

RESEARCH ARTICLE

# Evaluation of a 13-hexyl-berberine hydrochloride topical gel formulation

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## Abstract

13-hexyl-berberine hydrochloride (HB-13) is a derivative from berberine which finds widespread applications in the treatment of infectious pathogens including fungi, bacteria, parasites and viruses. As our continuing efforts for treatment of herpes simplex virus (HSV), we studied the topical delivery and safety of HB-13 in a gel formulation (0.5%) in a pig model. Our studies demonstrated the maximal HB-13 concentration was 2.51 µg/mL, which was more than the half maximal inhibitory concentration (IC<sub>50</sub>) as we previously reported. In addition, there was no sign of irritation or histological aberrance for stripped skin continuously applied with 0.5% HB-13 gel for 21 days. In conclusion, 0.5% HB-13 gel can achieve effective anti-HSV concentration in the dermis and it is safe to use.

**Keywords:** 13-hexyl-berberine hydrochloride (HB-13), microdialysis, safety, pig, gel

## Introduction

Berberine is a natural isoquinoline alkaloid extracted from medicinal herbs, which is commonly used as a dietary supplement in Chinese medicine. Although it has been shown that berberine has anti-replication of fungi (*Candida albicans* and yeast), parasites, bacteria and viruses<sup>1–5</sup>, the bioavailability of berberine is poor, which leads to low absorption of berberine in oral administration. To improve the bioavailability, various derivatives of berberine have been synthesized by introducing different functional groups to the base structure of berberine<sup>6,7</sup>. On the other hand, 13-hexyl-berberine hydrochloride (HB-13) has been synthesized to improve the bioactivity. HB-13 is generally prepared in two main steps, i.e. reduction of berberine to dihydroberberine in an alkali solution, and the reaction of dihydroberberine with hexanal<sup>8</sup>.

*In vitro* studies showed this compound strongly inhibited the replication of herpes simplex virus (HSV)<sup>9</sup> and human immunodeficiency virus type 1 (HIV-1)<sup>10</sup>, indicating its great potential applications against viruses. However, the cutaneous absorption and safety of HB-13 has not been studied yet.

HSV is one of neurotropic viruses and can establish life-long infection in human. Two types of HSV (i.e. HSV-1 and HSV-2) are of clinical importance. HSV-1 is generally associated with oral infections and encephalitis through non-sexual contact, whereas HSV-2 often causes genital infections in adults through sexual contact<sup>11</sup>. HSV enters the host through abraded skin or intact mucous membranes and causes watery blisters at the primary site. Meanwhile, HSV establishes latent infection in neurons and can cause sporadic episodes of

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viral reactivation, which leads to localized lesion on the skin with virus shedding. Recurrent HSV infection not only facilitates the transmission, but also increases the chances of coinfection of HIV through sexual contact<sup>12,13</sup>. Studies have shown that topical treatment can relieve the local symptoms and most importantly reduce the risk of coinfection with HIV<sup>14</sup>.

Two HB-13 topical formulations, 0.5% gel and 0.5% cream, have been developed in our lab for the topical treatment of HSV-induced lesions. Here, we report the topical penetration of 0.5% gel and safety in a pig model. The cutaneous penetration of 0.5% HB-13 gel on intact or stripped pig skin was evaluated using an *in vivo* dermal microdialysis method. The safety was evaluated in terms of skin irritation and histological observation after topical application of 0.5% HB-13 gel on stripped pig skin for 21 days. Our study demonstrated that 0.5% HB-13 gel is a safe topical formulation that could be potentially used for treatment of HSV lesions.

## Materials and methods

### Chemicals and reagents

13-hexyl berberine hydrochloride (HB-13, purity of 99.36%) was kindly provided by Prof. Naisan Li from China Pharmaceutical University (Nanjing, China). Both 0.5% HB-13 gel and 0.5% HB-13 cream used in this study were prepared at the Department of Pharmacology, Institute of Dermatology, Chinese Academy of Medical Sciences (Nanjing, China). 3M Tegaderm Transparent Dressings were purchased from 3M Corp. (Methuen, MA, USA). Normal saline was obtained from Xiaoying Pharmaceutical Factory (Nanjing, China). Methanol of liquid chromatographic grade was obtained from Hanbang Corp. (Nanjing, China). Triple distilled water was filtered using 0.45 µm disposable filters (3M Corp.) and was then used to prepare solutions.

### HPLC assay

HB-13 is a derivative from berberine with addition of a 13-hexyl group on aromatic hydrocarbon structure (Figure 1). The concentrations of HB-13 were analyzed using HPLC. The chromatographic system consisted of a chromatographic pump, an injector valve, a 20 µL sample loop and a 2487 ultraviolet detector (Waters Corp, Milford, MA, USA). The sample containing HB-13 was loaded onto a reverse-phase column (ODS C<sub>18</sub>, 5 µm, 4.6 × 150 mm, Dalian Elite Analytical Instruments Ltd,

Dalian China) and was then separated by the HPLC system. To facilitate the separation, a mobile phase consisting of water (contained 0.1% KH<sub>2</sub>PO<sub>4</sub>) and acetonitrile (40: 60, v/v), and 0.06 mL solution which contained 3 mmol sodium dodecyl sulfate (SDS) and 0.5% (v/v) triethylamine) was used at a flow rate of 1.0 mL/min. The separated HB-13 was subsequently detected at a wavelength of 345 nm. The retention time for HB-13 was 7.5 min. The peak area calculated within this retention time was highly correlated ( $r^2 > 0.99$ ) with HB-13 concentrations ranging from 0.041 to 20.54 µg/mL. Coefficient of variation (CV) was 14.68% at the lower limit of quantification (LLOQ) of 0.041 µg/mL.

### *In vitro* drug release

*In vitro* drug release of HB-13 from two formulations (i.e. gel and cream) was investigated using a ZRS4 dissolution test apparatus (Tianjin TDTF Technology Co., Tianjin, China) as previously reported<sup>15</sup>. 0.2 g 0.5% HB-13 gel or 0.5% HB-13 cream was evenly applied on the cellulose acetate membrane for up to 6 h to study the drug release. Every hour, 1 mL saline samples were collected and filtered (0.45 µm) prior to the quantification of HB-13 using HPLC.

### *In vitro* microdialysis

Prior to investigation of drug release *in vivo*, the microdialysis system was calibrated by a Zero-net-flux method as previously reported<sup>16</sup>. The used microdialysis system consisted of a microinjection pump controller, a microdialysis syringe pump, gastight syringes and LM-10 linear microdialysis probes (Bioanalytical Systems Inc, West Lafayette, IN, USA). The probe had a semipermeable membrane of 10 mm in length and a molecular weight cut-off of 20 kDa. For measuring the recovery efficiency, the window of the microdialysis probe was immersed in 0.2, 0.4, 0.6, 2.0 and 4.0 µg/mL of HB-13 solution and perfused with 1.0 µg/mL of HB-13 solution at a flow rate of 1.0 µL/min. The dialysate was collected every 30 min and subject to HPLC analysis.

### *In vivo* microdialysis

In this study, pigs were used as the animal model, since the pig skin has properties similar to human skin<sup>17,18</sup>. Six pigs (Suzhong line 1, 20–30-day-old, weighing 5.5–6.5 kg) were obtained from Jiangsu Academy of Agricultural Science, China. They were used for *in vivo* study following the requirements of the National Act on the Use of Experimental Animals (People's Republic of China). Pigs

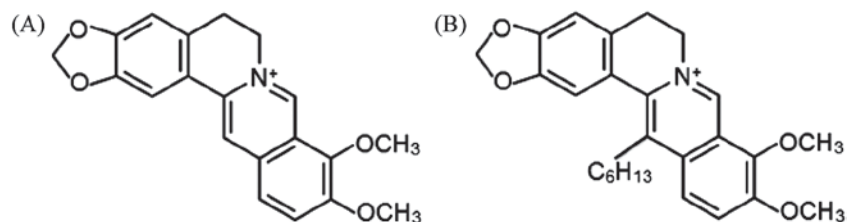


Figure 1. Structure of berberine and 13-hexyl berberine<sup>8</sup>. (A) Berberine. (B) 13-hexyl berberine.

were intraperitoneally injected with 30% urethane, which was prepared by adding 30 g of urethane in 100 mL of phosphate buffered saline (PBS), with a dosage of 3 mL/kg for anesthesia 30 min prior to the experiment. Two parallel positions at the dorsum were shaved with an electrical hair clipper. The stratum corneum at one clipped area was stripped using a standard method described in FDA guideline<sup>19</sup>, while the other clipped area was kept intact. Two linear microdialysis probes were inserted in parallel into the skin at the clipped areas. The probes were left on site for 90 min to allow for the release of insertion microtrauma. The probes were perfused with 1.0 µg/mL of HB-13 solution at a flow rate of 1.0 µL/min for 60 min. Microdialysate samples were collected in duplicate to calculate *in vivo* recovery. The probes were then perfused with normal saline for 40 min to remove residual HB-13 in the skin tissue before topical administration of HB-13 gel. A dosage of 0.2 g/cm<sup>2</sup> of HB-13 gel (0.5%, w/w) was applied to an area of 1.5 × 2.0 cm<sup>2</sup> (stripped and intact skin), which was covered with 3M Tegaderm Transparent Dressing. The residual HB-13 gel was carefully removed after application for 6 h using cotton swabs. Perfusate samples were collected every 30 min up to 8 h since the application of HB-13 gel.

### Measurement of HB-13 concentration in stripped skin tissue

To study the skin penetration of HB-13 (0.5%, w/w), the concentration of HB-13 in the epidermis was measured using HPLC. The stratum corneum of at the dorsa was stripped as described above. After topical application of HB-13 gel at a dosage of 0.2 g/cm<sup>2</sup> for 0, 2, 4, 6 and 8 h, HB-13 gel was carefully cleaned with three swabs. The stripped skin tissue (an area of 1.5 × 2.0 cm<sup>2</sup>) was then harvested using a surgeon lancet. The subcutaneous tissue was removed using a scalpel. The epidermis was finely cut and soaked into 3 mL of methanol (100%) overnight. The sample was centrifuged at 10,000 rpm for 10 min and 20 µL of the supernatant was analyzed by HPLC to measure the HB-13 concentration.

### Histological analysis

The HB-13 gel or the used drug-free gel was applied twice a day to stripped skin (1.5 × 2.0 cm<sup>2</sup>) at the pig dorsa for 21 days. Following this, the skin tissue at the stripped area treated with the HB-13 gel or drug-free gel, as well as the skin tissue at the non-stripped area (no application of HB-13 gel) was harvested using a surgeon lancet. These skin tissues were then fixed in 10% buffered formalin and analyzed by Hematoxylin and Eosin (H & E) staining under a microscope (Olympus CX40) with a magnification of 40×.

### Data analysis

Pharmacokinetic software 3P97 (Chinese Pharmacological Society, Professional Committee of Mathematics) was used to calculate the pharmacokinetic parameters. SPSS 12.0 software (SPSS, Inc, Chicago, IL, USA) was used for statistical analysis (independent *t*-test).

## Results and discussion

### *In vitro* drug release

To investigate the effect of formulation on the HB-13 release, we checked the cumulative release of HB-13 with time *in vitro* from two formulations, i.e. 0.5% gel and 0.5% cream (Figure 2). We observed that the accumulative amount of HB-13 released from both 0.5% gel and 0.5% cream yielded a linear regression as a function of time, with an *r*<sup>2</sup> of 0.98 and 0.99, respectively. The lag time was 0.99 h for 0.5% HB-13 gel and 0.38 h for 0.5% HB-13 cream. Despite a shorter lag time, 0.5% HB-13 cream demonstrated a lower cumulative release (Figure 2). The released HB-13 from 0.5% gel and 0.5% cream over the time of 6 h was 168.59 µg/cm<sup>2</sup> and 104.06 µg/cm<sup>2</sup>, respectively. The average release rate of HB-13 released from the gel and the cream over 6 h was 28.37 µg/cm<sup>2</sup> h and 17.24 µg/cm<sup>2</sup> h, respectively. These results indicate that HB-13 is fully dissolved or suspended in these two formulations and that they are continuously released with a relatively stable release rate. We deduced that these two HB-13 formulations would exhibit a stable release pattern *in vivo*, providing a concentration gradient across the epidermis is maintained. Since HB-13 was better released from 0.5% gel than 0.5% cream, we used the gel formulation for the following study.

### Calibration of the microdialysis probe *in vitro* and *in vivo*

To ensure that retrodialysis can be used to calculate the HB-13 concentration in the dermis, we calibrated the *in vitro* recovery of microdialysis probes using the Zero-net-flux method (Figure 3). We checked the difference of HB-13 concentration in the perfusate and the dialysate as a function of HB-13 concentrations in

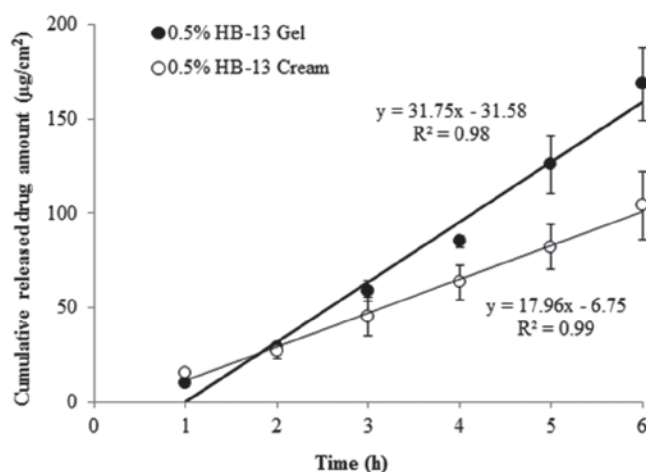


Figure 2. *In vitro* release profile of HB-13 from 0.5% gel and 0.5% cream formulations. An equal amount (0.2 g) of 0.5% HB-13 gel and cream was applied onto a cellular acetate membrane. HB-13 in these two formulations was released into phosphate buffered saline (PBS) for 6 h. The PBS containing released HB-13 was hourly collected. These samples were analyzed using HPLC. The cumulative amount of HB-13 was plotted as a function of time. Data are presented as mean ± SD (*n* = 9).

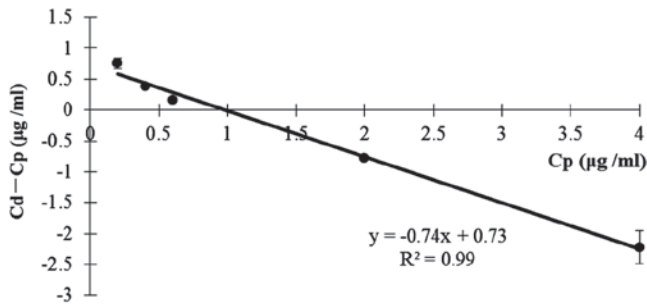


Figure 3. *In vitro* recovery of HB-13 measured by the no-net-flux method ( $C_m = 1.0 \mu\text{g/mL}$ ).  $C_p$  is the drug concentration in the perfusate,  $C_d$  is the drug concentration in the dialysate, and  $C_m$  is the drug concentration surrounding the microdialysis probe. Data are presented mean  $\pm$  SD ( $n = 3$ ).

the perfusate. We observed a linear regression slope ( $r^2 = 0.99$ ). This result indicates the delivery and recovery of microdialysis probes was 74.34% over the tested HB-13 concentrations. The linearity of the slope demonstrated that HB-13 molecules freely diffused across the probe membrane according to the concentration gradient. As such, retrodialysis can be used to calibrate the recovery microdialysis probes *in vivo*.

The *in vivo* recovery of microdialysis probes was measured by retrodialysis. The result showed that the *in vivo* recovery was  $44.02 \pm 12.61\%$ , ( $n = 8$ ) which was lower than that measured *in vitro*. The difference in the recovery may be due to the clearance of HB-13 under epidermis, which is affected by the interaction between HB-13 and local tissue, as well as the flow rate in capillary blood vessels. The difference of the probe recovery between *in vitro* and *in vivo* was also documented in previous studies<sup>15,20</sup>.

### Pharmacokinetic assessment

To investigate the topical absorption of HB-13 gel, we monitored the pharmacokinetics of HB-13 in the dermis using microdialysis (Figure 4). For both stripped and intact skin, the concentrations of HB-13 reached the plateau at 2 h, and it was kept at a high level till the formulation was removed at 6 h. During this period of time, the concentration of HB-13 ranged  $0.78\text{--}1.06 \mu\text{g/mL}$  and  $1.83\text{--}2.51 \mu\text{g/mL}$  for the intact and stripped skin, respectively. Following the removal of HB-13 gel, the HB-13 concentration in the dermis rapidly decreased to  $0 \mu\text{g/mL}$  in 2 h. The area under the curve (AUC) of HB-13 for stripped skin was  $13.04 \mu\text{g h/mL}$ , which was significantly higher than that for intact skin ( $5.31 \mu\text{g h/mL}$ ) ( $p < 0.05$ ), indicating that that stripped skin allowed for higher absorption of HB-13 than intact skin. This comparison is important to guide safety evaluation in patients with herpes virus infection where the stratum corneum is often broken.

The concentration of HB-13 in skin tissue was investigated after topical application of 0.5% HB-13 gel on stripped pig skin. As shown in Table 1, the HB-13 concentrations in the skin tissue reached the plateau ranging from  $24.53$  to  $27.77 \mu\text{g/mL}$  during the period of 2–6 h, which highly correlated with the microdialysis

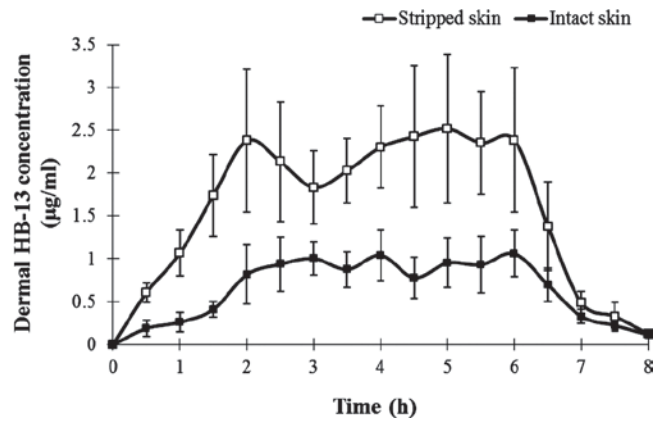


Figure 4. Concentration-time profile of HB-13 applied on intact and stripped pig skin. 0.5% HB-13 gel was applied on the dorsa of pigs (intact or stripped) at a dosage of  $0.2 \text{ g/cm}^2$  for 6 h. Microdialysate samples were continuously collected every 30 min up to 8 h. The concentration of HB-13 in microdialysis samples was measured by HPLC. Data are presented mean  $\pm$  SD ( $n = 4$ ).

Table 1. HB-13 concentration in skin tissue after topical application of 0.5 % HB-13 gel.

Application time (h)	HB-13 concentration ( $\mu\text{g/mL}$ , mean $\pm$ SD)
0	ND
2	$26.67 \pm 2.94$
4	$24.53 \pm 2.80$
6	$27.77 \pm 1.39$
8	$1.69 \pm 0.52$

0.5% HB-13 gel was applied on the stripped dorsa of pigs at a dosage of  $0.2 \text{ g/cm}^2$  for 0, 2, 4, 6 and 8 hours. The HB-13 concentration in the skin tissue was quantified by HPLC. Data are presented mean  $\pm$  SD ( $n = 4$ ).

ND, not detected; HB-13, 13-hexyl-berberine hydrochloride.

findings (Figure 4). It should be noted that the HB-13 concentrations during the period of 2–6 h in the skin tissue were significantly higher than those in the collected microdialysis samples. It may be due to the formation of large-sized drug-protein complexes<sup>21</sup>, which cannot be collected by microdialysis probes, penetration of HB-13 into surrounding cells, or a combination of these two possibilities. The plasma protein binding rate was reported as 48% for berberine in mice<sup>22</sup>, which indicates that HB-13 may have a higher protein binding rate due to the added 13-hexyl group.

### Cumulative skin irritation *in vivo*

To evaluate the safety, the pig skin applied with 0.5% HB-13 gel for 21 days ( $0.2 \text{ g}$ , twice per day) was observed for irritation, and the tissue was histologically studied. After 21 days of topical application, we did not observe obvious sign of cumulative skin irritation (rash or lesion). Neither the control treated with the gel base nor the non-treated control showed sign of abnormal appearance. Histologically, there was no aberrant aggregation of neutrophils or lymphocytes in the HB-13 applied skin (Figure 5A) compared to the base-treated (Figure 5B) and non-treated (Figure 5C) controls. These data suggest

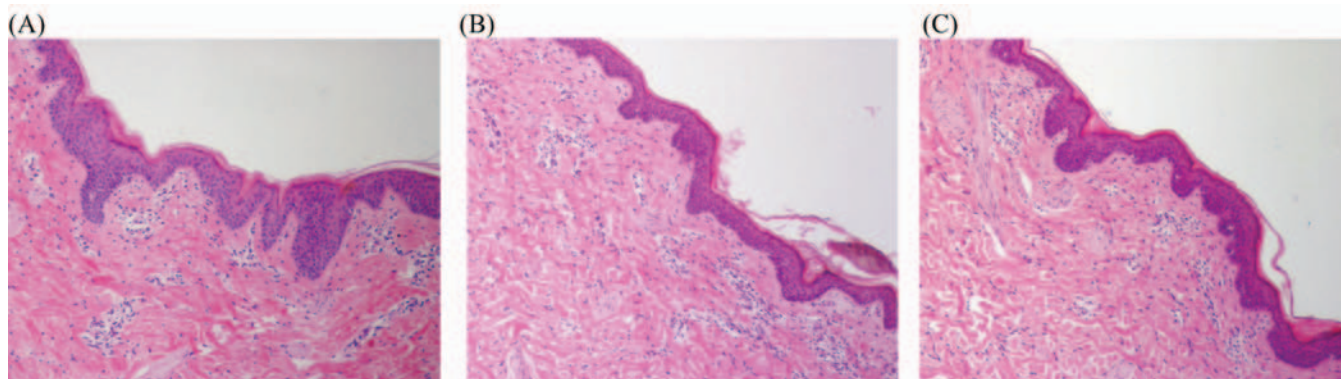


Figure 5. Histological analysis of pig skin treated with 0.5% HB-13 gel. (A) The skin tissue treated with 0.5% HB-13 gel for 21 days (0.2 g, twice per day). (B) Gel base-treated control. (C) No-treated control. Sections were stained by hematoxylin and eosin (H & E) and investigated under a 40× objective. No significant difference was observed in the treated samples and the non-treated control.

that 0.5% HB-13 gel is safe to be applied over a prolonged period up to 21 days.

Dermal microdialysis (DMD) has proven to be useful to evaluate transdermal delivery of topical drugs in pre-clinical trials and clinical trials<sup>23–25</sup>. Via this technique, we for the first time studied the topical penetration of 0.5% HB-13 gel in a pig model for potential dermatological applications. As previously reported, the pig DMD model can be used as an alternative to human skin microdialysis to evaluate new topical formulations to avoid unwanted side effects in humans<sup>15,16</sup>. HB-13, as a derivative from berberine, was modified to improve bioavailability, which may inherently increase the chances of causing side effects and/or intensify the severity. One study has shown that extending the alkyl side chain at position 8 or 13 results in strongly increased the cytotoxicity in *protoberberine-type alkaloids*<sup>26</sup>. Thus, we evaluated the transdermal delivery of HB-13 and its associated safety prior to clinical trial.

Cutaneous absorption and cumulative skin irritation test are two important aspects to ensure the efficiency and safety for topical application of the newly developed HB-13 gel. As such, we first compared the topical penetration on intact and stripped skin, because the stratum corneum is often disrupted for a variety of skin diseases such as HSV infection. As reported, the cutaneous absorption can be enhanced for skin without stratum corneum compared to intact skin<sup>27,28</sup>. In our study, we observed that the AUC of HB-13 for stratum corneum stripped skin was 13.04  $\mu\text{g h/mL}$ , which was 2.46-folds higher than that for intact skin (5.31  $\mu\text{g h/mL}$ ) ( $p < 0.05$ ). Then, we evaluated cumulative skin irritation of 0.5% HB-13 gel in the presence of disrupted stratum corneum, and the results demonstrated that HB-13 did not cause any accumulative toxic effects because of the following observations. First, the HB-13 concentration in the dermis rapidly decreased upon the removal of 0.5% HB-13 gel (Figure 4). Second, there was no sign of skin irritation after continuous application (twice per day) for 21 days on the stripped skin. Third, there was no histological difference between stripped skin and intact skin applied with 0.5% HB-13 gel

for 21 days, compared to the non-treated skin (Figure 5). These data indicate that 0.5% HB-13 gel is safe as a topical formulation in the pig model.

## Conclusions

In conclusion, we demonstrated the safety of a new compound HB-13, a derivative from berberine, in a 0.5% gel formulation topically applied to stripped pig skin and integer skin. Effective cutaneous absorption and satisfactory safety enables the potential application of 0.5% HB-13 gel for treatment of HSV lesions. The efficacy of 0.5% HB-13 gel for treatment of HSV lesions needs to be further clinically evaluated.

## Declaration of interest

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