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In Vitro and In Vivo Evaluation of Topical Formulations of Spantide II

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ABSTRACT

The purpose of this study was to develop and evaluate topical formulations of Spantide II, a neurokinin-1 receptor (NK-1R) antagonist, for the treatment of inflammatory skin disorders. Spantide II lotion and gel was formulated with and without n-methyl-2-pyrrolidone (NMP) as a penetration enhancer. The release of Spantide II from gels was evaluated using microporous polyethylene and polypropylene membranes in a Franz Diffusion cell setup. In vitro percutaneous absorption of Spantide II from lotion and gel formulations was evaluated using the above setup by replacing the membranes with hairless rat skin. The in vivo anti-inflammatory activity of Spantide II formulations was evaluated in an allergic contact dermatitis (ACD) mouse model. Among different gels studied, PF127 gel showed highest (70-fold) release of Spantide II compared with hydroxypropyl methylcellulose (HPMC) and hydroxypropyl cellulose (HPC) gels. Lotion and gel formulations with or without NMP showed no detectable levels of Spantide II in the receiver compartment of the Franz diffusion cell until 24 hours. However, Spantide II showed significant retention in epidermis and dermis from lotion and gel formulations at 24 hours. The dermal levels increased ~3.5- and 2-fold when the lotion and gel formulations contained NMP as compared with the formulation with no NMP ($P < .05$). The in vivo studies indicated that Spantide II formulations with NMP were effective in significantly reducing ACD response, similar to dexamethasone (0.5 mM). In conclusion, Spantide II was stable as a topical formulation and delivered to target skin tissue (epidermis and dermis) for the treatment of ACD. In addition this study supports the role of cutaneous neurosensory system in modulating inflammatory responses in the skin.

KEYWORDS: Spantide II, peptide, skin permeation, dermatitis, gel, lotion.

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INTRODUCTION

Delivery of drugs to the skin is an effective and targeted therapy for local dermatological disorders. This route of drug delivery has gained popularity because it avoids first-pass effects, gastrointestinal irritation, and metabolic degradation associated with oral administration. Topical gel formulations provide a suitable delivery system for drugs because they are less greasy and can be easily removed from the skin. Percutaneous absorption of drugs from topical formulations involves the release of the drug from the formulation and permeation through skin to reach the target tissue. The release of the drug from topical preparations depends on the physicochemical properties of the vehicle and the drug employed. In order to enhance drug release and skin permeation, methods such as the selection of a suitable vehicle,[1] co-administration of a chemical enhancer,[2] and iontophoresis[3] have been studied. There are various topical gel formulations on the market for both local and systemic delivery of drugs and several others are in clinical trials.[4] These formulations are targeted to deliver both small molecules and macromolecules.

Neuropeptides and their receptors play a significant role in mediating inflammatory responses in the skin. Spantide II is an anti-inflammatory peptide that blocks the inflammatory effects of substance P by competitively binding to neurokinin-1 (NK-1) receptors on cutaneous cells. It is proposed that Spantide II can be formulated as a stable topical agent that provides adequate skin permeability for the treatment of inflammatory skin disorders such as dermatitis and psoriasis. Various delivery systems, such as liposomes,[5] biodegradable microspheres,[6,7] and hydrogels[8] have been investigated in order to circumvent the susceptibility of peptides to degradation during formulation, storage, and administration. Proteins and peptides are compatible with hydrogels, such as nonionic cellulose polymers and poloxamer gels, which are nontoxic and exhibit reversible thermal characteristics. Poloxamers have been reported as suitable gel systems for various proteins such as insulin,[9] alpha chymotrypsin, and lactate dehydrogenase[10] and peptides such as deslorelin and gonadotrophin-releasing hormone (GnRH).[11] Pluronic F127 (PF127), a type of poloxamer, has been shown to enhance the stability of proteins such as urease, and interleukin-2.[12]

The objective of the present study was to develop and evaluate lotion and gel formulations of Spantide II using various vehicles and gelling agents such as PF127, hydroxypropyl methylcellulose (HPMC), and hydroxypropyl cellulose (HPC), which can be used as novel therapies for inflammatory skin diseases that may have a significant neuro-inflammatory component. In order to evaluate the feasibility of developing Spantide II lotion and gel formulations, the stability of Spantide II formulations, release from gels (through microporous membranes), percutaneous absorption through hairless rat skin, and the in vivo efficacy of formulations in the treatment of allergic contact dermatitis (ACD) in a mouse model were studied.

Materials and Methods

Materials

Spantide II (purity > 95.5%) was custom synthesized by Bio Peptide Co (San Diego, CA) and used without further purification. Trifluoroacetic acid (TFA), n-methyl-2-pyrrolidone (NMP), HPMC, HPC (high viscosity grade), and PF127 were procured from Sigma Chemical Co (St Louis, MO). Ethanol (ETOH) *United States Pharmacopeia (USP)* (200 proof) was obtained from Florida Distillers Co (Lake Alfred, FL). Water, acetonitrile and methanol (high-performance liquid chromatography [HPLC] grade) were purchased from Fisher Scientific (Atlanta, GA). Male Hairless rats (CD hrBi) and C57BL/6 male and female mice were obtained from Charles River Laboratories (Wilmington, MA) and Jackson laboratories (Bar Harbor, ME), respectively.

High-Performance Liquid Chromatography Assay

An HPLC system (Waters Corp, Milford, MA) along with a Vydac reverse phase C_{18} (300 Å pore size silica) analytical column (5 μm, 4.6 × 250 mm) (GraceVydac, Columbia, MD) were used for the analysis of Spantide II. The HPLC system with a photodiode array (PDA) UV detector interfaced with Empower software (Waters Corp, Milford, MA) was used. The mobile phases used were 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) and they were run at a gradient of 68%: 32% to 32%: 68% (solvent A:B, respectively) for 30 minutes, with a flow rate of 1 mL/min. Spantide II content in the samples was determined at 230 nm. The detection limit of Spantide II by this method was 0.1 μg/mL.[13]

Preparation of Spantide II Formulations

Lotion formulations were prepared by dissolving Spantide II at concentrations of 0.25% and 0.5% wt/vol in ethanol with and without 10% wt/vol NMP as an enhancer. First, Spantide II was added to the ethanol and mixed until it dissolved; then, where applicable, NMP was added and mixed to uniformity. Spantide II gel formulations were prepared using HPMC, HPC, and PF127 polymers. Spantide II, 0.5% wt/vol, was dissolved in ethanol and, where applicable, 10% wt/vol NMP was added. In the case of HPMC and HPC, 3% wt/vol and 2.5% wt/vol of the respective polymers were weighed, mixed with Spantide II solution, then diluted with water (to get 1:1 ethanol:water ratio), and stirred overnight. For PF127 gels, Spantide II at 0.5% wt/vol was dissolved in ethanol and, where necessary, 10% wt/vol NMP was added and 25% wt/vol PF127 was added to the mixture and stirred well. Finally, the mixture was diluted with water (to get a 1:1 ethanol:water ratio) and stirred well. The resulting solution was then stored at 4°C overnight to ensure complete polymer dissolution.

In Vitro Release Studies

The in vitro release of Spantide II from different gel formulations was measured through microporous polypropylene membrane (Waters) and 9711 CoTran microporous polyethylene membrane (3M Pharmaceuticals, St Paul, MN). The membrane was mounted between the donor and receptor compartments of Franz diffusion cells. The gel formulations (~0.5 g) were applied evenly on the surface of the membrane in the donor compartment. The receptor compartment was filled with pH 7.4 phosphate buffer solution (PBS) containing 10% ethanol, stirred at 300 rpm, and maintained at 37°C ± 0.5°C using a circulating water bath. Ethanol was added to PBS to increase the solubility of Spantide II for maintaining sink conditions. At predetermined time intervals 0.5-mL samples were taken from the receptor compartment, and Spantide II content of the samples was analyzed by HPLC.

Stability Studies

The lotion and gel formulation samples were stored in well-sealed glass vials for a period of 90 days at 40°C or 50°C and control formulations were stored at 4°C. At predetermined intervals, samples were collected and physical appearance was evaluated. Spantide II content of samples was determined by HPLC. Prior to analysis of gel samples, a weighed amount was reconstituted with 20% ethanol: water in order to allow ease of injection in the HPLC.

In Vitro Permeation and Distribution Studies

Hairless rats 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 on Franz diffusion cells with the epidermis facing the donor compartment. The skin

permeation studies were performed by the procedure as described under "release studies."

For the skin retention studies, the donor cell was removed, and the excess formulation was removed from the surface of the skin using a cotton swab. The skin was then washed with 50% ethanol:water and blotted dry with lint-free absorbent wipes. The entire dosing area (0.636 cm^2) was collected with a biopsy punch. The epidermis was separated from the dermis, and the tissues were minced using a dissection blade. Where applicable, the stratum corneum (SC) was stripped 20 times using breathable medical tape and the stripped skin was used to conduct permeation and skin retention experiments. Spantide II content of epidermis and dermis was extracted using a previously reported method.[13] Briefly, the samples were homogenized and boiled for 10 minutes in acetic acid (1M). The samples were then centrifuged and the supernatant was collected for analysis of Spantide II by HPLC. The experiments were repeated at least 3 times using the skins from different rats.

Induction and Treatment of Allergic Contact Dermatitis

The animals were cared for in accordance with the institutional guidelines (Northwestern University), and the experiments were performed as per the approved animal protocols. ACD was induced by a method as described previously.[13] Mice were sensitized on day zero by applying 25 μL of 0.5% 2,4-dinitro-1-flourobenzene (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) on the right ear in order to induce an ACD response. The left ears were treated with vehicle alone (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 with that of the vehicle-treated contra-lateral ear before DNFB challenge, and at 0 to 72 hours after challenge as measured with a micrometer (7309, Mitutoyo, Tokyo, Japan). Right ears of the mice were treated with topically applied Spantide II formulations, or dexamethasone 0.5 mM solution (DXM), 2 hours after antigen challenge and 3 times a day thereafter for 3 days. The ACD response was determined by measuring the ear swelling of the test drug-treated ears compared with that of vehicle-treated contra-lateral ears before DNFB challenge and at 12, 24, 48, and 72 hours after the challenge with allergen. Each treatment group consisted of 5 animals.

Histological sections of mice ears were performed using hematoxylin and eosin (H&E) staining. The sections of each ear were photographed at different representative sites using a microscope (Olympus America, Melville, NY).

Data Analysis

The Spantide II content of the skin tissue was expressed as mean (mg/g of the skin) ± standard error of mean (SEM). The ACD response was expressed as ear thickness in μm (mean ± SEM). Differences between multiple groups were examined using analysis of variance (ANOVA) and Tukey multiple comparison test. Mean differences with $P \leq .05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Release of Spantide II From Gel Formulations Through Synthetic Membranes

The influence of gel formulation on the release of Spantide II is shown in Figure 1. The release rate of Spantide II from PF127 gel though polypropylene membrane (Figure 1A) was ~70 fold higher compared with HPMC and HPC gels. There was no significant difference between the release profiles of Spantide II from HPMC and HPC gel formulations ($P > .05$). Similar gel release profiles were observed using polyethylene membrane (Figure 1B) indicating that the synthetic membrane used was not a rate-limiting factor in the release of Spantide II from the gels. Table 1 summarizes the slopes representing diffusion rates, correlation coefficients, and the amount of peptide released in 24 hours. The release profiles of gels followed matrix diffusion kinetics (ie, quantity released [Q] is proportional to square root of time [$t^{-1/2}$]) in accordance with the diffusion model proposed by Higuchi.[14] This relationship is observed in systems in which the drug is fully dissolved or suspended in the gel, and thus the membrane used has no significant effect on the release of the drug.[15] Due to the porous nature of the polyethylene membrane, it provides the least resistance to the diffusion of drug molecules, as the permeation through the membrane is governed by diffusion of drug molecules through the liquid retained in the pores of the membrane. The results indicate that Spantide II release from HPMC and HPC gels was retarded as a result of its slow diffusion through the gel matrix, suggesting some type of interaction between Spantide II and the polymer.

Spantide II lotion and PF127 gels with and without NMP showed good physical stability, as there was no discoloration, precipitation, or any other physical changes after storage. The chemical stability of Spantide II in lotion and gel formulations is shown in Figure 2. The lotion formulations were stored at 4°C and 40°C for a period of 60 days, while gel formulations were stored at 4°C and 50°C for a period of 90 days. Spantide II showed good chemical stability in the lotion and gel formulations. The gel stability results are similar to published studies, which show that the stability of urease, interleukin-2,[12] and recombinant human growth hormone[16] was enhanced when

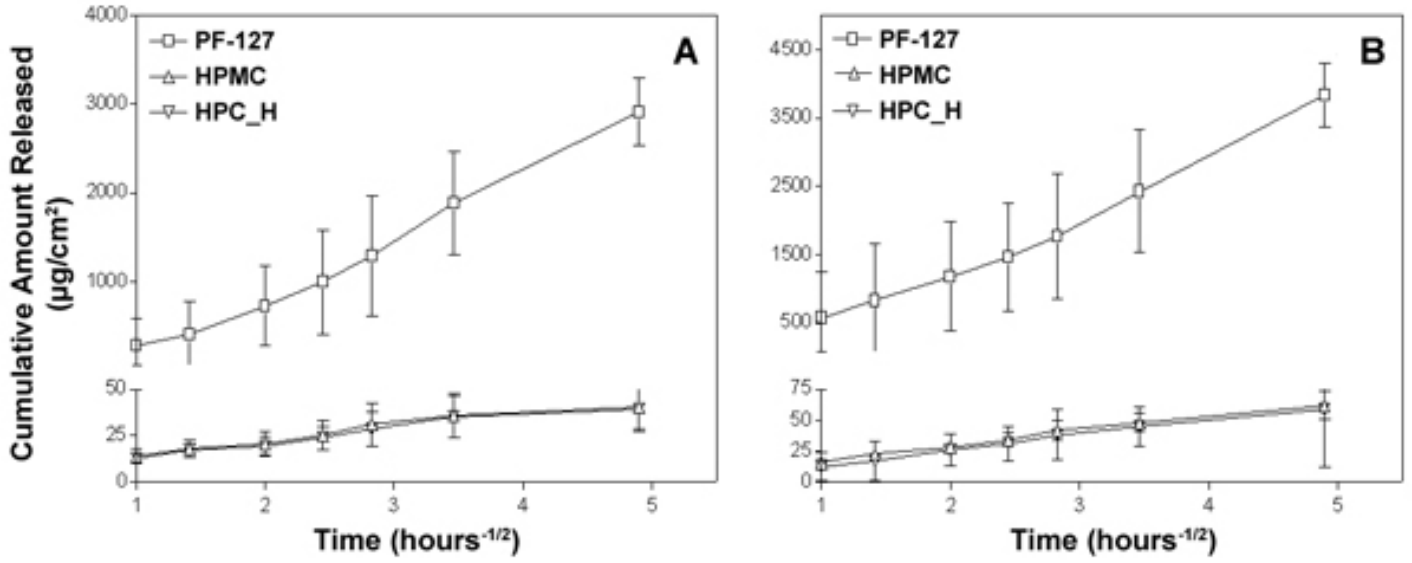

Figure 1. Release profiles of Spantide II from various gel formulations through synthetic membranes: (A) Microporous polypropylene membrane and (B) 9711 CoTran-polyethylene membrane. Data represent mean ± SEM, n=3.

formulated in PF127. This is a nontoxic block polymer with a molecular weight of 13 500 that exhibits reversible thermal gelation.[17] At low temperature, PF127 exists as micellar subunits in solution, and with an increase in temperature it undergoes micellar swelling, forming a cross-linked network[18] that is capable of protecting protein drugs from degradation.[10]

Permeation and Distribution Studies of Spantide II Formulations Through Hairless Rat Skin

Penetration enhancers have been widely used in the topical formulations of peptide and nonpeptide drugs.[19,20] In addition to affecting the drug solubility, enhancers may alter the skin barrier and modify penetration rates of drugs, thereby affecting both drug release and percutaneous absorption of drugs.[21] In the present study, NMP was used at 10% wt/vol concentrations as a penetration enhancer to increase Spantide II absorption into the epidermal and dermal layers of the skin. Pyrrolidone is a component of natural moisturizing factor, therefore its derivatives and structurally related compounds are widely studied as penetration enhancers.[22] These enhancers are known to improve transport of drugs of varied lipophilic/hydrophilic properties.[23,24] NMP exerts its direct influence on the aqueous regions between the polar lipid head groups of the bilayer. It penetrates into this region of tissue in such amounts that they alter the solubilizing ability of this site, thereby promoting drug partition into skin, which subsequently results in increased flux of the penetrant.[25] Many of the studies used pyrrolidones either as neat solvents or in combination with cosolvents; the drug was generally present in the solution form. NMP is believed to facilitate the transport of Spantide II in skin layers. Spantide II was formulated as a soluble form in both liquid (lotion) and semisolid (gel) state in hydro-alcoholic solution containing NMP. The poor skin permeability of Spantide II was taken as an advantage for local delivery into epidermal and dermal layers for its anti-inflammatory effect in disease situations such as dermatitis and psoriasis. The permeation

Table 1. Effect of Formulation of Gel on Diffusion Rates, Correlation Coefficients and Cumulative Amount of Spantide II Released After 24 Hours Through Synthetic Membranes*

	Diffusion Rates ($mg.cm^{-2}.hr^{-1/2}$)	Correlation Coefficient	Released Drug After 24 Hours (mg/cm^2)
Polypropylene			
PF 127	0.644	0.9700	2.909 ± 0.221
HPMC	0.008	0.9803	0.040 ± 0.007
HPC	0.009	0.9800	0.039 ± 0.006
Polyethylene			
PF 127	0.731	0.9838	3.833 ± 2.691
HPMC	0.013	0.9905	0.062 ± 0.006
HPC	0.013	0.9951	0.058 ± 0.027

*HPMC indicates hydroxypropyl methylcellulose; and HPC, hydroxypropyl cellulose.

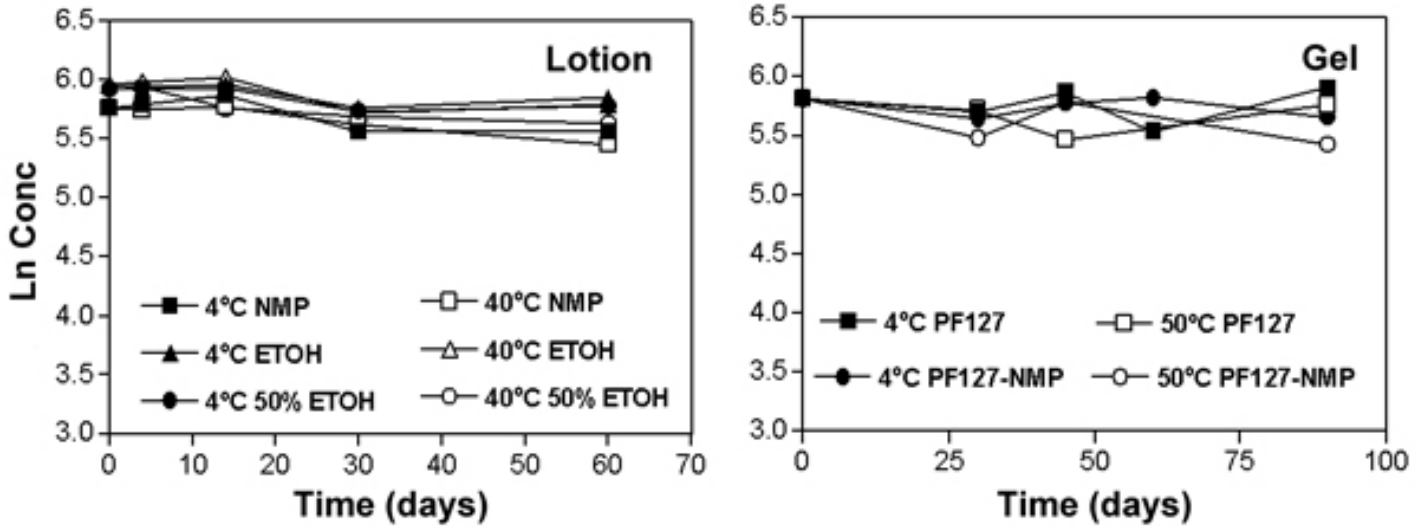

Figure 2. Stability of Spantide II after storage at elevated temperatures from Lotion and Gel formulations. Data represent mean ± SEM, n=3.

experiments of Spantide II from both lotion and gel formulations show no detectable level of Spantide II that permeated through hairless rat skin until 24 hours. The skin retention profiles of lotion and gel formulations are shown in Figure 3. Spantide II preferentially distributed into the epidermal tissue compared with the dermal tissue. It has been reported that NMP facilitates the permeation and deposition of drugs in the lipophilic regions of the skin,[22] and therefore it is believe that Spantide II absorption in the skin was facilitated by NMP. A combination of ethanol and NMP facilitated the partition of Spantide II across the SC, which is the rate-limiting step in transdermal delivery, while the molecular weight of Spantide II help to retain it in the target tissues. Under disease situations such as dermatitis and psoriasis, the skin is inflamed and SC is disrupted. Therefore a study was conducted on SC-stripped skin in order to understand the permeation behavior of Spantide II under skin-barrier damage situations. As shown in Figure 3, at 24 hours the epidermal levels of Spantide II by the lotion formulation was increased 1.8-fold by NMP ($P < .05$); the stripped skin also demonstrated 1.9-fold increase in the epidermal levels as compared with the intact skin. It is worthy to note that, in case of intact skin, more than 90% of Spantide II was retained in SC by the

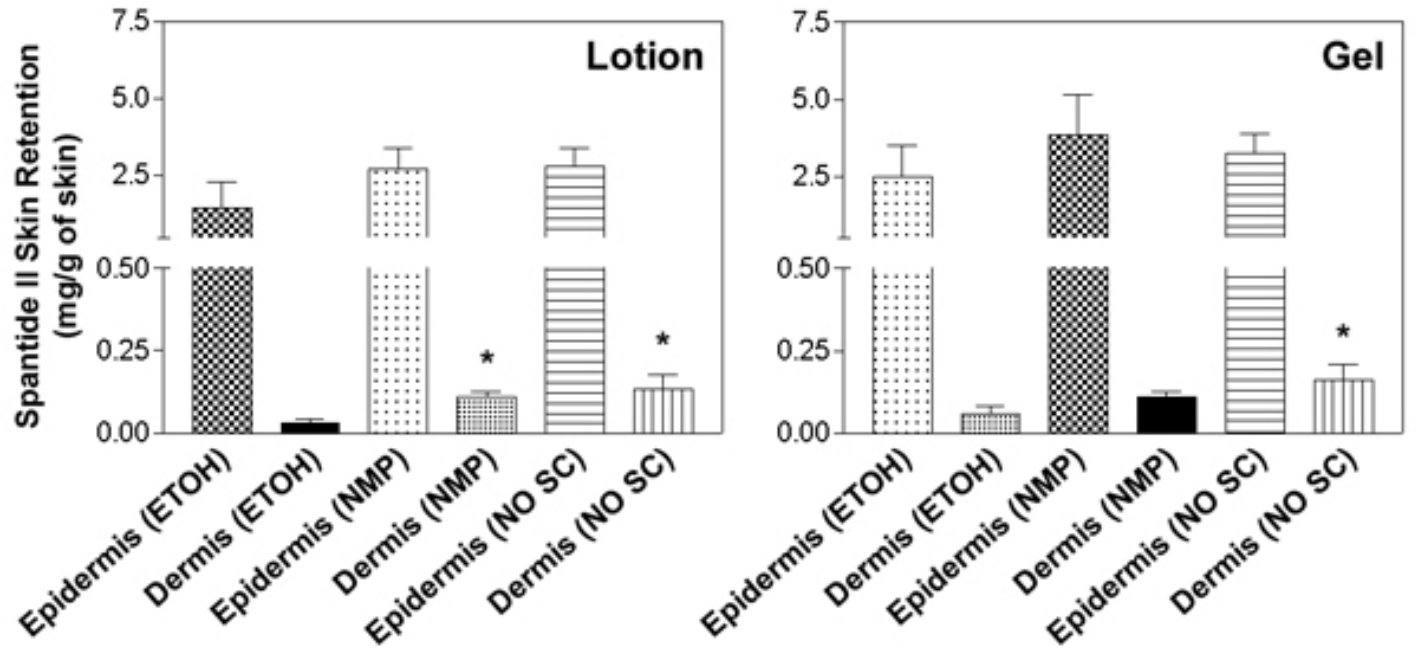

Figure 3. Skin retention profiles of Spantide II from Lotion and Gel formulations. Data represent mean ± SEM, n=3. * $P<.05$ versus dermis (ethanol).

formulations with or without NMP as an enhancer (data not shown). This finding indicates that the amount of Spantide II reaching to viable epidermis is very low as compared with the SC-stripped skin. On the other hand, Spantide II levels in dermis were increased 3.5-fold by NMP ($P < .05$), and the stripped skin demonstrated 4.2-fold higher dermal levels compared with the levels obtained by the intact skin. Since the SC barrier was removed, Spantide II was in direct contact with the viable epidermis, therefore demonstrating higher levels in both epidermis and dermis. It is difficult for Spantide II, an 11-amino-acid peptide, to penetrate through skin even if the SC barrier is removed completely, as demonstrated by the tape-stripping experiment in which no detectible level of Spantide II permeated through the stripped skin. The skin retention profiles of Spantide II from PF127 gel formulations are shown in Figure 3. The gel formulation (ethanol) demonstrated ~1.5- and 2-fold higher retention levels in epidermis and dermis, respectively, as compared with corresponding lotion formulation. In general, both the gel formulations (with or without NMP) demonstrated higher epidermal and dermal levels ($P < .05$) as compared with corresponding lotion formulations. Furthermore, epidermal levels of Spantide II gel increased 1.6-fold by NMP; the stripped skin also demonstrated 1.3-fold increase in the epidermal levels as compared with that obtained by the intact skin. From Figure 3, lotion formulations with ethanol showed comparable deposition profiles to the gel formulations containing ethanol. The enhanced deposition profile with the lotion formulations could be due to the enhancer effect of ethanol.[22] Ethanol interacts with the lipids of the skin by solubilizing them, thus allowing for promotion of drug partition into the skin, which subsequently results in increased flux of the drug. In addition, Spantide II gel showed ~1.8-fold increase in dermal levels by NMP, and the stripped skin demonstrated 2-fold higher dermal levels as compared with the intact skin. Pluronic F127 forms a thermo-reversible hydrogel, which is suitable for delivery of both hydrophilic and hydrophobic drugs. Pluronic F127 is known to provide sustained release and increased stability of drug molecules including peptides[9,11,26] and nonpeptides.[27] Our studies (unpublished data, 2005) on differential scanning analysis of PF127 gels containing Spantide II show no interaction of this peptide with the gel matrix. Therefore, this polymer offers better compatibility and release characteristics of Spantide II. The percutaneous absorption data generated in the present study will be further validated using human skin for permeation and distribution kinetics of Spantide II.

Effect of Spantide II Formulations on the Treatment of Allergic Contact Dermatitis

The reduction of ear swelling in ACD mice was used to monitor the treatment of inflammation after application of Spantide II formulations. The effect of Spantide II lotion formulations on the reduction of ear swelling is shown in Figure 4. Lotion formulations containing 0.25% and 0.5% wt/vol Spantide II without NMP (Figure 4) reduced the ACD response but not as effectively as 0.5 mM DXM. All lotion formulations containing Spantide II concentrations ranging from 0.05% to 1% wt/vol and 10% wt/vol NMP (Figure 4) were effective in the reduction of the ACD response, and this reduction was not significantly different ($P > .05$) compared with that of DXM (0.5 mM). The reduction in ear swelling by Spantide II formulations was similar to DXM. This study demonstrates that the in vitro skin retention data of Spantide II can be correlated to the in vivo anti-inflammatory effect data of ACD mouse model.

The reduction of ACD by Spantide II formulations was further characterized by cutaneous histological examination, and the results are presented in Figure 5. Spantide II

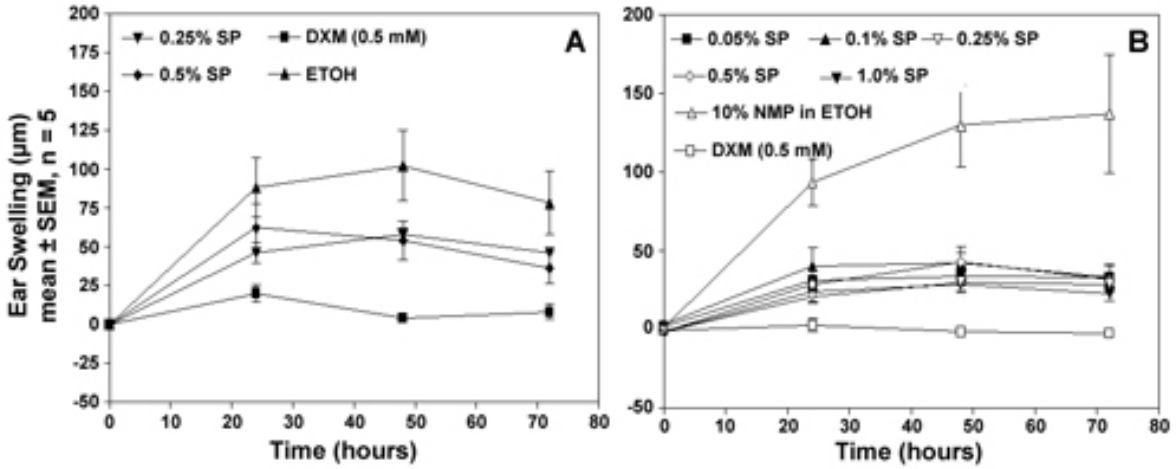

Figure 4. Effect of Spantide II lotion formulations in the treatment of ACD response in C57/BL mice: (A) Lotion formulation without NMP (B) Lotion formulations with NMP. Data represent mean ± SEM, n=3.

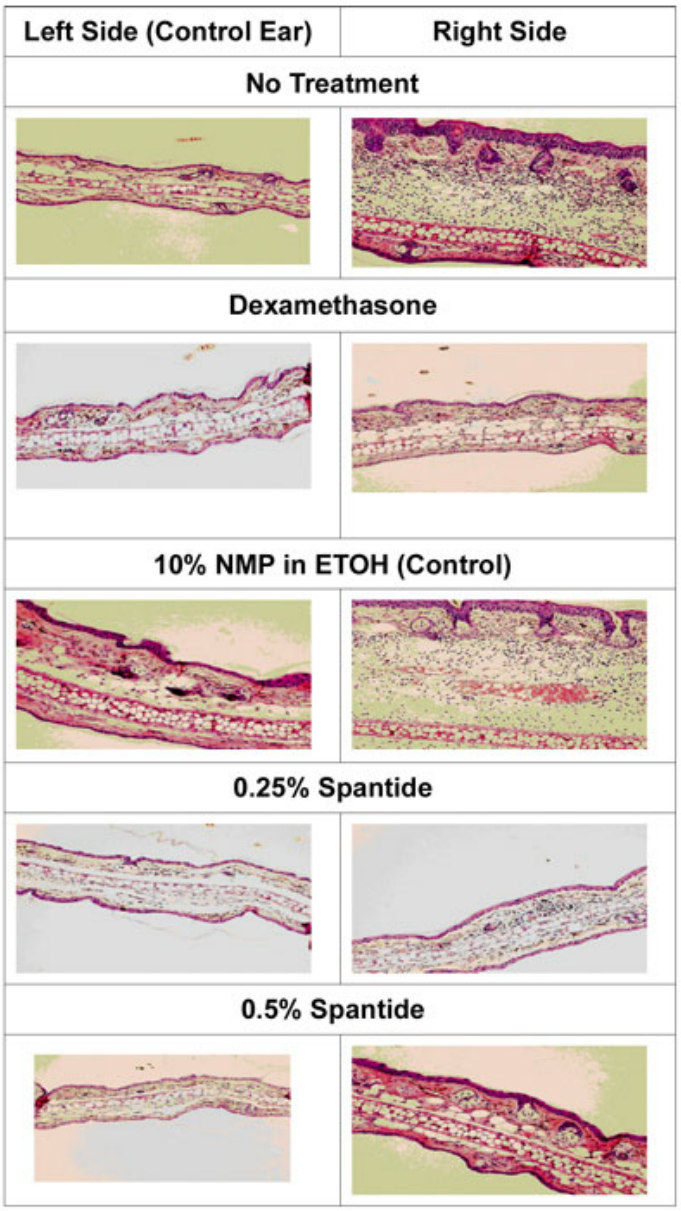

Figure 5. H&E histological staining of ACD C57/BL mice ears after treatment with Spantide II lotion formulations with NMP as a penetration enhancer.

topical treatment of sensitized mice after DNFB challenge resulted in a reduction in both cutaneous edema and in the number of leukocytes infiltrating into the skin compared with the ACD response in mice not treated with Spantide II. As illustrated in Figure 5, Spantide II was highly effective in the treatment of ACD by the reduction of ear swelling as compared with vehicle control and no treatment ears. The evaluation of anti-inflammatory activity of various neuromodulatory compounds in a mouse model of ACD is well established.[13] Although the epidermal and dermal levels of Spantide II were very low with the formulations containing no enhancer, these formulations still demonstrated a significant response in lowering ACD. However, incorporation of 10% NMP in Spantide II formulations facilitated diffusion of Spantide II through skin layers, therefore all the formulations containing Spantide II at a concentration of 0.05% to 1.0% showed a response similar to DXM (0.5 mM) in lowering the ACD in mice.

Conclusion

Spantide II was stable as a topical formulation and delivered to target skin tissue (epidermis and dermis) for the treatment of ACD. In addition this study supports the role of cutaneous neurosensory system in modulating inflammatory responses in the skin.

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