Growth factor mimetics for skin regeneration: In vitro profiling of primary human fibroblasts and keratinocytes

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Funding information
CAPES Foundation, Ministry of Education of Brazil, Grant/Award Number: 11718/13-7; European Union’s EuroSkinGraft project, Grant/Award Number: 279024

Abstract
Small molecules have gained considerable interest in regenerative medicine, as they can effectively modulate cell fates in a spatiotemporal controllable fashion. A continuous challenge in the field represents genuine mimicry or activation of growth factor signaling with small molecules. Here, we selected and profiled three compounds for their capacity to directly or indirectly activate endogenous FGF-2, VEGF, or SHH signaling events in the context of skin regeneration. Phenotypic and functional analysis of primary skin fibroblasts and keratinocytes revealed unique, cell-specific activity profiles for the FGF-2 mimetic SUN11602 and the putative VEGF mimetic ONO-1301. Whereas SUN11602 exclusively stimulated keratinocyte differentiation, ONO-1301 mainly affected the proliferation and migration behavior of fibroblasts. In each skin cell type, both compounds selectively enhanced the expression of MMP1 and VEGFA. A combined small molecule FGF-2/VEGF mimicry may not only improve angiogenesis-related microcirculation but also reduce early fibrosis while facilitating wound remodeling at later stages. SUN11602 and ONO-1301 represent valuable tools for improving the management of difficult-to-heal wounds, particularly for the design and development of small molecule-functionalized, next-generation, engineered skin substitutes.

KEYWORDS
ONO-1301, purmorphamine, small molecules, SUN11602, wound healing

1 INTRODUCTION

Skin wound healing features a significant clinical and socio-economic challenge worldwide. In Europe, chronic wounds affect around 1.5–2 million people and the costs with wound management can easily reach 3% of total healthcare expenditure. In the few last years, several bioengineered skin substitutes have been developed and medical devices are available in clinics. However, none result in complete regeneration. Despite efforts to achieve innovative regenerative approaches, nonhealing wounds are still associated with massive expenditures. Also, patients with complex wounds, such as burns, heal in a process that results in repair rather than regeneration. Limitations include poor integration, insufficient vascularization, scar formation, wound contraction, and immune rejection.
Inclusion of signaling molecules such as growth factors represents a promising strategy for enhanced skin graft performance. Although several clinical trials suggest that growth factors successfully affect hard-to-heal wounds, the risk–benefit ratio of using these biologicals is a matter of debate. Moreover, like other biologicals, growth factors comprise intrinsic pharmaceutical and pharmacokinetic shortcomings, including low stabilities and short half-lives as well as high production costs and batch-to-batch variations in quality.

Small molecular modalities have attracted attention as a more stable and less complex alternative to biological agents in the field of regenerative medicine. However, the utility of small molecules in skin regeneration has not been explored in detail. Here, we assessed several compounds for their potential to directly or indirectly mimic three distinct signaling pathways that are known to be involved in key processes of skin regeneration (Figure 1).

Sonic hedgehog (SHH) signaling is a regulator of skin tissue homeostasis, drives skin cell proliferation, and promotes angiogenesis. Upon injury, it stimulates hair follicle neogenesis. Purmorphamine is a purine derivative that activates SHH signaling by direct binding and activation of Smoothened, an essential component of the hedgehog pathway. Fibroblast growth factor-2 (FGF-2) is also known to significantly improve wound healing and scar quality in different types of wounds. SUN11602 is a small molecule that mimics functions of FGF-2 in a mechanism linked to fibroblast growth factor receptor 1 (FGFR1) activation, followed by downstream activation of the MEK/ERK-cascade and expression of signaling-related target genes. However, these FGF-2-mimetic features have so far only been probed in the context of neuroprotection. Moreover, prostacyclin (i.e., prostaglandin I2, PGI2) is a prostanoid with well-established roles in angiogenesis and regenerative responses in several tissues such as skeletal muscle, bone, and nerves. Its angiogenic and antiﬁbrotic activities are well documented in preclinical studies. ONO-1301 has been developed as a synthetic, chemically and biologically more stable PGI2 mimetic. It is a PGI2 receptor agonist that activates the cAMP/PKA signaling cascade, triggering the release of proangiogenic factors such as vascular endothelial growth factor (VEGF).

Given their different growth factor mimetic features, the three small molecules were applied to primary human skin cells to study effects on proliferation, differentiation, and migration to explore their potential as putative regenerative modalities in engineered skin substitutes.

### RESULTS AND DISCUSSION

Fibroblast proliferation is an essential process within the wound healing proliferative phase. Once safe dose ranges were determined for all compounds on commonly used fibroblast and keratinocyte cell lines (Figure S1), we evaluated three selected small molecules regarding proliferation of primary normal human fibroblasts (NHF) from three different donors (Figure 2a). For each compound, time-dependent proliferation profiles were generated (Figure S2). After 5 days of compound exposure, ONO-1301 (2 µM) and purmorphamine (0.5 µM) significantly increased the number of viable NHF cells from all donors by twofold, whereas SUN11602 had no effect.

We next performed gene expression analyses by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of compound-treated primary NHFs (Figure 2b) to assess several characteristic markers of different states, that is, proliferation (i.e., CCND1, MKI67), differentiation (i.e., ACTA2), and overall fibroblast activation (i.e., COL1A1, ELN). In addition, VEGF and MMP1 expression levels were monitored as relevant markers and mediators for healing responses. Data in Figure 2b show that, after 48 h of compound exposure, only ONO-1301 (2 µM) stimulated the expression of VEGFA and MMP1; however, SUN11602 (100 µM) did not significantly alter any of the monitored target genes and purmorphamine (0.5 µM) reduced the expression of VEGFA, COL1A1, and ELN. Figure S3 shows the full dose-dependent expression profiles after 48 h.

![Chemical structures of selected small molecule growth factor signaling mimetics.](image)

**Figure 1** Chemical structures of selected small molecule growth factor signaling mimetics. FGF-2, fibroblast growth factor-2; FGF-R1, fibroblast growth factor receptor 1; PGI2-R, prostaglandin I2 receptor; SHH, sonic hedgehog; Smo, smoothened; VEGF, vascular endothelial growth factor.
FIGURE 2  Functional and mechanistic profiling of growth factor mimetics using primary normal human fibroblasts (NHF). (a) Effects on cell viability (proliferation): NHF cells from three different donors (NHF1, NHF2, and NHF3) were exposed to SUN11602 (SUN, 100 µM), ONO-1301 (ONO, 2 µM), or purmorphamine (PUR, 0.5 µM) for 5 days before cell viability assessment. (b) Experimental setup and gene expression analysis of small molecule-treated NHF cells. Cells were exposed to SUN (100 µM), ONO (2 µM), or PUR (0.5 µM) for 48 h before RNA isolation and reverse transcription-quantitative polymerase chain reaction analysis. ACTA2, actin alpha 2, smooth muscle; CCND1, cyclin D1; COL1A1, collagen type I alpha 1 chain; ELN, elastin; MKI67, marker of proliferation Ki-67; MMP1, matrix metalloproteinase 1; VEGFA, vascular endothelial growth factor A. (c) Scratch assay results with NHF cells for cell migration and wound closure. A confluent layer of cells was scratched and exposed to SUN (100 µM), ONO (2 µM), or PUR (0.5 µM) for 48 h. Representative micrographs of untreated cells (vehicle control) and small molecule and fetal bovine serum (FBS)-treated cells after 48 h are shown. Scale bar = 0.5 mm. For quantitative analysis, pictures were taken at 0 h and 48 h, and the number of cells per scratch area was determined by image analysis. Independent biological replicates were carried out for all experiments (n = 3). Data are shown as means ± SD. For data in (a) and (c), statistical significances were evaluated by one-way analysis of variance complemented by Dunnett posthoc testing, whereas Student’s t test was applied for gene expression data in (b). Significances are expressed as *p < .05, **p < .01, and ***p < .001
Furthermore, the effect of all compounds on the migratory activity of primary NHF cells was evaluated using a wound healing assay (Figure 2c). High serum (i.e., 10% fetal bovine serum [FBS]) served as a positive control, increasing the migration of NHF cells by ca. twofold, compared with the low serum (i.e., 0.1% FBS) basal medium control, expressed as % closure of the scratched area. None of the tested compounds dramatically affected scratch wound closure by cell migration. Only ONO-1301 exhibited a very mild, but significant effect and reduced the migratory capacity of these cells.

Together, these data suggest that the synthetic prostacyclin mimetic ONO-1301 efficiently stimulated fibroblast proliferation and reduced cell migration, which is in line with a reported PKA-dependent inhibition of fibroblast migration by prostaglandin analogs. This activity may contribute to a general antifibrotic mode of action for ONO-1301. Moreover, ONO-1301 was the only compound that triggered the expression of MMP1 and VEGFA, thereby indirectly performing as a small molecule “VEGF mimic” on primary human fibroblasts. High VEGF levels typically accelerate and improve skin healing by angiogenesis that re-establishes microcirculation and rescues tissue perfusion in damaged tissue. Increased expression of matrix metalloproteinase 1 (MMP1) further underlines the antifibrotic and wound remodeling potential, keeping skin fibrosis and excessive scar formation at bay.

We next focused on functional profiling of the putative three small molecule growth factor mimetics on skin keratinocytes. Differentiation and cellular activity states of primary normal keratinocytes (NHKs) were captured by RT-qPCR-based gene expression analysis, as outlined in Figure 3a, whereas Figure 54 shows the full dose-dependent expression profiles after 48 h. In analogy to our studies in human fibroblasts, the expression levels of VEGFA and MMP1 were monitored as markers for wound healing–relevant regenerative cell responses upon compound treatment. Keratins-10 and -14 (i.e., KRT10, KRT14), involucrin (IVL), loricrin (LORICRIN), and transglutaminase 1 (TGM1) were analyzed as differentiation markers for keratinocytes.

Interestingly, only the putative FGF-2 mimetic SUN11602 (100 μM) increased keratinocyte differentiation, as almost all expression markers (i.e., KRT10, IVL, LORICRIN, TGM1) were significantly altered. At the same time, both VEGFA and MMP1 expression were enhanced. Thus, SUN11602 mimics a typical FGF-2 activity on skin cells. It should be noted that only the threefold increased VEGFA levels were statistically significant. Still, the extent of MMP1 expression stimulated by SUN11602 (ca. 16-fold) was unparalleled among the herein investigated small molecules and may be beneficial for mechanisms of wound remodeling. In contrast, ONO-1301 (2 μM) and purmorphamine (0.5 μM) only weakly enhanced the expression of MMP1. In line with these results, ONO-1301 and purmorphamine were both completely inactive concerning the migratory capacity of NHKs that was verified by our established wound healing assay (Figure 3b). However, SUN11602 inhibited keratinocyte migration by ca. twofold, which is in agreement with its potent stimulation of cell differentiation. In fact, during later stages of wound healing, keratinocytes are required to stop migrating to start the differentiation process. Taken together, SUN11602 appears to uniquely affect keratinocyte phenotypes that may be beneficial for skin regeneration. Stimulation of keratinocyte differentiation is recognized for late-stage wound healing via restoration of the skin barrier and the impermeable external epidermal layers. These features, combined with VEGF-triggered angiogenesis and an MMP-1-mediated promotion of the remodeling phase of wound healing via degradation of excessive extracellular matrix, may qualify SUN11602 as an efficient tool for later stages of wound healing.

Large, difficult-to-heal, and/or chronic wounds with different etiology remain a major therapeutic challenge. Aside from state-of-the-art wound care, split-thickness autografts represent the current gold standard for complex wound management. Novel approaches from regenerative medicine include the development of bioengineered skin grafts. However, the quality and effectiveness of such skin substitutes require the incorporation of molecules that support tissue regeneration on multiple levels. Growth factor and cytokine replacement by highly potent small molecule mimetics or activators of the underlying signaling events could provide favorable pharmacokinetic/pharmacodynamic profiles for next-generation, chemically defined engineered skin grafts. Even in cases when genuine, fully functional growth factor mimicry is not possible, a small molecule-mediated enhancement or sustained downstream signaling activity could be of therapeutic value. Here, we assessed three small molecules for their potential to mimic or mediate the biological activities of SHH, FGF-2, or VEGF signaling as key pathways involved in skin regeneration. Phenotypic and functional analysis of cultured primary skin fibroblasts and keratinocytes revealed unique, cell-specific, and complementary activity profiles for the FGF-2 mimetic SUN11602 and the putative VEGF mimetic ONO-1301, whereas purmorphamine turned out largely inactive. The prostacyclin receptor agonist ONO-1301 mainly affected skin fibroblasts, as it promoted their proliferation, inhibited migration, and stimulated the expression of MMP1 and VEGFA. In contrast, SUN11602 exclusively stimulated skin keratinocyte differentiation and their expression of MMP1 and VEGFA. These features resemble processes that are all relevant for efficient skin regeneration upon injury. A combined small molecule FGF-2/VEGF mimicry may not only improve angiogenesis-related microcirculation but also reduce early, excessive fibrosis (ONO-1301) while facilitating wound remodeling at later stages (SUN11602).

3 | CONCLUSION

Concluding from our findings, SUN11602 and ONO-1301 together may act as a valuable tool for improving the quality of wound healing. Building on these results, additional studies will have to further delineate the best application methods for these molecules, as well as their stability and compatibility with commonly applied biomaterials in tissue engineering.

4 | EXPERIMENTAL

4.1 | Small molecules

SUN11602 (Cat. No. 4826) and Purmorphamine (Cat. No. 4551) were purchased from Tocris Bioscience. ONO-1301 (Cat. No. O2264)
was purchased as a mixture of E/Z-isomers from Sigma-Aldrich. Stock solutions were prepared by dissolving the compounds in dimethyl sulfoxide (DMSO) (Boom B.V. Meppel), as indicated by the manufacturer. All cell groups received the same concentration of the vehicle (0.45% DMSO).

### 4.2 General cell culture methods

Primary normal human keratinocytes (NHKs) were obtained from skin explants after abdominoplasty, as previously described,[26] and were cultured using keratinocyte growth medium.
medium (KGM; Lonza) and differentiated by depletion of growth factors, as previously described. Primary normal human dermal fibroblasts (NHF) from healthy patients were isolated, as previously described, and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Life Technologies, Inc.) containing 10% FBS (Gold [Australian origin], PAA Laboratories GmbH) and 1% penicillin/streptomycin (10,000 U/100 mg/ml; Gibco, Life Technologies, Inc.). Cells were maintained at 37°C with 5% CO₂ in a humidified atmosphere.

4.3 Cell proliferation assay

NHF cells were seeded at a density of 2000 cells/well in 96-well plates and allowed to adhere for 24 h at 37°C. Standard medium was replaced by low-serum medium containing 0.1% FBS and the plate was incubated for 16 h, after which different concentrations of small molecules were added to the cells: SUN11602 (25–100 µM), ONO-1301 (0.5–2 µM), and purmorphamine (0.1–0.5 µM). The plates were incubated for a maximum period of 120 h and cell viability was assessed every 24 h using Alamar Blue® Cell Viability Assay (Thermo Fisher Scientific, Inc.), following the manufacturer’s instructions. Briefly, culture medium was replaced by Alamar Blue solution and plates were incubated for 4 h. After incubation, fluorescence was assessed at an excitation wavelength of 540 nm and emission of 620 nm in a microplate reader (Synergy 2; BioTek).

4.4 Quantification of messenger RNA expression levels

For gene expression analysis, NHFs were seeded in 24-well plates (3 × 10⁴ cells/well) in DMEM 10% FBS and incubated at 37°C. After 24 h, medium was replaced by DMEM + 0.5% FBS containing SUN11602 (25–100 µM), ONO-1301 (0.5–2 µM), or purmorphamine (0.1–0.5 µM). NHKs were seeded in 24-well plates (2 × 10⁴ cells/well) in keratinocyte growth medium (KGM; Lonza) and cultured until confluence. The medium was replaced by keratinocyte basal medium containing SUN11602 (25–100 µM), ONO-1301 (0.5–2 µM), or purmorphamine (0.1–0.5 µM). Control cells were exposed to medium containing 0.45% DMSO (vehicle). Cells were harvested for RNA isolation after 48 h of treatment. The following genes were assessed for keratinocytes: VEGFA, MMP1, KRT14, KRT10, IVL, LORICRIN, and TGM1. For fibroblasts, VEGFA, MMP1, CCND1, MKI67, ACTA2, COL1A1, and ELN were analyzed (Table S1).

4.5 RNA isolation and quantitative polymerase chain reactions

To isolate RNA, the cells were washed with PBS, followed by the addition of 250 µl TRIzol reagent (Invitrogen). After vigorous pipetting, lysed cells were transferred to 1.5 ml Eppendorf tubes. Hereafter, 1/5 volume of chloroform (Boom B.V. Meppel) was added, followed by vortexing for 30 s. After centrifugation at 17,000g for 20 min at 4°C, 150 µl of the water layer was transferred to a 1.5 ml Eppendorf tube. An equal volume of 70% ethanol (Boom B.V. Meppel) was added to the water layer and mixed shortly by inverting. Subsequently, the samples were purified using RNeasy mini kit columns (Qiagen GmbH) according to the manufacturer’s protocol. After washing, an on-column DNase I (RNase-free DNase I; Qiagen) treatment was performed. The yield was quantified spectrophotometrically at 260 nm using the NanoDrop (Thermo Fisher Scientific). Gene expression levels were measured using quantitative polymerase chain reactions (qPCR). Complementary DNA (cDNA) synthesis was performed in a 20-µl reaction volume on 50 ng of RNA (in 15 µl RNase-free water) using the iScript cDNA synthesis kit (iScript cDNA synthesis kit; Bio-Rad), as described by the manufacturer. As a no-reverse transcription control, 15 µl water was used. Hereafter, cDNA was diluted 20× in RNase-free water and used as a template for qPCR amplification (CFX real-time detection system; Bio-Rad) of the genes listed in Table S1. The reaction was performed in a total volume of 12.5 µl containing 2.5 µl cDNA (1.6 ng), 0.6 µM primers (see Table S1), and 6.25 µl iQ SYBR Green super mix (Bio-Rad). After incubation for 3 min at 95°C, amplification was carried out for 40 cycles of 15 s at 95°C and 30 s at 60°C. To indicate the purity of the product, the melting temperature was measured by increasing the temperature from 65°C to 95°C with 0.5°C increments every 10 s. The quantification cycles (Cq) for the genes of interest were normalized to the Cq value of the reference gene ribosomal protein large P0 (RPLP0). To calculate the relative quantity, the 2−ΔΔCt method was used, where cells treated with only the vehicle DMSO (0.45%) were taken as a control. No-template controls (2.5 µl water instead of cDNA) and no-reverse transcription controls were taken along on each plate for each gene. Quantification cycles (Cq) higher than 38 were not used for analysis. Primers were obtained from Biolegio (Nijmegen). All primers were validated to assess the PCR amplification efficiency (E), as described in the MIQE guidelines.[28] Primers with an efficiency of 100% ± 10% were used.

4.6 Scratch wound closure assay

NHK or NHF cells were cultured in 24-well plates until confluence. A single scratch wound was created using a 200 µm micropipette tip into a fully confluent cell layer. Cells were washed three times with PBS to remove cell debris, and medium containing SUN11602 (100 µM), ONO-1301 (2 µM), or purmorphamine (0.5 µM) was added. Control cells were exposed to the medium containing 0.45% DMSO (vehicle). FBS (10%) was used as a positive control. Microscopical images were taken at 0 and 48 h after scratching, and the area of the gaps was evaluated. NHF cells do not migrate in an orderly way, which makes it difficult to calculate the remaining open area after cell migration. So, for NHF, wound closure was calculated comparing the number of cells at the scratched area and at no scratched area (100% closure) after 48 h. For NHK, it was possible to calculate the area without cells. So, the percentage of wound closure area was calculated comparing the scratched area at 48 h with the initial
scratched area (0h). Images were captured by Leica DMi1 (Leica Microsystems) inverted optical microscope (×5 magnification) and the scratched area or the number of cells per area was determined by analyzing the images with ImageJ Software version 1.46r (National Institute of Health).

4.7 | Statistical analysis

Independent triplicates were carried out for all experiments (N = 3). The results were expressed as mean ± SD and data were evaluated by one-way analysis of variance complemented by Dunnett posthoc testing, except for gene expression analysis where Student’s t test was applied. p < .05 was considered as indicative of significance. All values were obtained using the statistical software GraphPad Prism version 6.00.

Additional methods’ information is described in the electronic supporting information.

ACKNOWLEDGMENTS

The authors would like to thank Katrien Brouwer and Marcel Vlij from the Association of Dutch Burn Centers, Beverwijk, The Netherlands, for kindly providing the primary fibroblasts, and Ellen H. van den Bogaard and Joost Schalkwijk from the Department of Dermatology of Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, for the primary keratinocytes. This study was funded by the CAPES Foundation, Ministry of Education of Brazil (process 11718/13-7), and the European Union’s EuroSkinGraft project (grant number 279024), TOC graphic was created with BioRender.com.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interests.

AUTHOR CONTRIBUTIONS

Cintia D. S. Horinouchi, Dennis Schade, Willeke F. Daamen, and Toin H. van Kuppevelt designed the research; Cintia D. S. Horinouchi and Corien Oostendorp performed the research and analyzed the data; Cintia D. S. Horinouchi, Corien Oostendorp, Dennis Schade, and Willeke F. Daamen wrote the paper; Dennis Schade, Willeke F. Daamen, and Toin H. van Kuppevelt critically revised the paper. All authors have read and approved the final manuscript.

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