Dracorhodin perchlorate enhances wound healing via β-catenin, ERK/p38, and AKT signaling in human HaCaT keratinocytes

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Abstract. Dracorhodin can be isolated from the exudates of the fruit of *Daemonorops draco*. Previous studies suggested that dracorhodin perchlorate can promote fibroblast proliferation and enhance angiogenesis during wound healing. In the present study, the potential bioactivity of dracorhodin perchlorate in human HaCaT keratinocytes, were investigated *in vitro*, with specific focus on HaCaT wound healing. The results of *in vitro* scratch assay demonstrated the progressive closure of the wound after treatment with dracorhodin perchlorate in a time-dependent manner. An MTT assay and propidium iodide exclusion detected using flow cytometry were used to detect cell viability of HaCaT cells. Potential signaling pathways underlying the effects mediated by dracorhodin perchlorate in HaCaT cells were clarified by western blot analysis and kinase activity assays. Dracorhodin perchlorate significantly increased the protein expression levels of β-catenin and activation of AKT, ERK and p38 in HaCaT cells. In addition, dracorhodin perchlorate did not induce HaCaT cell proliferation but promoted cell migration. Other mechanisms may yet be involved in the dracorhodin perchlorate-induced wound healing process of human keratinocytes. In summary, dracorhodin perchlorate may serve to be a potential molecularly-targeted phytochemical that can improve skin wound healing.

Introduction

Dragon blood is a type of deep-red resin that has been used as a form of traditional medicine worldwide since ancient times (1). Families of plants with the ability to produce dragon blood can be classified into four different categories: Asparagaceae, Arecaceae, Chamaesyce and Fabaceae (Table I) (2-4). The earliest record of dragon blood being used for medicine was performed using the plant genus *Dracaena* in Socotra Island in Yemen (5). Due to being situated in ideal location and the rapid growth rate of the palm tree species *Daemonorops draco*, dragon blood extracted from this tree became the mainstream product in the traditional Chinese medicine (TCM) market after the Ming Dynasty in China (6). Therefore, red resin from *Daemonorops draco* is recognized as authentic dragon blood in the official documents of China, Hong Kong and Taiwan (7-9). In particular, dracorhodin has become the reference standard of dragon blood from the palm tree *D. draco* (10), where the content of dracorhodin in dragon blood for medical use is recommended to be >1% (9).

Dragon blood has been included in recipes for wound treatment in various ancient traditional Chinese medical guides of the 14-18th century (6). Accumulating evidence has also indicated that dragon blood possesses wound healing activities

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both *in vivo* and *in vitro* (1,11-13). Accordingly, a previous study showed that the herbal complex 'Jinchuang Ointment' can successfully treat non-healing wounds on patients with diabetes, where dragon blood from *D. draco* is one of the components (2.1%) in this ointment (14).

Dracorhodin perchlorate is a synthetic chemical analog of dracorhodin (Fig. 1) (10,15-18) that has been shown to exhibit angiogenic activity on human umbilical vein endothelial cells (10,19). Recently, both dracorhodin perchlorate and methanol extracts of crude dragon blood have demonstrated *in vivo* pro-angiogenic activity in zebrafish embryos (10). Additionally, previous studies have revealed bioactivities of dracorhodin perchlorate in human cancer cells, where it can induce apoptotic cell death in different types of cancer cells, including U87MG and T98G glioma cells (20), SK-MES-1 human lung squamous carcinoma cells (21), MCF-7 human breast cancer cells (22), SGC-7901 human gastric tumor cells (23), PC-3 human prostate cancer cells (18), HL-60 leukemia cells (16), A375-S2 human melanoma cells (24) and HeLa human cervical cancer cells (15,25). Furthermore, dracorhodin perchlorate was reported to promote NIH-3T3 fibroblast cell proliferation *in vitro* and induce rat wound healing *in vivo* (26). Dracorhodin perchlorate has also been shown to accelerate skin wound healing in Wistar rats *in vivo* (27). However, the effects of dracorhodin perchlorate on wound healing in human HaCaT keratinocytes remain poorly understood. Therefore, in the present study the underlying mechanism of the wounding healing regulation and associated signaling pathways mediated by dracorhodin perchlorate in HaCaT keratinocytes was investigated *in vitro*.

Materials and methods

Reagents and chemicals. DMEM, FBS, penicillin/strep-tomycin and L-glutamine were purchased from HyClone, Cytiva. All primary antibodies and anti-mouse/-rabbit immunoglobulin IgG HRP-linked secondary antibodies were procured from GeneTex International Corporation. Reagent grade DMSO, thiazolyl blue tetrazolium bromide (MTT), PBS, propidium iodide (PI), wortmannin (an AKT inhibitor), U0126 (an ERK inhibitor) and SB203580 (a p38 inhibitor), were obtained from Sigma-Aldrich, Merck KGaA unless otherwise specified. Dracorhodin perchlorate (batch no. 110811-201506; purity, 98.6%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), which was dissolved in DMSO and stored at -20˚C freezer before use. The vehicle control was kept below 0.1% DMSO in culture medium.

Cell culture. Human skin HaCaT keratinocytes were obtained from CLS Cell Lines Service GmbH and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine in a humidi-fied atmosphere at 37˚C in 5% CO_2/95% air as previously described (28,29). HaCaT cells were cultured until passage 40 (20 weeks) and did not exceed 40 generations.

Dynamic cell migration assay. HaCaT cells ($1x10^4$ cells/well) were seeded into a 96-well plate overnight before the wound area (700-800-μm wide) was created using Incucyte 96-Well Woundmaker Tool (cat. no. 4563; Essen BioScience). The scratched cells were then exposed to 0, 1 and 2 μg/ml dracorhodin perchlorate in serum-free DMEM (HyClone; Cytiva). The cell migration experiment was conducted over 24 h with data collection every 3 h at 37˚C. The cell images and wound width were monitored using the IncuCyte ZOOM System instrument by light microscopy (magnification, x100) [(Incucyte S3 Live-Cell Analysis System and Incucyte Scratch Wound Analysis Software Module (cat. no. 9600-0012; Essen BioScience)] as previously described (30,31).

Cell viability assay. HaCaT cells ($2.5x10^5$ cells/well) were plated into 24-well plates and treated with or without 0.5, 1 and 2 μg/ml dracorhodin perchlorate for 24 h at 37˚C. Cell viability was determined using MTT assay as previously described (32-34). In brief, MTT solution (0.5 mg/ml) was added to each well for 2 h at 37˚C before the blue formazan crystals were dissolved in DMSO (500 μl/well) by constant shaking for 10 min. The absorbance of each well was measured using an ELISA plate reader at a test wavelength of 570 nm with a reference wavelength of 620 nm.

For the PI exclusion assay, $2.5x10^5$ cells/well were harvested and plated into 24-well plates and resuspended in 500 μl of 5 μg/ml PI for 5 min at 4˚C. The numbers of viable cells and dead cells were determined using a BD FACSCalibur™ Flow Cytometry System and BD CellQuest Pro Software version 6.0 (both BD Biosciences), where the PI dye solution was applied to specifically stain dead cells. Cell viability was calcu-lated as follows: % Cell viability = (PIem of test)/(PIem of control) x100%. Where, PIem = gated cells of PI fluorescence emission ($<10^2$ fluorescence).

Western blot analysis. HaCaT cells ($5x10^6$ cells per 75T flask) were incubated with or without 1 and 2 μg/ml dracorhodin perchlorate for 12 h at 37˚C. Cell samples were lysed in Trident RIPA Lysis Buffer (GeneTex International Corporation) and collected as previously described (35,36). Protein concentration was determined using the Pierce bicin-choninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of the protein sample (40 μg) were prepared, and 10-12% SDS-PAGE was performed. Proteins were transferred onto an Immobilon-P polyvinylidene difluoride transfer membrane prior to blocking with Trident Universal Protein Blocking Reagent (GeneTex International Corporation) for 1 h at room temperature. The membrane was subsequently incubated with primary antibodies against β-catenin (cat. no. GTX101435), phosphorylated (p)-AKT (Ser473) (cat. no. GTX28932), AKT (cat. no. GTX121937), p-ERK (cat. no. GTX59568), ERK (cat. no. GTX59618), p38 (cat. no. GTX110720), p-p38 (cat. no. GTX48614) and β-actin (cat. no. GTX109639) at a dilution of 1:1,000 at 4˚C overnight. The membranes were then incubated with the appropriate anti-mouse (cat. no. GTX213111-01) and anti-rabbit (cat. no. GTX213110-01) IgG HRP-linked secondary anti-bodies at a dilution of 1:10,000 for 1 h at room temperature. Blot visualization was performed using the Immobilon Western Chemiluminescent HRP Substrate (Merck KGaA) and all bands of immunoblots were normalized to the densi-tometric value of β-actin. These experiments were conducted in duplicate. The bands were quantified by densitometry using

Table I. Plant species that contain dragon blood and their respective geographical origins (4).

Plant family	Plant species	Origin
Asparagaceae	*Dracaena cinnabari*	Socotra Island, Yemen
	Dracaena draco	Canary Islands, Spain
	Dracaena cochinchinensis	Yunnan Province, China
Arecaceae	*Daemonorops draco*	Indonesia and Malaysia
Chamaesyce	*Croton salutaris*	
	C. lechleri	Amazon basin
	C. draconoides	
Fabaceae	*Pterocarpus draco*	India
	P. marsupium	

ImageJ software version 1.41 (National Institutes of Health) as previously described (33,37-39).

Sandwich ELISA assay for p-protein kinases. HaCaT cells ($5x10^6$ cells per T75 flask) were treated with or without 0.5, 1 and 2 μg/ml dracorhodin perchlorate for 12 h at 37°C. The cells were harvested and total proteins were collected using Trident RIPA Lysis Buffer (GeneTex International Corporation). The samples were incubated in microwells coated with the appropriate antibody for 2 h at 37°C (for p-ERK) or overnight at 4°C (for p-Akt) according to the manufacturer's protocols of the PathScan Sandwich ELISA kits [p-Akt (Thr308; cat. no. 7252C) and p-ERK (Thr202/Tyr204; cat. no. 7177C); Cell Signaling Technology, Inc.]. The secondary antibodies and the reagents used for the chemical reactions were provided in these kits. Absorbance was measured using an ELISA reader (Anthos 2001; Anthos Labtec Instruments GmbH) at a wavelength of 450 nm, as previously described (38-40).

Wound healing assay. HaCaT cells were placed in a six-well tissue culture plates for 24 h at 37°C and cultured to 90% confluence. Individual wells were then scratched with a 200-μl micropipette tip to create a denuded zone of constant width (1 mm). Cells were then cultured in serum-free DMEM (HyClone; Cytiva) and incubated with or without dracorhodin perchlorate (1 μg/ml) or with specific protein kinase inhibitors [10 μM wortmannin (AKT inhibitor), 10 μM U0126 (ERK inhibitor) or 10 μM SB203580 (p38 inhibitor)] and 0.1% DMSO as a control for 24 h at 37°C. To determine cell migration, cell images were captured after 24 h treatment under phase-contrast microscopy (magnification, x100; Leica DMIL microscope; type 090-135.001; Leica Microsystems GmbH), as previously described (38). Image analysis was performed by using AxioVision LE64 software (version 4.9.1.0; Carl Zeiss AG). The ability of cell migration was calculated as follows: Cell migration (% of control) = [Gw (0 h) of test-Gw (24 h) of test]/[Gw (0 h) of control-Gw (24 h) of control] x100%. Where, Gw (0 h) = gap width at 0 h, and Gw (24 h) = gap width at 24 h.

Statistical analysis. All results are expressed as the mean ± standard deviation (three experimental repeats per assay). Differences among groups were determined by one-way analysis of variance followed by Tukey's or Dunnett's post hoc
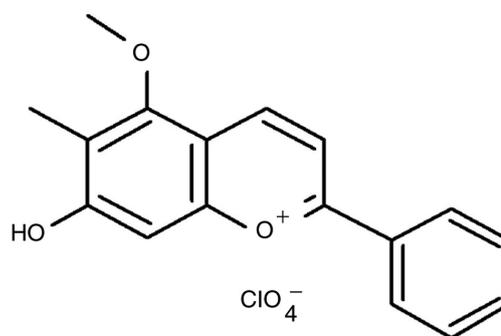

Figure 1. Chemical structure of dracorhodin perchlorate.

test with SPSS software version 16.0 (SPSS, Inc.). $P<0.05$ or $P<0.001$ was considered to indicate a statistically significant difference.

Results

Dracorhodin perchlorate promotes cell migration in HaCaT keratinocytes. Wound healing activity was first investigated, which mimics the migration of keratinocytes toward wound margins *in vitro*. As shown in Fig. 2A, the wound was closing progressively following treatment with dracorhodin perchlorate in HaCaT cells. No significant difference was found between 1 and 2 μg/ml groups. Therefore, the optimal dracorhodin perchlorate concentration to monitor wound healing activity was found to be 1 μg/ml (Fig. 2B). Quantitative data indicated that dracorhodin perchlorate at 1 μg/ml significantly reduced the wound width at the time point of 24 h and markedly promoted HaCaT cell migration compared with those in control cells (Fig. 2C). Therefore, this suggests that dracorhodin perchlorate enhanced cell migration in HaCaT keratinocytes.

Dracorhodin perchlorate does not enhance cell viability in HaCaT keratinocytes. As previously reported, the cell viability of human primary fibroblast proliferation significantly increased by dracorhodin perchlorate treatment (41). Therefore, MTT assay and PI exclusion method were performed to investigate the cell viability and cytotoxicity of HaCaT keratinocytes after dracorhodin perchlorate exposure. Dracorhodin perchlorate did
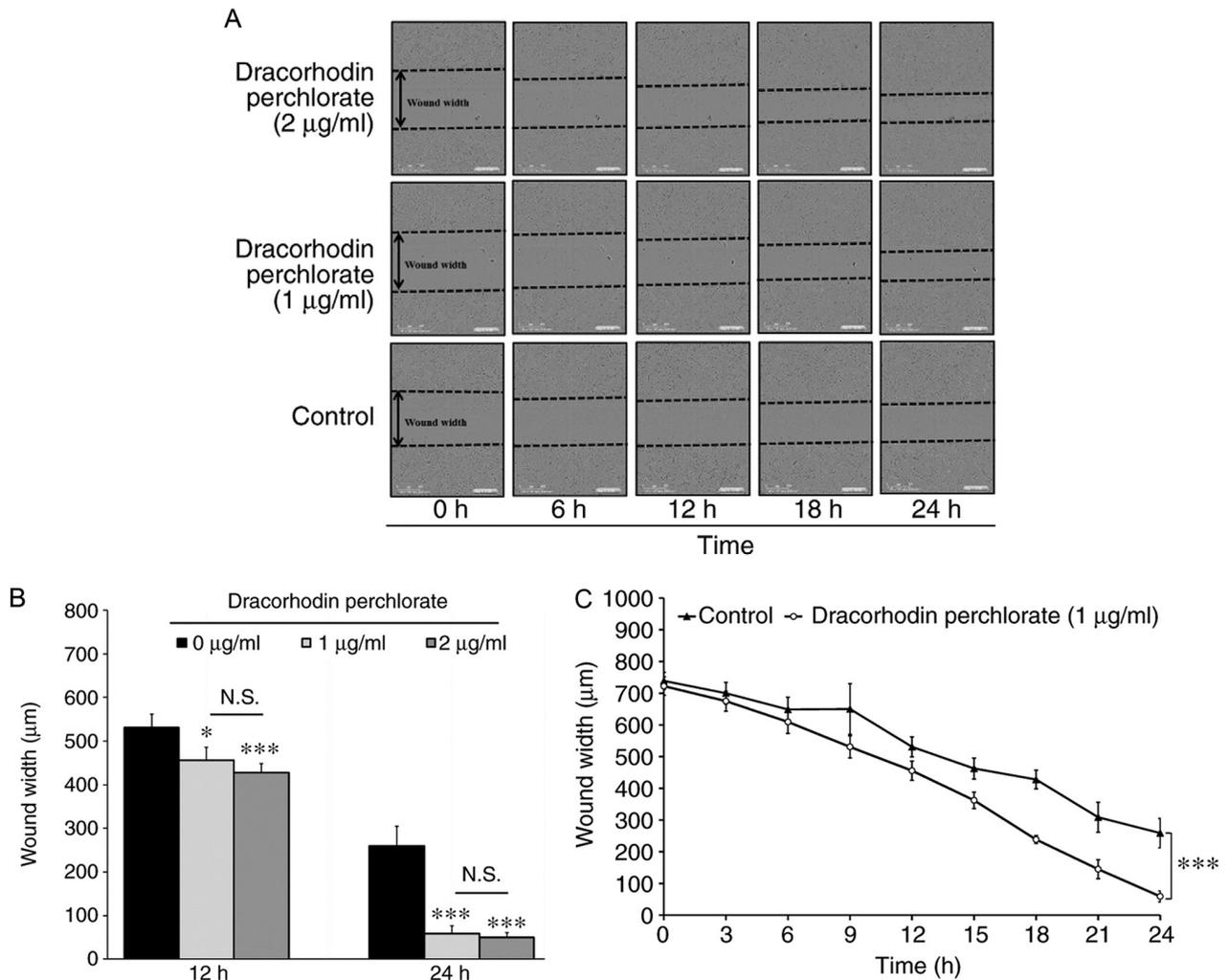
Figure 2. Determination of cell migration in HaCaT keratinocytes. (A) Effects of dracorhodin perchlorate on wound healing of HaCaT keratinocytes. Cells ($1x10^4$ cells/well) in 96-well plates were scratched and incubated with or without 1 and 2 μg/ml dracorhodin perchlorate for indicated time. (B) Cells ($1x10^4$ cells/well) in a 96-well plate were scratched and treated with or without 1 and 2 μg/ml dracorhodin perchlorate for 12 and 24 h. The wound width of HaCaT keratinocytes was determined using the IncuCyte ZOOM System instrument and then quantified. (C) Time courses of the relative wound widths of HaCaT keratinocytes after wound generation. Data are presented as the mean ± standard deviation, n=3. Tukey's post hoc test after ANOVA. *P<0.05 and ***P<0.001 vs. 0 μg/ml untreated control. N.S., not significant.

not affect the viability of HaCaT cells at the highest concentration tested in this study, which was 2 μg/ml (Fig. 3A). As shown in Fig. 3B and C, viable cells were generally excluded from PI because of low emission fluorescence. No significant change was observed in dracorhodin perchlorate-treated HaCaT keratinocytes (Fig. 3B). Moreover, flow cytometry results showed that the total counts of dead and viable cells were <10 and >90%, respectively (Fig. 3C). Thus, the migration ability of HaCaT cells caused by dracorhodin perchlorate was not due to the cell viability of HaCaT keratinocytes.

Dracorhodin perchlorate increases the protein expression of β-catenin and phosphorylation of Akt, p38 and ERK in HaCaT keratinocytes. Previous studies have suggested that wound healing is closely associated with β-catenin signaling (42,43), where keratinocyte mobility can be stimulated through the MAPK and AKT signaling pathways (44-46). Western blotting experiments were therefore performed to investigate the effects of dracorhodin perchlorate treatment on the protein

expression of β-catenin and both phosphorylation of ERK and AKT. Dracorhodin perchlorate treatment (2 μg/ml for 12 h) markedly upregulated the protein levels of β-catenin, phosphorylated ERK, phosphorylated Akt and phosphorylated p38 in HaCaT keratinocytes compared with those in control cells (Fig. 4). This was subsequently verified using sandwich ELISA. The levels of both phosphorylated AKT and ERK significantly increased after dracorhodin perchlorate treatment in a dose-dependent manner (Fig. 5).

To confirm further that the mechanism of wound healing underlying the effects of dracorhodin perchlorate, wortmannin, U0126 and SB203580, inhibitors of AKT, ERK and p38 MAPK, respectively, were used. As shown in Fig. 6, inhibitors of AKT, ERK and p38 MAPK could all significantly inhibit the migration of dracorhodin perchlorate-treated keratocytes. Therefore, it could be concluded that dracorhodin perchlorate activated wound healing by upregulating the ERK, MAPK and AKT signaling pathways in human HaCaT keratinocytes *in vitro*.
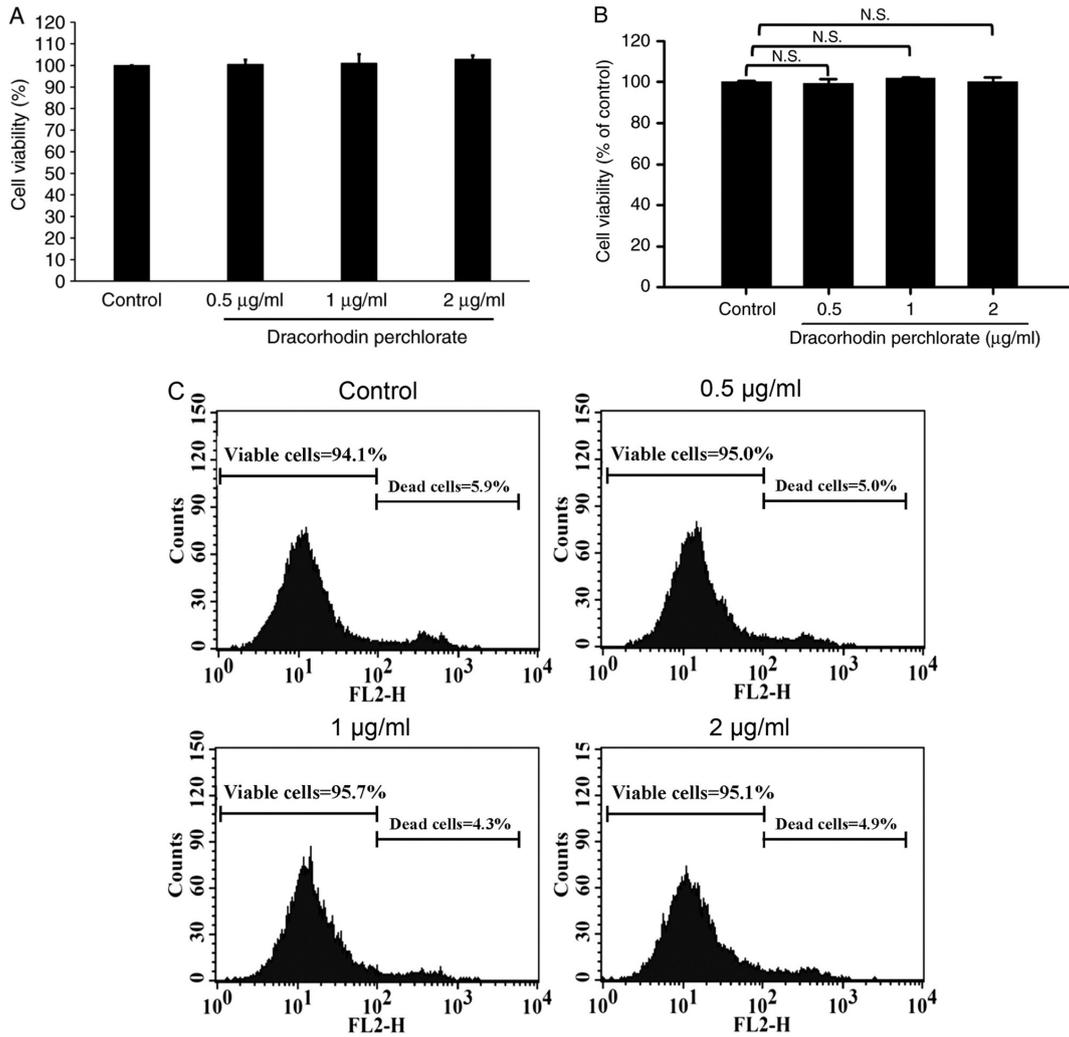
Figure 3. Effects of dracorhodin perchlorate on cell viability in HaCaT keratinocytes. Cells (2.5×10^5 cells/well) in 24-well plates were incubated with 0, 0.5, 1 and 2 μg/ml dracorhodin perchlorate for 24 h. The cell viability was determined by (A) MTT assay, (B) propidium iodide exclusion method, and (C) a representative histogram of the quantified flow cytometry plots. Data are presented as the mean ± standard deviation, n=3. Dunnett's post hoc test after ANOVA. N.S., not significant.
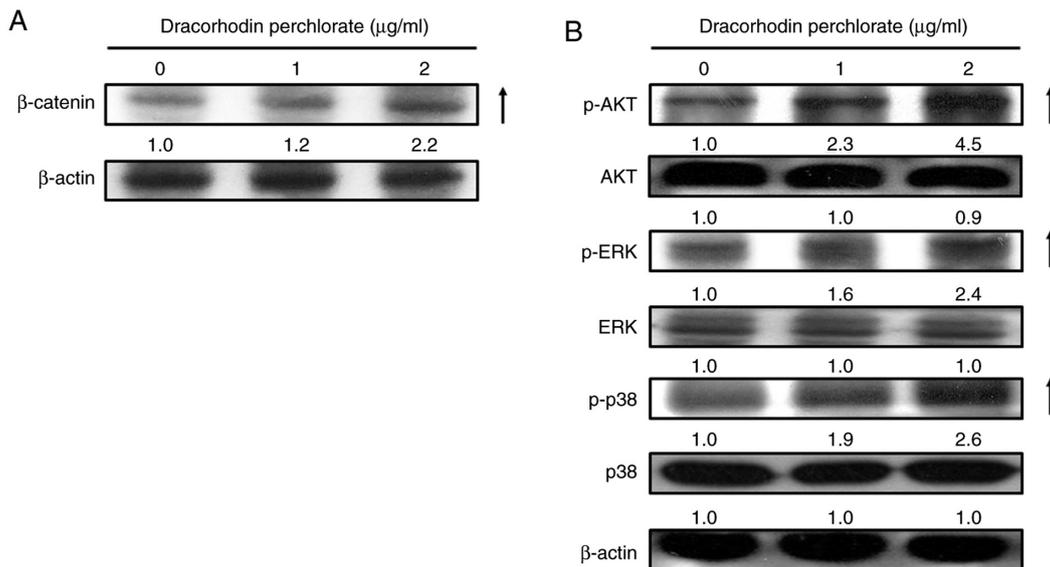

Figure 4. Effects of dracorhodin perchlorate on the protein expression levels of β-catenin, AKT, ERK and p38 proteins in HaCaT keratinocytes. Cells were incubated with 0, 1 and 2 μg/ml dracorhodin perchlorate for 12 h. Whole cell lysates were prepared before the levels of cell migration-related proteins were analyzed by western blot analysis. The protein levels of (A) β-catenin, (B) p-AKT, AKT, p-ERK, ERK, p-p38 and p38 were detected. All blots were normalized to β-actin to ensure equal loading.
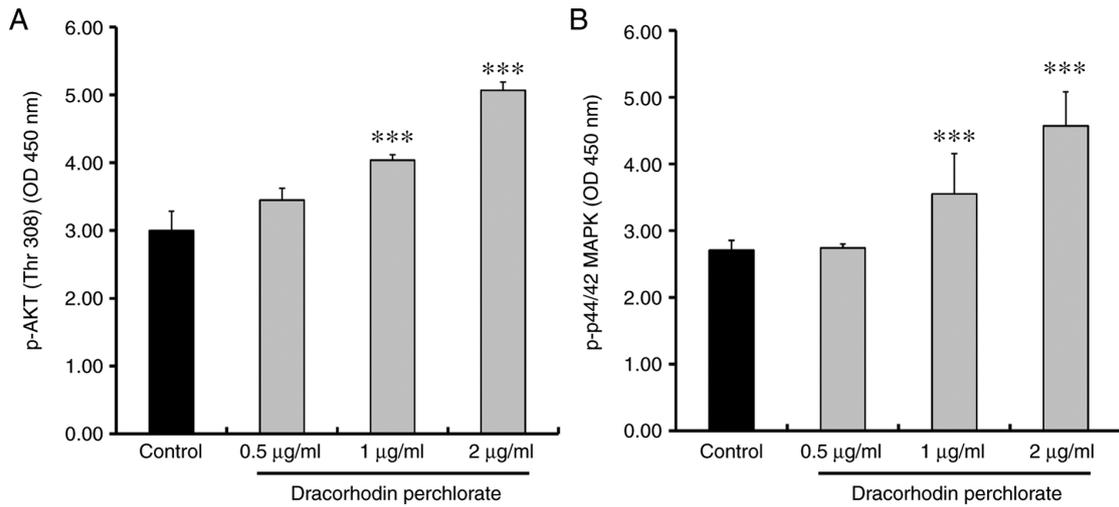
Figure 5. Effects of dracorhodin perchlorate on the AKT and ERK kinase activities in HaCaT keratinocytes. Cells were incubated with 0, 0.5, 1 and 2 μg/ml dracorhodin perchlorate for 12 h. Whole cell lysates were then prepared and the (A) AKT and (B) ERK kinase activities were analyzed by sandwich p-AKT and p-ERK ELISA. Data are presented as the mean ± standard deviation, n=3. Dunnett's post hoc test after ANOVA. ***P<0.001 vs. Control. OD, optical density.
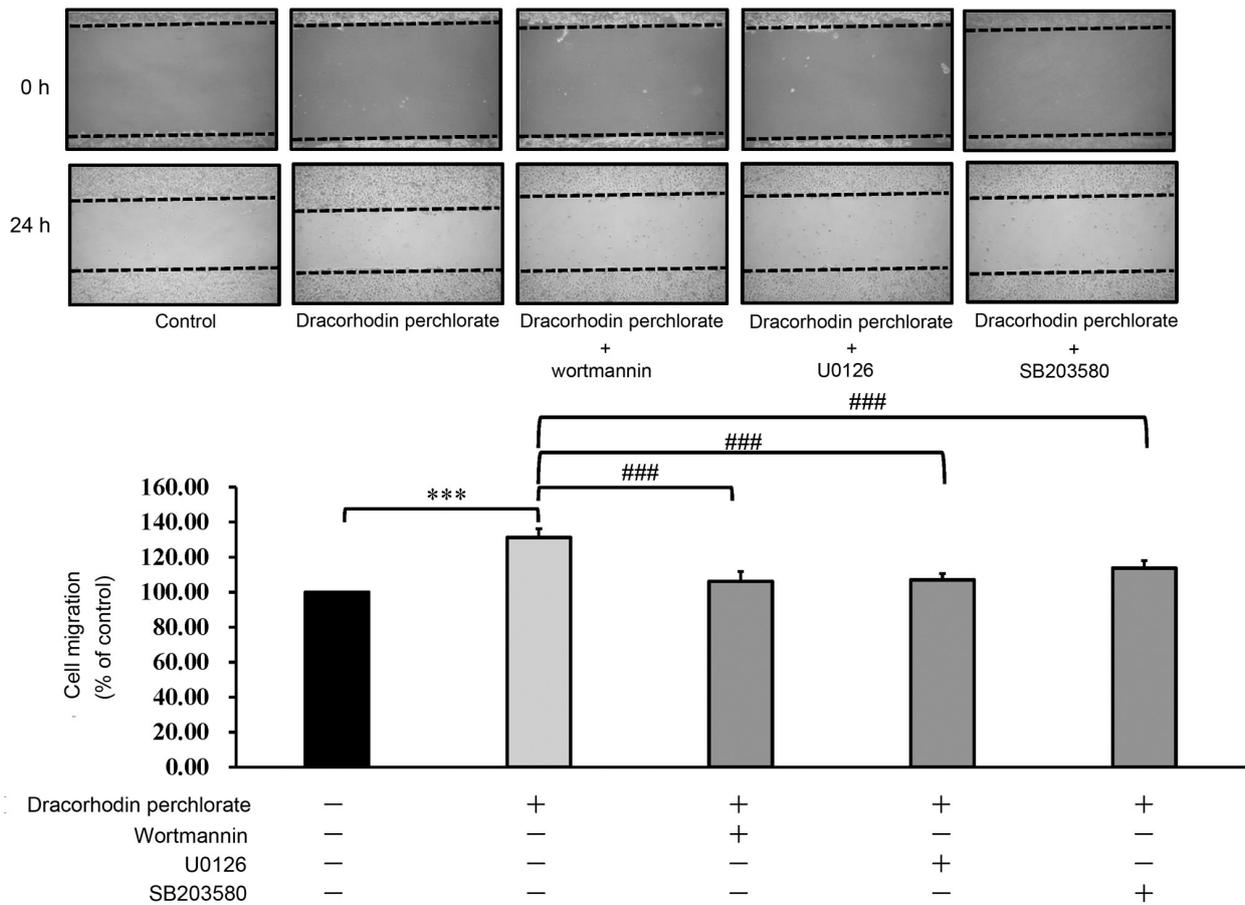

Figure 6. Effect of protein kinase inhibitors on the cell migration of dracorhodin perchlorate-treated keratinocytes. Cells were incubated with or without dracorhodin perchlorate (1 μg/ml) and three protein kinase inhibitors for 24 h. Wortmannin (10 μM), U0126 (10 μM) and SB203580 (10 μM) are AKT, ERK, and p38 inhibitors, respectively. Data are presented as the mean ± standard deviation, n=3. Tukey's post hoc test after ANOVA. ***P<0.001 and ###P<0.001.

Discussion

Dracorhodin perchlorate is a new synthetic chemical analog of dracorhodin (10,15-18). Dracorhodin is originally the fruit extract of dragon blood, which can be used as a TCM (2,10). Dragon blood confers a number of bioactivities, including antibacterial, anti-diarrheal, anti-inflammatory, anti-oxidant, antitumor, anti-ulcer, antiviral, immune-modulatory and
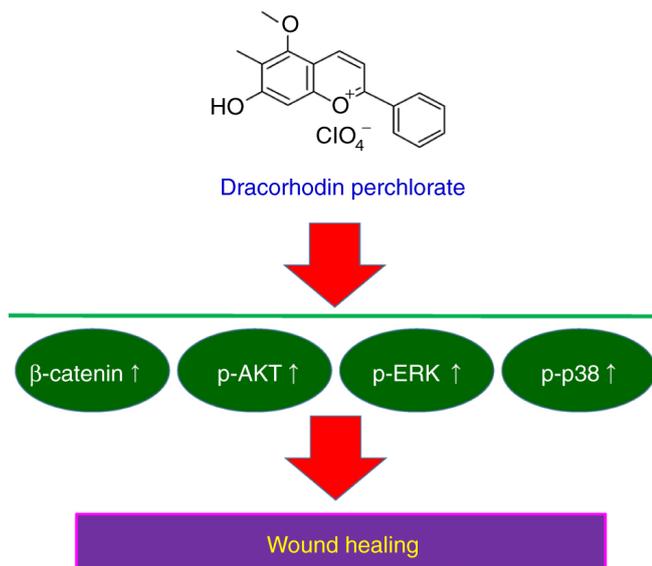
Figure 7. Summary of the possible mechanism underlying the wound healing of dracorhodin perchlorate in HaCaT keratinocytes.

wound healing functions (1). Previous studies have demonstrated that dragon blood can promote the proliferation of fibroblasts, increase the ratio of cells in the S-phase of the cell cycle and collagen formation, in addition to enhancing wound healing (1,11,12). In the present study, dracorhodin perchlorate was found to significantly promote wound healing in human HaCaT keratinocytes. However treatment with dracorhodin perchlorate (0.5-2 μg/ml) in HaCaT keratinocytes exerted no statistically significant effects on keratinocyte viability. To the best of our knowledge, the present study is the first time an *in vitro* scratch assay was utilized using dynamic analysis facilitated by the IncuCyte Kinetic Live Cell Imaging System to evaluate the effects on wound closure in dracorhodin perchlorate-treated HaCaT cells. Furthermore, visible gaps were observed in the monolayer of cells at the start of the assay. In accordance with this result, the cell number did not increase.

Previous studies have shown that activation of the Wnt/β-catenin signaling pathway serves an important role in the wound healing process of human keratinocytes (47). During the wound healing of keratinocytes, cell migration and invasion are regulated by the activation of the PI3K/AKT, ERK, p38/MAPK and Wnt/β-catenin signaling cascades (48-51). Additionally, activation of the ERK and p38/MAPK pathways in cell migration is crucial for wound healing (52,53). In accordance with these previous findings aforementioned, results from the present study showed that dracorhodin perchlorate treatment markedly increased the phosphorylation of AKT, ERK and p38 and kinase activities in HaCaT cells. In addition, dracorhodin perchlorate-treated HaCaT cells were selectively incubated with the AKT inhibitor wortmannin, the ERK inhibitor U0126 or the p38 inhibitor SB203580 for 24 h. Cell migration was found to be inhibited by all three inhibitors, suggesting that the ERK, p38 MAPK and AKT signaling pathways were all involved. All these results indicated that AKT, ERK and p38 MAPK were activated by dracorhodin perchlorate treatment during the wound healing

process in HaCaT cells in a dose-dependent manner within the concentration range of 0 and 2 μg/ml. However, the optimal dracorhodin perchlorate concentration for wound healing activity was found in the present study to be 1 μg/ml rather than 2 μg/ml. Therefore, other mechanisms may be involved in this dracorhodin perchlorate-induced wound healing process in HaCaT cells.

A proposed summary of the wound healing mechanism underlying the effects mediated by dracorhodin perchlorate in HaCaT cells is shown in Fig. 7. It was found that dracorhodin perchlorate promoted the wound healing activity of HaCaT keratinocytes through the β-catenin, ERK, p38 MAPK and AKT signaling pathways. These findings provide novel insights into the mechanistic information in the wound healing activity of dracorhodin perchlorate in human HaCaT keratinocytes.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Conceptualization and study design: TJH and HPC. Cell migration, cell viability, western blotting and protein kinase activity: CCL, JSY and YJC. Protein kinase inhibition: CCL and YNJ. Wound healing assay and acquisition of data: YMH, MCY and FJT. Statistical analysis of all data and interpretation of results: FJT, TJH and HPC. CCL and HPC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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