Effects of topical application of CHF6467, a mutated form of human nerve growth factor, on skin wound healing in diabetic mice.


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HE, Haemotoxylin and Eosin staining; NGF, nerve growth factor; p75, p75 neurotrophin receptor; PECAM-1, Platelet endothelial cell adhesion molecule-1; PGP9.5, Protein gene product 9.5; TrkA, neurotrophic tyrosine kinase receptor A.

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ABSTRACT

Nerve growth factor (NGF) is the protein responsible for the development and maintenance of sensory skin innervation. Given the role of appropriate innervation in skin healing, NGF has been indicated as a possible pro-healing treatment in pathological conditions characterized by nerve ending loss, such as chronic ulcers in diabetes, however its use as a therapeutic agent is limited by its hyperalgesic effect. We tested the effect of topical application of the non-algogenic NGF derivative hNGFP61S/R100E in two models of skin ulcer induced in db/db diabetic mice, investigating healing time, skin histology, reinnervation and angiogenesis using morphological and molecular approaches. We showed that the topical administration of CHF6467, a recombinant human NGF in which an amino acid substitution (R100E) abolished the hyperalgesic effect usually associated with NGF, accelerates skin repair in experimental wounds (full-excision and pressure ulcer) induced in diabetic mice (db/db). CHF6467-induced acceleration of wound healing was accompanied by increased re-epithelization, re-innervation and re-vascularization, as assessed by histology, immunohistochemistry and image analysis. Bioinformatic analysis of differentially expressed genes and signaling pathways in the wound tissues showed that Akt-mTOR was the most regulated pathway. In spite of the transdermal absorption leading to measurable, dose-dependent increases in CHF6467 plasma levels, no systemic thermal or local mechanical hyperalgesia was observed in treated mice. When tested in vitro in human cell lines, CHF6467 stimulated keratinocyte and fibroblast proliferation and tube formation by endothelial cells. Collectively, these results support a possible use of CHF6467 as a pro-healing agent in skin lesions in diabetes.

SIGNIFICANCE STATEMENT

Topical application of CHF6467 accelerates re-innervation, neoangiogenesis and wound healing in diabetic mice in both full-thickness skin excision and pressure ulcer models through the Akt/mTOR pathway, and does not induce hyperalgesia.
INTRODUCTION

Wound healing is a complex biological process involving different cell types which interact in a defined spatial and temporal sequence (Rodrigues et al. 2016). When a skin wound does not repair within an established time window, the lesion becomes a “chronic ulcer”, a phenomenon usually associated with co-morbidities such as diabetes, obesity, cardiovascular diseases, or simply aging (Jaul et al. 2018), and as complication of surgical wounds (Nenna et al. 2019). In diabetic patients, the incidence of chronic non-healing wounds is fairly high, with an estimated 2% of this population in developed countries suffering from non-healing injuries, while complications cause 90% of limb amputations (Rodrigues et al. 2016). Moreover, the quality of the repaired skin, and the occurrence of wound relapse, are still open issues (Martinengo et al. 2019). The problem of chronic skin wounds is further complicated by the worldwide increase in diabetes prevalence, contributing to 9% of global mortality (Shaw et al. 2010).

Despite the efforts made to establish guidelines for acute and chronic skin wound treatment (Powers et al. 2016; Westby et al. 2017; Blume and Wu 2018; Everett and Mathioudakis 2018), only three FDA-approved therapies are available: two are skin substitutes, and a recombinant human platelet-derived growth factor (PDGF-BB, becaplermin, Regranex®). Given that 50% of diabetic ulcers fail to heal (Lebrun et al. 2010), new solutions are needed to shorten healing time, guarantee healing in severe, non-healing lesions, and prevent ulcer relapse where possible.

One target for new products is the improvement of endogenous regeneration (Wells and Watt 2018) through growth factors (Werner and Grose 2003). Originally described as a “neurotrophic factor” by Rita Levi-Montalcini (Levi-Montalcini 1987), Nerve Growth Factor (NGF) supports a number of physiological processes in the human body (Levi-Montalcini 1987). In the peripheral nervous system, the development of thinly myelinated Aδ or unmyelinated C-fibers which innervate the skin is an NGF-dependent process (Indo 2010), and half of the body’s nociceptive sensory neurons remain dependent on NGF in adulthood (Snider and McMahon 1998). Notably, diabetic skin is characterized by reduced epidermal innervation (McCarthy et al. 1995; Kennedy et al. 1996), a factor considered to be one of the main cause of diabetic neuropathy (Ebenezer and Polydefkis 2014) (Ebenezer and Polydefkis, 2014) supporting chronic ulcers (Laverdet et al. 2015).

NGF is now considered a “pleiotropic molecule” (Aloe and Calzà 2004). Inflammatory and immune cells produce NGF and express NGF high- (trkA) and low- (p75) affinity receptors, while inflammation enhances the
synthesis of NGF in various tissues (Calzà et al. 1997) and in fibroblasts, epithelial, endothelial, and immune cells (Prencipe et al. 2014; Gostynska et al. 2019). NGF has been indicated as an angiogenic molecule which stimulates nitric oxide synthase (NOS) and vascular endothelial growth factor (VEGF) production (Calzà et al. 2001), and induces a variety of effects on endothelial cells by autocrine and/or paracrine mechanisms (Nico et al. 2008; Gostynska et al. 2019). In the skin microenvironment, NGF appears to be a major player in the communication between sensory neurons and skin cells (Chéret et al. 2013).

A role of endogenous NGF in wound healing has been suggested by in vitro, animal, and human studies (Werner and Grose 2003; Kawamoto and Matsuda 2004; Chéret et al. 2013), which have led to the approval of NGF eye drops for the treatment of corneal neurotrophic ulcers (Sacchetti et al. 2020), and which have demonstrated the role of NGF as a peripheral pain mediator, particularly in inflammatory pain states (Pezet and McMahon 2006; Lewin et al. 2014).

In order to distinguish the positive properties of NGF from its detrimental effects, several trkA-binding agents have been developed (Carleton et al. 2018; Bagal et al. 2019). The rare autosomal recessive neuropathy known as hereditary sensory and autonomic neuropathy type V (HSAN V) is caused by a mutation in the NGF gene (R100W), and HSAN V homozygous patients display a congenital indifference to painful events, with deficits in peripheral nociceptors (Einarsdottir et al. 2004). Similarly, replacement of the arginine residue at position 100 with a serine residue has been shown to abolish NGF-induced hyperalgesia without affecting downstream trkA-mediated signaling (Testa et al. 2019). The additional replacement of the proline residue at position 61 of NGF with a serine residue leads to a non-algogenic derivative of hNGF (hNGFP61S/R100E, CHF6467) (Severini et al. 2017).

The aim of this study was to test the efficacy of topical CHF6467 in two skin ulcer models in diabetic mice. Healing time, repaired skin histology, reinnervation and angiogenesis were investigated using morphological and molecular approaches.

**MATERIALS AND METHODS**

*Animals and group composition*
All animal protocols described herein were carried out according to the European Community Council Directives (2010/63/EU), complied with the ARRIVE guidelines and the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Ministry of Health (authorization no. 391/2017-PR). Ten eleven-week-old genetically diabetic C57BL/KsJ-m+/+Leprdb (db/db) male mice were included in the experiments (Charles River Laboratories -Calco-Lecco). The animals were housed with food pellets and water ad libitum, and a dark-light cycle of 12 hours. Glycemia was measured prior to treatment, the day after the final treatment and prior to sacrifice in non-fasting animals, between 9 and 10AM (Contour XT, Bayer, Basel, Switzerland).

For skin excision lesions, the following groups were included (N=8 each, according to the power analysis of sample size calculation based on a pilot experiment):
- db/db, wound + vehicle
- db/db, wound + CHF6467 1µg/cm²
- db/db, wound + CHF6467 10µg/cm²
- db/db, wound + CHF6467 30µg/cm²
- db/db, wound + mNGF, 10µg/cm²
- db/db, wound + hNGF, 10µg/cm²

For pressure ulcers, the following groups were included (N=15 each, according to the power analysis of sample size calculation based on a pilot experiment):
- db/db, wound + vehicle
- db/db, wound + CHF6467 1µg/cm²
- db/db, wound + CHF6467 10µg/cm²
- db/db, wound + CHF6467 100µg/cm²

Intact mice were also included for histology and immunohistochemistry experiments. For each model, mice were randomly assigned to the experimental groups following lesion induction.

On the day of sacrifice, mice were deeply anesthetized and skin samples (1cm × 1cm) taken from the area of the wound. For the skin excision study, 4 samples were collected from each group for immunohistochemistry and 4 samples were collected for histology. For the pressure ulcer study, a 6 mm skin area was taken using an excisional punch (wound area). A 6 mm area of intact skin was collected for an exploratory tumorigenic study.

For the pathway array experiments, 6 mice from each group were used.

**Drugs, surgery, wound dressing and monitoring**
The following compounds were included in the study: CHF6467, provided by Chiesi (produced by AGC Biologics, Heidelberg, Germany); human \( \beta \)-NGF, recombinant, E.coli. (hrNGF, N-245, Alomone, Jerusalem, Israel), and mouse NGF, recombinant (moNGF, 1156-NG, R&D System). The CHF6467 production process is completely devoid of additives of animal or human origin, and the purification process has incorporated technological advances, including precursor and multimodal chromatography resins. The recombinant NGF protein was expressed in E. coli, refolded from inclusion bodies, purified, and proteolytically processed to NGF, as previously described (Patent PCT/EP2019/060733). The vehicle was a solution of purified water, acetic acid 20mM, and L-methionine 20mM, pH5.5.

For the skin excision model, a 6mm circular full-thickness wound was created by dermal punch biopsy on the midback of the animals, and the wound area immediately covered with a Tegaderm dressing (Tegaderm Roll - 3M Health Care, St.Paul, MN, USA). A 26-gauge needle was used to infuse 50μl of medication through the Tegaderm into the wound bed. Compounds and vehicle were administered daily for a total of 7 days from the time of surgery onwards (Fig. 1A) (Landi et al. 2003), and animals were monitored daily for dressing integrity and infection. The Tegaderm was changed weekly in all animals until wound healing was complete. Photographs were taken to monitor the wounds and the area was measured using Nis-Elements AR 3.2 software (Nikon Corporation, Tokyo).

Pressure ulcers were induced using two magnetic disks of 12 mm diameter and a thickness of 5.0 mm, with an average weight of 2.4 g and 1000 G magnetic force (Algamagnetic, Italy). A skin fold was gently raised and placed between the two magnets. This procedure left a 1.0 cm skin bridge between the magnets creating about 50 mm Hg compressive pressure between the two plates (Stadler et al. 2004). Three ischemia-reperfusion (I/R) cycles were used, where a single I/R cycle consisted of a period of 12 h for the application of the magnets followed by a rest period of 12 hours without magnets. Application of the compounds (60μl in each ulcer) began following wound curettage (at day 3 after the last I/R cycle) and was continued daily until 50% closure at the same dosage (day 14), then twice a week (40 μl day 17, 20 μl from day 19) until 100% closure at the same dosage (Fig. 5A). Treatment of the wound following surgical curettage consisted of removal of the exudate by gentle application of sterile gauze soaked in sterile saline solution. The wounds were dressed with Tegaderm which was replaced as above. The lesions were monitored via visual inspection by two independent operators to establish day of closure, without Tegaderm removal.
**Hyperalgesia**

Thermal hyperalgesia was evaluated in freely moving animals by Hargreave's Method using the Thermal Plantar Test Instrument (Ugo Basile, Comerio, Varese) on day 0 (before surgery) and day 7 (the day after the final NGF application). The mean of four latency to paw retraction measurements was used for statistical analysis.

Local mechanical hyperalgesia was evaluated using the Electronic Von Frey apparatus (Bioseb, Vitrolles Cedex, France) fitted with an elastic spring tip at the edge of the wound on a gently constricted animal. The mean of four measurements were used for the statistical analysis.

**Total NGF plasma assay**

The Human Adipokine Magnetic Bead Panel 2 kit (HADK2MAG-61K, EMD Millipore Corporation, Billerica, MA, USA) was used to quantify the hNGF in the plasma samples using xMAP technology and a MAGPIX Luminex platform. The assay was performed according to the manufacturer’s specifications, with some in-house modifications. In brief, following incubation of the bead population with plasma samples (25 μl) overnight at RT, the beads were washed and incubated, initially with detection antibody solution for 1h at RT, then with the streptavidin–phycoerythrin conjugated solution for 30 min RT. After washing, the beads were resuspended in 100 μl of Drive fluid and read on the MAGPIX instrument. The data was analyzed using xPONENT 4.2 ® software and the results expressed as pg/mL. We obtained values within the dynamic range of the standard curve (from 10000 to 0.128 pg/mL) for all the samples. Standard curves had a correlation coefficient (R2) value >0.98. The detection limit of NGF-β was 0.3-0.7 pg/mL.

With this kit, a very small amount of mouse NGF-β was quantified in the vehicle group due to antibody species cross-reactivity. Moreover, since the recombinant mouse NGF-β used for treatment is a homodimer of two 120 amino acid polypeptides which shares approximately 90% amino acid homology with human NGF-β, NGF was also quantified in moNGF-β-treated mice.

**Immunohistochemistry and quantitative analysis**

The samples collected for histology were fixed in paraformaldehyde 4% (w/v) and picric acid saturated aqueous solution in Sörensen buffer 0.1 M pH 7, embedded in paraffin, sectioned at 4μm, and stained using hematoxylin
and eosin (HE). The samples collected for immunohistochemistry were rapidly removed and fixed as above for 24h, then washed for at least 48h in 5% sucrose in 0.1 M phosphate buffer. Cryostat sections (14μm thick) were incubated overnight at 4°C with the primary antibodies: Laminin (Rabbit, Sigma, 1:1000), PGP9.5 (Rabbit, Boheringer, 1:2000), and PECAM-1 (Goat, R&D systems, 1:150). After rinsing in PBS for 20 min, the sections were incubated with the secondary antisera conjugated with Rhodamine Red™-X-conjugated - affinity-pure Donkey anti-Rabbit IgG (Jackson Immunoresearch) or secondary DyLight 488 Donkey anti-Goat IgG (Thermo Fisher Scientific), diluted in 0.3% Triton / PBS. The sections were then rinsed in PBS mounted in glycerol containing 1,4-phenyldiamine (0.1 g/L).

Immunofluorescence images were captured using a Nikon Eclipse E600 microscope equipped with the Q Imaging Retiga-2000RV digital CCD camera (Q Imaging, Surrey, BC, Canada) and a z-axis motorized stage. Analyses were performed using the Nis-Elements AR 3.2 software, by applying the same procedure to all images under comparison. Briefly, five z-stakes every 2 μm were collected from each image (300x500 μm), and the maximum intensity projection was used to calculate the immunoreactive area. For all morphological analyses, five images and two levels/animal sampled in the epidermal papillae at the center of the repaired ulcer were analyzed in each animal. All analyses were performed blindly. The immunoreactive area was calculated as area/fraction (percentage of Laminin/PECAM-1 and PGP9.5 over 400x300μm area). The measurement of repaired epidermal thickness was performed on histological sections (4μm thick, HE) in the same area. The mean value of five measurements/section and two sections for animal was used for the statistical analysis. In preparing the figures, the immunofluorescence images were occasionally contrasted. Where this is the case, the same procedure was applied to all images under comparison.

RT-PCR and STRING analysis

Samples for RT2 Profiler PCR Arrays (Qiagen) were collected from the full-thickness skin excision experiment at the core of the lesion (6 mm diameter) and RNA was extracted from all animals (6 animals per group), quantified and pooled. Pooled RNAs were retrotranscribed using the RT2 First Strand Synthesis Kit (Qiagen). Each pooled group was tested using a single PCR array, using the CFX96 real time PCR instrument (BioRad).
Qiagen mouse angiogenesis, extracellular matrix and adhesion protein, neurotrophins and growth factors were used to profile the expression of 250 genes. The possible tumorigenesis effect was also investigated using the dedicated array. The relative gene expression was calculated using the $2^{\Delta \Delta Cq}$ comparative method.

The dedicated Qiagen online data analysis software for the relative quantification of gene expression was used to perform the analysis and generate the graphs. The differentially expressed genes identified were then plotted in the STRING network analysis software (v.10; http://string-db.org/) to analyze the functional interactions between their biological functions.

**Cell culture, immunocytochemistry and image analysis**

To test the CHF6467 activity in skin cell lines, adult human primary epidermal keratinocytes (HEKa, ATCC® PCS-200-011™), human skin fibroblast cell lines (BJ, ATCC® CRL-2522™), and human umbilical vein endothelial cells (HUVECp, umbilical vein endothelial cells pooled from multiple isolates, Invitrogen C-015-5C, Thermo Fisher Scientific, Waltham, MA, USA) were used and cultured according to the respective conventional protocols (Gostynska et al., 2020).

For the cell proliferation assay, cells were seeded in 96-well plates (BJ, $3.5 \times 10^3$ cells/well, FBS 2%; HEK, $2 \times 10^3$ cells/well, no FBS) and treated with CHF6467 at 0, 50, 100 or 200 ng/ml, changing the medium every second day. Every two days, for 8 or 10 total days for HEK and BJ cells respectively, cells were detached by trypsinization and counted using a Bürker chamber (3 wells per time point, 2 counts per well).

HUVECs were used for the tube formation assay. Cells were seeded overnight at $9 \times 10^4$ cells/well in 24-well plates in culture medium containing 0, 50, 100 or 200 ng/ml of CHF6467. The next day, the cells were detached by Trypsin/EDTA and seeded on Geltrex™ LDEV-free matrix (Thermo Fisher Scientific) in culture medium containing the same concentration of CHF6467. Tube formation analysis was performed after 8 hours. Cells were fixed, washed, incubated with blocking solution for 1 hour and then overnight at 4°C with primary antibody (rabbit actin, Santa Cruz, 1: 200). After 2 washes in PBS, cells were incubated with secondary antibody (Donkey anti-rabbit Alexa Fluor 488, Molecular Probes, 1:500) and Hoechst nuclear staining at 37°C for 30 minutes, and finally washed twice in PBS. Cultures were analyzed using the cell-based High Content Screening technology (CellInsight™ CX5 HCS Platform, Thermo Scientific). The angiogenesis algorithm of the CellInsight software permits detection of all cells present in the well by identification of the nuclei (Hoechst staining), and each cell body by structural protein staining (actin). The angiogenesis index is automatically
calculated as follows: angiogenesis index = (1000 * total area of connected tubes) / total image area = (% of the image area covered by connected tubes) * 10.

HUVEC and BJ cells cultured in high-glucose conditions and treated with CHF6467 200 ng/ml for 48 hours were used for Western Blot analysis.

**Western Blot**

Western blot analysis was performed to quantify the Akt protein expression in cell cultures and skin. For skin tissue samples, the analysis was performed on the same samples used for RT-PCR and STRING analysis, and from intact skin. The skin tissue was homogenized in RIPA buffer and protease inhibitor (1x cocktail inhibitor Sigma, 1 mM PMSF, 10 mM sodium fluoride, 1mM sodium orthovanadate) with a homogenization ratio 1:8 (mg:µL) and centrifuged at 12000 g for 20 min at 4°C. Cells were detached from the culture plates, centrifuged, and the pellet resuspended in RIPA buffer and protease inhibitor. Total protein concentration was estimated using a standard colorimetric method. For each experiment, the same quantity of proteins was loaded (tissues 25 µg; HUVECs, 15 µg; BJ, 0.5 µg) and the marker protein (Precision Plus Protein Standards, Bio-Rad) added. A solution of Laemmli / β-mercaptoethanol was added to each sample, and after heating treatment (100 °C, 5 minutes), the proteins were resolved in 4-20% Mini-PROTEAN TGX Stain-Free Gels (Bio-Rad) and transferred to Amersham Protran 0.45µm Nitrocellulose Blotting Membrane (Bio-Rad). After blocking in Tris Buffer Saline solution containing 1% Tween20 (TBST) and 2.5% BSA, membranes were incubated with the primary antibody (rabbit pan-Akt, Cell Signaling -Leiden, The Netherlands- 1:1000; mouse β-actin, Santa Cruz Biotechnology -Dallas, Texas-, 1:150) overnight at 4°C. After washing three times in TBST, the membranes were incubated with HRP-conjugated secondary antibodies (swine anti-rabbit, Dako, 1:5000; swine anti-mouse, Dako, 1:5000) and HRP-conjugated protein for marker visualization (Precision Protein SrepTactin HRP-conjugate, Bio-Rad, 1:10000) for 1 hour at RT. The membranes were then washed three times with TBST. The immunoreactive signal was detected by incubating the membranes with Clarity Western ECL Substrate (Bio-Rad) for 5 minutes at RT in darkness and using the BioRad Chemi DOC MP imaging systems.

Western blot signals were measured by densitometry using ImageJ (Fiji) software. The Akt signals were normalized on β-actin in all the analyzed samples. Akt quantification was further normalized on intact skin values to obtain the variation index (intact skin = 1). For cell cultures samples, normalized Akt values were used for the analysis. Western blot signals were measured by densitometry using ImageJ (Fiji) software.
Statistical analysis

Data is expressed as mean±SEM. Statistical comparison of the rate of skin ulcer healing over time between treatment groups was performed with the log-rank test that compares the hazard functions of the different groups at each observed event time (Kaplan–Meier analysis). The Holm-Sidak test was used for multiple comparisons versus a control group (vehicle) (SigmaPlot 11, Systat Software Inc., San Jose, CA, USA). Histological and immunohistochemical results are reported as mean ± SEM. Prism software (GraphPad Software, San Diego, CA, USA) was used for the statistical analyses of this data, and for graph generation. The Student’s t-test or one-way ANOVA were used to analyze the data. Results were considered significant when the probability of their occurrence as a result of chance alone was less than 5% (p < 0.05).

RESULTS

PC12 in vitro assay

CHF6467 biological activity in PC12 cells was compared to that of moNGF and hrNGF using the neurite elongation test (Radio et al. 2008), performed by HCS measuring the average neurite length per cell, total neurite length per cell, and the percentage of cells showing a neurite equal to or longer than the cell body length. Results are presented in Fig. 1, supplementary materials. Initially, all NGF treatments were compared to the vehicle group and, from this analysis, only the most effective concentration for each NGF treatment was selected for further analysis (Fig. 2, supplementary materials). Compared to the vehicle treated group, all effective NGF treatment doses showed a significantly higher mean neurite length (moNGF, P = 0.0394; hrNGF, P = 0.0196; CHF6467, P = 0.0033) and mean total neurite length (moNGF, P = 0.0338; hrNGF, P = 0.0006; CHF6467, P = 0.0211). Only CHF6467 was found to significantly increase the percentage of cells showing long neurites (P = 0.0367), compared to the vehicle.

Animal monitoring

Animals were monitored for glycemia levels and body weight (Tables I and II). All diabetic mice showed very high blood glucose levels. No differences between the treatment groups were observed at the different times, either in the full-thickness excision or pressure ulcer model.
**Full-thickness excision skin ulcer**

**Wound healing**

The study schedule is shown in Fig 1A. Pictures of the wounds from representative mice from the vehicle, CHF6467, moNGF and hrNGF 10 μg/cm² groups are shown in Fig. 1B. The time course of mean percentage reduction in ulcer area compared to baseline is given in Fig. 1C, and shows that all NGF treatments significantly (two-way ANOVA, p < 0.0001) accelerated ulcer healing compared to vehicle (post-hoc test vs vehicle: CHF6467 1 μg/cm² p<0.0001; CHF6467 10 μg/cm² p<0.0001; CHF6467 30 μg/cm² p<0.0001; moNGF 10 μg/cm² p=0.0001; hrNGF 10 μg/cm² p<0.0001). The day-of-closure in individual mice is given in Table I, supplementary materials. At Day 29, complete healing was observed in all animals treated with hrNGF 10 μg/cm² or CHF6467 30 μg/cm². Fig. 1D shows the proportion of animals not completely healed over time, with moNGF, hrNGF and CHF6467 at 30 μg/cm² significantly accelerating the healing process compared to the vehicle.

**Pain threshold**

Thermal hyperalgesia was evaluated before skin lesion induction (d0) and after the final NGF application (d7). Results are shown in Fig. 2A. A significant reduction in pain threshold was observed in hrNGF-treated animals. No differences were observed in the other groups.

**Total NGF plasma levels**

Blood was collected 24 hours after the final application of NGF and at sacrifice (see Fig. 1A). Results are shown in Fig. 2B. The kit used to quantify NGF includes a capture monoclonal antibody that recognizes the epitope Ala 46-Asn 62, thus we quantified the total NGF, i.e. mouse endogenous NGF (since human antibody cross reacts with mouse NGF) and the CHF6467 present in the plasma of the treated animals.

A dose-dependent, transient increase in total NGF plasma level is observed in CHF6467-treated mice, reaching an average value of 350 pg/ml at d7. An increase in total NGF plasma levels is also observed in the moNGF-treated group. This is not surprising, since the recombinant mouse NGF-β used for treatments shares approximately 90% homology with human NGF-β at amino acid level (Ullrich et al. 1983). This increase is reversible, as indicated by the low levels of total NGF at sacrifice (12.80 ± 3.3 pg/ml). Very low levels of
endogenous NGF-β were detected in the vehicle-treated mice (11.0 ± 1.2 pg/ml), due to human antibody cross-reactivity with the mouse protein.

**Histology and immunohistochemistry**

The entire area of the skin ulcer, including a 5 mm margin of intact skin, was excised, embedded in paraffin and serially sectioned from the border to the centrum. The sections were then stained (HE), and representative low-power images taken at the different levels of the wound are shown in Fig. 3A (border of the lesion) and 3D (core of the lesion). High-power micrographs show the re-epithelization process at the wound border (Fig. 3B), where the epidermis migrating tongue (MET) and the extensive granulation tissue in the derma below the epidermal layer, characterized by inflammation, cell proliferation, matrix deposition and angiogenesis, are evident (Fig. 3E). Re-epithelization has been evaluated by measuring the epidermal layer thickness in the central area of the repaired wound. Non-healing wounds, as monitored in Table I (supplementary) were excluded from the analysis. Representative images from intact, vehicle- and CHF6467 30μg/cm²-treated mice are presented in 3 F-H, respectively, and the results are shown in Fig 3I. CHF6467 induced a dose-dependent thickening of the epidermal layer and a comparable effect was observed after treatment with matching doses of both moNGF and hrNGF. In treated mice, the basal layer of the epidermis was characterized by hypercellularity of the basal and spinous layers, possibly reflecting cell proliferation. Moreover, the dermis was also thicker and strongly stained, suggesting a higher extracellular matrix deposition, and showed the presence of skin annexes (glands and hair follicles).

Skin reinnervation was analyzed by immunostaining for the protein PGP9.5. The anatomy of the skin innervation is presented in Fig. 3C, where PGP9.5 –IR fibers in the intact mouse skin can be seen in the subcutaneous, deep cutaneous and sub-epidermal fibers. Radial fibers can be seen between the deep-subcutaneous and subependymal plexus, while the sub-epidermal plexus supplies the free epidermal nerve endings to the epidermis.

Fig. 3J-L illustrates PGP9.5-IR at sacrifice in intact, vehicle, and CHF6467 30μg/cm²-treated mice, respectively. Results from the morphometric analysis are presented as an immunoreactive percentage area in Fig. 3M. No differences in skin innervation were present between the intact animals, CHF6467 (all dosages) and moNGF groups, and there is no evidence of innervation restoration in vehicle-treated mice. On the contrary, a hyperinnervation is observed using hrNGF.
The effect of topical NGF application on angiogenesis was estimated using laminin as basal membrane marker, thus marking the outer endothelial cell membrane. Representative images are given in Fig. 3N-P, while the results are shown in Fig. 3Q. CHF6467 30µg/cm² induces a significant increase in laminin-IR, as hrNGF, possibly reflecting angiogenesis in progress.

Transcriptome analysis and validation

The aim of this experiment was to explore molecular mechanisms supporting the positive effect of CHF6467 on wound healing in diabetic mice, focusing on inflammation, extracellular matrix deposition, innervation, and angiogenesis. For this purpose, an exploratory strategy was used to map mRNA alterations of 252 key genes involved in angiogenesis, ECM, and growth factors (GFs) in the wound at 50% of the repair process (see Table III, supplementary material, for the full list). According to the data analysis software, the Thbs1 gene was used as the housekeeping gene. The results for angiogenesis (A), extracellular matrix (B) and growth factor (C) genes are presented in Fig 4 as a scatter plot analysis. Compared to vehicle-treated mice, the CHF6467 30µg/cm² treatment down-regulated several genes, including Akt, Ccl2 (chemokine (C-C motif) ligand 2), Ctgf (connective tissue growth factor), Hif1a (hypoxia-inducible factor 1-alpha), Mmp14 (matrix metalloproteinases-14), Thbs2 (thrombospondin 2), Tnc (Tenascin C), and up-regulated Mmp9 (matrix metalloproteinases-9).

We then analyzed the array data by STRING software analysis for pathway discovery, considering all the differentially expressed genes identified (web-software STRING 10 and Gene Ontology databases) to predict protein–protein interactions. We adopted stringent inclusion criteria by including genes whose expression changes are greater than the selected boundary (≥ 3). Analysis criteria were also stringent: confidence = 0.9 (highest, KEGG database); 1st shell. The results are shown in Fig. 4D, indicating a strong cluster around Akt-mTOR, which also includes Hifa and Hsp90.

In order to confirm the Akt mRNA down-regulation by the CHF6467 compared to vehicle-treated mice, we quantified the Akt protein in the same samples. Values were normalized on the intact skin of the same animals. Western blot analysis confirmed that CHF6467 treatment blocked the ulcer-mediated increase of Akt (Student’s t-test, p = 0.0049, Fig. 4E-F).

Pressure ulcer

Wound healing
The schedule of the experiment is presented in Fig. 5A. Representative images from the experimental groups are shown in Fig. 5B. “Day of closure” was established based on visual inspection and photo analysis by two independent operators (see Table II, supplementary materials for individual data). The clinical diagnosis of “non-healing wound” was confirmed by histological analysis of the specimens. The log-rank analysis of the healing curves indicated that all three doses (1, 10 and 100 µg/cm²) of topical CHF6467 significantly accelerated the healing process compared to vehicle (Fig. 5C), with 35 out of 36 skin lesions (97%) healed at Day 28 in the 10 µg/cm² group.

Pain threshold
Thermal hyperalgesia results are shown in Fig. 5D. A significant reduction in paw withdrawal latency was observed in CHF6467 1µg/cm²- compared to vehicle-treated mice. No hyperalgesia to thermal stimuli was observed in the other groups. Local mechanical hyperalgesia was also evaluated the day after the final test compound application (on day 15). No hyperalgesia to locally applied mechanic stimuli was observed in the CHF6467-treated groups (data not shown).

Histology and immunohistochemistry
Samples of the skin containing the area of the ulcer, including a margin of intact skin, were excised, embedded in paraffin, serially sectioned to obtain the “equator” of the repaired ulcer and stained for HE. A representative low-power image at the ulcer “equator” at sacrifice is shown in Fig. 6A. The histological structure of the epidermis, derma and hypodermis of the intact skin (peripheral parts) is shown, including the ulcer area (rectangle), where the epidermis is visible, but the derma is still incompletely restored, as indicated by the prevalence of collagen fibers and the paucity of dermal annexes (glands and hair follicles). The re-epithelization was analyzed by measuring the epidermal thickness at the equator of the lesioned area. Representative images in vehicle- and CHF6467-treated animals and respective results are shown in Fig. 6B-F. The histological analysis confirmed the clinical data (Table II, supplementary), showing that the new epidermal layer is complete in 18 out of 30 ulcers in vehicle-treated mice; in 29 out of 36 ulcers in CHF6467 1µg/cm²- treated mice; in 35 out of 36 ulcers in CHF6467 10µg/cm²- treated mice, and in 32 out of 36 ulcers in CHF6467 100µg/cm²- treated mice. The thickness of the newly formed epidermis sampled at the lesion equator progressively increases in a dose-dependent trend in CHF6467-treated mice compared to vehicle, resulting as being significantly thicker in CHF6467 100µg /cm²- treated mice (Student’s t-test, vehicle vs CHF6467 100µg/cm², P=0.0347).
The effect of topical CHF6467 application on nerve ending re-growth in the repaired skin has been analyzed as PGP9.5-IR in the epidermal papillae of the repaired area. Representative images and results from the morphometric analysis are given in Fig. 6G-K, respectively. A slight dose-dependent increase in innervation is observed (one-way ANOVA, F (3, 63) = 3.716, P=0.016), resulting in a significant effect in CHF6467 100μg /cm²–treated mice.

The effect of topical CHF6467 application on angiogenesis was estimated using PECAM-1 (CD31) as a marker. Representative images and results from the morphometric analysis are shown in Fig. 6L-P. An increase in capillary density, as evaluated by PECAM-1-IR, is observed in the dermis of CHF6467-treated mice (one-way ANOVA, F (3, 63) = 15.48, P < 0.0001), resulting in a significant effect in CHF6467 10μg /cm² and CHF6467 100 μg /cm²–treated mice.

**CHF6467 effect on in vitro population doubling of HEK and BJ cells and angiogenesis assays in HUVEC cells**

The aim of these experiments was to investigate the effect of CHF6467 on the three different cell types involved in the wound healing process, i.e. keratinocytes, fibroblasts and endothelial cells, considering proliferation and in vitro angiogenesis as respective readouts. We used three extensively used human cell lines, i.e. HEK keratinocytes, BJ fibroblasts, and HUVEC endothelial cells. Fig. 7 A and B illustrate HEK cultures in the investigated experimental conditions. CHF6467 induced a concentration-dependent increase in cell proliferation up to the 192-hour time point (two-way ANOVA, treatment F(3, 100) p < 0.0001, time F(4, 100) p < 0.0001, interaction F(12, 100) p < 0.0001) compared to the respective vehicle, and at the last time-point, all tested CHF6467 concentrations resulted in increased cell numbers compared to the vehicle-treated group (Dunnett’s post-hoc; 50, 100 and 200 ng/ml, p < 0.0001) (Fig. 7C).

Fig. 7D and E illustrate BJ cultures in the investigated experimental conditions. CHF6467 induced a concentration-dependent increase in cell proliferation in fibroblasts compared to the respective vehicle up to the 240-hour time point in culture (two-way ANOVA, treatment F(3, 100) p < 0.0001, time F(4, 100) p < 0.0001, interaction F(12, 100) p < 0.0001), and at the last time point, only the highest concentration of CHF6467 (200 ng/ml) produced an increase in cell number compared to cells exposed to vehicle (Dunnett’s post-hoc, p < 0.0001) (Fig. 7F).
The effect of CHF6467 on angiogenesis was tested on HUVEC cells stained for actin, using the conventional angiogenic assay protocol for cell growth and analysis. DAPT 5μM was used as reference compound. Fig. 7 G and H illustrate the effect of CHF6467 at the highest tested dose on angiogenesis (H) compared to vehicle-treated cells (G). The results indicate robust pro-angiogenic effects of CHF6467 (Fig. 7I; one-way ANOVA, F(4, 5) = 13.89, p = 0.0064), whose response at 100 and 200nM (Dunnett’s post-test, compared to vehicle, 100 ng/ml, p = 0.006; 200 ng/ml, p = 0.0051) was similar to the DAPT reference compound (Dunnett’s post-hoc, compared to vehicle, p = 0.0062).

To further support the mechanism identified by the bioinformatic analysis and confirmed by the protein analysis in the skin, we also quantified the Akt protein in BJ and HUVEC cell high-glucose cultures treated with vehicle or the highest dose of CHF6467 for 48 hours (200 ng/ml). BJ cultures (Fig. 8A) show a decrease in Akt protein quantity when treated with CHF6467 (Student’s t test, p = 0.0071; Fig. 8B-C), while the same treatment in HUVEC (Fig. 8D) resulted in no change in protein level (Fig. 8E-F).

**Potential tumorigenic effect: exploratory study**

To explore whether topical application of CHF6467 and the stimulating effect on cell proliferation activate aberrant, cancer-related pathways, we performed a pathway-focused gene expression analysis on tissue samples obtained immediately after CHF6467 discontinuation (day 14), corresponding to 50-70% of the repair process (see Table III, supplementary material, for the list of the investigated genes). The expression analysis was performed considering only the mean expression of B2m, or Acly, Xrcc4, Casp2, Stmn1, Igfbp3 as house-keeping genes, according to the Qiagen data analysis software recommendation. In both cases, the expression level of only one of the 84 genes (Ercc3) was down-regulated at the 100 μg/cm² concentration of CHF6467 (Fig. 2, supplementary materials), while the expression of the other genes was unchanged.

**DISCUSSION**

In this study, we showed that the topical application of CHF6467, a non-algogenic derivative of human NGF, accelerates skin repair in experimental wounds induced in diabetic mice. The main results of the study can be
summarized as follows: (i) CHF6467 accelerates wound healing in both the full-thickness skin excision model and in the model of pressure ulcer induced by repeated ischemia-reperfusion cycles; (ii) topical CHF6467 application does not induce systemic thermal or local mechanical hyperalgesia; (iii) CHF6467 improves re-epithelization, re-innervation and re-vascularization, as assessed by histology, immunohistochemistry and image analysis; (iv) the underlying molecular mechanisms involve the Akt-mTOR pathway; (v) when tested in vitro in human cell lines, CHF6467 stimulates keratinocyte and fibroblast proliferation, and tube formation by endothelial cells.

The study was performed in db/db mice, the most widely used mouse model of type 2 diabetes mellitus (Alpers and Hudkins, 2011). These mice develop progressive sensory loss, electrophysiological impairments and skin innervation loss (De Gregorio et al. 2018), as well as hyperalgesia to thermal and mechanical stimuli (Shi et al. 2013; Tang et al. 2019), aspects which reflect at least some of the features observed in human diabetic neuropathy (Yorek 2016).

Wound healing in db/db mice includes all phases of the process, i.e. inflammation (phase 1); proliferation (including re-epithelization), granulation tissue formation mainly by fibroblasts, neovascularization (phase 2), and remodeling, with scar maturation and reorganization of the extracellular matrix (phase 3). However, the repair process is much slower in db/db than in db/−, particularly in wounds covered by Tegaderm as in our experimental conditions (Sullivan et al. 2007), making this mouse model very appropriate for testing pro-healing compounds.

We observed that the topical application of CHF6467 accelerates wound healing in both ulcer models in db/db mice. Given the different pathogenic mechanisms supporting the ulcer (surgery and ischemia/reperfusion, respectively), and the differences in ulcer severity, we used two different application schemes. In the full-thickness ulcer, in which the lesion diameter is around 6 mm, we applied CHF6467 for 7 consecutive days starting immediately following lesion induction, therefore during the inflammatory phase of the process. Whilst in the pressure ulcers in which the lesion diameter is around 12 mm after debriding, we applied CHF6467 for 12 consecutive days, then every 2 days up to day 26, therefore in the inflammatory, but also in the proliferative phase of the process.

In the full-thickness skin excision model, the healing curve in the CHF6467-treated animal begins to differ from the other groups at 11 days post-lesion, and healing is complete in almost all animals treated with 10 and 30
μg/cm² of CHF6467 at 18 days post-lesion. Histological and immunohistochemical analysis reveals that NGF treatment (with native mouse and human forms of NGF and CHF6467) improves re-epithelization, re-innervation and angiogenesis in the repaired skin, confirming the previously described effects of the biologically active 2.5S NGF subunit on wound healing in full-thickness excision lesions in diabetic mice (Muangman et al. 2004).

To confirm the effectiveness of CHF6467 in skin wound healing in diabetic mice, we tested three different dosages (1, 10, 100μg/cm²) in a pressure ulcer model based on ischemia-reperfusion cycles (Stadler et al. 2004; Huggenberger and Detmar 2011), a model which has also been used to test new pharmacological and non-pharmacological treatments in dbdb mice (Duscher et al. 2015; Danigo et al. 2015; Cronk et al. 2015). Topical CHF6467 application accelerated the healing rate and time of ulcer repair, stimulating re-epithelization, re-innervation and angiogenesis processes in this model also.

Notably, none of the CHF6467 doses decreased the pain threshold, measured 24 hours after the final application, despite the dose-dependent increase in total plasma NGF levels compared to baseline, an increase which is transient and which normalized 22 days after CHF6467/NGF treatment withdrawal. On the contrary, an increase in pain sensitivity was observed in hrNGF-treated mice, allowing us to conclude that transdermal absorption of the mutated NGF CHF6467 does not induce pain threshold variations for thermal stimuli after multiple dose administration.

From these experiments, we can conclude that the topical application of CHF6467, a modified human recombinant NGF that does not elicit hyperalgesia at therapeutic doses, accelerates wound healing in diabetic mice. Improved re-innervation of the wound area may be a supporting mechanism: indeed, a central rationale for using NGF to promote healing in diabetes stems from a feature of the disease known as “small-fiber neuropathy” and characterized by a reduced number of nerve endings in the epidermis (Ebenezer and Polydefkis 2014). Appropriate skin innervation plays a preeminent role in wound healing (Kiya and Kubo 2019), and a reduction in neurotrophic support to peripheral neurons has been described in diabetic skin (Sima 2003). NGF is the neurotrophin responsible for establishing sensory innervation of the skin during development, and for its maintenance in adulthood (Indo 2010). Thus, the topical application of NGF has been considered as a possible therapy for accelerating skin wound healing in pressure ulcers (Aloe 2004) and post-chronic vasculitis ulcers (Tuveri et al. 2000).
Several cell types in the skin are NGF-sensitive (Sofroniew et al. 2001) and may be involved in the positive effect on wound healing exerted by the topical application of CHF6467. CHF6467, when applied in vitro, induces a concentration-dependent increase in keratinocyte and fibroblast proliferation. This confirms recent results demonstrating that moNGF stimulates in vitro keratinocyte proliferation and migration, and reverses the impairment induced by high d-glucose concentrations in the culture medium (Gostynska et al. 2019).

NGF plays a role in angiogenesis, as suggested by in vivo studies in several tissues under physiological and pathological conditions. It also promotes VEGF synthesis and secretion (Calzà et al. 2001; Li et al. 2018; Ahluwalia et al. 2018); indeed, an NGF defect has been associated with impaired angiogenesis and defective mucosa repair (Ahluwalia et al. 2018). A protective and proangiogenic role of NGF on endothelial cells has been also described in vitro (Ahluwalia et al. 2017; Lazarovici et al. 2018), and we demonstrated that both moNGF and CHF6467 increase endothelial cell proliferation and in vitro angiogenesis in a concentration-dependent manner, as well as restoring the tube formation capacity disrupted by high glucose concentrations in the culture medium (Gostynska et al. 2019).

Given the substantial hypertrophy observed in repaired skin epithelium, and the disputed role of NGF in cancer (Demir et al. 2016), we also explored the regulation of 80 cancer-related genes at 50% of the wound healing process in the pressure ulcer model. The only down-regulated gene in mice treated with the highest CHF6467 dose is Ercc3, a gene involved in DNA repair but also in hair follicle physiology (Yu et al. 2012).

Wound healing involves dozens of molecules which interact with the different cell types concerned. We therefore adopted an exploratory, bioinformatic strategy to identify possible primary molecular pathways involved in the pro-healing effect of CHF6467, comparing gene expression regulation in CHF6467 and vehicle-treated mice in the full-thickness excision model at 50% of the repair process, when ECM deposition, keratinocyte proliferation and angiogenesis are occurring simultaneously. From the analysis of 240 genes related to angiogenesis, ECM, and growth factors, we observed that matrix metallopeptidase 9 (Mmp9), an enzyme involved in tissue remodeling and required for the recruitment of endothelial stem cells during angiogenesis (Heissig et al. 2002), is up-regulated in CHF6467- mice compared to vehicle-treated. Conversely, Akt1, Ccl2, Ctgf, HIF1a, Mmp14, Thbs2 and Tnc were all down-regulated by CHF6467 treatment. Notably, a hyperactivity of these genes seems to play a role in pathological wound healing in diabetes and other conditions (Bornstein et al. 2000; Botusan et al. 2008; Liu et al. 2012; Ishikawa et al. 2015; Catrina and Zheng 2016; Zigrino et al. 2016; Abu El-Asrar et al. 2018; Gale et al. 2018; Kunkemoeller et al. 2019).
The bioinformatic analysis performed by STRING software indicates that the Akt-mTOR pathway is involved in the pro-healing effects of CHF6467, as also confirmed by protein analysis, and fibroblasts are the cells in which this regulation occurs. Akt and mTOR are considered survival and cellular growth in response to lesions. NGF, via trkA, activates PI3K/Akt and ERK/MAPK signaling pathways and the downstream mTOR, and this pathway mediates several NGF effects, such as endothelial cell invasion and cord formation during development (Park et al. 2007), angiogenesis in subchondral bone (Yu et al. 2019), and neuroprotection (Elsherbiny et al. 2019). Moreover, NGF-enhanced secretion of VEGF is also mediated by the ERK1/2 and PI3K/Akt pathways (Wang et al. 2016). An impairment of the Akt-mTOR pathway has been indicated as a possible cause of wound healing impairment in diabetic mice (Huang et al. 2015; Jere et al. 2019), and transient pharmacological activation of the PI3K-Akt-mTOR signaling axis has been considered a novel clinical intervention strategy to accelerate wound (ulcer) healing (Squarize et al. 2010).

In conclusion, we showed that the topical application of CHF6467, a non-algogenic hrNGF derivative, accelerates skin wound healing in diabetic mice, in both full-thickness excision and pressure ulcer models, also improving angiogenesis and re-innervation, without inducing hyperalgesia. We suggest that the Akt/mTOR pathway may be activated by CHF6467.

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AUTHOR CONTRIBUTIONS

Participated in research design: Calzà L., Giardino L. Imbimbo B.P., Villetti G.,
Conducted experiments: Alastra G., Cescatti M., Fernandez M., Flagelli A., Giuliani A., Lorenzini L., Pannella M.,

Contributed new reagents or analytic tools: Villetti G.

Performed data analysis: All Authors

Wrote or contributed to the writing of the manuscript: Baldassarro V. A., Calzà L., Giardino L., Imbimbo B.P., Villetti G.

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67:907–916


FOOTNOTES

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Legend to the figures

Figure 1

Full-thickness excision lesion: schedule of the experiment and healing curves.

A. Schedule of the experiment. B. Representative photos of the ulcers at different post-lesion times in vehicle, CHF6467, moNGF, hrNGF, 10μg/cm²-treated dbdb mice. C. Ulcer areas in the experimental groups, expressed as a percentage of the skin lesion area at day 0. Statistical analysis: two-way ANOVA indicates a time effect (F (50,434)=417.3, p<0.0001), a treatment effect (F (5,434)=21.45, p<0.0001), and an interaction (F (50,434)=1.638, p=0.0055). D. Kaplan-Meier curve expressing the non-healing probability in the experimental groups.

Figure 2

Pain threshold and NGF plasma levels in full-thickness excision lesion.

A. Thermal hyperalgesia measured before the first topical treatment (d0) and immediately after the final application (d7) in all experimental groups. Statistical analysis: Student’s t-test, *p<0.05. B. Total NGF plasma level 2 hours (day 7) and 21 days (day 28) after the last topical NGF application. The horizontal lines refer to NGF plasma levels in vehicle-treated mice. Statistical analysis: one-way ANOVA and Dunnett’s post-hoc test, ***p<0.001

Figure 3

Histological and immunohistochemical analysis of the repaired skin in the full-thickness excision model.

A, D: HE staining of the skin biopsy at experimental day 8, tangential to the lesion (A) and at the lesion equator. (D); B: HE staining showing the epithelial cell migration at the lesion border (epidermis migrating tongue) and (E) the underlying extensive granulation and angiogenesis. C: PGP-9.5 immunostaining in intact skin, illustrating the skin innervation anatomy.

F-I: representative HE stained section of the repaired skin in intact (F), vehicle (G), CHF6467 30μg/cm²-treated dbdb mice (H), and morphometric evaluation of epidermal thickness (I, grey horizontal bar corresponds to intact).
J-M: representative micrographs of PGP 9.5-IR fibers in repaired skin in intact (J), vehicle (K), CHF6467 30μg/cm²-treated dbdb mice (L), and morphometric evaluation of PGP 9.5-positive percentage area (M, white empty circle corresponds to intact).

N-Q: representative micrographs of laminin-IR in repaired skin in intact (N), vehicle (O), CHF6467 30μg/cm²-treated dbdb mice (P), and morphometric evaluation of laminin-positive percentage area (Q, white empty circle corresponds to intact).

Statistical analysis: one-way ANOVA and Dunnett’s post-hoc test, *p<0.05, **p<0.01, ****p<0.0001.

Bars: A,D 500μm; B,E 50μm; C, F-P 100μm.

**Figure 4**

RT2 Profiler PCR arrays analysis.

A-C: Scatter plot representation of gene expression analysis of RT2 Profiler PCR arrays for specific pathways: angiogenesis (A), extracellular matrix and adhesion molecules (B) and neurotrophins and growth factors (C) analyzed in the samples taken from the core of lesions of dbdb mice treated with CHF 30 μg/cm² vs dbdb mice treated with vehicle, at 50% of closure. The table of the differentially expressed genes (fold of change > 3) is included in each graph.

D: STRING software analysis of protein interactions. Proteins coded by the differentially expressed genes were included in the analysis and a 1st shell of interacting proteins was added.

E-F: Ulcer-induced variation expression of Akt protein levels quantified (E). Quantification was performed by Western Blot on samples taken from core of ulcers (6 mm) of animals treated with vehicle or CHF6467 (n = 6) and on intact skin of the same animals (n = 3) (F). Akt quantification was normalized on b-Actin levels for each sample and the obtained Akt levels of ulcers were further normalized on intact skin values to obtain the variation index (intact skin = 1, horizontal dotted line in graph E).

Statistical analysis. Student’s t-test, ** p < 0.01.

**Figure 5**

Pressure ulcer lesion: schedule of the experiment and healing curve.

A. Schedule of the experiment. B. Representative photos of the ulcers at different post-lesion times in vehicle, CHF6467 1, 10, 30μg/cm²-treated dbdb mice. C. Kaplan-Meier curve expressing the non-healing probability in
the experimental groups. D. Thermal hyperalgesia measured at day 15 in all experimental groups. Statistical analysis: one-way ANOVA and Dunnett’s post-hoc test, *p<0.05

**Figure 6**

Histological and immunohistochemical analysis of the skin in the pressure ulcer model.

A: HE staining of the skin biopsy at experimental day 28, showing the central area (dotted square) considered for morphometry.

B-F: representative HE stained section of the repaired skin in vehicle (B), CHF6467 1 (C), 10 (D), 100µg/cm² - (E) treated dbdb mice, and morphometric evaluation of epidermal thickness (F).

G-K: representative micrographs of PGP 9.5-IR fibers in vehicle (G), CHF6467 1 (H), 10 (I), 100µg/cm² -(J) treated dbdb mice, and morphometric evaluation of PGP 9.5-positive percentage area (K).

L-P: representative micrographs of PECAM-1-IR in repaired skin in vehicle (L), CHF6467 1 (M), 10 (N), 100µg/cm² -(O) treated dbdb mice, and morphometric evaluation of PECAM-1-positive percentage area (P).

Statistical analysis: one-way ANOVA and Dunnett’s post-hoc test, *p<0.05, **p<0.01, ****p<0.0001.

Bars: A,D 500µm; B,O 50µm.

**Figure 7**

Effect of CHF6467 exposure in human cell cultures.

A-C: Representative images of HEK cells exposed to vehicle (A) or CHF6467 200 ng/ml (B) at 192 hours in cultures and quantification of cell number at 5 different time points (every 48 hours) of cells exposed to vehicle or CHF6467 at three different concentrations (50, 100, 200 ng/ml; C). Scale bar: 20 µm.

D-F: Representative images of BJ cells exposed to vehicle (D) or CHF6467 200 ng/ml (F) at 240 hours in cultures and quantification of cell number at 6 different time points (every 48 hours) of cells exposed to vehicle or CHF6467 at three different concentrations (50, 100, 200 ng/ml; F). Scale bar: 20 µm.

G-I: Representative images (cell-based HCS montage representing the whole well) of HUVEC cells exposed to vehicle (G) or CHF6467 200 ng/ml (H), and quantification of angiogenesis index at 8 hours of cells exposed to vehicle or CHF6467 at three different concentrations (50, 100, 200 ng/ml; F). Scale bar: 200 µm.

Statistical analysis. Two-way ANOVA (C, F) or one-way ANOVA (I) followed by Dunnett’s post-hoc. Asterisks represent differences compared to vehicle-treated cultures (** p < 0.01; **** p > 0.0001).
Figure 8

Akt protein quantification in cell cultures.

A-C: Akt protein levels quantification in BJ cells high-glucose cultures (A) exposed to vehicle or CHF6467 200 ng/ml (B) by Western Blot (C).

D-F: Akt protein levels quantification in HUVEC high-glucose cultures (D) exposed to vehicle or CHF6467 200 ng/ml (E) by Western Blot (F).

Scale bar: 10 µm.

Statistical analysis. Student’s t-test, ** p < 0.01.
Table I.

Body weight and glycemia of animals included in the full-thickness excision study

<table>
<thead>
<tr>
<th>Groups, N=8</th>
<th>Body weight, day -1, gr</th>
<th>Body weight, day 26, gr</th>
<th>Glycemia day -1, g/ml</th>
<th>Glycemia day 26, g/ml</th>
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<tr>
<td>Intact</td>
<td>42.4 ± 1.7</td>
<td>43.1 ± 1.5</td>
<td>434 ± 33</td>
<td>562 ± 29</td>
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<tr>
<td>Vehicle</td>
<td>38.9 ± 0.4</td>
<td>36.5 ± 1.3</td>
<td>406 ± 35</td>
<td>552 ± 30</td>
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<tr>
<td>CHF6467, 1µg/cm²</td>
<td>40.3 ± 1.4</td>
<td>38.9 ± 1.2</td>
<td>398 ± 37</td>
<td>512 ± 45</td>
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<tr>
<td>CHF6467, 10µg/cm²</td>
<td>39.8 ± 1.2</td>
<td>35.8 ± 2.3</td>
<td>482 ± 27</td>
<td>585 ± 17</td>
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<td>CHF6467, 30µg/cm²</td>
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<td>37.9 ± 1.6</td>
<td>360 ± 64</td>
<td>578 ± 14</td>
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<td>moNGF, 10µg/cm²</td>
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<td>38.9 ± 1.6</td>
<td>391 ± 36</td>
<td>471 ± 45</td>
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<td>hNGF, 10µg/cm²</td>
<td>39.1 ± 8.9</td>
<td>35.4 ± 1.8</td>
<td>404 ± 60</td>
<td>573 ± 17</td>
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Table II.

Body weight and glycemia of animals included in the pressure ulcer study

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<th>Groups</th>
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<th>Body weight, day 26, gr</th>
<th>Glycemia day -1, g/ml</th>
<th>Glycemia day 26, g/ml</th>
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<td>Vehicle, N=15</td>
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<td>339 ± 31</td>
<td>553 ± 19</td>
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<td>CHF6467, 1mg/cm², N=18</td>
<td>39.0 ± 0.7</td>
<td>37.3 ± 1.0</td>
<td>361 ± 29</td>
<td>575 ± 11</td>
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<tr>
<td>CHF6467, 10mg/cm², N=18</td>
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<td>37.3 ± 1.0</td>
<td>330 ± 24</td>
<td>527 ± 32</td>
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<tr>
<td>CHF6467, 100mg/cm², N=12</td>
<td>39.4 ± 1.1</td>
<td>37.0 ± 1.2</td>
<td>364 ± 41</td>
<td>518 ± 42</td>
</tr>
</tbody>
</table>
Fig. 1
A. Thermal hyperalgesia

B. Total NGF plasma levels

Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig 8