore sought to evaluate SIG1273 clinically to assess its tolerance upon topical application. SIG1273 at 0.25% was formulated in a cream and was evaluated in a 31 subject study, where it was applied once daily for 4 weeks. Prior to this study, SIG1273 cream was tested clinically in a 100 subjects human repeated insult patch test and

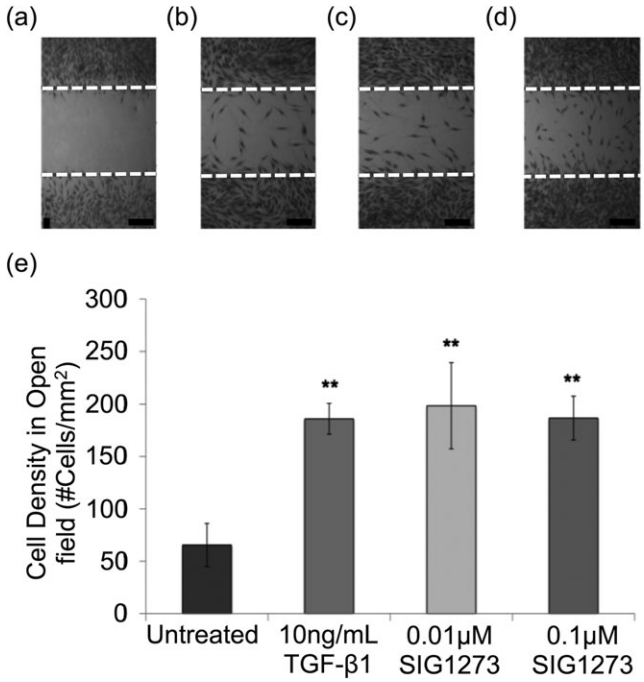

Figure 3 SIG1273 facilitates skin repair in human fibroblasts *in vitro*. A 900-μm cell-free gap (between dotted lines) was produced in confluent cell cultures and was left untreated or treated with SIG1273 or TGF-β1 for 8 h. After 8 h incubation, cells were fixed, stained, and observed by microscopic analysis (see Materials and Methods section). (a) Untreated; (b) 10 ng/mL TGF-β1; (c) 0.01 μM SIG1273; (d) 0.1 μM SIG1273. Scale bar = 225 μm. (e) Data graph of cell densities in open field represents the mean ± SD of two independent experiments. $^{**}P \leq 0.01$ indicates a statistically significant difference compared to untreated cells.

was found to be nonsensitizing and nonirritating (data not shown).

Twenty-nine of thirty-one enrolled subjects (Table 2) completed the 4-week study. Two subjects were dropped for noncompliance (one was on more than one clinical study at the same time, and the other missed the final visit). No signs of erythema, dryness, edema, and/or peeling were observed by the investigator at baseline and week 4, demonstrating the SIG1273 cream was well tolerated (data not shown). These results match previous reports indicating SIG1273 is well tolerated when applied topically.[2] At each specific time point, subjects participated in Objective Tolerance Evaluations assessing their face for the presence of burning, stinging, itching, and tingling and noticed no change, suggesting the subjects did not experience any self-perceived tolerability issues.

Next, we sought to determine what benefits SIG1273 cream could provide by measuring its activity *vs.* several skin-aging parameters (Table 3). Subjects assessed visually by an expert evaluator demonstrated significant improvement in the appearance of fine lines (−9.8%), coarse wrinkles (−12.5%), radiance/luminosity (−17.0%), and pore size skin (−21.6%) (Table 3). Furthermore, texture/smoothness was determined by touch and showed improvement (−39.3%) (Table 3). In addition to clinical evaluations, at baseline and week 4, subjects had Cutometer measurements taken in the center of both cheeks to determine elasticity and hydration/firmness of the skin. Results demonstrate those applying SIG1273 cream had a 20.7% improvement in elasticity and 24.1% improvement in hydration/firmness (Table 4). For all endpoints measured, there was a significant improvement from baseline (Tables 3 and 4) as subjects demonstrated visual improvement in the signs of aging from baseline to week 4 (Fig. 4). Given these encouraging initial results, we next plan to perform a 12-week clinical study.

Table 2 Demographics

Characteristic	Completed (n = 29)
Age (±SD)	53.6 (7.4)
Range	37–66
Gender (male/female)	2/27
Ethnicity (Caucasian/Hispanic)	24/5
Fitzpatrick skin type (I–V)	I – 1
	II – 5
	III – 20
	IV – 2
	V – 1
Skin type	Dry – 3
	Normal – 11
	Oily – 1
	Combination – 14

Table 3 Summary of skin-aging endpoints*

Parameter measured	% Change improvement
Fine lines	−9.8% (41.4%)
Coarse wrinkles	−12.5% (41.4%)
Skin radiance/brightness/luminosity	17.0% (58.6%)
Skin texture/smoothness	39.34% (69.0%)
Pore size	−21.6% (55.2%)

The number in parenthesis indicates the percent of the population that showed an improvement.
*Percent change improvement is relative to baseline. A negative number indicates a reduction.

Summary

SIG1273 has previously been shown to possess anti-inflammatory and antimicrobial properties *in vitro* and *in vivo*. Results here demonstrate SIG1273 inhibits UVA-induced MMP-1 production and promotes wound healing in HDFs. Moreover, SIG1273 was shown to be

Table 4 Summary of cutometer measurements

Parameter measured	% Change improvement
Elasticity	20.7
Firmness/hydration	24.1

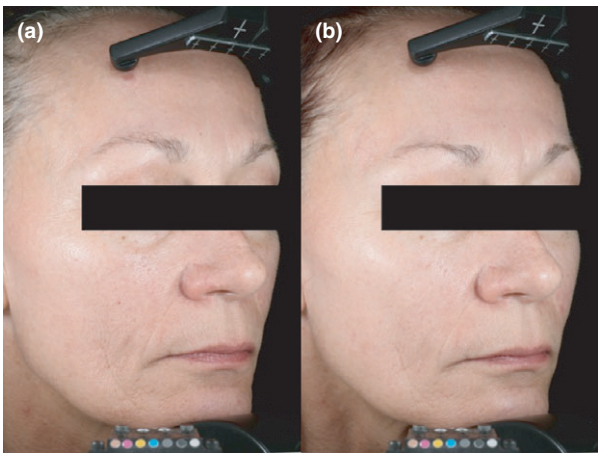

Figure 4 SIG1273 cream improves appearance of facial wrinkles. Application of a 0.25% SIG1273 cream results in clinical improvement in several signs of aging. Standardized photographs of the face of a 50-year-old woman at (a) baseline and (b) after 4-week application of 0.25% SIG1273 cream.

a radical scavenging antioxidant, intracellularly as well as in a cell-free system. Clinical assessment of SIG1273 cream (0.25%) showed it was well tolerated with significant improvement in the appearance of fine lines, coarse wrinkles, radiance/luminosity, pore size, texture/smoothness hydration, and increased firmness. Altogether, these results indicate SIG1273 *in vitro* and when applied topically provides benefits to individuals with aging skin.

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