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**Astragaloside IV and ginsenoside Rb1 combination improves diabetic foot ulcer
healing by downregulating the IRF1-ZBP1 axis in keratinocytes**

Running title: DFU healing and PANoptosis

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19

20 **Abstract**

21 **Background:** Diabetic foot ulcer (DFU) represents a prevalent and refractory complication of
22 diabetes mellitus. Active components from *Astragalus membranaceus* and *Panax ginseng* have
23 shown potential in treating diabetic complications. The present study aimed to investigate the
24 combined effects of astragaloside IV (AS-IV) and ginsenoside Rb1 (GsRb1) in DFU healing.

25 **Methods:** An in vitro DFU model was induced by high glucose (HG) in HaCaT
26 keratinocytes, which were treated with AS-IV and/or GsRb1. Cell viability, cell death patterns
27 (apoptosis, pyroptosis, and necroptosis), inflammatory reactions, and oxidative stress were
28 determined. A rat DFU model was established and treated with AS-IV and GsRb1. The
29 binding relationship between AS-IV/GsRb1 and potential targets was verified via molecular
30 docking, a cellular thermal shift assay (CETSA), and drug affinity responsive target stability
31 (DARTS). Additionally, the regulatory roles of ZBP1 and IRF1 in HaCaT keratinocytes were
32 clarified by downregulating or upregulating ZBP1 and IRF1.

33 **Results:** HaCaT keratinocytes treated with HG exhibited significantly elevated apoptosis,
34 pyroptosis, necroptosis, and oxidative stress. The combined application of AS-IV and GsRb1
35 at a 2:3 ratio demonstrated optimal protective effects. In the STZ-induced diabetic rat DFU
36 model, combined administration of AS-IV and GsRb1 significantly accelerated wound healing
37 and enhanced granulation tissue formation, angiogenesis, and collagen deposition, while
38 notably reducing neutrophil infiltration. Molecular docking analysis revealed that AS-IV and

GsRb1 potentially bind to core proteins involved in PANoptosis. CETSA and DARTS verified that AS-IV and GsRb1 could directly bind to ZBP1 and decrease its protein level. Co-administration of AS-IV and GsRb1 suppressed activation of ZBP1-mediated PANoptosis. ZBP1 knockdown showed no additional protective effects of AS-IV and GsRb1. Overexpression of ZBP1 or its upstream regulator IRF1 exacerbated HaCaT keratinocyte injuries, which were reversed by AS-IV and GsRb1.

Conclusions: AS-IV and GsRb1 combination synergistically promotes wound healing in DFU via targeting the IRF1-ZBP1 axis-mediated PANoptosis, providing a potential therapeutic strategy for DFU.

Keywords: Astragaloside-IV; ginsenoside Rb1; PANoptosis; diabetic foot ulcer; neutrophils; wound healing.

ZBP1

1. Introduction

As a chronic metabolic disorder, diabetes mellitus (DM) has experienced a continuous and dramatic increase in global prevalence. The number of adults living with diabetes worldwide has reached 529 million, and this figure is projected to increase to 1.31 billion by 2050, with the age-standardized prevalence expected to exceed 10% [1]. This increase is reflected not only in the increasing number of confirmed cases but also in the profound impacts of its various

severe complications on individual health and social healthcare resources [2]. Among multiple DM-associated complications, diabetic foot ulcer (DFU) has attracted considerable attention. Globally, approximately 18.6 million people are affected by DFU each year, and approximately one-fourth of diabetic patients will develop DFU during the course of their disease [3]. The clinical management of DFU is extremely challenging and is characterized by prolonged nonhealing, high susceptibility to secondary infections, and a high recurrence rate—with the 5-year recurrence rate reaching 65% [3]. In developed countries, DFU accounts for 12%–15% of the total healthcare expenditure on diabetes, while this proportion can reach 40% in resource-limited regions [4,5]. Current standard treatments for DFU include debridement, offloading, revascularization, and antibiotic therapy [5]. However, these interventions achieve limited efficacy. Therefore, identifying the molecular mechanisms of DFU and developing more effective therapies are critical.

Normal wound healing is a highly coordinated dynamic process that encompasses four phases: inflammation, cell proliferation, angiogenesis, and tissue remodeling [6]. As a core cell type in the innate immune response, neutrophils play a crucial role in the early inflammatory phase. Through phagocytosis, reactive oxygen species (ROS) generation, degranulation, and the release of neutrophil extracellular traps (NETs), neutrophils clear pathogens and initiate the repair process [7,8]. Under physiological conditions, neutrophils undergo programmed cell death processes such as apoptosis, which are subsequently cleared by macrophages. This sequence of events facilitates the resolution of inflammation and promotes tissue regeneration [9]. In the hyperglycemic environment of diabetes, neutrophils exhibit functional dysregulation. Although there is no significant increase in the number of circulating neutrophils, their subset

distribution and activity are markedly abnormal [10]. The proportion of proinflammatory low-density neutrophils (LDNs) is increased, and these cells extensively release inflammatory factors such as interleukin-6 (IL-6), IL-8, and C-reactive protein (CRP) while also forming excessive neutrophil extracellular traps (NETs) [11-12]. Hyperglycemia impairs the bactericidal function of neutrophils while promoting excessive NET formation [13-15], which severely hinders the ulcer healing process by inducing endothelial–mesenchymal transition (EMT) and inhibiting angiogenesis [16]. On the other hand, DFU wounds undergo multiple programmed cell death (PCD) patterns, including apoptosis, autophagy-mediated cell death, ferroptosis, pyroptosis, necroptosis, and NETosis. These processes do not operate independently; instead, they are interconnected through complex molecular interactions and signal crosstalk. Under pathological conditions, the amplified effect of such interactions may exacerbate tissue damage and inflammatory responses, making it difficult to restore tissue homeostasis by inhibiting a single pathway [17-19].

In recent years, PANoptosis—a novel form of inflammatory cell death—has gradually been revealed. It integrates the molecular features of pyroptosis, apoptosis, and necroptosis and is regulated by the PANoptosome complex, which comprises components such as ZBP1 (Z-DNA binding protein 1), AIM2 (absent in melanoma-2), RIPK (receptor-interacting protein kinase) 1/3, and ASC (apoptosis-associated speckle-like protein) (also known as PYCARD) [20]. This complex can simultaneously activate key effector molecules such as caspase-1/-3 and MLKL, mediating multiple death effects and amplifying inflammatory signals. ZBP1 is considered a crucial upstream sensor molecule in the assembly and function of the PANoptosome. It collaborates with molecules, including AIM2, RIPK1/3, ASC, FADD, and

caspace-8, to integrate and transmit damage- and pathogen-associated signals [21]. Studies have shown that targeting ZBP1 can effectively inhibit PANoptosis, thereby improving the dysfunction of keratinocytes and endothelial cells and promoting wound healing [19,20,22-23].

The traditional Chinese medicine (TCM) system has a long-standing practical foundation and unique advantages in the comprehensive management of diabetes and its complications through immune regulation, anti-inflammatory effects, antioxidant activity, and the promotion of angiogenesis [24-26]. For example, glycyrrhizic acid, a specific inhibitor of high-mobility group box 1 (HMGB1), can inhibit NET formation and alleviate various acute organ injuries [25,26]. Astragaloside IV (AS-IV), a key saponin active component in *Astragalus membranaceus*, possesses biological activities such as anti-inflammatory effects, antioxidant effects, improvements in endothelial function, and the promotion of angiogenesis [27]. Ginsenoside Rb1 (GsRb1) is one of the main active ingredients in *Panax ginseng*. It has been reported to exert effects, including immune regulation, anti-inflammatory effects, and the promotion of tissue repair [28]. Studies have indicated that natural products can facilitate wound healing by reducing the expression of proinflammatory factors, inhibiting oxidative stress, and improving endothelial cell function [29,30].

Thus, we propose that AS-IV and GsRb1 could improve wound healing in diabetic patients by mediating neutrophil dysfunction. To mimic the DFU environment, an in vitro HaCaT keratinocyte injury model was induced by high glucose (HG). AS-IV and GsRb1 were used to establish an in vitro DFU model and a rat DFU model. Mechanistic studies should be conducted to investigate the ZBP1-mediated PANoptosis pathway mediated by AS-IV and GsRb1. Overall,

this study aims to provide molecular pathological evidence for DFU adjuvant therapy strategies based on natural products.

2. Materials and methods

2.1 Drug Preparation

AS-IV (#HY-N0431) and GsRb1 (#HY-N0039) were purchased from MedChemExpress (USA) and dissolved in dimethyl sulfoxide (DMSO, #A13280.36, Thermo Fisher Scientific, USA) to prepare 100 mM stock solutions, which were stored at -20°C . The working concentrations were prepared by diluting the stock solutions with high-glucose DMEM (containing 25 mM glucose, #11965092, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, #10099-141, Gibco, USA), with a final concentration (of DMSO) $\leq 0.1\%$.

2.3 Cell Culture

The HaCaT human keratinocyte cell line was acquired from the Shanghai Cell Bank of the Chinese Academy of Sciences. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (100 U/mL, Cat. No. #15140122, Gibco, USA). The culture was maintained in a controlled environment at 37°C with 5% carbon dioxide (CO_2) and saturated humidity. For cell passaging, trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA, 0.25%, Cat. No. #25200-72, Gibco, USA) was used to dissociate the adherent cells.

2.4 Drug Treatment

An in vitro DFU injury model was established by treating HaCaT cells with HG. HaCaT cells were seeded into 96-well plates at a density of 5×10^3 cells per well and then treated with high-glucose DMEM containing D-glucose at a concentration of 25 mM D-glucose for 24 hours [31]. To evaluate the pharmaceutical effects, the following experimental groups were set up: the normal control group (Ctrl), model group (HG), AS-IV group, GsRb1 group, and combined drug group (AS-IV+GsRb1). After 24 hours of drug intervention, subsequent detection was performed.

To determine the optimal ratio of AS-IV to GsRb1, gradient screening of single-drug and combined-drug treatments was performed in the in vitro DFU injury model to confirm the concentrations used in subsequent experiments. Single-drug screening: HaCaT cells were seeded into 96-well plates at a density of 5×10^3 cells/well. Under model conditions, the cells were treated with different concentrations of AS-IV (0, 5, 10, 20, or 50 μ M) or with different concentrations of GsRb1 (0, 5, 10, 20, or 50 μ M) for 24 hours. Combined-drug ratio screening: Under model conditions, HaCaT cells were treated with mixed solutions of AS-IV and GsRb1 at different molar ratios (AS-IV: GsRb1 = 5:0, 4:1, 3:2, 1:1, 2:3, 1:4, 0:5) for 24 hours. For the mixed solutions, the total concentration of the two drugs was set at gradient levels (0, 5, 10, 20, or 50 μ M).

2.5 Rat DFU Model

Male Sprague–Dawley (SD) rats (4–6 weeks old, initial body weight 180–200 g) were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. All the rats were housed in an SPF-grade barrier environment at the Laboratory Animal Center of

Nanchang University under controlled conditions: temperature (22 ± 2) °C, relative humidity (55 ± 10)%, and a 12 h/12 h light/dark cycle, with free access to food and water. The animal experimental protocol was approved by the Animal Ethics Committee of Nanchang University (Approval No. KY20230112) and complied with the NIH Guidelines for the Care and Use of Laboratory Animals.

After 1 week of adaptive feeding, the SD rats were randomly divided into a normal control group ($n=10$) and a model group ($n=40$). The model group was fed a high-fat and high-sugar diet for 4 weeks. The rats in the model group were subsequently fasted for 12 hours with free access to water, followed by a single intraperitoneal injection of freshly prepared 1% streptozotocin (STZ, 55 mg/kg, i.p., dissolved in citrate buffer at pH 4.5) (Cat. No. #S80535; Shanghai Angte Biotechnology Co., Ltd., China) [32]. The rats in the control group were injected with an equal volume of citrate buffer. At 72 hours after STZ injection, random blood glucose levels in the tail vein of the rats were measured via a blood glucose meter. A T2DM model was considered successfully established if the blood glucose level was ≥ 16.7 mmol/L for 3 consecutive days.

At 8 weeks after the successful establishment of the T2DM model (at this stage, rats typically exhibit obvious polydipsia, polyuria, and slow weight gain or weight loss), skin ulceration was induced in 40 T2DM rats. The rats were anesthetized with 3% isoflurane and fixed in the prone position, and their dorsal hair was shaved via an electric hair clipper. The dorsal skin was disinfected with 75% ethanol. Two full-thickness skin defect wounds (deep to the fascia layer) were created symmetrically on both sides of the dorsal spine via a circular skin biopsy

punch (1 cm in diameter) precooled to -20°C . Immediately after wound creation, 50 μL of 50% glacial acetic acid was applied locally to the wounds for 5 minutes; this treatment was repeated once daily for 7 consecutive days to simulate the nonhealing microenvironment of diabetic ulcers.

2.6 Animal experimental grouping and drug administration

Rats in which the DFU model was successfully established were randomly divided into 4 groups, with 10 rats in each group. DFU group (model control group): DFU model rats were given an equal volume of normal saline solution by gavage once daily. AS-IV group: DFU model rats were administered AS-IV (12 mg/kg body weight) by gavage once daily [33]. GsRb1 group: DFU model rats were administered GsRb1 (18 mg/kg body weight) by gavage once daily [34]. AS-IV+GsRb1 group: DFU model rats were given a mixture of AS-IV and GsRb1 (12 mg/kg AS-IV + 18 mg/kg GsRb1, on the basis of body weight) by gavage once daily. The gavage volume for all groups was 10 mL/kg body weight, and drug administration was conducted once daily for 12 consecutive days. Additionally, a sham operation group (sham group, n=10) was established: nondiabetic rats, after wound creation on the dorsal skin, were given an equal volume of 0.5% CMC-Na normal saline solution by gavage once daily.

2.7 Genetic Intervention

For transient knockdown, a small interfering RNA (siRNA) targeting human ZBP1 (si-ZBP1) and a nontargeting siRNA (si-NC, which served as a negative control) were purchased. si-ZBP1 was synthesized by Thermo Fisher Scientific (Cat. No. #130954) and purified via

ethanol precipitation. In 6-well plates, Lipofectamine 3000 (Cat. No. #L3000001; Thermo Fisher Scientific, USA) was used to transfect the siRNAs at a final concentration of 50 nM, following the manufacturer's instructions. The transfection volume and the ratio of DNA/RNA complexes were configured as recommended by the manufacturer, and the knockdown efficiency was detected 24–48 hours post-transfection. For the overexpression of ZBP1 and IRF1, full-length human cDNAs were cloned and inserted into the CD510B (pCDH-CMV-MCS-EF1-Puro) vector (QP1171, Qiyun Biotechnology, China). HaCaT cells were infected with the recombinant vectors via a lentiviral packaging system (VSVG and psPAX2 packaging plasmids), with the empty vector used as the control (OE-NC). At 72 hours post-infection, stable cell lines were selected with 2 µg/mL puromycin (ant-pr-1; InvivoGen, France).

2.8 Detection of Cell Viability and Death

Cell viability was assessed via the Cell Counting Kit-8 (CCK-8) assay (#C0037, Beyotime, China). After treatment, 10 µL of CCK-8 reagent was added to each well of HaCaT cells, followed by incubation at 37°C with 5% CO₂ for 2 hours. The absorbance was measured at 450 nm via a microplate reader (SpectraMax M5, Molecular Devices, USA).

The calcein-AM/PI double-staining method (#C2015S, Beyotime, China) was employed to evaluate the ratio of viable to dead cells. The cells were stained with 2 µM calcein-AM and 4 µM PI, incubated for 30 minutes, and then observed under a fluorescence microscope (Leica DMi8, Germany) to detect the fluorescence intensity. For apoptosis analysis, an Annexin V-FITC Apoptosis Detection Kit (#CA1020, Solarbio Science & Technology Co., Ltd., China)

was used. The cells were harvested, resuspended in binding buffer, and stained sequentially with Annexin V-FITC and PI. After 15 minutes of incubation in the dark, the cells were analyzed via a flow cytometer (BD Biosciences, USA) to determine the proportions of early apoptotic cells (Annexin V⁺PI⁻) and late apoptotic/necrotic cells (Annexin V⁺PI⁺). All the experiments were performed in triplicate and were repeated three times independently.

For cell toxicity detection, the LDH Cytotoxicity Assay Kit (#C0016, Beyotime, China) was used following the manufacturer's instructions. Briefly, 100 μ L of culture supernatant was collected and mixed with LDH working solution, and the absorbance was measured at 490 nm via a microplate reader. All the experiments were performed in triplicate and were repeated three times independently.

2.9 Detection of oxidative stress

The level of reactive oxygen species (ROS) was detected via the DCFH-DA method (#S0035S, Beyotime, China). The treated cells were incubated with 10 μ M DCFH-DA at 37°C for 30 minutes. After staining, flow cytometry was performed for semiquantitative analysis. The level of lipid peroxidation was measured via an MDA assay kit (#S0131S, Beyotime, China). The cell lysates were reacted with thiobarbituric acid (TBA), and the absorbance was measured at 535 nm via a microplate reader. Superoxide dismutase (SOD) activity was determined via the WST-8 method (#S0101S, Beyotime, China). The cell lysates were reacted with WST-8 working solution, and the absorbance was measured at 450 nm via a microplate reader. All the experiments were performed in triplicate and were repeated three times independently.

2.10 Assessment of Wound Healing and Collection of Tissue Samples

On days 0, 3, 6, 9, and 12 following the initiation of drug administration, photographs of the treated wounds in the rats were captured via a digital camera (Canon EOS, Japan) under standardized conditions of fixed distance and illumination. Wound areas were measured via ImageJ software (NIH, USA), and the wound healing rate was calculated via the following formula: Wound healing rate (%) = [(Wound area on day 0 - Wound area on day n)/Wound area on day 0] × 100%.

Two hours after the final administration (day 12), the experimental rats were anesthetized with pentobarbital sodium (100 mg/kg, intraperitoneal injection) and subsequently euthanized. Wound tissues along with adjacent surrounding tissues (approximately 1.5 cm × 1.5 cm) were excised along the wound margins. A portion of the harvested tissues was fixed in 4% paraformaldehyde for histological analysis, while another portion was snap-frozen at -80°C for molecular biology assays. All tissue processing and analyses were independently performed by two observers blinded to the experimental groups.

2.11 Histopathological Staining

Wound tissues and adjacent cutaneous tissues were collected and fixed in 10% neutral buffered formalin (pH 7.4, Sigma–Aldrich, USA) for 24 hours, followed by routine dehydration, paraffin embedding, and sectioning into slices with a thickness of approximately 4 µm. To evaluate wound tissue architecture and inflammatory cell infiltration, hematoxylin–eosin (H&E) staining was performed via a commercial kit (#C0105S, Beyotime, China). Under microscopic

observation, inflammatory infiltration and granulation tissue formation were assessed, whereas epidermal thickness was quantified via threshold segmentation using ImageJ software. For the assessment of collagen fiber deposition in wound tissues, Masson's trichrome staining was conducted with a dedicated kit (#C0189S, Beyotime, China). The stained sections were photographed via a light microscope (Olympus BX53, Japan) and subjected to semiquantitative analysis via ImageJ software.

2.12 Immunohistochemistry (IHC)

Immunohistochemical staining was performed on 5 μ m thick tissue sections. The paraffin-embedded sections were first subjected to deparaffinization and rehydration, followed by antigen retrieval via sodium citrate buffer. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes, and nonspecific binding was inhibited by blocking with 5% bovine serum albumin (BSA). The sections were subsequently incubated with primary antibodies, including anti-ZBP1 (1:500, #13285-1-AP, Proteintech, USA), anti-CD31 (1:2000, #11265-1-AP, Proteintech, USA), anti-Ki67 (1:4000, #27309-1-AP, Proteintech, USA), anti- α -SMA (1:1500, #14395-1-AP, Proteintech, USA), and anti-collagen III (1:500, #22734-1-AP, Proteintech, USA), at 4°C overnight. On the following day, the sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000, #SA00001-2, Proteintech, USA) at room temperature for 1 hour, developed with 3,3'-diaminobenzidine (DAB) substrate, and counterstained with hematoxylin to visualize the cell nuclei. The sections were then mounted with neutral balsam. The distribution and intensity of positive staining (brown–yellow) were observed under a light microscope (Olympus, Japan),

and semiquantitative analysis was performed via ImageJ software.

2.13 Immunofluorescence

Immunofluorescence staining was performed on either tissue sections or cell culture coverslips. For the paraffin-embedded tissue sections, deparaffinization and rehydration were conducted; for the cells, fixation was carried out in 4% paraformaldehyde for 20 minutes. Subsequently, permeabilization was performed via incubation with 0.2% Triton X-100 (Sigma–Aldrich) for 5 minutes, followed by blocking with 5% bovine serum albumin (BSA, Sigma–Aldrich) for 1 hour to block nonspecific binding. The sections or coverslips were then incubated with primary antibodies, including anti-ZBP1 (1:500), anti-IRF1 (1:500, #11335-1-AP, Proteintech, USA), anti-MPO (1:500, #22225-1-AP, Proteintech, USA), and anti-CitH3 (1:600, #17168-1-AP, Proteintech, USA), at 4°C overnight. The next day, after washing with Tris-buffered saline containing Tween-20 (TBST), Alexa Fluor 488/594-conjugated secondary antibodies (1:500, #gb2AF488, Proteintech, USA) were added dropwise. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and the sections/coverslips were mounted with anti-fluorescence quenching mounting medium. The fluorescent signals were visualized and imaged via a laser scanning confocal microscope (Leica Microsystems, Germany).

2.14 Western blotting

The tissue or cell samples were lysed in precooled RIPA lysis buffer supplemented with protease/phosphatase inhibitors (Thermo Fisher Scientific, USA) on ice for 30 minutes. The

lysates were subsequently centrifuged at $12,000 \times g$ at 4°C for 15 minutes, after which the supernatants were collected. The protein concentration was determined via the bicinchoninic acid (BCA) assay. Equal amounts of protein (20–40 μg per sample) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk at room temperature for 1 hour, followed by incubation with the following primary antibodies at 4°C overnight: anti-CitH3 (1:2000), anti-PAD4 (1:1000, #17373-1-AP, Proteintech, USA), anti-ZBP1 (1:1000), anti-AIM2 (1:1000, #20590-1-AP, Proteintech, USA), anti-IRF1 (1:500), anti-phosphorylated RIPK1 (p-RIPK1, 1:2000, #66854-1-Ig, Proteintech, USA), anti-RIPK1 (1:1000, #17519-1-AP, Proteintech, USA), anti-phosphorylated MLKL (p-MLKL, 1:5000, #82090-2-RR, Proteintech, USA), anti-MLKL (1:1000, #21066-1-AP, Proteintech, USA), anti-NLRP3 (1:2000, #30109-1-AP, Proteintech, USA), anti-ASC (1:5000, #10500-1-AP, Proteintech, USA), and anti-Caspase-1 (Casp1, 1: The following day, after being washed with Tris-buffered saline with Tween-20 (TBST), the membranes were incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000) at room temperature for 1 hour. The protein bands were visualized via an enhanced chemiluminescence (ECL) detection kit (Thermo Fisher Scientific, USA), and images were captured via a chemiluminescence imaging system (Bio-Rad, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5000, #10494-1-AP, Proteintech, USA) was used as the internal reference. The gray intensity of each band was quantified via ImageJ software, and the relative protein expression levels were calculated.

2.15 Enzyme-Linked Immunosorbent Assay

Supernatants of tissue homogenates (postcentrifugation) or cell culture supernatants were collected, and the ELISA procedure was performed in strict accordance with the manufacturers' instructions provided with the kits. Briefly, standards or samples were added to the wells of a microplate. Subsequently, biotin-conjugated antibodies, horseradish peroxidase (HRP)-labeled streptavidin, and 3,3',5,5'-tetramethylbenzidine (TMB) substrate were sequentially added to the wells for color development. The reaction was terminated by the addition of 2 M sulfuric acid (H₂SO₄). The absorbance (optical density, OD value) was measured at a wavelength of 450 nm via a microplate reader. The concentrations of high-sensitivity C-reactive protein (hs-CRP, Cat. No. #ml106583), interleukin-1 β (IL-1 β , Cat. No. #ml058059), interleukin-18 (IL-18, Cat. No. #ml058055), tumor necrosis factor- α (TNF- α , Cat. No. #ml077385), Syndecan-1 (Cat. No. #ml027595), and citrullinated histone H3 (CitH3, Cat. No. #ml105339) were calculated on the basis of the standard curve. The ELISA kits for these molecules were purchased from Shanghai Enzyme-Linked Biotechnology Co., Ltd. (China), while the kit for high-mobility group box 1 (HMGB1, Cat. No. #EEL047) was obtained from Thermo Fisher Scientific (USA).

2.16 Quantitative real-time polymerase chain reaction

Total RNA was extracted from HaCaT cells or lesional tissues via TRIzol reagent (#NR0046, Invitrogen, USA). The concentration and purity of the RNA were determined via a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), with an optimal OD₂₆₀/OD₂₈₀ ratio of approximately 1.8–2.0. Reverse transcription was performed to synthesize complementary DNA (cDNA) via the PrimeScript™ RT Reagent Kit (#RR037A, Takara, Japan). Quantitative

real-time PCR was conducted on an ABI 7500 Real-Time PCR System (Applied Biosystems) using TB Green® Premix Ex Taq™ II (#RR820L, Takara, Japan). The total volume of the qPCR system was 20 µL, consisting of 10 µL of TB Green Premix Ex Taq II (2×), 0.8 µL each of forward and reverse primers (10 µM), 2 µL of cDNA template, and 6.4 µL of double-distilled water (ddH₂O). The thermal cycling program was set as follows: initial denaturation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing/extension at 60°C for 30 seconds. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference gene. Each sample was analyzed in triplicate, and the relative mRNA expression levels of the target genes were calculated via the $2^{-\Delta\Delta C_t}$ method. All primer sequences were purchased from OriGene Technologies, Inc., with the following details. ZBP1: Forward (F) 5'-GCAAACCTCCGAAGCCATCCAGA-3', Reverse (R) 5'-CCAAGTTGAGGAATCACCTGGTG-3' (#HP215403); IRF1: F 5'-GAGGAGGTGAAAGACCAGAGCA-3', R 5'-TAGCATCTCGGCTGGACTTCGA-3' (#HP205934); AIM2: F 5'-GCTGCACCAAAAGTCTCTCCTC-3', R 5'-CTGCTTGCCTTCTTGGGTCTCA-3' (#HP208094); GAPDH: F 5'-GTCTCCTCTGACTTCAACAGCG-3', R 5'-ACCACCCTGTTGCTGTAGCCAA-3' (#HP205798).

2.17 Cellular thermal shift assay (CETSA)

For the CETSA experiment, HaCaT cells were first treated with AS-IV or GsRb1, following a previously established protocol [35]. After treatment, the cells were harvested and lysed via freeze-thaw cycles. The lysates were centrifuged, and the supernatants were collected. These

supernatants were evenly distributed into multiple tubes, which were then heated at preset gradient temperatures (ranging from 42°C to 70°C, with 8 temperature points in total) for 3 minutes to induce protein denaturation. Immediately after heating, the samples were placed on ice for cooling to terminate the denaturation process. The samples were subsequently treated with lysis buffer and centrifuged again to remove insoluble precipitates. Finally, the supernatants were collected, and the remaining amount of ZBP1 protein under different temperature treatments was detected via Western blotting to evaluate its thermal stability. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference, and semiquantitative analysis was performed via ImageJ software.

2.17 Drug affinity-responsive target stability assay (DARTS)

For the DARTS experiment, lysates of HaCaT cells without drug treatment were incubated with AS-IV (10, 20, or 50 μ M) or GsRb1 (10, 20, or 50 μ M) at different concentrations for 1 hour at room temperature, allowing sufficient binding between small molecules and potential targets [36]. Pronase protease (#53702-25KU, enzyme-to-substrate ratio of 1:1600, Sigma–Aldrich, USA) was subsequently added to each tube, and the mixture was incubated at room temperature for 30 minutes to digest proteins not protected by small molecules. After incubation, a protease inhibitor cocktail was added to terminate the reaction. Finally, the degradation pattern of the ZBP1 protein was analyzed via Western blotting, and its degradation trend with increasing small molecule concentrations was observed to reveal the interaction and binding specificity between small molecules and the ZBP1 protein.

2.18 Molecular docking

For molecular docking validation, the molecular structures of the main active components (AS-IV and GsRb1) were downloaded from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) and saved in the Structure Data File (SDF) format. The three-dimensional (3D) crystal structures of the target proteins (ZBP1, AIM2, NLRP3, PYCARD, CASP1, GSDMD, RIPK1, RIPK3, MLKL, CASP3, CASP7, CASP8, and IRF1) were retrieved from the Protein Data Bank (PDB) database (<http://www.rcsb.org/>) and stored in PDB format.

PyMOL software was used to remove water molecules and ligands from the proteins, which were then resaved in PDB format. The Getbox plug-in was employed to obtain the parameters of the docking pockets. The processed protein and active component files were imported into AutoDock Tools 1.5.6 (The Scripps Research Institute, USA) and converted to PDBQT format. Molecular docking simulations were performed via AutoDock Vina 1.1.2. Finally, the molecular docking results were visualized via PyMOL 2.6.0 software (Schrödinger, USA) [37].

2.19 Statistical methods

The quantitative data are presented as the means $\pm$ standard deviations (means $\pm$ SDs). For normally distributed data, one-way analysis of variance (one-way ANOVA) was used, followed by Tukey's or Bonferroni post hoc correction for multiple comparisons. Repeated-measures ANOVA was applied to analyze the time series data. For nonnormally distributed data, the Kruskal–Wallis test was performed. Statistical analyses were conducted via GraphPad Prism or SPSS software, and a P value < 0.05 was considered statistically

significant.

3. Results

3.1 AS-IV and GsRb1 Exert a Synergistic Protective Effect on the HG-induced Injury Model

To evaluate the safety and protective effects of AS-IV and GsRb1, we studied HaCaT cells in an in vitro DFU model. First, we assessed the effects of different concentrations of AS-IV (0–50 μ M) and GsRb1 (0–50 μ M) on HaCaT cell viability. Toxicity tests revealed that neither the drug alone caused significant toxicity to normal HaCaT cells within the tested concentration range (0–50 μ M) (Fig. S1A-B) nor their combination at the tested ratios (AS-IV: GsRb1 from 5:0-0:5) (Fig. S1C). Next, we analyzed the protective effects of AS-IV and GsRb1 at different concentrations on the in vitro DFU model. The CCK-8 results demonstrated that both drugs alone increased the cell survival rate in a dose-dependent manner (vs. the HG group, $p < 0.05$) (Fig. 1A–B). To further explore the synergistic effect, cells were treated with the two drugs at different molar ratios (AS-IV:GsRb1 from 5:0 to 0:5). The strongest synergistic effect was observed when AS-IV and GsRb1 were combined at a 2:3 molar ratio, which resulted in the highest cell survival rate and the lowest LDH release (vs. the HG group, $p < 0.001$) (Fig. 1C–D). These results indicate that at a specific ratio, AS-IV and GsRb1 exert a synergistic protective effect on keratinocytes in a high-glucose and inflammatory microenvironment.

We further analyzed the concentration-dependent effect and cell protective role of the combined drugs. At a total concentration of 50 μ M, two groups were established: the AG-H

group (20 μ M AS-IV + 30 μ M GsRb1) and the AG-L group (10 μ M AS-IV + 15 μ M GsRb1). Calcein-AM/PI staining revealed that the proportion of dead cells was significantly lower in the AG-H group than in the AG-L group (Fig. 1E-F), and flow cytometry further confirmed that it markedly decreased the apoptosis/necrosis rate, with the protective effect in the AG-L group being weaker than that in the AG-H group (Fig. 1G-H). ELISA further revealed that the levels of IL-1 β and IL-18 in the supernatants of the combined treatment groups were lower than those in the supernatants of the HG group, and the reduction in the AG-L group was less pronounced than that in the AG-H group (Fig. 1I-J). Combined drug treatment also significantly inhibited the oxidative stress induced by HG, reducing the ROS fluorescence intensity and MDA content while significantly increasing the SOD activity (vs. the HG group, $p < 0.05$) (Fig. 1K-N). Similarly, the AG-L group was less effective at inhibiting oxidative stress than the AG-H group was. Collectively, these results demonstrate that the combination of AS-IV and GsRb1 at a ratio of 20 μ M + 30 μ M (AG-H group) exerts a synergistic protective effect in the HG-induced HaCaT injury model.

3.2 AS-IV + GsRb1 Promotes Wound Healing and Improves Tissue Repair in a Rat DFU Model

A 1 cm-diameter dorsal skin defect was created in diabetic rats via a high-fat and high-sugar diet combined with STZ, and 50% glacial acetic acid was used to simulate ulceration. The rats were divided into five groups: the sham, DFU, AS-IV, GsRb1, and AS-IV + GsRb1 groups. After 12 consecutive days of treatment, the combined drug group presented a significant reduction in the wound area and a marked increase in the wound closure rate (Fig. 2A). The

percentage of wound closure in this group was significantly greater than that in the DFU group and the single-drug treatment groups on days 3, 6, 9, and 12 (Fig. 2B). Hematoxylin–eosin (H&E) staining for histopathological analysis revealed that the combined group exhibited the mildest inflammatory cell infiltration, the thickest granulation tissue with a more uniform structure (Fig. 2C), and a lower epidermal thickness (Fig. 2D). Masson’s trichrome staining results indicated that the density and compactness of collagen fiber deposition in the combined group were greater than those in the DFU group and the single-drug groups (Fig. 2E).

To further explore the cellular and molecular events associated with wound healing, IHC and IF were performed. The results revealed that the number of CD31-positive microvessels in the wound tissue of the combined group was significantly greater than that in the other groups (Fig. 2F–G). Moreover, the expression of the cell proliferation marker Ki67 (Fig. 2H–I), the fibroblast marker α -SMA (Fig. 2J), and the extracellular matrix component Collagen III (Fig. 2K) was significantly increased. These findings suggest that the combined treatment enhances vascular endothelial cell formation, cell proliferation, myofibroblast activity, and reconstruction of the basic collagen structure. In addition, the immunofluorescence results demonstrated that the fluorescence signal intensities of MPO, a marker of neutrophil infiltration, and CitH3, a key marker of NETs, were significantly attenuated in the combined group (Fig. 2L), indicating that the combined treatment can inhibit neutrophil activation and NET formation. The western blot results further confirmed that the expression levels of the CitH3, H3, and PAD4 proteins in the combined group were significantly lower than those in the DFU group and the single-drug groups (Fig. 2M). Collectively, these results indicate that the combined application of AS-IV and GsRb1 can synergistically accelerate DFU wound healing by promoting angiogenesis,

enhancing tissue repair, and inhibiting NET formation.

3.3 AS-IV and GsRb1 Target ZBP1 and AIM2

To investigate whether AS-IV and GsRb1 target the PANoptosis pathway, we first evaluated their binding affinity to 13 core PANoptosis-related proteins via molecular docking analysis. The results revealed that both AS-IV and GsRb1 exhibited favorable binding affinities for ZBP1, with binding energies of -7.8 kcal/mol and -8.3 kcal/mol, respectively (Fig. 3A). Additionally, the two compounds also displayed high binding activity to key proteins such as AIM2, NLRP3, CASP8, and RIPK3. Visualization of the binding conformations further revealed that both compounds could embed into the potential functional pockets of the ZBP1 protein, forming hydrogen bonds and hydrophobic interactions (Fig. 3B). To verify the interaction between the small molecules and target proteins, DARTS and CETSA were performed. In the CETSA experiment, the binding effect was validated under a temperature gradient of 42°C to 70°C. The results revealed that the detectable levels of ZBP1 and AIM2 under high-temperature conditions were significantly altered in the AS-IV or GsRb1 treatment groups, and semiquantitative analysis suggested that drug treatment changed the thermal stability of ZBP1 and AIM2 (Fig. 3C–D). We also used the DARTS method to assess protease sensitivity under a drug concentration gradient of 0–50 μ M. The results demonstrated that AS-IV and GsRb1 reduced the susceptibility of ZBP1 and AIM2 to protease degradation in a dose-dependent manner, indicating that the drugs might achieve binding by altering the conformation of ZBP1 and AIM2 or mediating the formation of their complexes (Fig. 3E–F). In the *in vitro* DFU model, single-drug treatment with either AS-IV or GsRb1 reduced the mRNA and protein

expression of ZBP1 and AIM2 in a dose-dependent manner (Fig. 3 H-N). Collectively, these results support that both AS-IV and GsRb1 have the potential to act on ZBP1 and AIM2, providing molecular evidence for subsequent functional validation.

3.4 AS-IV and GsRb1 Combination Inhibited the Activation of the PANoptosis Signaling Pathway

To clarify the targeting relationship, we further explored the effect of drug treatment on the ZBP1/PANoptosis pathway. IF results revealed that the ZBP1 fluorescence intensity in the drug treatment groups was significantly weaker than that in the HG model group, and the fluorescence intensity in the AG-H group was lower than that in the AG-L group (Fig. 4A). Western blot analysis revealed that combined drug treatment not only downregulated the protein levels of ZBP1 and AIM2 (Fig. 4B) but also significantly inhibited the activation of key PANoptosis molecules, including RIPK1 and MLKL in the necroptosis pathway; NLRP3, ASC, Casp-1, and GSDMD, which are related to pyroptosis; and Casp-3/7/8, which are associated with apoptosis (Fig. 4C). In the rat DFU model, IHC results revealed that the proportion of ZBP1-positive cells in the AS-IV + GsRb1 (AG) group was significantly lower than that in the AS-IV or GsRb1 single-drug treatment groups (Fig. 4D). Western blot results further confirmed that single-drug treatment with either AS-IV or GsRb1 could partially reduce the levels of ZBP1 and AIM2, whereas combined drug treatment had a more significant effect (Fig. 4E), accompanied by an overall decrease in the protein expression of downstream PANoptosis effector molecules (Fig. 4F). These results indicate that AS-IV and GsRb1 not only directly target the ZBP1 protein but also alleviate inflammatory cell death in DFUs by inhibiting the

activation of the ZBP1-mediated PANoptosis signaling pathway.

3.5 ZBP1 knockdown exhibited cytoprotective effects of against HG-induced injury

HaCaT cells were transfected with si-ZBP1 to knock down ZBP1 expression. Western blot validation revealed that both si-ZBP1#1 and si-ZBP1#2 effectively reduced ZBP1 protein expression compared with that in the control si-NC group, indicating high and reproducible knockdown efficiency (Fig. 5A). In the cellular DFU model, ZBP1 protein expression was significantly decreased in both the si-ZBP1 group and the AG-H group, while the si-ZBP1 + AG-H group maintained ZBP1 protein expression levels (Fig. 5B). Under the HG condition, compared with the si-NC group, the si-ZBP1 group presented an increased cell survival rate and decreased LDH release, indicating that ZBP1 knockdown itself attenuated cell membrane damage and cell death (Fig. 5C–D). The staining and flow cytometry results further supported this conclusion: Calcein AM/PI staining revealed a significant reduction in the proportion of PI-positive cells in the si-ZBP1 group (Fig. 5E–F), and the Annexin V/PI assay revealed that the total proportion of early apoptotic and late apoptotic/necrotic cells in the si-ZBP1 group was lower than that in the si-NC group (Fig. 5G–H). We also observed that the cytoprotective effects of AG-H and si-ZBP1 on HaCaT cells were comparable, with no significant difference. Notably, after ZBP1 knockdown, the inhibitory effects of AS-IV + GsRb1 (AG-H) treatment on cell death and apoptosis remained unchanged (Fig. 5C–H). We further evaluated the impact of ZBP1 knockdown on the anti-inflammatory and antioxidant effects of the drugs. The ELISA results revealed that both the AG-H group and the si-ZBP1 group presented significantly decreased IL-1 β and IL-18 levels in HaCaT cells. However, after ZBP1 knockdown, the

inhibitory effect of AG-H treatment on the levels of IL-1 β and IL-18 in the supernatant did not significantly change (Fig. 5I–J). In addition, compared with the si-NC group, the si-ZBP1 group and the AG-H group presented significantly decreased ROS levels (Fig. 5K–L) and MDA content (Fig. 5M), as well as increased SOD activity (Fig. 5N). Similarly, in the context of si-ZBP1, the scavenging effect of combined drug (AG-H) treatment on ROS accumulation remained unaffected. Compared with the si-ZBP1 + AG-H group, the si-ZBP1 + AG-H group presented similar effects on reducing MDA levels and increasing SOD activity, with no significant difference; however, the cytoprotective effects in both groups were significantly greater than those in the si-ZBP1 group. Western blot analysis further demonstrated that both ZBP1 knockdown and AS-IV + GsRb1 treatment significantly downregulated the key effector proteins of PANoptosis. Nevertheless, under si-ZBP1 conditions, the downregulatory effect of AG-H treatment on PANoptosis-related key proteins remained unchanged (Fig. 5O). Collectively, these results indicate that ZBP1 is a key molecular target for the cytoprotective effect of AS-IV + GsRb1, yet its knockdown has no significant effect on the cytoprotective effect of the drugs.

3.6 AS-IV and GsRb1 combination suppressed IRF1 expression

As a pivotal upstream regulator of PANoptosis, the expression profile of IRF1 in the pathological microenvironment of DFU was first investigated. Quantitative real-time polymerase chain reaction (qPCR) assays demonstrated that the mRNA level of IRF1 in HaCaT cells subjected to HG treatment was significantly elevated compared with that in the ctrl group. Consistently, IF staining and Western blot analysis further validated the increased protein

expression of IRF1 under the same experimental conditions (Fig. 6A–C). Further mechanistic analysis revealed that the combined administration of AS-IV and GsRb1 potently inhibited the HG-induced upregulation of IRF1 in the in vitro injury model. Notably, in the AG-H group (20 μ M AS-IV + 30 μ M GsRb1), the protein expression level of IRF1 was markedly lower than that in the HG group (Fig. 6D). In the rat DFU model, the IRF1 protein was substantially upregulated in lesional tissues. However, this aberrant upregulation was mitigated by treatment with AS-IV, GsRb1, or their combination. Among these treatment strategies, combined therapy exhibited the most prominent inhibitory effect (Fig. 6E). Collectively, these findings suggest that IRF1 is aberrantly activated during the progression of DFU-induced injury and may function as an upstream regulatory molecule of ZBP1 to mediate the pathological development of DFU.

3.7 AS-IV + GsRb1 combination reversed cellular damage induced by overexpression of ZBP1 and IRF1

To assess the effect of ZBP1 overexpression on HG-induced HaCaT cell injury, we established ZBP1- or IRF1-overexpressing models. ZBP1 and IRF1 were separately introduced into HaCaT cells via plasmid-mediated gene overexpression. The quantitative real-time polymerase chain reaction (qPCR) results demonstrated that the mRNA level of ZBP1 in the OE-ZBP1 (ZBP1 overexpression) and OE-IRF1 (IRF1 overexpression) groups was significantly greater than that in the empty vector control group (OE-NC) (Fig. 7A). In contrast, the mRNA level of IRF1 was notably greater in the OE-IRF1 group than in the OE-NC group, whereas no significant change in the IRF1 mRNA level was detected in the OE-ZBP1 group (Fig. 7B). Western blot analysis

further confirmed that the protein expression levels of ZBP1 and IRF1 were significantly elevated in the respective overexpression groups (Fig. 7C), indicating that increased IRF1 levels lead to increased ZBP1 levels. In the HG group, the protein expression level of ZBP1 was significantly increased in both the OE-ZBP1 and OE-IRF1 groups (Fig. 7D). Compared with those in the OE-NC group, both OE-ZBP1 and OE-IRF1 induced more severe cell injury, which was characterized by a significant decrease in cell viability (Fig. 7E), increased lactate dehydrogenase (LDH) release (Fig. 8F), and a marked increase in the proportion of dead cells, as detected by calcein AM/PI staining and the Annexin V/PI assay (Fig. 7G–J). Moreover, the levels of interleukin-1 β (IL-1 β) and interleukin-18 (IL-18) in the supernatant (Fig. 7K–L), the level of intracellular reactive oxygen species (ROS) (Fig. 7M–N), and the malondialdehyde (MDA) content (Fig. 7O) in the OE-ZBP1 and OE-IRF1 groups were significantly greater than those in the OE-NC group, whereas the activity of SOD was further inhibited (Fig. 7P). Western blot analysis revealed that the overexpression of either ZBP1 or IRF1 significantly upregulated the expression of key effector proteins in the PANoptosis pathway (Fig. 7Q).

4. Discussion

DFU is one of the most severe and therapeutically complex complications of diabetes. This characteristic of chronic nonhealing is closely associated with persistent inflammatory responses, abnormal immune cell infiltration, and interactions among multiple PCD pathways [38–40]. In this study, we explored the combined application of AS-IV and GsRb1 promotes DFU healing. This combined strategy improved wound healing by mediating inflammation, oxidative stress, and PANoptosis (Fig. 8), providing new evidence for the application of natural

products in DFU.

Keratinocytes are the primary cells responsible for re-epithelialization during wound healing. In DFU, multiple keratinocyte dysfunctions have been observed. Proinflammatory reactions mediated by immune cells play essential roles in those alterations [41]. NETs are fibrous networks composed of chromatin and granular proteins released by neutrophils. They can clear pathogens, but excessive accumulation exacerbates tissue damage [42]. Patients with DFU exhibit a significant systemic inflammatory state and increased NET formation, which may be closely associated with excessive neutrophil activation in a high-glucose environment. Wong et al. demonstrated that under diabetic conditions, neutrophils are more prone to NETosis, and this process inhibits wound healing [43]. NETs can release components such as CitH3, which impair vascular endothelial function and promote the persistence of inflammation. It has been reported that NETs can induce EMT and activate the Hippo signaling pathway, thereby delaying wound repair [44]. In the present study, the serum levels of CitH3, HMGB1, IL-1 β , and IL-18 were significantly elevated in the DFU group, and these indicators were positively correlated with the neutrophil count and inflammatory markers. These findings suggest that NETosis and the associated inflammatory response play key roles in the progression of DFU. Clinical cohort studies have also shown that NET levels are associated with poor healing and amputation risk [45, 46]. A study by Goswami et al. also indicated that NET formation after trauma is closely related to Syndecan-1 shedding, which supports the association between NETs and endothelial injury observed in this study [47]. The release of neutrophil extracellular traps and the upregulation of proinflammatory cytokines (IL-1 β and IL-18) may trigger more cell damage and cell death pathways. Previous studies have proposed that NLRP3 inflammasome-mediated

pyroptosis is an important mechanism in diabetic wounds and is closely associated with increased secretion of IL-1 β and IL-18 [8]. Zhu et al. reviewed the role of NETs in wound healing and noted that while NETs contribute to pathogen clearance in the early stage, excessive NETs inhibit angiogenesis, delay epithelialization, and exacerbate tissue fibrosis [48]. In mice with type 1 diabetes, inhibiting NET formation can improve vascular function, reduce endothelial injury, and thereby alleviate vascular dysfunction [49]. Therefore, inhibiting NETs not only directly restricts inflammation but also indirectly protects endothelial cells, which is conducive to wound regeneration.

PANoptosis, a novel form of inflammatory cell death proposed in recent years, integrates the molecular features of pyroptosis, apoptosis, and necroptosis. It plays a crucial role in infections, autoimmune diseases, and metabolic disorders [17, 50]. In the contexts of infection, tissue injury, and chronic inflammation, ZBP1 is recognized as a key regulatory protein of PANoptosis, which can trigger PANoptosome assembly by recognizing nucleic acid conformations [50-54]. For example, ZBP1 induced during coronavirus infection limits the efficacy of interferon (IFN) therapy by driving inflammatory cell death and lethality; thus, inhibiting ZBP1 activity may enhance the therapeutic effect of IFN [51]. In an acute injury model of pulmonary endothelial cells/vascular endothelial cells, activation of the C1RP-ZBP1 pathway triggers PANoptosis, leading to severe impairment of vascular barrier function [52]. In the present study, ZBP1 and AIM2 upregulation was observed in clinical samples, in vitro models, and rat wound tissues. Concurrently, simultaneous increases in proteins related to necroptosis (RIPK1/MLKL), pyroptosis (NLRP3/Casp-1/GSDMD), and apoptosis (Casp-3/7/8) were detected. These findings suggest that ZBP1, as a hub molecule of PANoptosis, actually participates in the

process of cell injury in DFU tissues. Notably, there may be multiple interactions between NETs and PANoptosis. Previous studies have confirmed that neutrophils under diabetic conditions are more prone to NETosis and promote vascular barrier injury through the release of Syndecan-1 [47, 55]. NETs can release damage-associated molecular patterns (DAMPs, e.g., HMGB1) and proinflammatory cytokines to increase the expression or activation of immune sensors (including AIM2 and ZBP1), thereby facilitating PANoptosome assembly [3, 11]. Conversely, cell rupture caused by PANoptosis results in the release of more DAMPs, which can also activate neutrophils and promote NETosis, forming a vicious cycle. Here, we found HG induced increased ROS levels, decreased SOD activity, and increased cell death in keratinocytes. These findings indicate that neutrophils in the DFU microenvironment may further exacerbate endothelial injury not only through NETs but also potentially via PANoptosis.

AS-IV has been shown to exert endothelial protective and anti-inflammatory effects in various models of cardiovascular diseases and diabetic complications. For example, AS-IV can reduce the proinflammatory response in endothelial cells by inhibiting TAK1 activation [56]. It can also suppress pyroptosis in human umbilical vein endothelial cells (HUVECs) via the BCL2/BAX/ROS pathway [57]. Additionally, AS-IV can regulate exosome secretion to inhibit pyroptosis and oxidative stress in endothelial cells under diabetic conditions [58]. As a classic natural product with antidiabetic properties GsRb1 has been reported to improve insulin sensitivity, lower blood glucose, and alleviate inflammation [59, 60]. Although direct evidence for the regulation of PANoptosis by AS-IV and GsRb1 is currently lacking, their antioxidant and anti-inflammatory properties provide a theoretical basis for the protective effects observed in this study. In this study, in vitro ratio screening revealed that AS-IV and GsRb1 had a

significant synergistic effect when they were combined at a 2:3 molar ratio. In the in vitro model, their combined action maximized the cell survival rate, reduced ROS and LDH levels, and decreased the secretion of IL-1 β and interleukin-18 (IL-18). Animal experiments further verified this synergistic effect, which manifested as accelerated wound closure, increased angiogenesis, and reduced NETs—representing more significant advantages than traditional single-drug intervention. Notably, experiments such as molecular docking, a CETSA, and DARTS demonstrated that AS-IV and GsRb1 can bind to ZBP1 and AIM2. Moreover, drug treatment reduced the expression of ZBP1/AIM2 and inhibited the activation of downstream PANoptosis effector molecules. This in vitro and in vivo evidence suggests that the two compounds act not only indirectly by generally alleviating inflammation/oxidative stress but also potentially directly by interfering with the assembly or stability of the PANoptosome, thereby suppressing the synergistic activation of multiple cell death pathways. Similar mechanisms have been reported for other natural products. For example, baicalin—a flavonoid component—can block the assembly of the ZBP1-PANoptosome, thereby inhibiting PANoptosis in macrophages [61]. Salvianolic acid B inhibits ZBP1-mediated PANoptosis by acting on tumor necrosis factor receptor 1 (TNFR1), reducing tissue damage in *Mycobacterium tuberculosis* infection [62]. Danggui Buxue Decoction, a traditional Chinese medicine formula, has also been confirmed to inhibit ZBP1-PANoptosis in a cardiotoxicity model [63]. These studies corroborate the findings of the present study, indicating that ZBP1 is a targetable key molecule and that natural products possess potential PANoptosis-regulating effects.

The inhibitory effect of the combined drugs on NETs was also prominent in this study. In the wound tissues of the AS-IV + GsRb1 combination group, the expression of CitH3 and PAD4

was decreased, and the immunofluorescence signals of MPO and CitH3 were attenuated; these observations confirm that the combined drugs exert a functional effect on inhibiting NETosis. A reduction in NET levels can weaken the persistent activation of the NLRP3 inflammasome and other immune sensors, thereby decreasing the trigger signals for PANoptosis. This synergistic reduction in both NETs and PANoptosis is conducive to the survival of endothelial and epithelial cells and promotes angiogenesis [48, 49]. In addition, the combined drug treatment significantly accelerated wound closure in the rat DFU model, which was accompanied by increased granulation tissue formation, increased collagen deposition, and accelerated angiogenesis. These effects were closely associated with the inhibition of local NET formation (evidenced by decreased MPO and CitH3 expression) and the suppression of key PANoptosis molecule activation. Notably, the combined therapy exhibited superior efficacy in promoting re-epithelialization and matrix remodeling compared with the single-drug treatment groups. The aforementioned molecular and histological changes were consistent with the accelerated wound healing process, suggesting that the combined drugs interrupt the positive feedback loop between inflammation and cell death through dual targeting (inhibiting both NETs and PANoptosis). Therefore, the combined inhibition of NETs and PANoptosis may offer greater advantages than single-target intervention.

The transcription factor IRF1 plays a crucial role in inflammatory diseases. Studies have shown that IRF1 can promote the expression of inflammatory sensors such as ZBP1 and AIM2 and enhance the activation of the PANoptosis pathway [64]. The results of this study demonstrated that IRF1 was upregulated in both DFU clinical samples and in vitro models. Moreover, overexpression of IRF1 or ZBP1 (OE-IRF1/OE-ZBP1) exacerbated the injury

phenotype, whereas AG-H treatment partially reversed this effect, suggesting that these drugs exert a functional effect on the inhibition of the IRF1-ZBP1 axis. These findings not only confirm the pathogenic driving role of the IRF1/ZBP1 axis in DFU but also indicate that AS-IV + GsRb1 exerts a therapeutic effect by suppressing this pathway.

Clinically, the treatment of DFU remains a significant challenge. Current therapeutic strategies rely primarily on wound care, anti-infective therapy, and blood glucose control; however, a considerable proportion of patients still experience nonhealing wounds or progress to amputation [40]. Natural medicines, characterized by their multicomponent and multitarget actions, possess unique advantages in the treatment of complex diseases. Although this study clearly elucidates the mechanism by which the AS-IV + GsRb1 combination regulates PANoptosis via ZBP1, several aspects require further exploration. First, whether this combination affects other cell types (e.g., macrophages and fibroblasts) and overall remodeling of the immune microenvironment remains to be investigated. Second, the in vitro model used HaCaT keratinocytes as a representative cell type—while it can simulate part of the epithelial–endothelial crosstalk mechanism, it does not fully recapitulate the complex wound microenvironment in vivo. Third, impaired wound healing in diabetes involves crosstalk among multiple forms of cell death; future studies should focus on the roles of additional programmed cell death (PCD) modalities (e.g., ferroptosis and autophagy) in DFUs and the regulatory effects of the AS-IV + GsRb1 combination on these processes. Notably, although this study confirmed drug–target interactions via a CETSA and DARTS, further experiments, such as surface plasmon resonance (SPR) or isothermal titration calorimetry (ITC), are needed to verify the binding affinity and specificity between the drugs and their targets. Additionally, the long-term

safety of the AS-IV + GsRb1 combination and its optimal administration regimen have not been evaluated in long-term animal models or human studies, which also warrants further investigation.

Conclusion

In summary, this study systematically illustrates the molecular mechanism by which the combined application of AS-IV and GsRb1 improves DFU wound healing, specifically by inhibiting the ZBP1-mediated PANoptosis, thereby attenuating neutrophil activation and NET formation (Figure8). This research expands the understanding of the core role of ZBP1 as a key node linking inflammation and programmed cell death (PCD) in DFUs and provides solid preclinical evidence for the development of natural drug combinations for the treatment of chronic nonhealing ulcers. Future studies could further explore other cell death crosstalk networks and clinical translation trials to promote the practical application of this combined strategy. Specifically, it will be necessary to validate these mechanisms and therapeutic effects in clinical samples and optimize the administration mode and safety profile, with the goal of applying this strategy to improve wound healing and reduce complications in DFU patients.

Author contributions

ZF: Methodology, Investigation, Conceptualization, Project administration, Writing – original draft; XZ: Methodology, Visualization, Project administration, Investigation, Writing – original draft; ZJ: Methodology, Software, Data analysis, Writing – original draft;

JS: Methodology, Software, Writing – original draft; XL: Validation, Methodology, Data curation, Writing – original draft; HL: Formal analysis, Conceptualization, Writing – original draft; ZX: Writing – review & editing, Supervision, Funding acquisition. All authors reviewed the manuscript.

Ethics statement

The research on animals was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University (No.KY20230112) and conducted according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Extensive efforts were made to minimize the suffering of the animals involved. All the authors agreed to be published.

Declaration of Competing Interest

The authors declare that they have no known competing interests.

Data availability

Data will be made available on request from the corresponding author.

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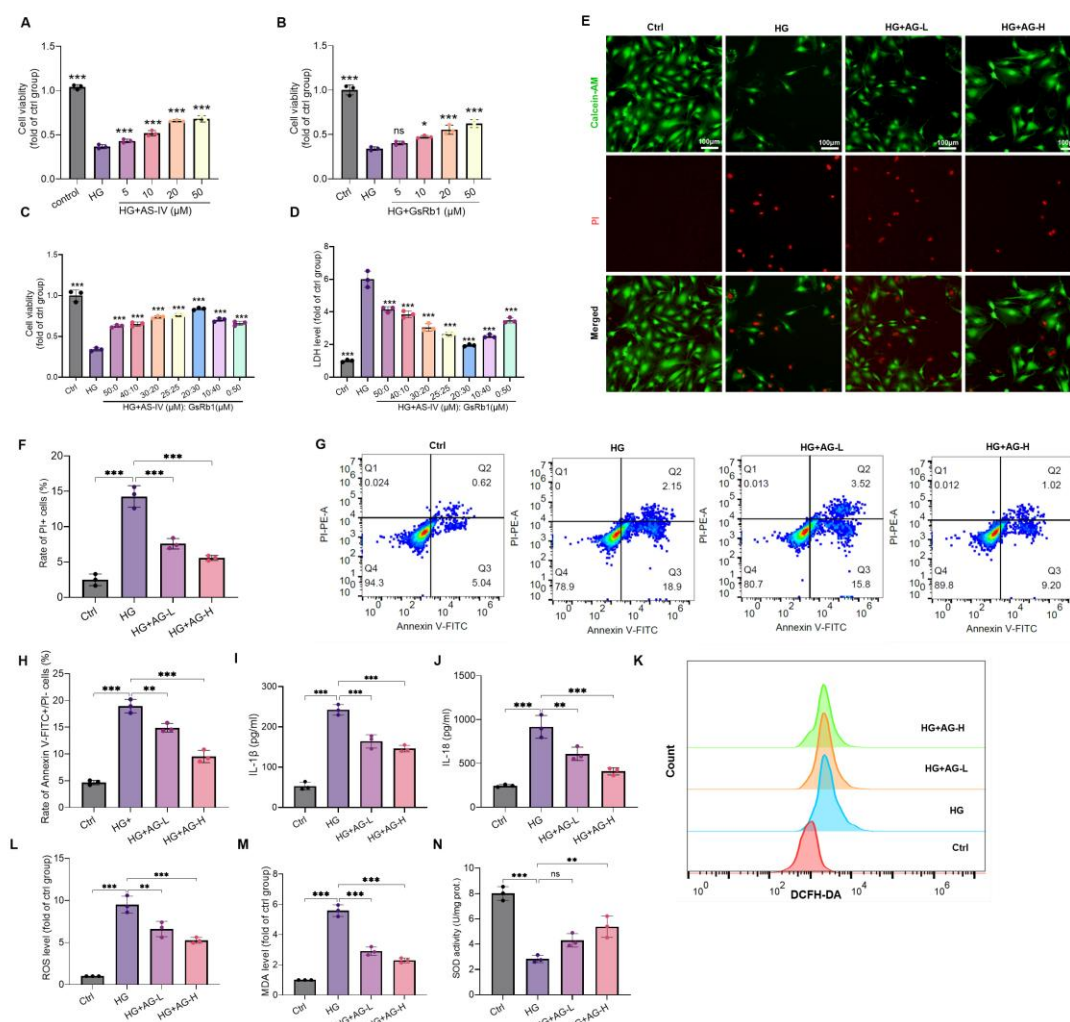
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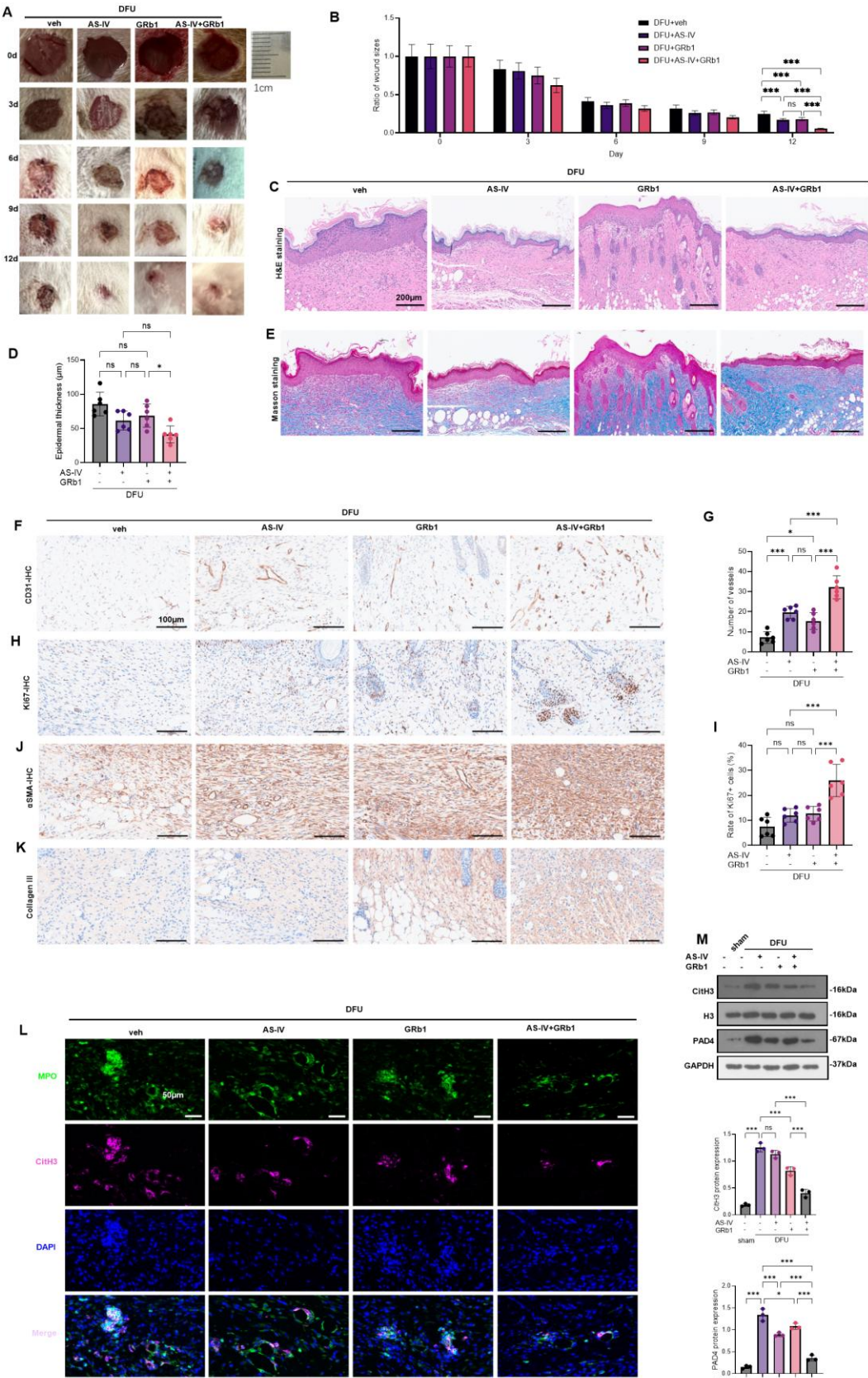
Figure legends

Figure 1. The effects of AS-IV and GsRb1 combination on HG-induced HaCaT cells.



levels in HaCaT cells. (M, N) Biochemical assays measuring the MDA content and SOD activity in HaCaT cells from different treatment groups. The data are presented as the means $\pm$ standard deviations; n = 3 independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant vs. the HG group. AG-L: 10 μ M AS-IV + 15 μ M GsRb1; AG-H: 20 μ M AS-IV + 30 μ M GsRb1.

Figure 2. The effects of AS-IV and GsRb1 combination on wound healing in a rat DFU model.

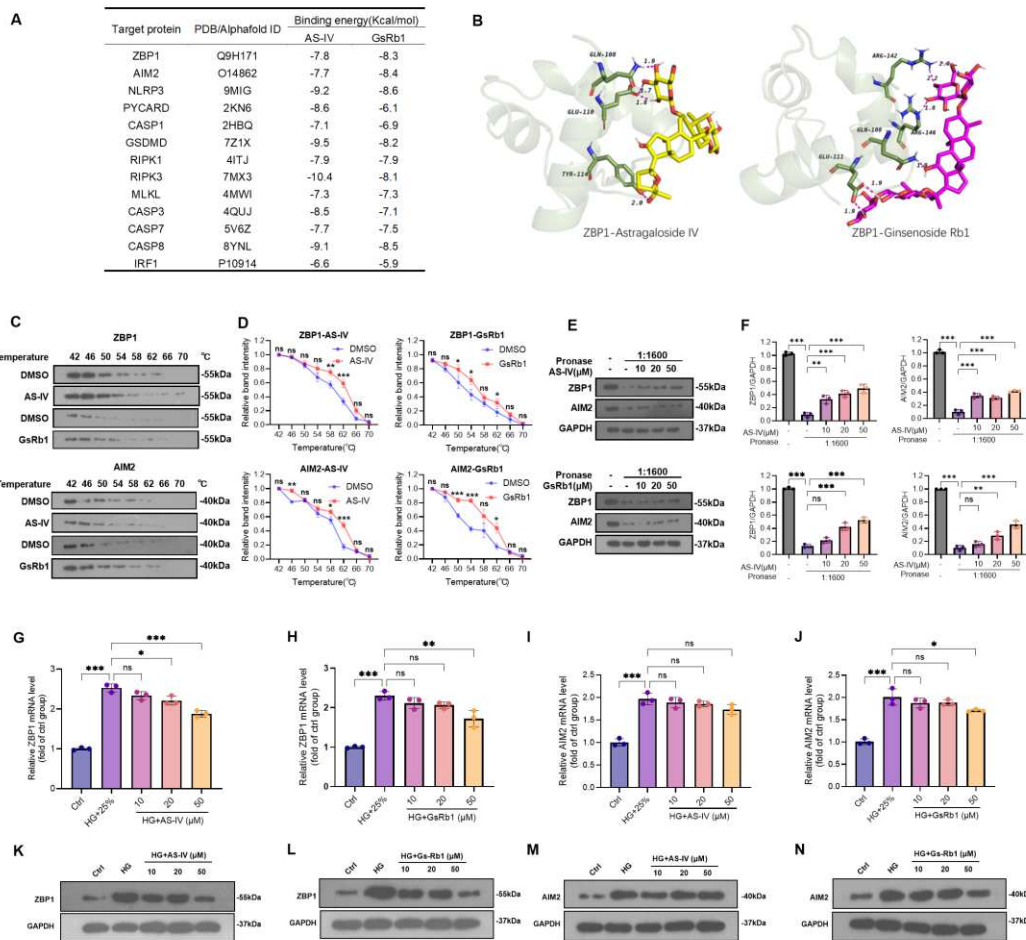


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1006 A rat DFU model was constructed and treated with AS-IV (12 mg/kg) and or GsRb1 (18 mg/kg).

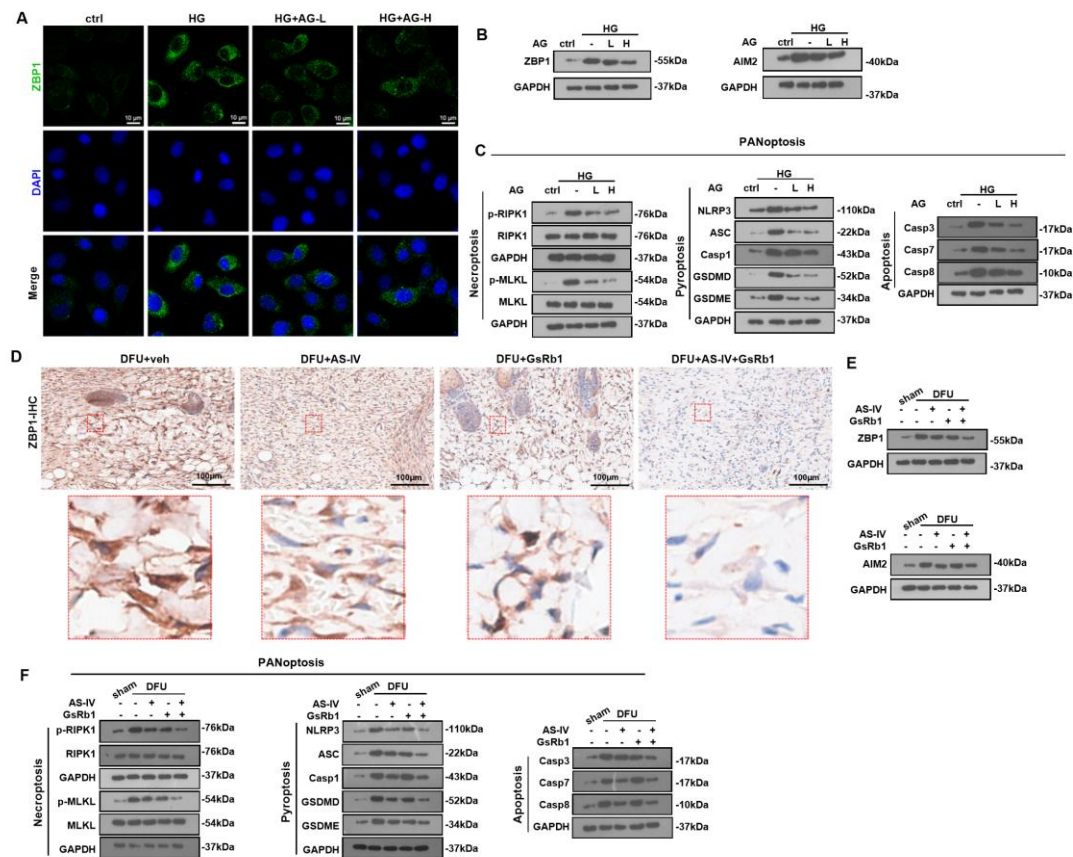
(A, B) Representative images of wound areas at days 0, 3, 6, 9, and 12 after treatment and quantification of the percentage of wound closure at different time points. Scale bar: 1 cm. (C, D) H&E staining of wound tissues on day 12, showing the degree of inflammatory cell infiltration and granulation tissue formation (C) and quantification of epidermal thickness (D). Scale bar: 200 μ m. (E) Masson's trichrome staining of wound tissues on day 12, demonstrating collagen fiber deposition. Scale bar: 200 μ m. (F) Immunohistochemical staining for CD31 was used to detect microvessel density. Scale bar: 100 μ m. (G) Quantification of the number of CD31-positive vessels. (H) Immunohistochemical staining for Ki67 was performed to assess cell proliferation. Scale bar: 100 μ m. (I) Quantification of the percentage of Ki67-positive cells. (J) Immunohistochemical staining for α SMA to identify fibroblasts. Scale bar: 100 μ m. (K) Immunohistochemical staining for collagen III was used to evaluate extracellular matrix reconstruction. Scale bar: 100 μ m. (L) Immunofluorescence staining for MPO and CitH3 to assess neutrophil infiltration and NET formation; nuclei were counterstained with DAPI (blue). Scale bar: 50 μ m. (M) Western blot analysis of CitH3, H3, and PAD4 protein expression levels in wound tissues. The data are presented as the means $\pm$ SDs; n = 6 independent experiments. Statistical significance was determined by one-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant.

Figure 3. The binding relationships between AS-IV/ GsRb1 and ZBP1/AIM2



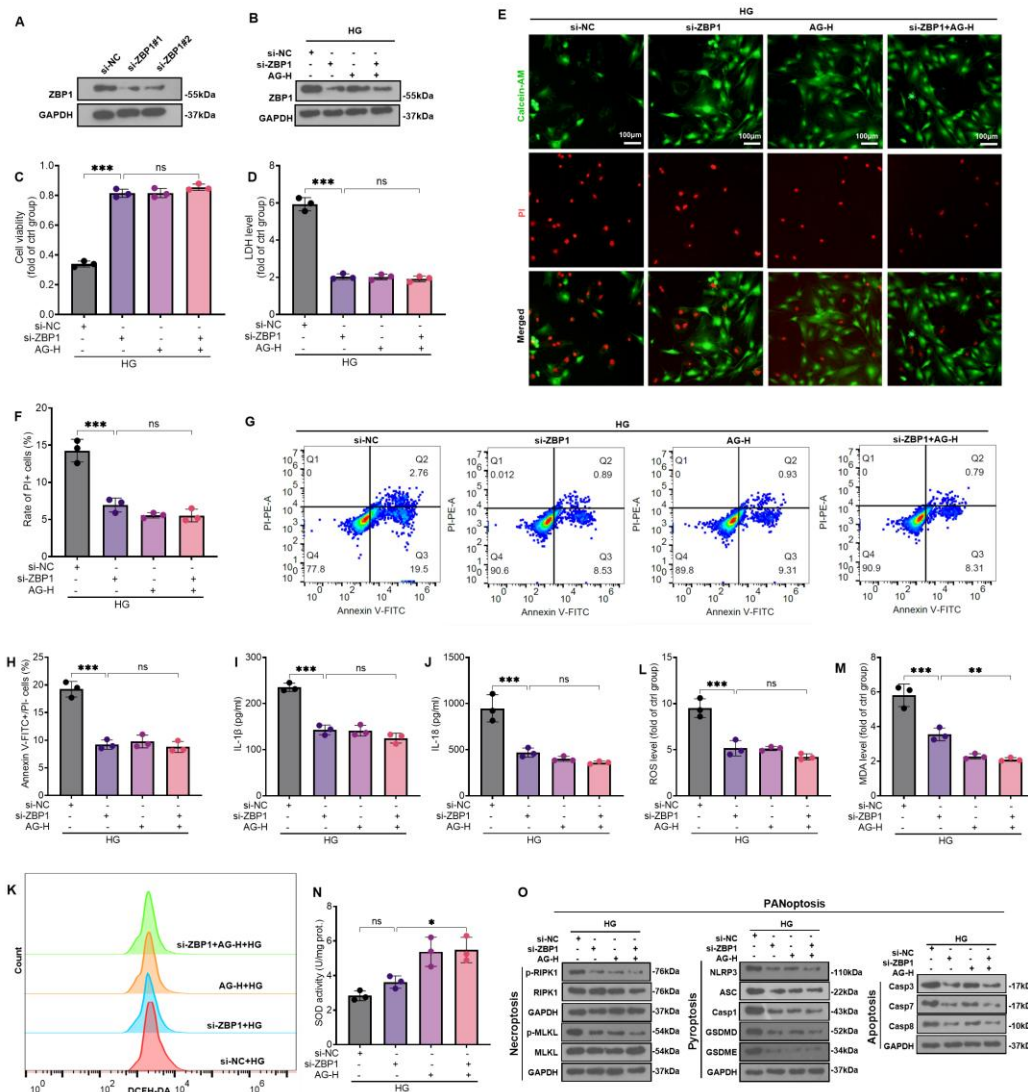
(A) Molecular docking analysis showing the binding energy (kcal/mol) of AS-IV and GsRb1 with 13 core PANoptosis-related proteins. (B) Visualization of the binding conformations of AS-IV and GsRb1 with ZBP1, showing hydrogen bonds and hydrophobic interactions. (C-D) CETSA analysis of ZBP1 and AIM2 protein stability under a temperature gradient (42–70 °C) with or without drug treatment. (E, F) DARTS assay evaluating the proteolytic sensitivity of ZBP1 and AIM2 after treatment with AS-IV or GsRb1 (0–50 μM). (G, J) qRT–PCR results showing the relative mRNA levels of ZBP1 and AIM2. (K–N) Western blot analysis of ZBP1 and AIM2 protein expression. The data are presented as the means ± SDs; n = 3; *p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant vs. the HG group.

Figure 4. The effects of AS-IV and GsRb1 combination on PANoptosis



(A) Immunofluorescence images showing ZBP1 expression levels in HaCaT cells treated with HG and AS-IV plus GsRb1. Scale bar: 10 μ m. (B) Western blot analysis of ZBP1 and AIM2 protein levels in HaCaT cells. (C) Western blot analysis of key proteins of PANoptosis in HaCaT cells. (D) Immunohistochemistry (IHC) images showing ZBP1 expression in the DFU rat model. Scale bar: 100 μ m. (E) Western blot analysis of ZBP1 and AIM2 protein levels in the DFU rat model. (F) Western blot analysis of PANoptosis-related proteins in the DFU rat model.

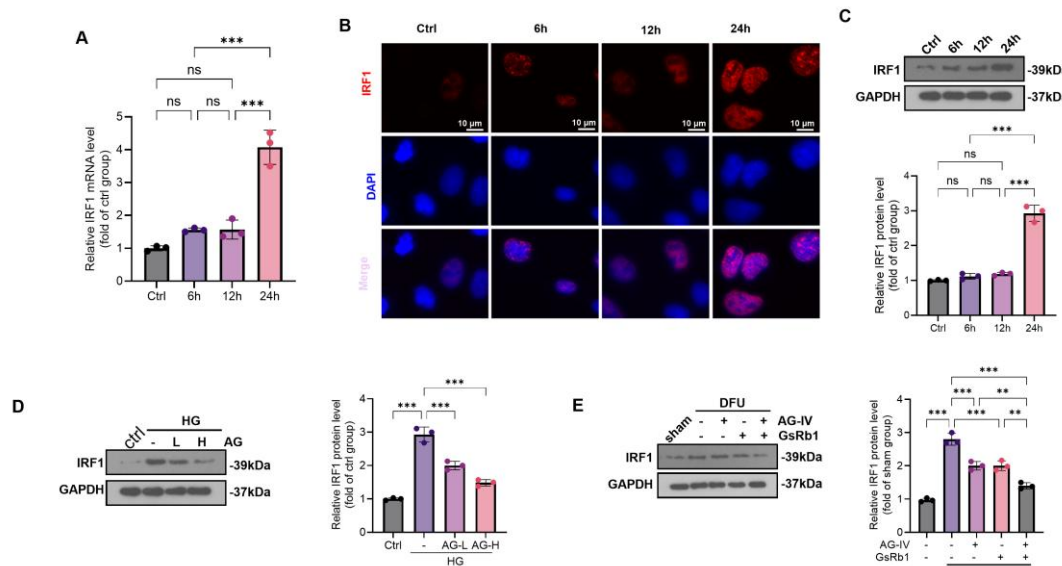
Figure5. Effects of ZBP1 knockdown on HG-induced injury in HaCaT cells.



(A) Western blot analysis of ZBP1 protein expression in HaCaT cells transfected with si-NC, si-ZBP1#1, or si-ZBP1#2. (B) ZBP1 protein levels were determined by western blot. (C) Cell viability was assessed via a CCK-8 assay. (D) LDH release was measured. (E, F) Representative calcein-AM/PI staining images showing live cells (green) and dead cells (red) in different treatment groups. Scale bar: 100 μ m. (G, H) Annexin V-FITC/PI staining was performed, followed by flow cytometry, to quantify early and late apoptotic/necrotic cell populations (G) and perform semiquantitative analysis (H). (I, J) The levels of IL-1 β and IL-18 in the culture supernatant were measured via ELISA. (K, L) ROS levels were detected by flow cytometry via

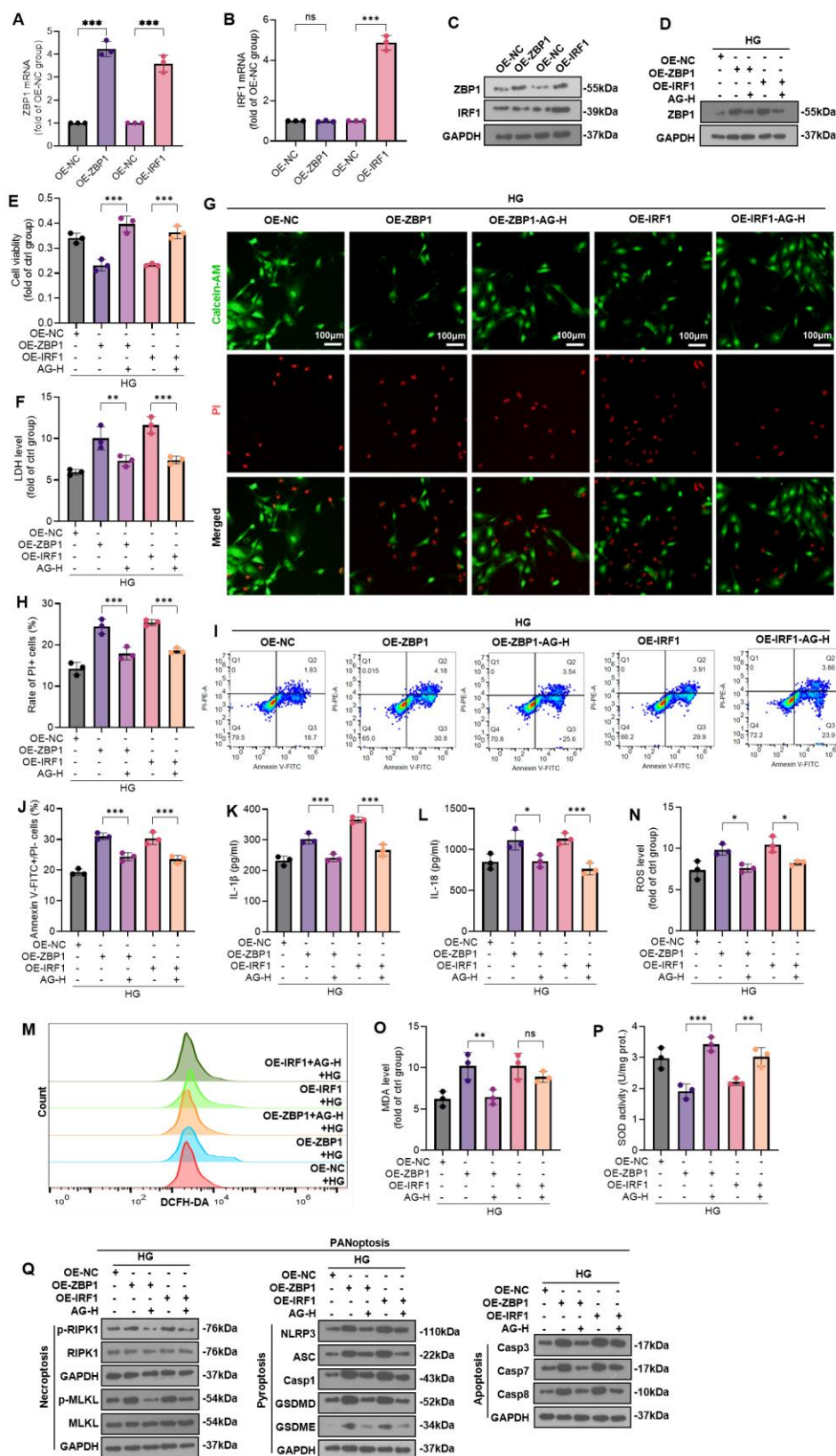
DCFH-DA staining (K) and semiquantitative analysis (L). (M, N) MDA content and SOD activity were measured to assess oxidative stress and antioxidant capacity, respectively. (O) Western blot analysis of PANoptosis-related proteins (necroptosis, pyroptosis, and apoptosis) in response to different treatments. The data are presented as the means $\pm$ SDs; $n = 3$ independent experiments; * $p < 0.05$, ** $p < 0.01$, ns, not significant vs. the HG+ si-ZBP1 group.

Figure6. IRF1 alteration in HaCaT cells under HG conditions and treated with AS-IV and GsRb1.



(A) qRT-PCR analysis of IRF1 mRNA expression in HaCaT cells under HG conditions for different time points. (B) Representative IF images of IRF1 (red) in HaCaT cells under the indicated conditions; nuclei were stained with DAPI (blue). Scale bar: 10 μ m. (C-E) Western blot analysis of IRF1 protein expression in HaCaT cells subjected to different treatments. The data are presented as the means $\pm$ SDs; $n = 3$ independent experiments; ** $p < 0.01$, *** $p < 0.001$, ns: not significant.

1073 **Figure 7** Effects of AS-IV and GsRb1 in HaCaT cells with overexpression of ZBP1 or IRF1.

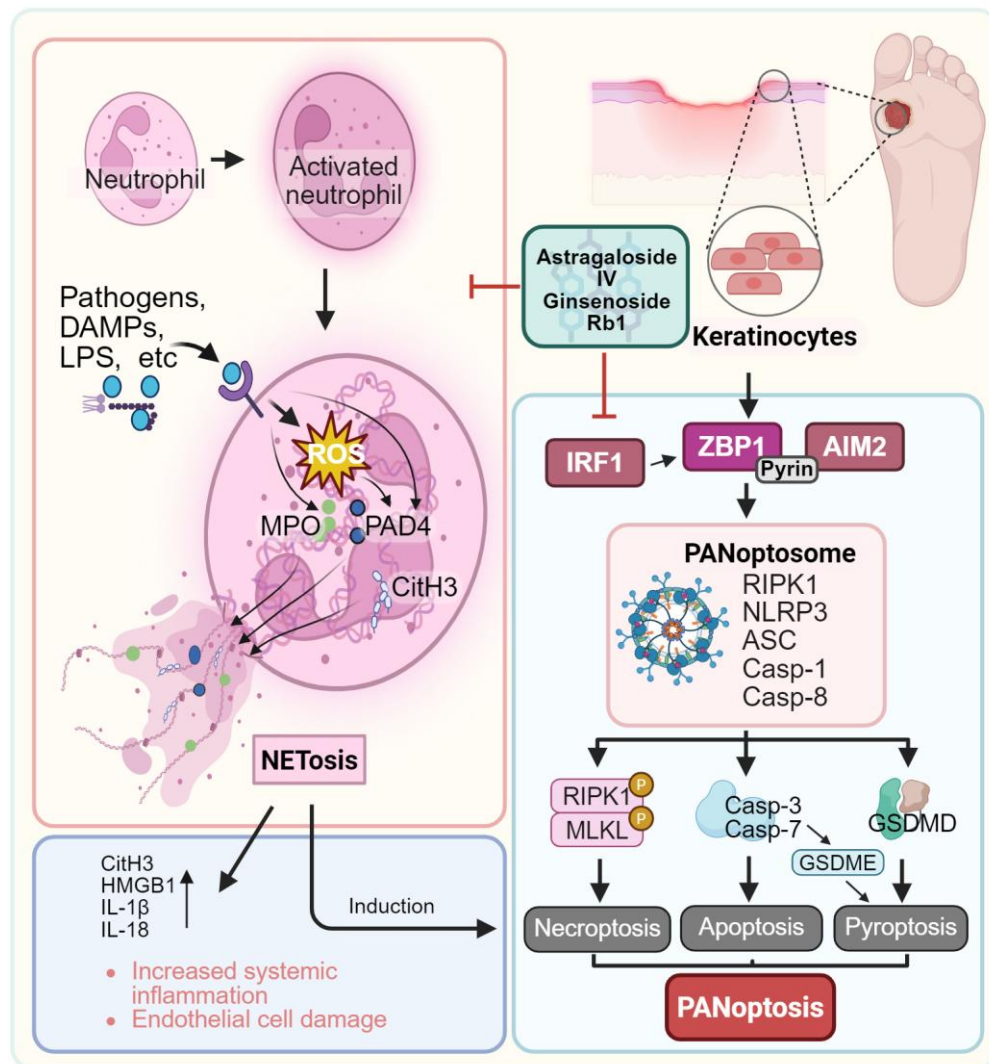


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1075 (A-B) qPCR analysis of ZBP1 and IRF1 mRNA expression in HaCaT cells overexpressing

ZBP1 (OE-ZBP1) or IRF1 (OE-IRF1). (C) Western blot validation of ZBP1 and IRF1 protein overexpression in transfected HaCaT cells. (D) Western blot analysis of ZBP1 protein expression. (E) Cell viability was assessed by a CCK-8 assay. (F) LDH release was measured to evaluate cell membrane damage. (G, H) Calcein-AM/PI staining showing live (green) and dead (red) cells in different treatment groups. (I, J) Annexin V-FITC/PI staining followed by flow cytometry was used to quantify apoptotic/necrotic cell populations (I), and semiquantitative analysis was performed (J). Scale bar: 100 μ m. (K, L) ELISA analysis of IL-1 β and IL-18 levels in cell culture supernatants from different treatment groups. (M, N) Intracellular ROS levels detected by DCFH-DA fluorescence (M) and semiquantitative analysis (N). (O, P) MDA content and SOD activity under different treatments. (Q) Western blot analysis of PANoptosis-related proteins (necroptosis, pyroptosis, and apoptosis) in different treatment groups. The data are presented as the means $\pm$ SDs; n = 3 independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant.

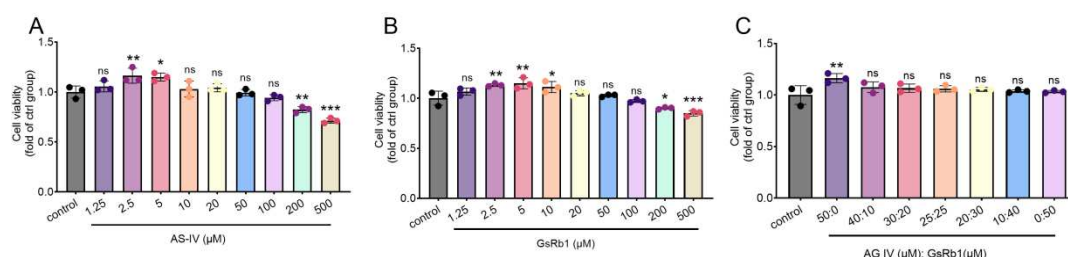
Figure 8. The mechanism by which the combined effects of AS-IV and GsRb1 in DFU healing by inhibiting ZBP1-dependent PANoptosis.



In the diabetic environment, neutrophils are activated and may undergo NETosis or release proinflammatory molecules. Pathological stimuli induce the upregulation of ZBP1 in endothelial cells. ZBP1, through its downstream signaling pathways (including RIPK3/MLKL, caspase-8, and caspase-1), triggers PANoptosis (a hybrid cell death pathway that integrates active cell death pathways, including apoptosis, necrosis/necroptosis-like death, and inflammatory apoptosis/pyroptosis). PANoptosis leads to endothelial cell damage/death, impaired angiogenesis, increased vascular permeability, and loss of barrier function, thereby slowing granulation tissue formation in wounds, prolonging the inflammatory phase, delaying wound healing, and even causing tissue necrosis. In terms of intervention, the combination of

astragaloside-IV and ginsenoside Rb1 can inhibit neutrophil activation/NETosis/proinflammatory cytokine release, suppress oxidative stress, downregulate ZBP1 expression or inhibit its activation, and block or reduce downstream execution pathways of PANoptosis, thereby enhancing endothelial cell survival and functional recovery, including angiogenesis and barrier repair. These effects ultimately lead to a reduced wound area, increased granulation tissue, tissue remodeling, and reduced inflammation in diabetic foot ulcers, resulting in improved clinical healing.

Supplementary Figure 1. Assessment of the toxicity of AS-IV and GsRb1 to normal HaCaT cells.



(A) CCK-8 assay showing the effects of different concentrations of AS-IV (0–50 μ M) on the viability of normal HaCaT cells. (B) CCK-8 assay showing the effects of different concentrations of GsRb1 (0–50 μ M) on the viability of normal HaCaT cells. (C) CCK-8 assay showing the effect of combined treatment with AS-IV and GsRb1 at different molar ratios (AS-IV:GsRb1 from 5:0 to 0:5) on the viability of normal HaCaT cells. The data are presented as the means $\pm$ standard deviations; n = 3 independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant vs. the ctrl group.