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Transdermal Delivery Enhanced by Antimicrobial Peptides

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Magainin antimicrobial peptide has been shown to increase skin permeability by perturbing *stratum corneum* lipids in the skin. In this study, we hypothesized that skin permeation enhancement depends on peptide structure. We therefore measured skin permeability enhancement by modified magainin derivitives and 20 different antimicrobial peptides in a formulation containing ethanol and N-lauroyl sarcosine (NLS). We found that modification of magainin structure did not improve skin permeability enhancement. Although all six magainin-based peptides had alpha-helical structure and fluidized *stratum corneum* lipids, only magainin and a Gly-Ala substituted magainin with NLS and ethanol significantly increased skin permeability. Among the 20 antimicrobial peptides, only magainin itself and a Lys-Leu analog peptide showed enhancement. Overall, this is the first study to survey skin permeability enhancement by antimicrobial peptides. We conclude that over the range of conditions studied here, most antimicrobial peptides did not enhance skin permeability and that magainin peptide provided the optimal structure.

Keywords: Antimicrobial Pore-Forming Peptide, Magainin Peptide, Chemical Enhancer, Transdermal Drug Delivery, Skin.

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1. INTRODUCTION

Transdermal drug delivery has many advantages over conventional oral delivery and injection, but drug delivery across the skin has been restricted due to the skin's barrier to drug permeation.[1] The skin barrier properties reside in skin's outermost layer, *stratum corneum*. In order to overcome this barrier, various physical and chemical methods have been tested to increase the permeability of the *stratum corneum* to drugs. Research on transdermal delivery using chemical agents has received the most attention and has yielded some successful formulations.[2] In our previous study, the combination of an anionic surfactant, N-lauroyl sarcosine (NLS), and ethanol synergistically increased skin permeability as chemical enhancers by increasing the fluidity of *stratum corneum* lipid structure.[3] As a novel approach, biochemical molecules, such as peptides, have recently been studied. Most of this research has focused on the conjugation of certain peptides with drugs.[4,5] For example, a conjugate of heptarginine oligomers to cyclosporine A was successfully delivered topically and was shown to enter target tissue T-cells, which resulted in functional inhibition of cutaneous inflammation.[5] In another study, TD-1 peptide was identified by phage display and shown to enhance delivery of insulin into the skin. The mechanism behind this phenomenon has not yet been elucidated, but it is thought that TD-1 may create transient pathways in the skin that enable insulin penetration possibly via hair follicles and thereby access the systemic circulation.[6]

Our previous work was the first application of a peptide as a percutaneous enhancer that was not attached to the drug and whose mechanism was similar to chemical enhancers involving lipid disruption in the *stratum corneum*.[7,8] The exact mechanism for transdermal delivery enhancement has not yet been fully elucidated, but the pore-forming peptide, magainin, demonstrated enabled enhancement of skin permeability. Antimicrobial activity caused by magainin has been shown to act by disrupting cell membranes, subsequently causing an increase in membrane permeability and often leading to cell lysis.[9]

Magainin is just one of many antimicrobial peptides found in nature. Animals and plants are vulnerably exposed to harmful pathogens and they can fight against infection by innate defense systems such as gene-encoded antimicrobial peptides.[10,11] So far, more than 750 different antimicrobial peptides, either inducible or constitutive, have been identified and investigated in a wide range of

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eukaryotic organisms including humans.[12] Naturally occurring antimicrobial peptides are normally 12–50 amino acids long. They are poly-cationic with positive net charge of more than +2 and form amphipathic structures with both positively charged and hydrophobic regions.[13]

Because pathogenic organisms are increasingly resistant to conventional antibiotics, antimicrobial peptides have been considered as novel antibiotic drugs, because these peptides act via specific permeabilization of microbial membranes.[10, 14]

In this study, we hypothesize that skin permeation enhancement depends on peptide structure and therefore measured skin permeability after exposure to six different magainin analogues. In addition, we screened 20 different antimicrobial peptides as percutaneous enhancers to determine if other antimicrobial and cell-penetrating peptides might enhance skin permeability better than magainin.

2. MATERIALS AND METHODS

2.1. Modified Magainin Peptides and Other Antimicrobial Peptides

All peptides were synthesized by Shanghai GL Biochemicals (Shanghai, China) with standard solid-phase FMOC methods using an automatic peptide synthesizer (CS Bio, Menlo Park, CA, USA). The sequences and characteristics of modified magainin peptides are shown in Table I. The sequences and characteristics of antimicrobial and cell-penetrating peptides are shown in Table II.

2.2. Skin Preparation

Human cadaver skin was obtained from Emory University School of Medicine (Atlanta, GA, USA) with approval from the Georgia Tech Institutional Review Board. After storage at −75 °C,[15] whole skin was thawed in deionized water at 30 °C for 1 h. Intact epidermis was isolated from dermis using the heat separation method, in which thawed whole skin was immersed in deionized water for 2 min at 60 °C and the epidermis was then carefully peeled away from the dermis using a spatula.[16]

2.3. Skin Permeability Measurement

Before measuring skin permeability, skin was pretreated with magainin and other control formulations. Epidermis was placed in a vertical, glass Franz diffusion cell apparatus (PermeGear, Bethlehem, PA, USA) with 0.7 cm^2 exposed skin surface area. The receiver chamber was completely filled with phosphate-buffered saline (PBS) and the donor chamber was filled with 0.3 ml of a formulation in PBS containing one or more of the following: 1 mM antimicrobial peptide, 50% (v/v) ethanol, and 2% (w/v) N-lauroyl sarcosine (NLS, 98%, Fluka, Buchs, Switzerland). In our previous study, optimal permeation enhancement was achieved using NLS in 50% ethanol solution.[3] All permeation studies using antimicrobial peptides were performed with NLS in 50% ethanol solution. In previous studies, safety concerns about ethanol and NLS were reported.[17, 18] Additional experiments on safety and skin irritation will be necessary in the future.

After a 12-h exposure to one of these formulations at 4 °C, the Franz cell was transferred to a heater/stirrer block (PermeGear) maintained at 32 °C and stirred at 455 rpm for 3 h. The 12-h exposure was selected for experimental convenience, even though exposures between 6–15 h provided similar results (data not shown). This exposure was carried out at 4 °C to minimize skin degradation, although preliminary experiments carried out at 25 °C showed similar behavior (data not shown). The subsequent 3-h exposure was selected to produce sufficient time to re-equilibrate the skin at 32 °C. After this pretreatment, the receiver chamber was emptied and filled with fresh PBS and the donor chamber was emptied and filled with 0.3 ml of 1 mM fluorescein (Sigma Aldrich, St. Louis, MO, USA) in PBS.

Every hour for 5 h, the receiver chamber was sampled by emptying it and refilling with fresh PBS. Samples were analyzed by calibrated spectrofluorimetry (Photon Technologies International, Birmingham, NJ, USA) to determine transdermal flux and permeability. If the fluorescence signal is similar to PBS solution (background) or saturation, we assume that it is out of range. Based on this approach, the detection limit was 10^{-3} μM.

2.4. Circular Dichroism (CD) Spectra

Solutions were prepared containing 50 μM peptide in 50% (v/v) aqueous ethanol. Solutions were placed in a capped quartz optical cell (1 mm path length; Starna cells, Atascadero, CA, USA) and circular dichroism spectra were acquired on a JASCO J-720 CD spectropolarimeter

Table I. Properties of modified magainin peptides.

Peptide	Modification	Characteristic	Sequence	Ref.
Magainin	—	Antimicrobial activity	GIGKFLHSAKKFGKAFVGEIMNS	[25]
Anti-magainin	Charge of magainin modified to negative	Unknown	GIGEFLHSAEEFGEAFVGEIMNS	
MK5E	Increased charge to +5	Greater antimicrobial activity	GIGKFIHAVKKWGKTFIGEIAKS	[26]
Magainin H	Replaced L-Ala with D-Ala	No antimicrobial or hemolytic activity	GIGKFLHSaKKFaKAFVaEIMNS	[27]
Magainin F	Substituted Gly^{13} and Gly^{18} with Ala	Greater antimicrobial activity	GIGKFLHSAKKFAKAFVAEIMNS	[27]
$I^6A^8L^{15}I^{17}$	Increased hydrophobicity	Greater antimicrobial and hemolytic activity	GIGKFIHAAKKFGKLFIGEIMNS	[22, 28]

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Table II. Properties of antimicrobial and cell-penetrating peptides.

Peptide	Characteristic	Sequence	Ref.
A Magainin	Pore-forming antimicrobial peptide	GIGKFLHSAKKFGKAFVGEIMNS	[25]
B TD-1	Insulin delivery peptide	ACSSSPSKHCG	[6]
C Maximin H5	Anionic antimicrobial peptide	ILGPVLGLVSDTLDDVLGIL-NH2	[29]
D Pandinin 2	Pore-forming antimicrobial peptide	FWGALAKGALKLIPSLFSSFSKKD	[30]
E Androctonin	Cysteine-rich antimicrobial peptide	RSVCRQIKICRRRGGCYYKCTNRPY	[31]
F Hexapeptide	Short antimicrobial peptide	Ac-RRWWCF-NH2	[32]
G Thanatin	Non-pore forming antimicrobial peptide	GSKKPVPIIYCNRRTGKCQRM	[33]
H Polyphemusin 1	Anti-parallel β–hairpin antimicrobial peptide	RRWCFRVCYRGFCYRKCR-$CONH_2$	[34]
I Misgurin	Pore-forming antimicrobial peptide	RQRVEELSKFSKKGAAARRRK	[35]
J Penetratin	Cell-penetrating peptide	RQIKIWFQNRRMKWKK	[36]
K Tachyplesin	Antimicrobial peptide with disulfide bond	KWCFRVCYRGICYRRCR-$CONH_2$	[37]
L Protegrin	β-sheet-forming antimicrobial peptide	NH_2-RGGRLCYCRRRFCVCVGR-$CONH_2$	[38]
M P5	Antimicrobial peptide	KWKKLLKKPLLKKLLKKL-NH_2	[39]
N Clavanin A	α-helix-forming antimicrobial peptide	VFQFLGKIIHHVGNFVHGFSHVF-$CONH_2$	[40]
O Indolicidin	Bovine antimicrobial peptide	ILPWKWPWWPWRR-NH_2	[41]
P Oxyopinin 1	Pore-forming antimicrobial peptide	FRGLAKLLKIGLKSFARVLKKVLPKAAKAGKALAKSMADENAIRQQNQ	[30]
Q LL-37	Human antibiotic peptide	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	[42]
R Fall-39	Human antibiotic peptide	FALLGDFFRKSKEKIGKEFKRIVORIKDFLRNLVPRTS	[43]
S Dermcidin	Human antibiotic peptide	SSLLEKGLDGAKKAVGGLGKLGKDAVEDL ESVGKGAVHDVKDVLDSV	[44]
T Lys-Leu peptide	High positive charge antimicrobial peptide	NH_2-KLLLKLLLKLLKLWLKLLKLLLK-COOH	[45]
U Melittin	Antimicrobial and hemolytic peptide	GIGAVLKVLTTGLPALISWIKRKRQQ	[46]

(JASCO, Easton, MD, USA). Spectra were obtained at room temperature by scanning five times from 250 nm to 190 nm at a rate of 500 nm/min in continuous scanning mode.

2.5. Fourier Transform Infrared Spectroscopy (FTIR)

Prior to spectral analysis by FTIR, several pieces of *stratum corneum* were each incubated in various peptide formulations for 15 h at 4 °C and then washed with PBS. FTIR spectra were then taken with skin still in a fully hydrated state. Using a Magma-IR 560 FTIR spectrometer (Nicolet, Thermo Electron Corporation, Waltham, MA, USA), all spectra (2 cm^{-1} resolution, representing the average of 64 scans) were obtained in the frequency range 4000–1000 cm^{-1}. OMNIC professional software (Thermo Electron Corporation) was used to determine the peak position and area under each peak. Although the FTIR had a data collection spacing of 2 cm^{-1}, interpolation between points is reliable because the noise level is so low and the reproducibility of FTIR spectra is so high. This permits one to determine the location of a peak, even if it exists between data points that were actually collected. This is well established in the spectroscopy literature[19] and is consistent with many previous studies involving FTIR analysis of skin, where peak shifts much smaller than the data collection spacing are reported.[20, 21]

2.6. Statistical Analysis

Enhancement of skin permeabilty to fluorescein and FTIR spectroscopy measurements were made using at least three replicate skin samples at each condition, from which the mean and standard error of the mean were calculated. A two-tailed Student's t-test was performed when comparing two different conditions. When comparing three or more conditions, a one-way analysis of variance (ANOVA) was performed.

3. RESULTS AND DISCUSSION

3.1. Effect of Modified-Magainin Peptides on Skin Permeability

To test our hypothesis that skin permeation enhancement depends on peptide structure, we exposed skin to six different modified magainin peptides in a formulation containing ethanol and N-lauroyl sarcosine (NLS) and measured subsequent skin permeability to fluorescein. Each of these peptides has specific characteristics such as charge, hydrophobicity, and hemolytic activity (Table I).

We first ran a control experiment using a solution containing NLS in 50% ethanol and no peptide. Consistent with previous findings,[3] this formulation caused a 16 fold increase in skin permeability (Fig. 1). Also consistent with our previous work,[7, 8] the addition of magainin peptide increased skin permeability 33 fold (Fig. 1), which was almost two times greater than NLS alone (Student's t-test, $p < 0.05$).

To assess the role of charge, we studied two different modified magainins. Magainin has a charge of +4.[11] First, we replaced all of the positively charged lysines in magainin with negatively charged glutamic acid to form "anti-magainin" with a −4 charge. This modified peptide not only did not increase skin permeability, but significantly decreased skin permeability enhancement relative to the NLS-alone control. This suggests that positive charge is needed for enhancement of negatively charged fluorescein

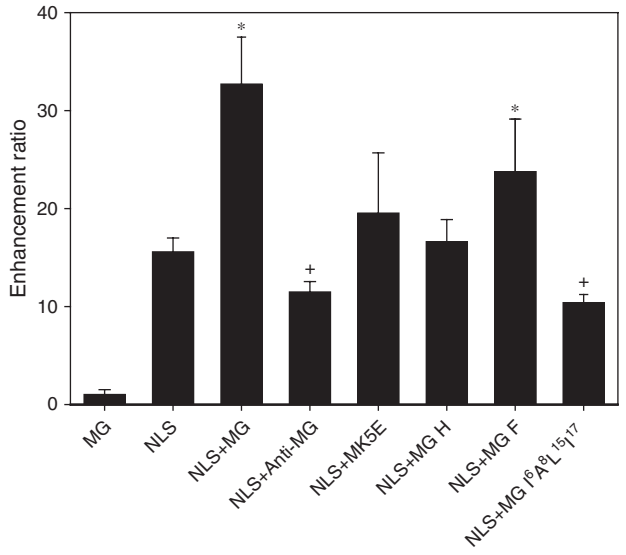

Fig. 1. Enhancement ratio of skin permeability to fluorescein in skin samples treated with magainin only (MG); with N-lauroyl sarcosine without peptide (NLS); and with NLS and magainin (NLS + MG), antimagainin (NLS + Anti-MG), MK5E (NLS + MK5E), magainin H (NLS + MG H), magainin F (NLS + MG F) and $I^6A^8L^{15}I^{17}$ (NLS + MG $I^6A^8L^{15}I^{17}$). Enhancement ratio is defined as the cumulative transdermal transport of fluorescein after 5 h in treated human epidermis relative to untreated human epidermis. Data represent averages of $n \geq 3$ samples with standard error of the mean. The $*$ symbol identifies enhancement ratio significantly larger than NLS treated skin and the $+$ symbol identifies enhancement ratio significantly smaller than NLS treated skin (Student's t-test, $p < 0.05$).

diffusion across the skin. Our previous work suggested an interaction between magainin-mediated pathways across the skin and the molecule diffusing through.[8] Thus, the anti-magainin may have reduced fluorescein permeation due to electrostatic repulsion by the two negatively charged molecules and possibly additional electrostatic repulsion between the anti-magainin and negatively charged skin lipids.

Secondly, we replaced eight amino acids to produce a modified magainin with a +5 charge and strong antimicrobial activity, as demonstrated in previous studies.[22] This modified magainin peptide (MK5E) increased skin permeability 20 fold, but was not statistically greater than NLS alone. This suggests that skin permeation enhancement is very sensitive to peptide structure and that strong antimicrobial activity does not necessarily correlate with skin permeation enhancement.

We next tested three additional modified magainins known from the literature. Magainin H has been shown to have no antimicrobial or hemolytic activity. This peptide did not show any enhancement effect relative to the NLS formulation. Magainin F, in which Gly was replaced with Ala to increase helix formation and produce strong antimicrobial activity, demonstrated increased skin permeability by 24 fold, which was significantly enhanced relative to the NLS control formulation, but not better than the enhancement by the unmodified magainin peptide. Finally, we increased hydrophobicity by substitution of four amino acids, which has been shown to have strong antimicrobial and hemolytic activity. This modified peptide ($I^6A^8L^{15}I^{17}$) resulted in no enhancement and rather decreased skin permeability relative to the NLS control.

Overall, the original magainin peptide and alpha-helix modified magainin (magainin F) showed significant transdermal delivery enhancement, the negatively charged antimagainin and hydrophobic modified magainin ($I^6A^8L^{15}I^{17}$) impeded transport, perhaps due to detrimental interactions with fluorescein, and the remaining two magainins had no significant effect. Furthermore, there was no apparent correlation between antimicrobial activity of peptides and skin permeability.

3.2. Circular Dichroism Spectra and Fourier Transform Infrared Spectroscopy

Magainin's antimicrobial activity is believed to be associated with its alpha-helical structure[10] and may similarly be required to increase skin permeability. We therefore assessed secondary structure of the modified magainins using CD spectroscopy. As shown in Figure 2, even though alpha-helicity was different, all modified magainin peptides exhibited alpha-helical structure in a hydrophobic environment. Therefore, while alpha-helical structure may (or may not) be necessary for skin permeability enhancement, it is not sufficient and does not provide an explanation for the different behaviors of the magainin derivatives.

FTIR was used to investigate *stratum corneum* lipid structural changes caused by different magainin peptides.

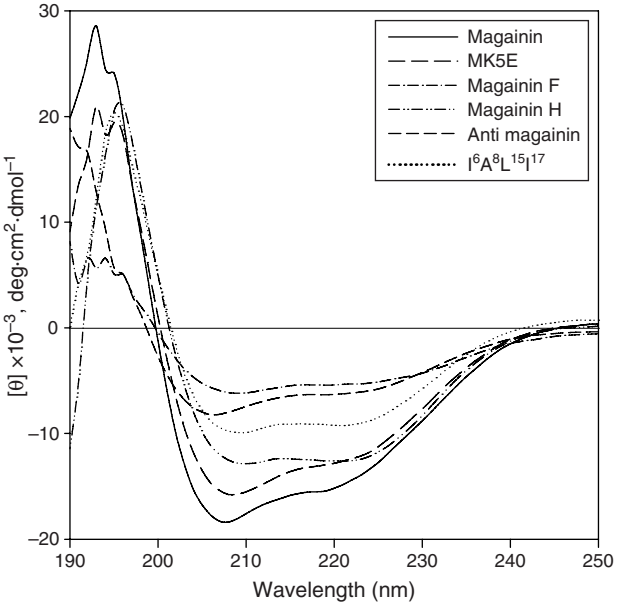

Fig. 2. Circular dichroism spectra of modified magainins in 50% ethanol.

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Our previous studies showed that increased skin permeability caused by magainin correlated with increased *stratum corneum* lipid fluidity, as measured at two characteristic CH_2 stretching peaks.[3,7,8] Representative IR absorbance spectra from 2970–2820 cm^{-1} of human *stratum corneum* samples treated with modified magainins are displayed in Figure 3(A). The wave-number positions of the two characteristic spectral peaks are shown in Figure 3(B).

These data show that the NLS formulation significantly increased skin fluidity relative to the PBS control and that the magainin formulation increased skin fluidity further. These increases correlate with increases in skin permeability, in agreement with previous findings.[7] All modified magainins, except anti-magainin also significantly increased skin lipid fluidity relative to the NLS control, but were indistinguishable from the original magainin. From this result, we conclude that increased lipid fluidity may be associated with increased skin permeability by magainins, but is not sufficient. The observation that anti-magainin did not increase lipid fluidity suggests that the electrostatic repulsion between anti-magainin and skin lipids may prevent anti-magainin from effectively interacting with those lipids.

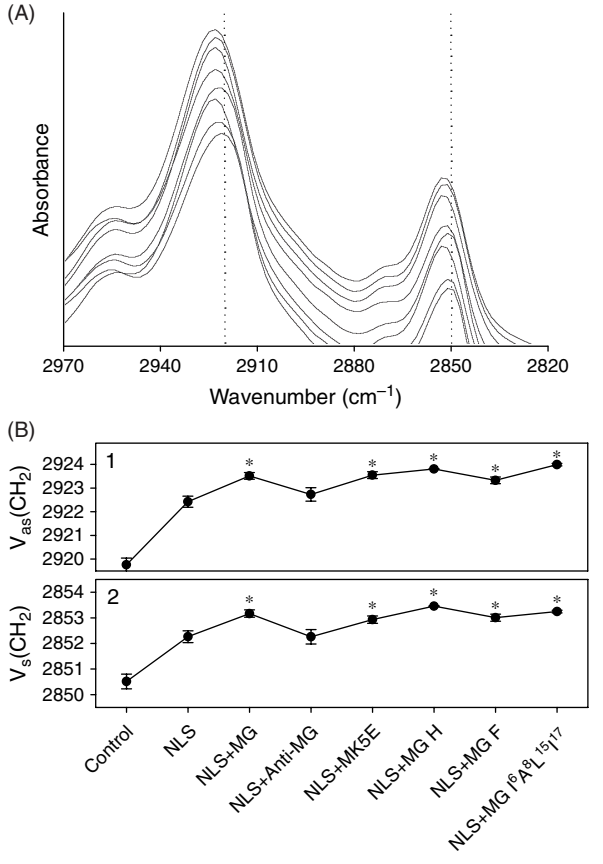

Fig. 3. Fourier-transform infrared spectroscopy analysis of human *stratum corneum* treated with different formulations. (A) Spectra showing wavenumbers characteristic of CH_2 stretching in lipids treated with (from bottom to top): PBS; NLS alone; and magainin, anti-magainin, MK5E, magainin H, magainin F, and magainin $I^6A^8L^{15}I^{17}$ with NLS in 50% ethanol. Dashed lines indicate peaks of interest. Graphs are representative of $n \geq 3$ replicate samples. (B) Change of CH_2 asymmetric stretching frequency (V_{as}) and CH_2 symmetric stretching frequency (V_s). Data represent averages of $n \geq 3$ samples with standard error of the mean. The $*$ symbol identifies wavenumbers after exposure to NLS and peptide that are significantly larger than exposure to just NLS (Student's t-test, $p < 0.05$).

3.3. Screening Antimicrobial and Cell-Penetrating Peptides for Effects on Skin Permeability

Magainin is just one of hundreds of different antimicrobial and cell-penetrating peptides known in the literature.[11] We hypothesized that other peptides might similarly enhance skin permeability and may be even better than magainin. We therefore screened 20 different kinds of antimicrobial and cell-penetrating peptides for skin permeation enhancement. Table II shows the sequences and representative characteristics of each peptide.

As shown in Figure 4, only one of tested peptides, other than magainin, significantly increased skin permeability better than the NLS control, but this enhancement was still significantly less than magainin. This one peptide is the highly positively charged lysine-leucine synthetic peptide (T). This shows that among the diverse set of peptides screened, magainin provided the optimal structure.

In Figure 5, we looked for alpha-helical structure among the 20 peptides using CD spectroscopy and found that about half of them showed alpha-helicity: magainin (A),

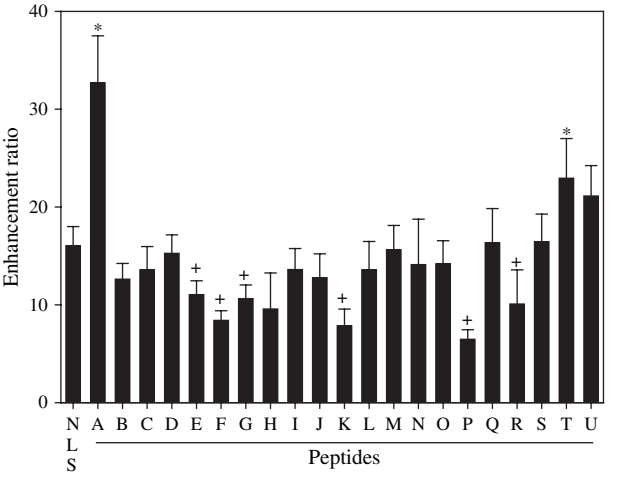

Fig. 4. Enhancement ratio of skin permeability to fluorescein in skin samples treated with NLS and different antimicrobial or cell-penetrating peptides. All permeation studies using antimicrobial peptides were performed with NLSin 50% ethanol solution. The lettering scheme identifies the peptides as listed in Table II. Data represent averages of $n \geq 3$ samples with standard error of the mean. The $*$ symbol identifies skin penetration enhancement ratio significantly larger than NLS treated skin and the $+$ symbol identifies enhancement ratio significantly smaller than NLS treated skin (Student's t-test, $p < 0.05$).

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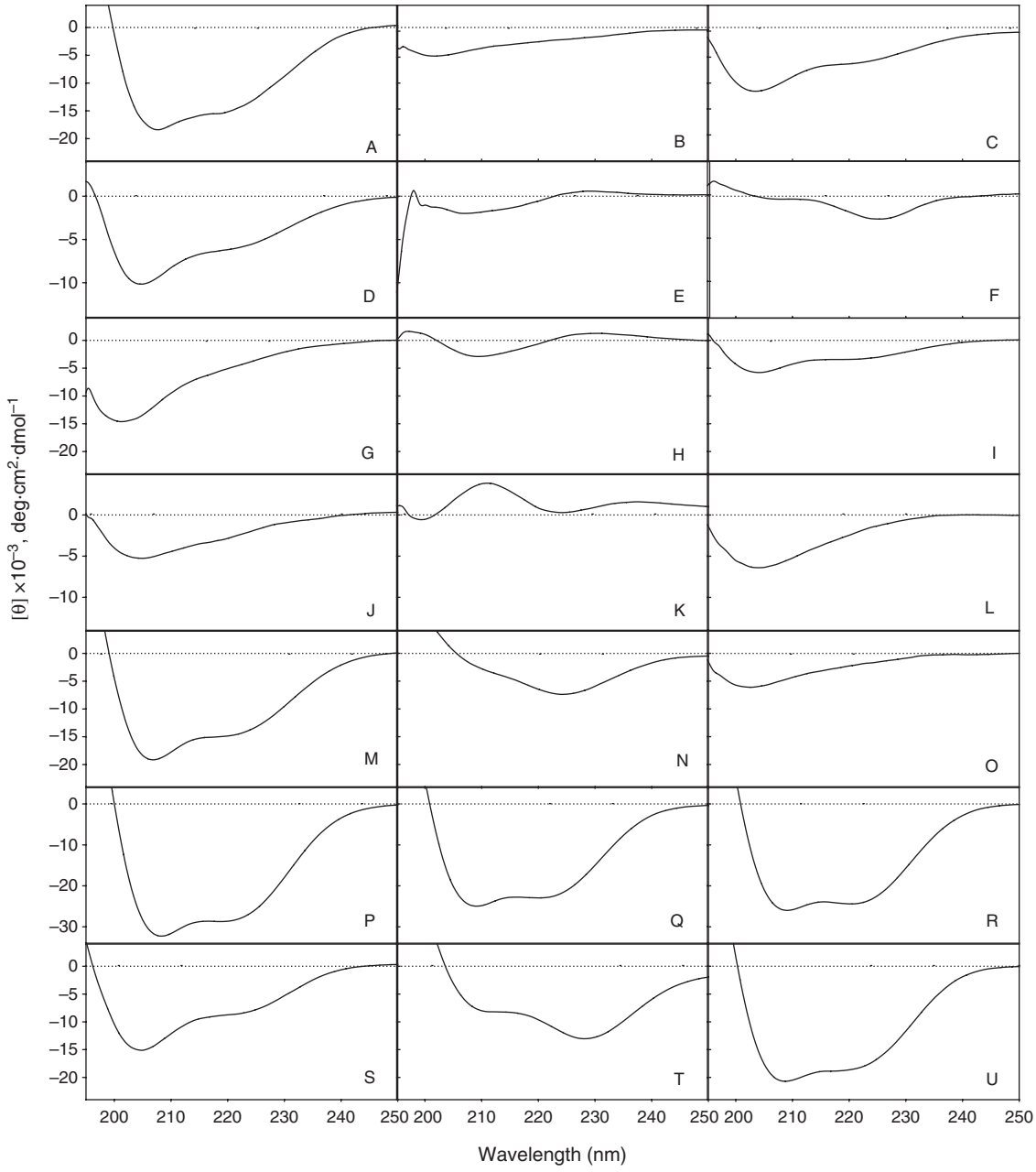

Fig. 5. Circular dichroism spectra of antimicrobial and cell-penetrating peptides in 50% ethanol. The lettering scheme identifies the peptides as listed in Table II.

maximin H5 (C), pandinin 2 (D), misugurin (I), P5 (M), oxkil (P), LL-37 (Q), fall-39 (R), dermcidin (S), Lys-Leu peptide (T), and melittin (U). Once again, however, there was no correlation between alpha-helicity and skin permeability enhancement.

3.4. Effect of Peptide Concentration on Skin Permeability

We were concerned that the reason why other peptides did not increase skin permeability well was because the formulation had been optimized for magainin and not for other peptides. For example, we previously observed that increased skin permeability was strongly dependent on magainin concentration.[23] As shown in Figure 6(A), increasing magainin concentration up to 1 mM increased skin permeability (ANOVA, $p < 0.05$). However, further increase in magainin concentration to 2 mM reduced enhancement below that of the NLS control, as reported previously.[23] This effect may be explained by aggregation of high-concentration magainin in the *stratum corneum* lipids, which may disrupt and occlude the expected pore structures formed by lower-concentration magainin.[24]

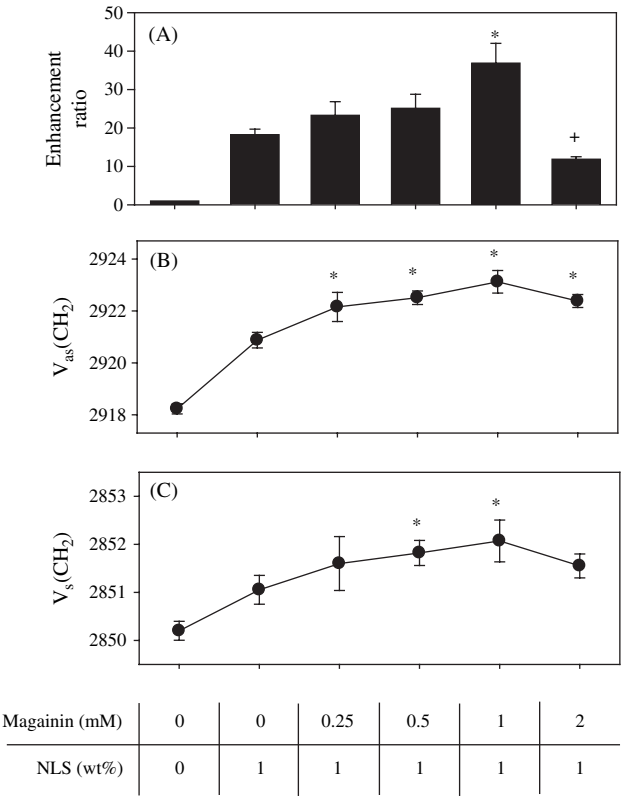

Fig. 6. Effect of magainin concentration on skin permeability. (All sample treated with NLS). (A) Enhancement ratio of skin permeability to fluorescein. Peak wavenumber of characteristic FTIR spectral peaks from human *stratum corneum* treated with magainin at different concentrations corresponding to (B) asymmetric CH_2 stretching and (C) symmetric CH_2 stretching as a function of magainin concentration. The $*$ symbol identifies skin penetration enhancement ratio or wavenumbers significantly larger than NLS treated skin (Student's t-test, $p < 0.05$).

In order to investigate the correlation of enhancement with lipid structure disruption, we measure FTIR spectra to assess lipid fluidity. As shown in Figure 6(B), addition of magainin peptide increased lipid fluidization relative to the NLS control, but increasing magainin concentration from 0.25 mM to 1 mM did not have significant effects (ANOVA, $p > 0.05$). However, 2 mM magainin caused decreased lipid fluidity compared to the NLS control as shown by the symmetric CH_2 stretching mode, which is consistent with the skin permeability experimental result.

Guided by these observations, we examined the effect of concentration of three representative peptides: Lys-Leu peptide, which significantly enhanced skin permeability, and dermcidin and pandinin 2, which had no significant effect (Fig. 7). For all three peptides, up to 1 mM skin permeability was statistically unchanged relative to the NLS control, but plunged significantly at 2 mM. We conclude that suboptimal choice of peptide concentration does not appear to explain the inferiority of other peptides to magainin's ability to increase skin permeability.

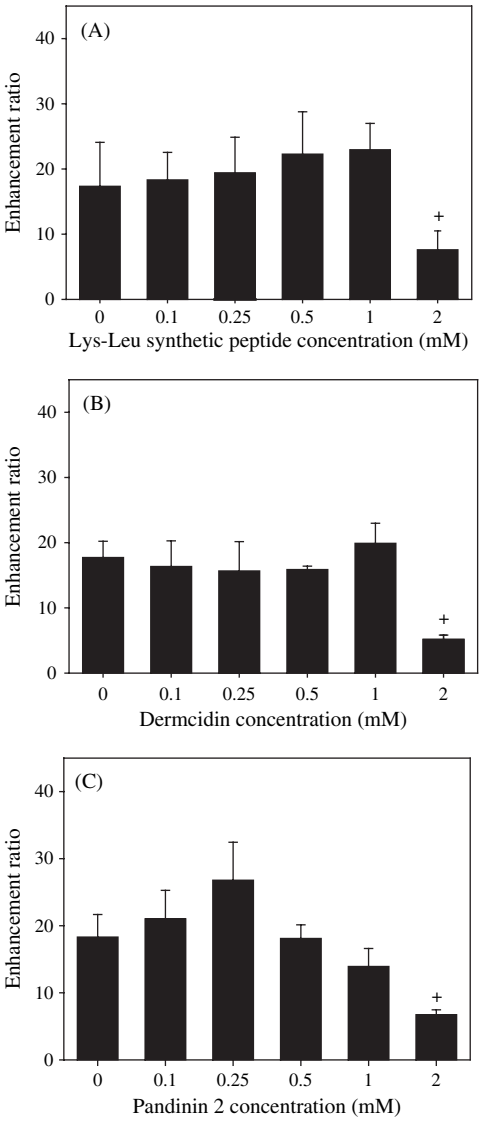

Fig. 7. Effect of peptide concentration on skin permeability in skin samples treated with NLS and selected peptides: (A) Lys-Leu synthetic peptide, (B) dermcidin and (C) pandinin 2. Data represent averages of $n \geq 3$ samples with standard error of the mean. The $+$ symbol identifies enhancement ratio significantly lower than NLS treated skin (Student's t-test, $p < 0.05$).

4. CONCLUSIONS

This study supported the hypothesis that skin permeation enhancement depends on peptide structure. Modification of magainin structure significantly influenced transdermal enhancement characteristics of the peptide. Screening 20 different antimicrobial and cell-penetrating peptides showed that only magainin and lysine-leucine synthetic peptide increased skin permeability under the conditions of this study. *Stratum cornuem* lipid fluidization and alpha helical peptide structure were associated with increased skin permeability, but were not predictive of permeation enhancement. Finally, transdermal enhancement by magainin peptide was found to be concentration dependent.

Overall, magainin was the only naturally occurring antimicrobial peptide that increased skin permeability and further optimization of magainin structure was not possible over the range of conditions studied.

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