UNIVERSIDAD AUTÓNOMA DE SAN LUIS POTOSÍ

FACULTAD DE CIENCIAS QUÍMICAS
POSGRADO EN CIENCIAS FARMACOBIOLÓGICAS

“Búsqueda de inductores de moléculas involucradas en la cicatrización como opción terapéutica para las úlceras de pie diabético”

Tesis para obtener el grado de:

Doctor en Ciencias Farmacobiológicas

Presenta:

Santos Mena Alan Orlando

Co-Directores de Tesis

Dr. Bruno T. Rivas Santiago

Dra. Diana Patricia Portales Pérez

SAN LUIS POTOSÍ, S.L.P.

FECHA: 06 de enero de 2025

UNIVERSIDAD AUTÓNOMA DE SAN LUIS POTOSÍ
Facultad de Ciencias Químicas
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Alan Orlando Santos Mena

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Agradezco sinceramente su valioso tiempo y dedicación para llevar a cabo una exhaustiva revisión de la tesis. Quedo a su disposición para cualquier consulta o inquietud que pueda surgir en el proceso.

Sin más por el momento, le envío un cordial saludo.

A T E N T A M E N T E

Dr. Sergio Zarazúa Guzmán
Coordinador Académico del Posgrado
en Ciencias Farmacobiológicas

Agradecimientos:

En esta última etapa de mi formación académica quiero dedicarle y agradecerle este logro a mi familia, a mi amada esposa Lizeth, que desde hace más de 10 años me ha acompañado incondicionalmente a lo largo de este proceso, a mi hijo Liam, que su sonrisa hace que todo valga la pena.

A mis papás Eduardo Santos y Silvia Mena, mis más grandes ejemplos a seguir, a pesar de ya no vivir con ellos, me siguen amando y cuidando, espero algún día ser tan buen padre como ustedes y poder darle el mismo amor a mi hijo Liam.

A mis hermanos, Erika, Eduardo y Aldo, que sepan que, sin importar el lugar y la fecha, siempre los voy a llevar conmigo en mis pensamientos, ya que somos el legado de mis abuelos y mis padres.

A mis sobrinos, Angel, Camila, Mateo, Miguel, Gerardo, Fernanda, Mónica, Alejandro, Caleb, Jacqueline, Victor, Jared, Ian, Jafet, Alondra, Giss y Sarita, que sepan que este logro es tan suyo como mío.

A mi familia Santos Zamarripa y Mena Alvarado, en quienes encontré inspiración para llegar hasta aquí, sobre todo a mis abuelos, Don Mundo, Doña Toña, Mamá Nena y Papá Pino, para que en esta tesis vean hasta donde llega su legado

A mi suegra Silvia De Ávila, quien, a excepción de mi madre, la considero y la quiero como a una segunda madre.

A mis cuñados y cuñadas, a quienes veo y quiero como si fueran mis hermanos. “La magia que tiene el amor de la familia y la resilencia que Dios me permite tener, han sido la clave de este éxito”

A mis amigos de la Preparatoria 1, quienes no importa cuánto duremos sin vernos, el tiempo con ellos se detuvo hace 18 años, durante el primer día de clases.

A mis Directores y asesores de tesis, sobre todo al Dr. Bruno Rivas Santiago quien permitió que durante 8 años desarrollara mis ideas y mi trabajo de tesis en su laboratorio.

A mis amigos de laboratorio, por transformar cada seminario y experimento en risas. Su amistad son de las cosas que más atesoro durante el doctorado.

A mis alumnos de Ammadeus y el Colegio del Centro, muchas gracias por las palabras de ánimo y el afecto que me dieron, fueron ese empujón que finalizó esta última etapa de mi vida.

Resumen

A nivel mundial, las heridas crónicas afectan la salud de millones de personas, afectando negativamente la calidad de vida. Las vías necesarias para la cicatrización son la respuesta a hipoxia y la expresión de péptidos de defensa del huésped, ambos disminuidos en enfermedades relacionadas con heridas crónicas, como la diabetes mellitus y enfermedades cardiovasculares. Recientemente se ha demostrado que los inhibidores de histona desacetilasa pueden inducir la expresión de estos péptidos mediante la activación del factor inducible por hipoxia 1-α, promoviendo la cicatrización, desafortunadamente, el alto costo y los efectos secundarios limitan la investigación clínica de estos inhibidores. Con la ayuda de herramientas bioinformáticas, encontramos potenciales inhibidores de la histona desacetilasa los cuales fueron tomados de una base de datos FDA, 3 candidatos DiPU, Ace y N-boc, mostraron tener efectos cicatrizantes en la línea celular HaCaT, aumentando la migración y activando al factor inducible por hipoxia 1-α que a su vez, induce la expresión de LL-37 y VEGF, en un ensayo en anillo de ratón utilizando Ace y N-boc se indujo la angiogénesis posiblemente por la activación de vías hipóxicas. En un modelo de queratinocitos primarios de donantes sin DM, con DM y de úlceras del pie diabético, se observó que sólo DiPU es capaz de inducir LL-37 en los 3 grupos. A pesar de la poca información sobre los inhibidores de la histona desacetilasa sobre la cicatrización, en este artículo se observaron resultados prometedores y específicos, enfocados en la inhibición de la histona desacetilasa 1.

Palabras Clave: Cicatrización, Heridas crónicas, HDAC, HIF-1α, Peptidos Antimicrobianos

Abstract

Globally, chronic wounds impact the health of millions of people, negatively affecting quality of life and healthcare budgets. Some of the crucial steps and pathways in healing mechanisms are the hypoxic response and the expression of host defense peptides, which are decreased in diseases related to chronic wounds such as diabetes mellitus and cardiovascular diseases. It has been shown that histone deacetylase inhibitors can induce the expression of host defense peptides by inducing the stabilization and activation of hypoxia-inducible factor 1-α, promoting wound healing pathways, although their high cost and side effects limit clinical research. With the help of bioinformatics tools, we found potential histone deacetylase inhibitor candidates in an FDA-approved drugs database, the candidates, DiPU, Ace, and N-boc, show wound healing effects in HaCaT, increasing cell migration possibly via hypoxia-inducible factor 1-α activation, inducing the expression of LL-37 and vascular endothelial growth factor, while in a mouse ring angiogenesis model, Ace and N-boc have angiogenic effects. In a model of basal primary keratinocytes from donors with DM, without DM, and from Diabetic Foot Ulcers, it was observed that only DiPU is capable of inducing LL-37 in all scenarios. There is limited information about histone deacetylase inhibitors and wound healing but in this paper, we observe promising results and a proposed mechanism that involved specifically Histone Deacetylase 1 inhibition.

Keywords: Wound healing, chronic wounds, HDAC, HIF-1α, host defense peptides, epigenetic modifications.

Índice tésis

Introducción

La piel constituye uno de los órganos más importantes en la defensa contra patógenos, siendo la barrera física más grande del cuerpo, cuando la integridad de esta barrera se deteriora, un mecanismo de cicatrización de heridas bien orquestado se encarga de recuperar la homeostasis alrededor de las 2 semanas[26]. Aquellas heridas con 4-12 semanas sin signos de curación se denominan Heridas Crónicas (HC) y comúnmente son complicaciones de otras enfermedades como la Diabetes mellitus (DM) o las Enfermedades Cardiovasculares (ECV)[1153]. Los desafíos que plantea el cuidado clínico y personal de las HC ejercen una presión significativa sobre la calidad de vida, los recursos y presupuesto sanitarios. Por ejemplo, en países como Australia, Singapur y varias naciones europeas y escandinavas, entre un 2% y un 4% del gasto sanitario total se asigna exclusivamente al tratamiento de heridas crónicas, con una tendencia a que aumente en los próximos años debido a la creciente cantidad de pacientes con DM y ECV en el mundo[20]. Las etapas clave del proceso de curación de heridas implican la resolución de la inflamación y el inicio de la proliferación por parte de las células de la piel[5]. Sin embargo, en ciertos tipos de heridas, como las úlceras de pie diabético (UPD) las cuales se asocian con la diabetes mellitus (DM), son interrumpidas estas fases debido a infecciones persistentes y la regulación negativa en las vías de señalización para la cicatrización de heridas como lo son proteínas hipóxicas como HIF-1α, VEGF, ANG, así como los péptidos de defensa del huésped (HDP), como LL-37 y HBD-3, se ha demostrado que restablecer cualquiera de estas vías mejora los procesos de cicatrización[323982]. En los últimos años se ha demostrado que una clase de fármacos conocidos como inhibidores de histona desacetilasa (HDACi) muestran diversos efectos dependiendo del tejido específico al que se dirigen[58]. Si bien se han sugerido por su potencial como agentes antitumorales, los inhibidores de HDAC también demuestran impactos favorables en el sistema inmunológico[64]. Esto se logra mediante la activación de varias vías, incluida la regulación positiva de HIF-1α, que induce a una mayor expresión de HDP, como LL-37[4870]. Gracias a esto podemos obtener candidatos prometedores para el tratamiento de heridas crónicas

mediante la activación de vías que involucran tanto a la hipoxia como la expresión de los HDP[31]. Sin embargo, aún resulta complicado proponerlos como un posible tratamiento por sus posibles efectos secundarios y el elevado costo asociado con ciertos medicamentos inhibidores de histona desacetilasa (HDACi). En este trabajo, propusimos la exploración de moléculas HDACi reposicionadas e investigamos sus efectos en varios modelos de cicatrización, que sean asequibles y seguras con el objetivo de mejorar la cicatrización.

Antecedentes

La piel es el órgano más grande en el ser humano, tiene un peso promedio de 10 kilogramos y abarca una superficie de hasta 2 metros cuadrados, cumple con diversas funciones como órgano sensorial además de poseer actividad glandular, aunque, su función más remarcable, es la defensa del organismo como el órgano de barrera más grande[46]. La piel evita el paso de patógenos dentro del organismo gracias al complejo sistema de defensa y su alta capacidad de regeneración, el cual lo componen principalmente péptidos de defensa del huesped (HDP), péptidos producidos la mayoría de los tejidos del cuerpo y especializados en el combate contra microogranismos y la modulación de la respuesta de una gran variedad de células inmunes, siendo capaces de inducir pocesos de inflamación y antinflamación, siendo un componente clave en la homeostasis de este órgano[6280]. Cuando la integridad de la piel se ve comprometida, la piel divide la fase de cicatrización en 3: Inflamación, proliferación y remodelamiento; durante la primera fase, la inflamación toma sitio en la herida, donde tanto células del sistema inmune como neutrófilos y macrófagos migran gracias a la actividad de diversas quimiocinas y de HDP como HBD-2 o LL-37. La función de estas células es la eliminación de posibles patógenos con ayuda de los HDP, además de producir interleucina 6 (IL-6) para inducir el cambio de fenotipo M1 a M2 en macrófagos, dando fin al proceso de inflamación[29-3038]. La IL-6 juega un papel importante en la cicatrización, ya que esta citocina activa al transductor de señal y activador de la transcripción 3 (STAT3), promoviendo la proliferación y diferenciación tanto de queratinocitos como de endotelio. Durante esta fase, el proceso de hipoxia también se encuentra presente,

ya que el tejido dañado es privado del O_2 por el daño ocasionado, esta condición estabiliza al factor de transcripción inducible por hipoxia 1 alfa (HIF-1α), induciendo angiogénesis y producción de HDP entre la fase inflamatoria y proliferativa por parte de las células implicadas[33]. La segunda fase está mediada predominantemente por fibroblastos, queratinoticos y macrófagos M2 (antiinflamatorios), ya que se encargan de cerrar la herida mediante la promoción de la proliferación y diferenciación de queratinocitos hacia corneocitos, la activación de HIF-1α induce la producción de proteínas asociadas a la angiogénesis, como el Factor de Crecimiento Vascuar Endotelial (VEGF) o Angiogenina (ANG) o HDP relacionados a cicatrización como LL-37 o HBD-3 por parte de queratinocitos y de macrófagos M2, activando al endotelio, promoviendo la angiogénesis en el sitio de proliferación[166075]. Finalmente, la fase de remodelación es mediada por la producción de matriz extracelular por parte de los fibroblastos, los cuales se encargan de sintetizar el colágeno donde van depositadas proteínas como el factor de crecimiento transformante beta (TGF-β) o Trombospondinas (TSBP 1-4), para el mantenimiento y correcto cierre de la herida[16], este proceso se resume en la figura 1.

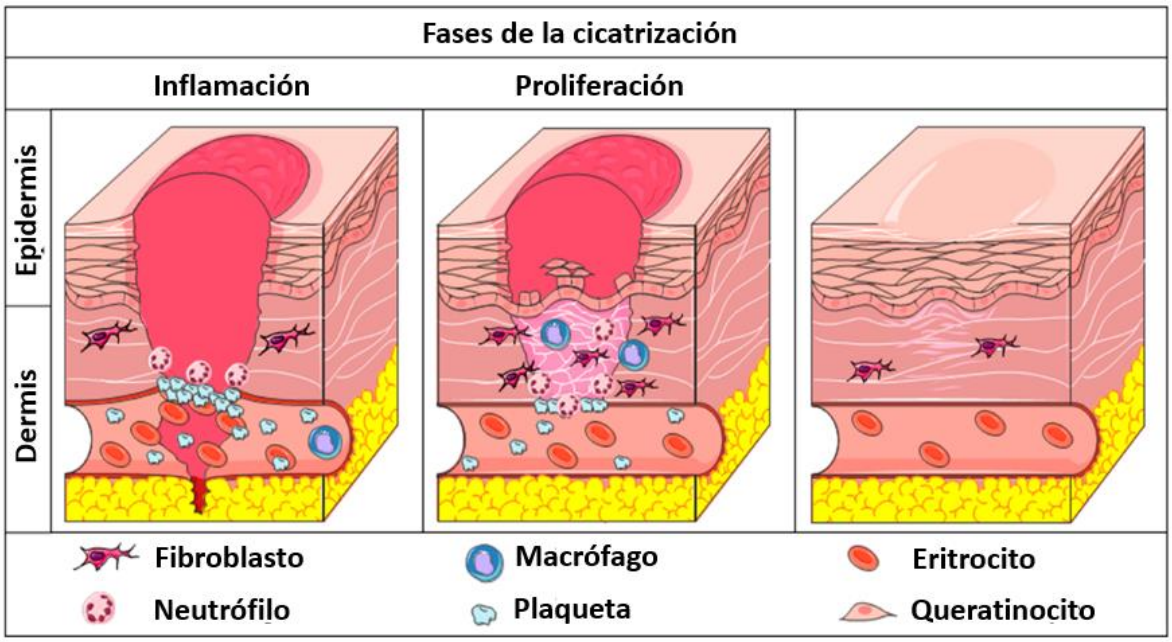

Figura 1.- Fases de la cicatrización. Esquematización de las 3 fases de la cicatrización y las células que participan en el. Figura traducida de la referencia [27]

El proceso de cicatrización dura aproximadamente 4 semanas, desde la fase

imflamatoria hasta la remodelación en individuos sin ninguna conmorbilidad, variables como la edad y enfermedades crónicas como diabetes mellitus (DM) o enfermedades cardiovasculares hacen que este tipo de heridas puedan estancarse en la primera fase, quedando el organismo totalmente expuesto al contacto con patógenos (**figura 2**) [212]. A esta falta del proceso de cicatrización se le denomina como herida crónica o úlcera, la cual resulta ser la complicación más problemática y seria que pueda cursar una persona con DM o CVD, ya que es la principal causa de amputación. Las fisiopatologías de las heridas crónicas comienzan cuando la herida queda expuesta el tiempo suficiente para que microorganismos como *S. aureus*, *E. coli* o *K. peumoniae* colonizan y proliferan en esta, alargando la fase inflamatoria, estancando el proceso de cicatriazición[474]. Las heridas crónicas predominan en pacientes con DM o pacientes con largas estadías en hospitales, donde reciben el nombre de úlceras de pie diabético o ulceras por presión respectivamente, la constante inflamación y el pobre control bacteriano compromenten la vida del paciente por el riesgo a osteomielitis y sepsis[4247]. El principal factor que obstaculiza la cicatrización es la marcada reducción de HDP cicatrizantes como LL-37 y HBD-3, lo que disminuye el correcto control bacteriano, además se ha observado que tanto la presión constante y altos niveles de glucosa, alteran el proceso de quimiotaxis de los macrófagos, estableciendoce en el sitio de la herida ya cuando el proceso de inflamación es crónico, los altos niveles de IL-17 promueven la polarización a un fenotipo de macrófagos M1 en piel[51-5262]. Una característica que poseen las personas con DM, es que la hiperglicemia disminuye significativamente la activación de HIF-1α, siendo uno de los principales factores que favorecen la progresión de una úlcera de pie diabético[877].

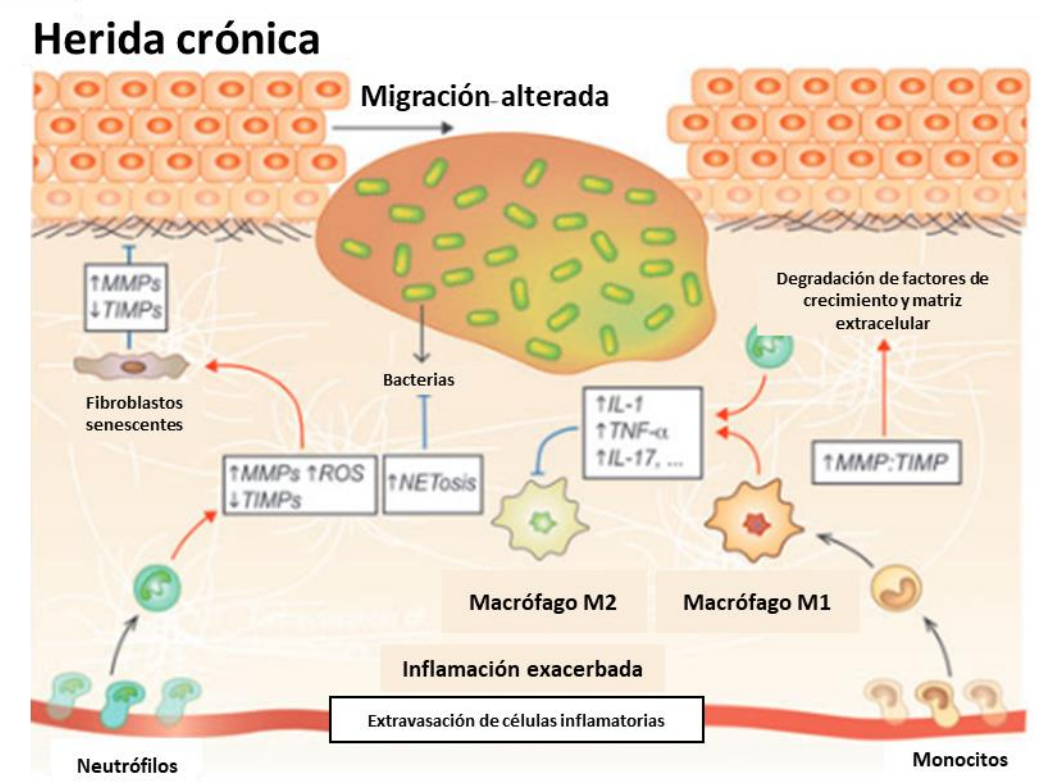

Figura 2.- Vías de inflamación en una herida crónica. En la imagen se muestran los principales puntos que hacen que se estanque el proceso de cicatrización en la fase inflamatoria. Figura modificada de la referencia[36].

Justificación

Las heridas crónicas constituyen un serio problema de salud pública ya en su mayoría, son complicaciones derivadas de enfermedades como DM o CVD, dificultando el control y manejo de las mismas, siendo la principal causa de amputación y sepsis en este grupo de pacientes. El retraso o la falta de cicatrización se ve obstaculizado por diversos factores, como las infecciones persistentes y la disminución de diversos componentes como los son los péptidos de defensa del huésped, péptidos especializados en el control de microorganismos y el factor inducible por hipoxia 1-α, encargado de la transcripción de genes y proteínas asociadas al proceso de angiogénesis. Tomando en cuenta estos puntos, el restablecimiento de estos se consideran como prometedores blancos terapéuticos. En los últimos años, el uso de un grupo de medicamentos conocidos como inhibidores de histonas deacetilasas, han demostrado activar la vía de señalización de HIF-1α e incrementar el HDP LL-37, el problema que tienen estos medicamentos

son los diversos efectos secundarios que ocasionan, ya que originalmente están clasificados como antitumorales. Gracias a la bioinformática, se pueden tomar medicamentos con aprobación por la “Food and Drug Administration” y mediante acoplamiento molecular filtrar y reposicionar compuestos como posibles inhibidores de histonas deacetilasas, esto con el propósito de poder aplicarse en heridas crónicas al inducir la vía de señalización de HIF-1α y mejorar la cicatrización.

Hipótesis

Los inhibidores de histonas deacetilasas son capaces de inducir la cicatrización mediante la activación de HIF-1α y expresión de péptidos de defensa del huesped en diferentes modelos de heridas.

Objetivos

Evaluar mediante diferentes modelos de cicatrización el efecto de los HDACi reposicionados en la expresión genética de genes y proteínas asociadas a la cicatrización en diferentes cultivos de queratinocitos humanos, su migración mediante un ensayo de raspado y la capacidad angiogénica en un ensayo de anillos aórticos de ratón.

Wound healing effect of HDACi repositioned molecules in the therapy for chronic wounds models

Short title: HDACi as wound healing promoters

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Word count	**4212**
Tables	**3**
Figures	**5**
Supplementary figures	**3**

Conflict of interest: None

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Abstract

Globally, chronic wounds impact the health of millions of people, negatively affecting quality of life and healthcare budgets. Some of the crucial steps and pathways in healing mechanisms are the hypoxic response and the expression of host defense peptides, which are decreased in diseases related to chronic wounds such as diabetes mellitus and cardiovascular diseases. It has been shown that histone deacetylase inhibitors can induce the expression of host defense peptides by inducing the stabilization and activation of hypoxia-inducible factor 1-α, promoting wound healing pathways, although their high cost and side effects limit clinical research. With the help of bioinformatics tools, we found potential histone deacetylase inhibitor candidates in an FDA-approved drugs database, the candidates, DiPU, Ace, and N-boc, show wound healing effects in HaCaT, increasing cell migration possibly via hypoxia-inducible factor 1-α activation, inducing the expression of LL-37 and vascular endothelial growth factor, while in a mouse ring angiogenesis model, Ace and N-boc have angiogenic effects. In a model of basal primary keratinocytes from donors with DM, without DM, and from Diabetic Foot Ulcers, it was observed that only DiPU is capable of inducing LL-37 in all scenarios. There is limited information about histone deacetylase inhibitors and wound healing but in this paper, we observe promising results and a proposed mechanism that involved specifically Histone Deacetylase 1 inhibition.

KEYWORDS: Wound healing, chronic wounds, HDAC, HIF-1α, host defense peptides, epigenetic modifications.

INTRODUCTION

The skin constitutes one of the most important organs in the defense against pathogens, being the largest physical barrier in the body, when the integrity of this barrier is impaired, a well-orchestrated wound healing mechanism is responsible for regaining homeostasis around 2 weeks [1726]. Those wounds with 4-12 weeks without any healing signs are called chronic wounds (CW) and are commonly the complications of other diseases such as diabetes mellitus (DM) or cardiovascular diseases (CVD) [556]. The challenges posed by both clinical and personal care of CW exert a significant strain on the quality of life, healthcare resources, and budgets. For instance, in countries such as Australia, Singapore, and several European and Scandinavian nations, a substantial 2-4% of the overall healthcare expenditure is allocated exclusively to managing chronic wounds. This allocation is expected to rise in the coming years due to the increasing prevalence of DM and CVD in the population [75472]. The key stages of the wound healing process involve the resolution of inflammation and the initiation of proliferation among skin-associated cells. However, in certain types of wounds, such as Diabetic Foot Ulcers (DFU) associated with DM, these phases are disrupted due to persistent infections and the downregulation of critical wound healing and hypoxic proteins such the Hypoxia Inducible Factor 1-α (HIF-1α), Vascular Endothelial Growth Factor (VEGF), Angiogenin (ANG), as well as Host Defense Peptides (HDP) such as LL-37 and HBD-3 [6278]. Is widely support that the expression of any of these molecules improves wound healing processes [223985]. In recent years, a class of drugs known as Histone Deacetylase inhibitors (HDACi) has emerged, exhibiting diverse effects contingent on the specific tissue they target. Originally HDACi have been used as antitumoral agents due his capacity to increase indirectly the histone acetylation, increasing the gene transcription of some downregulated antitumoral mechanisms [40], but also, they have shown a favorable impact on the immune system [49]. The HDACi are not restricted to increase the histone acetylation, they can also increase the acetylation of some non-histone proteins activating various pathways, like the upregulation of HIF-1α, leading to increased expression of LL-37 [486469]. These discoveries offer promising candidates for CW treatment through the activation of

dual pathways involving hypoxia and HDP. However, a significant challenge arises from the potential side effects and the elevated cost associated with certain HDACi drugs. In our research, we put forth the exploration of repurposed HDACi molecules and investigate their effects on various wound healing models, aiming to reach both efficacy and affordability concerns in the pursuit of improved therapeutic strategies.

MATERIALS AND METHODS

Searching of potential inhibitors of HDACi

The ZINC15 database (https://zinc15.docking.org) was employed to identify structural molecules akin to phenylenediamine or entinostat (ENT), with a Tanimoto index similarity value of ≥ 0.60. Subsequently, molecules resembling phenylenediamine or entinostat were assessed for their ability to inhibit the HDAC1-3 isoforms using the SEA program (Similarity Ensemble Approach, http://sea.bkslab.org/). These findings were corroborated through molecular docking with Autodock Vina software (http://vina.scripps.edu/), with the prototype molecule (entinostat) serving as the reference for binding affinity. The interaction between molecules and enzymes was gauged, selecting only those with a binding energy (ΔG) of ≤ -5.00 kcal/mol. Additionally, considerations were given to adverse effects and acquisition cost. Following these bioinformatic analyses, three molecules: 2-aminoacetanilide (Ace), N-Boc-1,2-phenylenediamine (N-Boc), and 1,3-Diphenylurea (DiPU)—were chosen due to their cost-effectiveness and lower adverse effects, to determinate the amino acids interaction against the HDAC enzymes, a LIGPLOT (LigPlot+ v.2.2.8) [37] analysis was used to generate a schematic diagram of protein-ligand for each selected enzymes.

Cell line and primary keratinocyte culture

The human keratinocytes cell line HaCaT (Cell Lines Service, Eppelheim, Germany) was cultured with DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA USA) supplemented with Fetal Bovine Serum at 10% (FBS, Biowest, Riverside USA) 100 U.I/ml of penicillin and 100 mg/ml of streptomycin (Corning, Manassas, VA, USA). HaCaT cells were seeded at $2.0x10^5$ cells per well for furthers stimulations with the HDACi molecules.

The primary keratinocyte culture was stablished from patient's biopsies obtained from the "Hospital General del Instituto Mexicano del Seguro Social, Zacatecas, México" with the institutional register **R-2019-3301-012**. All the participants signed an informed consent before taking biopsies only from lower extremities. 10 samples were collected from patients without diabetes and 10 diagnosed with diabetes (**table**

3), the biopsies were divided mechanically with a scalpel obtaining small explants and cultured in 25cm^2 cell culture flask (Corning, Manassas, VA, USA) during 4 days with DMEM supplemented with 10% SFB and penicillin-streptomycin until some cells were observed growing from the explants, at this point the DMEM was replaced with Keratinocyte Free Serum Media (Gibco, Thermo Fisher Scientific, Waltham, MA USA) supplemented with EGF 50ng/mL and Bovine Pituitary Extract 50µg/mL (Gibco, Thermo Fisher Scientific, Waltham, MA USA), until the flask was confluent obtaining primary basal keratinocytes, phenotype confirmed by the expression of cytokeratin-5 (KRT5) (Supplementary information) the primary basal keratinocytes where seeded in 2.0x10^5 cells per well for furthers experiments with the candidate's molecules.

RT-PCR

Primary basal keratinocytes and HaCaT cells were seeded during 18 h with KFSM and DMEM respectively, after that time the cells were washed with sterile PBS and stimulated during 24 h with the HDACi candidates ACE, N-boc and DiPU at the lower concentration (25µM), the HDACi control Entinostat (ENT) and the HIF-Hydroxylase Inhibitor, DMOG 1mM (Sigma-Aldrich; St. Louis, USA). The total RNA was extracted using Trizol reagent (Invitrogen, Auckland New Zealand) according to the manufacturer's instructions and used to synthesize cDNA in the Applied Biosystems™ Veriti™ Thermal Cycler, using the RevertAid Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA USA), subsequently with the LightCycler® 480 Real-Time PCR System (Roche LifeScience, USA) the following transcripts where evaluated using the indicated oligonucleotides for the next genes: *Hypoxanthine phosphoribosyl transferase hprt* Rv 5'-CGAGCAAGACGTTGAGCTCT-3' Fw 5'-TGACCTTGATTTATTTTGCATACC-3', *Cathelicidin antimicrobial peptide* CAMP Rv 5'-GTCTGGGTCCCCATCCAT-3' Fw 5'-TCGGATGCTAACCTCTACGG-3', *Defensin Beta 103A* DEFB103A Rv 5'-CGATCTGTTCCTCCTTTGGA-3' Fw 5'-TCATCATGGCCATCAAAACA-3' *Vascular Endothelial Growth Factor* VEGF Rv 5'-GCAGCTTGAGTTAAACGAACG-3' Fw 5'-GGTTCCCGAAACCCTGAG-3' and *Angiogenin* ANG Rv 5'-

CATTGTCCTGCCCGTTTC-3' Fw 5'-CAGCACGAAGACCAACAACA-3' (**table 1**). All data were analyzed using the expression of HPRT as a reference gene and internal control. Relative quantification of gene expression was performed by the comparative quantification cycle (Cq) method, using the formula, $2^{-\Delta\Delta CT}$ as previously described [45] . This method is based on the expression levels of a target gene vs a reference gene (HPRT), the comparative threshold cycle method was used to assess relative changes in mRNA levels between untreated cells (control) reflected in fold changes. Thus, untreated cells were uniformly normalized to a value of 1.

Western Blot of angiogenic proteins

To observe the protein expression on both, HaCat and primary basal keratinocytes, were stimulated with DMOG at 0.1 mM as hypoxic control, Ace 25µM, N-boc 25µM, DiPU 25µM, Entinostat 10µM and DMOG 1mM during 24 h the cells were washed with cold PBS and lysate using RIPA buffer (10 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% NP40, 0.1% SDS, 10 mM NaF, 0.05 mM PMSF) to obtain total proteins with protease inhibitor cocktail (Sigma-Aldrich; St. Louis, USA), the samples were handled at 4°C to minimize the protein degradation, after that were centrifuged at 13,000 rpm 4°C, taking only the supernatant and denaturing at 95°C with loading buffer during 10 minutes, the samples were electrophoresed in a 10% polyacrylamide gel and blotted into a nitrocellulose membranes 0.22µm (Biorad, Hercules,CA). Using a 5% milk solution the nitrocellulose membrane was blocked during 30 minutes and incubated with the next monoclonal antibodies: HIF-1α antibody (1:400; Abcam, Kendal Square, Cambridge, UK), anti-human VEGFA-165 (1:500; Abcam, Kendal Square, Cambridge, UK), anti-human KRT5 (1:500, Abcam, Kendal Square, Cambridge, UK) and anti-human β-actin (1:3000, Sigma-aldrich, San Luis, Misuri) during 18 h. at 4°C, the membranes were washed with TBST 1X and incubated with a the Goat anti-mouse fluorescent secondary antibody (1:5000, Thermo Scientific,Rockford, IL) and with Goat anti-rabbit, (1:8000 Thermo Scientific, Rockford, IL) for 1 h. The membrane was revealed using the Odyssey® CLx (LI-COR Lincoln, Nebraska USA) measuring the band density.

In vitro wound closure assay

To assess the direct wound healing impact of the HDACi, we conducted experiments using HaCaT cells. In a 24-well plate, $2.0x10^5$ cells were cultured in DMEM, FBS 1%, and penicillin-streptomycin during 18 h to form confluent cell monolayer, after that the cells were treated with mitomycin C at a concentration of 5µg/mL for 1 h to inhibit cell proliferation and measure only the migratory effect. The mitomycin C were retired and the excess removed adding to the cell monolayer PBS. The scratch was made in the center of each well using a 200µL sterile pipette tip and retiring the PBS with the generated debris. The wells were then recoated with fibronectin (5 µg/mL) for 1 h, and the initial image was captured at 0 hours using an inverted microscope (Leica, Germany). Subsequently, the cells were stimulated with DMEM at 1% FBS, being the control, the HDACi molecules Ace, N-Boc, and DiPU, along with Vitamin D as a positive control, for a period of 24 hours. After this incubation, a second image was obtained using an inverted microscope (Leica, Germany). To quantify the closed area, each image was analyzed using ImageJ software developed by Wayne Rasband at NIH, USA. The results were reported in percentage of Wound closure, measuring the pixels and their difference between the 0 hours and the 24 hours previously reported [6].

Ex vivo angiogenesis aortic ring assay

To assess the angiogenic impact of the compounds, the BALB/c aorta was utilized to gauge endothelial cell proliferation via the aortic ring assay [79]. Twelve 6-month-old male BALB/c mice were euthanized through cervical dislocation, followed by dissection and extraction of the aorta. The obtained aorta was meticulously cleaned and sectioned into small 1mm rings. In a 24-well plate, 100 µl of Corning Matrigel Growth Factor Reduced (Corning, NY, USA) was centrally placed and the aortic rings were embedded and incubated for one hour to allow Matrigel polymerization. Subsequently, the aortic rings were incubated with DMEM 10% FBS for 3 days. The viable aortas were stimulated with Vitamin D ($1x10^{-7}$M) as our positive control, while Ace, N-boc, and DiPU, Mitomycin C (3.5 µg/mL) was used as negative proliferative controls. Endothelial cell proliferation was documented using the Leica MC120

(Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) over 3 days, and the area was quantified using ImageJ software. The initial aortic area was denoted as AT0, the proliferation area at day 1 as AT1, and the proliferation area at day 3 as AT2. The results are expressed as the fold outgrowth, calculating the angiogenesis with the following formula:

$$Fold\ outgrowth\ = \frac{(AT2 - AT1)}{AT0}$$

RESULTS

HDACi candidates determined by in silico analysis

Candidates were chosen based on in silico analysis, as outlined in Table 2. The interaction between proteins and ligands was scrutinized using LIGPLOT, with a comparative evaluation against the positive control, ENT, and its crystallized ligands. The findings indicate that the candidates exhibit dissimilar interaction profiles compared to ENT across HDAC1-3 isoforms. Notably, upon comparing results with crystallized ligands, it becomes evident that the HDACi candidates demonstrate specificity towards certain isoforms, as detailed in Table 2. For instance, ENT functions as an inhibitor for HDAC1-3, whereas Ace displays a higher selectivity for HDAC2 and HDAC3, N-boc targets HDAC3, and DiPU is effective against HDAC1 and HDAC2. Supplementary Figure 1 illustrates the LIGPLOT interactions.

Effect on histone H3 acetylation with the repositioned HDACi candidates

After establishing the potential impact of our HDACi candidates on histones in silico, we proceeded to investigate their effects on histones in keratinocytes (see Figure 1a). Notably, we observed an increase in H3 acetylation with ENT, as compared to our control. Strikingly, this pattern was consistently replicated across all our candidates, indicating a uniform trend of acetylation enhancement (see Figure 1b). This collective outcome strongly suggests that the observed acetylation increment is likely attributable to the inhibition of HDACs by our candidates.

Effect on Expression of Wound Healing-Related Molecules in Keratinocytes

Following the observed acetylation increment across all HDACi candidates, we sought to investigate whether the repurposed HDACi compounds also influenced the HIF-1α pathway. To assess this, we utilized the prolyl-4-hydroxylase inhibitor DMOG, known for stabilizing and upregulating HIF-1α, as our positive control. As anticipated, our control compounds, ENT and DMOG, demonstrated a promotion of

HIF-1α expression. Intriguingly, our repositioned HDACi, DiPU, exhibited a similar induction of HIF-1α comparable to both controls (see Figure 2A). While N-boc and Ace did not exhibit statistically significant differences when compared with controls, both demonstrated an induction of HIF-1α.

Subsequently, we assessed the expression of various wound healing-related genes, including LL-37 and HBD-3, recognized as human antimicrobial peptides, and the angiogenic proteins associated with HIF-α activation (VEGF and ANG). Our findings revealed that only DiPU increased the expression of LL-37 and HBD-3 in HaCaT cells (see Figure 2b), whereas both Ace and DiPU promoted the expression of VEGF and ANG (see Figure 2c).
Wound healing effect on keratinocyte cell line HaCaT and aortic rings assay with HDACi candidates.

Wound healing effect on keratinocyte cell line HaCaT and aortic rings assay with repositioned HDACi

We aimed to evaluate the wound healing potential based on the expression of wound healing-related genes derived from our previous findings. Two distinct models were employed for this purpose: a keratinocyte migration assay with an in vitro wound closure approach using HaCat cells (refer to Figure 3), and an angiogenesis assessment through the mice aortic rings assay (refer to Figure 4). All cell types were treated with our HDACi candidates, with Vitamin D (VitD) serving as the positive control, since previous results from our group have shown the VitD effectiveness to promote wound healing.

Our results indicated a significant enhancement in wound closure, both with Vitamin D and with the HDACi candidates Ace and DiPU (refer to Figure 3a). However, no statistical differences were observed in N-boc stimulated cells (refer to Figure 3b and Figure 3e). In regards with the angiogenesis model, our findings demonstrated increased cell outgrowth in aortas stimulated with Ace, N-boc, and the positive control VitD, but not with DiPU. Despite the observed positive effects of the HDACi

candidates individually, we investigated the possibility of synergy through co-stimulation with VitD. Remarkably, there were no notable differences between HDACi alone and HDACi-VitD co-stimulation (refer to Figure 4a).

Representative images of HDACi stimulation without VitD after 48 hours are presented in Figure 4b-e, while those of HDACi with VitD co-stimulation are shown in Figure 4f-i. To validate that the aortas' outgrowth is attributed to a proliferative mechanism, we stimulated aortic rings with mitomycin C, resulting in a significant reduction in aorta outgrowth (refer to Supplementary Figure 2). This observation confirms that the HDACi candidates induce endothelial proliferation.

Effect on wound healing expression proteins on healthy, diabetic and chronic wound primary human keratinocytes

Building upon the outcomes in HaCaT, we sought to elucidate whether the effects of HDACi candidates extend to keratinocytes derived from skin biopsies. To emulate conditions prevalent in chronic wounds, we isolated basal keratinocytes from donors representing non-diabetic, diabetic, and diabetic foot ulcer cases (refer to Table 3). To ensure adherence to our protocol for working with a basal phenotype, we assessed the presence of Keratin-5, confirming that the isolated skin cells are indeed basal and proliferative keratinocytes (refer to Supplementary Figure 3).

Results from non-diabetic keratinocytes revealed that DMOG, ENT, and DiPU induce HIF-1α (refer to Figure 5a). Regarding human defense peptide (HDP) expression, LL-37 levels appear elevated with both DiPU and ENT, while HBD-3 levels only increased with ENT (refer to Figure 5b). Analyzing angiogenic gene proteins, only our positive control, DMOG, exhibited an increase in ANG expression (refer to Figure 5c). The patterns observed in diabetic donor's keratinocytes were analogous, where ENT and DiPU induced HIF-1α (refer to Figure 5d). HDACi candidate DiPU demonstrated increased expression of LL-37 and HBD-3 (refer to Figure 5e), while ANG expression was elevated only with ENT, which also increased

VEGF expression (refer to Figure 5f).

With insights from non-diabetic and diabetic donor keratinocytes, we proceeded to evaluate HDACi responses in keratinocytes derived directly from chronic wound skin biopsies of diabetic foot ulcers (DFU) Interestingly, no significant differences were observed in HIF-1α stabilization under any condition (refer to Figure 5g). However, delving into HDP gene expression, we noted an intriguing increase in LL-37 and HBD-3 with DiPU and ENT, respectively (refer to Figure 5h). Examining angiogenic gene proteins, both Ace and ENT increased ANG expression, with ENT additionally impacting VEGF expression (refer to Figure 5i).

DISCUSSION

Chronic wounds represent a prevalent challenge in healthcare, often accompanying nontransmissible and persistent conditions like diabetes mellitus (DM) and cardiovascular diseases (CDV), which are leading causes of global mortality according to WHO data [1161]. While not directly causing mortality in DM and CDV, chronic wounds significantly heighten the risk of complications such as sepsis and necessitate amputation, profoundly impacting patients' quality of life, healthcare budgets, and institutional medical resources [1468]. Given the susceptibility of chronic wounds to infections, compounded by the growing threat of antimicrobial resistance, there is a pressing global concern regarding wound healing efficacy. With the anticipated rise in patients affected by DM, CDV, and antimicrobial resistance over the next decade, it becomes imperative to explore novel strategies that bolster both the body's regenerative responses and antimicrobial defenses, thereby enhancing the wound healing process [245072].

A promising approach to elicit both proliferative and antimicrobial responses involves stabilizing HIF-1α and inducing host defense peptides (HDP) using histone deacetylase inhibitor (HDACi) drugs, typically employed in anti-tumoral therapy [3583]. Studies have revealed that butyrate, a mild HDACi, prompts HDP expression in the skin and intestine. Additionally, research indicates that entinostat, initially used in breast cancer treatment, robustly induces LL-37 [48], However, a key challenge in considering these drugs for treat chronic wound (CW) infections lies in butyrate's limited HDP expression and entinostat's high cost and associated side effects. Thus, our focus shifted towards repurposing FDA-approved HDACi drugs with minimal cost and fewer side effects for CW treatment.

As previously discussed, both entinostat and butyrate stimulate the expression of host defense peptides (HDP) through STAT3/HIF-1α activation. However, a notable distinction between these two compounds lies in their ability to inhibit histone deacetylase (HDAC) enzymes. Entinostat demonstrates greater efficacy in this regard due to the presence of a functional group known as aroilated phenylenediamine (APD) [4887]. Upon comparing the in-silico results of entinostat with the crystalized ligands of each HDAC isoform, we noted superior ΔG values.

Establishing a stringent cutoff based on the presence of the aroilated phenylenediamine (APD) group and entinostat's binding affinity, our initial screening exclusively identified benzamide class I HDAC inhibitor drugs (data not presented). However, these compounds were excluded due to their high costs and associated side effects. We selected only those compounds containing the aroilated phenylenediamine (APD) functional group and exhibiting superior binding affinity against crystalized ligands. While APD serves as a zinc-binding group (ZBG) crucial for HDAC inhibition, the presence of a linker and cap group enhances selectivity and stabilizes the hydrophobic channel and the outer rim, which are structures within the HDAC inhibition pocket. Our selected candidates, Ace and N-boc, lack both the linker and cap groups, leading to a considerable reduction in binding affinity compared to entinostat. However, the presence of APD in the Ace candidate imparts selectivity to HDAC2-3 due to its zinc-binding capability. Conversely, although N-boc shares a similar structure, the tert-butyl group impedes interaction with the zinc of all isoforms, yet it displays certain selectivity against HDAC3 by binding only to the entrance of the inhibition pocket. As for DiPU, the presence of two phenyl groups linked by the APD can serve as cap groups, a structural characteristic of reported inhibitors targeting HDAC1-2, elucidating its selectivity against these isoforms [41].

Drug repositioning through molecular docking has emerged as a valuable strategy in the quest for treatments against emerging and rare diseases. However, a significant challenge lies in bridging the gap between in silico predictions and in vitro experimental results [2365]. To elucidate the in-silico findings, we indirectly assess HDAC inhibition by measuring the increase in acetylated H3 histones. Previous studies conducted in the human cell line A549 (type 2 pneumocytes) by our group revealed that only cells stimulated with Ace and ENT demonstrated a significant increase in H3 acetylation. Consistent with the in-silico predictions in the present study, both compounds act as inhibitors of HDAC2, an isoform notably overexpressed in A549 cells [156381]. In the skin, HDAC1-3 isoforms are abundantly expressed in keratinocytes, particularly those in the basal layers. This observation elucidates why all our candidates can augment H3 acetylation compared to our control [7389].

Transcription factors STAT3 and HIF1-α, both pivotal in wound healing processes, are susceptible to regulation by HDAC inhibitors (HDACi). Despite the intricate details of these regulatory mechanisms remaining elusive, existing evidence suggests that acetylation may prompt STAT3 phosphorylation (via HDAC1-2 inhibition) and stabilize HIF-1α (through HDAC1 inhibition) [1921]. Our findings reveal that DiPU treatment elicits the upregulation of LL-37 and VEGF, whereas ACE treatment exclusively induces VEGF expression. This dichotomy underscores the essential role of HIF-1α in LL-37 induction, while VEGF expression appears to be modulated by both transcription factors. However, the available evidence does not permit a definitive conclusion regarding whether the induction of VEGF under both conditions primarily correlates with STAT3 activation or involves other regulatory factors [132855].

While various transcription factors can trigger the expression of human defensins, there is scant evidence linking HBD-3 expression specifically to STAT3 or HIF-1α activation mediated by HDAC inhibitors. Some studies implicate the MAPK and AP-1 pathways in butyrate-induced HBD-3 expression, an endogenous HDAC inhibitor. However, the precise association between HBD-3 expression in human keratinocytes and a particular HDAC isoform remains uncertain [6467].

The literature consistently reports that HDAC inhibitors (HDACi) can enhance cell migration, thereby promoting wound healing through diverse molecular pathways. Our experimental results corroborate this, demonstrating that HDACi capable of inducing LL-37 and VEGF expression exhibit significantly enhanced wound closure in scratch assays. This observed effect suggests a potential mechanism involving the activation of the STAT3-HIF-1α pathway, analogous to findings reported in studies utilizing ENT [36]. However, our comparative analysis is hindered by limitations inherent to our model. Specifically, our use of HaCaT cells precludes direct comparison with our lead compound due to their heightened sensitivity to elevated concentrations of ENT. Notably, the reported IC50 values for ENT fall below the concentrations typically employed in assays assessing human defense peptide (HDP) induction[57596686].

Angiogenesis studies involving HDAC inhibitors have yielded conflicting results,

primarily due to their known anti-proliferative effects. Presently, the only relevant evidence stems from studies utilizing butyrate, an endogenous HDAC inhibitor [92543]. Our angiogenesis assay data revealed that DiPU failed to increase vascular proliferation, despite its observed positive effects in HaCaT cells. This discrepancy may suggest that HDAC1-2 inhibition lacks significant influence in this specific tissue, or alternatively, that DiPU's physicochemical properties lead to its sequestration by extracellular matrix proteins, thereby limiting its efficacy on matrigel-coated surfaces and impeding its action on aortic rings [1888]. Intriguingly, our N-boc candidate (a weak HDAC3 inhibitor) exhibited angiogenic potential comparable to ACE in murine aortic tissues. However, unlike the results observed in keratinocytes, we cannot definitively conclude whether this angiogenic response is associated with the expression of murine HDPs such as mBD-14 (the ortholog of HBD-3) and CRAMP (the ortholog of LL-37) via HIF-1α signaling [75], or if it involves the induction of angiogenic proteins like VEGF or ANG [71]. Furthermore, it is important to note that while HDAC1-3 isoforms exhibit a 99% similarity between humans and mice, our understanding of the epigenetic profile of mouse vascular endothelium and the specific HDAC isoforms involved in the angiogenesis process remains incomplete [1].

Our previous data elucidates that our HDAC inhibitor (HDACi) candidates effectively induce the expression of proteins crucial for wound closure. One proposed mechanism underlying this effect may involve the stabilization of HIF-1α in keratinocytes. While the exact impact on proliferation in aortic vascular endothelium remains unclear, our preliminary results suggest that these compounds hold promise for promoting wound healing. However, devising treatments based on epigenetic regulation poses a challenge, given the variability of HDACs and their upregulation in diseases such as diabetes and cardiovascular disease (CVD), which could potentially influence the response to our candidates [76].

Our analysis reveals that DiPU induces LL-37 expression in all three groups of keratinocytes, with a notable association with HIF-1α observed specifically in keratinocytes from non-diabetic and diabetic donors. In contrast, compared to ENT, keratinocytes from patients with diabetes exhibit diminished LL-37 induction despite

the controlled glycemic status of the donors. This discrepancy may be attributed to the overexpression of certain HDAC isoforms in the keratinocytes from diabetic and diabetic foot ulcers (DFU) donors, such as HDAC8 or HDAC9, which could potentially interfere with LL-37 transcription [344484]. Furthermore, in keratinocytes from DFU, ENT strongly induces the expression of HBD-3, ANG, and VEGF, possibly through HDAC1 inhibition—an isoform known to be overexpressed in DFU, and paradoxically, a repressor of angiogenesis [10].

Despite the promising outcomes, further studies are warranted before considering the use of HDAC inhibitors in chronic wound therapy. The complexity of HDAC expression regulation and its modulation by various factors needs to be further clinical evaluated using topical or localized approaches to avoid systemic implications. Altogether, our findings suggest that targeted inhibition against specific HDAC isoforms may offer a more effective strategy to yield positive therapeutic outcomes for chronic wounds therapy.

ACKNOWLEDGEMENT

The present study was supported by the Mexican Institute of Social Security-IMSS grant FIS/IMSS/PRIO/13/025 and for the Consejo Nacional de Ciencia y Tecnología (CONACyT) grant 320582. We also acknowledge Fundación IMSS for their laborious and outstanding administrative work. ASM had a scholarship (930503) from CONACyT.

CONFLICT OF INTEREST

The authors have declared no conflicting interests.

AUTHOR CONTRIBUTIONS

ASM, OEGM and ARG performed the research. BRS and ARC designed the research study. CRM, MVN, GSM, RC, IGC, YR and DPP contributed essential reagents or tools. YMJD, ASM, LAJG and VTP analysed the data. BRS and ASM wrote the paper.

Conclusiones

Las moléculas reposicionadas lograron inducir un efecto de cicatrización en línea celular, aortas de ratón, y queratinocitos de donadores sin diabetes por una vía de modificación epigenética. Los resultados de los queratinocitos de pacientes con diabetes y úlceras se necesita una mayor comprensión de la huella epigenética para concluir el posible efecto de nuevas moléculas.

Tables

Gene name	Protein	Right primer	Left primer
HPRT	HPRT	CGAGCAAGACGTTGAGCTCT	TGACCTTGATTTATTTTGCATACC
CAMP	LL-37	GTCTGGGTCCCCATCCAT	TCGGATGCTAACCTCTACGG
DEFB103A	HBD3	CGATCTGTTCCTCCTTTGGA	TCATCATGGCCATCAAAACA
VEGF	VEGF	GCAGCTTGAGTTAAACGAACG	GGTTCCCGAAACCCTGAG
ANG	ANG	CATTGTCCTGCCCGTTTC	CAGCACGAAGACCAACAACA

Table 1.- Sequence of primers

Table 1.- Sequence primer

Table 2.- Zinc interaction of the HDACi candidates with HDAC1-3 isoforms

Canonical SMILE	Name	Binding energy (kcal/mol)			Zinc interaction		
		HDAC1	HDAC2	HDAC3	HDAC1	HDAC2	HDAC3
	GLY-ALA-6A0-ARG-HIS	-5.6			Yes	*	*
CN(C)C(=O)C(CC1=CC=C(C=C1)F)[NH3+]	[(2~{R})-1-(dimethylamino)-3-(4-fluorophenyl)-1-oxidanylidene-propan-2-yl]azanium		-4.2		*	Yes	*
CC(=O)O	Acetate Ion			-3.3	*	*	Yes
C1=CC=C(C(=C1)N)NC(=O)C2=CC=C(C=C2)CNC(=O)OCC3=CN=CC=C3	Entinostat	-7.6	-9.2	-8.2	Yes	Yes	Yes
CC(=O)NC1=CC=CC=C1N	2'-Aminoacetanilide	-5.3	-5.9	-5.8	No	Yes	Yes
CC(C)(C)OC(=O)NC1=CC=CC=C1N	Tert-butyl (2-aminophenyl) carbamate	-5.3	-5.6	-5.9	No	No	No
C1=CC=C(C=C1)NC(=O)NC2=CC=CC=C2	1,3-Diphenylurea	-6.3	-6.9	-6.2	Yes	Yes	No

Table 2.- Interaction of the HDACi candidates and their crystallized controls. The HDAC structures with the crystallized ligands of the HDAC1, HDAC2 and HDAC2 were obtained from PDB with the ID 5CIN, 7ZZO and 4A69 respectively.

Table 3.- Clinical characteristics

Clinical characteristics	Non-DM2 donnors	D2 donnors	DFU donnors	*P value* Non-DM2/D2	*P value* Non-DM2/DFU
Number of participants	10	10	3	-	-
Age (years)	40±12.01	53.1±7.9	53.33±8.33	0.005**	0.005**
Gender (male/female	8\2	6\4	1\2	-	-
Glucose (mg/dl)	98.55±7.9	166.22±66.7	268.533±84.63	0.002**	0.0001**
DM evolution (years)	NA	8±6.2	10.67±4	-	-

Table 3.- Clinical characteristics. The samples were obtained from lower extremities.

Figures

Figure 1

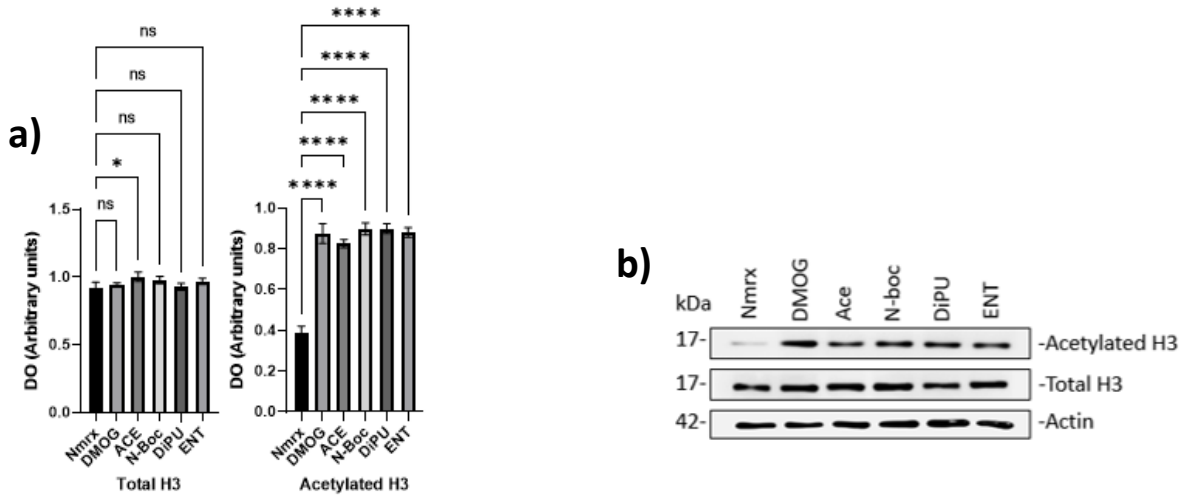


Figure 1.- Total H3 vs acetylated H3 confirming the mechanism of the proposed HDACi. Total histone H3 and acetylated histone H3 levels were assessed to validate the proposed mechanism of action of the HDAC inhibitor (HDACi) in primary human keratinocytes. Panel A displays the levels of total H3 and acetylated H3 under various concentrations of DMOG (1 mM), ACE (25 µM), N-boc (25 µM), DiPU (25 µM), and ENT (10 µM). Statistical significance was determined with *p≤ 0.05 and ****p≤0.0001. Panel B depicts representative Western blot results showing the band density for each treatment condition. Untreated cells served as the control (Nmrx).

Figure 2

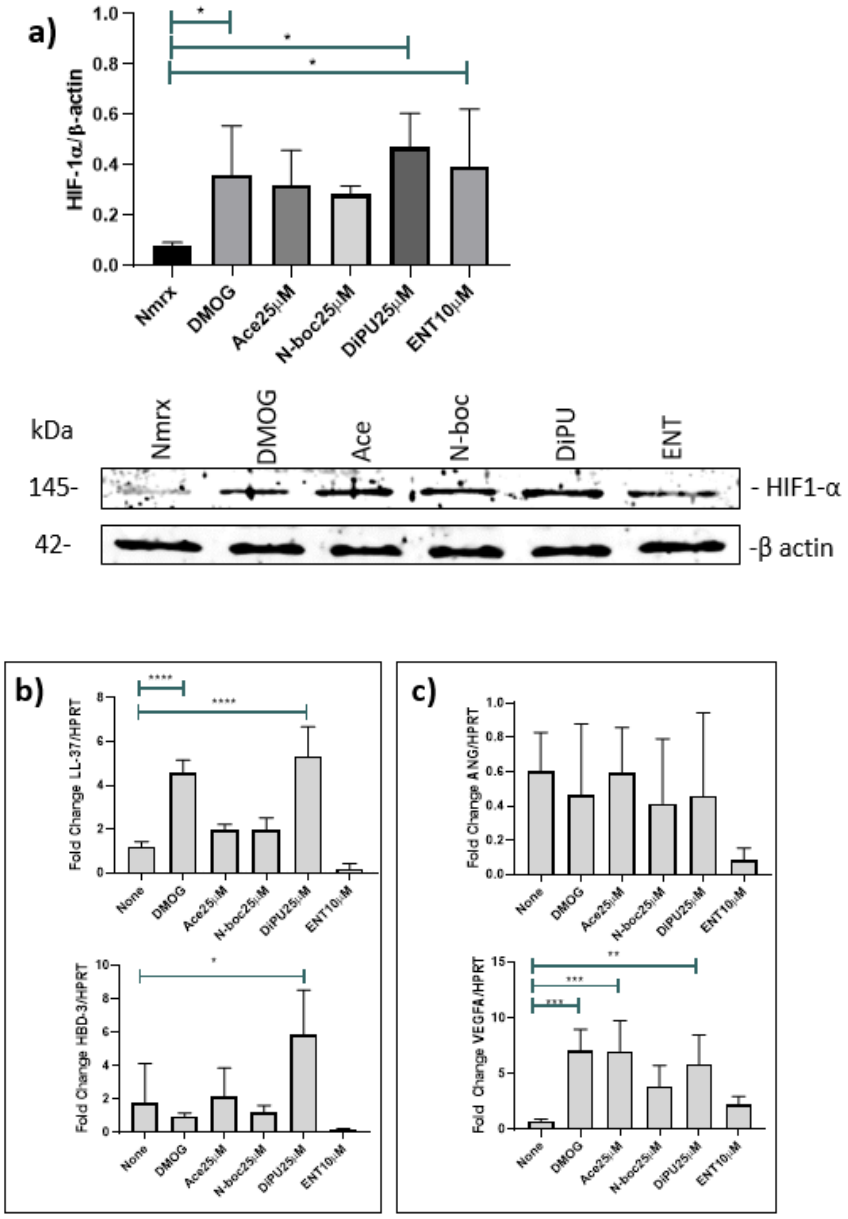


Figure 2.- Hypoxia-inducible factor 1 alpha (HIF-1α) stabilization leads to increased expression of angiogenic and host defense peptide (HDP) genes. Panel A presents a Western blot illustrating HIF-1α stabilization, with DMOG (prolyl-4-hydroxylase inhibitor) serving as a positive control. Results are depicted as the disparity in band density between HIF-1α and β-actin. Panel B demonstrates the gene expression levels of host defense peptides LL-37 and HBD-3. Panel C depicts the gene expression of angiogenic proteins VEGF and ANG, associated with HIF-1α stabilization. Each experiment was conducted with a sample size of at least n=3, and statistical significance was denoted by *p≤ 0.05, **p≤0.01, ***p≤0.001, and ****p≤0.0001.

Figure 3

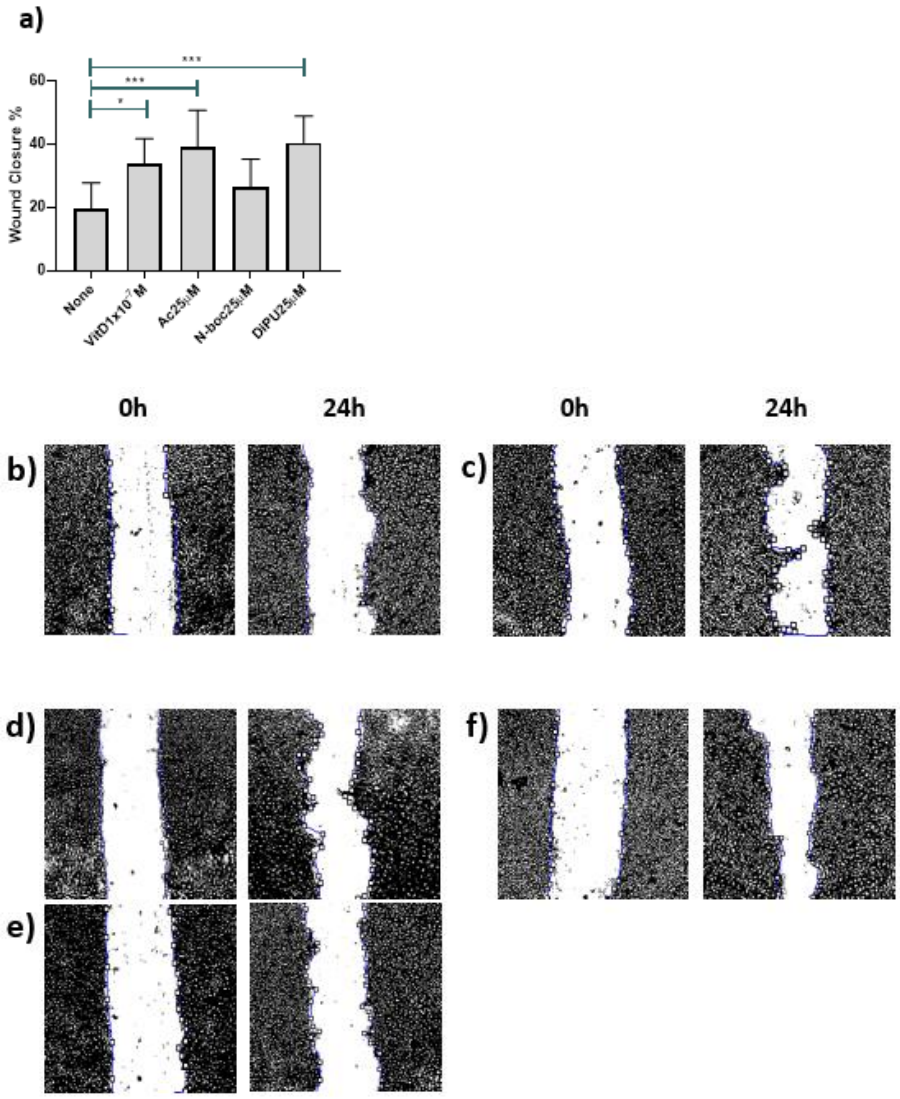


Figure 3.- Wound healing effect on keratinocyte cell line HaCaT. Panel a displays a graphical representation depicting the percentage of wound closure by HaCaT cells. To assess migration exclusively, cells were treated with mitomycin C to inhibit proliferation. Panels b and c exhibit representative images of the untreated (none) and positive control (Vitamin D, 1x10^-7 M) at 0 and 24 hours, respectively. Panels d, e, and f depict representative images of potential HDAC inhibitor (HDACi) candidates (Ace, N-boc, and DiPU) at 0 and 24 hours, respectively. . Each experiment included a sample size of n=6, with statistical significance indicated by *p≤ 0.05 and ***p≤0.001.

Figure 4

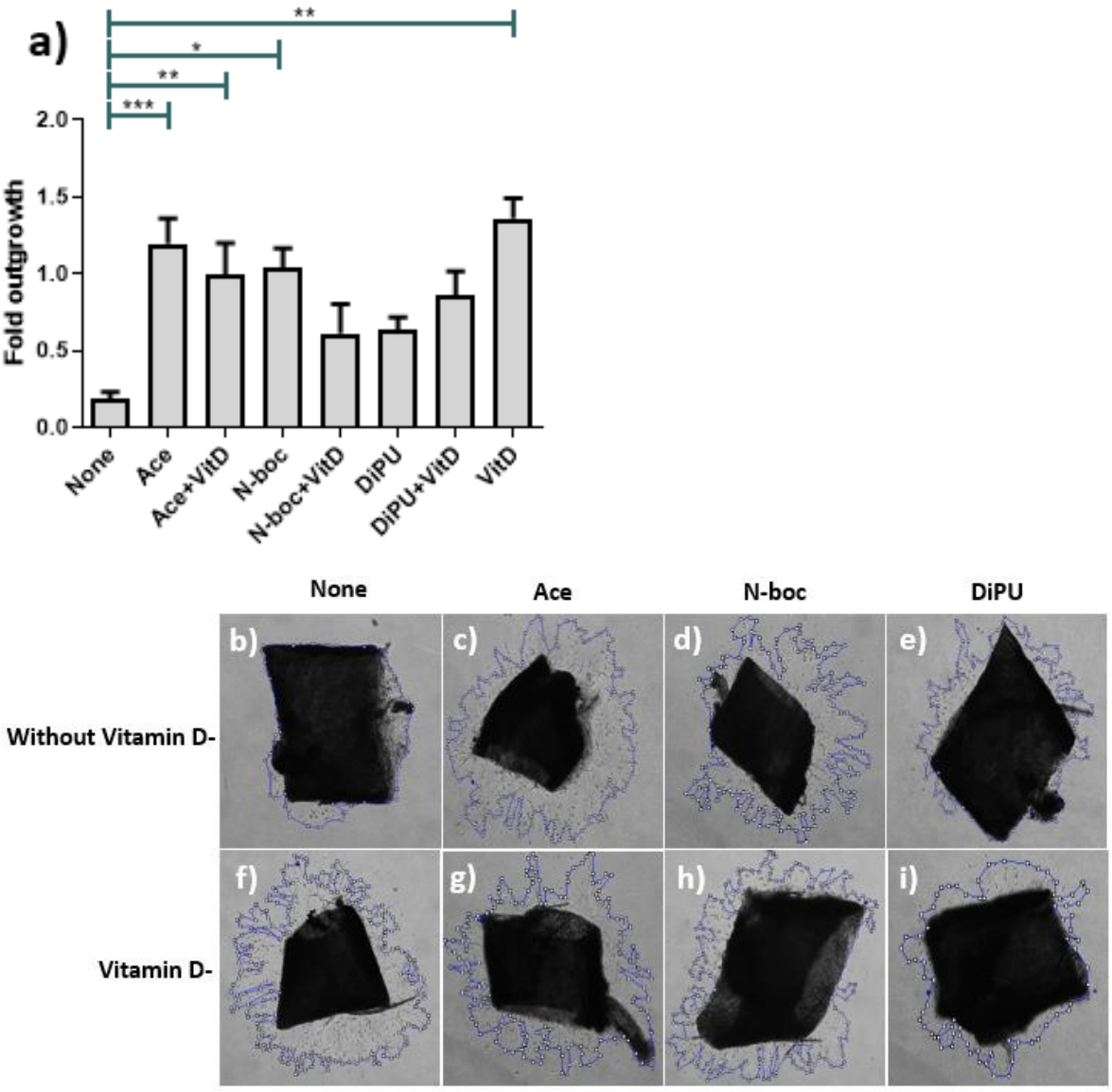
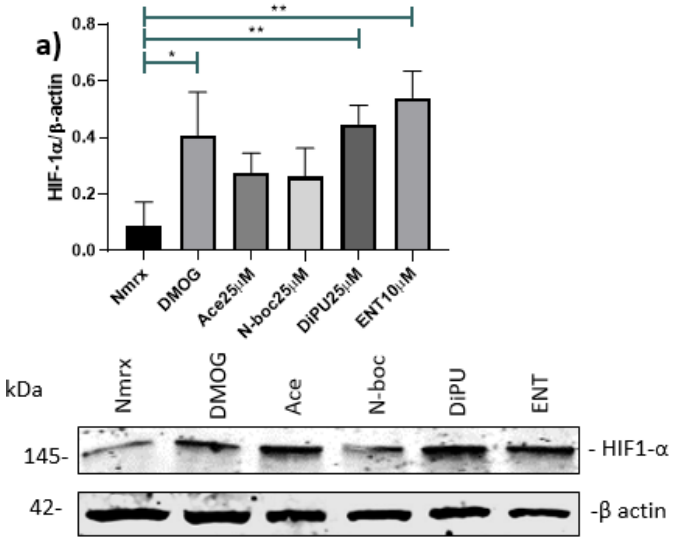
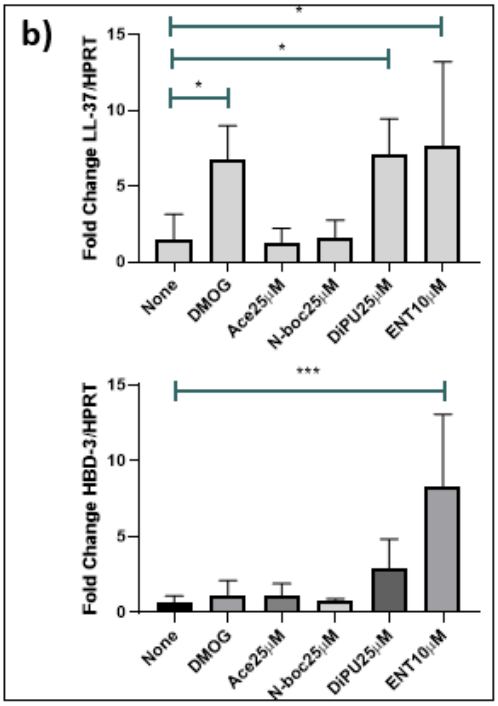
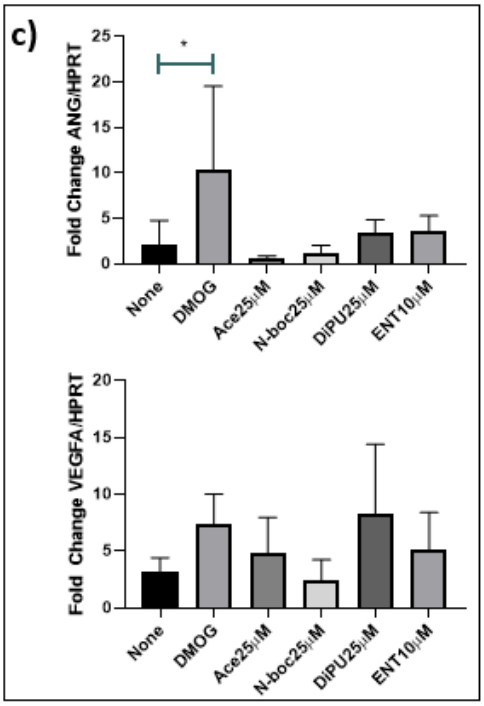


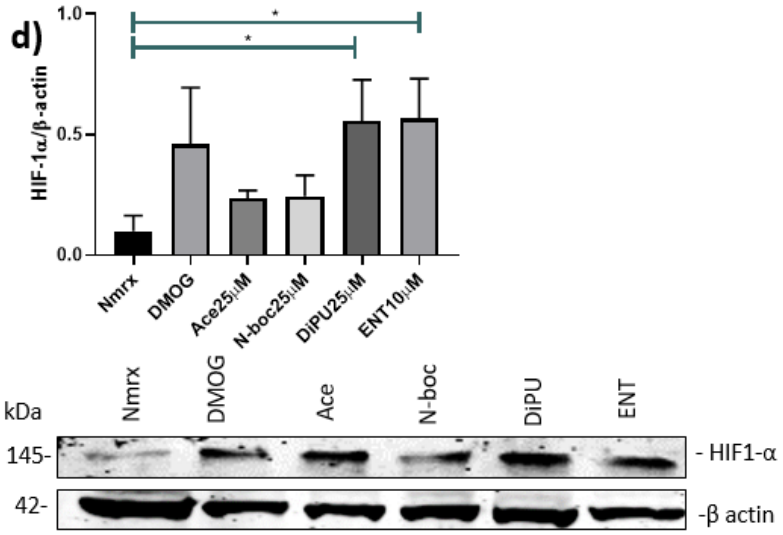
Figure 4.- Angiogenesis effect of the HDACi candidates. Panel a) presents a graphical representation of the fold outgrowth, with vitamin D utilized as the positive control. Panels b)-e) display representative images of mouse aortas treated with the HDACi candidates. Panels f)-i) depict representative images of mouse aortas subjected to co-stimulation with HDACi candidates and vitamin D. The experiment included a sample size of n=9, and statistical significance was indicated by *p≤ 0.05, **p≤0.01, and ****p≤0.0001.

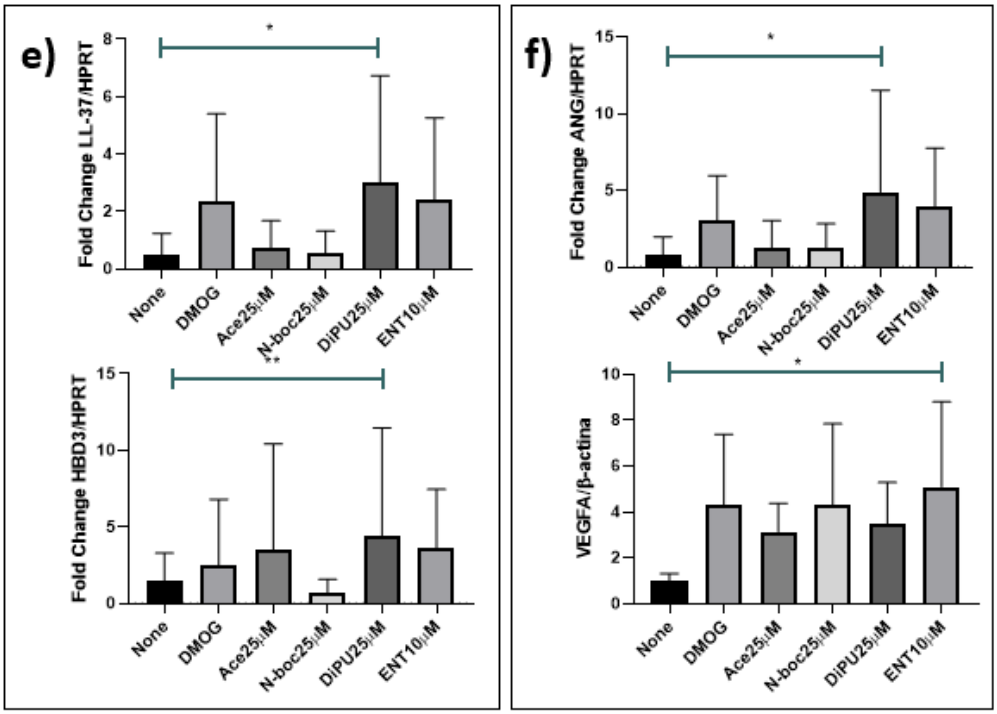
Figure 5

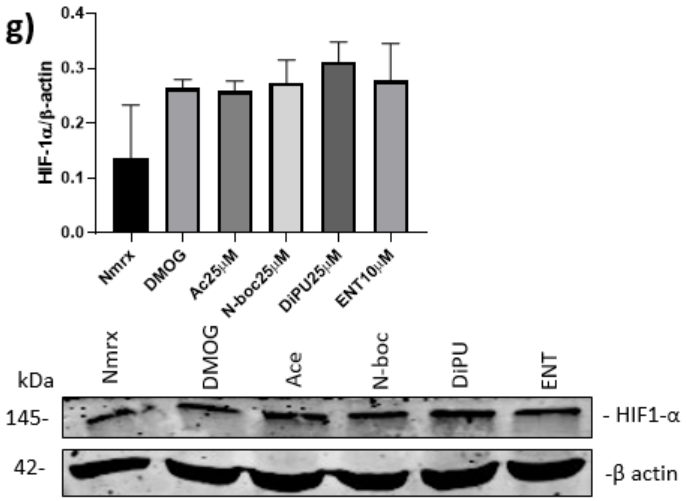

a)
HIF-1α/β-actin
0.0
0.2
0.4
0.6
0.8
*
**
**
Nmrx
DMOG
Ace25μM
N-boc25μM
DiPU25μM
ENT10μM
kDa
Nmrx
DMOG
Ace
N-boc
DiPU
ENT
145-
- HIF1-α
42-
-β actin


b)
Fold Change LL-37/HPRT
0
5
10
15
*
*
*
None
DMOG
Ace25μM
N-boc25μM
DiPU25μM
ENT10μM
Fold Change HBD-3/HPRT
0
5
10
15
***
None
DMOG
Ace25μM
N-boc25μM
DiPU25μM
ENT10μM


c)
Fold Change ANG/HPRT
0
5
10
15
20
25
*
None
DMOG
Ace25μM
N-boc25μM
DiPU25μM
ENT10μM
Fold Change VEGFA/HPRT
0
5
10
15
20
None
DMOG
Ace25μM
N-boc25μM
DiPU25μM
ENT10μM


d)
HIF-1α/β-actin
1.0
0.5
0.0
*
*
Nmrx
DMOG
Ace25μM
N-boc25μM
DiPU25μM
ENT10μM
kDa
Nmrx
DMOG
Ace
N-boc
DiPU
ENT
145-
- HIF1-α
42-
-β actin


e)
Fold Change LL-37/HPRT
Fold Change HBD3/HPRT
None
DMOG
Ace25μM
N-boc25μM
DiPU25μM
ENT10μM
f)
Fold Change ANG/HPRT
VEGFA/β-actina



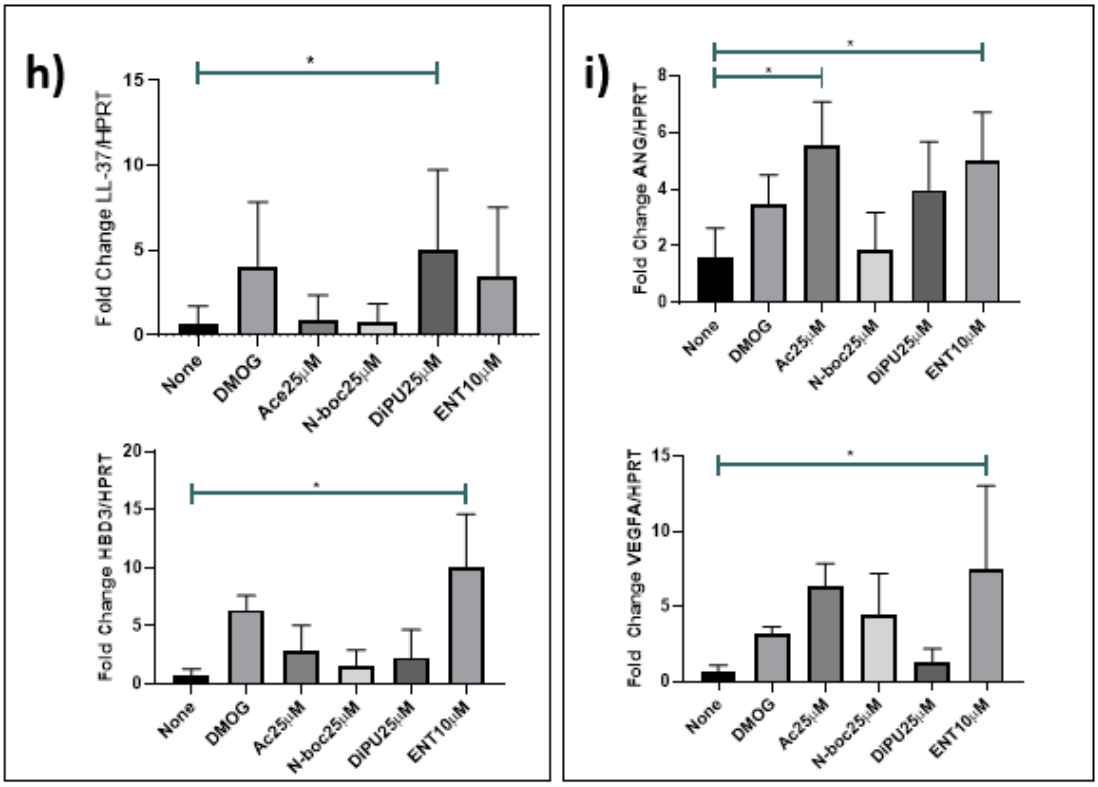
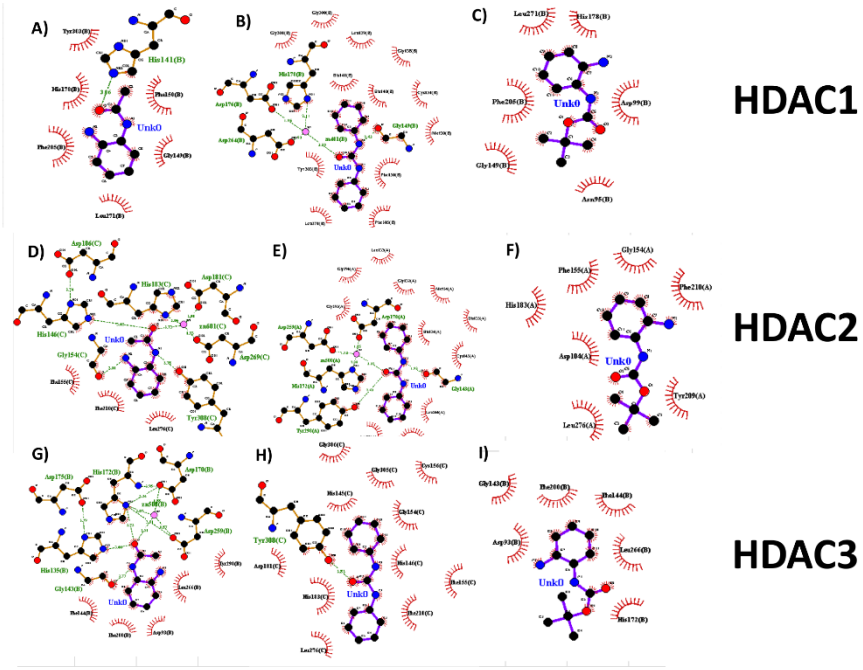


Figure 5.- Effect on wound healing expression proteins on healthy, diabetic and chronic wound primary human keratinocytes. a) HIF-1α stabilization was assessed in primary keratinocytes from non-diabetic donors. b-c) Expression levels of host defense peptides (HDP) and angiogenic genes were evaluated. A sample size of n=10 was used, with statistical significance indicated by *p≤ 0.05 and **p≤0.01. d) HIF-1α stabilization was examined in primary keratinocytes

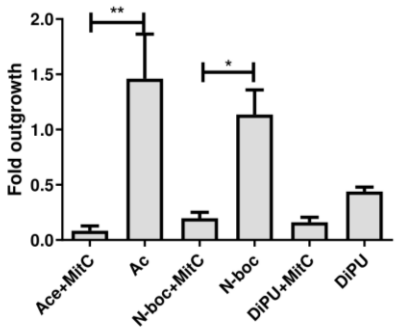
from diabetic donors. e-f) Expression levels of HDP and angiogenic genes were analyzed. The experiment included a sample size of n=10, with statistical significance denoted by *p≤ 0.05. g) HIF-1α stabilization was investigated in primary keratinocytes from donors with diabetic foot ulcers (DFU). h-i) Expression levels of HDP and angiogenic genes were assessed with a sample size of n=3, and statistical significance was indicated by *p≤ 0.05.

Supplementary figures



Supplementary Figure 1: LIGPLOT analysis of HDAC1-3 isoforms with DiPU, Ace, and N-boc.

Crystallized structures from HDAC1-3 isoforms with their ligands were obtained from Protein Data Bank (PDB) with the following PDB ID: 5CIN (HDAC1), 7ZZO (HDAC2), and 4A69 (HDAC3). Using PyMOL software 2 files were obtained, one of the HDAC without the ligand and the other with only the ligand, the files were exported to Autodock Tools to establish the interaction coordinates according to each ligand from PDB crystallized structures, and docking was running with Autodock Vina using DiPU, Ace and N-boc candidates were with their respective configuration. The result was exported as .pdb and analyzed with LigPlus+ software, to observe the ligand interaction with a LIGPLOT analysis. We observe the interaction of each HDACi candidate with the HDAC1-3 isoforms, showing that DiPU binds HDAC1-2 zinc, Ace to HDAC2-3 zinc, and N-boc don't bind to any HDAC isoforms zinc. The obtained data suggest a certain selectivity coming from the HDACi candidates.



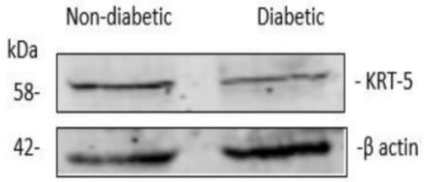
Supplementary Figure 2: Proliferative effect of HDACi candidates in Aortic ring assay

To confirm that HDACi candidates exert proliferative and not migratory effects, we measure the fold

outgrowth with aortic rings stimulated with and without Mitomycin C to inhibit proliferation. Three 6-month-old male BALB/c mice were euthanized through cervical dislocation, followed by dissection and extraction of the aorta. The obtained aorta was meticulously cleaned and sectioned into small 1mm rings. In a 24-well plate, 100 µl of Corning Matrigel Growth Factor Reduced (Corning, NY, USA) was centrally placed and the aortic rings were embedded and incubated for one hour to allow Matrigel polymerization. Subsequently, the aortic rings were incubated with DMEM 10% FBS for 3 days. The viable aortas were stimulated with HDACi candidates DiPU, Ace, and N-boc with and without Mitomicyn C (3.5 µg/mL). The fold outgrowth was measured using the following formula.

$$Fold\ outgrowth\ = \frac{(AT2 - AT1)}{AT0}$$

The data obtained shows that the adition of Mytomicin C significantly reduces the fold outgrowth, showing a proliferative effect from the HDACi.



Supplementary Figure 3: Primary culture phenotype of human basal epidermal keratinocytes.

Wound healing mechanisms such as re-epithelialization and production of angiogenesis proteins, are mainly directed by the transcriptional activity of primary basal keratinocytes, to confirm the phenotype of our primary cultured keratinocytes we observed the expression of cytokeratin-5 (KRT5) with western blot, incubating the membrane with anti-human KRT5 (1:500, Abcam, Kendal Square, Cambridge, UK) and anti-human β-actin (1:3000, Sigma-Aldrich, San Luis, Misuri) as a loading control, KRT5 its a marker highly expressed in basal keratinocytes. The membrane was revealed using the Odyssey® CLx (LI-COR Lincoln, Nebraska USA) measuring the band density.

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