fect on reducing skin inflammatory aging by blockade of a novel target HMGB1

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1. Introduction

Skin is the largest organ of the human body and serves as the primary interface with the external environment and is thus continually subjected to various environmental stressors. These stressors can initiate a cascade of events that lead to inflammation, a physiological response aimed at protecting the body from potential harm. However, when inflammation becomes chronic, it can have detrimental effects on skin health and function [1,2]. Chronic inflammation results in multiple skin-related issues. One such issue is skin barrier dysfunction, which impairs the skin's ability to retain moisture and repel allergens and pathogens, exacerbating the condition. Additionally, the persistent inflammation leads to pruritus, or itching, a symptom that is not only uncomfortable but can also further damage the skin when scratched, creating a vicious cycle of irritation and inflammation. Moreover, during this inflammatory process, the skin releases pro-inflammatory cytokines. Cytokines are small proteins that play a significant role in cell signaling and immune responses. In the case of atopic d ermatitis, the overproduction of these cytokines not only sustains the inflammatory state but also contributes to the process of skin aging, or "inflammaging." This term encapsulates the idea that chronic inflammation accelerates the visible and cellular signs of aging, such as fine lines, wrinkles, and a loss of skin elasticity.
High-mobility group box 1 protein (HMGB1) is a nuclear protein that is actively released by immune cells during times of inflammation [3]. HMGB1 has been identified as a pivotal factor in the pathogenesis of inflammatory diseases. It acts as a late mediator of inflammation, amplifying the immune response and prolonging the inflammatory process. Given the significant role of HMGB1 in inflammation, the current study aimed to explore the therapeutic potential of modulating HMGB1 activity. A novel HMGB1-binding peptide, designated as cIY8, was developed. This peptide was designed to specifically bind to HMGB1 and potentially inhibit its pro-inflammatory effects. In parallel, the study also investigated the effects of glycyrrhizin, a small molecule known for its anti-inflammatory properties. Glycyrrhizin has been shown to inhibit HMGB1 release and activity, suggesting its potential utility in treating inflammatory conditions. In this case, the study aimed to alleviate inflammatory symptoms, improve skin barrier function, and potentially slow down the process of skin aging associated with chronic inflammation. Our research could pave the way for novel therapeutic interventions for inflammatory skin conditions. By targeting HMGB1, the study seeks to address the root cause of inflammation and its downstream effects on skin health and appearance, offering a promising avenue for the development of more effective treatments.

2. Materials and Methods

1.Molecular modeling of protein and peptides performed by molecular simulations.
Three-dimensional structures of HMGB1 and related proteins were initially downloaded from two reputable databases: the RCSB Protein Data Bank (RCSB PDB) [https://www.rcsb.org] and the AlphaFold Protein Structure Database [https://alphafold.ebi.ac.uk/], which offers access to over 200 million protein structure predictions. Following the acquisition of these structures, molecular dynamics (MD) simulations of peptides and peptide-protein complexes were conducted using the GROMACS software [4]. This high-throughput, highly parallel

open-source molecular simulation toolkit is based on molecular mechanical force fields. All simulations were executed for a duration of 3.0 nanoseconds in a cubic water box, applying periodic boundary conditions at a temperature of 300 Kelvin. Subsequently, the secondary structures of the peptides and the refined structures of the peptide-protein complexes were analyzed using Chimera and PyMol softwares. The Chemical Sketch tool was employed to present the two-dimensional diagrams of peptide chemical structures. Predicted binding poses of the peptide-protein complexes were generated by employing AlphaFold2 Multimer and utilizing a reduced database [5]. The quality of these predictions was assessed through the analyzed predicted aligned error and the predicted local distance difference test on Cα, performed with ALPHAPICKLE. Finally, an in silico molecular interaction analysis of the complex structures was comprehensively implemented and further analyzed using the same Chimera and PyMol software.

2.Peptide solid-phase synthesis.

Candidate peptides were synthesized by GenScript Biotech Co., Ltd using an automated peptide synthesizer. The synthesis was conducted employing Fmoc [N-(9-fluorenyl) methoxycarbonyl] chemistry and custom protocols, which are available upon request. A key modification in the synthesis process involved the introduction of the palmitoyl (Pam) side chain. This was achieved by coupling palmitic acid with HATU (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxid hexafluorophosphate) and DIPEA (N,N-Diisopropylethylamine). These reagents were dissolved to a concentration of 0.5 M in DMF (Dimethylformamide) and the mixture was shaken for 3 hours at room temperature (RT) to facilitate the coupling reaction. Following the coupling process, the resin was thoroughly washed in a sequential manner with five rinses using different solvents: DMF, CH2Cl2 (Methylene Chloride), and Et2O (Diethyl Ether). The next step was the cleavage of the peptides from the resin. This was done by adding a mixture of CH3CN (Acetonitrile), thiamazole, and 1,2-ethanedithiol to the resin, which was then shaken for an additional 3 hours at RT. Post-cleavage, the peptide was precipitated out from the solution using ice-cold Et2O. The precipitated peptide was washed five times with Et2O and subsequently dried under vacuum conditions to remove any residual solvents. Finally, the purified peptide was dissolved in a mixture of H2O (Water) and tBuOH (tert-Butyl Alcohol) and subjected to lyophilization to obtain the peptide in a solid, stable form. The synthesized peptides were then rigorously evaluated for their quality and purity. Analysis was performed using two complementary techniques: Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry and High-Performance Liquid Chromatography (HPLC).

3.Real-time quantitative PCR for gene expression analysis.

The process of extracting and analyzing RNA began with the isolation of total RNA from the sample using the TRIzol Reagent, which was sourced from Invitrogen. Following the RNA extraction, the next step involved the synthesis of complementary DNA (cDNA). This was achieved by employing the cDNA reverse transcription kit provided by Applied Biosystems, which facilitated the conversion of RNA into cDNA. Once the cDNA was synthesized, its quantity and quality were assessed using a SYBR Green Master Mix, a reagent also supplied by Roche. The SYBR Green Master Mix binds to the DNA and exhibits fluorescence, which is proportional to the amount of DNA present, allowing for the quantification of cDNA. The quantification of cDNA was performed utilizing the StepOnePlus real-time PCR (Polymerase Chain Reaction) system, another product of Applied Biosystems. This advanced system enabled the precise measurement of cDNA through real-time monitoring of the PCR amplification process.

4.Volunteer clinical testing of clinical scores for aging and inflammation.
A clinical study involving 30 volunteers who exhibited signs of inflammation and aging was conducted. These participants were treated with a lotion containing 7.5 parts per million (ppm) of the compound cIY8. The treatment was compared against a placebo base to evaluate its effectiveness. The study aimed to measure the clinical scores related to aging, which included parameters such as skin redness, wrinkles, contour line and other items in participants treated with a lotion with 7.5ppm cIY8 compared with placebo group. These measurements were taken at two distinct time points of 14 and 28 days into the treatment period.

3. Results

In our quest to identify a protein that plays a pivotal role in the process of inflammaging, we employed network pharmacology strategies. Utilizing keywords intrinsically linked to the phenomenon of inflammaging, we conducted a comprehensive screening of targetable protein which is critical for inflammaging process. Among the extracellular proteins, HMGB1 emerged as a candidate of interest, ranking highly in our scoring system. HMGB1 acts as a potent mediator of inflammation [6]. It can be passively released from necrotic cells or actively secreted by immune cells such as macrophages in response to various stimuli [7]. Extracellular HMGB1 can activate immune cells, including macrophages and dendritic cells, by binding to receptors such as RAGE (Receptor for Advanced Glycation End-products) and TLRs (Toll-Like Receptors), particularly TLR2 and TLR4. This activation leads to the production of pro-inflammatory cytokines, which are integral to the inflammatory response seen in inflammaging. Besides, evidence has accumulated that HMGB1 has been implicated in various age-related diseases where inflammation plays a significant role. It is associated with conditions such as autoimmune diseases, diabetes, and neurodegenerative disorders, which are often exacerbated by the chronic inflammation of aging. Given its role in promoting inflammation, HMGB1 is considered a potential therapeutic target for interventions aimed at reducing the impact of inflammaging and related disease. In our analysis, the similarity of HMGB1 protein sequences among different species is very high, suggesting its evolutionary conservation (Figure 1b).

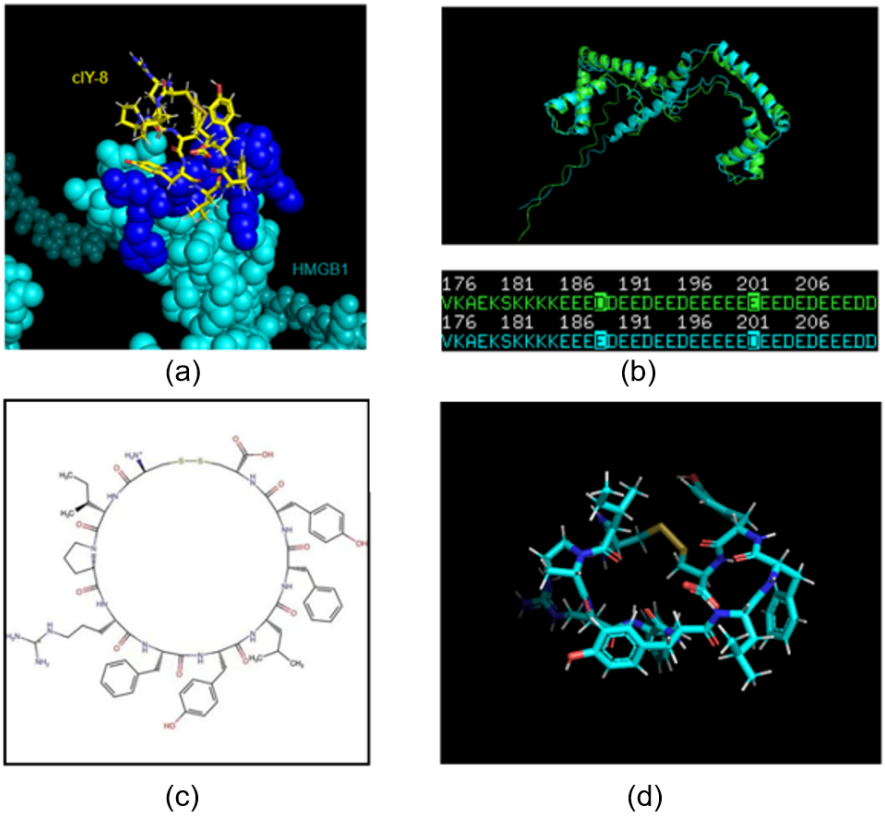

Figure 1. (a) Predictive binding mode of peptide cIY8 (shown in yellow) and HMGB1 (shown in cyan) with potential interactive regions presented (shown in marine). (b) Analysis of HMGB1 sequence and structural homology between human (Homo sapiens, shown in cyan) and mouse (Mus musculus, shown in green). (c) Two-dimensional diagram of peptide cIY8. (d) Three-dimensional structure of peptide cIY8 by molecular dynamics simulation.

Based on the important functions of HMGB1 and through a combination of peptide-protein binding prediction and force field-based molecular simulations, we identified a novel peptide inhibitor that targets HMGB1 (Figure 1a). This inhibitor, named cIY8, was found to have a cyclic structure facilitated by the formation of a disulfide bond (Figures 1c and 1d). The efficacy of cIY8 was experimentally validated. It was shown to effectively inhibit HMGB1-induced inflammation by reducing the cellular production of related inflammatory factors and mitigating the phenotype of skin inflammation and aging in the epidermis and dermis. Treatment with cIY8 led to a decrease in the levels of IL-1α, IL-17, IL-33, IFN-γ and notably inhibited the nuclear translocation of NF-kB signaling, underscoring its potential as a promising inhibitor of the skin's chronic inflammation process (Figure 2). Additionally, cIY8 significantly promotes the expression of various collagens, including I, IV, VII, XII, XVI, and XVIII. These results highlight the impact of HMGB1 on collagen synthesis, thereby exerting anti-aging effects (Figure 3).

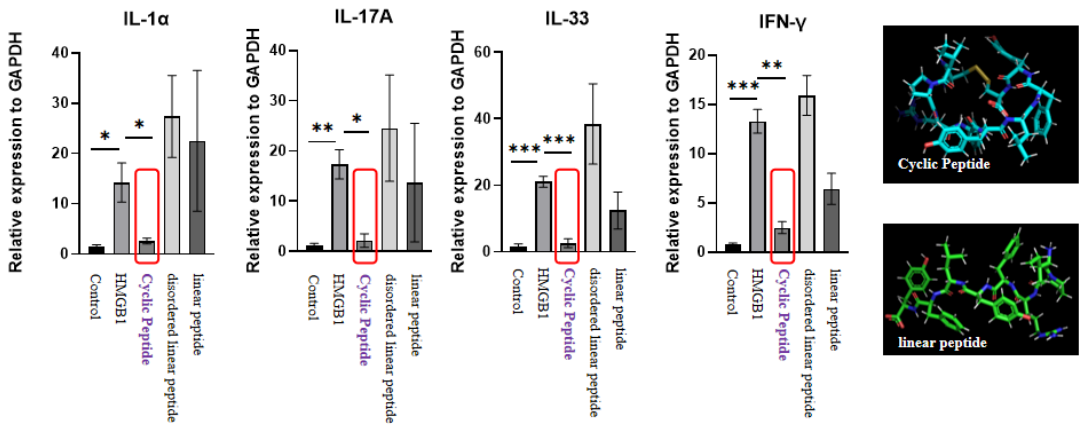

Figure 2. Quantitative PCR analysis of cytokine expression levels in response to HMGB1 inhibition by the peptide cIY8. The data represent the relative expression levels of IL-1α, IL-17, IL-33, IFN-γ (compared with linear peptide and disordered linear peptide). The results demonstrate a significant decrease in cytokine levels post-treatment with cIY8, indicating its inhibitory effect on the inflammatory response.

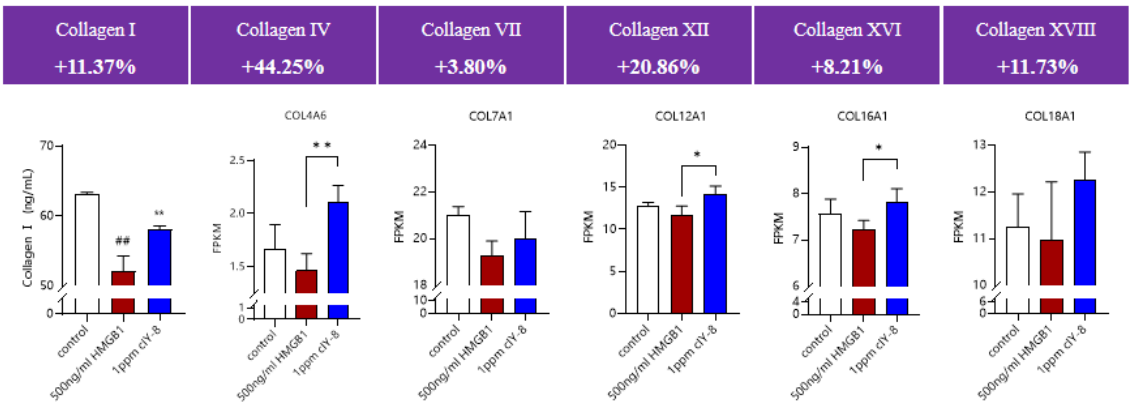

Figure 3. Peptide cIY8 at 1ppm increases the expression rates of various collagens as follows: Collagen I by 11.37%, Collagen IV by 44.25%, Collagen VII by 3.80%, Collagen XII by 20.86%, Collagen XVI by 8.21%, and Collagen XVIII by 11.73%, compared to the control group.

Additionally, we conducted a clinical study to evaluate the efficacy of the lotion in improving skin redness, wrinkles, and contour lines. A cohort of 30 volunteers with symptoms of skin inflammation and aging were enrolled. Participants were treated with a lotion containing 7.5 ppm of the cyclic peptide cIY8 and compared with a control group using a placebo lotion base without the active peptide. The primary outcomes included clinical assessments of skin redness, wrinkles, and contour lines, which were recorded at two distinct time points of 14 and 28 days post-treatment initiation.

For skin redness, objective measurements were taken using colorimeters. The results showed a significant reduction in skin redness in the cIY8-treated group compared to the placebo group, indicating an improvement in skin inflammation. Regarding wrinkles, a standardized method was employed to capture and analyze the depth and volume of wrinkles. High-resolution photography and profilometry were used to objectively quantify changes in

skin topography. The cIY8-treated group exhibited a significant decrease in wrinkle depth and volume after 14 and 28 days of treatment, demonstrating an improvement in skin quality and a reduction in aging signs. As for contourlines, the firmness and resilience of the skin were assessed using a durometer. The cIY8-treated group showed a significant increase in skin elasticity, which contributed to a more defined and youthful facial contour. The improvements in skin redness, wrinkles, and contour lines were statistically significant and clinically meaningful, highlighting the efficacy of the cIY8-containing lotion in enhancing overall skin health and appearance (Figure 4).

Detailed data also presents the efficacy of a lotion containing 7.5 ppm of cIY8 peptide compared to a placebo (Table 1). It shows significant improvements in various skin parameters, including crow's feet wrinkles, nasolabial folds, contour lines, and skin moisture content. The cIY8 peptide lotion demonstrated higher improvement rates than the placebo group, with notable reductions in erythema and sebum content. These results highlight the potential benefits of cIY8 peptide in enhancing skin health and appearance.

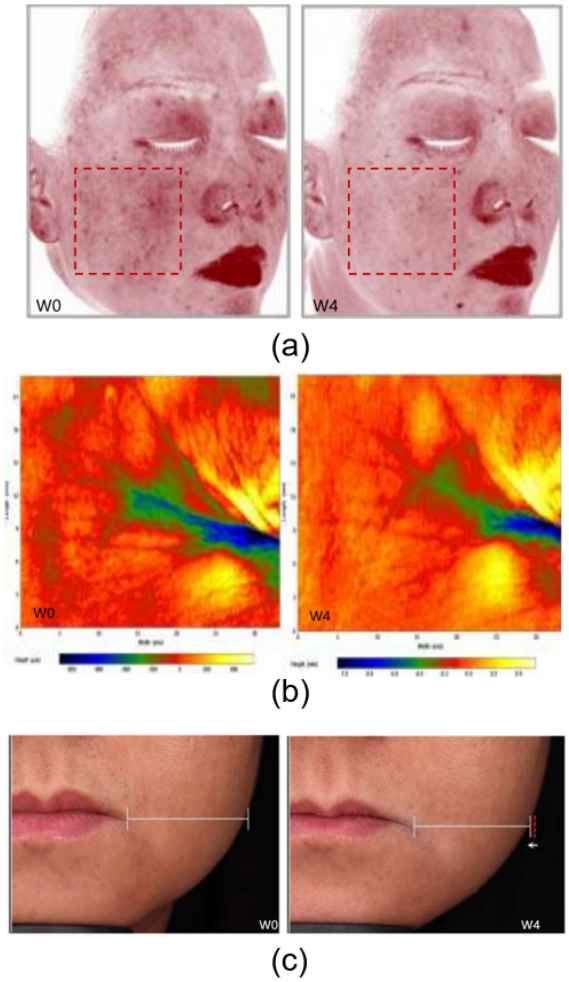

Figure 4. Clinical assessment for skin redness, wrinkles, contour line and other items in participants treated with a lotion with 7.5ppm cIY8 compared with placebo group. (a) Assessment for skin redness of the improvement rate (W4 vs W0). (b) Assessment for wrinkles of the improvement rate (W4 vs W0). (c) Assessment for contour line of the improvement rate (W4 vs W0).

Table 1. Efficacy of Lotion Containing 7.5 ppm cIY8 Peptide vs. Placebo

Item	Lotion Improvement Rate/Individual Maximum Improvement Rate	Lotion Improvement Rate/Placebo Group
Crow's feet wrinkles count	-58.90% / up to up -90.00%	-17.61%
Crow's feet wrinkles volume	-14.06% / up to up -53.12%	-14.02%
Crow's feet wrinkles area	-13.76% / up to up -30.39%	-4.47%
Crow's feet wrinkles length	-14.11% / up to up -50.72%	-5.01%
Nasolabial folds count	-54.95% / up to up -86.67%	-19.80%
Nasolabial folds volume	-7.02% / up to up -38.00%	-0.95%
Nasolabial folds area	-9.07% / up to up -22.93%	-5.34%
Nasolabial folds length	-11.45% / up to up -51.69%	-5.34%
Contour line	-4.55% / up to up -15.04%	-1.06%
F4	-13.74% / up to up -39.09%	-11.82%
R2	+3.43% / up to up +8.74%	+2.54%
Transepidermal water loss value	-5.13% / up to up -25.61%	-4.97%
Moisture content	+7.83% / up to up 57.82%	+7.00%
Erythema area	-15.48% / up to up -57.85%	-9.70%
EI value	-6.62% / up to up -36.21%	-6.23%
Sebum content (face)	-11.42% / up to up -46.98%	-9.50%
Sebum content (forehead)	-9.18% / up to up -24.71%	-8.65%

*Lotion Improvement Rate/Individual Maximum Improvement Rate represents Lotion (containing 7.5ppm cIY8 Peptide) Improvement Rate (W4 vs W0)/Individual Maximum Improvement Rate

*Lotion Improvement Rate/Placebo Group represents Lotion (containing 7.5ppm cIY8 Peptide) Improvement Rate/Placebo Group

4. Discussion

In recent years, the role of HMGB1 protein in the field of skincare has increasingly been recognized for its potential as a therapeutic target. HMGB1, traditionally known for its nuclear functions in chromatin remodeling and gene regulation, has emerged as a critical mediator in the inflammatory processes that contribute to skin aging and various dermatological conditions. In the context of skincare, Its extracellular actions are particularly pertinent. When released by damaged or stressed cells, HMGB1 can act as a potent pro-inflammatory cytokine, initiating and amplifying the inflammatory response. This can lead to a cascade of events that result in tissue damage, fibrosis, and the breakdown of the skin's structural integrity, all of which are hallmarks of aging skin. The clinical significance of HMGB1 in skincare is further highlighted by its involvement in the pathogenesis of several skin disorders, including acne, psoriasis, and atopic dermatitis, where excessive inflammation can exacerbate symptoms and impede the healing process. By modulating HMGB1 activity, therapeutic interventions may offer a novel approach to managing these conditions and improving patient outcomes.

HMGB1 consists of two structurally similar domains, the pro-inflammatory box (B-box) and the anti-inflammatory box (A-box). HMGB1 A-box and B-box individually activate TLR4 signaling to release cytokines/chemokines [8,9]. HMGB1 recognition cascade could be disrupted by blocking A-box with anti-HMGB1 mAb 2G7. We proposed that HMGB1 was a promising target for skin inflammaging process and therefore topically used the novel HMGB1-binding peptide cIY8 and demonstrated that blockade of HMGB1 significantly improved the symptoms and pruritus in AD or inflammation related aging. The development of cIY8, a cyc-

lic peptide inhibitor targeting HMGB1, represents a promising step forward in skincare research. By inhibiting HMGB1, cIY8 has demonstrated the ability to reduce the production of pro-inflammatory cytokines, such as IL-1α, IL-4, and IL-13, which are key players in the inflammatory response. This suggests that cIY8 could be effective in mitigating inflammation-mediated skin damage and promoting skin health. Moreover, NF-kB is a central regulator of genes involved in inflammation and cellular stress responses, and its inhibition by cIY8 could have far-reaching effects on skin health, potentially reducing erythema, pruritus, and sebum overproduction commonly associated with inflammatory skin conditions. The clinical improvements observed in skin elasticity and the reduction of wrinkles and redness with cIY8 treatment are indicative of its potential to address both the symptoms and underlying causes of skin aging. The clinical study involving the application of a lotion containing 7.5ppm cIY8 to volunteers exhibiting signs of inflammation and aging provides valuable insights into the potential therapeutic effects of this novel peptide inhibitor. The reduction in wrinkle depth and volume is particularly noteworthy, as these are some of the most visible indicators of aging skin. The assessment of skin elasticity further supports the notion that cIY8 may enhance the skin's structural integrity, potentially through the modulation of collagen and elastin production. This is a critical aspect of anti-aging treatments, as loss of elasticity is a common complaint among individuals seeking to improve their skin's appearance and texture. Inflammation markers such as redness, itching, and oil secretion also showed significant improvement in cIY8-containing group, highlighting its anti-inflammatory capabilities. This is consistent with the known role of HMGB1 in promoting inflammation and suggests that the inhibitory effect of cIY8 on HMGB1 activity can lead to a reduction in pro-inflammatory cytokines, thereby alleviating the symptoms of inflammation. These effects position cIY8 as a candidate for inclusion in anti-aging and anti-inflammatory skincare formulations. However, while the preliminary findings are encouraging, further research is necessary to fully elucidate the long-term effects and safety of HMGB1 inhibition in skincare. Studies with larger sample sizes, diverse demographics, and extended treatment durations will be crucial to establishing the efficacy and safety profile of cIY8 in various skin types and conditions. In conclusion, the role of HMGB1 in inflammation and its potential as a therapeutic target in skincare is a promising avenue of research. The development of cIY8 as a specific HMGB1 inhibitor offers new possibilities for treating skin aging and inflammatory skin disorders.

5. Conclusion

In summary, the relationship between HMGB1 and skincare has been brought to the forefront by recent research, highlighting its potential as a novel therapeutic target for various skin conditions. HMGB1 protein has now been implicated as a pivotal mediator in the inflammatory processes that underpin skin aging and inflammation-related conditions. To elucidate the roles of HMGB1, we employed peptide-protein binding predictions to discover a novel peptide inhibitor that targets HMGB1. This inhibitor, designated as cIY8, features a cyclic structure formed by a disulfide bond. Treatment with peptide cIY8 led to a reduction in Type 2 cytokines and a significant inhibition of NF-kB nuclear translocation. Furthermore, inhibition of HMGB1 can lead to the activation of major collagens and key proteins in the dermal-epidermal junction layer, thereby promoting anti-aging effects. Subsequent clinical trials demonstrated a marked decrease in signs of inflammaging compared to placebo, with significant improvements against wrinkle, skin redness, and other problems.

The development of cIY8, a cyclic peptide inhibitor of HMGB1, presents a significant advancement in our ability to modulate the inflammatory response in the skin. By targeting HMGB1, cIY8 has shown promise in reducing the levels of pro-inflammatory cytokines,

thereby potentially mitigating the signs of skin aging and improving the management of inflammatory skin conditions. Clinical improvements, as evidenced by the reduction in skin wrinkling, enhanced elasticity, and diminished signs of inflammation, suggest that cIY8 could be a viable candidate for integration into skincare products aimed at anti-aging and anti-inflammatory treatments. Nonetheless, as with any novel therapeutic approach, a measured and thorough investigation is necessary. Future studies could aim to address the long-term efficacy, safety, and impact of HMGB1 inhibition in a broader population and across various skin types. In conclusion, the modulation of HMGB1 activity by cIY8 represents a promising frontier in skincare science. As our understanding on the role of HMGB1 in skin health and disease continues to evolve, targeted therapies would be approached for the treatment and prevention of skin aging and inflammation.

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