Percutaneous Absorption and Anti-Inflammatory Effect of a Substance P Receptor Antagonist: Spantide II

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Purpose. There is accumulating evidence that neurogenic mediators such as substance P (SP) and α-melanocyte stimulating hormone (α-MSH) contribute to inflammation following chemical and thermal injuries or in disease conditions such as psoriasis and contact dermatitis. Spantide II is a peptide with a molecular weight of 1670.2 which binds to neurokinin-1 receptor (NKR-1) and blocks proinflammatory activities associated with SP. The aim of this study was to investigate *in vitro* permeation and distribution of spantide II through hairless rat skin and the anti-inflammatory effect of topically delivered spantide II in an allergic contact dermatitis (ACD) mouse model.

Methods. The *in vitro* permeation and distribution of spantide II with or without cysteine HCl (CH) as a penetration enhancer through hairless rat skin was studied using Franz diffusion cells. The anti-inflammatory effect of spantide II was studied by measuring the reduction of ACD in C57BL/6 mice after application of spantide II as a topical solution.

Results. The skin permeation experiments with or without cysteine HCl (as penetration enhancer) showed no detectable levels of spantide II permeation across rat skin over a period of 48 h. Cysteine HCl significantly increased the distribution of spantide II in skin layers; also, the reduction in ACD response was significantly higher with the formulation containing cysteine HCl ($p < 0.05$). Spantide II at different concentrations showed a dose-dependent reduction of ACD response in mice.

Conclusions. The current study demonstrates that spantide II can effectively be delivered to epidermis and dermis to exert a significant anti-inflammatory activity on the reduction of inflammation in a mouse model of ACD.

KEY WORDS: cysteine HCl; dermatitis; skin permeation; Spantide II.

INTRODUCTION

Inflammation is mediated by a series of events that are mediated by cellular and soluble components of the immune system. In addition, it has recently been recognized that the neurological system may modulate certain aspects of the inflammatory responses (1,2). Psoriasis and contact dermatitis are skin disorders that are characterized by long-term inflammation with a significant neurogenic component. Currently, these disorders are treated with topical corticosteroids that target a variety of pathways of the inflammation cascade (3,4). However, the corticosteroid therapy is associated with local side effects such as skin atrophy, telangiectasia, acne, and secondary infections which lead to contact dermatitis or perioral dermatitis. Neurogenic inflammation refers to the ability of the neurological system to evoke local inflammatory responses through the release of neuropeptides such as Substance P (SP), calcitonin gene–related peptide, and α-melanocyte stimulating hormone (α-MSH). There is accumulating evidence that released neuropeptides contribute to the inflammatory response following chemical or thermal injuries and disease conditions such as psoriasis and contact dermatitis (2). These neuromediators are capable of mediating cutaneous neurogenic inflammation by the induction of vasodilation, plasma extravasation, and augmentation of cytokine, chemokine, and cellular adhesion molecule expression (5). Substance P is a classical mediator of triple response consisting of erythema, edema, and itching. It is capable of upregulating production of the proinflammatory cytokines IL-1, IL-6, and IL-8 in murine and human keratinocytes (6). With the exception of capsaicin, there are no therapies that focus on the neurogenic mechanism of inflammation. Spantide II is a peptide that specifically binds to neurokinin-1 receptor (NKR-1) and blocks proinflammatory activity associated with SP (7). Spantide II has been reported to inhibit capsaicin-induced ear edema in mice (8) and substance P–induced plasma extravasation in mice by intradermal injection (9).

Several studies have demonstrated the capability of peptide drugs to penetrate across the skin (10,11). The basic requirement of transdermal delivery is for the drug to penetrate the outermost layers of the skin—the stratum corneum that is composed of keratin and lipid-rich cellular-derived material. The binding, metabolism, and clearance of peptides in the tissue below stratum corneum also influence the pattern of its local tissue distribution (12). The permeation of peptides through the skin can be influenced by the type of the vehicle, concentration of peptide, and penetration enhancers in the formulation (13,14). Recently, we have shown some sulfhydryl compounds such as cysteine HCl (CH) to be novel skin penetration enhancers (15). Spantide II is a peptide with 11 amino acids with the structure H-D-Lys(nicotinoyl)-Pro-β-(3-pyridyl)-Ala-Pro-3,4-dichloro-D-Phe-Asn-D-Trp-Phe-D-Trp-Leu-Nle-NH_2 (molecular weight, 1670.2; isoelectric point, 5.229) (16). In the current study, we hypothesize that topically delivered spantide II would prove to be an effective therapeutic agent for the treatment of inflammatory skin diseases. To test this possibility, the *in vitro* skin distribution and penetration of spantide II through hairless rat skin and the anti-inflammatory effect of topically applied spantide II in an allergic contact dermatitis (ACD) mouse model was examined.

MATERIALS AND METHODS

Spantide II (Assay >95%) was custom synthesized from Biopeptide Co. LLC (San Diego, CA, USA) and stored at –22°C as supplied. Ethanol USP (200 proof) was obtained from Florida Distillers Co. (Lake Alfred, FL, USA). Cysteine HCl and 2,4-dinitro-1-fluorobenzene (DNFB) were procured from Sigma Chemical Co. (St. Louis, MO, USA). All other

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chemicals used in the study were of reagent or high-performance liquid chromatography (HPLC) grade. Male hairless rats (CD HrBi) and mice (C57BL/6, male and female) were obtained from Charles River Laboratories (Wilmington, MA, USA) and Jackson Laboratories (Bar Harbor, ME, USA), respectively, and housed in a barrier facility with free access to water and food. Care of animals for *in vitro* and *in vivo* experiments was in accordance with the institutional guidelines, and the experiments were carried out as per the approved animal protocols.

Preparation of Spantide II Formulations

The composition of spantide II formulations is shown in Table I. The powder was weighed directly into a 2-ml graduated glass vial and dissolved in 50% ethanol. A weighed quantity of cysteine HCl was dissolved in the solution, and the volume of the vial was made up to 1.5 ml to get the spantide II concentration in the range from 5% to 0.05% as per Table I. Dexamethasone as 5 mM (0.02% w/v) solution in ethanol was used as a control formulation (DXM).

Extraction of Spantide II from the Skin Homogenate

A recovery (mass balance) experiment was conducted to validate the method of extraction of spantide II in the skin tissue. Hairless rats (CD HrBi, Male) were sacrificed by an overdose of halothane anesthesia. The skin from the dorsal surface was excised, and the adherent fat and subcutaneous tissue were removed. The skin was mounted between the donor and receptor compartments of Franz diffusion cells with the epidermis facing the donor compartment. Different quantities of spantide II (200–500 μg) were applied as an alcoholic solution into the donor compartments. The receptor compartments were filled with pH 7.4 phosphate-buffered saline (PBS) with 10% v/v ethanol and maintained at 37°C by a circulating water bath and stirred continuously with a magnetic bar at 600 rpm. At the end of 8 h, the receptor phase was sampled for analysis of spantide II. The exposed skin was collected and homogenized with 8 ml of 1 M acetic acid using a tissue homogenizer (VirTis Co., Gardiner, NY, USA) at 5000 rpm for 5 min. The homogenate was boiled for 10 min, and the evaporative losses were made up to 8 ml. The samples were centrifuged at 12,000 rpm for 15 min, and supernatant was collected for analysis. The extraction was repeated for two more times on the residue with 1 M acetic acid. The extracts were analyzed for spantide II content by HPLC. To ascertain stability of spantide II in acetic acid at 95°C, we prepared two sets of spantide II solutions in acetic acid. One set was heated at 95°C for 10 min, and the other set of solutions at room temperature were taken as the control. The calibration curves were made with these solutions. All the experiments were performed in triplicate.

HPLC Assay of Spantide II

The analysis of spantide II was performed using a Waters HPLC operated by Millennium[32] software. The HPLC system consisted of an autosampler (model 717 plus), two pumps (model 515), a photodiode array UV detector (model 996), and a reverse-phase C_{18} analytical column (5 μm, 4.6 × 250 mm, Vydac Co., Hesperia, CA, USA). A gradient system of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) was used as the mobile phase. Prior to use, the mobile phase was filtered, degassed by sonication, and run at a gradient of 32%:68% (solvent A:B, respectively), which was reversed to 68%:32% (solvent A:B, respectively) in 30 min, with a flow rate of 1 ml/min. The spantide II content of the samples was analyzed using the UV detector set at 230 nm. All injections were performed at room temperature.

In Vitro Skin Permeation and Distribution Studies

The hairless rat skin was excised and mounted between donor and receptor compartments as described in the recovery experiment. The volume of donor and receptor cells was 2.0 and 5.0 ml, respectively. The formulation (100 μl) was applied to the skin in the donor compartment and sealed with aluminum foil and Parafilm. The receptor compartment was filled with pH 7.4 PBS with 10% ethanol. Ethanol was included in the PBS to increase the solubility of spantide II in order to maintain sink conditions. At predetermined time intervals (2, 4, 8, 12, 24, 36, 48 h), samples (0.5 ml) were taken from the receptor compartment for analysis of spantide II. For skin distribution studies, the diffusion cells were set up as in skin permeation experiments. At defined time intervals, the receptor compartment was sampled, and the formulation in the donor compartment was removed by rinsing with 50% ethanol. The skin was collected, and the surface was gently wiped with a cotton swab dipped in 50% ethanol and rinsed with 2 × 0.5 ml of 50% ethanol using a transfer pipette and blotted dry with Kimwipes (17,18). The dosing area (0.636 cm^2) was cut with a biopsy punch. The stratum corneum was removed by tape stripping with Transpore tape, and 10 strips were collected. The underlying tissue was frozen at –60°C and sectioned with a Cryotome (Thermo-Shandon, 620 Electronic, Pittsburgh, PA, USA) into epidermis and dermis. The skin was placed between two cover slips to align it flat and frozen for horizontal sectioning. Four sections 25-μm-thick each were collected to represent the epidermis, and the remaining portion represented the dermis. The cover slips in contact with epidermal and dermal surfaces were washed with 100 μl 1 M acetic acid and combined into epidermis and dermis slices, respectively, before extraction. Spantide II was extracted from tape strips, epidermis, and dermis by the method described in the recovery experiment, except that the volume of 1 M acetic acid was reduced to 1.5 ml. Three successive

Table I. Composition of Different Formulations of Spantide II

Composition (%w/v)	Formulation code							
	SF1	SF2	SF3	SF4	SF5	SF6	SF7	SF8
Spantide II	1	0.5	5	1	0.5	0.25	0.1	0.05
Cysteine HCl	—	—	10	10	10	10	10	10
50% Ethanol q.s.	100	100	100	100	100	100	100	100

extractions were performed for complete recovery of spantide II from the skin samples. The spantide II content of the samples were analyzed by HPLC. The experiments were repeated at least three times using skin from different rats.

Induction and Treatment of Allergic Contact Dermatitis

Allergic contact dermatitis was induced by a method as previously described (19). Mice were sensitized on day 0 by applying 25 μl of 0.5% DNFB in acetone–olive oil 4:1 on the shaved abdomen. Mice were challenged on day 5 by epicutaneous application of 10 μl of 0.2% DNFB in acetone–olive oil (4:1) and served as an internal control for the studies. The ACD response was determined by the degree of ear swelling of the hapten-exposed ear compared to that of the vehicle-treated contralateral ear before DNFB challenge and at 0–72 h after challenge as measured with a micrometer. Right ears of the mice were treated with topically applied 10 μl of spantide II or dexamethasone solution 30 min after antigen challenge and three times a day thereafter for 4 days. The ACD response was determined by measuring the ear swelling of the test-drug-treated ears compared to that of vehicle-treated contralateral ears before DNFB challenge and at 12, 24, 48, and 72 h after the allergen challenge.

Statistical Analysis

The spantide II content of the skin tissue was expressed as milligrams per gram of the tissue. The ACD response was expressed as ear thickness in micrometers (mean ± standard error). The area under curve of ear thickness vs. time was determined to compare various treatment groups. Differences between multiple groups were examined using analysis of variance (ANOVA) and Tukey multiple comparison test. Mean differences with $p < 0.05$ were considered to be significant.

RESULTS

HPLC Assay of Spantide II

The retention time of spantide II was 22.6 min. Over the concentration range of 0.1–10 μg, the HPLC method demonstrated good linearity with a correlation coefficient of >0.9999. The detection limit of spantide II was 0.1 μg/ml.

Extraction of Spantide II from the Skin Homogenate

The recovery of spantide II, as shown in Table II, after two successive extractions was 80–85% of the theoretical input, indicating the extraction method was optimized to yield a reasonably good recovery. No further increase in the recovery values were observed due to third extraction. The slope and correlation coefficient of the calibration curve with or without heating were not significantly different, thus indicating that spantide II remained stable after heating in acetic acid (data not shown). The possible loss of about 15–20% of spantide II might be due to skin enzyme activity, metabolism and handling of the skin tissue, and homogenization and other processes in the experiment.

Table II. Recovery (Mass Balance) of Spantide II from the Skin Homogenate Followed by the Topical Application of Different Doses of Spantide II

Quantity of spantide II applied on the skin	Two extractions (n = 3) quantity recovery (% ± SD)	Three extractions (n = 3) quantity recovered (% ± SD)
0.5 mg	83.840 ± 17.82	84.186 ± 17.89
0.4 mg	83.153 ± 11.34	83.950 ± 12.95
0.3 mg	80.637 ± 12.77	82.226 ± 14.96
0.2 mg	83.100 ± 11.01	83.760 ± 16.80

In Vitro Skin Permeation and Distribution Studies

The skin permeation and distribution experiments with or without penetration enhancer showed no detectable levels of spantide II in the receptor solution (PBS). This indicated that spantide II did not permeate through full-thickness rat skin over a period of 48 h. The skin distribution profile of spantide II as a function of time is presented in Fig. 1. Spantide II absorbed preferentially into stratum corneum followed by epidermis and dermis. The spantide II levels increased steadily with time in all the skin layers. In the presence of cysteine HCl as enhancer (SF5), maximal levels of spantide II were attained in SC within 8 h (8.732 ± 1.47 mg/g) and plateaued thereafter. The concentration of spantide II in epidermis and dermis steadily increased up to 48 h (4.452 ± 1.86 and 0.348 ± 0.16 mg/g in epidermis and dermis, respectively, at 48 h). Cysteine HCl enhanced the diffusion of spantide II to 4-fold within 4 h, and the SC levels of spantide II due to cysteine HCl were significantly higher at all time points of the study ($p < 0.05$). Similarly, the levels of spantide II in the epidermis and dermis were 4.2- and 20-fold higher due to cysteine HCl ($p < 0.05$) and the levels were maintained at consistently higher levels at all the time points of the distri-

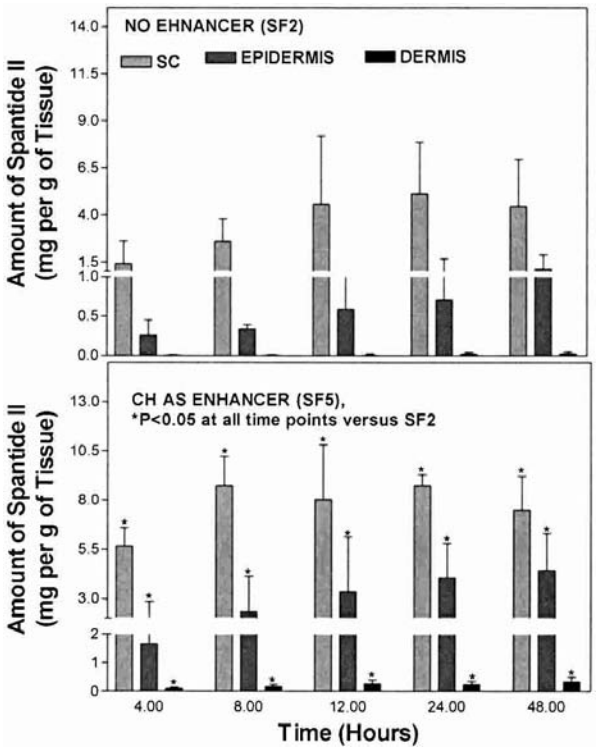

Fig. 1. Distribution of spantide II in different layers of the skin.

bution study ($p < 0.05$). Thus, cysteine HCl was effective as a penetration enhancer to increase the spantide II levels in the skin layers as compared to the formulation with no enhancer (SF2).

Anti-Inflammatory Activity of Spantide II in Mice

Figure 2 shows the effect of spantide II formulations with or without enhancer on the reduction of ACD in C57BL/6 mice. The area under curve (AUC) of ear thickness vs. time data is presented as an inset in Fig. 2. Notably, the ACD response was significantly lowered in all the formulations (SF1, $p < 0.01$, F4 and DXM, $p < 0.001$ vs. control) at different time points after DNFB sensitization and challenge. The formulation with cysteine HCl as an enhancer (SF4) showed greater ACD response than the formulation with no enhancer (SF1) ($p < 0.05$). The effect of concentration of spantide II on the reduction of ACD is shown in Figs. 3 and 4. The AUC of ear thickness vs. time data are presented as insets in the respective figures. Spantide II at concentrations (5.0% to 0.5%, SF3 to SF5) showed significant decrease in the ACD responses, which were comparable to DXM treatment ($p > 0.05$). The AUC of all formulations (SF3 to SF5 and DXM) were significantly lower than the control group ($p < 0.001$). Further lowering the concentration of spantide (0.25 to 0.05%, SF6 to SF8) also reduced the ACD response dose-dependently, but the difference in the response among different concentrations of spantide II (as shown by AUC values) was not statistically significant ($F_{3, 16} = 2.297$, $p > 0.05$).

DISCUSSION

The diffusion of a drug through the skin and accumulation in tissues below the surface is a combination of interactions between the vehicle or formulation, the drug, and the skin structures. The principal barrier function is contained in the superficial layer of the epidermis, the stratum corneum. This is demonstrated in many studies by the fact that gradual stripping away of the stratum corneum produces gradual increase in skin permeability (20). Penetration enhancers may promote drug diffusion through skin by interacting reversibly with stratum corneum and by increasing the drug solubility in the vehicle. Ethanol is an approved vehicle in topical and transdermal systems (e.g., Estraderm patch, Nimulid gel, Topicaine gel, and so forth), which reduces skin barrier function by solubilizing the SC lipids. Cysteine HCl is a pharmaceutically acceptable sulfhydryl compound and is a component of skin depilatory preparations; also, it is believed to interact with keratin to reduce the barrier function of skin (21). Topically applied antioxidants (e.g., cysteine HCl or *N*-acetyl cysteine) have been proven to be remarkably efficient in protecting the skin against UVR-induced photooxidative damage (22,23), and this photoprotective effect of the antioxidants has been associated with their ability to quench free-radical formation (24). Furthermore, the generation of reactive oxygen species (ROS) leads to the expression of specific genes involved in the development of cutaneous inflammation (25). We did not study the permeation enhancement effect of cysteine HCl alone as a penetration enhancer because of very low aqueous solubility of spantide II; consequently, we used ethanol–water mixture (1:1) to keep both spantide II and cysteine HCl in solution. Our earlier studies (15,26) demonstrated that cysteine HCl was effective as a skin penetration enhancer at 1%, 2.5%, and 5.0% (in 50% ethanol), and a concentration-dependent increase in the permeation rate was observed for other drugs. In the current study, preliminary experiments showed that cysteine HCl at 5% concentration was not very effective as a penetration enhancer for spantide II across hairless rat skin (data not shown). Spantide II being a large-molecular-weight peptide, we decided to use the enhancer at 10% concentration. We did not observe any

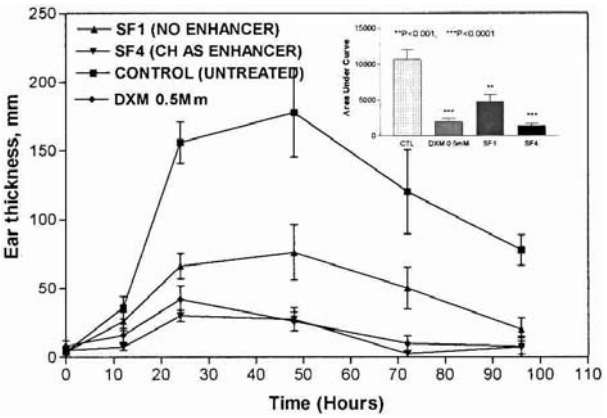

Fig. 2. Effect of spantide II (with or without enhancer in the formulation) on the reduction of allergic contact dermatitis in C57BL/6 mice.

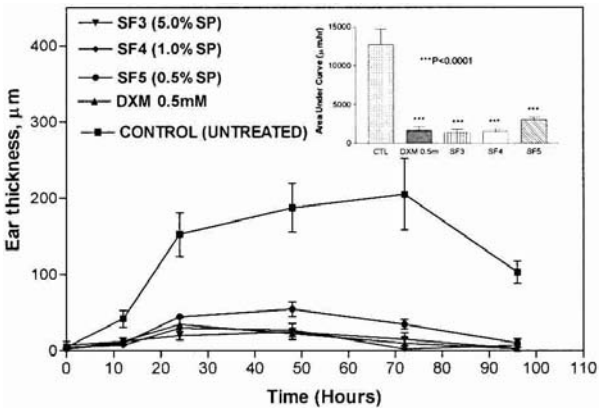

Fig. 3. Effect of spantide II concentration on the reduction of allergic contact dermatitis in C57BL/6 mice.

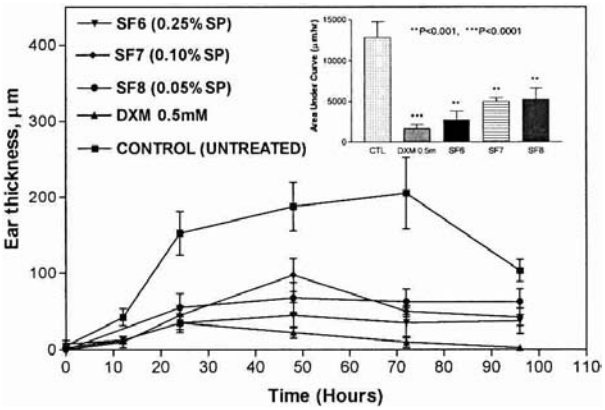

Fig. 4. Effect of spantide II concentration on the reduction of allergic contact dermatitis in C57BL/6 mice.

physicochemical interaction of cysteine HCl with spantide II during skin permeation or bioactivity studies. The samples did not show color change or any signs of degradation for several weeks at room temperature. We are currently investigating the preformulation stability of spantide II in various vehicles including cysteine HCl.

Currently, there is no validated method available on the extraction of peptides from skin tissue. One of the major requirements of such a method is to demonstrate a reasonably good recovery of the peptide from the biological tissue. We validated the method of extraction of spantide II from skin homogenate after incubating the skin with different concentrations of spantide II in the diffusion cells. Acetic acid has been used to extract neuropeptides such as SP and vasoactive intestinal peptide from skin of patients with disease conditions like psoriasis and atopic dermatitis (27,28). We observed consistent recovery values in experiments with different concentrations of spantide II. Two successive extraction steps were adequate for complete recovery, as a third extraction did not produce any further increase in the recovery of spantide II.

No detectable levels of spantide II in the receptor solution were seen for 48 h in the skin permeation and distribution studies. The entire receptor cell content (5.0 ml) of skin permeation as well as distribution experiments at 24 h and 48 h were freeze-dried and reconstituted to 500 μl and analyzed by HPLC in order to detect the spantide II levels in the receptor fluid. No spantide II was detected indicating that there was no permeation of spantide II across rat skin. Drugs with higher molecular weight are poor candidates for delivery by transdermal route to achieve therapeutic blood levels. However, for topical drug delivery, accumulation of drug in the skin with minimal permeation is desired. Hence, drugs with moderate and low permeability can also be considered for topical use. Moreover, in topical products, incorporation of skin penetration enhancers and modulation of drug release is easier than transdermal delivery systems, where the multiple components make it difficult to configure the enhancers in the formulations (29). The epidermal and dermal levels of spantide II were increased several-fold by cysteine HCl at all the time points of the skin distribution study ($p < 0.05$). This indicates that the barrier property of the SC was reduced by cysteine HCl, thus increasing the levels of spantide II in the epidermis and dermis. Sulfhydryl compounds are believed to interact with keratin of SC cells to reduce the barrier to transcellular pathway. It is believed that hydrophilic drugs mainly follow the transcellular pathway (30). Peptides are generally polar due to hydrophilic amino acids, and hence spantide II probably follows the transcellular pathway, thus increasing its skin distribution significantly by cysteine HCl. Our previous studies on permeation enhancement of melatonin with several sulfhydryl compounds have shown that cysteine HCl significantly increased permeation of melatonin through hairless rat and human cadaver skin (15).

The evaluation of anti-inflammatory activity of various neuromodulatory compounds in a mouse model of ACD is well established (19,31). To determine the effectiveness of topically applied neuromodulatory agent spantide II, we compared the ACD response in spantide II treated and untreated C57BL/6 mice. In DNFB sensitized mice, application of DNFB to the right ear induced marked swelling in C57BL/6 mice. The ACD response was significantly lowered by SF1 (no enhancer, $p < 0.01$), SF4 (with cysteine HCl as enhancer), and DXM ($p < 0.001$) at all time points after DNFB sensitization and challenge. Even though the epidermal and dermal levels of spantide II were very low with SF1, it still demonstrated a significant response in lowering ACD. Spantide II at a concentration of 0.5% with enhancer (SF5) showed a response similar to DXM in lowering the ACD in mice.

The cutaneous neurosensory system releases neuropeptides such as SP, vasoactive intestinal peptide, calcitonin gene–related peptide, and so forth, which appear to play an important role in inflammation and wound healing (32,33). We demonstrated the anti-inflammatory effect of SP antagonist spantide II by topical application. The results of the current study further support the role of the cutaneous neurogenic system in skin inflammatory conditions and demonstrates the therapeutic potential of neuropeptides in their treatment. The permeation of penetrants through rat skin is generally several-fold higher than the human skin; therefore, the data of the current study may be an overestimate of percutaneous absorption through healthy human skin (34). However, it is pertinent to mention here that the human skin in disease situations like psoriasis and contact dermatitis is inflamed and more permeable (possibly due to disruption of SC), thus the *in vitro* distribution data with rat skin may be helpful for understanding the topical delivery of spantide II in disease states in humans. Currently, *in vitro* skin permeation and distribution experiments using human cadaver skin are in progress in our laboratory to establish a correlation between the human skin and rat skin for permeation and distribution kinetics of spantide II.

CONCLUSIONS

In vitro permeation studies indicate no detectable levels of spantide II permeated through hairless rat skin. However, spantide II retained in different skin layers and cysteine HCl as a penetration enhancer significantly increased distribution of spantide II in epidermis and dermis. The current study demonstrates that spantide II can be delivered to different skin layers (site of action) to show significant anti-inflammatory activity in allergic contact dermatitis mice.

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