Accepted: 9 May 2018
DOI: 10.1111/exd.13692

ORIGINAL ARTICLE

WILEY Experimental Dermatology

SIG1459: A novel phytyl-cysteine derived TLR2 modulator with in vitro and clinical anti-acne activity

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Abstract

Cutibacterium (formerly *Propionibacterium acnes*) is a major contributor to the pathogenesis of acne. *C. acnes* initiates an innate immune response in keratinocytes via recognition and activation of toll-like receptor-2 (TLR2), a key step in comedogenesis. Tetramethyl-hexadecenyl-cysteine-formylprolinate (SIG1459), a novel anti-acne isoprenylcysteine (IPC) small molecule, is shown in this study to have direct antibacterial activity and inhibit TLR2 inflammatory signalling. In vitro antibacterial activity of SIG1459 against *C. acnes* was established demonstrating minimal inhibitory concentration (MIC = 8.5 μmol\L), minimal bactericidal concentration (MBC = 16.1 μmol\L) and minimal biofilm eradication concentration (MBEC = 12.5 μmol\L). To assess SIG1459's anti-inflammatory activity, human keratinocytes were exposed to *C. acnes* and different TLR2 ligands (peptidoglycan, FSL-1, Pam3CSK4) that induce pro-inflammatory cytokine IL-8 and IL-1α production. Results demonstrate SIG1459 inhibits TLR2-induced IL-8 release from TLR2/TLR2 (IC_{50} = 0.086 μmol\L), TLR2/6 (IC_{50} = 0.209 μmol\L) and IL-1α from TLR2/TLR2 (IC_{50} = 0.050 μmol\L). To assess the safety and in vivo anti-acne activity of SIG1459, a vehicle controlled clinical study was conducted applying 1% SIG1459 topically (n = 35 subjects) in a head-to-head comparison against 3% BPO (n = 15 subjects). Utilizing the Investigator Global Assessment scale for acne as primary endpoint, results demonstrate 1% SIG1459 significantly outperformed 3% BPO over 8 weeks, resulting in 79% improvement as compared to 56% for BPO. Additionally, 1% SIG1459 was well tolerated. Thus, SIG1459 and phytyl IPC compounds represent a novel anti-acne technology that provides a safe dual modulating benefit by killing *C. acnes* and reducing the inflammation it triggers via TLR2 signalling.

KEYWORDS
acne, anti-inflammatory, *Cutibacterium acnes*, isoprenylcysteine analog, toll-like receptor

1 | INTRODUCTION

Acne vulgaris is one of the most common skin diseases, affecting ~50 million people in the US alone.[1] An inflammatory disease of the pilosebaceous unit, acne's pathological process starts with hypersecretion of lipid-rich sebum providing a medium for the growth of *Cutibacterium* (formerly *Propionibacterium acnes*).[2] *C. acnes* hydrolyse sebum lipids releasing unsaturated fatty acids

capable of inducing hyperkeratinization of the follicular epithelium resulting in comedone formation.[3] The comedone entrapped *C. acnes* are postulated to undergo a virulent transformation and interact with keratinocytes, initiating the production and release of pro-inflammatory mediators attracting neutrophils and macrophages.[4,5] These events progress to comedone wall rupture, releasing bacteria to surrounding inflammatory cells, further amplifying inflammation.[6,7] Hyperkeratinization and the inflammatory activation of keratinocytes and macrophages have all been shown to be mediated through toll-like receptor-2 (TLR2), whose signalling also plays a key role in the pathogenesis of several dermatological diseases.[8,9] Moreover, it has recently been demonstrated that TLR2 formation of TLR2 homodimers and/or TLR2/6 heterodimers are required for *C. acnes* recognition in human cells.[10]

Current treatments for acne and/or acne prone skin include topical and oral retinoids, antibiotics, and topical antimicrobials, all of which have potential adverse effects. In addition, long-term exposure to antibiotics can result in selection and growth of antibiotic-resistant bacteria. Furthermore, the oxidizing agent benzoyl peroxide (BPO) for which resistance does not occur can result in skin bleaching, dryness and stinging. Thus, development of safe, new compounds which provide relief to individuals with skin prone to acne is desired.

Isoprenylcysteine (IPC) analogs represent a novel class of topical non-steroidal anti-inflammatories,[11] which have also been previously reported to have antibacterial and anti-acne properties.[12] IPC analogs contain a 15- or 20-carbon side chain linked to the amino acid cysteine, thereby mimicking the C-terminus of processed CAAX proteins.[13] Introducing chemical modifications to both the fatty acid tail and cysteine moieties of the IPC backbone, yielded a novel phytyl IPC compound: Tetramethyl-hexadecenyl-cysteine-formylprolinate (SIG1459) (Supplemental data, Figure S1). In this study, we report that SIG1459 possesses in vitro antibacterial and anti-inflammatory properties inhibiting two TLR2 family members required for *C. acnes* recognition, TLR2 homodimer and TLR2/6 heterodimer. Furthermore, clinical results in subjects with acne prone skin demonstrate SIG1459 is well-tolerated and significantly outperforms benzoyl peroxide (BPO) when applied topically for 8 weeks.

2 | METHODS

2.1 | Reagents

BPO, salicylic acid and organic solvents were purchased from Fisher Scientific (Hampton, NH). Pam3CSK4 and FSL-1 were obtained from InvivoGen (San Diego, CA, USA). CU-CPT22 was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). SIG1459 was synthesized according to methods as described in US patent application US 12/616,781. Compounds were analysed by LC/MS (Agilent 1100), ^{1}H and ^{13}C NMR (500 MHz and 125 MHz, Bruker) for structural identity and confirmed to be >95% purity by analytical HPLC (Agilent 1200; Santa Clara, CA, USA).

2.2 | Antimicrobial assays

Cutibacterium acnes strains (ATCC® 6919™, ATCC® 11827™) were cultured in Reinforced Clostridial or Brain Heart Infusion broth media (BD Diagnostics; Sparks, MD) under anaerobic conditions (37°C, under shaking at 175 rpm). Compounds to be tested for antibacterial activity were added dissolved in water or DMSO, diluted by 2-fold serial titrations and then 7.5 μL added to 142.5 μL of a 7.5×10^5 CFU/mL bacteria suspension and incubated for 72 hours. Turbidity (OD_{595}) was measured using a microplate reader (PerkinElmer; Houston, TX, USA). The Minimum Inhibitory concentration (MIC) was defined as the lowest concentration of an agent that achieved ≥90% growth inhibition. The Minimum Bactericidal concentration (MBC) was determined by sub-culturing broth samples after test material incubation from the MIC assay to agar plates. MBC was defined as the lowest antimicrobial concentration that completely inhibited colony formation. For Minimum Biofilm Eradication concentration (MBEC) determination, we followed protocols previously described[14] using the crystal violet staining assay. MBEC was defined as the minimum concentration necessary to achieve ≥80% eradication of attached biofilm compared to vehicle control.

2.3 | Cell culture and assays

Primary NHEKs from neonatal origin, purchased from Thermo-Fisher (Carlsbad, CA, USA) were cultured at low calcium (0.06 mmol\L) for 24 hours in full growth factor-supplemented EpiLife™ medium (Gibco; Gaithersburg, MD, USA). Twenty-four hours before treatment, medium was replaced with growth factor-depleted medium. NHEKs were exposed to *C. acnes* (1×10^7 CFU/mL), PGN (10 μg/mL), Pam3CSK4 (10 μg/mL) or FSL-1 (0.1 μg/mL) and incubated for 24 hours with test compounds. Cell proliferative capacity was measured after compound treatments by MTS reduction assay. Only non-toxic concentrations (≤1 μmol/L) were used for anti-cytokine experiments (Supplemental data, Figure S2). Media supernatants were then harvested for cytokine measurements using human interleukin-8 (BD Biosciences; San Jose, CA, USA) and interleukin-1α (R&D Systems; Minneapolis, MN, USA) levels were measured by ELISA following manufacturer's protocols. Inhibition activity for each compound was calculated by the difference between the vehicle-only and inducer plus vehicle treated cells. IC_{50} values were determined from concentration-dependent curves using 4-parameter logistic equation in Sigma Plot (Systat Software; San Jose, CA, USA).

2.4 | Clinical study

A multi-site study was conducted at Princeton Consumer Research Group (PCR; Princeton, NJ and UK) 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, and/or PCR Standard Operating Procedures. This was a single-blinded vehicle-controlled study, with 65 of the 69 subjects enrolled completing the study (two subjects in the 1% SIG1459 group and two subjects

in the vehicle control group were lost to follow-up). The subjects were 18 years and above. Subjects reported to the testing facility for baseline screening at which time Informed Consent and demographics were obtained. Inclusion/Exclusion criteria were verified, using expert clinical grading evaluations to determine eligibility. Subjects had to exhibit mild to moderate acne on their face (mild to moderate acne on the Investigator Global Assessment (IGA) acne scale = 2 or 3, where 2 is mild severity; some non-inflammatory lesions with no more than a few inflammatory lesions (papules/pustules only, no nodular lesions) and 3 is moderate severity; up to many non-inflammatory lesions and may have some inflammatory lesions, but no more than one small nodular lesion). Thirty-five subjects received 1% SIG1459 cream, 15 subjects 3% benzoyl peroxide (BPO) cream and 15 subjects the vehicle cream for facial application (Supplemental data, Table S1 for subject demographics). The test formulations were applied *bid*, morning and evening and subjects were instructed to use no other skin-care products on their faces during Study participation, except for water washable eye makeup and lipstick. Subjects received a written explanation of the nature of the study and the possibility of being in the vehicle control group.

Study duration was for 8 weeks with four evaluations: at enrolment (Baseline), Week 2, Week 4 and Week 8. At each visit, subjects underwent expert clinical grading and completed a self-perception questionnaire at the end of the study. Photography was completed for subjects in treatment cell 1% SIG1459 only.

2.5 | Statistical analysis

For all antibacterial measurements and cytokine levels, samples were assayed in triplicates. Statistical significance was determined by ANOVA followed by a Dunnett multiple comparisons test using *P*-values less than .05 as significant difference. Cytokine concentration-response curves were generated by fitting data with the logistic, four-parameter equation using the Sigma Plot software, from which the IC_{50} and maximum inhibition were determined. For the Clinical study, the paired one–way ANOVA was used to assess within-subject improvements over baseline, and the unpaired one–way ANOVA to compare responses between SIG1459, BPO and vehicle treatments. $P < .05$ was considered significant.

3 | RESULTS

3.1 | SIG1459 possesses antibacterial activity against *Cutibacterium acnes*

Previously, we demonstrated that IPCs possessed antimicrobial activity vs *Cutibacterium acnes*, exhibiting a significant minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC).[12] To investigate the antibacterial properties of the new phytyl IPC compound, identified as SIG1459 and compare its activity to commonly used anti-acne cosmetic actives and drug compounds, we determined MIC and MBC values vs two common commercially available *C. acnes* strains isolated from facial acne (ATCC® 6919™ and ATCC® 11827™). SIG1459 demonstrated antibacterial activity against the two *C. acnes* strains as did BPO and clindamycin. SIG1459's MIC value of 8.5 μmol\L was ~200 times more potent than BPO (1.75 mmol\L) and it's MBC value of 16.1 μmol\L, was ~800 times more active than BPO (Table 1). Clindamycin demonstrated the strongest antibacterial activity (MIC = 0.3 μmol\L, MBC = 2 μmol\L) (Table 1). As expected, salicylic acid had a negligible effect against *C. acnes*.

Recently, it has been suggested that *C. acnes* cells residing within the follicles grow as a biofilm, which in turn can make them more resistant to antimicrobial agents.[15] Thus, in addition to demonstrating strong MIC and MBC activity, we explored for the first time the potential of IPC compounds such as SIG1459 to specifically eradicate biofilms. Our results demonstrate SIG1459 disrupts *C. acnes* biofilms with a minimal biofilm eradication concentration (MBEC) of 12.5 μmol\L, once again significantly outperforming BPO and salicylic acid (MBEC > 10 mmol\L) (Table 1). As was the case with MIC and MBC determination, clindamycin was the most potent with an MBEC = 0.6 μmol\L (Table 1).

3.2 | SIG1459 inhibits TLR2-activated cytokine release from human keratinocytes

TLR2 expression is increased in acne lesions, facilitating recognition of *Cutibacterium acnes* and contributing to the inflammatory response.[6,16] *C. acnes* activates TLR2 signalling in keratinocytes of the intact comedone wall and macrophages after it is released to the surrounding dermis following comedone rupture. Our results demonstrate that SIG1459 strongly inhibits *C. acnes*-induced IL-8 levels in NHEKs with an IC_{50} = 0.003 μmol\L (Figure 1), several orders

TABLE 1 Antimicrobial activity against *Cutibacterium acnes*

Compound	ATCC® 6919™ MIC[a] (μmol\L)	ATCC® 11827™ MIC[a] (μmol\L)	ATCC® 6919™ MBC[b] (μmol\L)	ATCC® 6919™ MBEC[c] (μmol\L)
Benzoyl Peroxide	1754	1515	13 211	>13 211
Salicylic Acid	7240	7240	14 480	>57 921
SIG1459	8.5	8.5	16.1	12.5
Clindamycin HCl	0.3	0.3	2	0.6

[a]*C. acnes* (ATCC® 6919™, ATCC® 11827™) were incubated with compounds in 5% vehicle under anaerobic conditions at 37°C for 72 h. MIC was defined as the lowest concentration of an agent that achieved ≥90% growth inhibition.

[b]MBC was defined as the lowest antimicrobial concentration that completely inhibited colony formation on I agar plates.

[c]MBEC was determined using the crystal violet assay (as detailed in the Materials and Methods). MBEC was defined as the minimum concentration necessary to achieve ≥80% eradication of attached biofilm compared to vehicle control.

of magnitude more potent than BPO and salicylic acid (>1 μmol\L) (Supplemental data, Figure S3, Table S2). No inhibitory activity was found for *C. acnes*-induced IL-1α levels (Table S2). Furthermore, given SIG1459's IC_{50} value for inhibiting *C. acnes*-induced IL-8 is ~3000 times lower than its MIC, MBC and MBEC values (Table 1), it is unlikely that its anti-inflammatory activity is directly a consequence of bacterial killing.

Recently, it has been demonstrated that TLR2 formation of TLR2/6 heterodimers is required for *C. acnes* recognition in human cells.[10] To investigate SIG1459's potential anti-inflammatory activity via inhibition of TLR2 signalling in keratinocytes, we utilized three different TLR2 ligands specific to each of the dimers, peptidoglycan (PGN) (TLR2/2 homodimer ligand), FSL-1 (TLR2/TRL6 agonist) and Pam_3CSK_4 (TLR2/TRL1 agonist). Our results show that IL-8 was induced by *C. acnes*, TLR2, TLR2/TRL6, TLR2/TRL1 agonists, while IL-1α was only induced by *C. acnes* and PGN (Supplemental data, Figure S4). SIG1459 reduces FSL-1 (IC_{50} = 0.209 μmol\L) and PGN-induced (IC_{50} = 0.086 μmol\L) IL-8 production (Figure S3, Table 2). Furthermore, SIG1459 inhibits IL-1α production (IC_{50} = 0.050 μmol\L) from normal human epidermal keratinocytes (Table S2), once again outperforming BPO and salicylic acid (>1 μmol\L). Interestingly, SIG1459 did not inhibit Pam_3CSK_4-induced IL-8 production (Table 2), demonstrating it acts through selective TLR2 receptor dimers. Furthermore, we used the TLR2/TLR1 inhibitor CU-CPT22 and inhibited Pam_3CSK_4-induced (IC_{50} = 0.03 μmol\L) IL-8 production (Figure S3), thus validating the experimental conditions (Figure S3D). Altogether, these results also suggest that while BPO and salicylic acid have been reported to possess anti-inflammatory properties,[17,18] they are unlikely to confer this activity by inhibiting *C. acnes*-TLR2 signalling.

3.3 | SIG1459 is well-tolerated and outperforms BPO in human subjects

In view of SIG1459's in vitro anti-acne potential, we sought to determine its tolerability and activity in human subjects. 1% SIG1459 was first tested clinically in a Human Repeated Insult Patch Test and was found to cause no skin sensitization or irritation (PCR, data not shown). Given this result, 1% SIG1459 in a topical cream was tested in a single-blinded vehicle controlled-study, to determine its tolerability in subjects with acne prone skin and to compare its activity vs 3% BPO, a commonly used anti-acne treatment. No adverse effects were reported for the 1% SIG1459 group, indicating again that it was well tolerated. Visual evaluation data for the 35 subjects completing use of 1% SIG1459 and the 15 subjects using 3% BPO, showed statistically significant improvement in average IGA scores at 2-week, 4-week and 8-week assessments as compared to baseline (Figure 2). However, after 8 weeks of use, the average assessment scores demonstrated 1% SIG1459 significantly outperformed the 3% BPO group with a 77% reduction in IGA score compared to baseline vs a 56% reduction in the average score for the BPO group (Figure 2). These results were even more impressive given that the 15 subjects in the vehicle control group worsened by ~30% on average over the same 8-week period (Figure 2). This reduction in IGA scores was confirmed in the photographs taken of the 1% SIG1459 group, where marked visual improvement in the signs and symptoms of acne from baseline to Week 8 (Supplemental data, Figure S2) were observed. Interestingly, photographs also taken using UV light illumination were utilized to visualize the production of porphyrins by *C. acnes* (fluorescence - orange-red dots). Subjects that applied SIG1459 exhibited a reduction of porphyrins on their skin, most notable in the nostrils and alar-facial areas (Supplemental data, Figure S5), an indirect measure of *C. acnes* killing confirming our in vitro antibacterial findings. Lastly, self-perception questionnaires (SPQs) completed by the subjects in the 1% SIG1459 group demonstrated that 76% of the users felt they noticed the number of blemishes on their face were visibly reduced and 74% of users felt that their skin appeared clearer.

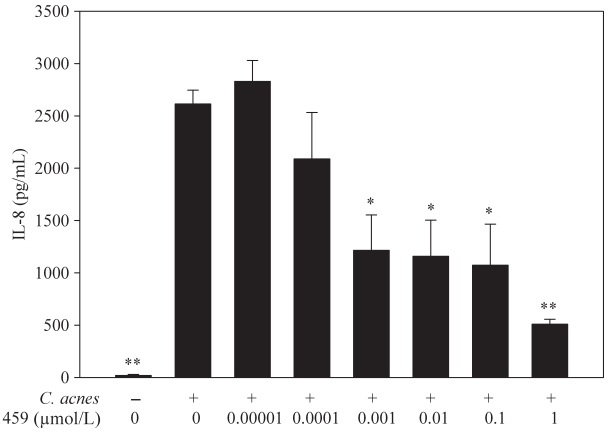

FIGURE 1 SIG1459 inhibits *Cutibacterium acnes* induced pro-inflammatory cytokine production. NHEKs were co-incubated with 1 × 10^7 CFU/mL (ATCC® 6919™) and SIG1459 in supplement-depleted media for 24 h. Pro-inflammatory cytokine (IL-8) levels in media supernatants were assayed by ELISA. The data represent the mean ± SE of cumulative from three independent experiments. *$P \leq .05$; **$P \leq .01$ indicates a statistically significant difference compared to *C. acnes*-only treated cells

TABLE 2 Anti-inflammatory activity against *Cutibacterium acnes* and TLR2 ligands[a]

Compound	*Cutibacterium acnes* IC_{50} (nmol\L)	TLR2 IC_{50} (nmol\L)	TLR2/6 IC_{50} (nmol\L)	TLR2/1 IC_{50} (nmol\L)
Benzoyl Peroxide	>1000	>1000	>1000	>1000
Salicylic Acid	>1000	>1000	>1000	>1000
SIG1459	3	86	209	>1000

[a]NHEKs were cotreated with compounds in combination with either *C. acnes* (10^7 CFU/mL), PGN (10 μg/mL), FSL-1 (0.1 μg/mL), or Pam3CSK4 (10 μg/mL) at 37°C and 5% CO_2 for 24 h. After incubation, media supernatants were harvested for Interleukin-8 (IL-8) assayed by ELISA. IC_{50} values were determined from the resulting concentration-dependent curves using 4-parameter logistic equation (Sigma Plot software).

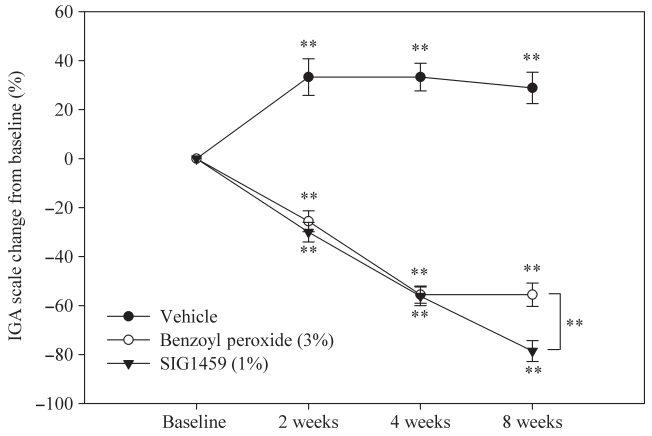

FIGURE 2 SIG1459 (1%) outperforms Benzoyl Peroxide (3%) in an 8-week acne clinical tolerance study. A multi-site use single-blinded study was conducted in healthy male and female subjects, aged ≥18 y. with evaluator assessed mild to moderate acne. Subjects used the assigned product at home for 8 wk. At 2, 4 and 8 wk of use subjects underwent expert clinical grading. Values are given as mean ± SE. ***P* value ≤ .01 by one-way ANOVA between group differences from IGA scale values from baseline

4 | DISCUSSION

Acne is a multifactorial disease caused primarily by increased sebum production, hyperkeratinization, inflammation and *Cutibacterium acnes* activity within the follicle. *C. acnes* is an anaerobic gram-positive commensal bacterium known for becoming virulent in the development of acne that affects ~85% of adolescents and in some instances, persists into adulthood.[19] In inflammatory acne lesions, *C. acnes* activates an innate immune response via TLR2.[6] In vivo studies demonstrate that TLR2 is increased in the epidermis of acne lesions and in vitro studies using human keratinocytes show incubation with *C. acnes* also results in an increase of TLR2 expression.[16,20] Additional studies performed in keratinocytes demonstrate that TLR2 activation is an initiating step in comedone formation[21] indicating a key role for *C. acnes* and TLR2 in the pathogenesis of acne.

Previously, we reported that IPC compounds inhibited TLR2- and TLR4-induced pro-inflammatory cytokine production of IL-6 and IL-8 and possessed antibacterial activity.[12,22] As demonstrated here, subsequent medicinal chemistry efforts yielded a novel phytyl IPC, SIG1459 (Tetramethyl-hexadecenyl-cysteine-formylprolinate) with antibacterial activity against the gram-positive organism, including eradicating biofilm formation and inhibiting TLR2-induced inflammation (Table 1 and 2). Phytol (3, 7, 11, 15-tetramethyl-2-h exadecen-1-ol), an isoprenoid isolated from chlorophyll and thus all green vegetables, is a diterpene alcohol attached to a cysteine residue in SIG1459 (Supplemental data Figure S1). Phytol has been reported to have antibacterial activity against several organisms including *Staphylococcus aureus*,[23] *Mycobacterium tuberculosis*[24] and *Pseudomonas aeruginosa*.[25] For *C. aeruginosa* it was proposed that phytol exerts its antibacterial properties by inducing oxidative stress.[25] However, we have previously demonstrated that phytyl-cysteine compounds similar in structure to SIG1459 possess antioxidant properties and reduce oxidative stress,[26] which would contrast with BPO's antibacterial mechanism of action (oxidation and the formation of free radicals decrease *C. acnes*). Thus, this is an unlikely mechanism for SIG1459 and its class of compounds to confer antibacterial activity.

Phytol has also been reported to induce leakage of potassium ions from *S. aureus* cell walls, thus damaging cell membranes and killing the bacteria.[23] Yet, in-house antibacterial studies testing SIG1459 and other phytyl IPC compounds against *S. aureus* show that they do not kill or inhibit the growth of this organism (data not shown). Given its lipophilic nature, SIG1459 may still disrupt *C. acnes* cell membranes as part of its mechanism of action for antibacterial activity, but results presented here suggest phytyl IPC compounds have a distinct and novel mode of action that is yet to be determined. Lastly, we recognize that there is strain heterogeneity in *C. acnes* virulence potency,[27–29] but for initial antibacterial screening we used two of the most commonly used commercially available *C. acnes* strains isolated from facial acne (ATCC® 6919™, ATCC® 11827™). In the future, we plan to determine SIG1459's antibacterial activity vs the most virulent *C. acnes* strains.

To begin to elucidate how SIG1459 successfully reduces *C. acnes*-induced inflammation in vitro and yielded positive clinical results in subjects with acne prone skin, we utilized specific molecularly defined TLR2 homodimer and heterodimer ligands to induce a pro-inflammatory response in NHEKs. IL-8 was previously shown to be induced by TLR2 and is one of the highest upregulated genes in acne lesions.[30] IL-1α was previously shown to be induced by *C. acnes*,[31] but not TLR2/TLR6 ligand.[32] Interestingly, SIG1459 was found to have selective, albeit potent anti-inflammatory response to TLR2/2 homodimers and TLR2/TLR6 heterodimers, identified by their known activating ligands. SIG1459 strongly inhibited PGN activated TLR2/2 homodimer (IL-8 IC_{50} = 86 nmol\L; IL-1α IC_{50} = 50 nmol\L), FSL-1 activated TLR2/6 heterodimer (IC_{50} = 209 nmol\L) and *C. acnes* induced IL-8 release with single digit nanomolar potency (IC_{50} = 3 nmol\L) (Table 2). However, using the TLR2/1 heterodimer ligand Pam_3CSK_4, SIG1459 tested up to 1 μmol\L did not inhibit IL-8 production. Not surprisingly, these results are consistent with the recently reported findings that *C. acnes* is mainly recognized by TLR2 and TLR6, but not TLR1.[10] This suggests that TLR2/2 and TLR2/6 dimers share a specific molecular conformation that is recognized by SIG1459 and is required for *C. acnes* induced inflammation. *C. acnes* preincubation with TLR2 and TLR6 but not TLR1 antibodies inhibits NFκB activation,[10] suggesting that SIG1459's target(s) is in these specific TLR-NFκB signalling modules. SIG1459 blocking PGN, but not *C. acnes* induced IL-1α is not surprising, as previous published results indicate that stimulation with *C. acnes* could also activate TLR2-independent pathways (ie PAR-2) to induce IL-1α expression.[33] As reported here, we were able to induce IL-1α with peptidoglycan (TLR2 ligand), suggesting that SIG1459 activity is dependent on TLR2 inhibition. IPC compounds have also been reported to modulate MEK/MAPK kinase[34] and ERK signalling.[35] Our next goal will be to elucidate where SIG1459 is targeting along the *C. acnes*-TLR pathway that leads to NFκB activation and ultimately cytokine release.

SIG1459's in vitro anti-inflammatory, antibacterial and safety profile suggested strong potential for clinical efficacy that would be well-tolerated when applied topically to acne prone skin (Figure 2). Clinical results presented here show SIG1459 is a novel anti-acne compound that not only outperforms BPO over 8 weeks, but also does not cause the stinging, burning or bleaching associated with BPO (data not shown). Current acne cosmetic and over-the-counter products predominantly utilize salicylic acid and BPO. Having a potentially new class of compounds to be utilized as a stand-alone active and/or in combination with other anti-acne actives that functions through a different dual action mechanism is a potentially significant addition for acne sufferers. We've demonstrated here that BPO and salicylic acid do not function via TLR2 inhibition. Previous studies using human skin explants from acne subjects showed that adapalene decreases TLR2 expression.[36] However, in-house studies treating keratinocytes with adapalene showed that like BPO and salicylic acid, it does not inhibit TLR2 heterodimer induced IL-8 production (data not shown). Adapalene's effect on TLR2 requires additional studies, though it appears as if any possible modulation of TLR2 by adapalene is upstream affecting the receptor's expression levels or is indirect by inducing modulators of TLR2 signalling, while SIG1459 and presumably other phytyl IPC compounds function directly downstream blocking TLR2 signalling and pro-inflammatory mediator release.

In addition, SIG1459's ability to modulate TLR2 raises the potential of it ameliorating a third important hallmark of acne, hyperkeratinization. *C. acnes* activated TLR2 signalling has been shown to induce epidermal hypercornification through the release of IL1α, which is a positive feedback stimulator of keratinocyte differentiation, in addition to its pro-inflammatory activity.[21] Oleic acid and other unsaturated fatty acids generated through sebum lipid hydrolysis have also been shown to induce epidermal hyperkeratinization[37] and to be TLR2 receptor ligands.[38,39] Perhaps oleic acid specifically activates TLR2/2 and does not involve direct TLR2/6 activation by *C. acnes* to initiate hyperkeratinization. Thus, in addition to its antimicrobial and anti-inflammatory properties, SIG1459 and other phytyl IPC derivatives may also inhibit another key factor of acne pathogenesis, hyperkeratinization. Additional studies need to be performed to explore this potential mechanism, though initial results are promising as SIG1459 inhibits TLR2 induced IL-1α production (Table S2). In conclusion, SIG1459 is a novel phytyl-cysteine derived TLR2 modulator that targets acne through its dual acting properties. The data presented here provides in vitro and in vivo proof-of-concept that phytyl IPC derivatives are effective for subjects with acne prone skin. Further research and development will potentially identify more potent new chemical entities that can treat at least two and perhaps three cardinal factors of acne.

CONFLICT OF INTEREST

All authors for this manuscript are paid employees or consultants for Signum Biosciences which is where the research was performed.

AUTHORS CONTRIBUTION

JRF, CW, KR performed the research. JRF, JSG, EP designed the research study. MV, JH, synthesized SIG1459 and MT designed the topical formulations. JRF, JSG, KH, MS, JBS and EP analysed the data and JRF, JSG and EP wrote the paper. There were no non-authors or scientific writers that contributed to this manuscript, only the listed authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Demographics Information for Subjects (n = 65)
Table S2 Anti- IL-1α activity against *C. acnes* and TLR2 ligand‡
Figure S1 SIG1459 Chemical Structure. Tetramethyl-hexadecenyl-cysteine-formylprolinate
Figure S2 Cell viability as measured by the proliferative capacity response of test materials measured using MTS assay. NHEKs were incubated with test compounds in supplement-depleted media for 24 hours. The data represent the mean ± SE of cumulative from three independent experiments as percentage of cell viability with respect to the corresponding vehicle. $^{**}P \leq .01$ indicates a statistically significant difference compared to untreated treated cells
Figure S3 IL-8 inhibition curves of test materials. NHEKs were co-treated with compounds in combination with either (A) *C. acnes* (10^7 CFU/mL), (B) PGN (10 μg/mL), (C) FSL-1 (0.1 μg/mL) or (D) Pam3CSK4 (10 μg/mL) at 37°C and 5% CO_2 for 24 hours. After incubation, media supernatants were harvested for Interleukin-8 (IL-8) assayed by ELISA. IC_{50} values were determined from the resulting concentration-dependent curves using 4-parameter logistic equation (Sigma Plot software). The data represent the mean ± SE of cumulative from three independent experiments
Figure S4 IL-1α induction by *C. acnes* and TLR2. NHEKs were treated with (A) *C. acnes* (10^7 CFU/mL), PGN (10 μg/mL), FSL-1 (0.1 μg/mL) or Pam3CSK4 (10 μg/mL) at 37°C and 5% CO_2 for 24 hours. (B) Cells were co-treated with compounds in combination with PGN (10 μg/mL). After incubation, media supernatants were harvested for Interleukin-1 alpha (IL-1α) assayed by ELISA. IC_{50} values were determined from the resulting concentration-dependent curves using 4-parameter logistic equation (Sigma Plot software). The data represent the mean ± SE of cumulative from three independent experiments. $^{**}P \leq .01$ indicates a statistically significant difference compared to *C. acnes* treated cells
Figure S5 Photographic Assessment of 1% SIG1459 Before and After 8 Weeks of Use. Subject applied 1% SIG1459 cream twice daily. (A) Photograph on left is photo taken at baseline and on right at the end of the study (week 8). Facial Cream (1% SIG1459) was tested in a randomized single-blind vehicle-controlled study (Active, n = 35; Vehicle, n = 15) to demonstrate the safety and tolerability in subjects with mild to moderate facial acne. (B) UV light mode was utilized to observe porphyrins fluorescence (orange-red dots) as an indirect measure of *C. acnes* killing. Insert of the nostrils and alar-facial groove areas shows notable porphyrin reduction. This is representative of subjects using the SIG1459 facial cream demonstrating marked visual improvement in the signs and symptoms of acne as well as reduction in porphyrins during and after weeks 2-8 of application

How to cite this article: Fernández JR, Webb C, Rouzard K, et al. SIG1459: A novel phytyl-cysteine derived TLR2 modulator with in vitro and clinical anti-acne activity. *Exp Dermatol*. 2018;00:1–7. https://doi.org/10.1111/exd.13692