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Acceleration of cutaneous wound healing by brassinosteroids

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Abstract

Brassinosteroids are plant growth hormones involved in cell growth, division and differentiation. Their effects in animals are largely unknown, although recent studies showed the anabolic properties of brassinosteroids possibly mediated through the phosphoinositide 3-kinase (PI3K)/ protein kinase B (Akt) signaling pathway. Here we examined biological activity of homobrassinolide (HB) and its synthetic analogues on *in vitro* proliferation and migration assays in murine fibroblast and primary keratinocyte cell culture. HB stimulated fibroblast proliferation and migration, and weakly induced keratinocyte proliferation in vitro. The effects of topical HB administration on progression of wound closure were further tested in the mouse model of cutaneous wound healing. C57BL/6J mice were given a full thickness dermal wound, and the rate of wound closure was assessed daily for 10 d alongside adenosine receptor agonist CGS-21680 as a positive control. Topical application of brassinosteroid significantly reduced wound size and accelerated wound healing in treated animals. mRNA levels of TGF-β and ICAM-1 were significantly lower, while TNF-α was nearly suppressed in the wounds from treated mice. Our data suggest that topical brassinosteroids accelerate wound healing by positively modulating inflammatory and re-epithelialization phases of the wound-repair process, in partby enhancing Akt signaling in the skin at the edges of the wound and enhancing migration of fibroblasts in a wounded area. Targeting this signaling pathway with brassinosteroids may represent a promising approach to the therapy of delayed wound healing.

Keywords

brassinosteroids; wound healing; inflammation; proliferation; tissue remodeling

INTRODUCTION

Impaired wound healing causes increased pain, suffering, and decreased mobility of the trauma and chronic metabolic disease (i.e. diabetes) patients. There are many treatments that promote wound healing, including topical hormones (1), growth factors (2), silver-containing antimicrobial agents (3), and natural complex remedies like pollen (4) and honey (5). However, the emergence of antibiotic resistant strains of bacteria and adverse reactions

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associated with silver sulfadiazine treatment underline the importance of finding more effective agents with shorter time of application and novel mechanisms of action.

Cutaneous wound healing is characterized by an initial inflammatory response, followed by tissue repair and remodeling. Recruitment of polymorphonuclear neutrophils and macrophages by release of inflammatory cytokines and chemokines with a peak concentration occurring slightly before maximum macrophage infiltration is a critical first step in wound healing (6). When this stage is compromised or excessive, the exaggerated inflammatory phase of wound healing may be causally related to slower wound healing and scarring. Indeed, wounds created in fetal mice that exhibit lower levels of inflammation heal faster and without scarring (7). PU1 null mice, lacking polymorphonuclear neutrophils and macrophages, have greatly reduced levels of inflammation in their wounds that are associated with enhanced healing (8). Exercise also improves cutaneous wound healing in aged mice in association with decreased levels of the tumor necrosis factor alpha (TNF-α) and proinflammatory chemokines in the wound tissue (9).

Protein kinase B (Akt) is a central element in the phosphoinositide 3-kinase (PI3K)/Akt network, and there is increasing evidence that this pathway plays a role in wound healing. The importance of PI3K in collagen gene regulation was previously demonstrated in human dermal fibroblasts (10), and analysis of the Akt-1 null mice skin revealed an impaired matrix organization with reduced amount of collagen (11). Inhibition of Akt upregulated production of the basal matrix metalloproteinase 1 and reversed the inhibitory effect of transforming growth factor beta (TGF-β) (12), thus establishing the link between Akt activation, inflammatory response, and wound healing. Recently we have shown that oral administration of homobrassinolide (HB, Fig. 1A) to healthy rats triggered a selective anabolic response that was associated with activation of Akt (13). HB belongs to the group of plant-derived polyhydroxylated derivatives of 5α-cholestane, structurally similar to cholesterol-derived animal steroid hormones and insect ecdysteroids. Brassinosteroids were isolated from all plant organs, however seeds and pollen contain highest levels of naturally available brassinosteroids (14). Growth promoting effect of brassinosteroids in plants is associated with the increased synthesis of nucleic acids and proteins (15, 16). Although the structural requirements for biological activity of brassinosteroids have been clearly recognized, a number of related compounds that are potent plant growth promoters have been synthesized recently (17). Structure-activity studies of brassinosteroids have revealed that 5α-configuration is required for optimum activity, but the B-ring tolerates considerable variation, providing that the presence of a polar functional group, which does not have to be a lactone, is maintained (18).

Following this principle, we previously synthesized a group of HB analogues (Fig. 1B) and compared them to several natural brassinosteroids in their ability to stimulate Akt (19). In this study we examined the possible structure-activity relationships and underlying therapeutic mechanisms in the fibroblast and keratinocyte cell cultures and further investigated the effects of topical brassinosteroid application in the mouse model of cutaneous wound healing.

METHODS

Reagents

HB [(22S, 23S, 24S)-2α, 3 α, 22,23-tetrahydroxy-24 ethyl-^{2}-homo-7-oxo-5α-cholestane-6-one] (Fig. 1A) was purchased from Waterstone Technology (Carmel, IN) and its structure was confirmed by ESI-LCMS and NMR. Brassinosteroid analogues 2–9 (Figure 1B), including homocastasterone (22S, 23S, 24S)- 2α, 3α, 22, 23-tetrahydroxy-24-ethyl-5α-cholestan-6-one [2], (22S,23S,24R)-3α-fluoro-22,23-dihydroxy-24-ethyl-^{2}-homo-7-oxa-5 α-

cholestan-6-one [3], (22S,23S,24S)-3α-fluoro-22,23-dihydroxy-24-ethyl-5 α-cholestan-6-one [4], (22S,23S,24S)-2α,3α,22,23-tetrahydroxy-24-ethyl-^{2}-homo-7-aza-5α-cholestan-6-one [5], (22S,23S,24S)-2α,3α,22,23-tetrahydroxy-24-ethyl-^{2}-homo-6-aza-5α-cholestan-7-one [6], (22R,23R,24S)-2α,3α,22,23-tetrahydroxy-^{2}-homo-7-oxa-5α-cholestan-6-one [7], (22S,23S,24R)-2α,3α,22,23-tetrahydroxy-24-methyl-^{2}-homo-7-oxa-5α-cholestan-6-one [8], and (22R,23R,24R)-2α,3α,22,23-tetrahydroxy-24-methyl-^{2}-homo-7-oxa-5α-cholestan-6-one [9] were synthesized or purchased previously (19), and are shown on Fig. 1B. All other chemicals and cell culture media were obtained from Sigma (Saint Louis, MO) or Invitrogen (Carlsbad, CA) unless specified otherwise.

Fibroblast culture

NIH 3T3 murine embryonic fibroblast cell line CCL-92 was obtained from ATCC (Manassas, VA). Cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 0.1% penicillin-streptomycin at 37°C and 5% CO_2 and passaged every 3–4 days. Cells were subcultured into 96 well plates for proliferation and viability assays, and 24 well plates for scratch wound closure studies (Greiner Bio One, Monroe, NC).

Fibroblast viability and proliferation assays

3T3 fibroblasts were seeded in a 96-well flat bottom plate at a density of 1×10^4 cells/well. Cell viability was measured by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in triplicate essentially as described (0.3–30 μM of test substance for 4 h) (20) and quantified spectrophotometrically at 550 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). The concentrations of test reagents that showed no changes in cell viability compared with that of the vehicle (0.1% ethanol) were selected for further studies. For cell proliferation studies, cells were treated in triplicate with 0.1–10 μM of test substance for 24 h and assayed using BrdU (5-bromo-2′-deoxyuridine) Cell Proliferation kit from Amersham (Uppsala, Sweden).

Scratch wound closure

3T3 Swiss fibroblast were seeded into 24-well tissue culture at a concentration of 3×10^5 cells/ml and cultured to nearly confluent cell monolayers. Then, a linear wound was generated in the monolayer with a sterile 100 μl plastic pipette tip. Any cellular debris was removed by washing with phosphate buffer saline. DMEM medium with vehicle (0.1% ethanol), FBS (1%, positive control), or various concentrations of the pure compounds was added to set of 3 wells per dose and incubated for 12 h at 37°C with 5% CO_2. The cells were visualized in 10% methylene blue for 5 minutes. Three representative images from each well of the scratched areas under each condition were photographed to estimate the relative migration of cells at 0 and 12 h past treatment. The data were analyzed using ImageJ software by calculating the percentage of scratch closure at each dose point relative to control.

Primary keratinocyte cell culture and proliferation assay

Primary mouse keratinocytes were isolated from the skin of C57Bl/6J newborn mice following the procedure described previously (21). Cells were cultured on 96-well collagen IV-coated plates in CnT-07 medium (CellNTec, Bern, Switzerland) to promote attachment and initial growth. Test compounds were dissolved in ethanol and diluted into CnT-02 (a reduced growth factor medium) for treatment of cells. Agent and vehicle treatments were performed in sextuplet. Cells were cultured at 37 C and 4.5% CO_2 until control wells reached approximately 80% confluency. Media was then removed and replaced with a solution of 5% Alamar Blue (InVitrogen) in CnT-07 and incubated a further 4 hours to

evaluate keratinocyte proliferation. Alamar Blue reduction was quantified by measuring absorbance at 570 nm and 650 nm on a Perkin-Elmer Victor2 multilabel plate reader (Perkin-Elmer, Waltham, MA) and calculated per the manufacturer's instructions.

Mouse model of cutaneous wound healing

All animal experiments were performed according to procedures approved by the Rutgers Institutional Animal Care and Use Committee in AAALAC accredited animal care facility. Twenty seven six-week-old male C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) were housed in individual chambers, in a room maintained at a constant temperature with 12 h light-dark cycle and had free access to food and water. Animals were allowed to adapt to new conditions for seven days and handling the animals was performed daily during this time to reduce the stress of physical manipulation. Animals were randomized into groups (n=9) according to body weight one day prior to dosing. Under volatile anesthesia (5% isoflurane to effect), the shoulder and back region of each animal was shaved. A sharp punch (ID 6 mm) over lumbar spine was applied to remove the skin including panniculus carnosus and adherent tissues. Test substance (control vehicle 1.5% carboxymethyl cellulose, or 10 μg/mouse of either HB or positive control CGS-21680) was administered topically, immediately following cutaneous injury, and then daily for 10 d. To investigate the kinetics of wound healing, wound size were photographed and measured every two days with ImageJ software. Time to wound closure was estimated by comparing the area of treated wounds to the area of control wounds. The percent closure of the wound (%) was calculated, and wound half-closure time (CT_{50}) was analyzed by linear regression. At the end of experiment, animals were euthanized by CO_2 gas inhalation, wounded tissue samples were collected by snap-freezing in the liquid nitrogen and stored at −80°C for wound healing factor assays or fixed in 4% paraformaldehyde for routine histological sectioning and staining using Mayers haematoxylin and eosin.

Real time PCR

Total RNA was extracted using Trizol (Invitrogen) and quantitative PCR was performed in duplicate essentially as described (22) using the following gene-specific primers (IDT, Coralville, IA) selected using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA): β-actin, forward primer 5′-GGG AAA TCG TGC GTG ACA TT-3′, reverse primer 5′-GCG GCA GTG GCC ATC TC-3′; TNF-α, forward primer 5′-TAC TGA ACT TCG GGG TGA TTG GTC C-3′, reverse primer 5′-CAG CCT TGT CCC TTG AAG AGA ACC-3′; ICAM-1, forward primer 5′-TGT TTC CTG CCT CTG AAG C-3′, reverse primer 5′-CTT CGT TTG TGA TCC TCC G-3′; TGF-β, forward primer 5′-GCT TCA GAC AGA AAC TCA CT-3′, reverse primer 5′-GAA CAC TAC TAC ATG CCA TTA T-3′. Samples were subjected to a melting curve analysis to confirm the amplification specificity. The relative change in the target gene with respect to the endogenous control gene was determined using 2ΔΔCT method (23).

Western blot analysis

Wound tissue extracts were prepared in ice-cold RIPA buffer supplemented with 10 mM sodium floride, 2mM sodium orthovanadate, 1 mM PMSF, and protease inhibitor cocktail (Sigma) and centrifuged at 12,000 g for 20 min at 4°C. Equal amounts of protein (50 μg) from the supernatants were separated on 10% SDS polyacrylamide gels and blotted onto the nitrocellulose membrane. Western blot detection was performed with monoclonal antibodies for the phospho-Akt1(Ser473) and Akt1 (EMD Millipore, Bedford, MA) according to the manufacturer's instructions. After washing, the blots were incubated with an anti-rabbit peroxidase-labeled secondary antibody and visualized using ECL Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ).

Statistical analysis

Data are represented as mean ± SEM. Statistical analyses were performed with GraphPad Prism 4.0 (San Diego, CA) using one-way ANOVA completed by a multicomparison Dunnett's test. Wound closure associated body weight change was analyzed by two-factor repeated-measures ANOVA, with time and treatment as independent variables. P-values of less than 0.05 were considered significant.

RESULTS

Effect of brassinosteroids on fibroblast viability and proliferation

To elucidate effects of brassinosteroid treatment on cell proliferation and determine the structure-activity requirements for their biological activity, we analyzed cytotoxic and cell proliferation effects of HB and its natural or synthetic analogues in 3T3 mouse fibroblast cell culture (19) (Fig. 1B). Compound 5 synthesized to carry a 7-aza substitution in the B ring of the molecule showed highest cytotoxicity with IC_{50} of 12.5μM. Two other synthetic brassinosteroids with fluorinated substitutes in the A ring of the molecule showed weak toxicity at 30 μM, the highest concentration tested. There was no correlation between compound's ability to induce cell proliferation and stimulation of cell migration (Table 1).

Effect of brassinosteroids on scratch wound closure

Microscopic observation of 3T3 fibroblasts demonstrated that HB promotes cell migration into a scratch wound zone with maximum efficacy of 30 ± 4.2% at 5 μM after 12 h of incubation. This compares favorably with 1% fetal bovine serum used as a positive control in this assay (41.5 ± 6.5%), as well as reference activity of the platelet-derived growth factor reported earlier (24). Several HB analogues showed similar or decreased scratch wound closure activity in this assay, with no specific reference to structural modifications (Table 1). The cytotoxic compound 5 showed no effect on fibroblast migration, as expected. A dose dependent migration activity was evaluated for all active compounds that significantly accelerated wound closure at concentrations of 0.1–10 μM. Compound 4 turned out to possess high activity similar to HB, while compound 6 showed highest activity at 3 μM, possibly due to weak cytotoxicity associated with the higher doses of this treatment (Fig. 2A–C).

Effect of brassinosteroids on proliferation of primary keratinocytes

The effects of biologically active brassinosteroids (compounds 1, 4, and 6) on proliferation of primary mouse keratinocytes were assessed using Alamar Blue. Treatment with 1–10 μM of HB (compound 1) resulted in a significant increase in the proliferation of primary keratinocytes at doses of 0.3 and 10 μM, but did not reach significance at 3 μM compared to cells that were treated with vehicle alone (ethanol). In addition, compounds 4 and 6 dose-dependently decreased primary keratinocyte proliferation, suggesting higher cytotoxicity of brassinosteroids towards the keratinocyte cells (Fig. 3). Based on this observation, HB (compound 1) was chosen for evaluation of wound healing properties of brassinosteroids *in vivo*.

Body weight and food intake

While there were no overall effects for body weight in the 10-day period following wounding, we noticed that all mice lost weight on d 2 post-wounding, with weight gain resuming on d 4. Mice lost 1.5 g of body weight by d 2, and there was no significant difference between the treatments, although there was a tendency for HB to reduce weight loss associated with injury (Fig. 4). There was also a transient reduction in food intake that

lasted for 48 h post-wounding with no significant differences noted between the treatments (data not shown).

HB treatment improves wound healing

We found that cutaneous wound healing was significantly improved in animals receiving 10 μg topical HB per mouse per day for 10 d compared with control group treated with vehicle alone (Fig. 5A). The brassinosteroid effect appeared to occur in the early phases up to d 8 post-wounding of wound healing (Fig. 5B). The CT_{50} time at which 50% of the cutaneous wound was closed, was significantly reduced by both HB (5.40.3 days) and positive control CGS-21680 (6.20.4 days) compared with vehicle controls (7.20.2 days). The strongest effect associated with HB treatment was observed on d 4. When wound data were expressed in terms of percent of original wound size, there was a 2-fold increase in speed of wound closure relative to control mice. Another interesting morphological observation associated with HB treatment was increased volume of the wound edges that reached prominence on d 4 and slowly subsided on d 6–8 to completely disappear on d 10 post-wounding.

HB reduces proinflammatory markers in healing wounds

We next examined mRNA levels of the proinflammatory cytokines TNF-α and TGF-β along with an adhesion chemokine ICAM-1 in the wound tissue of control and treated mice on d 10 post-wounding. Transcriptional regulation plays an important role in physiological relevance of these factors in the context of wound healing (25). Wound tissue from animals treated with adenosine receptor agonist CGS-21680 as a reference control (26) showed a remarkable suppression of TNF-α mRNA, but no effect on either TGF-β or ICAM-1 mRNA levels. Contrary to this, HB treatment was associated with weak effect on TGF-β, significant suppression of ICAM-1, as well as nearly complete downregulation of TNF-α. As expected from our previous studies, we also observed upregulation of Akt-1 phosphorylation in the HB-treated edge-of-the-wound tissue (Figure 6).

DISCUSSION

Previously we reported that orally applied brassinosteroids produced significant anabolic effects and improved physical fitness in healthy animals with minimal androgenic effects (27). Brassinosteroids are a class of plant hormones with a polyoxygenated steroid structure showing pronounced plant growth regulatory activity (28). They also exhibit striking structural similarities with animal sex hormones and arthropod hormones of the ecdysteroid type (Fig. 1) such as 20-hydroxyecdysone (29) that have been reported to produce anabolic effects in mammals (30). Animal sex hormones play a gender- and age-modified role in wound healing. For example, aged males were shown to have delayed healing of acute wounds when compared to aged females. A partial explanation for this is that the male androgens (testosterone and 5α-dihydrotestosterone), female estrogens (estrone and 17β-estradiol), and their steroid precursor dehydroepiandrosterone have significant effects on the wound-healing process (31). Estrogen affects wound healing by regulating a variety of genes associated with regeneration, matrix production, protease inhibition, epidermal function, and the genes primarily associated with inflammation (32). Conversely, oral mucosal wounds heal faster in males (33). The skin also acts as a steroidogenic tissue containing the full cytochrome P450 system required for the de novo production of sex steroids from cholesterol (34). This raises the possibility that bioactive steroid hormones within the wound microenvironment may also be important in healing.

Since brassinosteroids are plant steroid hormones that activate PI3K/Akt signaling pathway with minimal androgenic effects (27), we hypothesized that they may have potential applications to animal wound healing. Indeed, topical application of brassinosteroids

significantly reduced wound size in treated C57BL/6J mice (Fig. 4 and 5). On the contrary, testosterone appears to delay wound healing by directly altering wound cell populations and cytokine profiles, thereby enhancing the inflammatory response and reducing matrix deposition. This is supported by androgen receptor expression in keratinocytes, fibroblasts, and inflammatory cells within the wound (35).

Proinflammatory cytokines and chemokines initiate and coordinate the early inflammatory phase of wound healing (36). However, it is now well established that uncontrolled or elevated inflammation might be responsible for the delay in wound healing rates. We next examined whether the HB-induced alteration in wound healing in mice was related to a reduction in inflammatory cytokines and chemokines within wound tissue (Figure 6). We chose to examine the cytokine TNF-α since it has been shown to play an important function in the development of inflammation (37). TGF-β, on the other hand, acts as an antiproliferative factor in normal epithelial cells with potent regulatory and inflammatory activity that is context-specific (38). Finally, ICAM-1 regulates inflammation and vascular permeability that permits the excessive transfer of solutes to peripheral tissues (39). HB treatment was associated with a weak effect on TGF-β, significant suppression of ICAM-1, as well as nearly complete downregulation of TNF-α. This result has two possible explanations. On one hand, it may suggest that brassinosteroids (and more specifically, HB) have anti-inflammatory activity, however this conclusion was not supported by our preliminary data as no pro- or anti-inflammatory effect of HB on LPS-stimulated inflammation in macrophage cell culture was observed (not shown). However, another synthetic brassinosteroid analogue was shown to block NF-kB activation, reduce the secretion of TNF-α, but enhance IL-6 production in infected corneal and conjunctival cells *in vitro,* suggesting a possible direct immunomodulating effect of brassinosteroidsin certain cell types (40).

Significantly lower levels of TNF-α and ICAM-1 in the wound tissues could be also explained by the time of sampling and analysis. Since the strongest effect of HB was observed at the end of the early phase of skin wound healing when inflammatory and tissue repair stages overlap (day 4), it is possible that HB promotes wound healing by induction of the re-epithelialization phase. In this case, HB-treated wound tissues analyzed 10 days post-wounding will appear more advanced in wound healing process than the respective control samples. An interesting morphological feature that included increased volume of the wound edges and reached prominence on d 4 post wounding may also suggest that HB exerted its effect on wound healing early in the healing process (Fig. 5). This feature was absent in both negative and positive controls, and may represent enhanced epidermis re-grows from germinative cells left in the skin at the edges of the wound. This conclusion is partially supported by a prolonged activation of the Akt signaling in these tissues following the brassinosteroid application (Fig. 6B). On the other hand, application of adenosine receptor agonist CGS-21680 to the wound area typically stimulates angiogenesis, granulation tissues formation, and inflammatory vascular leakage and leukocyte accumulation resulting from increased vascularity, therefore promoting early inflammation phase unlike treatment with classic growth factors (26).

The hypothesis that HB promotes wound healing by possible stimulation of cell proliferation or migration into the wound area was further tested in the 3T3 mouse fibroblast cytotoxicity, proliferation, and scratch wound assays (24). While HB showed no cytotoxicity *in vitro* when tested up to 30 μM concentration, several brassinosteroid analogues containing either 6-aza group in the B ring of the molecule, or fluorinated substitutes in the A ring, showed weak toxicity at the highest concentrations tested (Table 1). This is in agreement with a previous study that analyzed cytotoxicity of various brassinosteroids against several human cancer cell lines despite having minimal effects on BJ human foreskin fibroblasts (41). For

example, (22R, 23R)-homocastasterone showed highest cytotoxicity (IC_{50} = 13 μM) against the T-lymphoblastic leukaemia CEM cells, while its synthetic counterpart (22S, 23S)-homocastasterone (compound 2 in this study) showed weak to no cytotoxicity below 50 μM. (22S, 23S)-homobrassinolide (HB or compound 1 in this study) had an IC_{50} of ~30 μM against CEM and RPMI 8226 cancer cells, but no cytotoxicity was observed towards the K562, A549, HeLa, and HOS cancer cell lines (41). Additionally, 24-epibrassinolide (compounds 8–9 in this study) increased the proportions of viable hybridoma mouse cells at nM concentrations (42). All four brassinosteroids tested in this study for their ability to induce cell proliferation at 5 μM, showed moderate biological activity that had no correlation to structural changes in either A or B ring of the molecule (Table 1). There was also no correlation between compound's ability to induce cell proliferation and stimulation of cell migration, as both R,R- and S,S-24-epibrassinolides promoted cell proliferation but not migration, while HB treatment resulted in significant increase in both parameters. However, there was a direct correlation between compound's ability to promote cell migration (Fig. 2–3) and to induce Akt phosphorylation reported in the previous study (19). Akt is a key enzyme in signal transduction pathways involved in cell survival, cell-cycle progression, and migration. Increasing evidence suggests that Akt may play a role in repair and collagen production by activated fibroblasts (12). Thus, while stimulating the components of the PI3K/Akt network ultimately leads to increased collagen deposition by fibroblasts and enhanced tissue repair, distinct cellular mechanisms may be involved in mediating the proliferation and migration effects.

In summary, our study shows that brassinosteroid analogues positively modulate inflammatory and re-epithelialization phases of the wound-repair process *in vivo* and *in vitro*, in partby enhancing Akt signaling in the skin at the edges of the wound and enhancing migration of fibroblasts in a wounded area *in vitro*. Brassinosteroids promote skin regeneration and, thus, may have applications in medicine and skin care. Further research is needed to address the precise underlying mechanisms of their action and to find the optimal therapeutic concentration for use in clinical practice. These results bring scientific support to potential applications of bioactive compounds from plant steroid analogues in regenerative medicine.

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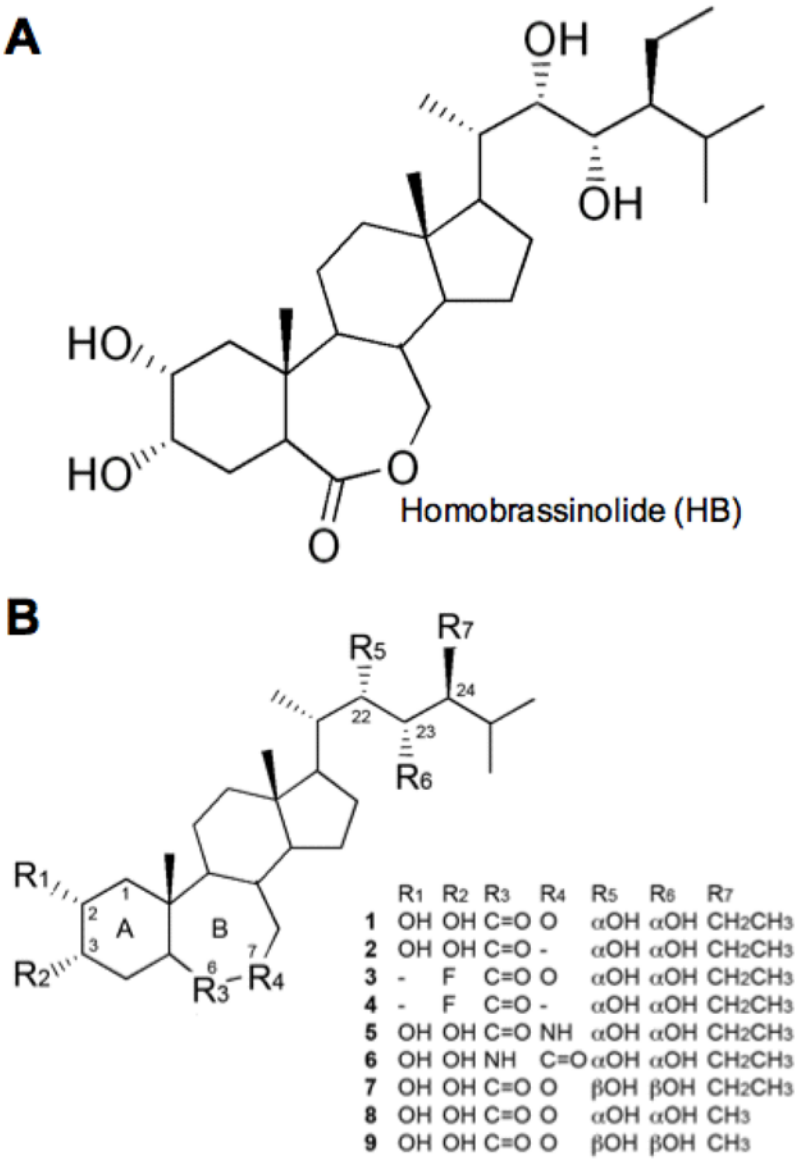
References

1. Ashcroft GS, Greenwell-Wild T, Horan MA, Wahl SM, Ferguson MW. Topical estrogen accelerates cutaneous wound healing in aged humans associated with an altered inflammatory response. Am J Pathol. 1999; 155(4):1137–46. [PubMed: 10514397]
2. Fu X, Li X, Cheng B, Chen W, Sheng Z. Engineered growth factors and cutaneous wound healing: success and possible questions in the past 10 years. Wound Repair Regen. 2005; 13(2):122–30. [PubMed: 15828936]
3. Mooney EK, Lippitt C, Friedman J. Silver dressings. Plast Reconstr Surg. 2006; 117(2):666–9. [PubMed: 16462356]
4. Khorasani G, Hosseinimehr SJ, Zamani P, Ghasemi M, Ahmadi A. The effect of saffron (Crocus sativus) extract for healing of second-degree burn wounds in rats. Keio J Med. 2008; 57(4):190–5. [PubMed: 19110531]
5. Lusby PE, Coombes A, Wilkinson JM. Honey: a potent agent for wound healing? J Wound Ostomy Continence Nurs. 2002; 29(6):295–300. [PubMed: 12439453]

6. Dipietro LA, Reintjes MG, Low QE, Levi B, Gamelli RL. Modulation of macrophage recruitment into wounds by monocyte chemoattractant protein-1. Wound Repair Regen. 2001; 9(1):28–33. [PubMed: 11350637]
7. Liechty KW, Kim HB, Adzick NS, Crombleholme TM. Fetal wound repair results in scar formation in interleukin-10-deficient mice in a syngeneic murine model of scarless fetal wound repair. J Pediatr Surg. 2000; 35(6):866–72. discussion 72–3. [PubMed: 10873028]
8. Martin P, Leibovich SJ. Inflammatory cells during wound repair: the good, the bad and the ugly. Trends Cell Biol. 2005; 15(11):599–607. [PubMed: 16202600]
9. Keylock KT, Vieira VJ, Wallig MA, DiPietro LA, Schrementi M, Woods JA. Exercise accelerates cutaneous wound healing and decreases wound inflammation in aged mice. Am J Physiol Regul Integr Comp Physiol. 2008; 294(1):R179–84. [PubMed: 18003791]
10. Asano Y, Ihn H, Yamane K, Jinnin M, Mimura Y, Tamaki K. Phosphatidylinositol 3-kinase is involved in alpha2(I) collagen gene expression in normal and scleroderma fibroblasts. J Immunol. 2004; 172(11):7123–35. [PubMed: 15153536]
11. Chen J, Somanath PR, Razorenova O, Chen WS, Hay N, Bornstein P, et al. Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. Nat Med. 2005; 11(11): 1188–96. [PubMed: 16227992]
12. Bujor AM, Pannu J, Bu S, Smith EA, Muise-Helmericks RC, Trojanowska M. Akt blockade downregulates collagen and upregulates MMP1 in human dermal fibroblasts. J Invest Dermatol. 2008; 128(8):1906–14. [PubMed: 18323784]
13. Esposito D, Komarnytsky S, Shapses S, Raskin I. Anabolic effect of plant brassinosteroid. FASEB J. 2011; 25(10):3708–19. [PubMed: 21746867]
14. Bajguz A, Tretyn A. The chemical characteristic and distribution of brassinosteroids in plants. Phytochemistry. 2003; 62(7):1027–46. [PubMed: 12591256]
15. Bajguz A. Effect of brassinosteroids on nucleic acids and protein content in cultured cells of Chlorella vulgaris. Plant Physiol Biochemy. 2000; 38(3):209–15.
16. Kartal G, Temel A, Arican E, Gozukirmizi N. Effects of brassinosteroids on barley root growth, antioxidant system and cell division. Plant Growth Regulation. 2009; 58(3):261–7.
17. Romero-Avila M, de Dios-Bravo G, Mendez-Stivalet JM, Rodriguez-Sotres R, Iglesias-Arteaga MA. Synthesis and biological activity of furostanic analogues of brassinosteroids bearing the 5alpha-hydroxy-6-oxo moiety. Steroids. 2007; 72(14):955–9. [PubMed: 17905389]
18. Andersen DL, Back TG, Janzen L, Michalak K, Pharis RP, Sung GC. Design, synthesis, and bioactivity of the first nonsteroidal mimetics of brassinolide. J Org Chem. 2001; 66(21):7129–41. [PubMed: 11597241]
19. Esposito D, Rathinasabapathy T, Poulev A, Komarnytsky S, Raskin I. Akt-dependent anabolic activity of natural and synthetic brassinosteroids in rat skeletal muscle cells. J Med Chem. 2011; 54(12):4057–66. [PubMed: 21491949]
20. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983; 65(1–2):55–63. [PubMed: 6606682]
21. Caldelari R, Suter MM, Baumann D, De Bruin A, Muller E. Long-term culture of murine epidermal keratinocytes. J Invest Dermatol. 2000; 114(5):1064–5. [PubMed: 10792572]
22. Komarnytsky S, Cook A, Raskin I. Potato protease inhibitors inhibit food intake and increase circulating cholecystokinin levels by a trypsin-dependent mechanism. Int J Obes (Lond). 2011; 35(2):236–43. [PubMed: 20820171]
23. Winer J, Jung CK, Shackel I, Williams PM. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem. 1999; 270(1):41–9. [PubMed: 10328763]
24. Schmidt C, Fronza M, Goettert M, Geller F, Luik S, Flores EM, et al. Biological studies on Brazilian plants used in wound healing. J Ethnopharmacol. 2009; 122(3):523–32. [PubMed: 19429323]
25. Bascom CC, Wolfshohl JR, Coffey RJ Jr, Madisen L, Webb NR, Purchio AR, et al. Complex regulation of transforming growth factor beta 1, beta 2, and beta 3 mRNA expression in mouse fibroblasts and keratinocytes by transforming growth factors beta 1 and beta 2. Mol Cell Biol. 1989; 9(12):5508–15. [PubMed: 2586525]

26. Valls MD, Cronstein BN, Montesinos MC. Adenosine receptor agonists for promotion of dermal wound healing. Biochem Pharmacol. 2009; 77(7):1117–24. [PubMed: 19041853]
27. Esposito D, Rathinasabapathy T, Poulev A, Komarnytsky S, Raskin I. Anabolic effect of plant brassinosteroid. Nat Chem Biol. 2011 (Submitted).
28. Zullo MAT, Adam G. Brassinosteroid phytohormones - structure, bioactivity and applications. Braz J Plant Physiol. 2002; 14(3):143–81.
29. Adler JH, Grebenok RJ. Biosynthesis and distribution of insect-molting hormones in plants--a review. Lipids. 1995; 30(3):257–62. [PubMed: 7791535]
30. Bathori M, Toth N, Hunyadi A, Marki A, Zador E. Phytoecdysteroids and anabolic-androgenic steroids--structure and effects on humans. Curr Med Chem. 2008; 15(1):75–91. [PubMed: 18220764]
31. Gilliver SC, Ashworth JJ, Ashcroft GS. The hormonal regulation of cutaneous wound healing. Clin Dermatol. 2007; 25(1):56–62. [PubMed: 17276202]
32. Hardman MJ, Ashcroft GS. Estrogen, not intrinsic aging, is the major regulator of delayed human wound healing in the elderly. Genome Biol. 2008; 9(5):R80. [PubMed: 18477406]
33. Engeland CG, Sabzehei B, Marucha PT. Sex hormones and mucosal wound healing. Brain Behav Immunol. 2009; 23(5):629–35.
34. Thiboutot D, Jabara S, McAllister JM, Sivarajah A, Gilliland K, Cong Z, et al. Human skin is a steroidogenic tissue: steroidogenic enzymes and cofactors are expressed in epidermis, normal sebocytes, and an immortalized sebocyte cell line (SEB-1). J Invest Dermatol. 2003; 120(6):905–14. [PubMed: 12787114]
35. Ashcroft GS, Mills SJ. Androgen receptor-mediated inhibition of cutaneous wound healing. J Clin Invest. 2002; 110(5):615–24. [PubMed: 12208862]
36. Efron PA, Moldawer LL. Cytokines and wound healing: the role of cytokine and anticytokine therapy in the repair response. J Burn Care Rehabil. 2004; 25(2):149–60. [PubMed: 15091141]
37. Sethi G, Sung B, Aggarwal BB. TNF: a master switch for inflammation to cancer. Front Biosci. 2008; 13:5094–107. [PubMed: 18508572]
38. Sanjabi S, Zenewicz LA, Kamanaka M, Flavell RA. Anti-inflammatory and pro-inflammatory roles of TGF-beta, IL-10, and IL-22 in immunity and autoimmunity. Curr Opin Pharmacol. 2009; 9(4):447–53. [PubMed: 19481975]
39. Sumagin R, Lomakina E, Sarelius IH. Leukocyte-endothelial cell interactions are linked to vascular permeability via ICAM-1-mediated signaling. Am J Physiol Heart Circulatory Physiol. 2008; 295(3):H969–H77.
40. Michelini FM, Berra A, Alche LE. The in vitro immunomodulatory activity of a synthetic brassinosteroid analogue would account for the improvement of herpetic stromal keratitis in mice. J Steroid Biochem Mol Biol. 2008; 108(1–2):164–70. [PubMed: 18054220]
41. Malikova J, Swaczynova J, Kolar Z, Strnad M. Anticancer and antiproliferative activity of natural brassinosteroids. Phytochemistry. 2008; 69(2):418–26. [PubMed: 17869317]
42. Franek R, Eckschlager T, Kohout L. 24-Epibrassinolide at subnanomolar concentrations modulates growth and production characteristics of a mouse hybridoma. Collect Czech Chem Commun. 2003; 68:2190–200.



	R1	R2	R3	R4	R5	R6	R7
1	OH	OH	C=O	O	αOH	αOH	CH_2CH_3
2	OH	OH	C=O	-	αOH	αOH	CH_2CH_3
3	-	F	C=O	O	αOH	αOH	CH_2CH_3
4	-	F	C=O	-	αOH	αOH	CH_2CH_3
5	OH	OH	C=O	NH	αOH	αOH	CH_2CH_3
6	OH	OH	NH	C=O	αOH	αOH	CH_2CH_3
7	OH	OH	C=O	O	βOH	βOH	CH_2CH_3
8	OH	OH	C=O	O	αOH	αOH	CH_3
9	OH	OH	C=O	O	βOH	βOH	CH_3

Figure 1.
Chemical structure of (A) 28-homobrassinolide, HB in comparison with (B) other synthetic and natural brassinosteroid analogues used in this study.

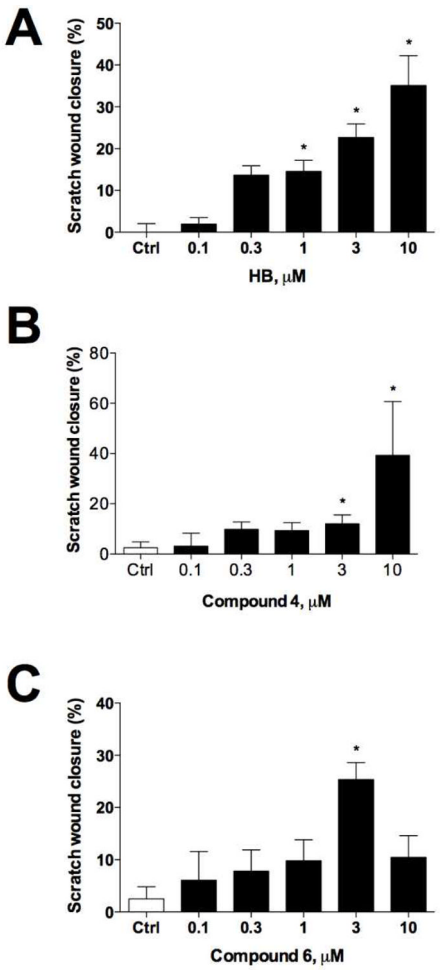


Figure 2. Dose dependent effect of brassinosteroid treatment on scratch wound closure *in vitro*

3T3 Swiss fibroblast monolayers were scratched with a sterile pipette tip and vehicle (0.1% ethanol), FBS (1%, positive control), or various concentrations of (**A**) HB, (**B**) (22S, 23S)-3α-fluoro-homocastasterone, or (**C**) (22S,23S)-7-aza-homobrassinolide were added to set of 3 wells per dose and incubated for 12 h. The data represent the average of 2 experiments ± SE. * $P<0.05$; ** $P<0.01$ (n=3) using one-way ANOVA and Dunnett's post-test.

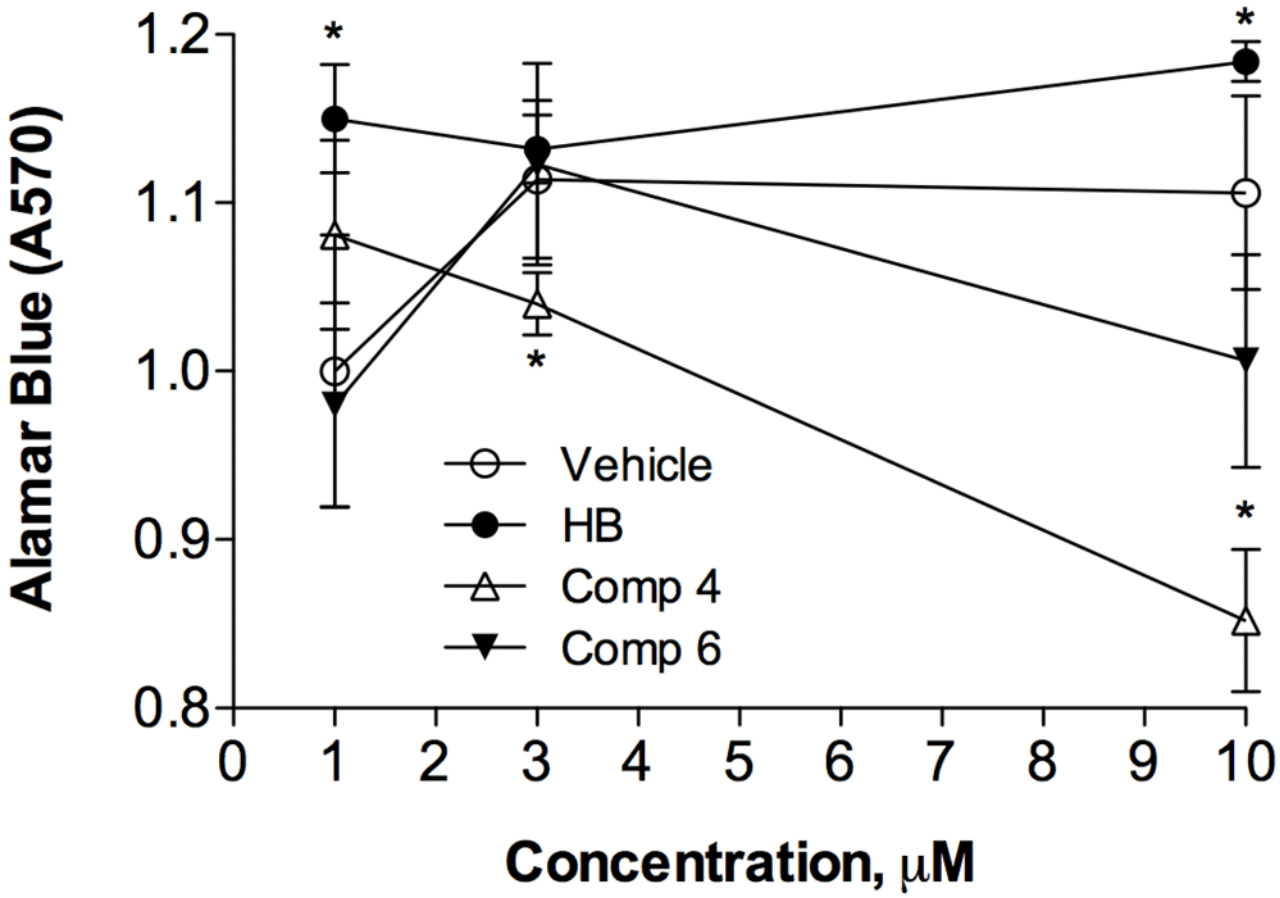


Figure 3. Proliferation of primary keratinocytes in response to brassinosteroids

The effect of brassinosteroids on the proliferation of primary mouse keratinocytes was determined using Alamar Blue dye. Cells were treated with respective doses of homobrassinolide (HB), compounds 4 (Comp 4) and 6 (Comp 6). Control cells were treated with vehicle alone (Vehicle). The reduction of the Alamar Blue dye, which is a measure of proliferation, was determined spectrophotometrically at 570 nm at the end of the 48-hour period. *P < 0.05.

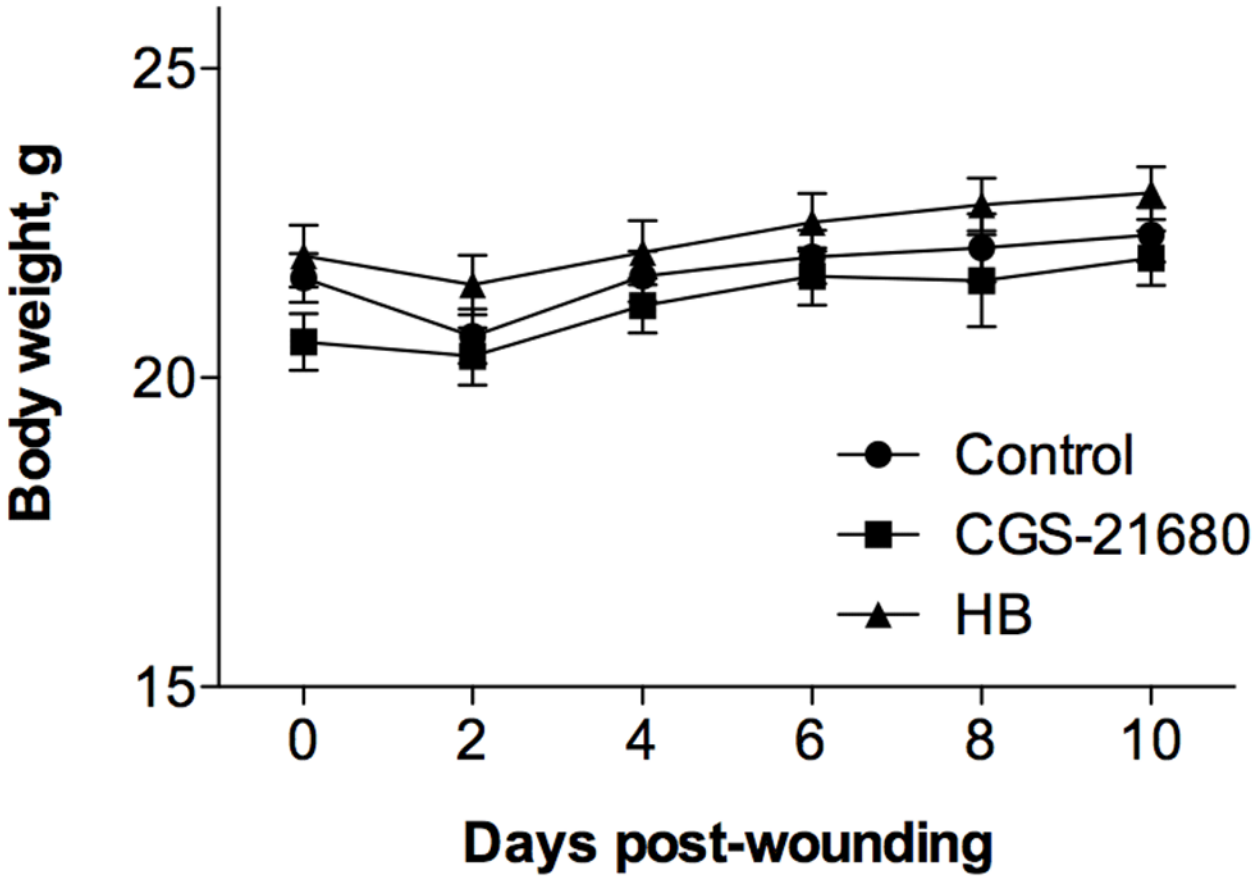


Figure 4. Effect of HB treatment on body weight change associated with wounding

A sharp punch over lumbar spine was applied to remove the skin and vehicle, or 10 μg/mouse of either HB or positive control CGS-21680 was administered topically daily for 10 d. Two-factor repeated-measures ANOVA, *P<0.05 (n=9).

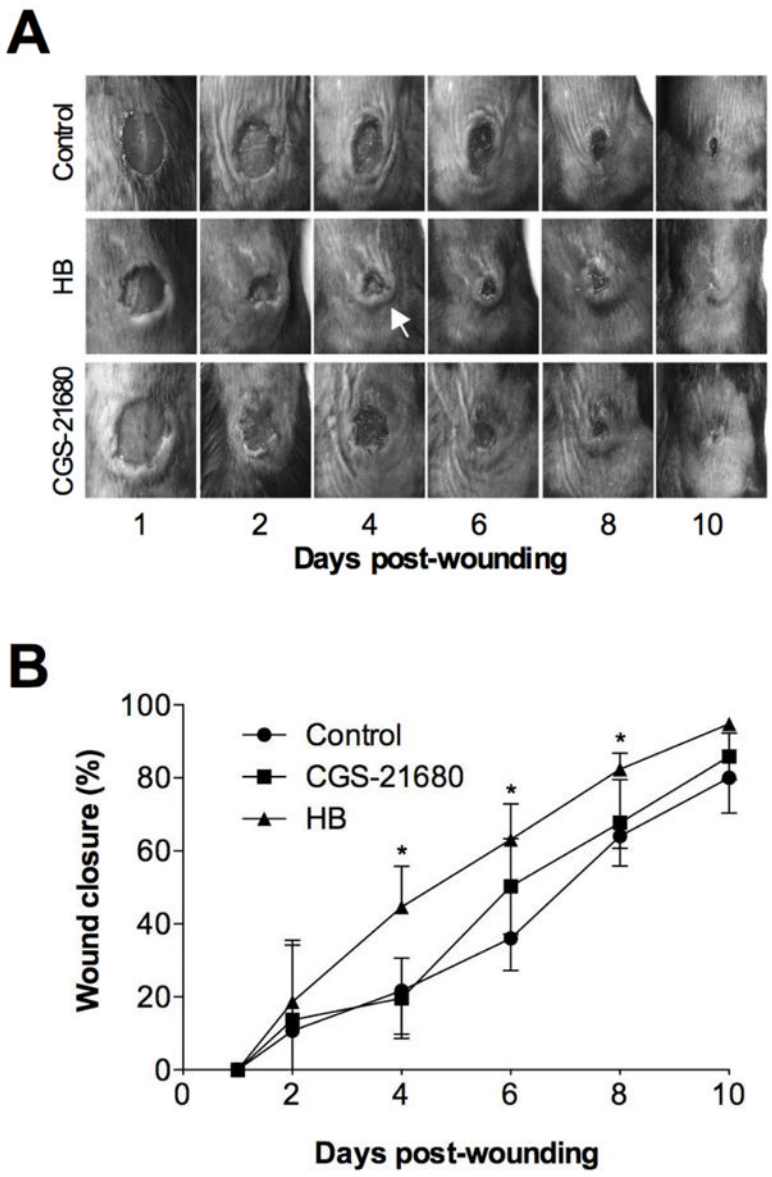


Figure 5. Time course of wound healing in mouse cutaneous injury model

(**A**)Wound sizes were photographed and measured every 2 d for 10 d. (**B**) The wound closure (%) relative to d 1 was determined every 2 d, and the wound half-closure time (CT_{50}) was calculated by linear regression. Two-factor repeated-measures ANOVA, *P<0.05 (n=9).

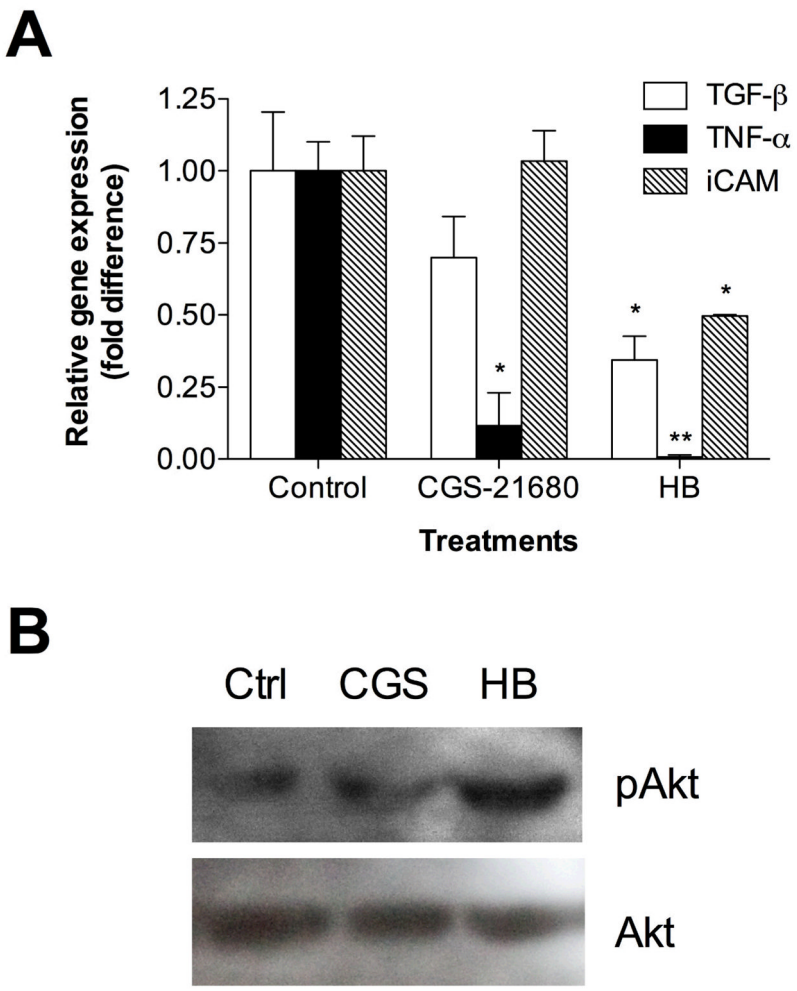


Figure 6. Effect of HB on cytokine and chemokine mRNA expression and Akt signaling in wounds of C57Bl/6J mice

(**A**)RNA was isolated from wound tissues collected 10 d post-wounding and mRNA levels for proinflammatory cytokines TNF-α, TGF-β, and an adhesion chemokine ICAM-1 were measure by qPCR. The target gene expression of the housekeeping gene (actin) was assigned a value of 1. *p<0.05, **p<0.01 significantly different from vehicle controls, one-way ANOVA with Dunnett's post-hoc test. (**B**) Activation of Akt phosphorylation in the wounds treated with vehicle (Ctr), positive control CGS-21680 (CGS), or HB. Edge-of-the-wound tissue lysates were analyzed by immunobloting with phospho- and nonphospho-specific Akt antibodies.

Table 1

Effect of HB [1] and its analogues [2–9] on cell liability, proliferation, and scratch wound closure in 3T3 mouse fibroblasts.

ID	Common name	Scratch wound closure at 5 μM, %	Cell proliferation at 5 μM, % of control	Cell cytotoxicity, IC
1	(22S,23S)-homobrassinolide (HB)	30 ± 4.2*	37.7 ± 3.4*	>30
2	(22S,23S)-homocastasterone	32.9 ± 4.1*	Nt	>30
3	(22S,23S)-3α-fluoro-homobrasinolide	15.3 ± 2.6	Nt	~30
4	(22S,23S)-3α-fluoro-homocastasterone	27.7 ± 4.0*	Nt	~30
5	(22S,23S)-6-aza-homobrassinolide	13.0 ± 0.7	Nt	12.5
6	(22S,23S)-7-aza-homobrassinolide	30.5 ± 4.2*	Nt	>30
7	(22R,23R)-homobrassinolide	16.5 ± 0.5	29.1 ± 3.4*	>30
8	(22S,23S)-epibrassinolide	13.2 ± 1.5	31.9 ± 5.3*	>30
9	(22R,23R)-epibrassinolide	8.1 ± 1.6	31.0 ± 2.6*	>30
Ref	FBS, 1%	41.5 ± 6.5*	39.3 ± 5.7*	--
Ref	PDGF, 2 nM (24)	64.8 ± 1.7*	--	--

Results are expressed as the mean ± SEM of determinations performed in triplicate (* $P<0.05$ when compared with control by one-way ANOVA and Dunnett's post-test). FBS (fetal bovine serum) and PDGF (platelet-derived growth factor) are shown as reference treatments. Nt, not tested.