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Anti-inflammatory and anti-bacterial properties of tetramethylhexadecenyl succinyl cysteine (TSC): a skin-protecting cosmetic functional ingredient

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Synopsis

BACKGROUND: The skin is the first line of defence against exposure to microbial, physical, environmental and chemical insults. In mobilizing a protective response, several different cell types located in our skin release and respond to pro-inflammatory cytokines ensuring skin homeostasis and health. However, chronic activation of this response eventually causes damage resulting in premature ageing. Diosodium tetramethylhexadecenyl succinyl cysteine (TSC or SIG1273), an isoprenylcysteine small molecule, down modulates these inflammatory signalling pathways in various cell types (keratinocytes, peripheral blood mononuclear cells (PBMCs) and endothelial cells) and possesses anti-bacterial properties. Thus, TSC represents a novel cosmetic functional ingredient that provides a broad spectrum of benefits for the skin.
OBJECTIVE: To assess the anti-inflammatory properties of TSC in several cutaneous cell types and further investigate its anti-microbial activity.
METHODS: Cultured normal human epidermal keratinocytes were exposed to chemical irritant phorbol 12-myrisate 13-acetate (TPA) or ultraviolet-B light (UVB) to induce pro-inflammatory cytokine (IL-6, IL-8 and TNF-α) production. T-cell receptor (TCR) activation of PBMCs and nickel (Ni^{2+}) treatments of human dermal microvascular endothelial cells (HDMECs) were performed resulting in IL-4, IL-6, IL-8 and IL-17 production. *Streptococcus pyogenes* were cultured to determine minimal inhibitory concentration values.
RESULTS: *In vitro* studies demonstrate TSC blocks TPA and UVB-induced cytokine production in cultured keratinocytes. Similarly, TSC inhibits overproduction of IL-4 and IL-17 in T-cell receptor (TCR)-activated PBMCs as well as nickel induction of IL-6 and IL-8 in HDMECs. Lastly, TSC demonstrated anti-microbial properties, inhibiting cell growth of *S. pyogenes*.
CONCLUSIONS: Tetramethylhexadecenyl succinyl cysteine represents a novel cosmetic functional ingredient that provides a dual modulating benefit of skin protection to individuals by reducing inflammation in keratinocytes, endothelial and mononuclear cell types and *S. pyogenes* counts.

Résumé

CONTEXTE: La peau est la première ligne de défense contre l'exposition aux microbes, les insultes, l'environnement physique et chimique. En mobilisant une réponse protectrice, plusieurs types différents de cellules situées dans notre peau sécrètent et/ou répondent à des cytokines pro-inflammatoires ainsi assurant l'homéostasie de la peau et la santé. Cependant, l'activation chronique de cette réponse, finalement provoque des dommages conduisant à un vieillissement prématuré. Le Diosodium Tetramethylhexadecenyl succinoyle cystéine (TSC ou SIG1273), une petite molécules d'isoprenylcysteine (IPC), diminue l'activité de ces voies de signalisation inflammatoires dans divers types de cellules (kératinocytes, des cellules mononucléées du sang (PBMC) et des cellules endothéliales) et possède des propriétés antibactériennes. Ainsi, le TSC représente un ingrédient fonctionnel cosmétique nouveau qui offre un large éventail d'avantages pour la peau.
OBJECTIF: évaluer les propriétés anti-inflammatoires du TSC dans plusieurs types de cellules cutanées et enquêter davantage sur son activité antimicrobienne.
MÉTHODES: Des cultures de kératinocytes épidermiques humains normaux (NHEK) ont été exposées au phorbol irritant, le 12-myrisate 13-acétate chimique (TPA) ou aux irradiations d'ultraviolet-B (UVB) pour induire la production des cytokines pro-inflammatoires (IL-6, IL-8 et TNF-α). L'activation des récepteurs des cellules T (TCR) des PBMC et le traitement au nickel (Ni^{2+}) des cellules endothéliales microvasculaires dermiques humaines (HDMECs) ont été réalisés, résultant en la production d'IL-4, IL-6, IL-8 et IL-17. Du *Streptococcus pyogenes* a été cultivé afin de déterminer la concentration minimale inhibitrice (CMI).
RÉSULTATS: Les études *in vitro* montrent que le TSC bloque la production des cytokines induite par TPA et UVB dans les kératinocytes en culture. De même, le TSC inhibe la surproduction d'IL-4 et IL-17 dans des cellules PBMS aux récepteurs T (TCR) activés ainsi que l'induction de l'IL-6 et IL-8 dans HDMECs par le nickel. Enfin, le TSC démontre des propriétés antimicrobiennes, l'inhibition de la croissance des cellules de *S. pyogenes*.
CONCLUSION: Le TSC représente un ingrédient fonctionnel cosmétique nouveau qui offre une double protection de la peau pour les personnes, en réduisant l'inflammation dans les kératinocytes et les cellules endothéliales et mononucléaires ainsi qu'en diminuant le nombre d' *S. pyogenes*.

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Introduction

Acute environmental insults stimulate cutaneous protective and reparative responses. Hyperactivity of these responses due to

chronic exposure to these environmental stressors damages the skin, which accelerates ageing. The immune system allows the skin to resist infection from pathogens and helps to combat extrinsic insults such as photo damage, pollution and chemical irritants. The main external culprit in skin ageing is ultraviolet (UV) radiation, which results in the production of oxidative reactive oxygen species (ROS), DNA damage and chronic inflammation promoted by pro-inflammatory cytokines [1]. In addition, bacteria and/or bacterial products directly activate host tissue resident inflammatory cells triggering inflammatory signalling pathways [2]. Identifying and developing new ingredients and/or compounds that modulate these signalling pathways are attractive candidates for providing the skin with the additional defence that is required.

Isoprenylcysteine (IPC) analogues have been identified as a novel class of anti-inflammatory and anti-microbial small molecules for topical use [3, 4]. IPC analogues contain a 15- or 20-carbon side chain attached to the amino acid cysteine, mimicking the C-terminus of processed CAAX proteins (where C is cysteine, A usually an aliphatic amino acid and X is any amino acid) [5]. In cells, proteins possessing a CAAX sequence are subject to a series of post-translational modifications that begin with the addition of a farnesyl or geranylgeranyl isoprenoid to the cysteine sulphur. The hydrophobic isoprenoid group localizes the protein to the endoplasmic reticulum where the 'AAX' C-terminal residues distal to the prenylated cysteine are cleaved. The exposed prenylcysteine alpha carboxyl is then subject to methylesterification and demethylation [5]. IPCs have been shown to inhibit signalling activation in the membrane, by competing with isoprenoid groups for prenyl-binding sites of interaction at the membrane [6–9]. In addition, IPCs modulate signal transduction by inhibiting heterotrimeric G-protein formation and/or presumably by blocking downstream G-protein-effector interactions [10, 11] and recently by binding and activating PPARγ [12]. Moreover, IPCs have been shown to abrogate toll-like receptor 2 inflammatory signalling [4], and below, we demonstrate toll-like receptor 4 modulation as well.

In vitro studies have shown IPC compounds to be effective down modulators of inflammatory responses in neutrophils, macrophages and platelets [13–15]. Treatment of endothelial cells with IPC derivatives blocks pro-inflammatory TNF-α stimulation of vascular cell adhesion molecule-1 (VCAM-1) by modulating Rac1 activity [16, 17] as well as suppressing GPCR-mediated pro-inflammatory cytokine release [18]. Moreover, topically applied IPC analogues demonstrate *in vivo* anti-inflammatory activity [3], including first in class cosmetic functional ingredient, *N*-acetyl-S-farnesyl-L-cysteine, which is currently present in several cosmetic products.

Similar to *N*-acetyl-S-farnesyl-L-cysteine, second generation IPC cosmetic functional ingredient, diosodium tetramethylhexadecenyl succinyl cysteine (TSC) (Fig. 1) possesses anti-inflammatory properties, but is also the first IPC reported to have anti-microbial activity [4]. TSC (using its compound ID number) was recently shown to kill *Propionibacterium acnes* (*P. acnes) in vitro* as well as on humans, where facial scrub samples demonstrate subjects applying TSC had almost a 1.0 logarithmic *P. acnes* colony reduction (-0.9Log_{10}) [4]. Moreover, TSC was shown to inhibit *P. acnes* and peptidoglycan-induced IL-8 secretion using normal human epidermal keratinocytes (NHEKs) as well as reducing inflammatory lesions and microcomedones in subjects with acne prone skin [4]. Here we demonstrate that TSC, a novel cosmetic functional ingredient, has a broader range of anti-inflammatory mechanisms and anti-bacterial properties that may confer additional protection for the skin.

Materials and methods

Reagents

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

Anti-microbial assays

Streptococcus pyogenes (ATCC 19615) was grown in tryptic soy agar with 5% BAP at 35–37°C, in carbon dioxide. Minimal inhibitory concentration (MIC) testing for *S. pyogenes* was performed by the CRO ATS Labs (Eagan, MN, U.S.A.). In short, TSC was diluted in DMSO to make 2 mg mL^{-1} stock solution. Aliquots of 10 μL of each TSC dilution (two-fold, e.g. 100, 50, 25, down to 0.8 μg mL^{-1}) were then added to two empty rows of eight wells on the microtitre plate. One hundred and ninety microlitre aliquot of diluted *S. pyogenes* was then added to each well. The microtitre well plate was then incubated and assayed for MIC.

Cell treatments

Human primary cells obtained from pooled donors were purchased from Cascade Biologics (NHEKs; Gibco, Carlsbad, CA, U.S.A.), ScienCell (HDMECs; Carlsbad, CA, U.S.A.) and 3H Biomedical [peripheral blood mononuclear cells (PBMCs); Uppsala, Sweden]. Cells were grown at normal conditions (5% CO_2; 37°C) and later pre-incubated for 2–6 h with TSC (0.1–30 μM; 1% v/v ethanol vehicle) in growth factor-depleted fresh media in triplicates. NHEKs were induced by 5 ng mL^{-1} TPA or irradiated with 25 mJ cm^{-2} broadband 305-12 nm UVB (Daavlin, Bryan, OH, U.S.A.). HDMECs and PBMCs were induced with 1 mM $NiSO_4$ (Sigma-Aldrich Co.) and Dynabeads T-activator anti-CD3/CD28 (Life Technologies, Carlsbad,

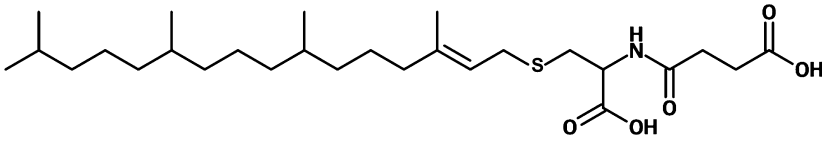

Figure 1 Diosodium tetramethylhexadecenyl succinyl cysteine (TSC) chemical structure.

CA, U.S.A.), respectively. Media supernatants were harvested after 24 h induction for cytokine measurements. Cells were subjected to viability tests by MTS assay (Promega, Madison, WI, U.S.A.).

Cytokine ELISA

The levels of human IL-4, IL-6, IL8, TNF-α from cell media supernatants were assayed by sandwich ELISA using appropriate standards and following manufacture's protocols (BD Biosciences, San Jose, CA, U.S.A.). Human IL-17a levels were measured using standard ELISA-kit from Antigenix America, Inc. (Huntington Station, NY, U.S.A.).

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. For all anti-bacterial measurements and cytokine levels, samples were assayed in triplicate. Cytokine 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

TSC inhibits UVB and chemical irritant (TPA)-induced IL-6, IL-8 and TNF-α release from human keratinocytes

Secretion of several pro-inflammatory cytokines and chemokines by epidermal keratinocytes (the main cellular constituent of the epidermis) is activated as part of the initial phase of the danger response which forms the basis of various inflammatory skin conditions when continually activated over time [19]. Two common environmental stressors that cause skin damage and ageing by chronically activating inflammatory signalling pathways are ultraviolet (UV) light and chemical irritation. Previous studies demonstrate human keratinocytes treated with chemical irritant, phorbol 12-myrisate 13-acetate (TPA), result in increased production of several pro-inflammatory mediators [19, 20] as does UVB irradiation. To determine TSC's skin protecting properties, we tested for its ability to mitigate UVB and TPA-induced inflammation. Our results show TSC inhibits UVB-induced IL-6 release from NHEKs in a dose-dependent manner (Fig. 2, Table I). Furthermore, TSC blocks TPA-induced IL-8 and TNF-α production from NHEKs with similar to or greater activity than clobetasol, a potent topical corticosteroid (Table I).

TSC blocks nickel-induced IL-6 and IL-8 release from human endothelial cells

Nickel-induced allergic contact dermatitis (ACD) is a delayed-type hypersensitive reaction. Approximately, 4–8% of males and 18–30% of females in the industrialized part of the world are sensitized to nickel, and recent reports indicate ACD occurs in subjects with eczema [21]. Nickel treatment of endothelial cells has been shown to activate toll-like receptor-4 mediated induction of pro-inflammatory cytokine release (IL-6, IL-8) [22]. Our results demonstrate human dermal microvascular endothelial cells (HDMECs), treated with nickel, induce the overproduction of IL-6 (Fig. 3) and IL-8 (Table II). Treatments with TSC reduced the release of these pro-inflammatory mediators (Fig. 3, Table II) dose dependently with

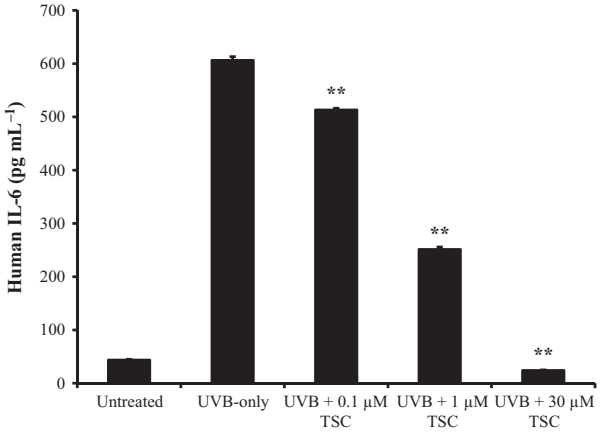

Figure 2 TSC inhibits UVB-induced IL-6 release from human keratinocytes 11(NHEKs). Media supernatants were collected 24 h after UVB irradiation, and the content of IL-6 was measured by ELISA. The data represent the mean SD of three independent experiments. *$P < 0.05$ and **$P < 0.01$ indicate a statistically significant difference compared to UVB-only irradiated cells.

Table I TSC anti-cytokine effects on UVB and TPA-treated keratinocytes (NHEKs)*

Compound	Inducer	IL-6 IC_{50} (μM)	Inducer	IL-8 IC_{50} (nM)	TNF-α IC_{50} (nM)
TSC	UVB	3	TPA	10	11
Clobetasol	UVB	ND	TPA	40	10

TSC, tetramethylhexadecenyl succinyl cysteine.
*NHEKs were cultured in the presence of TSC at various concentrations followed by addition of the stimulant 25 mJ cm^{-2} UVB or 5 ng mL^{-1} TPA. Media supernatants were collected and analysed by ELISA for IL-6, IL-8 and TNF-α. Cell cytotoxicity was not observed at concentrations tested. ND = not determined.

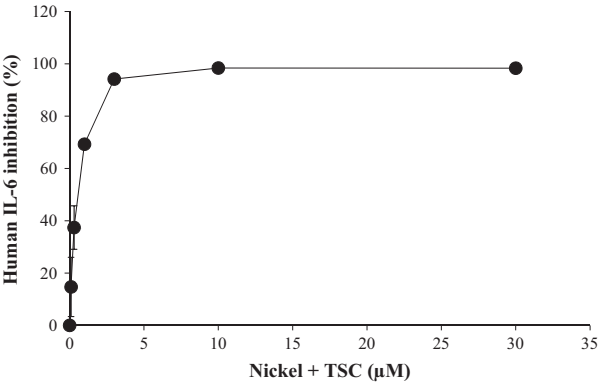

Figure 3 TSC inhibits Nickel-induced IL-6 release from human dermal microvascular endothelial cells (HDMECs). Media supernatants were collected 24 h after nickel induction, and the content of IL-6 was measured by ELISA. The inhibition data represent the mean ± SD of three independent experiments.

potency similar to clobetasol (Table II), suggesting a potential role for TSC in protecting the skin from metal-induced contact hypersensitivity.

TSC reduces T-cell receptor induced IL-4 and IL-17 release from human peripheral mononuclear cells

In addition to endothelial cells and keratinocytes, PBMCs are a critical component of the immune system, fighting off infection and adapting to foreign substances [23]. The cytokines produced in the skewed immune responses (e.g. IL-17 and IL-4) have received great attention as potential targets for dermal protection. Th2 and Th17 cell proliferation and the production of pro-inflammatory cytokines, IL-4 and IL-17 can be induced by utilizing anti-CD3/CD28 beads as a (TCR)-activator. Our results demonstrate treating PBMCs with TSC inhibits anti-CD3/CD28-induced cytokine release of both IL-4 and IL-17 in a dose-dependent manner (Fig. 4, Table III) and with greater potency than clobetasol (Table III).

TSC demonstrates anti-microbial activity against *S. pyogenes*

Bacteria invade normal skin, broken skin or wounds resulting in a variety of skin infections activating inflammatory responses. Common pathogens for inflammatory skin conditions are *P. acnes* [24] and *S. pyogenes* [25]. Previously, TSC was shown to inhibit *P. acnes* growth [4]. To investigate the anti-bacterial properties of TSC against other common skin microflora, cultures with *S. pyogenes* were prepared. TSC was tested in a concentration range of 0 to 100 μg mL^{-1} using two-fold serial dilutions and was determined to have a MIC of 2 μg mL^{-1} vs. *S. pyogenes* (MIC represents the lowest concentration to prevent bacterial growth). Thus, TSC demonstrates anti-microbial properties against two common skin bacteria.

Summary

Previously, TSC was shown to reduce inflammation and *P. acnes* counts in acne prone skin [4]. Results here show in cultured keratinocytes that TSC inhibits UVB and TPA-induced IL-6, IL-8 and TNF-α production secretion. Studies utilizing HDMECs demonstrate TSC blocks nickel-induced IL-6 and IL-8 cytokine release. Furthermore, studies using PBMCs, which are a key part of one's immune response, show that TSC reduces T-cell receptor-activated IL-4 and IL-17 overproduction. Anti-microbial studies demonstrate TSC to possess anti-bacterial properties against *S. pyogenes*. The mechanism of action of TSC, an IPC mimic, could be linked to

Table II Anti-inflammatory effects of TSC on Nickel-treated endothelial cells (HDMECs)*

Treatment	Inducer	IL-6 IC_{50} (μM)	IL-8 IC_{50} (μM)
TSC	Nickel	1	0.1
Clobetasol	Nickel	1	0.05

TSC, tetramethylhexadecenyl succinyl cysteine.
*HDMECs were cultured in the presence of each compound at various concentrations simultaneous to addition of Nickel (1 mM $NiSO_4$). Media supernatants were collected and analysed by ELISA for IL-8 and IL-6. Cell cytotoxicity was not observed at concentrations tested.

Table III Anti-cytokine effects of TSC on anti-CD3/CD28-treated mononuclear cells (PBMCs)*

Treatment	Inducer	IL-4 IC_{50} (μM)	IL-17 IC_{50} (μM)
TSC	Anti-CD3/28	0.7	0.4
Clobetasol	Anti-CD3/28	27	>100

TSC, tetramethylhexadecenyl succinyl cysteine.
*PBMCs were cultured in the presence of each compound at various concentrations followed by addition of the stimulant anti-CD3/CD28 beads. Media supernatants were collected and analysed by ELISA for IL-17 and IL-4. Cell cytotoxicity was not observed at concentrations tested.

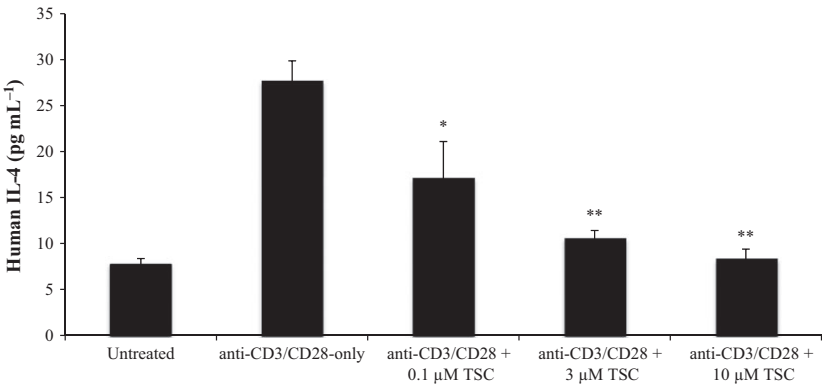

Figure 4 TSC inhibits TCR-induced IL-4 release from human peripheral blood mononuclear cells (PBMCs). Media supernatants were collected 24 h after anti-CD3/CD28-induction, and the content of IL-6 was measured by ELISA. The data represent the mean ± SD of three independent experiments. *$P < 0.05$ and **$P < 0.01$ indicate a statistically significant difference compared to anti-CD3/CD28-only treated cells.

the modulation of G-protein signal transduction or G-protein coupled receptors (GPCRs) translocation of activated inflammatory pathways as well as modulation of toll-receptor signalling. More studies are required to fully understand TSC's receptor targets. Altogether, these results highlight the broad action of TSC to protect against UV, chemical (TPA) and metal-induced inflammation. Paired with its ability to inhibit bacterial growth, TSC represents a novel cosmetic functional ingredient that can be used alone or in combination with other skin care molecules to protect the skin.

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Conflict of interest

JSG is a paid consultant for Signum Dermalogix, whereas JSF, KR, MV, MS, KLH and EP are employees. JBS serves on the board of directors. All authors have stock and/or stock options in the company.

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