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Short communication

Promotion of skin epithelial cell migration and wound healing by a 2-benzazepine derivative

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Abstract

Re-epithelialization is an important event in the healing of skin wounds. We have now shown that a 2-benzazepine derivative, *N*-(2,2,2-trifluoroethyl)-8-methoxy-4-methyl-2-benzazepin-3-one (compound A), facilitated the migration of human keratinocyte HaCat cells in an in vitro model of wound healing and inhibited the attachment of these cells to a collagen matrix. Topical application of compound A also promoted the healing of skin wounds in mice. Our results suggest that compound A promotes the repair of skin wounds by facilitating epithelial cell migration and that this 2-benzazepine derivative is a potential new drug for the treatment of such wounds.

Keywords: Skin; Wound healing; 2-Benzazepine; HaCat cell; Cell migration; Re-epithelialization

1. Introduction

The healing of skin wounds is a complex process that involves formation of a clot, an inflammatory response, accumulation of granulation tissue, as well as deposition and remodeling of extracellular matrix (Clark, 1996). It also requires the interaction of cells in the epidermis and dermis as well as mediators released from inflammatory cells, fibroblasts, and keratinocytes (Martin, 1997). Re-epithelialization by keratinocytes, which is achieved by migration and mitosis of cells in the epidermis proximal to the wound margin, is a central event in wound healing (Santoro and Gaudino, 2005). The many factors that influence wound healing include growth factors, cytokines, metalloproteinases, and extracellular matrix proteins (Coulombe, 2003; Martin, 1997; Santoro and Gaudino, 2005), and the exogenous application of some of these factors has been shown to be beneficial for the healing process (Werner and Grose, 2003).

Benzazepine consists of fused benzene and azepine rings. Many compounds that contain this structure interact with receptors for biogenic amines and exhibit a variety of pharmacological effects including antipsychotic (Iorio et al., 1983) and anticancer (Xia et al., 2000) actions. The 2-benzazepine derivatives SB223245 (Keenan et al., 1997) and (*S*)-3-oxo-8-[2-[6-(methylamino)-pyridin-2-yl]-1-ethoxy]-2-(2,2,2-trifluoroethyl)-2,3,4,5-tetrahydro-1*H*-2-benzazepine-4-acetic acid (compound 1) (Miller et al., 2000) were synthesized as nonpeptide Arg-Gly-Asp (RGD) mimetic antagonists of the vitronectin receptor ($\alpha v\beta 3$ integrin). These compounds inhibit integrin $\alpha v\beta 3$-mediated cell adhesion as well as osteoclast-mediated bone resorption in vitro (Lark et al., 1999; Lark et al., 2001; Miller et al., 1999; Miller et al., 2000). They also inhibit bone resorption in the acute thyroparathyroidectomized rat model of osteoporosis (Lark et al., 1999; Lark et al., 2001). We now show that one of two 2-benzazepine derivatives examined promoted not only re-epithelialization in a scratch wound assay performed in vitro with the spontaneously transformed, nontumorigenic human keratinocyte cell line HaCat (Boukamp et al., 1988) but also the healing of mouse skin wounds in vivo. Our results suggest that 2-benzazepine derivatives are potential new drugs for treatment of skin wounds.

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2. Materials and methods

2.1. Synthesis of 2-benzazepines

Two 2-benzazepines were synthesized as described previously (Kamimura et al., 2003). In brief, a solution of Bu_3SnH and α,α′-azobisisobutyronitrile in benzene was added to a solution of *N*-(2-bromo-5-methoxybenzyl)-*N*′-(2,2,2-trifluoroethyl)methacryl amide in benzene at 80 °C over 6 h and under a nitrogen atmosphere. The reaction mixture was then stirred at the same temperature for an additional 6 h. Benzene was removed from the mixture under reduced pressure, and the crude product was purified by flash chromatography (with hexane followed by hexane–ethyl acetate) to yield *N*-(2,2,2-trifluoroethyl)-8-methoxy-4-methyl-2-benzazepin-3-one (compound A) and *N*-butyl-8-methoxy-4-(methoxycarbonyl)methyl-2-benzazepin-3-one (compound B). Both compounds were dissolved in dimethyl sulfoxide (DMSO) for experiments.

2.2. In vitro wound healing assay

HaCat cells (2.5×10^4 per well) were seeded in a 96-well plate and grown until confluent. Each monolayer was scratched with the use of a 200-μl pipette tip to generate a cell-free zone (0.8 to 1 mm in width). After extensive washing with Dulbecco's modified Eagle's medium (DMEM), the cells were incubated for 24 h at 37 °C with DMEM containing various concentrations (0 to 100 μM) of compounds A or B (final DMSO concentration of 0.1%). The cells were photographed immediately after wounding, the wounded area was marked on the base of the plate, and the same field was photographed again after incubation for 24 h. The migration of cells into the wound area was evaluated. A similar assay was also performed with human epidermal keratinocytes from neonatal foreskin (Kurabo, Neyagawa, Japan) cultured in HuMedia-KB2 medium (Kurabo) instead of DMEM.

2.3. Assay of cell attachment

The wells of a 96-well half-area plate were coated overnight at 4 °C with type I collagen (0.1 mg/ml) (Sigma). HaCat cells (2.5×10^3 per well) were added to the plate in RPMI 1640 medium containing various concentrations of compounds A or B. After incubation for 1 h at 37 °C, the cells were washed three times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, stained with crystal violet, and counted with the use of a microscope.

2.4. In vivo wound healing assay

All procedures involving animals were performed in accordance with the National Institutes of Health guidelines and were approved by the Animal Ethics Committee of Yamaguchi University. Three full-thickness excisional wounds

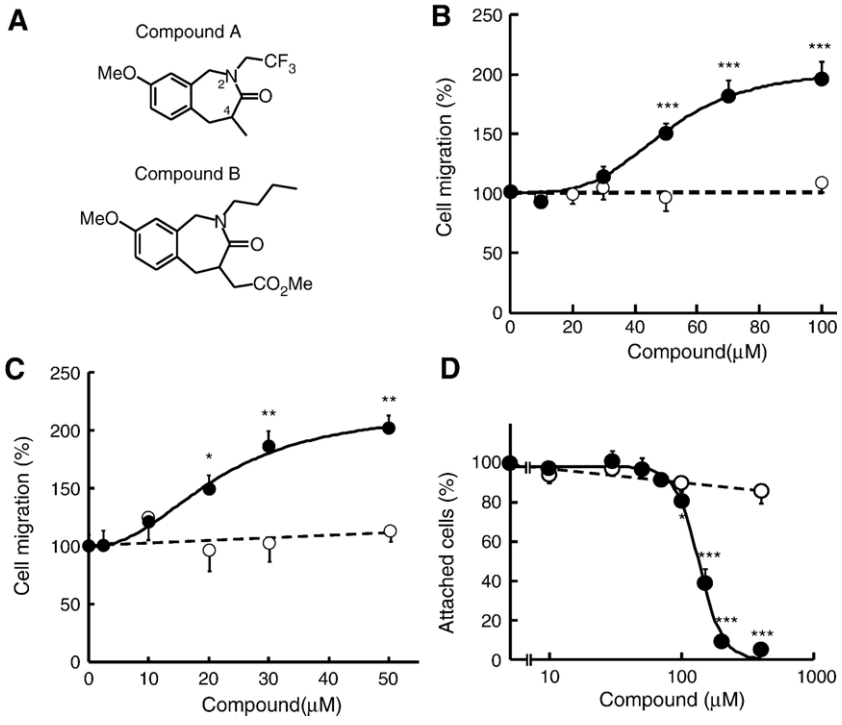

Fig. 1. Effects of compounds A and B on migration and attachment to collagen of HaCat cells or human epidermal keratinocytes. (A) Structure of compounds A and B. (B, C) Confluent cultures of HaCat cells (B) or human epidermal keratinocytes (C) were wounded and then incubated for 24 h at 37 °C with the indicated concentrations of compound A (closed circles) or compound B (open circles). The extent of cell migration into the wound area was then determined and expressed as a percentage of that for cultures incubated with vehicle (control). Data are means ± S.E.M. of eight (B) or six (C) independent determinations. *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus control (Student's *t* test). (D) HaCat cells were plated on collagen-coated wells and incubated for 1 h at 37 °C with the indicated concentrations of compound A (closed circles) or compound B (open circles). The number of attached cells was then counted and expressed as a percentage of the value for cells incubated with vehicle (control). Data are means ± S.E.M. of six independent determinations. *$P<0.05$, ***$P<0.001$ versus control (Student's *t* test).

(5 mm in diameter) were made with biopsy punches through the skin on the back of 6- to 8-week-old BALB/c mice that had been anesthetized with diethyl ether. The wounds were treated twice a day by topical application of 30 μl of compounds A or B (50 μM) or vehicle (0.1% DMSO in PBS). A digital image of each wound together with a calibration scale was recorded with a digital camera and stored on a personal computer in JPEG format. The open wound area was determined by image analysis with ImageJ 1.32i software (National Institutes of Health, Bethesda, MD). For histological analysis, mice were killed and the wounded tissue was excised together with a margin of ~10 mm, fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned. Sections were stained with hematoxylin–eosin.

3. Results

3.1. Effects of 2-benzazepines on migration of HaCat cells

In assays of potential biological activity of 2-benzazepine derivatives, we found that one of two such derivatives synthesized, *N*-(2,2,2-trifluoroethyl)-8-methoxy-4-methyl-2-benzazepin-3-one (compound A) (Fig. 1A), promoted the migration of human skin epithelial HaCat cells in an in vitro model of skin re-epithelialization (a scratch assay). Compound A thus increased the extent of migration of HaCat cells in a concentration-dependent manner (Fig. 1B), with this effect being half-maximal at 50 μM as determined by curve fitting. The other 2-benzazepine derivative, *N*-butyl-8-methoxy-4-

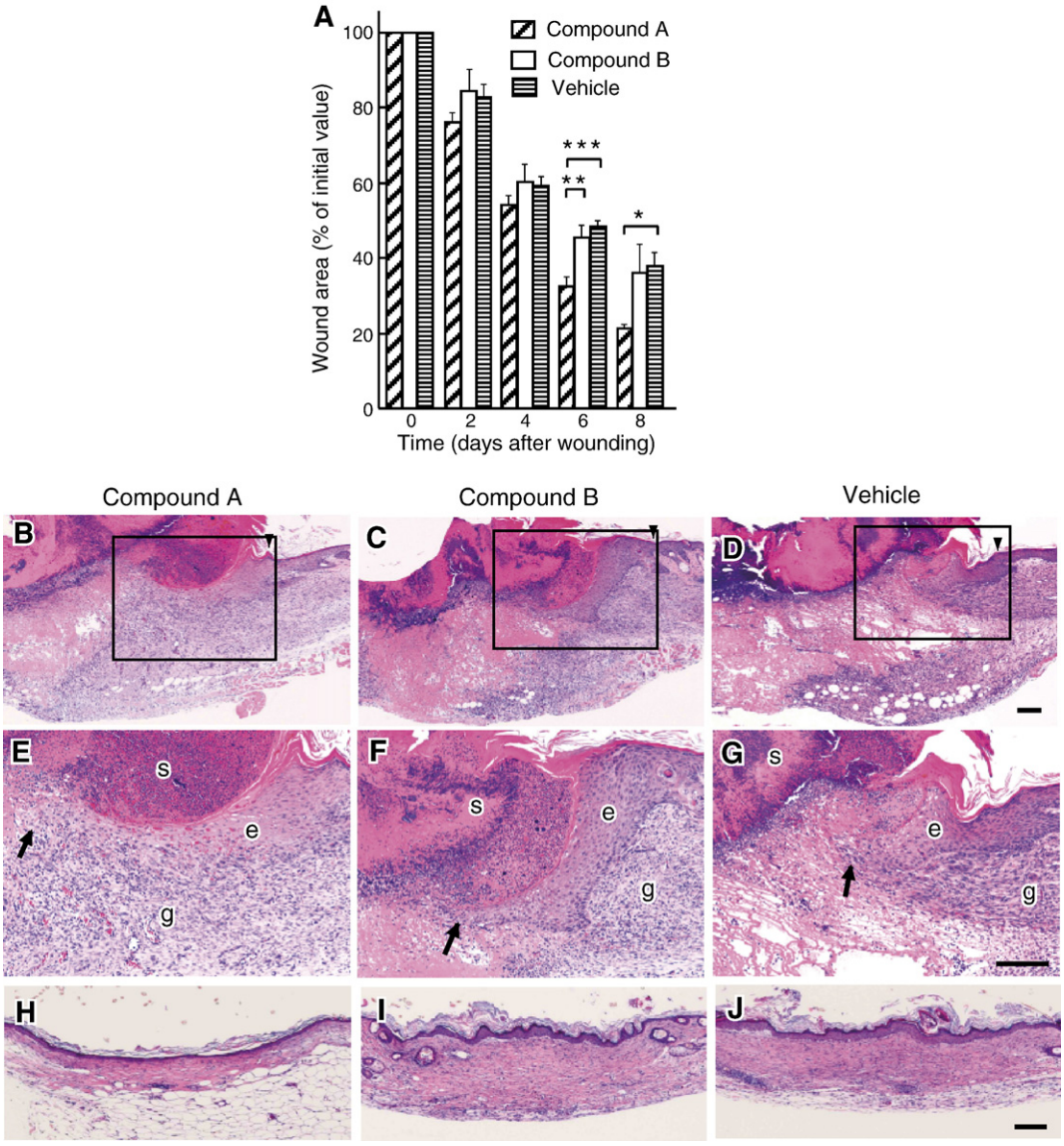

Fig. 2. Effects of compounds A and B on wound closure in vivo. (A) Full-thickness excisional wounds (5 mm in diameter) on the back of mice were treated twice a day by topical application of 30 μl of compounds A or B at 50 μM or vehicle. The open wound area was determined at the indicated times after injury and expressed as a percentage of the initial value. Data are means±S.E.M. of values from six to nine mice. *$P<0.05$, **$P<0.005$, ***$P<0.001$ for the indicated comparisons (Dunnett's test). (B–J) Sections of wounded tissue obtained from mice treated with compound A (B, E, H), compound B (C, F, I), or vehicle (D, G, J) for 7 days (B–G) or 19 days (H–J) after wounding were stained with hematoxylin–eosin. Boxed regions in (B) through (D) are shown enlarged in (E) through (G), respectively. Arrowheads and arrows indicate the original wound margin and the leading front of keratinocytes, respectively. e, epithelium; g, granulation tissue; s, scab. Bars, 100 μm.

(methoxycarbonyl)methyl-2-benzazepin-3-one (compound B) (Fig. 1A), had no effect on HaCat cell migration (Fig. 1B). Similar results were obtained with normal human epidermal keratinocytes with the effect of compound A being half-maximal at 22 μM (Fig. 1C). The stimulatory effect of compound A on cell migration was not due to enhancement of mitosis, given that neither compound A nor compound B at a concentration of 50 μM had a significant effect on the proliferation of HaCat cells (data not shown).

3.2. Effects of 2-benzazepines on HaCat cell attachment

We next examined the effects of these compounds on the attachment of HaCat cells to collagen. Compound A inhibited cell attachment to a collagen-coated plate in a concentration-dependent manner (Fig. 1D), with half-maximal inhibition apparent at a concentration of 135 μM as determined by curve fitting. Compound B had no effect on HaCat cell attachment at concentrations up to 400 μM (Fig. 1D).

3.3. Effects of 2-benzazepines on wound healing in vivo

We then examined the possible effects of the 2-benzazepines on the healing of skin wounds in vivo. Each compound was applied twice a day directly to circular full-thickness wounds on the backs of mice. Wound closure was significantly promoted in mice treated with compound A compared with that apparent in mice treated with vehicle (Fig. 2A). The wound area remaining open in the mice treated with compound A was 76, 54, 32, and 21% of the initial wound area after 2, 4, 6, and 8 days, respectively, whereas the corresponding values for vehicle-treated mice were 83, 59, 49, and 38%. The differences were statistically significant at 6 and 8 days after injury. Topical application of compound B did not affect wound closure (Fig. 2A).

Histological examination revealed that the healing of skin wounds, including infiltration of inflammatory cells, establishment of granulation tissue, and re-epithelialization, proceeded normally in mice treated with vehicle, compound A, or compound B (Fig. 2). At 7 days after wounding, the area of re-epithelialization was markedly increased in the wounds treated with compound A (Fig. 2B, E) compared with that in those treated with vehicle (Fig. 2D, G) or compound B (Fig. 2C, F). The wounds treated with compound A also established granulation tissue earlier than did those treated with vehicle or compound B. Immunostaining of Ki-67 revealed that the number of proliferative epithelial cells was not significantly changed in the wounds treated with compound A at 7 days after wounding compared with those treated with vehicle or compound B (data not shown). At 19 days after injury, when closure of all wounds was complete, the wounds treated with compound A appeared to have almost fully healed (Fig. 2H), although hair follicles were absent in the wound area. In contrast, the epidermis remained thicker and the dermis more abundant in the wound area of mice treated with compound B or vehicle (Fig. 2I, J), compared with those of mice treated with compound A. Healing was thus accelerated in the mice treated with compound A.

4. Discussion

We have shown that a 2-benzazepine derivative (compound A) promotes the healing of skin wounds in mice. With the exception of the difference in healing rate, the pattern of wound healing in mice treated with compound A was indistinguishable from that in mice treated with vehicle. Compound A also facilitated the migration of HaCat cells in a scratch assay in vitro at concentrations similar to that shown to be effective in promotion of wound healing in vivo. In addition, compound A inhibited the attachment of HaCat cells to a collagen matrix, although this effect was apparent at higher concentrations. Compound A did not have a significant effect on the proliferation of HaCat cells in vitro. Treatment with compound A did not significantly increase the number of proliferating epithelial cells at 7 days after wounding, although indirect effects on the proliferation cannot be ruled out at the earlier stages of the wound healing. Our results thus suggest that compound A promotes the repair of skin wounds through facilitation of epithelial cell migration and that this 2-benzazepine derivative is a potential new drug for the treatment of skin wounds.

Another 2-benzazepine derivative, compound B, did not manifest activities similar to those of compound A in vitro or in vivo, indicative of a high structural specificity for the effects of the latter compound. Comparison of the structures of compounds A and B (Fig. 1A) suggests that the functional groups at the N2 and C4 positions are important for the promotion of wound healing. Our data indicate that compound B is an appropriate negative control for studies of the mechanism by which compound A promotes epithelial cell migration and wound healing.

Biological activities have been previously demonstrated for two 2-benzazepine derivatives, SB223245 (Keenan et al., 1997; Lark et al., 1999) and compound 1 (Lark et al., 2001; Miller et al., 2000), although the possible effects of these compounds on the healing of skin wounds have not been described. Compound 1 was shown to inhibit bone resorption in vivo with a median effective circulating concentration of ~20 μM (Lark et al., 2001), similar to the concentration of compound A (50 μM) found to be effective in the promotion of wound healing in the present study. Both SB223245 and compound 1 mimic the RGD peptide sequence by specifically binding to αvβ3 and αvβ5 integrins (Keenan et al., 1997; Miller et al., 2000). Various integrins, including αvβ3, αvβ5, α3β1, and α6β4, contribute to re-epithelialization during wound healing (Nguyen et al., 2000; Santoro and Gaudino, 2005). Expression of the αv subunit was shown to be markedly increased and largely restricted to the basal region of basal keratinocytes during re-epithelialization of skin wounds in humans (Cavani et al., 1993). These observations suggest that the pharmacological target of compound A might also be αvβ3 or αvβ5 integrin expressed by keratinocytes during wound healing. The molecular size of compound A is about half that of SB223245 or compound 1, however. In addition, whereas these latter two compounds mimic the RGD peptide sequence (Keenan et al., 1997; Miller et al., 2000), compound A corresponds only to

Gly-Asp of this sequence. Furthermore, compound A lacks the carboxyl group corresponding to that of the Asp residue. Compound A might thus not be an effective mimic of the RGD peptide sequence. Further studies are thus necessary to elucidate the mechanism by which compound A promotes the healing of skin wounds.

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