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Cell Penetrating Peptide as a High Safety Anti-Inflammation Ingredient for Cosmetic Applications

Tse-Kai Fu ^{1,2,†}, Ping-Hsueh Kuo ^{1,†}, Yen-Chang Lu ¹, Hsing-Ni Lin ¹, Lily Hui-Ching Wang ^{1,3}, Yu-Chun Lin ⁴, Yu-Chen Kao ⁴, Huey-Min Lai ^{4,*} and Margaret Dah-Tsyr Chang ^{1,5,*}

¹ Institute of Molecular and Cellular Biology, National Tsing Hua University, Hsinchu 30013, Taiwan; gn00042366@gmail.com (T.-K.F.); s9980584nthu@gapp.nthu.edu.tw (P.-H.K.); johnnylu0629@gmail.com (Y.-C.L.); sisnin66@gmail.com (H.-N.L.); lilywang@life.nthu.edu.tw (L.H.-C.W.)

² Research and Development Department, Simpson Biotech Co., Ltd., Taoyuan 333, Taiwan

³ Department of Medical Science, National Tsing Hua University, Hsinchu 30013, Taiwan

⁴ Industrial Technology Research Institute, Hsinchu 30011, Taiwan; YuChunLin@itri.org.tw (Y.-C.L.); YCKao@itri.org.tw (Y.-C.K.)

⁵ Department of Life Science, National Tsing Hua University, Hsinchu 30013, Taiwan

* Correspondence: HMLai@itri.org.tw (H.-M.L.); dtchang@life.nthu.edu.tw (M.D.-T.C.); Tel.: +886-3-573-2765 (H.-M.L.); +886-3-574-2767 (M.D.-T.C.)

† These authors contributed equally to this work.

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Abstract: Cosmeceutical peptides have become an important topic in recent decades in both academic and industrial fields. Many natural or synthetic peptides with different biological functions including anti-ageing, anti-oxidation, anti-infection and anti-pigmentation have been developed and commercialized. Current cosmeceutical peptides have already satisfied most market demand, remaining: “cargos carrying skin penetrating peptide with high safety” still an un-met need. To this aim, a cell-penetrating peptide, CPP_{AIF}, which efficiently transported cargos into epithelial cells was examined. CPP_{AIF} was evaluated with cell model and 3D skin model following OECD guidelines without using animal models. As a highly stable peptide, CPP_{AIF} neither irritated nor sensitized skin, also did not disrupt skin barrier. In addition, such high safety peptide had anti-inflammation activity without allergic effect. Moreover, cargo carrying activity of CPP_{AIF} was assayed using HaCaT cell model and rapid CPP_{AIF} penetration was observed within 30 min. Finally, CPP_{AIF} possessed transepidermal activity in water in oil formulation without disruption of skin barrier. All evidences indicated that CPP_{AIF} was an ideal choice for skin penetrating and its anti-inflammatory activity could improve skin condition, which made CPP_{AIF} suitable and attractive for novel cosmeceutical product development.

Keywords: cell-penetrating peptides; cosmeceutical peptides; anti-inflammation

1. Introduction

Peptides, polymers composed of amino acids, are known to possess versatile biological functions such as promoting cell proliferation, migration, inflammation/anti-inflammation, angiogenesis, and melanogenesis [1], which causes numerous physiological procedures in human body [2]. The first synthetic peptide incorporated into skin care products in the late 80s was copper glycine-histidine-lysine (Cu-GHK) generated by Pickard in 1973 [3]. Since then, many short synthetic peptides playing roles in inflammation, extracellular matrix synthesis or pigmentation have been developed. These peptides are used for anti-oxidation, whitening effects, “Botox-like” wrinkle smoothing and collagen stimulation.

Cosmeceutical peptides usually have certain features. Historically, it has always been assumed that the molecular weight of a peptide should be less than 500 Da, if not it could not pass skin barrier [4]. In addition, the peptide should have water solubility over 1 mg/mL and have no or few polar centers in its sequence [5]. Copper tripeptides, tetrapeptide PKEK, manganese tripeptide-1, soybean peptide, black rice oligopeptides and silk fibroin peptide have been on the market for several decades, but less *in vivo* efficacy data is available [5]. In general, their substance mixtures are examined in cosmetic formulations, so as to the actual effect of individual peptides on the skin still remains unclear in many cases.

Scientific research on peptides usually focus on identification of functional mechanism, and practical application for cosmetic and/or pharmaceutical use. In the last decade, the development of active peptides has established a new field in cosmeceutical and pharmaceutical skin care. To generate safety profile and functional data of peptide, *in vivo* animal test was usually chosen in the past. However, correctness of results from animal model for human skin has always been questioned. This problem, using animals for testing purposes, finally leads to EU regulation (76/768/EEC, Feb. 2003), beginning in 2009, to prohibit use of animals to accumulating toxicological data for cosmetic ingredients.

As an alternative solution, artificial human skin models have been established and many of these are now commercially available. Several technologies have been introduced to design and develop artificial skin models which highly simulate complex structure of human skin [6,7]. The most common skin models were epidermis models using human skin epidermal cells, including EpiSkin[®] (L'Oreal, Levallois-Perret, France), EpiDerm[®] (MatTek Corporation, Ashland, MA, USA), SkinEthic[®] (SkinEthics, Lyon, France) and epiCS[®] (CellSystems, Troisdorf, Germany). Recently, some advanced skin models were commercialized, including Phenion[®] (Henkel, Düsseldorf, Germany) and NeoDerm[®] (Tegoscience, Seoul, Korea). These models have been proven to replace animal tests in pharmaceutical and cosmetic industries for evaluation of corrosion, skin irradiation and photo-toxicity [8]. Some of them can also be applied in basic research for clinical use [9].

Human skin gives protective, perceptive and communication functions to the body with resilient and relatively impermeable barrier. To develop agents to deliver pharmacy or active ingredients across skin tissues is a highly attractive topic in recent years. Using compounds or physical equipment to enhance cargo delivery causes some problems, for instance, skin toxicity, skin irritation, inconvenience and high costs [10,11]. Comparing with chemical or physical ways, peptide with cell or skin penetrating activity is an alternative choice. Cell-penetrating peptides (CPP) are peptides that can transport cargos such as chemical compounds, proteins, peptides, and nanoparticles into cells [12]. Most CPP sequences are rich in positively charged residues, and are internalized after interacting with negatively charged glycosaminoglycans (GAGs) and clustering on outer membrane surfaces [13]. For example, a modified PTD (Protein transduction domain) peptide from human immunodeficiency virus (HIV): tat (RKKRRQRRR) has been shown to have cell membrane penetration property and deliver therapeutic proteins into mammalian cells [14]. Another case is AID (arginine-rich intracellular delivery) peptides which successfully enter and deliver functional proteins into epidermis and dermis of mouse [15,16]. Many modified AID peptides (HGH6, TAT, R7) proven to penetrate into skin of living animals with cargos [17,18]. For example, R7-CsA could reach dermal lymphocytes and inhibit cutaneous inflammation [19]. These facts indicated a new approach for increasing delivery of poorly absorbed ingredients across skin tissue barriers.

Here a 10-residue peptide, covering major GAG binding motif of a human ribonuclease, is identified as a CPP_{AIF} (anti-inflammatory CPP). CPP_{AIF} has been shown to possess epithelial cell, GAG and lipid binding properties as well as cell penetrating activity through macropinocytosis [20,21]. Notably, CPP_{AIF} is able to deliver small fluorescent molecules, recombinant proteins, nanoparticles, and peptidomimetic drugs into cells [20]. Based on these facts, safety and potential of CPP_{AIF} for cosmeceutical application were examined with skin cell and 3D-skin models following the Organization for Economic Co-operation and Development (OECD) guidelines with special focus on stability, safety,

skin irritation, skin barrier function, *chemico* sensitization, bio-functions and transepidermal activity in this work.

2. Materials and Methods

2.1. Stability Test of CPP_{AIF} under Different Conditions

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). All cell lines were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). For dry powder stability test, CPP_{AIF} (NYRWRCKNQN with unmodified N- and C-termini; AIF: anti-inflammation, synthesized by Kelowna International Scientific, Taipei, Taiwan) and was dissolved in water, concentration of 1 mg/mL, and then freeze and dry into powder. Samples were separately incubated in 4 and 25 °C for 1, 3 and 7 days. These tubes were collected and stored at −80 °C. For solution stability test, CPP_{AIF} was dissolved in water to 1 mg/mL then used 0.2 µm filter filtered and dispense to 100 µL in each tubes. Samples were separately incubated in specific temperatures, including −20, 4, 30 and 50 °C for 1, 3, 7, 14, 21, 30 and 60 days. These tubes were collected and stored at −80 °C. Then, the remainder of CPP_{AIF} were tested with high-performance liquid chromatography (HPLC) (Waters, Milford, MA, USA) Separation was performed on XBridge C18 column (250 mm × 4.6 mm, particle size 5 µm, Waters). The HPLC condition and program: A buffer is ddH₂O with 0.1% TFA (trifluoroacetic acid), B buffer is acetonitrile with 0.1% TFA. The flow rate is 1 mL/min and acetonitrile gradient from 10 to 50% in 15 min and the percentage of acetonitrile raise to 100% from 16 to 20 min.

2.2. In Vitro Skin Irritation Test (OECD 439)

The 3D reconstructed human epidermis tissue model: SkinEthic™ RHE (SkinEthics, Lyon, France) was used to evaluate whether CPP_{AIF} cause skin irritation [8]. Testing procedure involved topical application of testing article (CPP_{AIF}) to surface of epidermis and subsequent assessment of effect on cell viability. All 3D-skin tissues were incubated with growth medium for 2 h and then CPP_{AIF} was add to final 1 mM for 42 min treatment. After treatment, testing substance was washed out by 25 times with 1 mL phosphate buffered saline (PBS) and tissues were further incubated in growth medium for 42 h. After incubation, growth medium was substituted by maintenance medium with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) agent for 3 h incubation. Next, insert (with tissue) were washed with PBS and air dried. Formazan in tissues was extracted with isopropanol and measured by determining the OD at 570 nm using a microplate spectrophotometer (iMark Microplate Absorbance Reader, Bio-Rad, Hercules, CA, USA). Cell viability of treated models was normalized to the negative control, which was set to 100%.

2.3. In Vitro Skin Barrier Function Test (Developed from OECD TG 439)

A test procedure was developed to test effect of CPP_{AIF} on the barrier function on SkinEthic™ reconstructed human epidermis (RHE) based on the relevant procedure mentioned in OECD TG 439. This testing procedure involved topical application of CPP_{AIF} onto surface of the epidermis model for 1 h. After 1 h of exposure, CPP_{AIF} was washed by PBS from the surface followed by application of detergent solution (1% Triton X-100) onto surface of the tissue for another 2 h. Then, washed the detergent solution and AlamarBlue cell viability assay (BUF012B, Bio-Rad, Hercules, CA, USA) was performed for assessment of cell viability. Cell viability of treated models was normalized to the negative control, which was set to 100%.

2.4. In Chemico Skin Sensitization: Direct Peptide Reactivity Assay (DPRA) (OECD 442C)

The direct peptide reactivity assay (DPRA) is an *in chemico* method which quantified the remaining concentration of cysteine- or lysine-containing peptide after 24 h incubation with the test chemical at 25 °C. Relative peptide concentration was measured by HPLC (Waters, Milford, MA, USA) with gradient elution and UV detection (220 nm). Cysteine and lysine peptide percent depletion values

were calculated for a prediction model (Table 1). This model allowed to classify the test chemical to one of four reactivity classes used to support the discrimination between sensitizers and non-sensitizers. The study is carried out according to the OECD guideline 442C (2015).

Table 1. Cysteine 1:10/lysine 1:50 prediction model

Mean of Cysteine and Lysine% Depletion	Reactivity Class	DPRA Prediction
$0\% \leq \text{mean\% depletion} \leq 6.38\%$	No or minimal reactivity	Negative
$6.38\% \leq \text{mean\% depletion} \leq 22.62\%$	Low reactivity	
$22.62\% \leq \text{mean\% depletion} \leq 42.47\%$	Moderate reactivity	Positive
$42.47\% \leq \text{mean\% depletion} \leq 100\%$	High reactivity	

2.5. Macrophage Inflammation Assay

Macrophage inflammatory assay were carried out to evaluate whether CPP_{AIF} displayed anti-inflammation potential [22–24]. In this experiment, macrophage (Raw264.7) cells were seeded in 96-well plates (5×10^5 cells/mL) and allowed to attach overnight. After attachment, cells were incubated with various concentrations of CPP_{AIF} for 1 h and followed by stimulation with 1 $\mu\text{g/mL}$ of LPS (lipopolysaccharide). No LPS-added cells were considered as control groups. After incubation, the amount of TNF- α and IL-6 in the medium were analyzed by enzyme linked immunosorbent assay (# KHC3011 and #EH2IL6, Thermo Fisher, Waltham, MA, USA). Cell viability was measured by AlamarBlue cell viability assay (BUF012B, Bio-Rad, Hercules, CA, USA).

2.6. Mast Cell Degranulation Assay

Inhibitory effects on release of β -hexosaminidase in RBL-2H3 (rat-basophilic leukemia cell line) were evaluated by a cell degranulation assay [25]. Briefly, 0.2 mL of 5×10^5 cells/mL RBL-2H3 cells were seed in 24-well plates (in Minimum Essential Medium (MEM) containing 10% Fetal Bovine Serum (FBS), streptomycin (100 $\mu\text{g/mL}$) and penicillin (100 units/mL)) and sensitized with anti-DNP IgE (0.45 $\mu\text{g/mL}$). These cells were washed with Siraganian buffer (5 mM KCl, 0.4 mM MgCl_2 , 119 mM NaCl, 25 mM piperazine-*N,N* 2-bis(2-ethane sulfonic acid (PIPES), and 40 mM NaOH, pH 7.2) with glucose, 1 mM CaCl_2 , and 0.1% bovine serum albumin (BSA). After washing, these cell were incubated in 160 μL of the PBS for 10 min at 37 $^\circ\text{C}$ and then 20 μL of test sample solution or calcimycin (final 1 mM) was added and further incubated for 10 min. The plate was cooling on ice for 10 min to stop reaction. 50 μL of supernatant was transferred to 96-well plate and added with 50 μL of substrate (1 mM *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37 $^\circ\text{C}$ for 2.5 h. The reaction was stopped by adding 200 μL of carbonate-bicarbonate buffer (0.1 M, pH 10.0). The value of A_{405} was measured with a microplate reader.

2.7. Internalization of CPP_{AIF} in Human Keratinocyte (HaCaT)

Confocal Laser-scanning Microscopy (CLSM, Zeiss Cell Observer-Z1, Baden-Württemberg, Germany) was performed to assess distribution of TMR-labeled CPP_{AIF} (tetramethylrhodamine-NYRWRCKNQ, synthesized by Kelowna International Scientific, Taipei, Taiwan) in human keratinocytes, HaCaT. First, apply a layer of type I collagen on a glass cover slip, dry and sterilize the cover slip with UV light. Then, culture the HaCaT cells (1×10^3 per slip) on the coverslip for 16 h. The attached HaCaT cells were cultured with 20 μM of the TMR- CPP_{AIF} for 30 min, observed with CLSM (63 \times oil-immersion objective lens), and photographed under same exposure conditions.

2.8. Transepidermal Measurement of CPP_{AIF} by Reconstructed Human Epidermis Tissue Model

3D human epidermis tissue model (SkinEthic™ RHE, SkinEthics, Lyon, France) and two kinds of CPP_{AIF}-containing emulsion systems (water-in-oil emulsion (W/O) and oil-in-water emulsion (O/W)) were prepared for the transepidermal measurements. Formulation contents of the W/O and O/W

are listed in Tables S1 and S2. All 3D-skin tissues were incubated with growth medium for 2 h and then 0.1 mL formulated CPP_{AIF} (0.1 mM) emulsions were added to top of the tissues for 1 h treatment. After treatment liquid beneath the model was collected for HPLC quantification. Transepidermal rate was calculated by measuring the amount of CPP_{AIF} in medium beneath the 3D model by HPLC ((concentration of the beneath liquid/concentration of the topical exposure) × 100).

2.9. Statistical Analyses

All statistical analyses were processed by GraphPad Prism version 5.01 for Windows 548 (GraphPad Software, La Jolla, CA, USA). Each value was the average of three measurements, where the presented data was the mean ± SD and all means were compared by one-way ANOVA.

3. Results

3.1. Stability of CPP_{AIF} under Different Conditions

CPP_{AIF} was incubated at a specified temperature (4 °C and 25 °C) and analyzed remaining quantity by HPLC. The molecular weight of CPP_{AIF} was validated by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry. The result indicated that m/z of CPP_{AIF} was 1381.1 as expected (Figure S1). The results in Figure 1 show that CPP_{AIF} maintained intact close to 100% in dry powder form at low temperature (4 °C) and normal temperature (25 °C) up to 1 week, indicating that preservation of CPP_{AIF} in dry powder form could effectively prevent peptide precipitation and fragmentation.

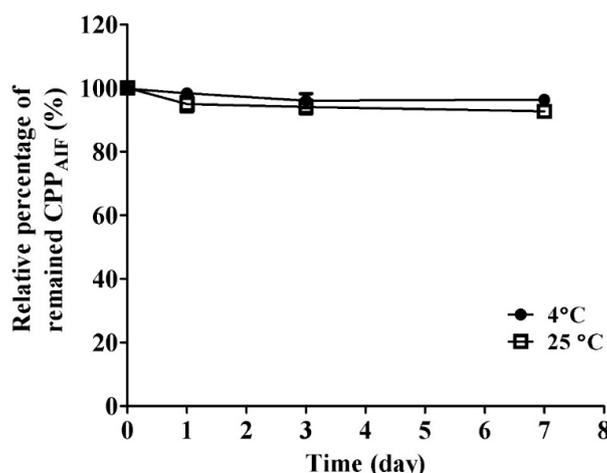


Figure 1. Stability of CPP_{AIF} stored as dry powder at different temperatures. Each sample was dissolved in milliQ H₂O, and filtered through 0.2 µm filter, then freeze dried into powder form. The samples (100 µL, 1 mg/mL) were separately incubated at 4 °C and 25 °C for 1, 3, and 7 days. The residual amount of CPP_{AIF} ingredient was quantified by HPLC equipped with C18 column.

CPP_{AIF} under different solutions or temperatures was further examined to explore suitable storage condition. It was dissolved in ddH₂O at different temperatures and time to examine which solution was suitable for storage. First, CPP_{AIF} was dissolved in water at a concentration of 1 mg/mL passed through 0.2 µm filter, and incubated at different temperatures. After one week, residual quantity of CPP_{AIF} was still more than 90% when tested at −20 °C and 4 °C, but that of CPP_{AIF} incubated at 30 °C and 50 °C dropped to about 70% (Figure 2). Moreover, after storage for 30 days intact CPP_{AIF} retained in solution was measured to be 81% at −20 °C, 73% at 4 °C, 36% at 30 °C, and 17% at 50 °C, indicating that CPP_{AIF} had better stability at low temperature environment (−20 °C and 4 °C) (Figure 2). MALDI-TOF mass spectrometry was applied to validate the molecular weight of residual CPP_{AIF} as shown in the signal of HPLC chromatogram (Figure S2).

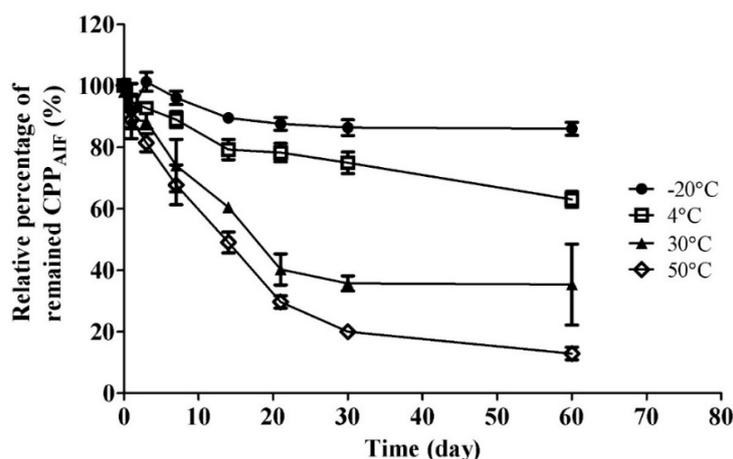


Figure 2. Stability of CPP_{AIF} stored in water at different temperature. Stability of CPP_{AIF} in water at various temperature (−20 °C, 4 °C, 30 °C, 50 °C) for different time duration. Each sample (100 μL, 1 mg/mL) was filtered through 0.2 μm filter, and residual amount of CPP_{AIF} ingredient was quantified by HPLC equipped with C18 column.

3.2. Safety of CPP_{AIF}

In vitro skin irritation test of CPP_{AIF} were carried out following OECD Test Guideline No. 439 (2015), using a reconstructed human epidermis test method.

As shown in Figure 3, relative viability of negative control (NC, PBS), positive control (PC, 5% sodium dodecyl sulphate, SDS), and CPP_{AIF} (1 mM) was determined to be respectively: $100.0 \pm 4.8\%$, $1.44 \pm 0.8\%$, and $90.3 \pm 3.2\%$, clearly suggesting that CPP_{AIF} (1 mM) did not cause skin irritation (no category) according to the classification of OECD 439.

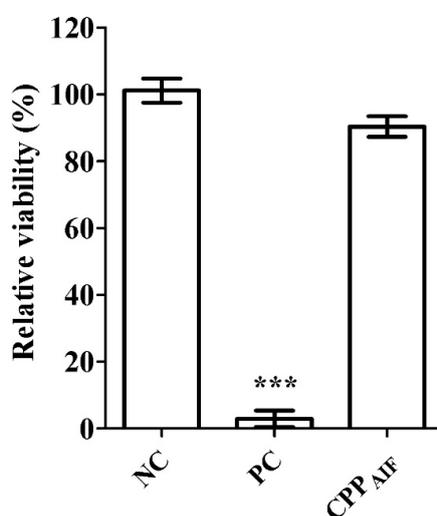


Figure 3. In vitro skin irritation test of CPP_{AIF} in SkinEthic™ RHE model. 3D reconstructed human epidermis tissues were incubated with growth medium for 2 h followed by treatment with NC, PC, and 1 mM CPP_{AIF} for 42 min. Then the tissues were washed and further incubated in growth medium for 42 h. Afterwards, the growth medium was substituted with maintenance medium containing MTT agent for an additional 3 h incubation. Next, the insert (with tissue) was washed with PBS and air dried. Formazan in tissues was extracted with isopropanol and measured by OD at 570 nm. PBS was applied as negative control (NC) and set as 100% (mock), 5% SDS was applied as positive control (PC). *** $p < 0.001$ versus the NC.

CPP_{AIF} was subsequently applied to in vitro skin barrier function test (developed from OECD TG 439). A normal stratum corneum was multilayered containing essential lipid profile to produce desired functional barrier with robustness to resist rapid penetration of cytotoxic chemicals, e.g., SDS or Triton X-100. Here, a 3D human epidermis tissue model, SkinEthic™ RHE, was used for skin barrier function tests. Tissues incubated with CPP_{AIF} (1 mM) remained cell viability over 80%, which was considered not influencing barrier function of the tissue. As shown in Figure 4, relative viability of negative control (NC, H₂O), positive control (PC, 5% SDS), and CPP_{AIF} (1 mM) was respectively 100.0 ± 6.2%, 24.0 ± 3.3%, and 95.6 ± 2.8%, evidently indicating that CPP_{AIF} would not impair the skin barrier function of the 3D epidermis tissue model.

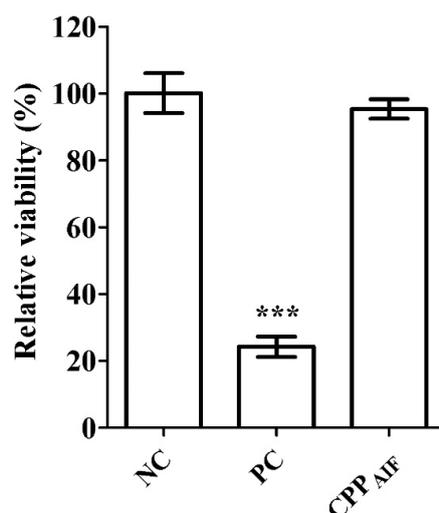


Figure 4. In vitro skin barrier function test of CPP_{AIF} in SkinEthic™ RHE model. 3Dreconstructed human epidermis tissues were exposed with NC, PC, and 1 mM CPP_{AIF} for 1 h. The CPP_{AIF} was washed with PBS from the surface followed by application of detergent solution (1% Triton X-100) onto surface of the tissues for another 2 h. The tissues were washed with PBS and cell viability was measured by AlamarBlue cell viability assay. PBS was applied as negative control (NC) set as 100% (mock) and 5% SDS was applied as positive control (PC). *** $p < 0.001$ versus the NC.

Finally, in chemico skin sensitization of CPP_{AIF} was tested following OECD Test Guideline No. 442C. The mean of cysteine and lysine % depletion of 100 mM cinnamaldehyde (positive control), phenoxyethanol, caprylyl glycol, hexalene glycol, 1,3-butanediol and 0.1 mM CPP_{AIF} was respectively calculated to be 65.07, 0.56, 2.53, 0.63, −0.12 and 0.74 (Table 2).

Table 2. DPRA calculation according to prediction model.

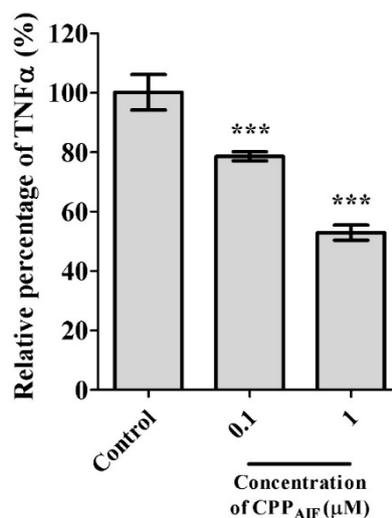
Test Item	Concentration (mM)	Lysine Depletion	Cysteine Depletion	Mean of Cysteine and Lysine % Depletion	DPRA *1 Prediction
Cinnamaldehyde	100	53.82	76.33	65.07	Sensitiser
Phenoxyethanol	100	−0.25	1.38	0.56	Non-sensitiser
Caprylyl glycol	100	2.34	2.72	2.53	Non-sensitiser
Hexalene glycol	100	−0.36	1.62	0.63	Non-sensitiser
1,3-Butanediol	100	−0.36	0.13	−0.12	Non-sensitiser
CPP _{AIF}	0.1	1.02	0.45	0.74	Non-sensitiser

*1: Direct Peptide Reactivity Assay (OECD Test Guideline No. 442C).

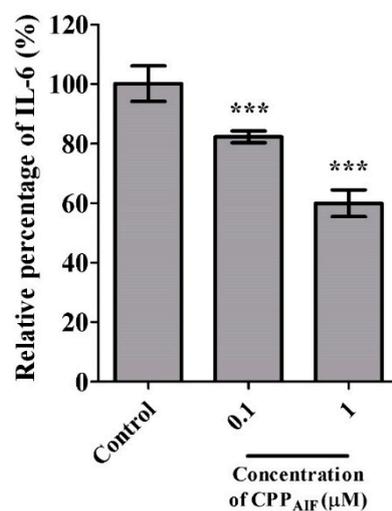
The HPLC chromatograms for cysteine and lysine depletion quantification of CPP_{AIF} were shown in Figure S3. The value of CPP_{AIF} was lower than 19% and thus classified as “Non-sensitizer” according to OECD 442C like other regulatory approved cosmetic ingredients.

3.3. Bio-Function of CPP_{AIF}: Anti-Inflammation without Sensitization

Inflammation inhibition effect of CPP_{AIF} on macrophage cells was tested at various concentrations of 1 and 0.1 μM . As shown in Figure 5A, 0.1 μM CPP_{AIF} inhibited $22.1 \pm 1.2\%$ TNF- α secretion and 1 μM CPP_{AIF} showed stronger inhibition effect of $56.7 \pm 2.5\%$. 0.1 μM and 1 μM CPP_{AIF} also inhibited $18.3 \pm 3.4\%$ and $40 \pm 4.2\%$ of IL-6 secretion, respectively (Figure 5B). These results implied a dose-dependent relationship between the concentration of CPP_{AIF} (from 0~1 μM) and the amount of TNF- α or IL-6 inhibition. Nevertheless, cell viability remained $85.2 \pm 4.25\%$ at CPP_{AIF} concentration of 1 μM . Taken together, CPP_{AIF} might be a potential ingredient with skin protectant function, especially anti-photo aging [26,27] related to the anti-inflammatory effects [28].



(A)



(B)

Figure 5. Anti-inflammation activity of CPP_{AIF}. Raw264.7 macrophage cells were seeded in 96-well plates (5×10^5 cells/mL) and allowed to attach overnight. After attachment, the cells were incubated with various concentrations of CPP_{AIF} for 1 h followed by stimulation with 1 $\mu\text{g}/\text{mL}$ of LPS. The control group was not treated with LPS and its cytokine secretion was set as 100% (mock). The amounts of TNF α (A) and IL-6 (B) in the medium were analyzed by ELISA. Cell viability was measured by AlamarBlue cell viability assay. *** $p < 0.001$ versus the control group.

The sensitization of CPP_{AIF} was evaluated by mast cell degranulation assay. Here RBL-2H3 cells were treated with various concentrations (0, 0.1, 1, 2, 5, 10 μ M) of CPP_{AIF}. Granule release represented by β -hexoaminidase was induced by A23187 (Calcimycin) as positive control (PC). In this experiment CPP_{AIF} under 10 μ M did not induce any mast cell degranulation release (Figure 6) while cell viability remained over 90%. This result indicated that CPP_{AIF} would not induce any allergic effect in epidermal tissue [29].

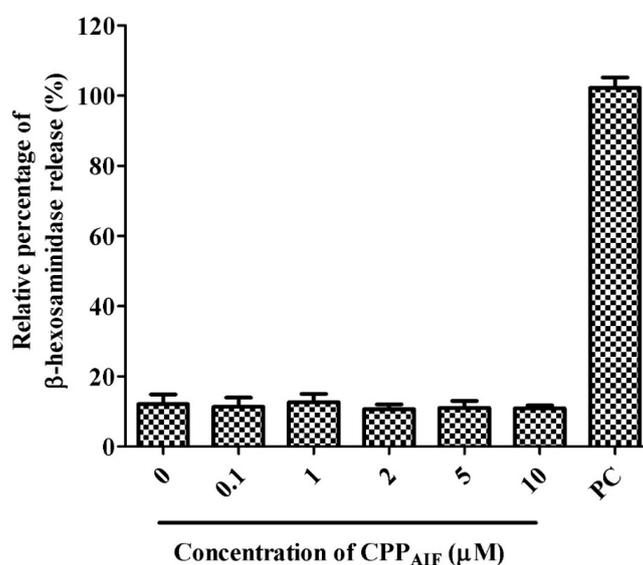


Figure 6. Sensitization test of CPP_{AIF}. The sensitization of CPP_{AIF} was evaluated by mast cell degranulation assay. RBL-2H3 mast cells (5×10^5) were treated with various concentrations (0, 0.1, 1, 2, 5, 10 μ M) of CPP_{AIF}. The positive control (PC) was treated with 1 mM A23187 (calcimycin) and the amount of its granule release was set as 100% (mock).

3.4. Cell Penetration and Transepidermal Test. of CPP_{AIF}:

First, epidermal cell penetration activity of CPP_{AIF} was measured in human keratinocyte HaCaT cells. The cells were incubated with 20 μ M TMR-CPP_{AIF} at 37 $^{\circ}$ C for 30 min prior to observation by CLSM (Scale bar: 10 μ m). Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). As shown in Figure 7, after addition of 20 μ M TMR-CPP_{AIF} (tetramethylrhodamine, TMR) for 30 min, TMR signal was clearly detected in the cells (Figure 7). A strong signal accumulation in the cytoplasm showed that TMR-CPP_{AIF} internalized into the cells. Such effect was observed while the skin barrier function still maintained well as investigated by 3D human epidermis tissue model. This result indicated that TMR-CPP_{AIF} could penetrate skin tissue without interrupting function of skin tissue.

After confirming the cell penetration activity of CPP_{AIF} to normal human keratinocyte, transepidermal activity of CPP_{AIF} in different formulations was evaluated by 3D reconstructed human epidermis tissue model. After 4 h of exposure the liquid at the bottom of the 3D model was collected and analyzed by HPLC. As shown in Figure S4, CPP_{AIF} appeared as a sharp peak at retention time of 9 min. It was also observed that both emulsion compositions did not influence stability of CPP_{AIF}, indicating that CPP_{AIF} could be added to properly designed formulation as a functional cosmetic ingredient.

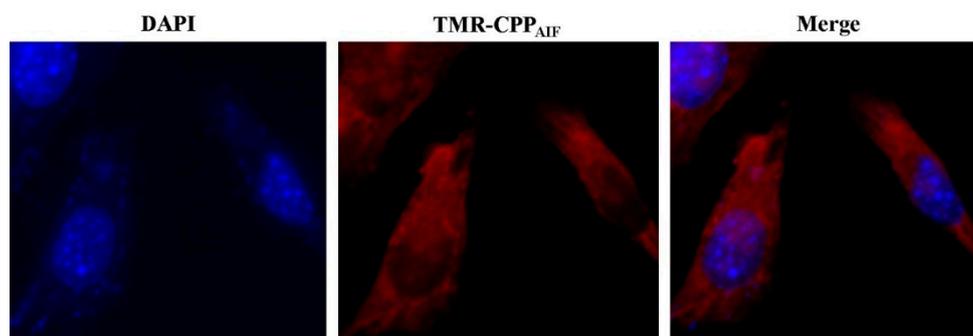


Figure 7. Internalization of TMR-CPP_{AIF}. HaCaT cells (1×10^3) were seeded on collagen I coated cover slide and incubated at 37 °C for 16 h. The cells were then incubated with 20 μ M of TMR-CPP_{AIF} at 37 °C for 30 min. After washing and fixing, the cells were mounted and monitored by confocal microscopy. Red: TMR; Blue: nuclear staining with DAPI. (Magnification: 63 \times).

Transepidermal degree of CPP_{AIF} was further calculated according to HPLC results. ((concentration of CPP_{AIF} in the beneath liquid/concentration of CPP_{AIF} in the topical exposure) * 100). As shown in Figure 8, after 4 h of exposure to the emulsions (100 μ L containing 100 μ M CPP_{AIF}), CPP_{AIF} present at the bottom of the 3D skin tissues W/O and O/W formulations was determined to be respectively $41.4 \pm 3.6\%$ and $28.4 \pm 1.7\%$ respectively in W/O and O/W formulations. The transepidermal activity of W/O CPP_{AIF} was evidently higher than that of O/W formulation. Taken together, appropriate formulation such as W/O emulsion in this study could evidently facilitate transepidermal penetration of CPP_{AIF}.

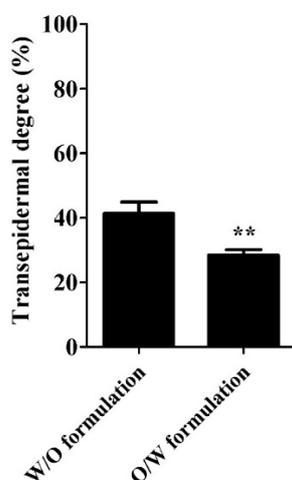


Figure 8. Transepidermal degree of formulated CPP_{AIF} in SkinEthic™ RHE model. All 3D reconstructed human epidermis tissues were incubated with growth medium for 2 h, then 0.1 mL formulated CPP_{AIF} (0.1 mM) emulsions were added to the top of the tissues and incubated for 1 h. After treatment the liquid beneath the 3D skin model was collected for HPLC quantification. Transepidermal degree was calculated by measuring the amount of CPP_{AIF} in the liquid beneath the 3D skin model using HPLC. ** $p < 0.01$ versus the W/O formulation group.

We further examined skin barrier function of the 3D model after 4 h of exposure to formulated CPP_{AIF}. As shown in Figure 9, appropriate W/O formulations promoted CPP_{AIF} penetration into stratum corneum while at the same time maintained the robustness barrier function of the epidermis. The 3D models incubated with W/O CPP_{AIF} showed a higher relative cell viability of $76.5 \pm 8.5\%$ than O/W CPP_{AIF} formulation ($43.3 \pm 7.8\%$). Relative cell viability of negative control (NC, H₂O) equaled to $100.0 \pm 9.6\%$. Thus, W/O CPP_{AIF} displayed a better transepidermal degree with slight disruption effect on disruption of skin barrier function.

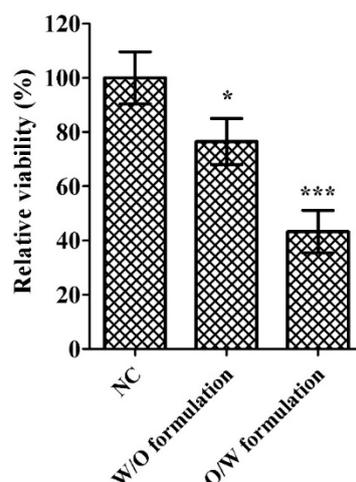


Figure 9. In vitro skin barrier function test of formulated CPP_{AIF} in SkinEthic™ RHE model. 3D reconstructed human epidermis tissues were exposed with formulated CPP_{AIF} (0.1 mM CPP_{AIF}) for 1 h. Then CPP_{AIF} was washed by PBS from the surface followed by application of detergent solution (1% Triton X-100) onto surface of the tissues for another 2 h. The tissues were washed and cell viability was measured by AlamarBlue cell viability assay. H₂O was applied as negative control (NC) in which cell viability was set as 100% (mock). * $p < 0.05$ and *** $p < 0.001$ versus the NC.

4. Discussion

Since 2000 the application of peptides in cosmeceutical products has rapidly increased, and this trend has sped up research and knowledge of physiological properties of peptides. Now, researchers have identified peptide sequences for penetration into skin layers or different cosmetic activities (e.g., anti-ageing, antioxidant, whitening) [2]. The commercial potential for these bio-functional peptides is high, especially for peptides with excellent stability and no toxicity. Many peptides were reported to have different cosmetic activities, but some clinical study results about these peptides were obtained using formulations containing peptides and other active ingredients. These trials did not clearly differentiate the role of peptide from other actives in the formulation. Hence, the results could not be claimed clearly to be the effect of the peptides for skin benefits [2].

Here CPP_{AIF} was tested with regulatory affair approved methods and clear formulations, therefore the safety datum and anti-inflammatory activity were highly credible. CPP_{AIF} in W/O or O/W formulation could pass through 3D human tissue model. Our CPP_{AIF} showed skin penetration activity without disrupting skin barrier function, and its anti-inflammatory activity might alleviate slight inflammation caused by conventional transepidermal methods [2]. As a CPP, this peptide could also carrier cargos into epidermal cell in skin tissue. Skin tissue is composed by four different layers: stratum corneum, viable epidermis, dermis and subcutaneous connective tissue [30]. This structure efficiently blocks penetration of extraneous molecules in to deeper tissue. It has been reported that TAT can be apply for topical drug-delivery, but high cell penetrating activity might increase some risks which is that TAT might bring drug penetrating cell-layers into deeper tissues [30]. Unlike TAT, our CPPAIF only penetrate into cytosol of epidermal cells without exocytosis property [21], hence it would not be a concern of drug effect. Environmental conditions of skin surface might be tough for bio-molecules (peptide). Cream formulation could provide a stable environment for cosmetic ingredients and remained longer on the skin surface. Some cosmeceutical peptides were also applied in cream formulation [2]. To reduce these challenges that might disrupt stability of CPPAIF, our strategy was applied peptide with W/O or O/W formulation as a mimic of cream in transepidermal test. This standard cosmetic formulation might prove a relatively stable condition for CPPAIF and slightly enhance transepidermal activity. Taken together, CPPAIF itself has been demonstrated to be safe and effective for cosmeceutical use. Comparison between W/O and O/W formulations revealed that the former was more suitable for further application, and the latter with reduced skin barrier function

presumably due to formulation components, which might be improved with alternative composition or process. With these facts, CPP_{AIF} was convinced to be a perfect choice for carrying active ingredients through skin tissue and that will be our next goal.

5. Conclusions

CPP_{AIF}, a GAG binding peptide, could penetrate cell membranes with cargos in living animals and was proven to be stable in powder form under room temperature or in water solution under $-20\text{ }^{\circ}\text{C}$ using HPLC analysis. Following OECD guidelines, CPP_{AIF} was evaluated and characterized without skin irritation, skin sensitization and did not disrupt skin barrier function using 3D skin model. In addition to high safety CPP_{AIF} was identified to inhibit inflammation by decreasing inflammatory cytokines, TNF- α and IL-6, using macrophage model. With this bio-function, CPP_{AIF} was further proven to not have sensitization effects. The result of penetration test in HaCaT cells verified CPP_{AIF} for cargo (TMR in this case) delivery into cell in less 30 min. Finally, two commonly used formulations were applied to evaluate transepidermal activity of CPP_{AIF} in 3D skin model in order to imitate real cosmetic applications, and W/O formulation of CPP_{AIF} was identified as a better choice to efficiently penetrate 3D skin with slight disruption of skin barrier function.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2218-273X/10/1/101/s1>, Figure S1: MALDI-TOF mass profile of CPP_{AIF}; Figure S2: MALDI-TOF mass profile of CPP_{AIF} in water under different temperature for 1, 3 and 7 days; Figure S3: The HPLC chromatograms for cysteine and lysine depletion quantification of CPP_{AIF}; Figure S4: HPLC chromatogram of CPP_{AIF} in different formulations applied in reconstructed human epidermis tissue model; Figure S5: Cytotoxicity test of CPP_{AIF} to HaCaT cells; Table S1: O/W formulation content

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