BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 33(3), 494-500 (2025)

Anti-Inflammatory Activity of Cell-Penetrating Peptide Nucleic Acids Targeting Indoleamine 2,3-Dioxygenase 1 in IFNγ-Treated Human Keratinocytes

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Abstract

Indoleamine 2,3-dioxygenase 1 (IDO1) is an enzyme that plays a pivotal role in immune regulation by metabolizing tryptophan into kynurenine, leading to T cell suppression and promoting immune tolerance. However, persistent activation of IDO1 can lead to prolonged immune stimulation in inflammatory conditions such as skin diseases and chronic inflammation. In this study, we developed modified peptide nucleic acids (PNAs) conjugated with cationic lipid chains to target IDO1 pre-mRNA and evaluated their anti-inflammatory effects in human keratinocytes. The modified PNAs demonstrated enhanced solubility, robust binding affinity, and effective penetration into keratinocytes. Quantitative PCR results showed significant downregulation of IDO1 and pro-inflammatory cytokines such as IL-6, IL-8, and PTGS2 in interferon γ (IFNγ)-treated keratinocytes. These findings suggest that cell-penetrating PNAs targeting IDO1 hold potential as a therapeutic approach for inflammatory skin disorders and chronic inflammation.

Key Words: Indoleamine 2,3-Dioxygenase 1, Anti-inflammation, Peptide nucleic acids, Cell penetration, Antisense oligonucleotides, Normal human keratinocytes

INTRODUCTION

Indoleamine 2,3-dioxygenase 1 (IDO1) is a key immunoregulatory enzyme that catalyzes the conversion of tryptophan to kynurenine, exerting substantial effects on immune cell behavior (Opitz *et al*., 2020). The IDO1-mediated reduction of tryptophan levels can lead to T-cell suppression, while kynurenine pathway metabolites further contribute to immunosuppressive effects on T-cell function (Zhai *et al*., 2018). IDO1 plays an essential role in maintaining immune homeostasis by limiting excessive immune responses (Opitz *et al*., 2020). However, its function in chronic inflammation remains controversial and paradoxical. Although IDO1 is typically immunosuppressive, sustained activation during chronic inflammation can contribute to persistent immune stimulation (Platten *et al*., 2019). In pathologies such as colitis, cirrhotic cardiomyopathy, and certain cancers, IDO1 has been implicated in the production of pro-inflammatory cytokines, exacerbating inflammation (Shon *et al*., 2015; Prendergast *et al*., 2017; Shayesteh *et al*., 2021). Conversely, IDO1 deficiency has been associated with anti-inflammatory outcomes in chronic viral myocarditis (Guo *et al*., 2019). Elevated IDO1 expression has also been linked to inflammatory skin conditions, including atopic dermatitis and psoriasis (Ito *et al*., 2015; Staudacher *et al*., 2015). Consequently, targeting IDO1 suppression in chronic inflammatory diseases has been suggested as a potential therapeutic strategy.

A variety of antisense RNA therapeutics, including antisense oligonucleotides (ASOs) and peptide nucleic acids (PNAs), has been developed to target specific RNA sequences. ASOs are classified into two primary types: RNase H-competent ASOs and steric-blocking ASOs (Roberts *et al*., 2020; Quemener *et al*., 2022). Steric-blocking ASOs, in particular, are designed to modulate RNA processing by targeting pre-mRNA, thus interfering with translational initiation (Lauffer *et al*., 2024). Structurally, PNAs consist of nucleobases attached to a peptide-like backbone, and they function similarly to steric-blocking ASOs. Compared to conventional ASOs, PNAs exhibit enhanced binding affinity for complementary nucleic acids and possess resistance to nuclease and protease

Open Access **https://doi.org/10.4062/biomolther.2024.207**

Received Nov 4, 2024 **Revised** Dec 10, 2024 **Accepted** Dec 12, 2024
Published Online Apr 30, 2025

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degradation, leading to greater stability *in vivo* (Terada *et al*., 2023). However, the clinical application of PNAs is challenged by their poor solubility in water. To address this, modifications such as the addition of substituents, like hydroxymethyl groups or diethylene glycol side chains, to the PNA backbone have been implemented (Dragulescu-Andrasi *et al*., 2006; Avitabile *et al*., 2012; Gupta *et al*., 2017). Furthermore, a key limitation of PNAs lies in their limited ability to cross the plasma membrane, which has been tackled through strategies like conjugation with cell-penetrating peptides or encapsulation within polymer nanoparticles (Öztürk *et al*., 2023; Zhai *et al*., 2024).

In this study, we synthesized modified PNAs incorporating cationic lipid groups into nucleobases to improve solubility, binding affinity, and cellular uptake (Fig. 1). These modified

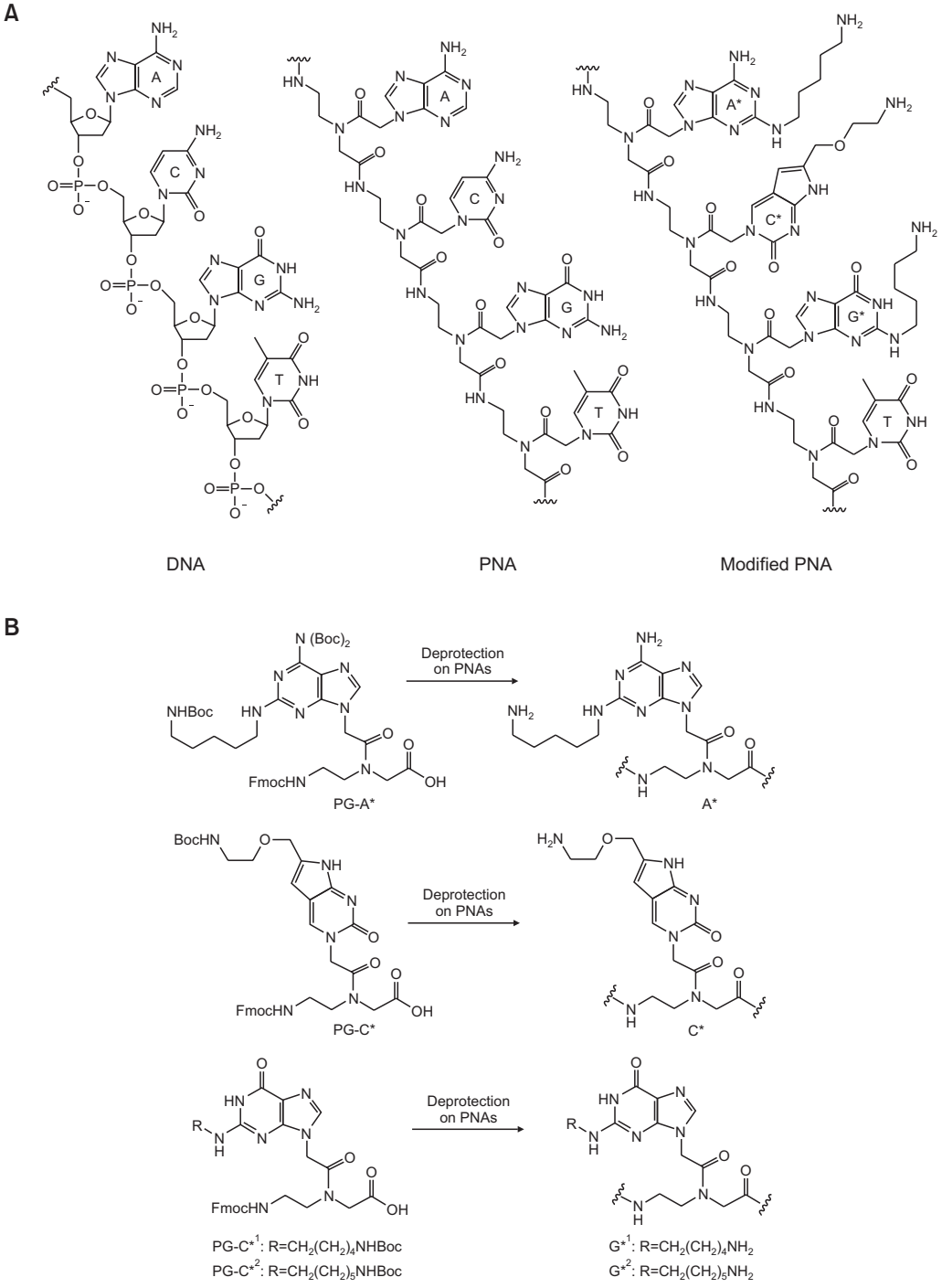


Fig. 1. Structures of cell-penetrable modified PNA. (A) Structural comparison of DNA, PNA, and modified PNA. (B) Summary of synthesis for modified PNA monomers PG-A*, PG-C*, PG-G*¹, and PG-G*² along with their deprotected forms.

PNAs were designed to target IDO1 pre-mRNA. We then assessed their effects on the expression of pro-inflammatory markers IL-6, IL-8, and prostaglandin E_2 (PGE_2) in interferon-γ (IFNγ)-stimulated human keratinocytes.

MATERIALS AND METHODS

Chemistry

The synthesis of modified PNA monomers and unmodified PNA monomers followed the methods described in previous studies (Shaikh *et al*., 2021; Podlaski *et al*., 2023). The synthesis and analytical data for adenine-modified PNA monomer (A*), cytosine-modified PNA monomer (C*), guanine-modified PNA monomers (G*[1], G*[2]), and unmodified PNA monomers are provided in the Supplemental Data. PNA oligomers were synthesized using solid-phase peptide synthesis (SPPS) based on Fmoc chemistry (Thomson *et al*., 1995; Nandhini *et al*., 2023). The analytical data for modified PNAs **1 - 8**, FAM-labeled PNA (**2**-FAM), and scrambled PNA (ScPNA: TC*CA*TTCA*TAC*TG*[2]C) are also described in the Supplemental Data.

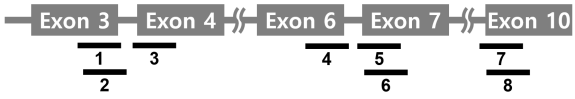
Melting temperature (T_m) measurements of modified PNAs

The binding affinity between PNA and DNA was assessed using T_m measurements. DNAs purchased from BIONEER (Daejeon, Korea) were used without further purification. A mixture containing 2 μM of modified PNA and 2 μM of DNA in 4 mL of buffer (10 mM sodium phosphate, pH 7.0, and 0.1 M NaCl) was heated in boiling water for 2 min and then allowed to cool to room temperature (Podlaski *et al*., 2023). Absorbance at 260 nm was monitored using an Agilent Cary 100 UV-Vis spectrophotometer (Agilent, CA, USA), with the temperature increased from 25°C to 90°C at a rate of 1.0°C per minute.

Cell culture and confocal microscopy

Human keratinocytes derived from neonatal foreskins were purchased from Lonza (Basel, Switzerland) and cultured in keratinocyte basal medium (KBM™ Gold™ Basal Medium) supplemented with hydrocortisone, transferrin, epinephrine, gentamicin, amphotericin, bovine pituitary extract, human epidermal growth factor, and insulin (KGM™ Gold™ SingleQuots™ supplements). To induce an inflammatory dermatological condition in human keratinocytes, recombinant human interferon γ (IFNγ; R&D systems, Minneapolis, MN, USA) was treated. The cell penetration activity of FAM-labeled PNA was evaluated using a confocal microscope (Leica, Wetzlar, Ger-

Table 1. The target sites, sequences, and binding affinities of modified PNAs



Cpd No.	Sequence (N'→C')	T_m (°C)[a]
1	CA*AA*CC*TTA*CGG*[2]A	81.02
2	CTC*CA*AA*CCTTA*CG*[1]	82.07
3	GG*[2]CAA*GA*CC*TGA*T	N.D. (>90)
4	CG*[2]TA*C*TTTGA*TTG*[2]	72.07
5	AA*TTA*CCTA*AA*AC*A	72.02
6	GG*[2]AA*TTA*CC*TAA*A	73.37
7	CA*TG*[1]TCC*TATA*GG*[1]A	84.22
8	AG*[2]CA*TG*[2]TC*CTA*TA	81.07

[a]The T_m values were measured using a 2 μM PNA-DNA duplex in a buffer containing 10 mM sodium phosphate at pH 7.0 and 0.1 M NaCl. 'N.D.' indicates that the T_m values were beyond the measurement range and could not be detected.

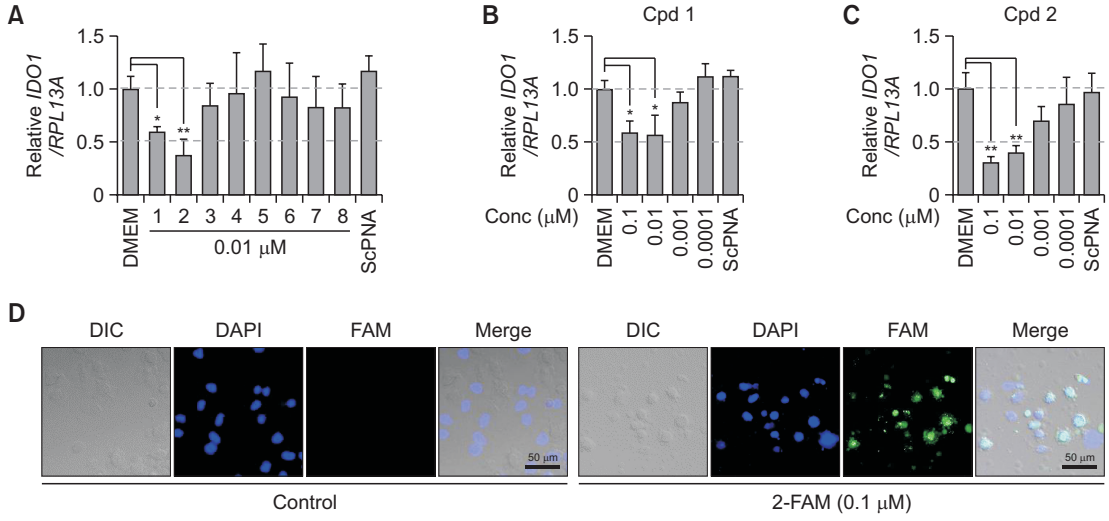


Fig. 2. The effect of modified PNA oligomers on IDO1 gene expression in human keratinocytes. (A) Total RNA samples treated with modified PNA oligomers and a scrambled PNA oligomer (ScPNA) were extracted using TRIzolTM. The gene expression of IDO1 was analyzed using q-RT-PCR. (B) Concentration-dependent analysis of compounds (B) 1 and (C) 2 was conducted using q-RT-PCR at non-cytotoxic concentrations. ScPNA was used as a control at 0.1 μM. (D) Confocal microscopy image of human keratinocytes treated with FAM-labeled 2. The values represent the mean expression level ± SD (n=3). *p<0.05; **p<0.01.

many). Human keratinocytes were seeded onto a 6-well cell culture plate for 24 h. The FAM-labeled PNA was treated for 24 h, and cell nuclei were stained with DAPI. The data analysis was conducted using Leica Application Suite X.

Quantitative real-time reverse transcription polymerase chain reaction (q-RT-PCR) and enzyme-linked immunosorbent assay (ELISA)

Total RNA samples were isolated using TRIzol™ (Invitrogen, Carlsbad, CA, USA) and subsequently purified with the RNeasy Mini Kit (Qiagen, Germantown, MD, USA). The cDNA was synthesized using the Maxima First Strand cDNA synthesis kit (Thermo Fisher Scientific, MA, USA). q-RT-PCR was conducted using the Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). TaqMan primer sets used in this study were indoleamine 2, 3-dioxygenase 1 (*IDO1*, Hs00984148_m1), interleukin-6 (*IL6*, Hs00985639_m1), C-X-C motif chemokine ligand 8 (CXCL8, Hs00174103_m1), prostaglandin-endoperoxide synthase 2 (*PTGS2*, Hs00153133_m1) and human ribosomal protein L13a (*RPL13A*, Hs04194366_g1). The relative expression of each gene was determined using the mathematical model developed by Pfaffl (Pfaffl *et al*., 2001). The protein levels of IL-6, IL-8, and PGE_2 were quantified using the DuoSet® ELISA Development system (R&D Systems).

Statistical analyses

Statistical analyses were performed using RStuio for windows (RStudio Inc., Boston, MA, USA). All data were expressed as means ± standard deviation (SD). Student's t-test was used for comparison between group means. Significance thresholds were set at (*,#) $p<0.05$ and (**,##) $p<0.01$.

RESULTS

Design of modified PNAs and evaluation of binding affinities

To modulate RNA processing of the IDO1 gene and generate non-productive mRNA species, we designed modified PNAs specifically targeting IDO1 pre-mRNA (GenBank accession no. NG_028155) (Table 1). The PNA structures incorporated strategically selected modified monomers—A*, C*, G*[1], and G*[2]—to enhance binding characteristics. Eight modified PNAs were synthesized, and their binding affinities to complementary DNAs were assessed using melting temperature (T_m) measurements. For short double-stranded DNAs (16–30 base pairs), T_m values are typically predicted to be below 50°C (Panjkovich and Melo, 2005). In contrast, all modified PNAs exhibited strong binding, with T_m values exceeding 70°C. Notably, compound 3 demonstrated exceptionally high affinity, with a T_m beyond the measurable range, precluding precise determination.

Effect of modified PNA oligomers on IDO1 expression in human keratinocytes

To evaluate the impact of modified PNA oligomers on IDO1 gene expression in human keratinocytes, we assessed their inhibitory activity at non-cytotoxic concentrations. The appropriate concentration range for these oligomers was determined through cell viability assays, which demonstrated that neither the modified PNAs nor their scrambled controls exhibited cytotoxicity at concentrations up to 1 μM (Supplemental Data Fig. 7). Among the oligomers tested, compounds 1 and 2 significantly downregulated IDO1 gene expression in human keratinocytes (Fig. 2A). In a concentration-dependent analysis, compound 2 exhibited the significant inhibitory effect, while compound 1 showed weak inhibition (Fig. 2B, 2C). To further investigate the cell-penetrating capability of the modified PNA targeting IDO1, a FAM-labeled version of compound 2 was synthesized and tested in human keratinocytes. Confocal microscopy revealed a green fluorescence signal in cells treated with FAM-labeled compound 2 (2-FAM), indicating successful cellular uptake compared to the control. These results suggest that modified PNA oligomers are capable of penetrating the plasma membrane independently of transfection reagents and modulating IDO1 gene expression in human keratinocytes.

Anti-inflammatory effects of modified PNA oligomers targeting IDO1 in IFNγ-treated human keratinocytes

The anti-inflammatory effects of modified PNA oligomers targeting IDO1 were examined in IFNγ-stimulated human keratinocytes. IFNγ, a key cytokine produced by Th1 cells, is

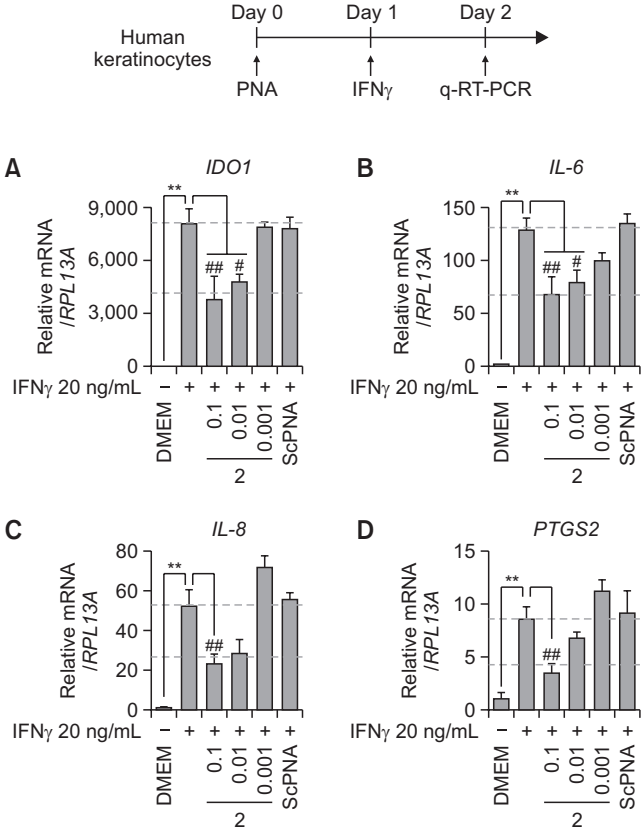


Fig. 3. The anti-inflammatory effects of modified PNA oligomer 2 in IFNγ-treated human keratinocytes. Compound 2 was treated to human keratinocytes (μM). After 24 h, IFNγ was treated in combination with keratinocyte basal medium (KBM Gold Basal Medium). Total RNA samples were isolated and q-RT-PCR was conducted for (A) IDO1, (B) IL-6, (C) IL-8, and (D) PTGS2. ScPNA was used as a control at 0.1 μM. The values represent the mean expression level ± SD (n=3). $^{\#}p<0.05$; $^{**,\#\#}p<0.01$.

closely associated with chronic skin inflammatory diseases, such as psoriasis. IFNγ treatment notably increased the expression of IDO1 and inflammatory response-related genes, including interleukin-6 (IL-6), IL-8, and prostaglandin synthase 2 (PTGS2) in these cells (Fig. 3). Compound 2 significantly downregulated the expression of these inflammatory genes in a concentration-dependent manner, demonstrating its potential anti-inflammatory activity. In contrast, the scrambled PNA (ScPNA) showed no effect on the expression of inflammation-associated genes in human keratinocytes.

To evaluate the effect of compound 2 on pro-inflammatory mediators, the levels of IL-6, IL-8, and PGE_2 were measured in the cell culture supernatants of IFNγ-treated human keratinocytes following treatment with compound 2 using enzyme-linked immunosorbent assay (ELISA) (Fig. 4). IFNγ stimulation significantly upregulated the expression of these pro-inflammatory mediators. Treatment with compound 2 effectively reduced IL-6, IL-8, and PGE_2 levels in a concentration-dependent manner. This significant reduction in IL-6, IL-8, and PGE_2 accumulation in cell culture supernatants following treatment with compound 2 underscores the regulatory role of IDO1 suppression in modulating inflammatory signaling pathways in IFNγ-stimulated keratinocytes. These results suggest that the downregulation of IDO1 expression effectively attenuates IFNγ-induced pro-inflammatory responses. Taken together, this study demonstrate that the cell-penetrating modified PNA compound 2 not only significantly inhibits IDO1 expression but also suppresses major pro-inflammatory mediators (Fig. 5).

DISCUSSION

Recently, several oligonucleotide therapeutics, including Mipomersen, Eteplirsen, Nusinersen, Givosiran, and Golodirsen, have received FDA approval (Roberts *et al*., 2020). Additionally, many nucleic acid-based therapies are in clinical trials for a wide range of diseases. Although limitations in physicochemical properties and cell permeability have challenged nucleic acid therapeutics, chemically modified PNAs have emerged as promising drug candidates. In this study, we introduce modified PNAs with significantly improved physicochemical properties and cell permeability, designed to regulate RNA processing in target genes effectively.

IDO1 is widely recognized for its role in inducing immune tolerance and suppressing immune responses; however, its involvement in chronic inflammation remains less well under-

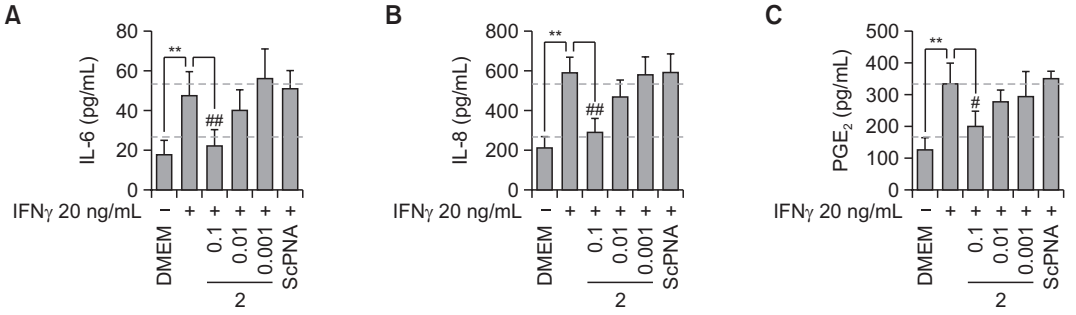


Fig. 4. Effects of pro-inflammatory mediators in IFNγ-treated human keratinocytes following compound 2 treatment (μM). Protein expression levels of (A) IL-6, (B) IL-8, and (C) PGE_2 were quantified by enzyme-linked immunosorbent assay (ELISA). ScPNA was used as a control at 0.1 μM. The values represent the mean expression level ± SD (n=3). $^{\#}p<0.05$; $^{**,\#\#}p<0.01$.

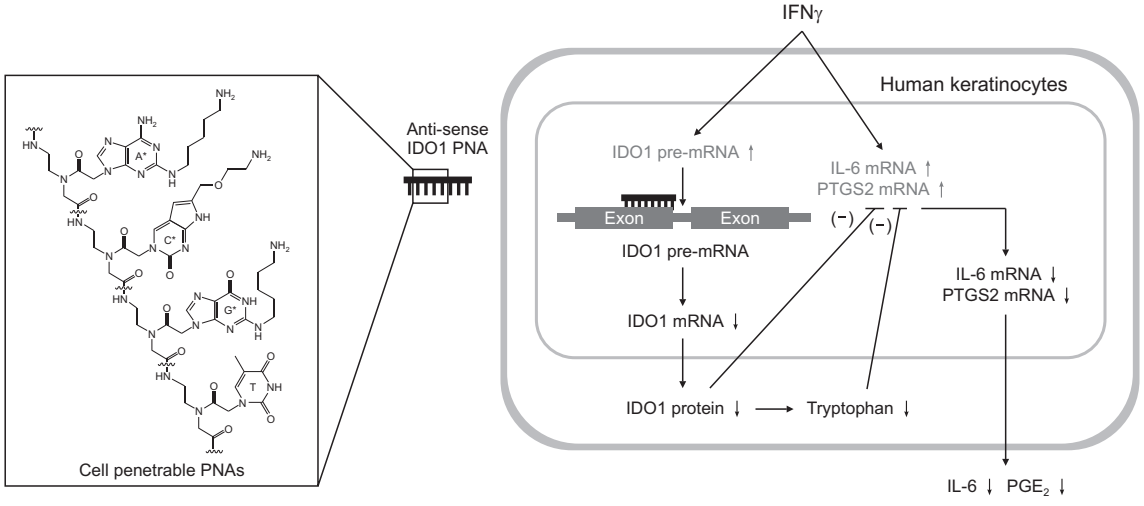


Fig. 5. Mechanism of anti-inflammatory effects of cell-penetrating PNA targeting IDO1 in human keratinocytes. IDO1, indoleamine 2,3-dioxygenase 1; IFN, inteferon; IL-6, interleukin 6; PNAs, peptide nucleic acids; PTGS2, prostaglandin endoperoxide synthase 2.

stood and remains further exploration (Cesario *et al*., 2011). Additionally, IDO1 may serve as a critical link between chronic inflammation and cancer progression, making it a target of interest in cancer immunotherapy (Fujiwara *et al*., 2022). Notably, in animal models examining the transition from chronic inflammation to skin carcinoma, IDO1-deficient mice did not develop skin carcinoma, unlike their wild-type counterparts (Muller *et al*., 2008). A reduced incidence of papilloma was also observed in IDO1-null mice following carcinogen and phorbol ester exposure (Prendergast *et al*., 2010). These findings underscore the potential of IDO1 inhibition as a therapeutic approach to prevent the progression from chronic inflammation to tumor development.

This study introduces cell-penetrating modified PNAs designed to regulate IDO1 expression. These PNAs exhibit enhanced cell permeability, improved physicochemical properties, and high binding affinity for complementary nucleic acids. In human keratinocytes, the modified PNAs effectively inhibited IDO1 expression and downregulated genes associated with the inflammatory response. The therapeutic potential of these IDO1-targeting PNAs for inflammatory skin conditions, such as atopic dermatitis and eczema, will be further evaluated in *in vivo* pharmacological studies. Additionally, the efficacy of IDO1-targeting PNAs in chronic inflammation, cancer-associated inflammation, and cancer progression is further explored. In conclusion, cell-penetrating IDO1-targeting PNAs show promise as therapeutic agents for treating inflammatory skin diseases and cancer-associated inflammation.

ACKNOWLEDGMENTS

This study was partly supported by the National Research Foundation (NRF) of Korea (and RS-2024-00399364) and by the Ministry of Health & Welfare, Republic of Korea (RS-2024-00351858).

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