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RESEARCH PAPER

Enhanced Transdermal Peptide Delivery and Stability by Lipid Conjugation: Epidermal Permeation, Stereoselectivity and Mechanistic Insights

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ABSTRACT

Purpose Efficient delivery of therapeutic peptides to the skin will facilitate better outcomes in dermatology. The tetrapeptide AAPV, an elastase inhibitor with potential utility in the management of psoriasis was coupled to short chain lipoamino acids (Laa: C6-C10) to enhance the peptide permeation into and through human epidermis.

Methods AAPV was conjugated to Laas by solid phase synthesis. Peptide stability, skin distribution and permeation, elastase activity and surface activity were determined.

Results Laas increased peptide permeation into the skin. The permeation lag time and amount of peptide remaining in the skin increased with the carbon chain length of the Laa conjugate. We also demonstrated stereoselective permeation enhancement in favour of the D-diastereomer. Importantly, the elastase inhibition activity of the peptide was largely retained after coupling to the Laa conjugates, showing potential therapeutic utility. The Laa-peptide structures were shown to be surface active, suggesting that this surfactant-like activity coupled with enhanced lipophilicity may contribute to their interaction with and permeation through the lipid domains of the *stratum corneum*.

Conclusions This study suggests that the Laa conjugation approach may be useful for enhancing the permeation of moderately sized peptide drugs with potential application in the treatment of skin disorders.

KEY WORDS dermatology · peptide delivery · percutaneous · psoriasis · transdermal · lipid conjugation

INTRODUCTION

Peptides with therapeutic potential in dermatology have been identified (1) but most do not have suitable physicochemical characteristics for effective skin permeation, thus limiting their therapeutic value. Various methods to enhance their delivery to the skin have been investigated (2). In this study we have demonstrated the potential for enhancing the skin permeability of a tetrapeptide that has potential utility in psoriasis. Our approach involves addition of a lipophilic moiety to the peptide to provide favourable physicochemical characteristics for permeation across the *stratum corneum* barrier. Lipoamino acids (Laa) are α-amino acids with a hydrocarbon side chain and have been shown to significantly increase the transport and biological half-life of peptides (3,4). They are a convenient method of incorporating lipid groups into peptides as they can be coupled to the peptide during solid phase synthesis using standard coupling techniques and can be incorporated at any place in the sequence. The strategy of adding a lipophilic chain has also been used in some commercially available cosmetic peptide products such as palmitoyl pentapeptide.

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Whilst these are very well accepted in the cosmetic industry we could not find any report in the scientific literature quantifying their skin permeation.

Human Neutrophil Elastase (HNE) is a member of the chymotrypsin family of serine proteases and is primarily located in the azurophil granules of polymorphonuclear leucocytes. It can cause degradation of a wide variety of bio-macromolecules such as elastin, collagen types I, II, III, IV, VIII, IX and XI, fibrin, fibronectin, cartilage proteoglycans, cytokines, the platelet IIb/IIIa receptor and cadherins (5). Active HNE is detected in psoriatic lesions and induces keratinocyte hyperproliferation *via* the EGFR signaling pathway (6). This imbalance can be restored by administration of natural inhibitor (α_1P_i) or low molecular weight peptidic reversible or irreversible inhibitors (7,8). Thus, the involvement of HNE in such pathological processes makes it an interesting target for the development of anti-inflammatory drugs and potentially useful in the management of psoriasis.

Peptidic HNE inhibitors have a common hydrophobic sequence that partially mimics certain amino acid sequences recognised by elastin. The Ala-Ala-Pro-Val (AAPV) tetrapeptide sequence fits the P-P1 subsites of elastase and inhibits HNE competitively. Toth *et al.* synthesized and analyzed the HNE inhibitory capacity of a series of lipopeptides of increasing lipophilic character keeping the AAPV peptide moiety constant. Enzyme inhibition increased with increased lipophilicity of the substances (9). In a preliminary study we showed that coupling to a racemic mixture of a short chain Laa resulted in two diastereomers of the Laa modified tetrapeptide, both exhibiting enhanced skin permeability compared to the parent peptide (10).

In the current study AAPV was conjugated to a series of Laas (carbon chain length C6 to C10) and the influence on permeation into and across human skin assessed. The stability of Laa conjugates and peptide in the presence of human skin was assessed. HNE inhibition assays were carried out to ensure that the lipophilic conjugates of AAPV retained the HNE inhibitory activity of the parent peptide AAPV. Molecular models were generated and assessment of surface activity was undertaken in order to provide insights into the mechanism of skin permeation enhancement of the peptide conjugates.

MATERIALS AND METHODS

Chemicals

AAPV (MW 355.4), C6(D,L)-Laa-AAPV (MW 468.6), C8(D,L)-Laa-AAPV (MW 496.6) and C10(D,L)-Laa-AAPV (MW 524.4) (structures shown in Fig. 5) were synthesised, purified and validated by mass spectrometry and analytical HPLC at the University of Queensland (see supplementary material for detailed descriptions). Dimethylformamide (DMF) and trifluoroacetic acid (TFA) of peptide synthesis grade were purchased from Auspep (Parkville, Australia). HPLC grade acetonitrile was purchased from Labscan Asia Co. Ltd. (Bangkok, Thailand). Fmoc-protected amino acids (Ala, Pro, Val, NIe) and Rink amide MBHA resin (100–200 mesh, 0.78 mmol/g loading) were obtained from Novabiochem (Melbourne, Australia). Piperidine was purchased from Auspep, Melbourne Victoria. Propylene glycol (PG) and dimethyl sulphoxide (DMSO) were obtained from BDH Chemical Pvt Ltd and Ajax FineChem, respectively. HPLC grade acetonitrile and methanol (MeOH) were used and all other chemicals were of analytical grade. Phosphate buffered saline solution (PBS) was prepared according to the United States Pharmacopoeia. All other chemicals were purchased from the Aldrich Chemical Company unless otherwise stated.

Lipoamino Acid Synthesis

N-*tert*-Butoxycarbonyl-2-amino-D,L-octanoic acid (C8Laa) and N-*tert*-butoxycarbonyl-2-amino-D,L-dodecanoic acid (C10Laa) were synthesised from diethyl acetamido malonate and the appropriate alkyl bromide followed by N-Boc protection using published procedures, and the spectral data for these compounds matched the reported data (10–12).

Peptide Synthesis

The parent peptide, AAPV, and the C8- and C10-Laa derivatives were assembled on Rink amide MBHA resin (100–200 mesh, 0.78 mmol/g loading) on a 0.5 mmol scale using HBTU/DIPEA activation and the *in situ* neutralisation protocol (13). The efficiency of each amino acid coupling was determined by the quantitative ninhydrin reaction (14) and couplings repeated until an efficiency > 99.6% was achieved.

When construction of the peptide was complete, the resin was washed with DMF, DCM and MeOH and then dried. The peptide was deprotected and cleaved from the resin by treatment with TFA:water:triisopropylsilane (TIS) (95:2.5:2.5 v/v, 25 mL) for 6 h. The resin was removed by filtration and washed with TFA. The solvent was removed from the peptide solution under a stream of nitrogen and the crude peptide precipitated with cold diethyl ether, collected and dissolved in 20% acetonitrile and lyophilized.

The purification of peptide analogues was achieved by preparative RP-HPLC on a Grace Vydac C18, 25 cm preparative column using a Shimadzu SCL-10AVP controller and pump with an SPD 10AVP UV–Vis detector set at a wavelength of 214 nm. The peptides were purified using a gradient elution profile from 100% Solvent A (0.1% TFA in

H_2O) to 90% solvent B (90% CH_3CN in water with 0.1% TFA) over 35 min at a flow rate of 5 mL/min. The peptides were purified to a single peak in the case of AAPV and two peaks in the case of the C8Laa and C10Laa-derivatives corresponding to the two diastereomers of the peptide, by analytical RPHPLC. The purity of peptide analogues was determined by analytical RP-HPLC on a Vydac C18, 25 cm analytical column using the Shimadzu SCL-10AVP system described above and a gradient of 100% Solvent A to 90% Solvent B over 30 min at a flow rate of 1 mL/min, electrospray ionization MS (ESI-MS; Perkin-Elmer Sciex API 3000) and LC/MS (Shimadzu LC-10AT HPLC, Perkin-Elmer Sciex API 3000). Example analytical HPLC traces and mass spectra of the purified Laa-AAPV conjugates are available in the supplementary material.

Synthesis of Nle(C6-Laa)-Ala-Ala-Pro-Val-NH_2

Both D- and L-norleucine (C6-Laa) were coupled to purified AAPV by a solution phase coupling procedure: Fmoc-D-Nle-OH (27.14 mg, 0.077 mmol) was activated by benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate (HBTU) (30.28 mg, 0.08 mmol) and hydroxybenzotriazole (HOBt) (14.89 mg, 0.11 mmol) in dimethylformamide (DMF) (5 mL). This solution was stirred for 25 min before being added to a stirred solution of AAPV (22.28 mg, 0.063 mmol), N,N-diisopropylethylamine (DIPEA) (40 μL, 0.23 mmol) in DMF (10 mL) under anhydrous conditions. The reaction was stirred at room temperature for 21 h. The solution was concentrated to dryness *in vacuo* at 40°C with residual DMF azeotropically removed using toluene to give dark yellow oil. The residue was dissolved in water (10 mL), the product extracted with ethyl acetate (3×25 mL) and combined layers successively washed with 10% citric acid (2×20 mL), 10% sodium bicarbonate (2×20 mL), brine (3×20 mL), dried magnesium sulfate ($MgSO_4$), filtered and concentrated to dryness *in vacuo* at 35°C. Fmoc deprotection was undertaken by addition of 20% piperidine solution (20% in DMF) (40 mL) to the crude Laa and stirred at room temperature for 3 h. The resultant solution was concentrated to dryness *in vacuo* at 35°C to give a yellow oil and dissolved in 1:1 CH_3CN/H_2O, filtered and lyophilized overnight to afford crude C6-AAPV.

Purification of C6-Laa-AAPV

Crude C6-AAPV was purified on strata-X-C 33 um polymeric strong cation 200 mg/3 mL sep-pack. The crude C6-AAPV was dissolved in H_2O (2 mL). Sep-pack was flushed with 2 mL H_2O before crude C6-AAPV was loaded. Sep-pack was successively flushed with H_2O (4 mL) and fractions collected and lyophilized from the following gradients; 20% CH_3CN/H_2O (6 mL), 50% CH_3CN/H_2O (6 mL) and 100% CH_3CN/H_2O (6 mL) to give purified C6-AAPV **1** as a white solid: LRESIMS m/z 491 $[M+Na^+]^+$; HRESIMS m/z 491.2961 ($M+Na^+$, $C_{22}H_{40}N_6O_5Na$ requires 491.2952).

Example analytical HPLC traces and mass spectra of the purified Laa-AAPV conjugates are available in the supplementary material.

HPLC Instrumentation and Analysis for Stability and Permeation Studies

AAPV and Laa conjugates were quantified by reverse phase HPLC (Agilent 1200 system) with separation achieved on a Phenomenex C18 column (5 μm, 4.6 mm×50 mm) using a mobile phase gradient protocol developed for each compound. Buffer A was 0.1% TFA and buffer B was 0.1% TFA in acetonitrile; flow rate of 1.0 ml/min; column temperature held at 25°C, injection volume 50 μL and UV absorbance detection at 214 nm. The chromatography was optimised to permit identification and quantification of the diastereomers of the Laa conjugates.

Skin Stability Experiment

The stability of AAPV and Laa conjugates was determined by placing human skin in a vial containing peptide solution to provide an estimate of their stability during the skin diffusion experiments. Vials containing 2 mL of 20 μg/mL AAPV, 10 μg/mL C6(D,L)-Laa-AAPV, 100 μg/mL C8(D,L)-Laa-AAPV and 30 μg/mL C10(D,L)- Laa-AAPV solutions were stored at 35°C. Samples (150 μL) were withdrawn at 0, 2, 4, 6 and 24 h and analysed by HPLC. The percentage of intact peptide remaining at each time point was calculated.

Skin Permeation of AAPV and Laa Conjugates

Human epidermal membranes were obtained by heat separation of whole skin (15) obtained from female donors following abdominal surgery at Perth Hospitals under approval from the Human Research Ethics Committee of Curtin University. The epidermal membrane was mounted on Franz-type diffusion cells (surface area 1.2 cm^2 and receptor volume 3.5 ml PBS at pH 7.4) which were partially immersed in a water bath maintained at 35°C to provide a skin surface temperature of 32°C. Membrane integrity was tested by resistance measurement with rejection of membranes showing resistance less than 20 kΩ. A solution of AAPV or Laa conjugate (3 mg in 300 μL of propylene glycol) was placed in the donor compartment and sealed with Parafilm. Samples (200 μL) were taken from the receptor compartment at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 24 h and replaced immediately with 200 μL of receptor medium pre-equilibrated at 35°C. At 24 h the epidermal surface was washed (with washings retained), the epidermal membrane retained and the AAPV

or conjugate content extracted using a validated procedure. This permitted mass balance to be calculated. AAPV and conjugate in all samples was identified and quantified by HPLC. The D- and L-diastereomers of the lipopeptide were identified by comparison with synthesised standards of the individual diastereomers of known configuration.

Surface Tension Measurement

The surface tension of AAPV, C6(D,L)-Laa-AAPV, C8(D,L)-Laa-AAPV and C10(D,L)-Laa-AAPV was measured by the ring method using a Du Nouy ring tensiometer at room temperature. Solutions were prepared in PBS at 0.5, 0.25, 0.125 and 0.0625 mg/mL and compared with PBS as a control. The surface activity of AAPV and its Laa conjugates in buffer was determined and an approximate critical micelle concentration (CMC) estimated from the graph of surface tension in dyne/cm *versus* concentration.

Elastase Inhibition Assays

Inhibition of HNE by AAPV and Laa conjugates was assessed using an EnzChek® Elastase Assay Kit (E-12056, Molecular Probes). The activity of the enzyme was measured in the presence of varying concentrations of the peptides using a final concentration of DQ elastin substrate of 6.25 and 25 μg/mL and of elastase of 0.25 and 0.5 U/mL. A one hour incubation period at room temperature and a fluorescence microplate reader equipped with standard fluorescein filters were used to detect the activity of elastase from porcine pancreas down to a final concentration of 5×10^{-3} U/mL (40 ng protein/mL). Correction was made for background fluorescence by subtracting the values derived from the no-enzyme control.

Statistical Analysis

Differences in the stability, permeation and biological activity of AAPV and the Laa conjugates were analysed for statistical significance ($p<0.05$) using Analysis of Variance (ANOVA).

RESULTS

Synthesis and Analysis of AAPV and Laa Conjugates

AAPV was conjugated to form C6(D,L)–Laa, C8(D,L)-Laa and C10(D,L)-Laa. The HPLC assay developed was able to detect the individual Laa conjugate diastereomers.

Skin Stability of AAPV and Laa Conjugates of the Tetrapeptide

AAPV demonstrated substantial degradation in the presence of skin at 35°C with the concentration of the peptide detected after 24 h being reduced to 31.93±7.73% of the initial concentration. Conjugation afforded significantly greater protection to the peptide ($p<0.0001$) with the% remaining at 24 h ranging from 73.60±3.88 to 94.30±0.02 (Fig. 1: mean ± SEM, $n=3$). The rank order of peptides from most to least stable was C8(D,L)-Laa-AAPV > C10(D)-Laa-AAPV > C6(D)-Laa-AAPV > C10(L)-Laa-AAPV > C6(L)-Laa-AAPV, although there was no significant difference in the stability between these conjugates. This data confirms previous reports that conjugation with long alkyl side chains protects the labile parent peptide from enzymatic attack (16).

In-Vitro Skin Diffusion of Tetrapeptide and Laa Conjugates Across Human Epidermis

Laa conjugation was used as an enhancement strategy to improve transdermal delivery of the HNE inhibitor AAPV. The cumulative amount of conjugated or native AAPV permeated through human epidermis (μg/cm^2/h) *versus* time (h) was plotted (Fig. 2) and the steady-state flux and permeability coefficients (cm/h) calculated. Conjugation substantially enhanced AAPV permeation for all Laa chain lengths (Table I).

Skin Permeability of AAPV and Laa Conjugates

C6(D,L)-Laa-AAPV

When C6(D,L)-Laa-AAPV was applied as a racemic mixture permeation was enhanced compared to the native AAPV peptide. Permeation of the L-diastereomer was substantially lower than that of the D-diastereomer (Fig. 2), which is consistent with our earlier work with C8(D,L)-Laa-AAPV racemate (10). Epidermal permeation data for cumulative amount, flux and permeability coefficients calculated over the 24 h experimental period are presented in Table I. Epidermal flux of C6(D)-Laa-AAPV was 2.29 μg/cm^2/h, 0.5 μg/cm^2/h for C6(L)-Laa-AAPV and 0.46 μg/cm^2/h for the unconjugated AAPV. Thus the D-diasteromer provided a 5-fold enhancement of peptide permeation. In contrast, there was no significant improvement in epidermal peptide permeation for the L-diastereomer. This suggests that the stereochemistry of the molecule is an important factor in skin permeation. Given the likelihood that the molecules are permeating within the lipid bilayers of the *stratum corneum*, the stereochemistry of the molecules could influence their interaction with the chiral ceramide molecules in the bilayers.

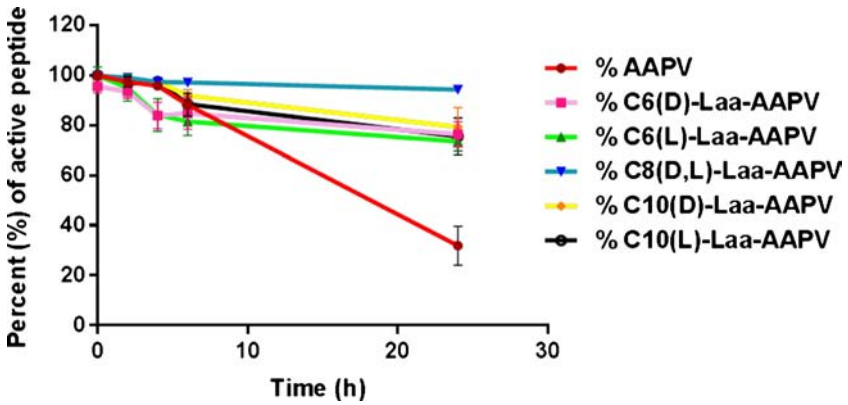

Fig. 1 Degradation profile of tetrapeptide and its lipoamino acid conjugates.

C8(D,L)-Laa-AAPV

C8-Laa-AAPV was applied as the racemate and as individual diastereomers. When the C8-Laa-AAPV was applied as a racemic mixture there was significantly increased epidermal permeation compared to AAPV (Table I). Over the 24 h experimental period the epidermal flux of C8(D,L)-Laa-AAPV was 10.08 μg/cm^2/h compared to 0.46 μg/cm^2/h for the unconjugated AAPV ($p<0.05$). The approximate lag time was 0.5 h. In addition the C8 chain length appears to have a greater permeation enhancement effect than the C6 chain (2.29 μg/cm^2/h for C6(D)-Laa-AAPV), suggesting that chain length and consequent increase in lipophilicity is an important parameter in epidermal permeation. The amount permeating into the skin was also higher (21.89 and 15.88 μg for C8 and C6(D,L)-Laa-AAPV respectively). When applied as individual stereoisomers (Fig. 2) the permeation of D-diastereomer (7.42 μg/cm^2/h) was significantly greater than the L-diastereomer (1.37 μg/cm^2/h: $p<0.05$). This is in agreement with the results for C6(D,L)-Laa-AAPV.

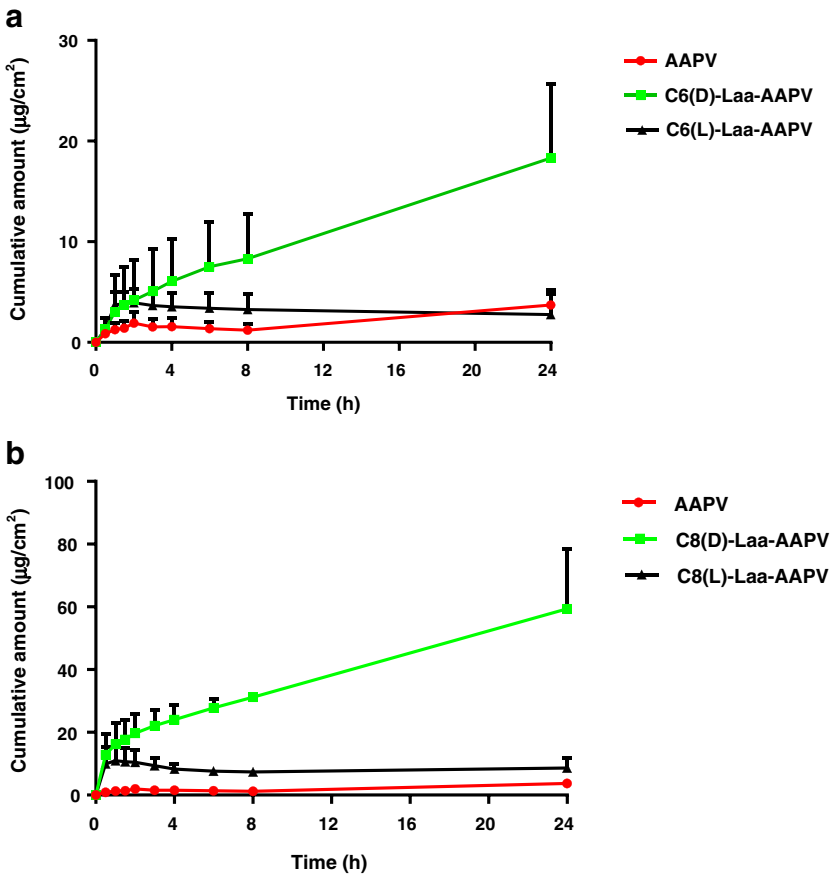

Fig. 2 (**a**) Permeation profiles for C6(D)-Laa-AAPV, C6(L)-Laa-AAPV and AAPV (mean±SEM; n=9) and (**b**) C8(D)-Laa-AAPV, C8(L)-Laa-AAPV and AAPV (mean±SEM; n=4) across human epidermis (3 mg in 300 μL propylene glycol in all cases).

Table I Comparison of Permeation Parameters of AAPV and Laa Conjugated AAPV (ND: Not Determined)

Peptide	Estimated C log P	Molecular weight	Cumulative amount ± SEM ($\mu g/cm^2$)	Epidermal flux ($\mu g/cm^2/h$)	Permeability coefficient K_p(cm/h)	Amount retained in the skin (μg)
AAPV	−1.272	355.4	3.70 (±1.09)	0.46	1.50×10^{-4}	9.1
C6(D)-Laa-AAPV	−0.107	468.6	18.32 (±7.40)	2.29	7.6×10^{-4}	15.88 (total D,L)
C6(L)-Laa- AAPV	−0.107	468.6	3.93 (±1.32)	0.50	1.6×10^{-4}	15.88 (total D,L)
C8(D,L)-Laa-AAPV	0.609	496.6	80.00 (±10.64)	10.08	3.3×10^{-3}	21.89
C8(D)-Laa-AAPV	0.609	496.6	59.38 (±19.14)	7.42	1.90×10^{-2}	ND
C8(L)-Laa-AAPV	0.609	496.6	10.99 (±5.20)	1.37	4.50×10^{-4}	ND
C10(D,L)-Laa-AAPV	1.325	524.4	71.43 (±40.3)	8.92	2.9×10^{-3}	63.83

C10(D,L)-Laa-AAPV

The C10Laa conjugate was more lipophilic (ClogP 1.325) than the C6-Laa-AAPV (ClogP -0.107) and C8-Laa-AAPV (ClogP 0.609) conjugated peptides. Consequently C10(D,L)-Laa-AAPV demonstrated a long lag time of approximately 8 h after which the cumulative permeation over 24 h was 71.43 $\mu g/cm^2$. The estimated epidermal flux value for C10(D,L)-Laa-AAPV of 8.92 $\mu g/cm^2/h$ compared to 0.24 $\mu g/cm^2/h$ for AAPV is based on the limited data points available. C10(D,L)-Laa-AAPV permeation was significantly increased as compared to AAPV permeation at 24 h ($p<0.05$) representing a 37-fold enhancement in the transdermal delivery of the tetrapeptide.

Recovery of Tetrapeptide and Laa Conjugates from Skin

The amounts of AAPV and Laa conjugates remaining in the skin after the permeation experiments are presented in Fig. 3 and Table I. The amount of AAPV remaining in the skin increased as the carbon chain length of Laa attached to the AAPV molecule increased. Amount remaining in the epidermis correlates with ClogP and suggests that the greater lipophilicity of the Laa conjugates facilitates partitioning into the lipid domains of the *stratum corneum* but slows upon partitioning to the more aqueous epidermal layers and into the receptor solution. Thus the lag time increases with increasing lipophilicity.

Surface Activity

We hypothesised that the Laa-peptide conjugates may have surface activity that could contribute to enhancement of skin permeability. A preliminary study was undertaken to determine the critical micelle concentration of the tetrapeptide and its lipophilic conjugates, thus providing a measure of their surface-active properties. The surface activity of AAPV and its Laa conjugates in buffer was determined and an approximate critical micelle concentration (CMC) estimated from the graph of surface tension in dyne/cm *versus* concentration. All Laa conjugates lowered surface tension significantly more than AAPV ($p<0.0001$). The estimated CMC for AAPV, C6(D,L)-Laa-AAPV, C8(D,L)-Laa-AAPV and C10(D,L)-Laa-AAPV was 0.125, 0.25, 0.5 and 0.5 mg/mL respectively. This preliminary data indicates that the tetrapeptide and its conjugates possess surface activity, but further work with more sensitive equipment is required to establish reliable CMC values.

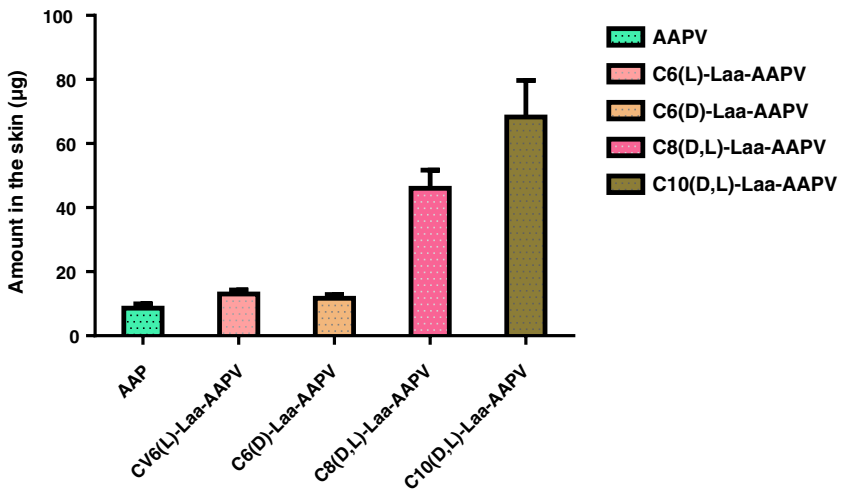

Fig. 3 Amount of AAPV and Laa conjugates in the skin after the permeation experiments: mean (±SEM: $n=4$).

HNE Inhibitory Activity

The biological activity of the Laa conjugates was assessed to determine if AAPV activity remains intact with the conjugate group attached. The elastase inhibitory activity of these peptides was assessed at two different substrate (6.25 μg/mL and 25 μg/mL) and enzyme concentrations (0.25 and 0.5 U/mL). The ability of elastase to digest the substrate decreased as the concentration of each of the inhibitors in the reaction mixture increased (Fig. 4). The half maximal inhibitory concentration (IC50) and Ki values were calculated for each of the peptides (Table II). All peptide/conjugates inhibited HNE activity with at least 70% reduction in the activity of elastase by the lipopeptides and tetrapeptide at 0.25 mM. Whilst there was a small decrease in activity of some Laa-conjugates compared to AAPV they clearly retain substantial activity. In a separate experiment, the activity of C6(L)-Laa-AAPV and C6(D)-Laa-AAPV was compared with AAPV, showing that the activity of the conjugates was comparable to the tetrapeptide at concentrations of 0.031, 0.062 and 0.125 mM. There was no significant difference in activity between the diastereomers.

DISCUSSION

The primary aim of the study was to investigate the effect of conjugating lipopamino acid chains of varied length on the skin permeation of the tetrapeptide Ala-Ala-Pro-Val. Our preliminary work had suggested that permeation could be increased and that the Laa conjugates showed stereoselective permeation enhancement (10). This study further investigated the permeation and skin retention of a series of Laa conjugates (C6-C10), established that the HNE inhibitory activity of the peptide was retained and also showed that the conjugates exhibit surface-active properties.

The permeability of the lipoamino acid conjugates of Ala-Ala-Pro-Val was much higher than the native tetrapeptide, with the following order of permeation when applied as

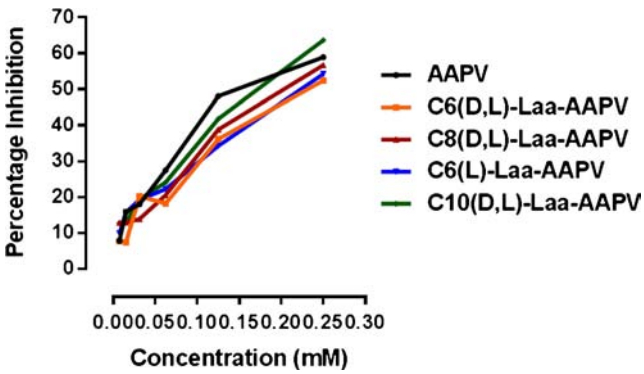

Fig. 4 Percent inhibition of elastase by AAPV and Laa conjugates at 25 μg/mL of substrate and 0.5 U/mL elastase.

Table II HNE Inhibition Activity: Half Maximal Inhibitory Concentration (IC50) and Ki Values AAPV and Lipoamino Acid Conjugates

Peptide	IC50	Ki
AAPV	173.63 nM	71 nM
C6 (D,L)-Laa-AAPV	217.05 nM	89.2 nM
C8 (D,L)-Laa-AAPV	204.30 nM	83.8 nM
C10 (D,L)-Laa-AAPV	178.29 nM	73.2 nM
C6 (L)-Laa-AAPV	219.85 nM	90.3 nM

racemic mixtures: C8(D,L)>C10(D,L)>C6(D and L)-Laa-AAPV (Table I). These results are in good agreement with the previous finding that the transdermal absorption of tetragastrin was enhanced by its chemical modification with fatty acids (17). The permeability of the more lipophilic C10-Laa showed a long lag time prior to permeation through the epidermis, suggesting that it has good solubility in the lipid domains of the *stratum corneum*. The poor permeability of native Ala-Ala-Pro-Val across the epidermis may be mainly attributed to its poor permeation through the stratum corneum due to its high polarity and its degradation in the skin. The linking of a lipophilic group to a polar molecule to enhance its membrane permeability characteristics is a strategy that has been employed for a number of routes of drug administration. Yamamoto and colleagues reported that the stability and permeability across full-thickness rat skin of a dipeptide (Phe–Gly) was improved by chemical conjugation with short chain fatty acids (C4, C6 and C8) (18). The permeability across intact skin was highest for the C6-Phe–Gly, whilst C8-Phe–Gly remaining in the intact skin was the highest of all the acyl derivatives studied, likely due to its high binding affinity to the skin. Our data showed a similar pattern, with enhanced permeability of the tetrapeptide greatest with the C8 Laa conjugate, whilst the C10 showed a longer lag time and greater binding in the *stratum corneum*. This clearly correlates with the increase in ClogP values (Table I) associated with the length of the carbon chain, which would be expected to influence both permeation through the skin and binding within the lipid-rich *stratum corneum*, thus resulting in increased lag time and skin retention. The low initial permeability of the more lipophilic derivative of Ala-Ala-Pro-Val [C10(D,L)-Laa-AAPV] is most likely due to its strong partitioning and binding to the *stratum corneum*. From these findings, it may be considered that there is an optimal lipophilicity (length of lipoamino acid chain) of the derivatives for improving their transdermal delivery.

Where the diastereomers could be clearly resolved by HPLC, the D-diastereomers of each of these peptides showed higher epidermal flux compared to the L-diastereomers, confirming our previous preliminary study (10). In this study, the tetrapeptide sequence was synthesized to generate the active conformation (using all L-amino acids) and the addition

of the Laa generated the two stereoisomers, therefore we did not expect a decrease in activity for either of the lipopeptide isomers generated. This was confirmed in our enzyme inhibition assays (Table II) that demonstrated HNE inhibitory activity by the Laas, and that there was no significant difference in activity between the diastereomers. The higher flux for the D- compared to the L-diastereomer suggests that the stereochemistry of the molecule is an important factor in skin permeation. Structural components of the *stratum corneum* are proposed to serve as potential sources of chiral discrimination that may result in differential diffusion rates, dependent on the stereochemistry of the solute (19). This has been demonstrated for enantiomers of small molecule drugs that have been shown to exhibit different permeation profiles across animal and human skin (19,20). This enantiomeric discrimination is likely due to interactions with the chiral components of the ceramide molecules in the *stratum corneum* of the skin, which constitutes the rate-limiting barrier in percutaneous penetration. For example, the head groups of ceramide chains have multiple hydroxylated carbon atoms with specific stereochemistry, giving rise to stereospecific interactions with chiral drug molecules (19–21). As a consequence, the different interactions of drug enantiomers with chiral ceramides gives rise to different permeation profiles. Other extrinsic factors have also been implicated, such as the physicochemical differences between enantiomers and their racemates, the presence of chiral permeation enhancers and stereoselective retardants in the drug vehicle, differences in the rate of hydrolysis of prodrugs of enantiomers in the epidermis/dermis, and carrier-mediated transport (19,20). Equally, there are a number of factors that might lead to increased drug diffusion through lipidic structures and hence to the loss of enantiomeric selectivity: high drug concentration (resulting in higher diffusion rate than that arising from the effect of chiral interactions), the presence of an organic solvent in the drug vehicle (which may induce changes in the lipidic structure of the stratum corneum that facilitate drug diffusion), and differences in the pH of the drug vehicle and the skin. In the current study the increase in lipophilicity clearly provided the main mechanism of enhanced permeation into the skin but enantiomeric selectivity is apparent, suggesting that interaction within the *stratum corneum* varies for the diastereomers and influences their ease of permeation.

The structure of the conjugates is surfactant-like with a polar head group provided by the peptide and a lipophilic chain provided by the Laa (Fig. 5).

We demonstrated the surface-active properties of the Laa conjugates in the study of surface tension changes. This correlates with the structure of the molecules (Fig. 5) and the balance between the lipophilic carbon chain and polar head group that is characteristic of surface-active molecules. Bio-surfactants-based antibiotics such as the cyclic lipopeptides and the powerful surfactant surfactin

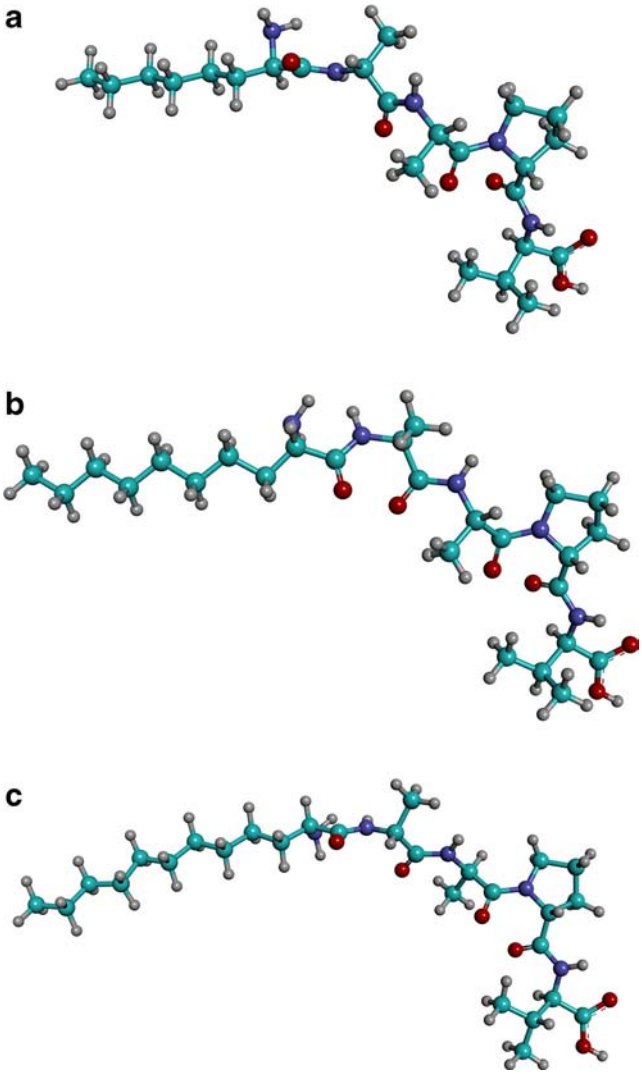

Fig. 5 Molecular models of (**a**) C6-Laa-AAPV, (**b**) C8-Laa-AAPV and (**c**) C10-Laa-AAPV.

have been known for over 30 years. The surface activity, aggregation properties and broad biological activity of surfactant lipopeptides allows them to permeate membranes and exert anti-bacterial, anti-viral, anti-fungal, anti-mycoplasma and hemolytic activities (22). Surfactants have been widely used as skin penetration enhancers due to their action as solubilisers and their effect on reducing the barrier function of the *stratum corneum* (23–26). The surfactant-like property may facilitate partitioning of the lipopeptides into the *stratum corneum* lipidic bilayers, thus contributing to enhanced permeability and accumulation in the epidermis. We conclude that although the increase in lipophilicity is the predominant mechanism in enhanced permeability, the surfactant-like property in the Laas may also facilitate their permeability into and across the lipid domains of the *stratum corneum*.

In summary, the main findings of this study were that coupling of the tetrapeptide AAPV to short chain Laas

enhanced both the permeation into and through human epidermis and the stability in the presence of skin. We also demonstrated stereoselective permeation enhancement with the D-diastereomer permeating through the epidermis to a greater extent. As expected, the amount of peptide remaining in the skin increased with the increase in carbon chain length of the Laa conjugate due to the greater lipophilicity of the conjugate and its tendency to associate with the lipid domains of the *stratum corneum*. This demonstrates that increased lipophilicity is important for enhancing skin permeation but also that there is an optimal chain length. The surface active properties conferred by the Laa-peptide structure may also contribute to their skin permeability through surfactant-like activity on the *stratum corneum* barrier.

Importantly, the elastase inhibition activity of the peptide was retained after coupling to the Laa conjugates. Whilst this activity was lower than the parent AAPV, the increased permeation and stability in the skin will provide an overall substantially increased biological activity within the skin. This preliminary study suggests that the Laa conjugation approach may be useful for enhancing the permeation of moderately sized peptide drugs, with potential application in the treatment of skin disorders such as psoriasis and dermatitis.

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