The effects of Tubacin, a HDAC6 inhibitor on skin wound healing and its mechanism

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Research Article

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Abstract

Background Wound healing is a common clinical pathological process that is interrupted when abnormal pathological factors are present, which can significantly delay wound healing and lead to complication. Epigenetic modification plays an important role in wound repair, including histone deacetylase HDAC6-mediated regulation of cell morphology, autophagy, migration, inflammation, and oxidative stress. Therefore, this study aimed to investigate how HDAC6 inhibitors affect the proliferation and migration of dermal fibroblasts in allogeneic skin wound repair.

Methods and results We effectively isolated primary skin fibroblasts from newborn rat skin tissue, and the effects of TGFB1 and different concentrations of HDAC6 inhibitor Tubacin on skin fibroblast growth and migration were detected using the MTT assay and scratch test. Tubacin was discovered to decrease fibroblast growth and migration. Tubacin down-regulated the expression levels of COL3, p-AKT, HDAC6, Col1a1, -SMA, and p-ERK, which were up-regulated by TGF-1, in fibroblasts treated with TGF-1 and different doses of Tubacin. Tubacin also increased the protein levels of ace–tubulin and CD31 (platelet endothelial cell adhesion molecule). To examine the impact of the HDAC6 inhibitor Tubacin in skin wound regeneration, we created a full-thickness wound model on the back of rats and used Western blot to assess the expression levels of HDAC6, acetylated -tubulin, COL1A1, COL3, and -SMA. The results demonstrated that trauma increased the expression levels of HDAC6 and acetylated -tubulin in the skin; these findings suggest that HDAC6 and acetylated -tubulin may be involved in wound repair. Tubacin, on the other hand, decreased the protein levels of HDAC6 and acetylated-tubulin, as well as the protein levels of COL1A1 and COL3. The mechanism could be that COL1A1, COL3, and -SMA expression, which are involved in pathological wound repair, are blocked via modulating the TGF-β-PI3K-Akt pathway and MAPK/ERK signaling. Thus, our results implies that inhibiting HDAC6 plays a beneficial function in wound healing and scar formation.

Conclusion Tubacin inhibits fibroblast proliferation and migration, as well as the expression of COL1A1, COL3, and -SMA, all of which are involved in pathological wound repair. It also promotes the expression of CD31, which is associated with inflammation or angiogenesis, by regulating the tgf-PI3K-Akt pathway and the MAPK/ERK signaling pathway.

1. Introduction

The skin's wound healing process is extremely complex, with three distinct and overlapping stages: inflammatory reaction, new tissue development, and tissue remodeling[1]. Multiple cellular events are involved in continuous process[2], including infiltration and apoptosis of inflammatory cells, epithelial-mesenchymal transition (EMT) of skin cells. In pathological conditions, the abnormal proliferation of fibroblasts and the massive synthesis of collagen fibers lead to excessive deposition of collagen, which eventually leads to various complications such as skin scar formation, skin fibrosis, delayed wound healing or incomplete healing[3, 4]. Skin scar and skin fibrosis are treated by a combination of surgery,
local injections of glucocorticoids and radiation therapy, but the effects of these treatments are unsatisfactory [5].

Recent EMT pathway research has revealed novel therapeutic targets, and a number of histone deacetylase inhibitors (HDACis) are being developed to target these pathways and improve current medical treatments. Pan-HDACi Trichostatin A (TSA) can significantly inhibit the synthesis of collagen, thereby promoting wound repair and inhibiting scar formation [6, 7]. At the same time, TSA can inhibit the development of skin inflammation [8]. Histone deacetylase 6 (HDAC6) regulates cell morphology, autophagy, migration and inflammation, and is also involved in oxidative stress protection. Tubastatin A is a specific HDAC6 inhibitor that prevents Bleomycin-induced lung fibrosis [9]. Tubastatin A inhibits peritoneal fibrosis by inhibiting the progression of EMT in peritoneal mesothelial cells [10]. Pan-HDACi has toxic side effects such as nausea, vomiting, and myelosuppression, while selective HDAC6 inhibitors have almost no cytotoxicity to normal cells [11]. The effects of HDAC6 inhibitor on the proliferation and migration of dermal fibroblasts and wound repair in full-thickness skin were studied.

2. Materials and methods

2.1. Isolation and culture of primary skin fibroblasts

The Southern Medical University Laboratory Animal Center authorized all animal research that followed National Institutes of Health rules and ethical principles. Animals used in experiments are given the care and respect they deserve. Newborn rats were euthanized, their skin obtained by sterile methods, and it was washed with PBS. Skin tissue was sliced into 1- to 3-millimeter pieces and digested for 45 minutes with 0.25% trypsin and 0.125% collagenase. After centrifugation at 2000 R/min for 15 minutes, the cells were resuspended in DMEM/F12 media (Gibco, USA) supplemented with 10% fetal bovine serum, 100 u/ml penicillin, and 100 mg/ml streptomycin (Solarbio, Beijing, China). The cells were then placed in a 25 cm2 dish and cultured in a 37°C CO2 incubator. The medium was changed every 48 hours and the cells were digested with 0.25% trypsin at 85% fusion and passaged, followed by 4–8 passages of fibroblasts.

2.2. Proliferation assay

The experiment involved enduing cells into 96-well plates at a viscosity of 4000 cells per well. After the cells were attached, the medium was removed, and the cells were treated with 10 ng/mL TGF-β1 (Peprotech, USA) for 6 hours. Different concentrations of tubacin (Sigma, USA) were added to each group, while the negative control group received the same volume of serum-free medium. After incubating for 24 hours, 5 g/L MTT solution (provided by Huamei Bio-Engineering, Beijing, China) was introduced to each well, followed by an additional 4-hour incubation period of the cells. The supernatant was then removed, and 200 µl DMSO (Solarbio, Beijing, China) was added to each well. The plate was shaken for 10 minutes to dissolve the crystals, and the OD value of each well was measured on a microplate reader at a wavelength of 490 nm. Cell viability was determined utilizing the formula: cell viability (%) = [(As-Ab)/(Ac-Ab)] × 100%, where As indicates the OD value of experimental wells, Ac indicates the OD value of control wells, and Ab indicates the OD value of the blank group. The data were presented as mean ± SD (n = 5)
and depicted using a graph, with the x-axis representing the concentration of tubacin and the y-axis indicating the rate of cell survival.

2.3. Wound healing assay

Skin fibroblasts were cultured at a density of $2 \times 10^5$ cells/mL in 24-well plates supplemented with DMEM/F12 medium containing 10% FBS and 10 ng/mL TGF-β1. Various concentrations of tubacin were incorporated into the medium to stimulate the cells. When the cells reached 100% confluence, a linear wound was formed by scratching the monolayer cells with a 10 L pipette, followed by 2–3 washes with PBS to remove the scraped cells, and it was incubated with fresh medium in the cell incubator for 24 hours. Finally, the simulated wound healing was observed under a microscope.

2.4. Western blot detection of protein expression levels of fibroblasts

Skin fibroblasts were treated with 10 ng/mL TGF-β1 or different concentrations of tubacin for 24 hours. Subsequently, the cells were lysed with RIPA buffer at 4°C and sonicated with an ultrasonic disruptor. Finally, the lysate was centrifuged at 12,000 rpm for 15 minutes at 4°C, and the supernatant was collected. The harvested samples were stored in a -80°C freezer for later use or immediately subjected to Western blot.

The antibodies used in this study, including HDAC6, acetylated α-tubulin, t-ERK, p-ERK, t-Akt, and p-Akt, were obtained from Cell Signaling Technology (USA). The antibodies Collagen I, Collagen III, a-SMA, and CD31 were obtained from Boster Biological Technology (China). Western blot results were analyzed by Image-Pro Plus 6.

2.5. Immunofluorescence detection of protein distributions

The coverslips inoculated with adherent cells were transferred from the incubator to the ultra-clean workbench in the laboratory. Discard the old medium, add 4% precooled paraformaldehyde into each well, and hold at 4°C for 10 minutes. The immobilized samples were then washed three times in PBS for five minutes each time. The samples were incubated with 0.3% Triton X-100 for 20 minutes to facilitate cell permeabilization. The cells were washed three times with PBS solution, each time for 5 minutes. Add 5% BSA protein to rule out non-specific staining. The samples were incubated for about 30 minutes. The primary antibody was dissolved in PBS containing 0.1% BSA and then added dropwise onto the slide. The slide was then placed horizontally and incubated for 2 hours at 37°C. After 2 hours, the samples were washed three times in PBS for 10 minutes each time to clean off excess primary antibodies. Incubate with the secondary antibody (Cy3-labeled or FITC-labeled goat anti-rabbit IgG) (Biyuntian Biotechnology Research Institute, Guangzhou, China) for 30 minutes at room temperature, protected from light. Rinse the samples three times for five minutes each in PBS to wash away excess secondary antibody, protected from light. DAPI was diluted appropriately in PBS, and nuclei were counterstained for 15 minutes at room temperature and in the dark. The sample was washed three times in PBS for 10 minutes at a time, and
mounting medium was applied to each hole and then fixed with a coverslip. The slides were kept in a dark environment at 4°C, and the images were observed and captured using a fluorescence microscope.

2.6. Flow cytometry detection of skin fibroblast cell cycle

Plated cells at proper density, when the cells reached a certain confluence, the original medium was discarded, and DMEM/F12 medium containing different concentrations of drugs was added to each well for 24 h, the digested cells were centrifuged at 1000 r/min for 5 min, washed with 10 ml of PBS, the precipitated cells were collected. The cells were then resuspended in 1 ml of PBS solution twice. Add 1 ml of pre-cooled 70% ethanol to the cell suspension and incubate the cells at 4°C for at least 2 hours or overnight. Finally, the cells are centrifuged again and resuspended in a solution containing propidium iodide and an RNA enzyme and incubated for 30 min at 37°C in a light-proof environment. A red laser (488 nm) is used to set up and adjust the mobile blood cell counter, and finally fluorescence is obtained and the cell cycle of each sample is analyzed.

2.7. Establishment of a full-thickness cutaneous wound healing model

Each rat was anesthetized by an intraperitoneal injection of 10% chloral hydrate, at dose of 3ml/kg body weight, respectively. The surgical area was disinfected using 75% ethanol. In each group, a circular wound of 2 cm in diameter was made on the left and right dorsal midlines. The resulting circular wound is a full-thickness wound in which the epidermis and dermis are removed until the subcutaneous connective tissue. Then the rats were divided into 2 groups, with 8 rats in each group: 1. Control group: 8 rats were subjected to subcutaneous injection of phosphate-buffered saline (PBS) .2. Tubacin group: eight wounded rats received 500ng/day Tubacin diluted in PBS subcutaneously for seven days. To prevent infection or further damage to the wounds, each animal was housed separately, and intraperitoneal injection of penicillin-streptomycin after surgery to prevent wound infection.

Record of wound healing: Recorded rat wounds at the 1st, 7th, 14th, 21st and 28th days after wound induction by a digital camera and observed the wound healing.

2.8. Measurement of skin healing rate

The wound shape was assessed by tracing the wound margin onto tracing paper on days 1, 7, 14, 21, and 28 following wound induction. Calculated wound area by using OPTPro image software, the percentage of wound healing = ((original wound area - current wound area on the n\textsuperscript{th} day)/original wound area) × 100%, n = days after wound induction.

2.9. Microscopic evaluation of wound healing

Full-thickness skin samples were taken from the healing wound and its surrounding area, fixed with 4% paraformaldehyde, and stored in 75% ethanol solution after 24 hours. The samples were then embedded in paraffin, sectioned to a thickness of 50 µm, and stained with HE-stained and Masson's trichrome. The staining results were observed and photographed under an optical microscope. Finally, the thickness of
the epidermis in 5 different areas was measured using Photoshop software from the HE-stained images for statistical analysis.

2.10. Western blot of skin tissue

Full-thickness skin samples were taken from the healing wound and its surrounding area. Tissue samples were homogenized and extracted proteins from tissues, electrophoresis and western blotting were carried out.

2.11. Data processing and statistical analysis

The above experimental results were repeated three times or more. Statistical analysis of the data was performed using SPSS 19. The numerical values are presented as mean ± SD deviation. The differences between groups were analyzed using either a one-way analysis of variance (ANOVA) or a t-test.

3. Results

3.1. The effect of various tubacin and TGF-β1 concentrations on skin fibroblast cell survival and cell cycle

Cultured skin fibroblasts reach 80–90% confluence in 3 or 4 days. With the increase of cell passage, the cells became mature gradually, the cell outline became clearer, the cytoplasm was more abundant, the nuclei were larger and clearly visible (Fig. 1A, B). The cells of the second generation were arranged radially or rotationally, and were fusiform or polygonal (Fig. 1C, D). With increasing tubacin concentrations, the cell viability also decreased. Compared with the control group, the cell viability of the 10 µm and 20 µm tubacin treated groups was significantly decreased ($P < 0.01$), and it peaked at a tubacin concentration of 20 µM, it was reduced to 60%, below 70%, indicating that the tubacin concentration of 20 µM has potential toxic effects on cells (Fig. 1E). Compared with the control group, there was no significant change in cell viability of 10 ng/mL TGF-β1 group. The cell viability also decreased significantly with increasing tubacin concentrations and 10 ng/mL TGF-β1 (Fig. 1F). Therefore, we selected 10 µM concentration of tubacin for follow-up experiments, the results showed that tubacin can inhibit the proliferation of fibroblasts.

3.2. Effect of TGF-β1 and tubacin on cell cycle of skin fibroblasts

To determine if tubacin reduced cell viability by affecting the cell cycle, we detected the effect of TGF-β1 and tubacin on cell cycle of skin fibroblasts. There was no significant difference between groups in the proportion of cells in the G0/G1 phase, S phase, or G2/M phase (Fig. 1G, H). This data showed that TGF-β1 and tubacin had no influence on cell cycle.

3.3. Effect of TGF-β1 and tubacin on migration of skin fibroblasts
The results of the scratch test are shown in Figure 2. At the same time, tubacin prevented the migration of the fibroblasts, preventing them from entering the scratched area, compared with the control group (p < 0.01). There was no significant difference in scratch area ratio between the TGF-β1 group and the control group. These results showed that TGF-β1 had no significant effect on cell migration, while tubacin significantly inhibited cell migration.

1. 3.4. Western blot detection of HDAC6, acetylated α-tubulin, COL1A1, COL3, CD31, p-ERK, t-ERK, p-AKT and t-AKT in skin fibroblasts

α-Tubulin, an important substrate molecule of HDAC6, can be expressed at a relatively stable level in different cells. Type III collagen (COL3) and type I collagen (COL1A1) were biomacromolecules in the extracellular matrix, which participated in wound filling and tissue reconstruction; α-SMA is the marker protein of myofibroblasts and also the marker of EMT. Tubacin could downregulate the protein levels of COL3, p-AKT, HDAC6, COL1A1, α-SMA and p-ERK, which were upregulated by TGF-β1. In addition, tubacin could upregulate the protein levels of ace-α-tubulin and CD31 significantly (Fig. 3). These results suggest that tubacin may be involved in the regulation of collagen expression and EMT via the ERK and AKT pathways.

3.5. Immunofluorescence detection of protein distributions

HDAC6 was mainly distributed in the cytoplasm, and it was more concentrated in the cytoplasm near the nucleus; α-SMA was mainly distributed in the cytoplasm filamentously. And HDAC6 and α-SMA had the same subcellular localization. The treatment of TGF-β1 and tubacin had no significant effect on the distribution of HDAC6 and α-SMA. (Fig. 2D)

3.6. Gross evaluation of wound healing

The photos showed that the wound was scarred at the 7th day, healed basically at the 21th day. And the wound healed completely at the 28th day, but the hair did not cover the skin completely. No significant variance in the process of wound healing was observed between the control and tubacin groups, as demonstrated in (Fig. 4A). Similarly, no disparity in the rate of wound healing was found between the two groups, as shown in (Fig. 4B). These outcomes indicate that the use of tubacin does not have a positive effect on wound healing.

3.7. Western blot detection of HDAC6, acetylated α-tubulin, COL1A1, COL3, α-SMA in skin

Trauma upregulated the protein levels of HDAC6 and acetylated α-tubulin in skin, and the protein levels of acetylated α-tubulin peaked at the 7th day (Fig. 4A, B, C). These results demonstrated that HDAC6 and acetylated α-tubulin may be involved in wound repair. Furthermore, there may be other enzymes that affect the acetylation of α-tubulin in the early stage of wound healing. In addition, tubacin could downregulate the protein levels of HDAC6 and acetylated α-tubulin, which could be heightened by trauma (Fig. 4D).
Meanwhile, tubacin could downregulate the protein levels of COL1A1 and COL3, which could be promoted by trauma. It is noteworthy that tubacin upregulated the protein levels of α-SMA, which is the marker protein of myofibroblasts and also the marker of EMT (Fig. 4E, F, G). These results showed that tubacin inhibited the expression of collagen caused by trauma.

### 3.8. Histological results

#### (A) Masson's trichrome stain

Collagen bundles were discovered in the regenerated dermis on day 7. On day 14, the skin revealed regular collagen bundles organized in various directions. At day 28, photographs reveal that the wound was filled with extensive layers of collagen and sediment bundles. Remarkably, the Tubacin treated group exhibited reduced collagen deposition compared to the Control group, indicating a superior and thorough process in wound healing (Fig. 5A). These results demonstrated that tubacin promoted functional healing of skin wounds.

#### (B) Haematoxylin and Eosin stain

The experimental results showed that on the first day, there was a small amount of inflammatory cell infiltration in the tissues. By the 14th day, inflammatory cells and blood vessels filled the wound. By day 21, the tubacin group showed a thinner layer of regenerated epidermis compared to the control group. (P < 0.01) And immature skin appendages were found in the dermis of wound. Furthermore, both groups showed skin appendages in the dermis of wound on the 28th day. Nonetheless, the skin appendages observed in the tubacin group displayed a greater degree of maturation in comparison to those seen in the control group, whereas the regenerative epithelium thickness was less in the tubacin group as opposed to the control group (P < 0.01) (Fig. 5B, C). From these results, we knew that tubacin promoted the transformation of regenerated epithelium into normal mature epithelium.

### 4. Discussion

The skin is the external epithelium of the body, capable of maintaining and repairing damage throughout life. The capacity of human skin to recover from injury is of paramount importance. The intricate process of skin wound healing necessitates a cohesive reaction involving immune cells, hematopoietic cells, and skin-resident cells[12]. Skin injuries that heal poorly can result in the development of scars. Scar tissue is formed due to an overabundance of extracellular matrix (ECM) elements, including collagen, which accumulate excessively in the dermis and subcutaneous tissue beyond the location of the initial wound.[13]. Keloids have been growing for years and rarely subside spontaneously[14]. Skin wound healing has broad relevance to medicine, public health and global disease burden[15]. Understanding the mechanisms of cellular activity involved in skin wound healing is crucial for enhancing the prevention and treatment of wounds. Among the numerous skin-related cells, fibroblasts play an essential part in wound healing and scarring by synthesizing collagen and other extracellular matrix (ECM) proteins. Furthermore, fibroblasts differentiate into myofibroblasts, which express α-smooth muscle actin (α-SMA).
and increase collagen synthesis and secretion, thus promoting wound healing[16]. Although wound healing is essential for tissue repair, the continued presence of fibroblasts and myofibroblasts may lead to the formation of hypertrophic scars[17]. This is especially true in keloids, where the activity of TGF-β1 in fibroblasts is believed to trigger collagen production and promote greater contractile ability than normal dermal fibroblasts[18, 19]. From a therapeutic point of view, it is of great clinical significance to block the fibrotic effects of the molecules involved in collagen synthesis and TGF-β1[20]. Several research studies have documented alterations in DNA methylation and histone acetylation patterns in fibroblasts within keloids, suggesting epigenetics modifications may have a role in their formation. Expanding research in the area of epigenetics may present new avenues for keloid treatment[21]. Reports suggest that Histone deacetylase (HDACs) have the ability to regulate transcription of collagen by binding with certain transcription factors and cofactors at the proximal promoter of collagen genes. As a result, they have the potential to serve as therapeutic strategies for the treatment of hypertrophic scars[22]. Research indicates that Trichostatin a (TSA), an HDAC inhibitor, has the ability to prevent the development of skin fibrosis. Moreover, administering TSA to keloid fibroblasts can notably hinder the production of collagen I after TGF-1 stimulation, suggesting that TSA may effectively suppress collagen synthesis in skin fibroblasts[23].

Tubastatin A (also known as tubacin), a selective inhibitor of HDAC6, has been reported to inhibit bleomycin-induced pulmonary fibrosis. We conjecture that tubacin is also able to promote wound repair and inhibit scar formation by inhibiting collagen synthesis. Therefore, we investigated the effect of tubacin, an HDAC6 inhibitor, on the proliferation and migration of dermal fibroblasts and its role in all-over skin wound repair, and found that tubacin inhibited fibroblast proliferation and significantly inhibited cell migration. Current evidence suggests that TGF-β1 can regulate the transformation of fibroblasts into myofibroblasts[24, 25]. We observed the effect of TGF-β1 and tubacin on the migration of skin fibroblasts by wound scratch test, and found that TGF-β1 had no obvious effect on cell migration, while tubacin could obviously inhibit cell migration. Collagen 1(COL1), collagen 3(COL3), α-SMA and other fibrosis-related proteins are all transcriptionally regulated by TGF-β1[26, 27]. We treated fibroblasts with TGF-β1 and different concentrations of tubacin and detected the protein expression levels in fibroblasts by Western blot, and found that tubacin downregulated the protein levels of COL3, p-AKT, HDAC6, COL1A1, α-SMA and p-ERK that were upregulated by TGF-β1. In addition, tubacin significantly upregulated the protein levels of ace-α-tubulin and CD31 (platelet endothelial cell adhesion molecule). HDAC6 mainly exists in cytoplasm and regulates cytoskeleton by deacetylation of ace-α-tubulin and cortactin[28]. These results suggest that tubacin may be involved in the regulation of collagen expression and EMT through the ERK pathway and AKT pathway.

To further investigate the role of tubacin, an HDAC6 inhibitor, in the repair of skin wounds, we established a rat dorsal skin all-over wound model, injected tubacin after wound formation, observed the wound healing at different time periods, and performed histological analysis. Mouse skin excision models benefit from pathological changes similar to wound healing and hypertrophic scars[29]. So we chose this model in our experiments. The expression levels of HDAC6, acetylated α-microtubulin, COL1A1, COL3, and α-SMA in skin were detected by protein blot analysis of proteins extracted from tissues of excised healing
wounds, and it was found that trauma upregulated the protein levels of HDAC6 and acetylated α-microtubulin in skin, indicating that HDAC6 and acetylated α-microtubulin may be involved in wound repair. In contrast, treatment with tubacin downregulated the protein levels of HDAC6 and acetylated α-tubulin; tubacin also downregulated the protein levels of COL1A1 and COL3. Because during wound healing, fibroblasts around the wound are able to differentiate into myofibroblasts that then migrate to the wound area, promoting wound healing by increasing collagen synthesis and secretion. After wound healing, myofibroblasts are eliminated by the body through apoptosis. However, under pathological conditions, if prolonged inflammation and infection are present, myofibroblasts will persist in the wound appendage, causing excessive deposition of ECM proteins and leading to scar formation[30, 31]. Therefore, inhibition of collagen synthesis and secretion during wound healing is important to promote wound healing and reduce scar formation.

In this study we demonstrated that the HDAC6 inhibitor tubacin inhibited the expression of COL1A1, COL3 and α-SMA during wound healing, suggesting that tubacin promotes functional wound healing. Masson trichrome staining of the histological results showed that collagen deposition was lower in the tubacin group than in the control group, indicating that tubacin treatment resulted in a better level of wound healing. Hematoxylin and eosin staining reveals that tubacin promotes the conversion of regenerating epithelial cells to normal mature epithelial cells. All of this evidence suggests that HDAC6 inhibition has a promising role in wound healing and scar formation, and therefore it may be able to be used as an alternative treatment for trauma management and as a scar formation prevention strategy.

In conclusion, treatment of fibroblasts with tubacin could inhibit the proliferation and migration of fibroblasts. In addition, tubacin may inhibit the expression of COL1A1, COL3, and α-SMA, which were involved in pathological wound repair, by regulating the TGF-β-PI3K-Akt pathway and the MAPK/Erk signaling pathway. At the same time, it promoted the expression of CD31, which may be involved in inflammation or angiogenesis. Last but not least, tubacin promotes functional wound healing, but the mechanisms involved need to be further elucidated and future studies will focus on the exact molecular mechanisms underlying the antifibrotic properties of selective inhibitors of HDAC6.

**Declarations**

**Declaration of competing interest**

The authors state that they do not have any known competing financial interests or personal ties that could appear to have influenced the work disclosed in this study.

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**Data Availability Statement**
No Data associated in the manuscript.

Compliance with Ethical Standards

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Competing interests:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval:

All animal manipulations were performed in accordance with NIH guidelines and ethical principles for the care and use of laboratory animals and were approved by the Southern Medical University Laboratory Animal Center. All of the experimental animals were treated humanely.

Author Contributions:

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Jiaqiang Liang and Tingyu Wang. The first draft of the manuscript was written by Jiaqiang Liang and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Consent to participate:

Non-applicable.

Consent to publish:

Non-applicable.

References


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Figures

**Figure 1**

Effects of different concentrations of tubacin and TGF-β1 on the viability and cell cycle of skin fibroblasts
Effects of different concentrations of tubacin and TGF-β1 on the viability and cell cycle of skin fibroblasts.

A B C D) Morphological observation of skin fibroblasts at different times. (E) With increasing tubacin concentrations, the cell viability also decreased; the cellular activity peaked at a tubacin concentration of 20 µM. (F) The cell viability also decreased significantly with increasing tubacin concentrations and 10ng/mL TGF-β1. (G H) The effects of TGF-β1 and tubacin on the cell cycle of skin fibroblasts were examined, and there was no significant difference in the proportion of cells in G0/G1, S or G2/M phases among the groups.
Figure 2

Effect of TGF-β1 and tubacin on migration of skin fibroblasts

Immunofluorescence detection of protein distributions

Effect of TGF-β1 and tubacin on migration of skin fibroblasts
Immunofluorescence detection of protein distributions.

(A B) The wound scratch test revealed that at the same time point, tubacin prevented fibroblasts from migrating across the wound edge to the scratch area. (C) The ratio of scratch area between different groups was statistically analyzed. (D) Immunofluorescence detection of protein distributions: HDAC6 was mainly distributed in the cytoplasm, and it was more concentrated in the cytoplasm near the nucleus; α-SMA was mainly distributed in the cytoplasm filamentously. The treatment of TGF-β1 and tubacin had no significant effect on the distribution of HDAC6 and α-SMA.
Figure 3

Protein expression in cells of each group

Protein expression in cells of each group.
Western blot detection of HDAC6, acetylated α-tubulin, COL1A1, COL3, CD31, p-ERK, t-ERK, p-AKT and t-AKT in skin fibroblasts. Tubacin could downregulate the protein levels of COL3, p-AKT, HDAC6, COL1A1, α-SMA and p-ERK. Tubacin could upregulate the protein levels of acetylated α-tubulin and CD31 significantly.

Quantitative analysis of Western blotting results for each group.

Figure 4
Effect of tubacin on skin wound healing and wound healing rate

Effect of tubacin on skin wound healing and wound healing rate. (A) Photographs show no significant difference in the degree of wound healing between the control and tubacin groups at days 7, 21, and 28. (B) There was no significant difference in the wound healing rate between the control and tubacin groups by statistical analysis.
**Figure 5**

Figure 4 Protein expression in skin

Figure 4. Western blot detection of HDAC6, acetylated α-tubulin, COL1A1, COL3, α-SMA in skin. (A B C) Trauma upregulated the protein levels of HDAC6 and acetylated α-tubulin in the skin, and the protein levels of acetylated α-tubulin peaked at day 7. (D) Tubacin could downregulate the protein levels of HDAC6 and acetylated α-tubulin, which could be heightened by trauma. (E) Tubacin could downregulate the protein levels of COL1A1 and COL3; tubacin upregulated the protein level of α-SMA, a marker protein of myofibroblasts and a marker of EMT.

**Figure 6**

Figure 5 Protein expression in cells of each group
Figure 5. Protein expression in cells of each group. (A) Haematoxylin and Eosin stain: Both groups showed regenerated epidermis covering the wound surface on the 21th day, but tubacin group showed wound surface covered by regenerated epidermis with reducing thickness than control group. The difference was statistically significant ($P < 0.01$). (B) The collagen deposition in the tubacin group was found to be lower than that in the control group when skin tissues were taken for Masson's trichrome staining on days 7, 14 and 28 of the experiment.