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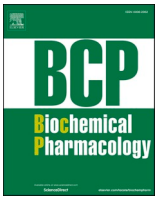

Epoxytiglianes induce keratinocyte wound healing responses via classical protein kinase C activation to promote skin re-epithelialization

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ABSTRACT

Epoxytiglianes are a novel class of diterpene esters. The prototype epoxytigliane, EBC-46 (tigilanol tiglate), is a potent anti-cancer agent in clinical development for local treatment of a range of human and animal tumors. EBC-46 also consistently promotes wound re-epithelialization at the treatment sites, mediated via activation of classical protein kinase C (PKC) isoforms. We have previously shown that epoxytiglianes stimulate proliferative and wound repopulation responses in immortalized human skin keratinocytes (HaCaTs) *in vitro*, abrogated by pan-PKC inhibitor, bisindolylmaleimide-1. In this study, we further investigate the specific PKC isoforms responsible for inducing such wound healing responses, following HaCaT treatment with 1.51 nM-15.1 μM EBC-46 or analogue, EBC-211. Classical PKC inhibition by GÖ6976 (1 μM), significantly attenuated epoxytigliane induced, HaCaT proliferation and wound repopulation at all epoxytigliane concentrations. PKC-βI/-βII isoform inhibition by enzastaurin (1 μM), significantly inhibited HaCaT proliferation and wound repopulation responses induced by both epoxytiglianes, especially at 1.51–151 nM. PKC-α inhibitor, Ro 31–8220 mesylate (10 nM), exerted lesser inhibitory effects on HaCaT responses. Epoxytigliane changes in key keratin (KRT17) and cell cycle (cyclin B1, CDKN1A) protein levels were partly attenuated by GÖ6976 and enzastaurin. GÖ6976 also inhibited increases in matrix metalloproteinase (MMP-1, MMP-7, MMP-10) activities. Phospho-PKC (p-PKC) studies confirmed that epoxytiglianes transiently activated classical PKC isoforms (p-PKCα, p-PKC-βI/-βII, p-PKCγ) in a dose- and time-dependent manner. By identifying how epoxytiglianes stimulate classical PKCs to facilitate keratinocyte healing responses and re-epithelialization, these findings support further epoxytigliane development as topical therapeutics for clinical situations involving impaired re-epithelialization, such as non-healing wounds in skin.

1. Introduction

Wound re-epithelialization is essential to the re-establishment of the protective barrier function of skin by the actions of epidermal keratinocytes, the predominant cell type within the epidermis [1,2]. Chronic wounds, including venous and diabetic ulcers, are examples of situations where these responses are impaired, thereby failing to restore the integrity of the epidermal barrier. Such dysfunctional healing greatly impacts on patients through the subsequent pain, immobility, psychological issues, and increased morbidity associated with these conditions

Abbreviations: BIM-1, bisindolylmaleimide-1; BSA, bovine serum albumin; CT, untreated controls; DAG, diacylglycerol; DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; FDA, US Food and Drug Administration; HaCaT, human immortalized skin keratinocytes; HRP, horseradish peroxidase; KRT, keratin; MMP, matrix metalloproteinase; MTT, [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide]; NHS, UK National Health Service; PBS, phosphate buffered saline; PKC, protein kinase C; p-PKC, phospho-PKC; PVDF, polyvinylidene difluoride; RFU, relative fluorescence units; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SD, standard deviation; TBS, Tris-buffered saline; TT, tigilanol tiglate.

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[3,4]. In addition to the humanistic impact, these wounds also pose extensive financial burdens on healthcare providers responsible for managing and treating these wounds. Indeed, managing these wounds is estimated to cost the UK National Health Service (NHS) approximately £5.6 billion per annum, with the cost of wound care increasing by approximately 8–9 % per year [5–7], with such incidence rising with the ever-increasing ageing population and prevalence of diabetes and obesity worldwide [4].

Intrinsic differences within keratinocytes contribute towards this inability to restore the skin barrier integrity and function, particularly through their dysfunctional hyper-proliferative and impaired migratory abilities, the two main responses required for successful wound re-epithelialization [8–11]. There are numerous treatment strategies currently available to manage these impaired healing scenarios, including wound dressings, negative pressure wound therapy, skin substitutes and growth factor therapy [12,13]. However, there remains considerable unmet medical need for new, cost-effective and efficacious treatments against all chronic wound types, in line with their ever-increasing prevalence [14,15].

Epoxytiglianes are a class of diterpene esters derived from the native Australian rainforest plant, *Fontainea picrosperma* [16,17]. The prototype epoxytigliane, EBC-46 (otherwise known as tigilanol tiglate, TT), possesses potent anti-cancer properties against solid tumors in a range of pre-clinical xenograft head and neck squamous cell carcinoma, melanoma and other mouse cancer models, in addition to various veterinary case studies involving carcinoma, sarcoma, sarcoid, and mast cell tumors; and has subsequently received approval as a veterinary pharmaceutical as a local treatment for canine mast cell tumors under the trade name, STELFONTA® [18–23]. The drug is also currently under evaluation in Phase II human clinical trials as a local treatment for head and neck cancers and soft tissue sarcomas [24–26].

EBC-46 has a multifactorial mode of action in tumor destruction, induced by a rapid, localized, inflammatory response and the loss of tumor vasculature integrity, resulting in hemorrhagic necrosis and tumor ablation, in part mediated via the activation of classical protein kinase C (PKC) isoforms (PKC-α, −βI, −βII and −γ), particularly PKC-βI and −βII [16–18]. More recent studies have further demonstrated that EBC-46 promotes endothelial and cancer cell death via pyroptosis [27], whilst it can further act as a PKC-dependent regulator of pro-tumorigenic c-MET and NGFRp75/TNFR16 signaling and nectin-1-mediated cell adhesion in head and neck cancer cells, potentially disrupting the mechanisms driving tumor progression [28].

EBC-46 can also stimulate exceptional dermal wound healing responses *in vivo* following tumor destruction, evident as accelerated wound re-epithelialization and closure over 1-month, post-treatment [23,29,30]. Our previous work has shown that EBC-46 and analogue, EBC-211, stimulate human immortalized keratinocyte (HaCaT) cell cycle progression/proliferation and cell migration/wound repopulation *in vitro* [31]. Microarray analyses showed that epoxytiglianes regulated keratin (KRT), DNA synthesis/replication, cell cycle/proliferation, migration, differentiation, matrix metalloproteinase (MMP) and cytokine/chemokine genes, to promote such responses. Furthermore, as with their anti-cancer effects, epoxytigliane stimulation of keratinocyte wound healing responses were confirmed as being mediated by PKC activation and significantly abrogated by pan-PKC inhibitor, bisindolylmaleimide-1 (BIM-1) [31].

The PKC family is a group of related serine/threonine protein kinases, broadly divided into three main categories, classical, novel, and atypical PKCs. Classical PKCs (−α, −βI, −βII and −γ) require diacylglycerol (DAG) and calcium for activation, novel PKCs (−δ, −ε, −η and −θ) also require DAG activation but are calcium independent; and atypical PKCs (−λ/ι and −ζ) are both DAG- and calcium-independent [32,33]. PKCs participate in many cell signaling pathways affecting proliferation, migration, morphology, and apoptosis, with different isoforms inducing contrasting responses. There are numerous PKC isoforms present in HaCaTs, namely PKC-α, −β, −γ, −δ, −ε, −ζ, −η and −θ [32,33]. PKC isoforms induce opposing responses in keratinocytes, with PKC-α and PKC-δ associated with reduced cell proliferation in favor of stimulated differentiation, whereas PKC-β increases keratinocyte proliferative abilities at the expense of differentiation [34,35]. Despite inhibited proliferative responses being associated with PKC-α activation, this isoform also plays a key role in stimulating wound re-epithelialization, due to its abundance within the epidermis and its role in regulating desmosome adhesiveness and cell–cell interactions [36,37]. In acute wounds, PKC-α is translocated to the desmosome and accumulates at the cell periphery, permitting keratinocyte migration at the wound edge. However, translocation is impaired in non-healing chronic wounds, preventing keratinocyte migration [38,39].

Although our previous studies have confirmed that PKCs play a key role in mediating the stimulatory effects of epoxytiglianes on keratinocyte proliferation and wound repopulation [31], to date, no studies have established which specific PKC isoforms participate in regulating epoxytigliane wound healing responses in keratinocytes. Therefore, this study aimed to identify the PKC isoform(s) involved in stimulating keratinocyte wound healing responses induced by epoxytiglianes, EBC-46 (Fig. 1A) and EBC-211 (formed by Payne re-arrangement of the epoxide group on the EBC-46 β ring, Fig. 1B) [17,31]. This study confirmed that epoxytiglianes primarily mediate enhanced proliferative and migratory responses in keratinocytes via classical PKC activation, such as the PKC-α, PKC-βI/-βII and PKC-γ isoforms. Identification of the specific PKC isoforms involved in facilitating these enhanced keratinocytes wound healing responses is essential to our ultimate aims of developing these small molecule entities as novel pharmaceutical therapies for clinical situations associated with impaired re-epithelialization and healing, such as chronic skin wounds.

2. Materials and Methods

2.1. Chemicals, reagents, and antibodies

Epoxytiglianes, EBC-46 and EBC-211, were provided by QBiotics Group (Yungaburra, Australia). Dulbecco's modified eagle medium (DMEM), antibiotics/antimycotics, fetal calf serum (FCS), L-glutamine, dimethyl sulfoxide (DMSO, ≥99.7 %) and RIPA buffer, were obtained from ThermoFisher Scientific (Paisley, UK). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin (BSA) and Tween 20 were purchased from Sigma-Aldrich (Poole, UK). Classical PKC (PKC-α and −βI) inhibitor, GÖ6976, was obtained from Merck Millipore (Watford, UK). The predominantly PKC-α and −β inhibitors, Ro 31–8220 mesylate and enzastaurin (LY317615) respectively, were obtained from Selleck Chemicals (Houston, TX, USA). Ro 31–8220 mesylate was used at a final concentration of 10 nM, in line with its IC_{50} for the selective inhibition of PKC-α [40]. Enzastaurin is established as a PKC-βI and −βII inhibitor at the final concentration used (1 μM) [41]. Primary antibodies against cytokeratin 17 (#ab109725), cyclin B1 (#ab32053), p21 (CDKN1A, #ab109520) and β-actin Loading Control (#ab8227) were purchased from Abcam (Cambridge, UK). Primary phospho-PKC (p-PKC) antibodies to p-PKC-α (targeting T497 residue, #ab76016) and p-PKC-βI (targeting T642 residue, #ab5782) were purchased from Abcam. p-PKC antibodies to p-PKC-βII (targeting S660 residue, #sc-365463) and p-PKC-γ (targeting T514 residue, #Ma5-37192) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and ThermoFisher Scientific, respectively [42]. Horseradish peroxidase (HRP)-conjugated secondary antibody (#P039901-2) was purchased from Dako (Ely, UK). All remaining reagents were of the highest grade and either supplied by ThermoFisher Scientific or Sigma-Aldrich.

2.2. Keratinocyte cell culture

The immortalized keratinocyte cell line, HaCaTs, were obtained from the German Cancer Research Centre (Heidelberg, Germany) and

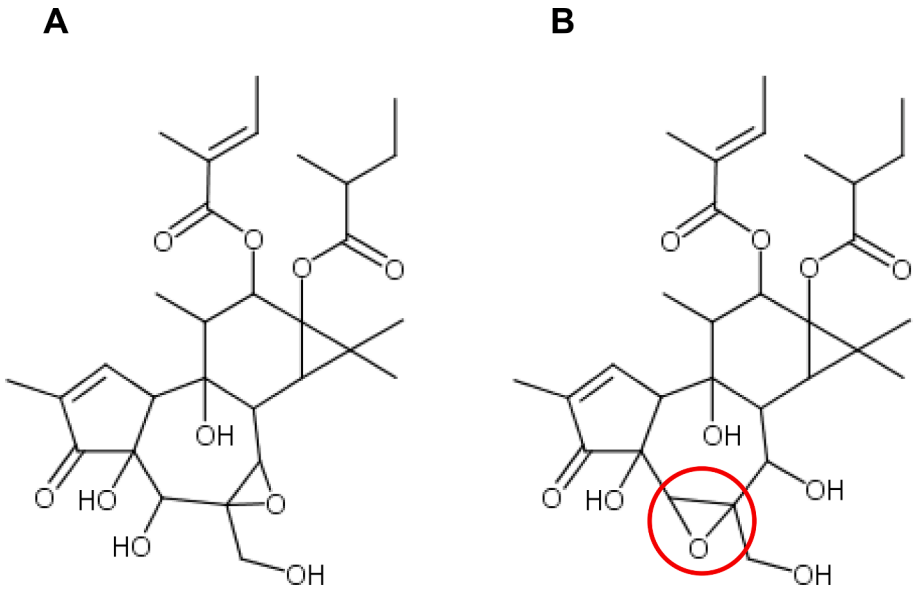

Fig. 1. Chemical structures of epoxytiglianes, (A) EBC-46 (tigilanol tiglate) and (B) EBC-46 analogue, EBC-211 (produced by a Payne re-arrangement of the epoxide group on the β ring of EBC-46, circled in red).

cultured in DMEM, supplemented with 1 % antibiotics/antimycotics (100 U/mL penicillin G sodium, 0.25 μg/mL amphotericin B and 100 μg/mL streptomycin sulfate), 2 mM L-glutamine and 10 % FCS [31]. HaCaTs were maintained at 37 °C in a humidified 5 % CO_2/95 % air atmosphere, with medium changed every 48 h.

EBC-46 and EBC-211 were solubilized in DMSO, at 151 mM (100 mg/mL) and 15.1 mM (10 mg/mL), respectively. Stock solutions of both epoxytiglianes were subsequently prepared and diluted in 1 % serum-containing DMEM to provide final EBC-46 and EBC-211 concentrations of 1.51 nM-15.1 μM, as previously described [31]. Untreated, vehicle-only control cultures were also supplemented with 0.1 % DMSO, to discount influences on HaCaT behavior in epoxytigliane treated cultures.

2.3. Keratinocyte proliferation assays

HaCaTs were seeded into 96-well plates in 10 % serum-containing DMEM at 5 × 10^3 cells/well for 24 h, followed by incubation in serum-free DMEM for a further 24 h. HaCaTs were subsequently incubated in 1 % serum-containing DMEM with EBC-46 or EBC-211 (1.51 nM, 151 nM and 15.1 μM) ± sub-lethal concentrations of classical PKC (−α and −βI) inhibitor, GÖ6976 (1 μM), the predominantly PKC-β inhibitor, enzastaurin (1 μM), or the PKC-α inhibitor, Ro 31–8220 mesylate (10 nM), used at concentrations which only inhibit PKC-α. Cultures were maintained at 37 °C in a humidified 5 % CO_2/95 % air atmosphere. HaCaT proliferation was assessed at 24 h, 48 h and 72 h, using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide] assay [31]. Absorbance values were measured using FLUOstar® Optima Microplate Reader (BMG Labtech, Aylesbury, UK), at 540 nm. Epoxytigliane effects on cell proliferation were expressed as percentage viable cells versus untreated, vehicle only controls, which were arbitrarily assigned a viability of 100 %.

2.4. Keratinocyte scratch wound repopulation

HaCaTs were seeded into 24-well plates in 10 % serum-containing DMEM at 7.5 × 10^4 cells/well for 48 h, followed by incubation in serum-free DMEM for a further 24 h. Serum-free DMEM was removed, and scratch wounds made to the confluent cultures using sterile pipettes. Following phosphate buffered saline (PBS) washing (×2), 1 % serum-containing DMEM with EBC-46 or EBC-211 (1.51 nM, 151 nM and 15.1 μM) were added ± sub-lethal concentrations of GÖ6976 (1 μM), enzastaurin (1 μM) or Ro 31–8220 mesylate (10 nM), as described above. Cultures were maintained at 37 °C in a humidified 5 % CO_2/95 % air atmosphere for 48 h. HaCaT migration and wound repopulation were monitored by Time-Lapse Confocal Microscopy (TCS SP5 Microscope, Leica Microsystems, Milton Keynes, UK), with digital images obtained and analyzed using ImageJ® Software, as previously described [31]. Data were expressed as percentage wound closure at 24 h and 48 h, versus wound areas at 0 h.

2.5. Western blot analysis

EBC-46 and EBC-211 effects on keratin (KRT17) and cell cycle/proliferation (cyclin B1, CDKN1A) protein levels, were confirmed by Western blotting. These proteins were analyzed based on their differential expression induced by epoxytiglianes, as previously described [31]. HaCaTs were seeded into T-25 tissue culture flasks in 10 % serum-containing DMEM at 1.33 × 10^5 cells/flask for 24 h, followed by incubation in serum-free DMEM for a further 24 h. Serum-free media was replaced with 1 % serum-containing DMEM, with EBC-46 or EBC-211 (1.51 nM, 151 nM and 15.1 μM) ± sub-lethal concentrations of GÖ6976 (1 μM) or enzastaurin (1 μM), as described above. Cultures were maintained at 37 °C in a humidified 5 % CO_2/95 % air atmosphere for 24 h and 48 h. Once the culture medium had been removed, HaCaT cultures were harvested into 1 mL RIPA buffer containing Complete Protease Inhibitor Cocktail Tablets (Roche, Burgess Hill, UK). Sonicated protein extracts (10 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), using pre-formed 4–15 % TGX™ gels (Mini-Protean® Tetra Cell System; Bio-Rad, Hemel Hempstead, UK). Gels were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Hybond™-P, ThermoFisher Scientific), using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad), as per manufacturer's instructions.

Membranes were blocked with 5 % semi-skimmed milk/1% Tween 20 in Tris-buffered saline (TBS) for 1 h at room temperature, followed by primary antibody incubation in 5 % semi-skimmed milk/1% Tween 20 in TBS, for 1 h at room temperature or 4 °C overnight. Membranes were immuno-probed with primary antibodies to cytokeratin 17 (1:1,000); cyclin B1 (1:1,000); and p21 (CDKN1A, 1:1,000). Normalized protein loading was confirmed by β-actin Loading Control (1:20,000). All membranes were washed (×3) in 1 % Tween 20 in TBS and incubated in

HRP-conjugated, swine anti-rabbit secondary antibody (1:3,000), in 5 % semi-skimmed milk/1% Tween 20 in TBS; for 1 h at room temperature. Membranes were washed (×3) as above, followed by additional 5 min washes (×1) in TBS. Membranes were subsequently incubated in ECL™ Prime Detection Reagent (Hyperfilm™-ECL, ThermoFisher Scientific) and autoradiographic films developed, per manufacturer's instructions. Immunoblot images were captured by densitometry. Densitometric analysis was performed using ImageJ® Software, as previously described [31].

2.6. MMP activity quantification

PKC inhibitor effects on MMP-1, −7 and −10 activities induced by EBC-46 and EBC-211 were quantified using MMP Activity Assays, as these were previously demonstrated to be significantly up-regulated by epoxytigliane treatment [31]. HaCaTs were seeded into T-25 tissue culture flasks in 10 % serum-containing DMEM at 1.33×10^5 cells/flask for 24 h, followed by incubation in serum-free DMEM for a further 24 h. Serum-free media was replaced with 1 % serum-containing DMEM, with EBC-46 or EBC-211 (1.51 nM, 151 nM and 15.1 μM) ± sub-lethal concentrations of GÖ6976 (1 μM), as described above. Cultures were maintained at 37 °C in a humidified 5 % CO_2/95 % air atmosphere, for 48 h. Culture media were collected, and MMP-1, MMP-7 and MMP-10 activities quantified using SensoLyte® 520 Activity Assays (Cambridge Bioscience, Cambridge, UK) at 490 nm/520 nm, as previously described [31]. Activities were expressed as relative fluorescence units (RFU).

2.7. Detection of PKC isoform phosphorylation and activation

To confirm p-PKC isoform (PKC-α, −βI, −βII, −γ) phosphorylation and activation following epoxytigliane treatment, HaCaTs were seeded into T-25 tissue culture flasks as described above; and treated with EBC-46 or EBC-211 (0 or 1.51 nM, 151 nM and 15.1 μM) for 0 h, 0.5 h, 1 h, 3 h, 6 h and 24 h. Cell extracts were harvested using RIPA lysis buffer containing Protease Inhibitor Cocktail Tablets, PhosStop™ tablets (Merck Millipore) and sodium orthovanadate (2.5 μg/mL, Sigma-Aldrich). Protein samples (10 μg) were separated and electroblotted onto nitrocellulose membranes (ThermoFisher Scientific), as above. Membranes were blocked in 5 % semi-skimmed milk/1% Tween 20 in TBS and immuno-probed with primary antibodies to p-PKC-α (1:1,000), p-PKC-βI (1:1,000), p-PKC-βII (1:1,000) and p-PKC-γ (1:1,000), in 5 % semi-skimmed milk/1% Tween 20 in TBS, at 4 °C overnight. Protein loading confirmation and secondary antibody incubations were performed, as described above. Immunoprobed proteins were analyzed by iBright™ 1500 Imaging System (ThermoFisher Scientific), using iBright Analysis Software. Densitometric analysis was performed using the calculated target protein/β-actin ratios, which were subsequently normalized to untreated control samples at each time-point.

2.8. Statistical analysis

All experiments were performed on n = 3 independent occasions, with data expressed as mean ± standard deviation (SD). Proliferation, migration and MMP activity data were analyzed by one-way ANOVA with post-Tukey test. Western blot densitometry data were analyzed by one-way ANOVA and unpaired Student's t tests. p-PKC isoform activation densitometric data were analyzed by two-way ANOVA with post-Dunnett's Multiple Comparisons Test. Significance was considered at $p < 0.05$.

3. Results

3.1. Classical PKC isoforms mediate epoxytigliane induced keratinocyte proliferation and wound repopulation responses

Our previous studies demonstrated that the enhanced HaCaT proliferation and wound repopulation responses induced by epoxytigliane treatments (1.51 nM-15.1 μM), were significantly abrogated by pharmacological inhibition of PKC activation by pan-PKC inhibitor, BIM-1 [31]. Thus, as with their established anti-cancer properties [16,17], we initially established whether classical PKC isoforms were also principally responsible for the enhanced HaCaT responses observed following epoxytigliane treatment, through inhibition of PKC-α and PKC-β activation by GÖ6976. Our results showed that both epoxytigliane induced proliferative responses were significantly abrogated by GÖ6976, as were the proliferative responses of untreated HaCaT controls ($p < 0.001$–0.01, Fig. 2A & B).

Additional comparisons of proliferative responses between EBC-46 treated and untreated HaCaT controls in the presence of GÖ6976, identified further significant increases in proliferation with 1.51 nM-15.1 μM EBC-46 at 48 h ($p < 0.001$–0.01) and at 72 h (all $p < 0.001$), compared to the corresponding untreated controls at these time-points with GÖ6976 (Fig. 2A). Similar proliferation comparisons between EBC-211 treated and untreated HaCaT controls in the presence of GÖ6976, showed decreased proliferation with 15.1 μM EBC-211 at 24 h ($p < 0.001$), but increased proliferation with 1.51–151 nM EBC-211 at 48 h and 72 h (all $p < 0.001$), versus the corresponding untreated controls at these time-points with GÖ6976 (Fig. 2B). In contrast, decreased proliferation was evident with 15.1 μM EBC-211 and GÖ6976 at all time-points ($p < 0.001$ at 24 h and 48 h, $p < 0.05$ at 72 h), versus the untreated controls with GÖ6976 (Fig. 2B).

We previously showed that EBC-46 and EBC-211 (1.51 nM-15.1 μM) also significantly enhanced scratch wound repopulation and closure, which was inhibited by BIM-1 [31]. Hence, we further assessed EBC-46 and EBC-211 (1.51 nM-15.1 μM) abilities to promote HaCaT migration and scratch wound repopulation in the presence of classical PKC inhibitor, GÖ6976, using automated Time-Lapse Microscopy and ImageJ® analysis over a 48 h period. Significant stimulatory effects on wound repopulation were induced by EBC-46 (1.51 nM, $p < 0.001$ at 48 h) and EBC-211 (1.51 nM-15.1 μM, $p < 0.001$–0.05 at 24 h and 48 h) without PKC inhibition versus untreated controls, as previously described [31] (Fig. 2C & D and Supplementary Videos [43]). However, GÖ6976 significantly inhibited these EBC-46 stimulated wound closure responses, particularly at 1.51 nM and 151 nM concentrations at 48 h ($p < 0.01$–0.05, Fig. 2C and Supplementary Videos [43]). The significant abrogation of enhanced wound repopulation responses by EBC-211 were also evident following GÖ6976 treatment, albeit over a wider concentration range (1.51–151 nM at 24 h and 1.51 nM-15.1 μM at 48 h, $p < 0.001$–0.05, Fig. 2D and Supplementary Videos [43]). GÖ6976 treatments were also largely shown to inhibit HaCaT wound repopulation responses in untreated controls ($p < 0.001$, Fig. 2D). Cross-statistical comparisons between the different EBC-46 treatment and untreated controls, further identified no significant reductions in GÖ6976 inhibition of HaCaT wound repopulation with EBC-46 (all $p > 0.05$), versus the corresponding untreated controls at all time-points with GÖ6976 (Fig. 2C). Similar reductions in GÖ6976 inhibition of HaCaT wound repopulation were further identified with 1.51–151 nM EBC-211 at 48 h ($p < 0.001$ and $p < 0.05$, respectively), compared to the corresponding untreated controls at these time-points with GÖ6976 (Fig. 2D).

3.2. PKC-α and −β isoform inhibition induces contrasting effects on epoxytigliane stimulated keratinocyte proliferation

Following the responses observed with GÖ6976, we next established the extent to which PKC-α and −β isoforms contribute to the induction of enhanced proliferative responses by epoxytiglianes. Treatment with PKC-α inhibitor, Ro 31–8220 mesylate, exerted no significant effects on HaCaT proliferative responses in untreated control and epoxytigliane treated cultures (all $p > 0.05$ at 24 h, 48 h and 72 h, Fig. 3A & B). In contrast, PKC-β inhibitor, enzastaurin, significantly abrogated EBC-46 stimulated HaCaT proliferative response at 48 h (1.51–151 nM, both $p < 0.001$), in addition to the proliferative responses of untreated HaCaT

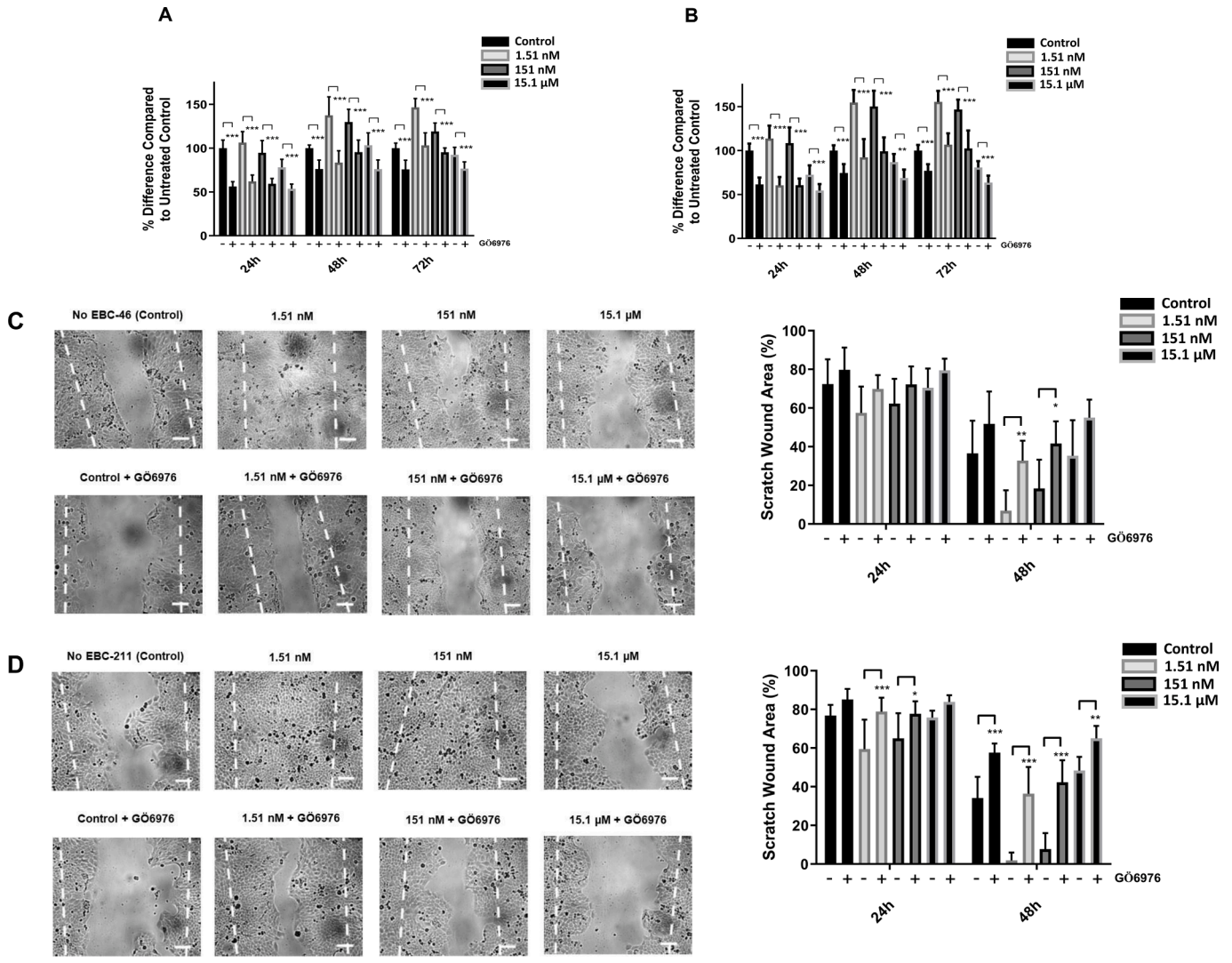

Fig. 2. Classical PKC activation mediates enhanced human skin keratinocyte (HaCaT) proliferative and migratory responses by epoxytiglianes. MTT analysis of proliferation, following HaCaT treatment with (A) EBC-46 and (B) EBC-211 (1.51 nM, 151 nM or 15.1 μM) over 72 h versus untreated HaCaTs, ± classical PKC inhibitor, GÖ6976 (1 μM). (C-D) Representative Time-Lapse Microscopy images and ImageJ® analysis of HaCaT scratch wound repopulation and closure rates at 24 h and 48 h, following treatment with EBC-46 and EBC-211 (1.51 nM, 151 nM or 15.1 μM), versus untreated HaCaTs, ± GÖ6976 (1 μM). White dashed lines show original scratch wound distances at 0 h. Scale bar = 100 μm. All Supplementary Videos are accessible at [43]. Results are presented as mean ± SD, n = 3 independent experiments. Significance at *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus corresponding GÖ6976-free controls.

controls at 48 h and 72 h ($p < 0.001$–0.01, Fig. 3C). Similar responses were evident in EBC-211 treated HaCaT cultures, with significant inhibition of HaCaT proliferation by enzastaurin at all EBC-211 concentrations (all $p < 0.001$, Fig. 3D), particularly at 1.51–151 nM concentrations, in addition to the proliferative responses of untreated HaCaT controls (all $p < 0.001$). Thus, PKC-β inhibition by enzastaurin had far greater repressive effects on epoxytigliane stimulated keratinocyte proliferation than PKC-α inhibitor, Ro 31–8220 mesylate, which exerted no inhibitory effects in comparison.

Additional statistical cross-comparisons of proliferative responses between EBC-46 treated and untreated HaCaT controls in the presence of Ro 31–8220 mesylate and enzastaurin supported such conclusions, as despite no significant differences in responses between untreated control and epoxytigliane treated cultures with Ro 31–8220 mesylate (all $p > 0.05$, Fig. 3A & B), significantly increased proliferation were identified with 1.51 nM-15.1 μM EBC-46 at 48 h and 72 h ($p < 0.001$–0.01), compared to the corresponding untreated controls at these time-points with enzastaurin (Fig. 3C). Similar proliferation comparisons between EBC-211 treated and untreated HaCaT controls in the presence of Ro 31–8220 mesylate and enzastaurin, only showed significant decreases in proliferation in enzastaurin treated cultures with 15.1 μM EBC-211 at 24 h, 48 h and 72 h ($p < 0.001$–0.05), in contrast to increased proliferation with 1.51–151 nM EBC-211 at 48 h and 72 h (all $p < 0.001$, Fig. 3D).

3.3. *Individual PKC isoform inhibition also promotes variable epoxytigliane stimulated keratinocyte wound repopulation*

We also ascertained the extent to which inhibition of individual PKC isoforms prevented the enhanced wound repopulation responses by epoxytiglianes. Following treatment with Ro 31–8220 mesylate, no significant inhibition of EBC-46 stimulation of wound repopulation responses were shown (all $p > 0.05$, Fig. 4A and Supplementary Videos [43]). However, EBC-211 enhancement of wound repopulation was significantly inhibited by Ro 31–8220 mesylate, over a wider concentration range of 1.51–151 nM at 24 h ($p < 0.01$–0.05) and 48 h (both $p < 0.001$, Fig. 4B and Supplementary Videos [43]). However, in both cases, Ro 31–8220 mesylate only exhibited minimal inhibitory effects on wound repopulation responses in untreated HaCaT controls ($p < 0.05$, Fig. 4B). Following enzastaurin treatment, it was apparent that the

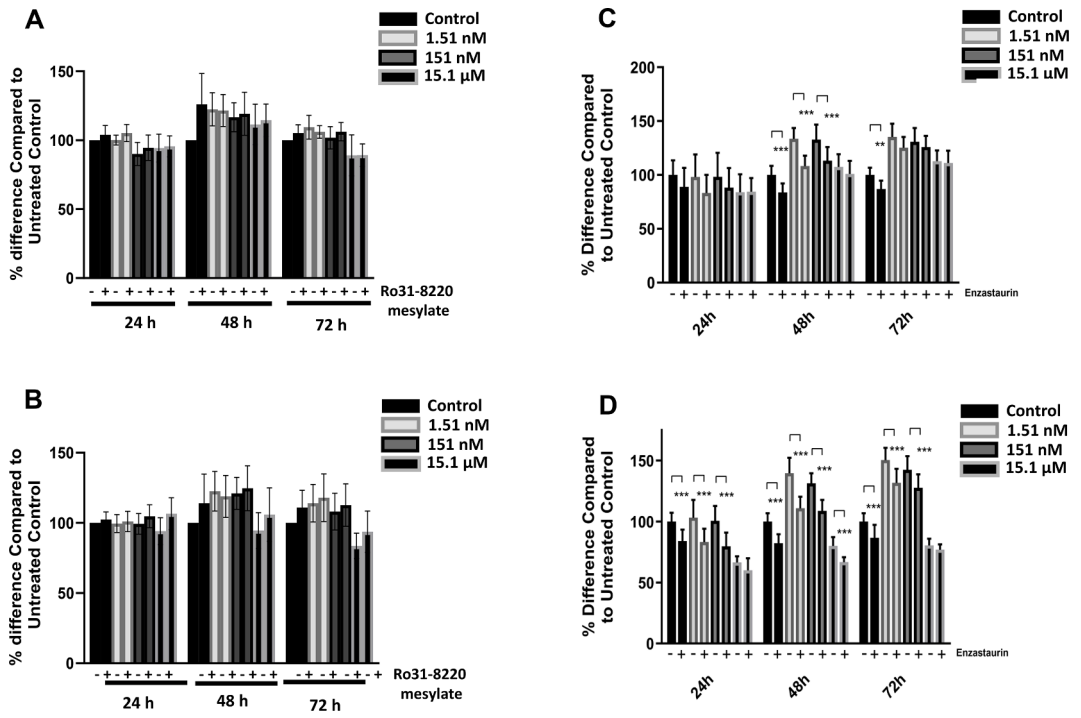

Fig. 3. PKC-β activation primarily mediates human skin keratinocyte (HaCaT) proliferative responses by epoxytiglianes. MTT analysis of proliferation, following HaCaT treatment with (A & C) EBC-46 or (B & D) EBC-211 (1.51 nM, 151 nM or 15.1 µM) over 72 h versus untreated HaCaTs, ± PKC-α inhibitor Ro 31–8220 mesylate (10 nM, A-B) or ± PKC-β inhibitor, enzastaurin (1 µM, C-D). Results are presented as mean ± SD, n = 3 independent experiments. Significance at **$p < 0.01$ and ***$p < 0.001$, versus corresponding Ro 31–8220 mesylate- or enzastaurin-free controls.

stimulation of wound repopulation responses induced by 1.51–151 nM EBC-46 were also diminished at 48 h ($p < 0.001$–0.05, Fig. 4C and Supplementary Videos [43]). EBC-211 enhancement of wound repopulation was also significantly inhibited by enzastaurin, especially at 1.51 nM at 24 h ($p < 0.001$) and 1.51 nM-151 nM at 48 h (both $p < 0.001$, Fig. 4D and Supplementary Videos [43]). Enzastaurin further exerted significant inhibitory effects on wound repopulation responses of untreated HaCaT controls at 48 h ($p < 0.001$, Fig. 4C & D), and to a much greater extent than the inhibitory effects observed following Ro 31–8220 mesylate treatment (Fig. 4A & B).

Further statistical cross-comparisons of wound repopulation responses between epoxytigliane treated and untreated HaCaT controls in the presence of Ro 31–8220 mesylate showed that no significant differences in the inhibition of EBC-induced wound repopulation were evident at between epoxytigliane treated keratinocytes at 24 h and 48 h (all $p > 0.05$), compared to the corresponding untreated controls at these time-points with Ro 31–8220 mesylate (Fig. 4A & B). In contrast, cross-statistical analyses of wound repopulation responses between epoxytigliane treated and untreated HaCaT controls in the presence of enzastaurin, identified significant decreases in inhibition with 1.51 nM EBC-46 at 48 h ($p < 0.01$) and 151 nM EBC-211 at both 24 h ($p < 0.05$) and 48 h ($p < 0.001$), compared to the corresponding untreated controls at these time-points with enzastaurin (Fig. 4C & D).

3.4. Classical PKC isoforms contribute to epoxytigliane induced changes in keratin- and cell cycle-related protein levels

We previously observed differential expression of certain keratin and cell cycle genes and protein levels following epoxytigliane treatment, which potentially contribute to their stimulatory effects on keratinocyte proliferation and migration [31]. As pan-PKC inhibitor, BIM-1, was also demonstrated to reverse these keratin and cell cycle-related protein levels induced by epoxytiglianes, such as KRT17, cyclin B1 and CDKN1A (also known as p21), we next focused on evaluating the effects of GÖ6976 and enzastaurin on the differential expression of these proteins following treatment with EBC-46 and EBC-211 (1.51 nM-15.1 µM).

GÖ6976 exhibited no significant inhibitory effects on EBC-46 induced decreases in KRT17 protein levels at 48 h ($p > 0.05$, [43]), although showed a significant increase in KRT17 protein levels at 15.1 µM EBC-46, at 24 h ($p < 0.01$, Fig. 5A). However, GÖ6976 showed no significant inhibitory effects on increased cyclin B1 or decreased CDKN1A levels at 24 h or 48 h with EBC-46 treatment (all $p > 0.05$, [43]). Similar responses were also seen with EBC-211. Although GÖ6976 exhibited no significant inhibitory effects on decreased KRT17 at 24 h with EBC-211 ($p > 0.05$, [43]), GÖ6976 significantly inhibited decreased KRT17 protein levels induced by 151 nM EBC-211 at 48 h ($p < 0.01$, Fig. 5B). GÖ6976 also had no significant effects on cyclin B1 levels at 24 h ($p > 0.05$, [43]), although GÖ6976 induced further increases in cyclin B1 with 151 nM EBC-211 at 48 h ($p < 0.05$, Fig. 5B). However, GÖ6976 showed no significant inhibitory effects on CDKN1A levels at 24 h or 48 h with EBC-211 treatment ($p > 0.05$, [43]).

Enzastaurin exerted no significant inhibitory effects on EBC-46 induced decreases in KRT17 protein levels at 24 h ($p > 0.05$, [43]), although a significant increase in KRT17 protein levels was determined at 151 nM EBC-46, at 48 h ($p < 0.05$, Fig. 5C). However, enzastaurin showed no significant inhibitory effects on increased cyclin B1 or reduced CDKN1A levels induced by EBC-46 at 24 h or 48 h ($p > 0.05$, [43]). Similar responses were observed with EBC-211, with GÖ6976 exhibiting no significant inhibitory effects on KRT17 at 24 h or 48 h ($p > 0.05$, [43]). However, although enzastaurin had no significant effects on cyclin B1 levels at 24 h ($p > 0.05$, [43]), enzastaurin significantly inhibited the EBC-211 induced increase in cyclin B1 with 1.51 nM EBC-211 at 48 h ($p < 0.01$, Fig. 5D). Enzastaurin showed no significant inhibitory effects on decreased CDKN1A levels associated with EBC-211 at 24 h or 48 h ($p > 0.05$, [43]).

3.5. Classical PKC isoform inhibition attenuates epoxytigliane induced matrix metalloproteinase activities

Our previous studies demonstrated the increased expression and

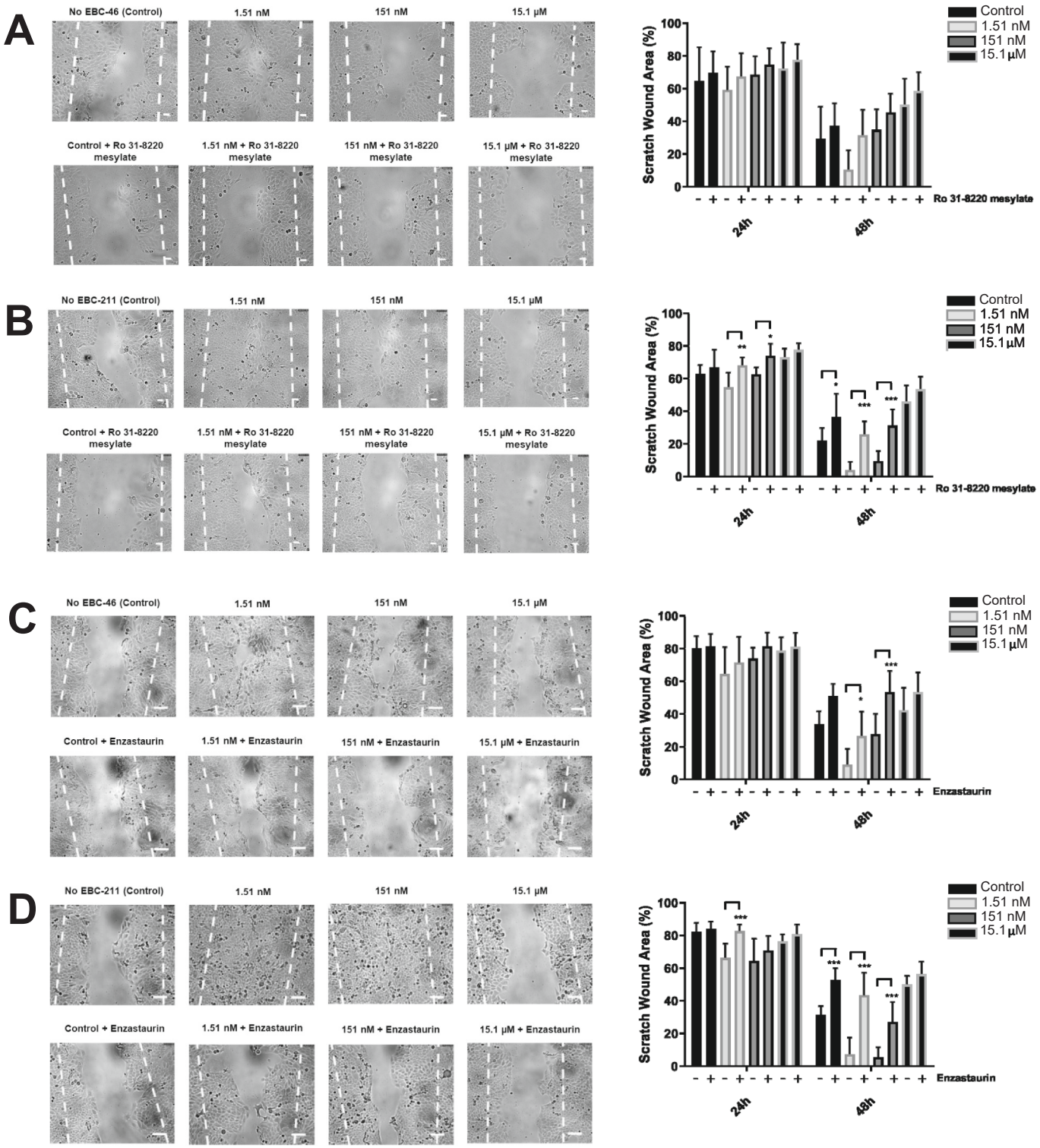

Fig. 4. PKC-α and PKC-β activation contribute to enhanced human skin keratinocyte (HaCaT) migratory responses by epoxytiglianes. Representative Time-Lapse Microscopy images and ImageJ® analysis of HaCaT scratch wound repopulation and closure rates at 24 h and 48 h, following treatment with (A & C) EBC-46 and (B & D) EBC-211 (1.51 nM, 151 nM or 15.1 μM), versus untreated HaCaTs ± PKC-α inhibitor Ro 31–8220 mesylate (10 nM, A-B) or ± PKC-β inhibitor, enzastaurin (1 μM, C-D). White dashed lines show original scratch wound distances at 0 h. Scale bar = 100 μm. All Supplementary Videos are accessible at [43]. Results are presented as mean ± SD, n = 3 independent experiments. Significance at *p < 0.05, **p < 0.01 and ***p < 0.001 versus corresponding Ro 31–8220 mesylate- or enzastaurin-free controls.

activities of MMP-1, MMP-7, and MMP-10, following epoxytigliane treatments (1.51 nM-15.1 μM), which were significantly inhibited by pan-PKC inhibitor, BIM-1 [31]. Therefore, we next evaluated the effects of GÖ6976 on MMP-1, MMP-7, and MMP-10 activities, following treatment with EBC-46 and EBC-211 (1.51 nM-15.1 μM). GÖ6976 significantly inhibited EBC-46 induced increases in the activities of MMP-1 (1.51 nM-15.1 μM, p < 0.001–0.01, Fig. 6A), MMP-7 (1.51–151 nM, p < 0.01–0.05, Fig. 6B); and MMP-10 (1.51 nM-15.1 μM, all p <

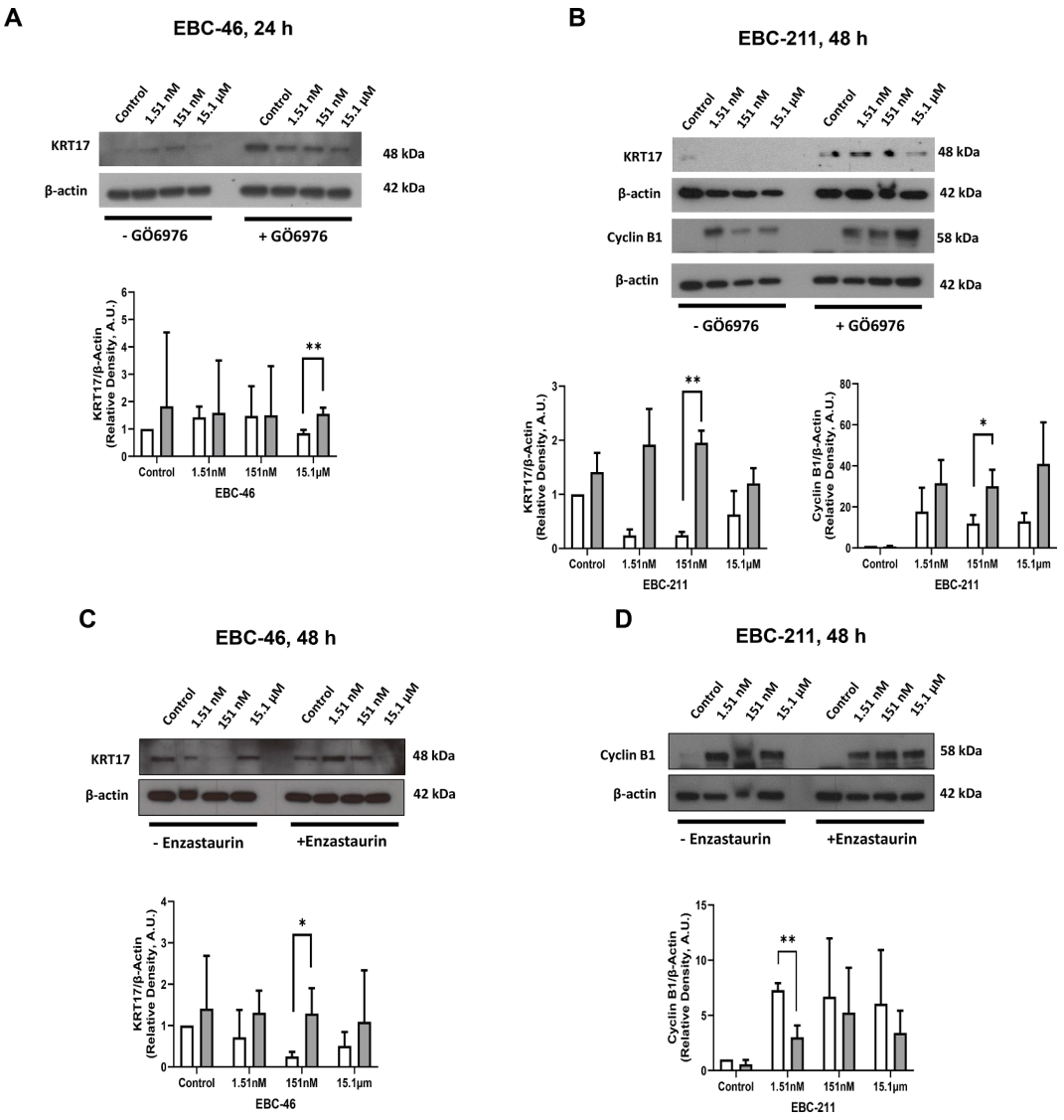

Fig. 5. Classical PKC activation, such as PKC-βI/-βII, modulates keratin- and cell cycle-related gene expression profiles by epoxytiglianes to enhance human skin keratinocyte (HaCaT) proliferation and migration. (A) Western blot images and ImageJ® densitometric analysis of KRT17 protein levels, following HaCaT treatment with EBC-46 (1.51 nM, 151 nM or 15.1 μM) for 24 h, versus untreated HaCaTs in the absence (*white bars*) and presence (*grey bars*) of GÖ6976 (1 μM). (B) Western blot images and ImageJ® densitometric analysis of KRT17 and cyclin B1 protein levels, following HaCaT treatment with EBC-211 (1.51 nM, 151 nM or 15.1 μM) for 48 h, versus untreated HaCaTs in the absence (*white bars*) and presence (*grey bars*) of GÖ6976 (1 μM). (C) Western blot images and ImageJ® densitometric analysis of KRT17 protein levels, following HaCaT treatment with EBC-46 (1.51 nM, 151 nM or 15.1 μM) for 48 h, versus untreated HaCaTs in the absence (*white bars*) and presence (*grey bars*) of enzastaurin (1 μM). (D) Western blot images and ImageJ® densitometric analysis of cyclin B1 protein levels, following HaCaT treatment with EBC-211 (1.51 nM, 151 nM or 15.1 μM) for 48 h, versus untreated HaCaTs in the absence (*white bars*) and presence (*grey bars*) of enzastaurin (1 μM). For all Western blots, images from one representative experiment of three are shown. Results are presented as mean ± SD, n = 3 independent experiments. Significance at *$p < 0.05$ and **$p < 0.01$, versus untreated controls.

0.001, Fig. 6C), at 48 h. Similarly, GÖ6976 significantly inhibited increased MMP-1, MMP-7, and MMP-10 activities (all $p < 0.001$, Fig. 6D-F, respectively), induced by EBC-211 (1.51 nM-15.1 μM) at 48 h. However, whereas GÖ6976 has no discernable effects on MMP-1 or MMP-10 activities in untreated HaCaT controls (all $p > 0.05$, Fig. 6A, C, D & F), GÖ6976 treatment significantly reduced MMP-7 ($p < 0.001$, Fig. 6E) in untreated HaCaT controls overall.

3.6. *Epoxytiglianes induce phosphorylation and activation of PKC-α, PKC-βI/-βII and PKC-γ isoforms in HaCaTs*

Our previous studies demonstrated the biphasic phosphorylation and activation of PKCs following epoxytigliane treatment (1.51 nM-15.1 μM), using pan-phosphoPKC detection [31]. Here, we utilized Western blot analysis to determine the relative levels of phosphorylation and activation of individual p-PKC isoforms.

Detection of p-PKC-α showed activation following EBC-46 treatment at 1 h (1.51 nM and 15.1 μM, Fig. 7A & B), which were further sustained by 1.51 nM EBC-46 concentrations at 3 h and 6 h time-points. However, p-PKC-α phosphorylation was significantly reduced at higher EBC-46 concentrations (15.1 μM) at the subsequent time-points, versus untreated HaCaT controls ($p < 0.05$ at 3 h and 6 h, Fig. 7B). p-PKC-α phosphorylation levels were comparable to controls by 24 h. Detection of p-PKC-βI demonstrated a dose-dependent response in activation between 0.5–3 h, especially at higher EBC-46 concentrations (151 nM-15.1 μM, Fig. 7C & D), which subsequently declined and returned to baseline levels at 24 h. In contrast, EBC-46 induced rapid PKC-βII phosphorylation over the initial 1 h (Fig. 7C & E), although p-PKC-βII

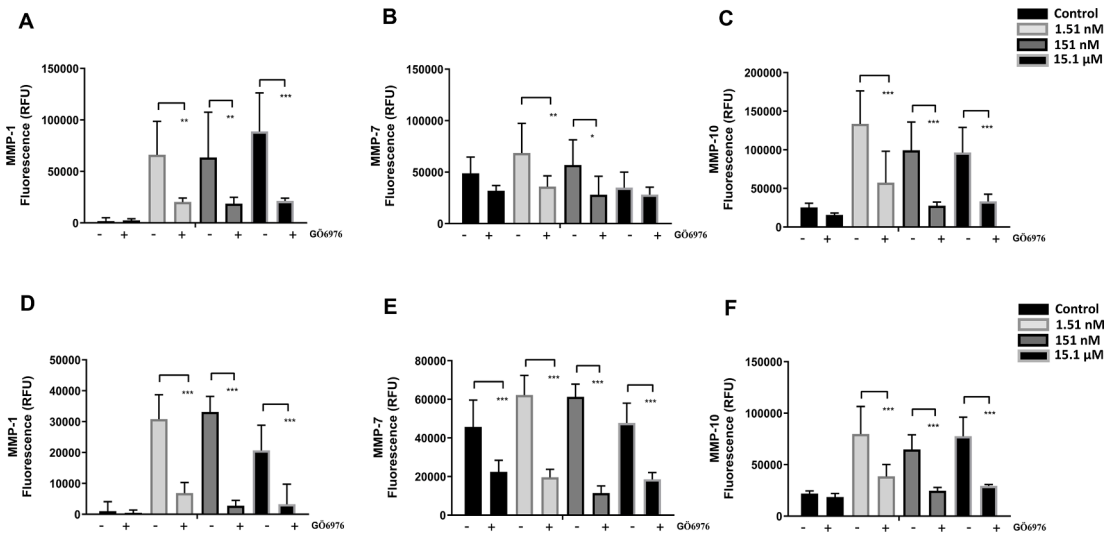

Fig. 6. Classical PKC activation modulates MMP expression and activity profiles by epoxytiglianes to enhance human skin keratinocyte (HaCaT) proliferation and migration. (A & D) MMP-1, (B & E) MMP-7 and (C & F) MMP-10 activities (activity assays), following HaCaT treatment with (A-C) EBC-46 or (D-F) EBC-211 (1.51 nM, 151 nM or 15.1 µM) for 48 h, versus untreated HaCaTs, ± GÖ6976 (1 µM). Results are presented as mean ± SD, n = 3 independent experiments. Significance at *p < 0.05, **p < 0.01 and ***p < 0.001, versus corresponding GÖ6976-free controls.

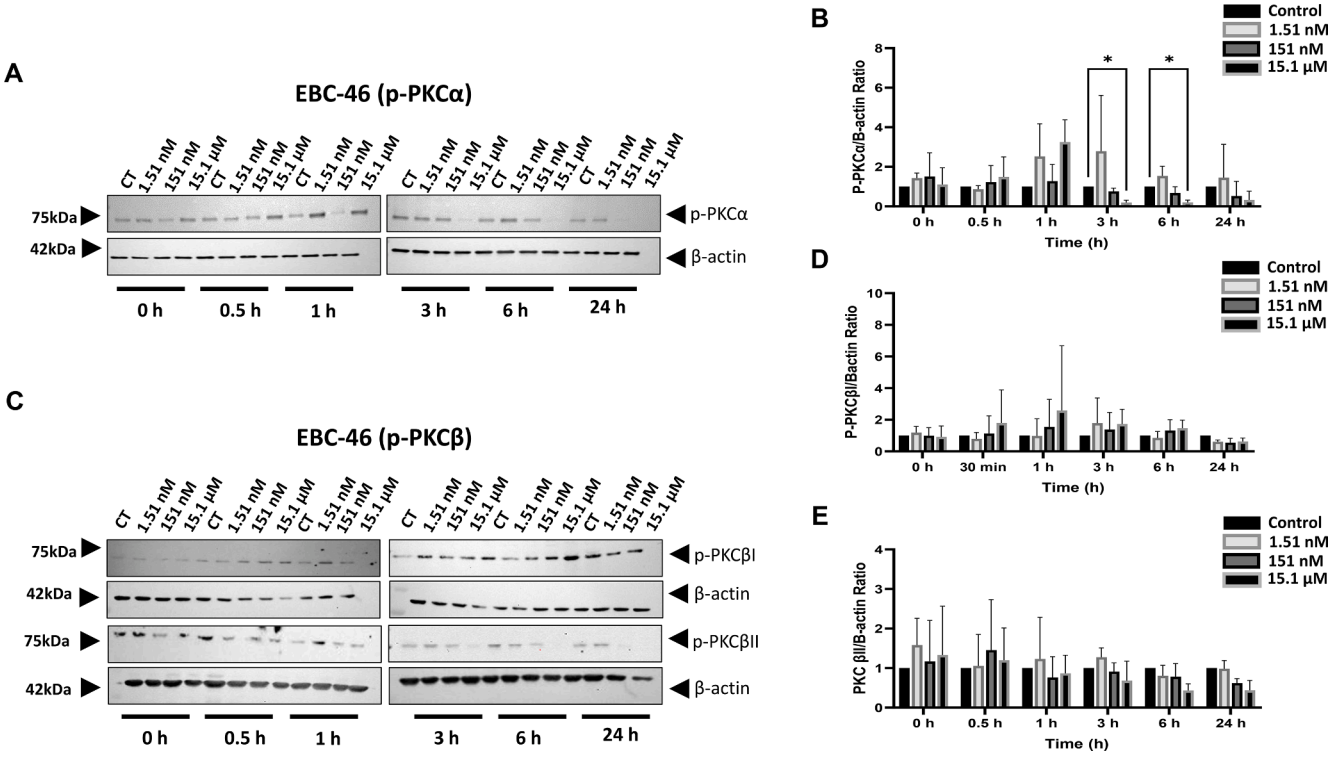

Fig. 7. Classical PKC-α, −βI and −βII phosphorylation and activation profiles induced by EBC-46 to facilitate enhanced human skin keratinocyte (HaCaT) proliferation and migration. Western blot images and densitometric analysis (iBright Analysis Software) of (A-B) PKC-α, (C-D) PKC-βI and (C & E) PKC-βII phosphorylation, following HaCaT treatment with 1.51 nM, 151 nM or 15.1 µM EBC-46 over 24 h, versus untreated HaCaTs. For all Western blots, images from one representative experiment of three are shown. Results are presented as mean ± SD, n = 3 independent experiments. CT = untreated controls. Significance at *p < 0.05, versus untreated controls.

declined beyond 1 h onwards, especially at higher EBC-46 concentrations (151 nM and 15.1 µM).

In contrast to EBC-46, p-PKC-α did not demonstrate any discernable activation following EBC-211 treatment until the latter 6 h and 24 h time-points, with significantly increased p-PKC-α induced by 151 nM EBC-211 versus untreated HaCaT controls at 24 h ($p < 0.05$, Fig. 8A & B). However, more rapid PKC-βI activation was evident at earlier time-points (0–0.5 h), especially at lower EBC-211 concentrations ($p < 0.01$ for 1.51 nM EBC-211 at 0.5 h, Fig. 8C & D). However, detectable PKC-βI phosphorylation levels returned to baseline from 1 h onwards. PKC-βII phosphorylation exhibited a similar profile to p-PKC-α (Fig. 8C & E), only demonstrating PKC-βII activation following EBC-211 treatment at later 24 h time-points ($p < 0.05$ for 1.51–151 nM EBC-211 at 24 h, Fig. 8C & D).

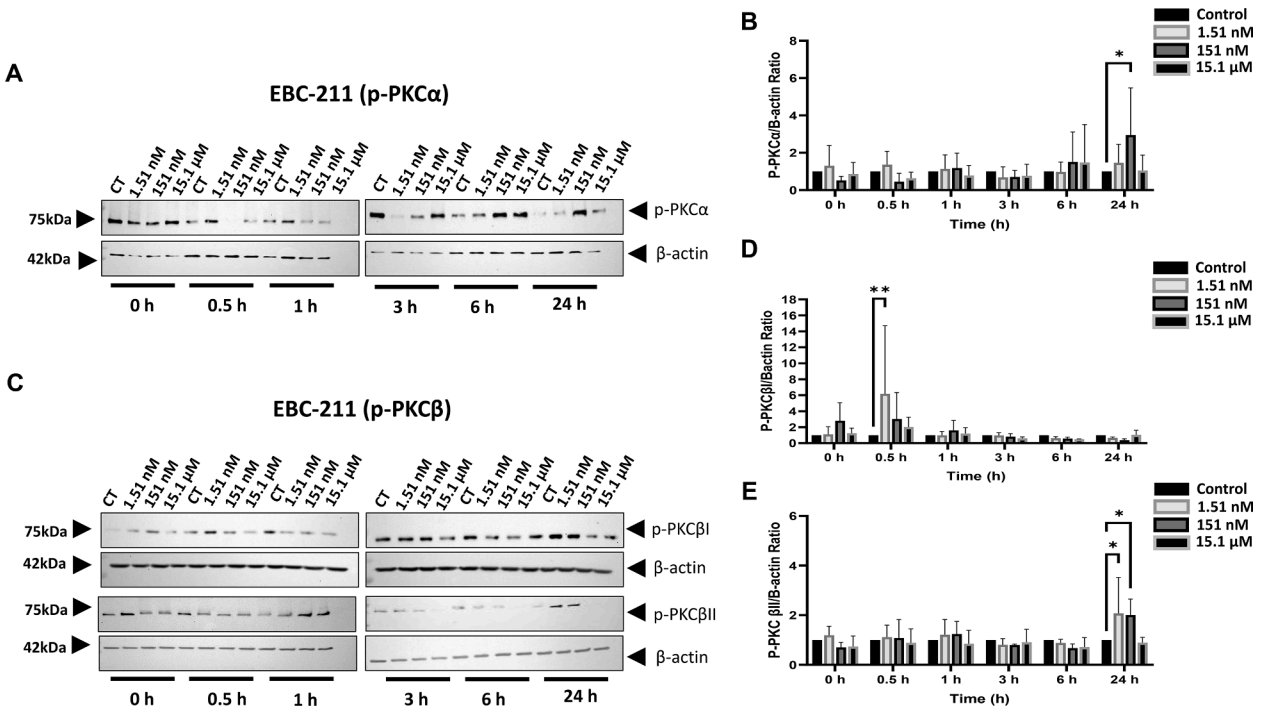

Fig. 8. Classical PKC-α, −βI and −βII phosphorylation and activation profiles induced by EBC-211 to facilitate enhanced human skin keratinocyte (HaCaT) proliferation and migration. Western blot images and densitometric analysis (iBright Analysis Software) of (A-B) PKC-α, (C-D) PKC-βI and (C & E) PKC-βII phosphorylation, following HaCaT treatment with 1.51 nM, 151 nM or 15.1 μM EBC-211 over 24 h, versus untreated HaCaTs. For all Western blots, images from one representative experiment of three are shown. Results are presented as mean ± SD, n = 3 independent experiments. CT = untreated controls. Significance at $^{**}p < 0.01$ and $^{*}p < 0.05$, versus untreated controls.

Although it is suggested that PKC-γ is undetectable in primary epidermal keratinocytes [44], it is recognized as a PKC isoform constituent in HaCaTs [32,35]. Rapid p-PKC-γ phosphorylation and activation was identifiable in HaCaTs, following treatment at 0–0.5 h at all EBC-46 concentrations ($p < 0.05$ at 151 nM-15.1 μM, Fig. 9A & B), compared to untreated controls, which subsequently declined to

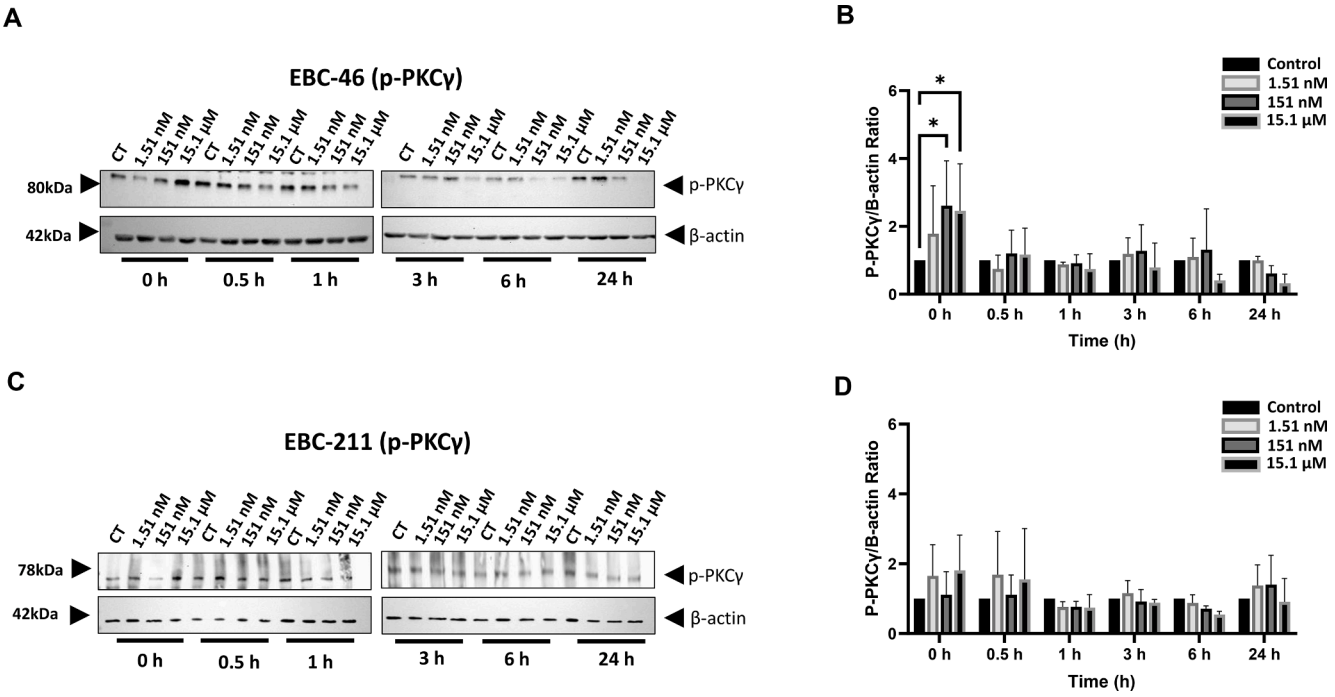

Fig. 9. Classical PKC-γ phosphorylation and activation profiles induced by EBC-46 and EBC-211 in treated human skin keratinocytes (HaCaTs). Western blot images and densitometric analysis (iBright Analysis Software) of PKC-γ phosphorylation, following HaCaT treatment with 1.51 nM, 151 nM or 15.1 μM (A-B) EBC-46 and (C-D) EBC-211 over 24 h, versus untreated HaCaTs. For all Western blots, images from one representative experiment of three are shown. Results are presented as mean ± SD, n = 3 independent experiments. CT = untreated controls. Significance at $^{*}p < 0.05$, versus untreated controls.

baseline levels and below at later time-points. Similar PKC-γ phosphorylation profiles were evident following HaCaT treatment with EBC-211, with early p-PKC-γ detection between 0–0.5 h time-points (Fig. 9C & D), although further PKC-γ activation was apparent at later 24 h time-points, especially at 1.51 nM-151 nM concentrations.

4. Discussion

Our previous findings suggested a prominent role for PKCs in mediating the stimulatory effects of epoxytiglianes on keratinocyte wound healing responses [31]. As such, the present study provides key mechanistic data confirming that the epoxytigliane enhancement of keratinocyte proliferative and wound repopulation responses are mediated via the activation of classical PKC isoforms, PKC-α, PKC-βI/-βII and PKC-γ; in accordance with accelerated wound re-epithelialization in epoxytigliane treated skin *in vivo* [23,29,30].

Chronic wounds are one predominant example of impaired healing, resulting in a compromised epidermal integrity [1,2]. In acute healing wounds, disassembled cell–cell/cell-matrix interactions initiate keratinocyte migration from the wound edge across denuded areas, whilst keratinocytes adjacent to the migrating front proliferate [1]. However, key changes occur in the epidermal keratinocytes of non-healing chronic wounds, associated with the activation and overexpression of c-myc by supra-basal keratinocytes, the non-migratory phenotype of wound edge keratinocytes; and dysregulated keratinocyte differentiation pathways [8–11]. Consequently, the resultant hyper-proliferative and non-migratory nature of keratinocyte wound healing responses contributes to the characteristic failure of re-epithelialization and inability to restore skin barrier function in chronic wounds.

Our previous studies implied that epoxytiglianes enhance keratinocyte proliferative and wound repopulation responses via the activation of PKCs; using pan-PKC inhibitor, BIM-1; and Western blot detection for pan-PKC phosphorylation and activation [31]. In the present study, classical PKC inhibitor, GÖ6976, strongly inhibited enhanced HaCaT proliferative responses at all epoxytigliane concentrations assessed, in a comparable manner to the inhibition observed previously with BIM-1, further suggesting that classical PKCs, particularly the PKC-α and PKC-β isoforms, participated in inducing these responses. Subsequently, these responses were further compared in the presence of more selective PKC-α and PKC-βI/-βII inhibitors, Ro 31–8220 mesylate and enzastaurin [40,41]. Studies confirmed that whilst Ro 31–8220 mesylate had no effects on the stimulation of HaCaT proliferation by either epoxytigliane, enzastaurin significantly inhibited EBC-46 stimulated proliferation at 48 h and EBC-211 induced proliferation at all time-points assessed, similar to previous observations with BIM-1 [31] and herein with GÖ6976. However, despite GÖ6976 and enzastaurin both evoking inhibition of epoxytigliane induced proliferation, enzastaurin induced less prominent inhibitory effects, possibly suggesting that PKC-βI/-βII isoforms are not solely responsible for promoting HaCaT proliferative responses induced by epoxytigliane treatment. Nonetheless, the absence of Ro 31–8220 mesylate inhibitory effects support PKC-α as not having a prominent role in mediating epoxytigliane stimulation of HaCaT proliferation, in accordance with its established anti-proliferative activities in keratinocytes [36,45,46]. In contrast, the significant inhibitory responses apparent with enzastaurin demonstrated that PKC-β isoforms are predominantly responsible for the promotion of keratinocyte proliferation, following epoxytigliane treatment [35,36,47].

The effects of GÖ6976, Ro 31–8220 mesylate and enzastaurin on the stimulatory effects of epoxytiglianes on HaCaT wound repopulation were further evaluated. As seen previously with pan-PKC inhibitor, BIM-1, GÖ6976 treatment resulted in the significant inhibition of the enhanced wound closure response induced by EBC-46 and EBC-211, especially at lower concentrations (1.51 nM-151 nM) [31]. As GÖ6976 primarily inhibits PKC-α and PKC-β, this indicated that one or both PKC isoforms were particularly involved in stimulating epoxytigliane wound repopulation responses. Like GÖ6976, Ro 31–8220 mesylate and enzastaurin also resulted in the significant inhibition of epoxytigliane induced wound repopulation, with both PKC inhibitors largely reducing HaCaT scratch wound repopulation to comparable levels, especially at lower EBC-46 and EBC-211 concentrations (1.51 nM-151 nM). However, as evident with the proliferative responses, the inhibitory effects of Ro 31–8220 mesylate and enzastaurin were less pronounced than the inhibition induced by GÖ6976. Therefore, the findings presented collectively imply that both PKC-α and PKC-βI/PKC-βII isoforms are involved in inducing epoxytigliane stimulated wound repopulation responses, supported by previous evidence demonstrating that PKCα and PKCβII facilitate keratinocyte motility and re-epithelialization at the wound edge [32,35–39,48], as evident following epoxytigliane wound treatment *in vivo* [23,29,30].

Previous expression profiling analyses and protein validation studies revealed that epoxytiglianes modulate a multitude of key genes associated with keratinocyte wound healing responses, to drive stimulated proliferation and migratory responses [31]. These included certain keratins, such as KRT17; and regulators of cell cycle progression and proliferation, such as cyclin B1 and CDKN1A. Although KRT17 plays a role in driving keratinocyte hyper-proliferation post-wounding, expression and protein levels were significantly downregulated by epoxytigliane treatments [31,49]. The findings herein demonstrate that both GÖ6976 and enzastaurin were capable of increasing KRT17 protein levels in the presence of EBC-46 and EBC-211, in both a time- and concentration-dependent manner, similar to previous findings with pan-PKC inhibitor, BIM-1 [31], implying that classical PKCs, such as PKC-β, are responsible for such responses in HaCaTs. Intriguingly, as GÖ6976 alterations in KRT17 proteins levels following EBC-46 treatment were identified at earlier time-points than with EBC-211, such observations may reflect the relative PKC activation potencies of these analogues, as EBC-46 is acknowledged to possess greater (≈10,000-fold) PKC isoform activation potential than EBC-211 [17,31]. However, despite these findings, no studies, to date, have reported an association between PKC-β activation and KRT17 expression in keratinocytes, although KRT17 is established to promote PKC-α activation during keratinocyte migration [37].

In accordance with epoxytiglianes stimulating HaCaT cell cycle progression and proliferation, cyclins B1, a promoter of G2/M cell cycle transitions, was also up-regulated by EBC-46 and EBC-211 [31,50]. In contrast, the cyclin-dependent inhibitor, CDKN1A, was significantly downregulated by epoxytiglianes, which acts by preventing cyclin complex formation with cyclin-dependent protein kinases, thereby inhibiting G1-S transition [31,51]. As classical PKCs, especially PKC-βI/βII, were implicated as participating in the enhancement of HaCaT proliferation by epoxytigliane treatments, GÖ6976 and enzastaurin were further evaluated in terms of the abilities to inhibit the cyclin B1 and CDKN1A protein level profiles induced by EBC-46 and EBC-211. From these findings, it was shown that GÖ6976 further increased cyclin B1 levels induced by EBC-211, as previously described with pan-PKC inhibitor, BIM-1 [31], whilst enzastaurin significantly decreased cyclin B1 levels at low EBC-211 concentrations (1.51 nM). However, neither PKC inhibitor induced significant changes in CDKN1A profiles following EBC-46 and EBC-211 treatments. PKC-α is known to interact with cyclin B1 to promote G2/M cell cycle progression [52]; whilst downregulated PKC-β signaling decreases cyclin B1 levels in cancer cells [53], hence suggesting that PKC-βI/βII signaling induces elevated cyclin B1 levels following epoxytigliane treatment. However, as both PKC-α and PKC-β activation are associated with the opposing up-regulation of CDKN1A and attenuation of proliferation in various cell types [54–57], this may partly explain the lack of significant CDKN1A up-regulation in epoxytigliane treated HaCaTs in the presence of GÖ6976 or enzastaurin. Nonetheless, as KRT17 and cyclin B1 are regarded as downstream targets of classical PKC activation, it is likely that these have subsequent roles in regulating HaCaT proliferative and migratory responses following PKC activation by epoxytigliane treatment, particularly PKC-βI/βII.

Our previous studies also demonstrated that epoxytiglianes up-regulate MMP-1, MMP-7, and MMP-10 activities, which play key roles in wound re-epithelialization through basement membrane degradation, allowing keratinocyte migration over the underlying matrix [58–60]. Additionally, we previously demonstrated that these epoxytigliane induced increases in MMP activities were PKC-dependent, being inhibited by BIM-1 [31]. MMP activity profiles with GÖ6976 showed responses to those apparent with BIM-1, with significant inhibition of MMP-1, MMP-7, and MMP-10. Such profound responses indicate that PKC-α or −PKC-βI/βII were particularly involved in inducing MMP expression in keratinocytes. Such conclusions are corroborated by studies which show induced MMP-1 expression in HaCaTs and other cell types is PKC-α dependent, whilst PKC-β signaling has no effects on the expression of these MMPs [61,62].

Variations in classical PKC isoform activation were identified overall, with EBC-46 inducing PKC-α phosphorylation at 1 h time-points at all concentrations, with sustained activation at 3–6 h with low EBC-46 concentrations (1.51 nM). PKC-βI phosphorylation demonstrated a dose-dependent response in activation between 0.5–3 h, especially at higher EBC-46 concentrations (151 nM-15.1 μM), whilst PKC-βII showed rapid phosphorylation over the initial 1 h before declining, especially at higher EBC-46 concentrations (151 nM-15.1 μM). In contrast to EBC-46, p-PKC-α did not demonstrate any discernable activation following EBC-211 treatment until 6–24 h time-points. However, more rapid PKC-βI activation was evident (0–0.5 h), especially at lower EBC-211 concentrations (1.51 nM-151 nM), before returning to baseline levels. PKC-βII phosphorylation exhibited a similar profile to p-PKC-α, only demonstrating p-PKC-βII activation following EBC-211 treatment at 24 h time-points. Although PKC-γ is undetectable in primary epidermal keratinocytes [44], it is present in HaCaTs [32,35]. Thus, further PKC-γ phosphorylation analyses identified rapid p-PKC-γ activation with EBC-46 treatment at 0–0.5 h at all concentrations, which subsequently declined to baseline levels. Similar PKC-γ phosphorylation profiles were evident with EBC-211, with early p-PKC-γ detection between 0–0.5 h time-points, although further PKC-γ activation was apparent at later 24 h time-points, especially at lower concentrations (1.51 nM-151 nM).

Such transient PKC-α, PKC-βI/-βII and PKC-γ isoform phosphorylation and activation profiles between epoxytigliane analogues and concentrations are likely consequences of their contrasting PKC activation capabilities and dose-dependent differences in HaCaT responses, as reported with other PKC activators [32]. Furthermore, short-term exposure or low PKC activator concentrations are established to promote rapid PKC activation, whilst continued exposure or higher activator concentrations result in constitutive activation, due to the induction of catalytic PKC domain release from the inhibitory PKC pseudo-substrate domain. Although prolonged activator exposure can downregulate PKC activity via catalytic PKC domain proteolysis, PKC-α is resistant to such degradation leading to sustained stimulation [63]. Together with our previous studies into pan-PKC phosphorylation following HaCaT treatment with these EBC-46 and EBC-211 concentrations, such p-PKC isoform profiles correlate well, as peaks in pan-PKC activation were detectable with EBC-46 at 1–3 h and 6–24 h time-points, and at 0.5–1 h and 6 h time-points with EBC-211 [31]. Therefore, phosphorylation of PKC-α, PKC-βI/-βII and PKC-γ may contribute to the initial pan-PKC activation peak at 1–3 h with EBC-46, whilst PKC-βI and PKC-γ are primarily responsible for the activation peak at 1–3 h following EBC-211 treatment, with PKC-α and PKC-βII additionally promoting pan-PKC activation at 6–24 h time-points. Classical PKC isoforms, such as PKC-α and PKC-βI/βII, are well-established to have opposing influences on keratinocyte functions, such as proliferation, migration, and differentiation [32,35–39,45–48], although less is known about the effects of PKC-γ on HaCaT wound healing responses. Nonetheless, the phosphorylation and activation of these PKC isoforms by epoxytiglianes, are likely to be primarily responsible for the enhanced keratinocyte proliferation, wound repopulation and re-epithelialization responses observed overall. As normal PKC-α signaling is impaired in non-healing chronic wounds, leading to dysfunctional keratinocyte activation and wound migration [37,38], PKC-α and other classical PKC isoform activation could be particularly beneficial in stimulating migratory responses in chronic wound keratinocytes, thereby leading to the restoration of impaired re-epithelialization responses.

Based on the collective findings of our previous *in vitro* and *in vivo* studies and those presented herein [23,29,30,31], epoxytiglianes promote classical PKC isoform activation leading to the downstream manipulation of expression profiles, such as KRT-, cell cycle/proliferation-, migration-, differentiation-, and MMP-related genes. Such events subsequently facilitate enhanced keratinocyte proliferative and migratory responses, at the expense of reduced keratinocyte differentiation, to re-establish the denuded wound epithelium which are subsequently proceeded by the resumption of keratinocyte differentiation and epidermal barrier restoration [23,29–31]. Therefore, as keratinocyte proliferative and migratory responses are impaired in non-healing chronic wounds, leading to delayed wound re-epithelialization and a failure to reinstate barrier integrity [8–11], such mechanisms may be restored by topical epoxytigliane treatment *in vivo*, thereby permitting wound re-epithelialization, closure and restoration of skin barrier function to occur.

Having confirmed that classical PKC isoforms are key contributors to enhanced keratinocyte proliferation and migration induced by epoxytiglianes, it is intriguing that comparable epoxytigliane concentrations induce both their anticancer and wound healing responses, especially since both involve classical PKC activation. From an *in vitro* perspective, PKC activation and their corresponding anti-cancer and wound healing properties have largely been observed at nM-μM epoxytigliane concentrations [16,17,28,30,31], whilst epoxytiglianes exhibit anti-cancer and pro-wound healing efficacies at μg/mL-mg/mL dosages in various *in vivo* animal models, following intra-tumoral or topical applications [16–23,29,30]. Thus, these findings collectively suggest that PKC activation and the anti-cancer/pro-wound healing capabilities of epoxytiglianes occur at comparable concentrations *in vitro* and *in vivo*. Additional studies are intended to investigate these responses further, in addition to delineating the cell signaling pathways downstream of PKC activation involved in mediating these responses.

As EBC-46 and EBC-211 comprise some of the > 100 different natural and semi-synthetic epoxytigliane analogues now available [17], we are also investigating their structure-functional relationships across this broader group of compounds relevant to PKC activity and wound healing responses, with the ultimate objective of selecting lead candidate(s) for development as novel wound healing pharmaceutics. Similarly, as genotypic/phenotypic differences are acknowledged to exist between HaCaTs and primary human epidermal keratinocytes, we intend to undertake complementary studies to evaluate epoxytigliane wound healing responses in primary human keratinocytes representative of *in vivo* epithelial repair in skin.

In summary, this study has demonstrated that like their anti-cancer properties, epoxytigliane activation of classical PKC isoforms, most notably PKC-α and PKC-βI/-βII, that are collectively responsible for the stimulation of keratinocyte proliferation and migratory responses; thereby explaining the enhanced wound re-epithelialization observed in epoxytigliane treated skin *in vivo*. Thus, such findings enhance our understanding of the underlying cell signaling by which epoxytiglianes promote rapid re-epithelialization in treated skin; and support the further development of these novel small molecules as topical therapeutics for clinical situations associated with impaired re-epithelialization, such as non-healing chronic skin wounds.

Author Contribution

R.L.M., E.L.W. and J.D. performed the experiments, data analysis and prepared the figures for the manuscript. J.P.J. isolated and purified epoxytiglianes for the studies described. R.L.M, E.L.W. and R.M. wrote the manuscript. R.M., R.S., P.R., G.M.B., E.L.W., V.K., J.D. and R.L.M. contributed to designing the experiments in this study and manuscript revision. P.R. and V.G. funded this study.

CRediT authorship contribution statement

Rachael L. Moses: Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Emma L. Woods:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Jordanna Dally:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Jenny P. Johns:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Formal analysis. **Vera Knäuper:** Writing – review & editing, Resources, Methodology, Conceptualization. **Glen M. Boyle:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Victoria Gordon:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Paul Reddell:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Robert Steadman:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Ryan Moseley:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: This work was conducted with financial support from QBiotics Group. P. R. and V.G. are employees and possess ownership interests in QBiotics Group. R.M., R.S., R.L.M., P.R., V.G., G.M.B. and QBiotics Group have filed patents on the work disclosed within this manuscript. E.L.W., J.D., J.P.J. and V.K. have no conflict of interests to declare.

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Data availability

Data will be made available on request.

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