



Article

γ -Amino Carboxylic Acid Modification Enhances the Efficacy of Peptide Nucleic Acids Targeting miR-221-3p in Lung Cancer Cell Lines

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Abstract

Peptide nucleic acids (PNAs) are versatile molecules with promising diagnostic and therapeutic applications, including gene expression regulation and miRNA targeting. However, their moderate biological efficacy limits their therapeutic application. This can be addressed by leveraging a key advantage of PNAs over other nucleic acids—the ease of modification, which enhances their functional properties. Notably, γ -modified PNAs have improved binding affinity and cellular uptake properties, underscoring the potential of backbone engineering. In this study, we introduced a novel γ -amino carboxylic acid modification into PNAs targeting miR-221-3p, a key miRNA implicated in various pathological processes. The binding affinity of the modified PNAs to their targets and their ability to inhibit miR-221-3p expression were considerably higher than those of unmodified PNAs in Lung cancer cell lines, leading to effective regulation of downstream gene and protein expression. These findings underscore the potential of γ -modified PNAs as a platform for developing miRNA-targeted therapeutics.

Keywords: peptide nucleic acids; γ -ACA modification; miRNA inhibition; miR-221-3p

1. Introduction

Peptide nucleic acids (PNAs) have emerged as powerful tools for a diverse range of applications, including gene silencing, diagnostics, and therapeutic interventions [1–5]. Their unique structure, characterized by a peptide-like backbone instead of the conventional sugar-phosphate backbone found in natural nucleic acids, confers distinct advantages, including enhanced binding affinity, stability, and specificity [1,2,6]. Unlike traditional DNA or RNA, PNAs are neutral, which eliminates electrostatic repulsion during hybridization, ensuring more flexible binding conditions [2,7]. These characteristics make PNAs particularly suitable for targeting genetic mutations and dysregulated gene expression, highlighting their strong potential for therapeutic applications in diseases such as cancer [5,8–11].

Despite their considerable therapeutic potential, the practical therapeutic applications of PNAs are limited by their poor aqueous solubility and low cellular uptake efficiency, primarily attributed to their neutral charge and limited interaction with lipid-rich cell



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membranes [9,10,12]. Furthermore, owing to their dose-dependent activity, rapid systemic clearance, poor tissue distribution, and limited cell permeability, high doses of PNAs are required to achieve therapeutic effects [13–15]. This high dosing requirement complicates clinical translation and is associated with side effects from repeated administration. In addition, PNAs are prone to aggregation in solution, further limiting their use in biological systems [16]. To overcome these limitations, structural modifications, such as the introduction of charged groups into the PNA backbone, have been explored to improve solubility, stability, and cellular uptake [17].

γ -Modified PNAs (γ -PNAs) contain a stereocenter at the γ carbon of the PNA backbone [18]. This modification improves solubility, cellular uptake, and binding affinity for DNA and RNA targets [19–24]. The right-handed helical conformation restricts molecular flexibility, leading to more stable and selective binding interactions [9,19,21]. For example, γ -guanidinyll-modified PNAs (γ -GPNAs) exhibit enhanced binding affinity and Hoogsteen-face selectivity for microRNA (miRNA) targets, while preventing the formation of undesired ternary complexes. This modification also facilitates internalization into living cancer cells, underscoring its therapeutic potential [21]. The structural preorganization and the presence of guanidine groups in γ -GPNAs further enhance hybridization with complementary nucleic acids and significantly enhance cellular uptake [19]. Similarly, γ -PNAs modified with mini-polyethylene glycol side chains improve aqueous solubility, strong nucleic acid binding, and low cytotoxicity, enabling effective targeting of double-stranded DNA via Watson–Crick base pairing [25]. These γ -modified PNAs have been successfully employed to silence oncogenic microRNAs, including miR-210, leading to significant tumor growth inhibition in vivo when delivered via poly(lactic-co-glycolic acid) (PLGA) nanoparticles [20]. Collectively, these advances highlight the potential of γ -PNAs for applications in various fields, including gene therapy and diagnostics.

miRNAs play critical roles in the regulation of gene expression and are frequently dysregulated in various pathological conditions, particularly cancer [26,27]. Aberrant miRNA expression contributes to tumor initiation, progression, and therapy resistance by modulating key pathways involved in cell proliferation, apoptosis, and differentiation. Among oncogenic miRNAs, miR-221-3p has been extensively reported to be overexpressed in multiple cancer types, where it promotes malignant phenotypes through the suppression of tumor suppressor genes such as CDKN1B (p27Kip1) and the dysregulation of cell-cycle control mechanisms [28–31]. Owing to these features, miR-221-3p is a representative and biologically relevant target for evaluating nucleic acid-based therapeutic strategies [26,32].

Therefore, this study introduces a novel γ -amino carboxylic acid (γ -ACA) modification designed to further enhance the PNA performance. Using miR-221-3p as a representative oncogenic miRNA target, we aim to evaluate the impact of γ -ACA modification on PNA binding affinity, cellular uptake, and functional miRNA inhibition. The findings of this study highlight the potential of γ -ACA-modified PNAs as an advanced platform for miRNA-targeted therapeutic applications.

2. Materials and Methods

2.1. Chemicals for PNA Synthesis

(6-Benzhydryloxycarbonylamino-purin-9-yl)-acetic acid (A(Bhoc)-acetic acid), (2-benzhydryloxycarbonylamino-6-oxo-1,6-dihydro-purin-9-yl)-acetic acid (G(Bhoc)-acetic acid), (4-N-(benzhydryloxycarbonyl)cytosine)-1-acetic acid (C(Bhoc)-acetic acid), and thymine-1-acetic acid were synthesized by HLB Panagene (Daejeon, Republic of Korea).

All chemical products: 2 M lithium aluminum hydride in tetrahydrofuran (THF) (LiAlH₄ in THF), sodium cyanoborohydride (NaBH₃CN), N- α -Fmoc-L-asparagine (Fmoc-Asn-OH), (diacetoxyiodo)benzene, di-tert-butyl dicarbonate((Boc)₂O), N,N,N',N'-tetramethyl-

O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate, O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), N,O-dimethylhydroxylamine·HCl, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, Boc-L-glutamic acid 1-tert-butyl ester (Boc-Glu-OtBu), 9-fluorenylmethyl-succinimidyl carbonate (Fmoc-Osu), 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine, trifluoroacetic acid (TFA), acetonitrile, ethyl acetate (EA), dichloromethane, N,N-dimethylformamide (DMF), diethyl ether, THF, methanol, sodium bicarbonate, sodium carbonate (Na_2CO_3), magnesium sulfate, 1 N hydrochloric, N,N-diisopropylethylamine (DIEA), acetic acid, lithium hydroxide (LiOH), and glycine ethyl ester hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Synthesis of γ -ACA-Modified PNA Monomers

The synthetic route for γ -ACA-modified PNA monomers is outlined in Figure 1C.

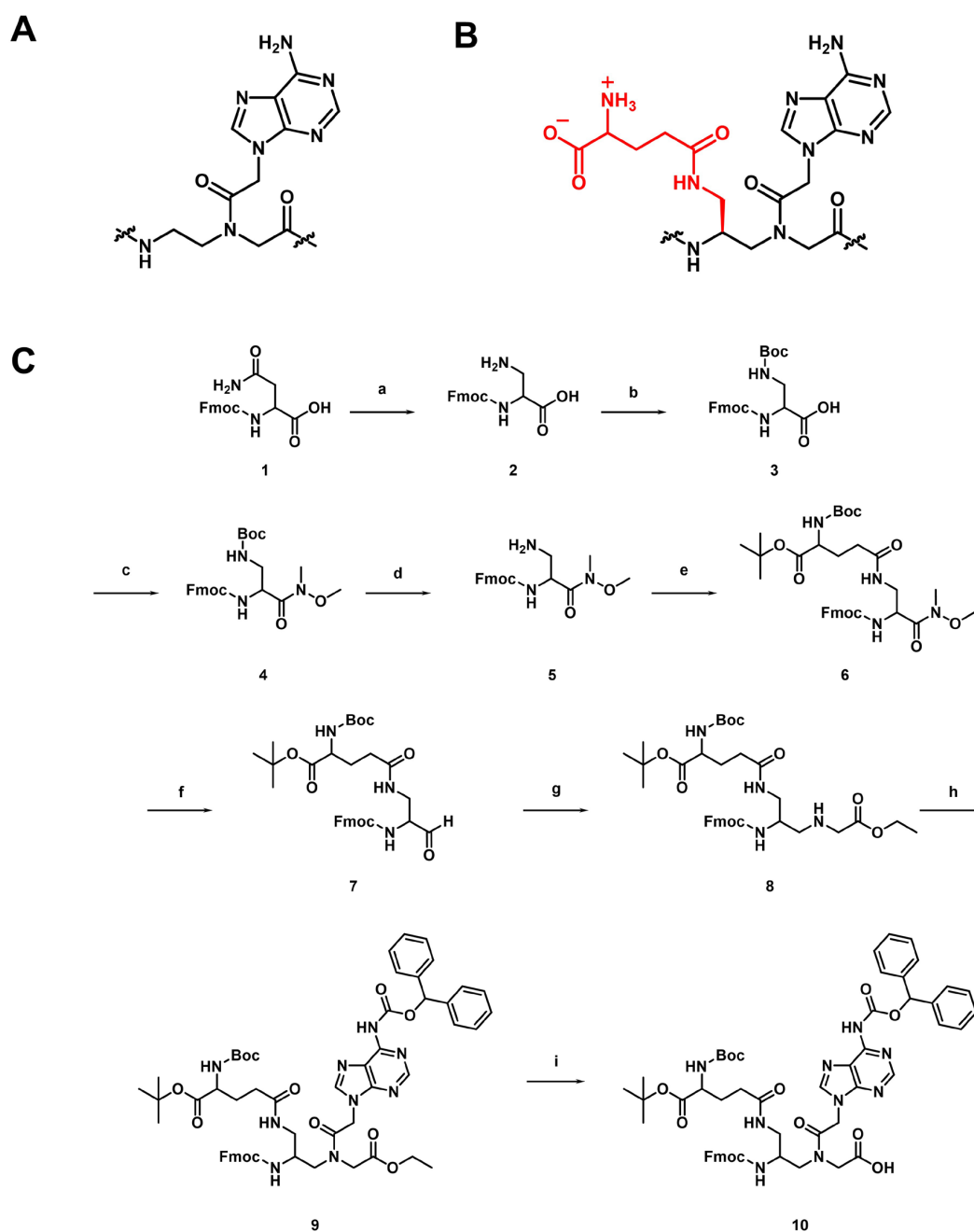


Figure 1. Chemical structures and synthetic scheme (A) Unmodified aeg peptide nucleic acid (PNA)-A (B) γ -(S-aminocarboxylic acid) aeg PNA-A. the γ -(S-aminocarboxylic acid) side chain introduced into

the PNA scaffold is highlighted in red. The wavy bonds in panels (A,B) indicate the points of continuation of the PNA backbone. (C) Chemical synthesis of the γ -ACA-modified PNA monomer. Reaction steps (a–i) correspond to the transformations indicated in panel C: (a) Iodosobenzene diacetate, EA/MeCN/H₂O, 4 h at room temperature (RT), 70%; (b) (Boc)₂O, sodium bicarbonate (NaHCO₃), tetrahydrofuran (THF)/H₂O, 3 h at RT, 95%; (c) N,O-dimethylhydroxylamine·HCl, O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), N,N-diisopropylethylamine (DIEA), N,N-dimethylformamide (DMF), 2 h at RT, 85%; (d) TFA/MC, 1.5 h at RT (crude); (e) Boc-L-glutamic acid 1-tert-butyl ester (Boc-Glu-OtBu), HBTU, DIEA, DMF, 4 h at RT, 92%; (f) 2 M LiAlH₄ in THF, 3 h at –55 °C, 97% (HPLC); (g) glycine ethyl ester hydrochloride, AcOH, sodium cyanoborohydride (NaBH₃CN), MeOH, 2 h at RT, 63%; (h) A(Bhoc)-acetic acid, DhbtOH, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, DMF, 2 h at RT, 71%; (i) 1 N Lithium hydroxide (LiOH) in H₂O/THF, 30 min at 5 °C; 9-fluorenylmethyl-succinimidyl carbonate (Fmoc-OSu), 10% sodium carbonate (Na₂CO₃), 2 h at RT, 98%. Numbers 1–10 in panel (C) represent key synthetic intermediates corresponding to reaction steps (a–i).

In the initial stage, the treatment of Fmoc-Asn-OH with (diacetoxyiodo)benzene effected a hypervalent iodine(III)-mediated Hofmann-type rearrangement resulting in amine derivative 2; the subsequent Boc protection of derivative 2 yielded compound 3, and the activation of the carboxylic acid, followed by coupling with N,O-dimethylhydroxylamine·HCl using HBTU and DIEA resulted in the corresponding Weinreb amide 4. The Boc deprotection of 4 with TFA yielded amine 5, which was then coupled with Boc-Glu-OtBu under HBTU/DIEA-mediated amide bond formation to give compound 6.

Next, a partial reduction reaction was performed using 2 M LiAlH₄ in THF to convert the intermediate to an aldehyde (–CHO). This was followed by a reductive amination reaction using glycine ethyl ester hydrochloride and NaBH₃CN, leading to the successful synthesis of the key intermediate (compound 8) containing both amine (–NH₂) and carboxylic acid (–COOH) functional groups at the γ -position.

From this intermediate stage, various nucleobase acetic acid derivatives were utilized in amide bond formation reactions, enabling the synthesis of PNA monomer derivatives with diverse basic substituents. Final hydrolysis and N-terminal Fmoc protection using Fmoc-OSu yielded γ -ACA-modified PNA monomers, which were subsequently used for PNA oligomer synthesis.

2.3. Synthesis of PNA Oligomers

The PNA oligomers used in this study were synthesized on a solid support (Rink amide AM resin) using standard Fmoc chemistry with an automatic synthesizer (HLB Panagene, Daejeon, Republic of Korea), incorporating both unmodified Fmoc PNA monomers and γ -ACA-modified PNA monomers [33–35]. The synthesized oligomers were cleaved from the resin using a cocktail solution of m-cresol and TFA (1:4), followed by precipitation with ether, purification, and analysis using reverse-phase high-performance liquid chromatography (Supplementary Figure S1) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Supplementary Figure S2) to confirm purity and molecular weight of the synthesized PNA oligomers.

2.4. Cell Culture and PNA Transfection

A549 human lung adenocarcinoma cells were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea), and NCI-H1975 human lung adenocarcinoma cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Cytiva, Wilmington, DE, USA) supplemented with 10% fetal bovine serum (Cytiva) and 1% penicillin/streptomycin (10,000 U/mL penicillin and 10,000 U/mL streptomycin; Cytiva) in a humidified CO₂

incubator at 37 °C. For PNA transfection, both cell lines were seeded in 6-well plates or 4-well glass slides and cultured overnight. The culture medium was replaced with fresh medium containing PNAs at final concentrations of 5 µM, unless otherwise specified. For dose-dependent experiments, PNAs were applied at concentrations of 0.25 to 2 µM. After transfection, the cells were harvested and used for subsequent experiments.

2.5. Thermal Melting Experiments

The melting temperatures were measured using UV spectrophotometry and quantitative polymerase chain reaction (qPCR). For UV spectrophotometry, PNA (4.5 µM) and its complementary DNA (4.5 µM; IDT, Coralville, IA, USA) or RNA (4.5 µM; IDT) were mixed in a 1:1 ratio in 1× Tris-EDTA buffer (PNA:DNA and PNA:RNA) and absorbance was measured at 260 nm using a Cary 3500 Multicell UV-Vis spectrophotometer (Agilent, Santa Clara, CA, USA). The temperature was decreased from 95 °C to 45 °C at a rate of 2 °C/min, and measurements were performed in triplicate. For qPCR, PNA (0.4 µM) and its complementary DNA (0.4 µM, IDT) or RNA (0.4 µM, IDT) were mixed in a 1:1 ratio and analyzed using the QuantStudio 5 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The temperature was increased from 40 °C to 99.9 °C at a rate of 0.5 °C every 5 s. Measurements were performed in triplicate.

2.6. Transfection Efficiency

2.6.1. Flow Cytometry Analysis

Cells were subcultured in either 6-well plates and incubated for 24 h before PNA treatment. After 24 h of incubation with PNA (5 µM), the culture medium was removed, and the cells were washed with sterile phosphate-buffered saline (PBS). Cells were detached using trypsin, washed with PBS, and suspended in cold PBS containing 10% fetal bovine serum. The samples were analyzed using a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) and quantified using the FlowJo software v10 (BD Biosciences).

2.6.2. Confocal Microscopy

Cells were cultured on 4-well glass slides and treated with PNA (5 µM) for 24 h. Following incubation, cells were stained with NucBlue™ Live ReadyProbes™ reagent (Thermo Fisher Scientific, Waltham, MA, USA) and CellMask™ Deep Red actin tracking stain (Invitrogen, Carlsbad, CA, USA), incubated at 37 °C with 5% CO₂ for 15 min, washed three times with HBSS, fixed using 4% paraformaldehyde, and observed using a LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.7. RNA Extraction

Modified PNA was transfected into cells, which were harvested using TRIzol™ reagent (Invitrogen) after 24 h, following the manufacturer's instructions. The extracted RNA was dissolved in nuclease-free water and quantified using a NanoDrop One Microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific) for subsequent experiments.

2.8. Reverse Transcription and qPCR

To quantify miR-221-3p and mRNA expression, cDNA was synthesized using two separate kits. For miR-221-3p, the miRNA first-strand cDNA synthesis kit (Agilent) was used, while mRNA was reverse-transcribed using the RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific) following the manufacturer's instructions. A universal reverse primer (Agilent) was used in both reactions. The reverse transcription was performed using the C1000 Touch thermal cycler (Bio-Rad, Hercules, CA, USA). qPCR was then performed using 20× EvaGreen™ (BIOFACT, Daejeon, Republic of Korea) on the QuantStudio 5 real-time PCR system (Applied Biosystems). All qPCR reactions were performed in

triplicate. miR-16-5p was used as the internal control for miR-221-3p quantification, while glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene for mRNA normalization.

The primers used for miRNA qPCR were as follows: The forward primer for miR-221-3p was TAGCAGCACGTAAATATTGGCG, and that for the miR-16-5p internal control was AGCTACATTGTCTGCTGGGTTTC. Both were used in combination with the Universal reverse primer provided in the Agilent miRNA quantification system.

The following primer pairs were used for mRNA quantification: CDKN1B forward, 5'-TAATTGGGGCTCCGGCTAACT-3'; reverse, 5'-TGCAGGTCGCTTCCTTATTCC-3'. ATF3 forward, 5'-GCTGGAAAGTGTGAATGCTG-3'; reverse, 5'-TTCTGAGCCCGGACAATACA-3'. GPX4 forward, 5'-GTAACCAGTTCGGGAAGCAG-3'; reverse, 5'-TGTCGATGAGGAAC TGTGGA-3'. SYVN1 forward, 5'-CCAACATCTCCTGGCTC TTTAC-3'; reverse, 5'-GTCAGGATGCTGTGATAGGCGT-3'. GAPDH forward, 5'-CTGGGCTAACTGAGCACC-3'; reverse, 5'-AAGTGGTCGTTGAGGGCAATG-3'.

2.9. Western Blot Analysis

Cells were washed with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer supplemented with a protease inhibitor (Thermo Fisher Scientific). Total protein lysates (30 µg) were loaded onto a 4–20% Mini-PROTEAN TGX precast gel (Bio-Rad) and electrophoresed at 100 V for 90 min. The separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) at 100 V for 90 min. The membranes were blocked with a blocking reagent for 1 h at room temperature (approximately 23 °C) and incubated overnight at 4 °C with anti-β-actin (mouse monoclonal, 1:5000; Sigma-Aldrich, Cat. No. A5441) or anti-p27 Kip1 (rabbit monoclonal, 1:2000; Cell Signaling Technology, Danvers, MA, USA, Cat. No. 3686S) primary antibodies. After washing with Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000; Abcam, Cambridge, UK, Cat. No. ab205719 and ab97051) for 1 h at room temperature. Protein bands were visualized using the SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific) and the ChemiDoc imaging system (Bio-Rad).

2.10. Analysis of Apoptosis and Necrosis

Apoptosis and necrosis were analyzed 72 h after PNA treatment (5 µM) using the dead cell apoptosis kit with Annexin V and propidium iodide (Thermo Fisher Scientific), following the manufacturer's instructions. Briefly, cells were trypsinized, washed with PBS, and resuspended in Annexin V binding buffer. The cells were then incubated with Annexin V and propidium iodide at room temperature for 15 min. Subsequently, Annexin V binding buffer was added to each sample, and the cells were analyzed using a BD FACS Canto II flow cytometer (BD Biosciences). Quantification of apoptotic and necrotic cells was performed using the FlowJo software version 10 (BD Biosciences).

2.11. Detection of Ferroptosis Using BODIPYTM 581/591 C11

Cells were seeded in a 6-well plate and incubated for 24 h, following which PNA was added to each well at a final concentration of 5 µM, and the cells were incubated for an additional 48 h. Following the 48-h treatment, the culture medium was removed, and the cells were labeled with BODIPYTM 581/591 C11 (Invitrogen) diluted in complete RPMI-1640 medium to a final concentration of 2 µM. The cells were incubated at 37 °C in a 5% CO₂ incubator for 30 min. After incubation, the cells were washed three times with sterile PBS and observed under a fluorescence microscope.

2.12. Statistical Analysis

Comparisons between two groups were performed using an unpaired Student's *t*-test, whereas comparisons among three or more groups were performed using one-way analysis of variance. $p < 0.05$ was considered statistically significant. Data were presented as group mean $\pm$ SD. Statistical analyses were performed using Prism version 10 (GraphPad Software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Characteristics of γ -ACA-Modified PNAs

The chemical structures of unmodified PNA and γ -ACA-modified PNA are shown in Figure 1A,B, with representative oligomer structures provided in Supplementary Figure S3. γ -ACA-modified PNAs were successfully generated by incorporating a γ -ACA monomer into the PNA backbone, and the synthetic route of the γ -ACA monomer is summarized in Figure 1C.

To assess the impact of γ -ACA modification on target binding affinity, melting temperature (T_m) was measured using new PNA oligomers designed to target miR-221-3p (Table 1). The antisense sequence was originally validated by Brognara et al., who demonstrated sequence specificity with both scrambled and mismatch control oligonucleotides [36]. The γ -ACA modification was applied to PNAs of varying lengths (18-mer, 15-mer, and 12-mer) to assess its universal applicability. The γ -ACA modification significantly increased T_m values for both DNA and RNA targets, with the 18-mer γ -ACA-PNA showing an increase of 7.4 °C for DNA and 7.5 °C for RNA compared to the unmodified PNA. Similar trends were observed for 15-mer and 12-mer PNAs, indicating a consistent stabilizing effect of γ -ACA modification across different PNA lengths. These trends were consistently observed in both UV spectrophotometry and fluorescence-based qPCR analyses, reinforcing the stabilizing effect of γ -ACA modification on PNA-target duplexes. Collectively, these findings highlight the potential of γ -ACA modification in improving PNA performance for therapeutic applications.

Table 1. Sequences and melting temperature of PNA oligomers. γ -ACA-modified sequences are underlined.

Name	N-Term	PNA (N-Term to C-Term)	C-Term	Length (mer)	Melting Temperature (°C)			
					UV Spec.		qPCR	
					DNA	RNA	DNA	RNA
PNA-1	Dabcyl	AAACCCAGCAGACAATGT	OK(FAM)	18	80.8 $\pm$ 0.5	78.7 $\pm$ 0.6	77.9 $\pm$ 0.1	76.1 $\pm$ 0.2
PNA-2	Dabcyl	<u>AAACCCAGCAGACA</u> ATGT	OK(FAM)		88.2 $\pm$ 1.0	86.1 $\pm$ 0.6	86.8 $\pm$ 0.2	83.7 $\pm$ 0.3
PNA-3	Dabcyl	CCCAGCAGACAATGT	OK(FAM)	15	74.7 $\pm$ 0.5	73.5 $\pm$ 0.2	72.0 $\pm$ 0.3	71.1 $\pm$ 0.1
PNA-4	Dabcyl	<u>CCCAGCAGACA</u> ATGT	OK(FAM)		83.0 $\pm$ 0.7	81.4 $\pm$ 0.9	80.3 $\pm$ 0.5	78.5 $\pm$ 0.3
PNA-5	Dabcyl	AGCAGACAATGT	OK(FAM)	12	65.7 $\pm$ 0.7	62.8 $\pm$ 0.4	61.0 $\pm$ 0.1	59.5 $\pm$ 0.0
PNA-6	Dabcyl	<u>AGCAGACA</u> ATGT	OK(FAM)		74.4 $\pm$ 0.5	71.7 $\pm$ 0.7	69.9 $\pm$ 0.6	66.3 $\pm$ 0.2

PNA, peptide nucleic acid; qPCR, quantitative polymerase chain reaction; FAM, Carboxyfluorescein.

To assess whether γ -ACA modification influences cellular uptake, flow cytometry analysis was performed on A549 and NCI-H1975 cells treated with unmodified PNA (PNA-1) and γ -ACA-modified PNA (PNA-2). γ -ACA modification alone negligibly affected cellular uptake, with fluorescence levels similar to those of unmodified PNA (Figure 2A,B). To enhance intracellular delivery, the Tat-modified peptide (RRRQRRKRR), a type of cell-penetrating peptide (CPP), was conjugated to γ -ACA-modified PNA (PNA-7), resulting in a substantial increase in cellular uptake. In contrast, conjugation with the Tat-modified peptide (PNA-7) resulted in a marked increase in cellular uptake, leading to near-complete internalization in both lung cancer cell lines (Figure 2A,B and Supplementary Figure S4).

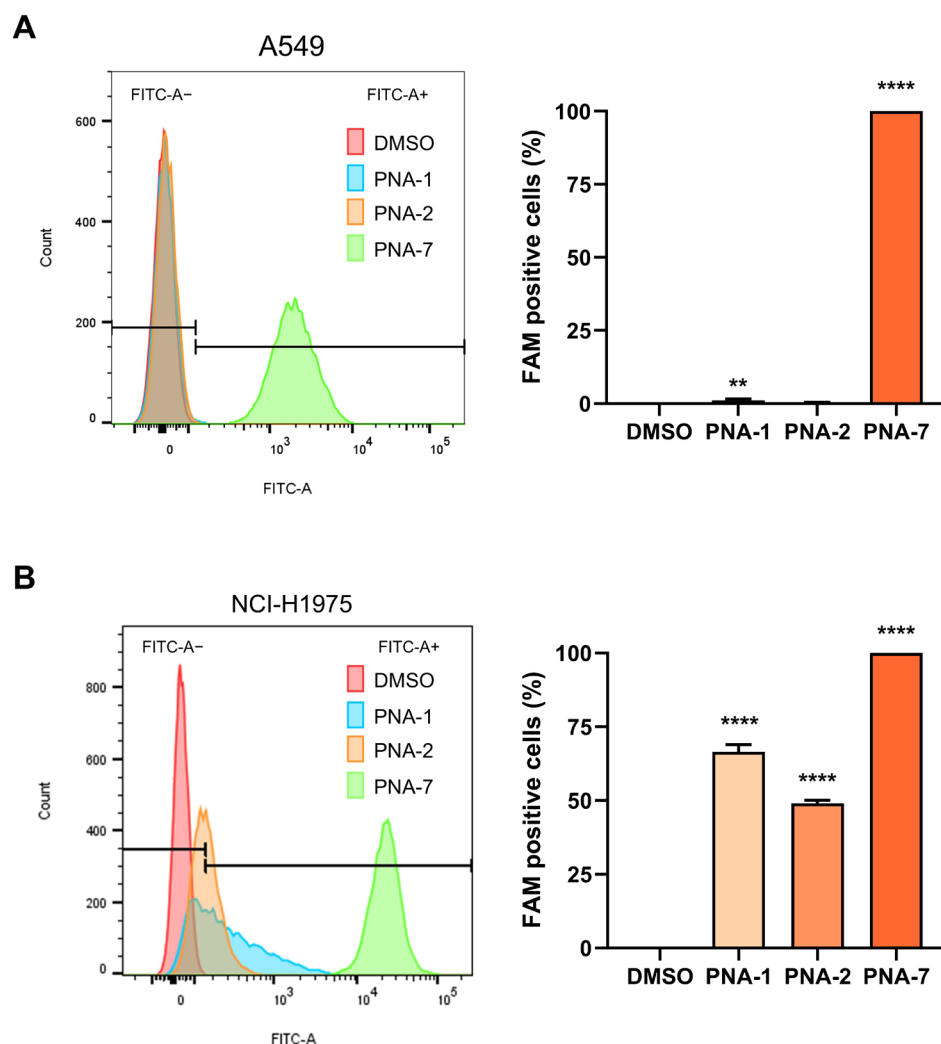


Figure 2. Evaluation of Cellular Uptake of γ -amino carboxylic acid (γ -ACA) Modified peptide nucleic acids (PNAs) in A549 Cells and NCI-H1975 cells. (**A,B**) A representative histogram showing the intracellular delivery efficiency of PNA-1, PNA-2, and PNA-7 (5 μ M, 24 h) in A549 (**A**) and NCI-H1975 (**B**) cells, and quantification of FAM-positive cells analyzed by flow cytometry. The horizontal black lines in the histograms indicate the gates used to distinguish between FAM-negative and FAM-positive populations. A total of 10,000 events were recorded for each sample. Results were reported as mean $\pm$ standard deviation ($n = 3$). Statistical analysis: ** $p < 0.01$, **** $p < 0.0001$ (One-way analysis of variance followed by Tukey's test). DMSO, Dimethyl Sulfoxide; FAM, Carboxyfluorescein.

3.2. Evaluation of miRNA Inhibition Following γ -ACA Modification

To further investigate the functional effect of γ -ACA modification, miRNA inhibition assays were performed using PNA-8 to PNA-13. These PNAs were selected to eliminate unnecessary components, such as fluorophores and quenchers, retaining only essential elements for precise inhibition analysis. A scrambled PNA (PNA-SC) was used as a negative control (Table 2). Cells were treated with 5 μ M of each PNA for 24 h, and miR-221-3p levels were quantified.

A modest reduction in miR-221-3p levels was achieved using PNA-8 (unmodified PNA), which exhibited the lowest inhibition efficiency among the tested PNAs, resulting in an approximately 5.3-fold decrease in A549 cells and a 2.0-fold decrease in NCI-H1975 cells compared with the untreated control (Figure 3A). The incorporation of CPP conjugation (PNA-9) led to a slightly improved inhibitory effect in both cell lines. Notably, PNA-10 (γ -ACA-modified PNA) induced a significantly greater inhibitory effect on miRNA expression than PNA-8 and PNA-9, achieving an approximately 33.5-fold reduction in A549 cells and

a 23.8-fold reduction in NCI-H1975 cells, suggesting that γ -ACA modification contributes more substantially to enhanced PNA activity than CPP-mediated uptake alone. Among the tested PNAs, PNA-11, which incorporated both γ -ACA modification and the Tat-modified peptide, showed the most pronounced inhibitory effect, reducing miR-221-3p expression by approximately 570-fold in A549 cells and 143-fold in NCI-H1975 cells, highlighting the synergistic impact of these modifications. In contrast, the scrambled control PNA (PNA-SC) did not show any inhibitory effect in either cell line, confirming the sequence-specific targeting of miR-221-3p by the γ -ACA-modified PNAs.

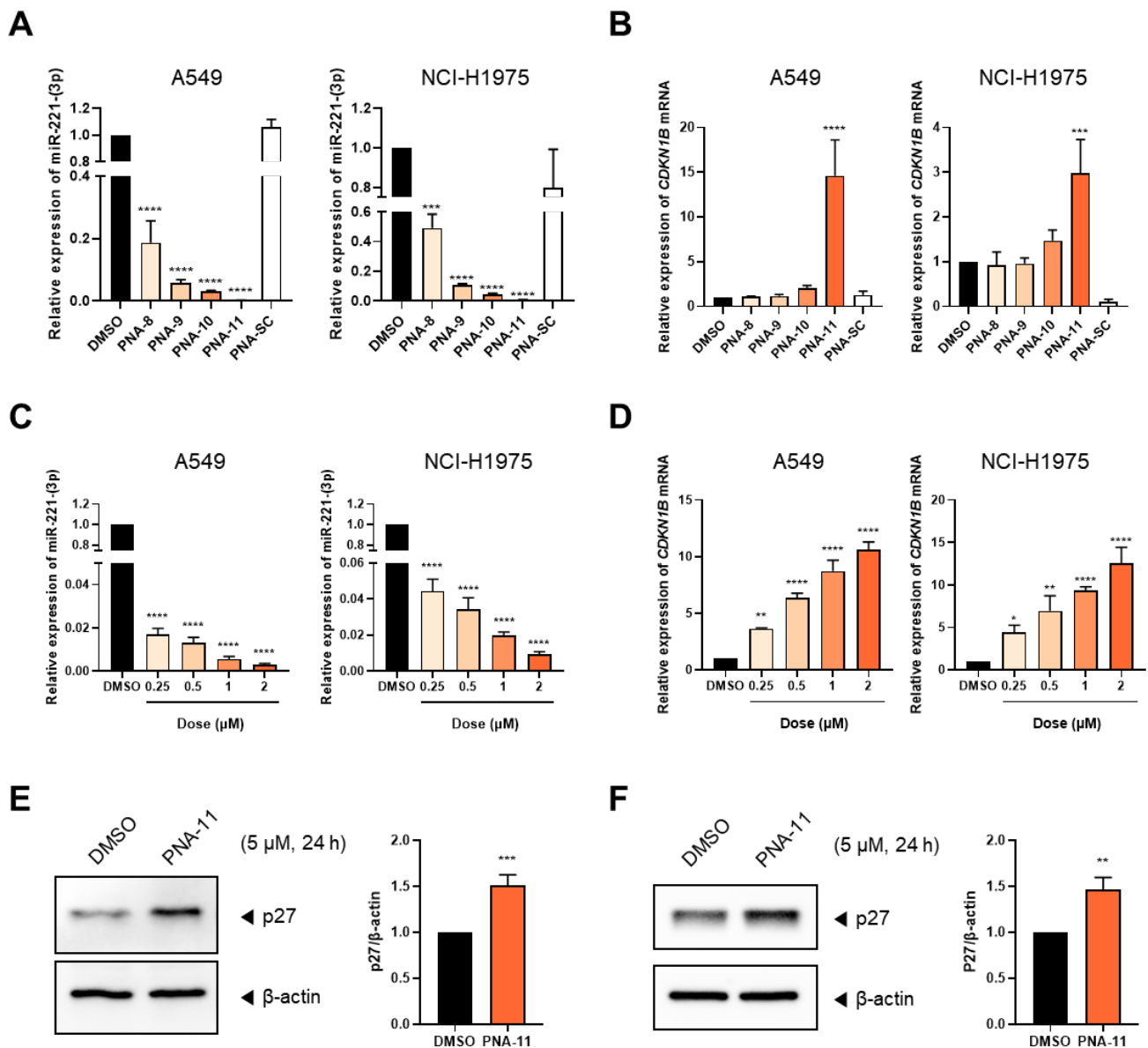


Figure 3. miR-221-3p Inhibition and Downstream Effects of γ -ACA Modified PNAs (A) Fold changes in miR-221-3p and (B) CDKN1B mRNA after treatment with PNA-8, PNA-9, PNA-10, PNA-11, and PNA-SC (5 μ M) for 24 h in A549 and NCI-H1975 cells (C) Fold changes in miR-221-3p and (D) CDKN1B gene expression of PNA-11 with different concentration (0.25, 0.5, 1, and 2 μ M) for 24 h in A549 and NCI-H1975 cells (E,F) Representative Western blot images and quantification of p27 protein expression in A549 (E) and NCI-H1975 (F) cells treated with DMSO or PNA-11 (5 μ M, 24 h). Protein levels were normalized to β -actin. Results were presented as mean $\pm$ standard deviation ($n = 3$). Statistical analysis: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. DMSO treated group (One-way analysis of variance followed by Tukey's test or un-paired Student's t -test). PNA, peptide nucleic acid; PNA-SC, scrambled PNA; DMSO, Dimethyl Sulfoxide; γ -ACA, γ -amino carboxylic acid.

Table 2. PNA oligomers with modified sequences targeting miR-221-3p. γ -ACA-modified sequences are underlined.

Name	N-Term	PNA (N-Term to C-Term)	C-Term	Length (mer)
PNA-7	Tat-modified +DabcyI	AA <u>ACC</u> CAGCAGACA <u>AT</u> GT	OK(FAM)	18
PNA-8	-	AAACCCAGCAGACAATGT	-	18
PNA-9	Tat-modified	AAACCCAGCAGACAATGT	-	18
PNA-10	-	AA <u>ACC</u> CAGCAGACA <u>AT</u> GT	-	18
PNA-11	Tat-modified	AA <u>ACC</u> CAGCAGACA <u>AT</u> GT	-	18
PNA-12	Tat-modified	CCCAGCAGACA <u>AT</u> GT	-	15
PNA-13	Tat-modified	AGCAGACA <u>AT</u> GT	-	12
PNA-SC	Tat-modified	GTAACGAGTGTGGTTGT	-	18

PNA, peptide nucleic acid; PNA-SC, scrambled PNA; γ -ACA; γ -amino carboxylic acid; FAM, Carboxyfluorescein.

The downstream effects of miR-221-3p inhibition were also assessed by measuring the expression of CDKN1B, a key gene regulated by miR-221-3p. Consistent with the results of the miRNA inhibition assay, PNA-11 treatment led to a marked upregulation of CDKN1B mRNA expression, resulting in an approximately 14.6-fold increase in A549 cells and a 3.0-fold increase in NCI-H1975 cells compared with the untreated control (Figure 3B). Importantly, this upregulation was dose-dependent, with PNA-11 inhibiting miRNA and inducing CDKN1B even at lower concentrations (0.25–2 μ M) (Figure 3C,D). Western blot analysis further confirmed these findings, showing a marked increase in the expression of p27, encoded by CDKN1B, following PNA-11 treatment (Figure 3E,F). Consistent with the mRNA expression data, p27 protein levels were increased by approximately 1.5-fold compared with the untreated control. Collectively, these results underscore the ability of γ -ACA modification to enhance both miRNA inhibition and activation of downstream targets, supporting the functional impact of γ -ACA modification on miRNA inhibition and downstream target regulation.

3.3. Influence of PNA Length on miRNA Inhibition

To determine the effect of PNA length on miR-221-3p inhibition, A549 and NCI-H1975 cells were treated with 5 μ M γ -ACA-modified PNAs of varying lengths (PNA-11: 18-mer, PNA-12: 15-mer, PNA-13: 12-mer) for 24 h. The PNAs were designed such that the C-terminal region was retained, which targets the 5' end of miR-221-3p, while sequentially truncating the N-terminal region. Notably, the 5' end contains the seed sequence crucial for miRNA target recognition and binding [37,38]. A clear correlation was observed between PNA length and inhibitory efficiency. In particular, the 18-mer PNA (PNA-11) exhibited the highest miRNA inhibition rate, whereas the 15-mer (PNA-12) showed a significant inhibition, and the 12-mer (PNA-13) exhibited the weakest inhibition (Figure 4A). Therefore, owing to their increased hybridization potential, longer PNAs may achieve stronger target binding and better inhibitory effects.

Consistent with miRNA inhibition data, CDKN1B expression was most significantly upregulated in cells treated with PNA-11, followed by those treated with PNA-12, whereas the lowest increase was observed in cells treated with PNA-13 (Figure 4B). Western blot analysis further demonstrated a significant increase in p27 protein levels following PNA-11 treatment (Figure 4C,D). This suggests that shorter PNAs may be insufficient to effectively inhibit miR-221-3p and elicit significant biological responses, underscoring the critical role of PNA length in achieving robust gene regulation.

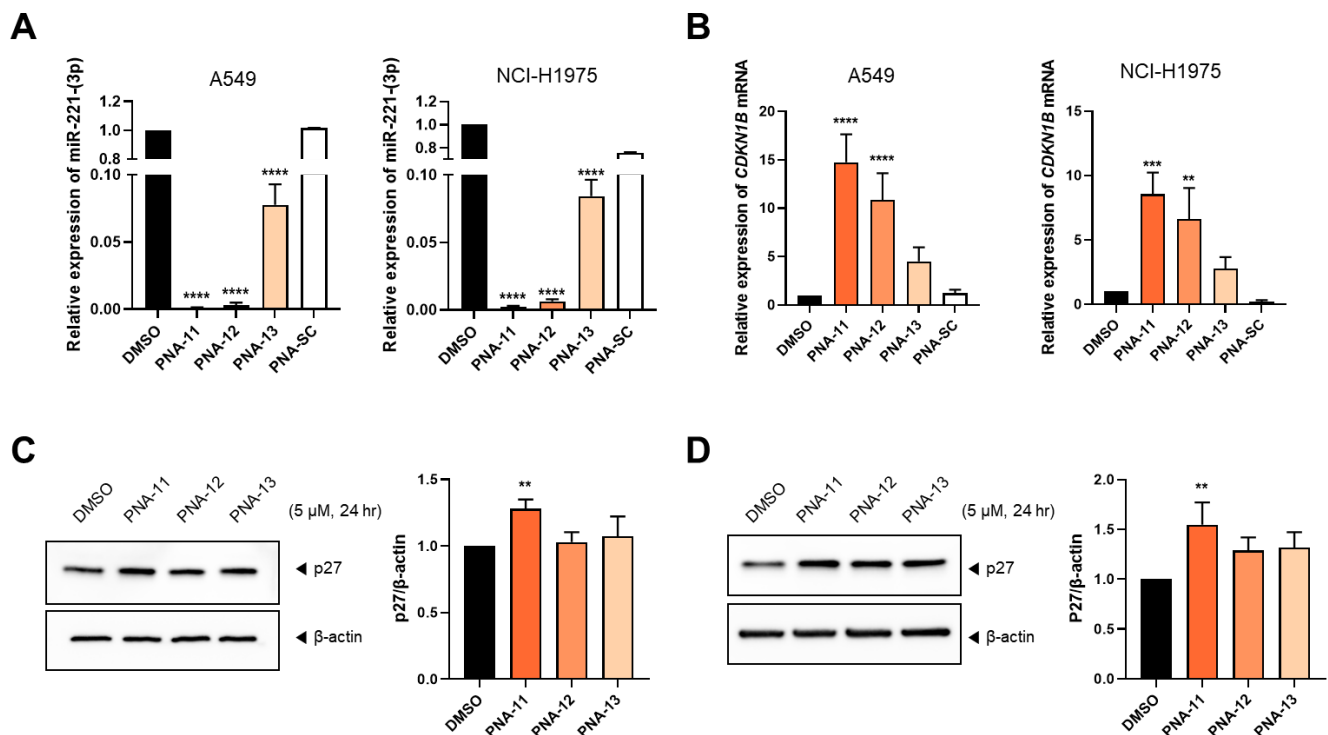


Figure 4. Impact of PNA Length on miR-221-3p Inhibition and Downstream Gene Expression (A) Fold change in miR-221-3p expression after PNA-11, PNA-12, PNA-13, and PNA-SC (5 μ M) treatment for 24 h in A549 and NCI-H1975 cells. miR-16-5p was used as control. (B) Relative expression of CDKN1B after PNA-11, PNA-12, PNA-13, and PNA-SC (5 μ M) treatment for 24 h in A549 and NCI-H1975 cells. GAPDH was used as control. (C,D) Representative Western blot images and quantification of p27 protein expression in A549 (C) and NCI-H1975 (D) cells treated with DMSO, PNA-11, PNA-12, or PNA-13 (5 μ M, 24 h). Protein levels were normalized to β -actin. Results were reported as mean $\pm$ standard deviation (n = 3). Statistical analysis: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. DMSO-treated group (One-way analysis of variance followed by Tukey's test or unpaired Student's t -test). PNA-SC; scrambled PNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PNA, peptide nucleic acid; DMSO, dimethyl sulfoxide.

3.4. PNA-11 Induces Cell Death via miR-221-3p Inhibition

Considering the strong inhibitory effect of PNA-11 on miR-221-3p and the associated CDKN1B upregulation, we examined whether these molecular changes result in cell death. Annexin V and propidium iodide staining demonstrated a significant increase in apoptotic cell populations in both A549 and NCI-H1975 cells following PNA-11 treatment (Figure 5A–C). This apoptotic response is consistent with the restoration of CDKN1B, a well-established cell cycle inhibitor and pro-apoptotic regulator.

In addition to apoptosis, necrotic cell fractions were also elevated in both cell lines upon PNA-11 treatment (Figure 5A,B,D). Given previous reports indicating a role for miR-221-3p in ferroptosis regulation [39], we further explored ferroptosis-related changes in cells. PNA-11 increased the expression of ATF3 and reduced GPX4 and SYVN1 levels (Supplementary Figure S5A), accompanied by a marked accumulation of lipid reactive oxygen species as detected by BODIPY staining in A549 cells (Supplementary Figure S5B).

Collectively, these findings demonstrate that γ -ACA-modified (PNA-11) primarily induces apoptosis through miR-221-3p inhibition and CDKN1B restoration, while necrotic and ferroptosis-associated changes are also observed, with the latter being evident in lung cancer cell lines.

Taken together, the comprehensive data confirm that the synergistic combination of γ -ACA modification and Tat-peptide conjugation yields PNA-11 with optimized properties,

resulting in robust, sequence-specific miRNA inhibition and predominant induction of anti-cancer effects.

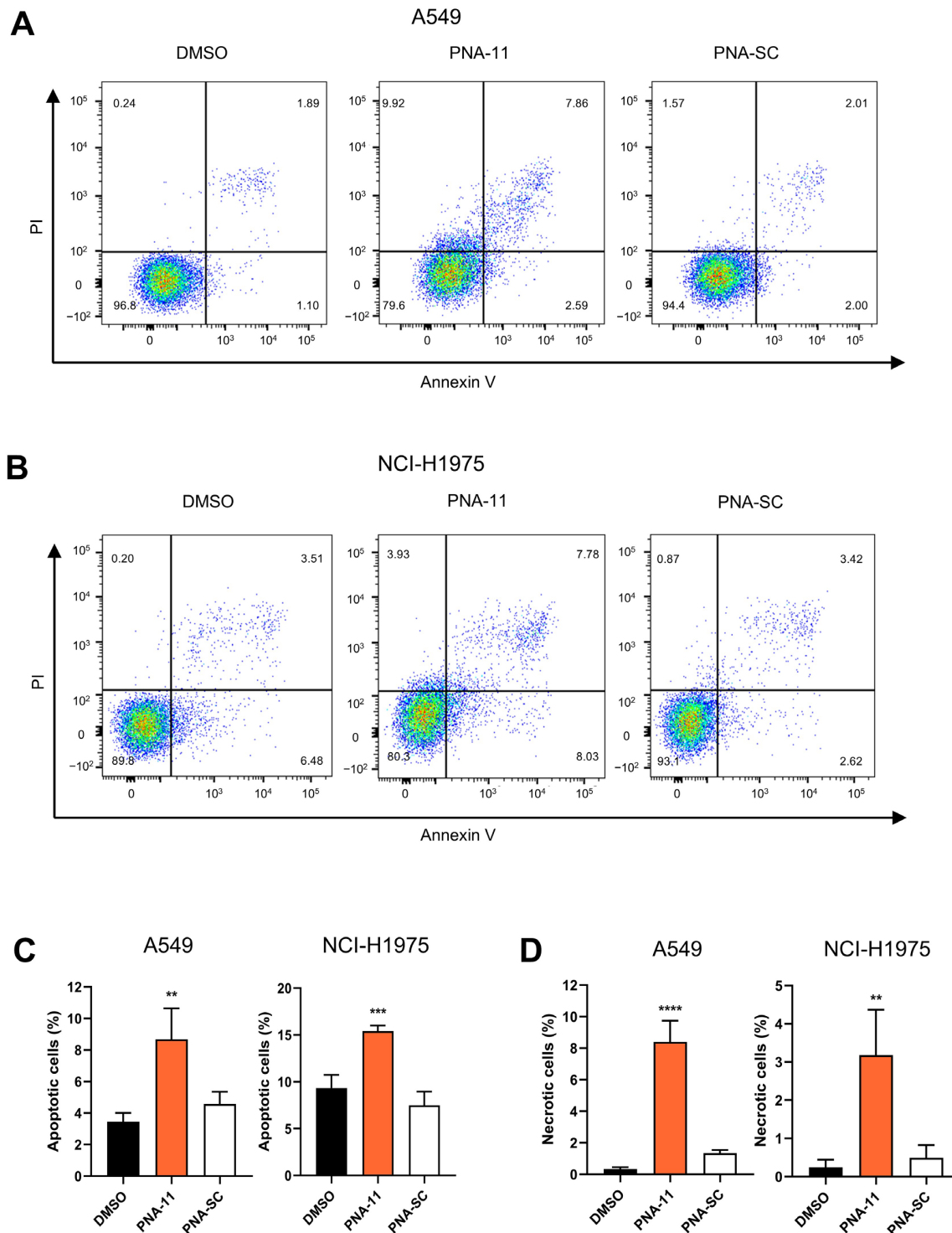


Figure 5. Analysis of apoptosis and necrosis induced by γ -ACA-modified PNAs (**A,B**) Flow cytometry analysis of apoptotic cells treated with PNA-11 and PNA-SC (5 μ M) for 72 h in A549 (**A**) and NCI-H1975 (**B**) cells (**C**) Quantification of apoptotic cells in A549 and NCI-H1975 cells based on flow cytometry data (**D**) Quantification of necrotic cells in A549 and NCI-H1975 cells based on flow cytometry data. Results were reported as mean $\pm$ standard deviation ($n = 3$). Statistical analysis: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. DMSO-treated group (One-way analysis of variance followed by Tukey's test or unpaired Student's t -test). PNA-SC; scrambled PNA; PNA, peptide nucleic acid; DMSO, dimethyl sulfoxide; γ -ACA, γ -amino carboxylic acid.

4. Discussion

The use of PNAs to modulate miRNA expression is attracting considerable interest as part of ongoing efforts to develop more effective gene regulation strategies. In this study, we demonstrated that a newly designed PNA oligomer with a γ -ACA modification effectively inhibits miR-221-3p expression in A549 and NCI-H1975 cells. This backbone modification significantly improved target binding affinity, resulting in enhanced miR-221-3p suppression and subsequent upregulation of CDKN1B. Notably, despite its relatively poor cellular uptake, the γ -ACA-modified PNA exhibited superior target inhibition compared to regular PNA conjugated with the Tat-modified peptide. While CPP conjugation enhances the miRNA inhibition efficacy of PNAs [40]; our findings indicate that the γ -ACA modification alone is sufficient to achieve higher target suppression. These findings suggest that optimizing binding affinity through backbone engineering may be a more effective strategy than enhancing cellular uptake alone to improve the therapeutic efficacy of PNAs. In particular, this study introduces a newly designed γ -ACA PNA backbone and demonstrates its functional relevance for miRNA inhibition through systematic physicochemical and biological evaluation.

Although γ -modified PNAs have been reported, the γ -ACA backbone modification investigated herein was newly designed and synthesized as a backbone engineering strategy, and its impact on PNA–miRNA interactions was systematically evaluated. To our knowledge, the application of γ -ACA–modified PNAs to miRNA targeting and the integrated assessment of their physicochemical properties and functional miRNA inhibition have not been previously reported. The unique contribution of this work lies in establishing γ -ACA modification as a rational backbone engineering strategy that enhances duplex stability and enables effective miRNA inhibition without relying on additional delivery-enhancing conjugates.

The observed increase in CDKN1B expression following miR-221-3p inhibition supports the hypothesis that the modified PNA effectively disrupts miRNA-mediated suppression. CDKN1B is a critical regulator of cell cycle progression, and the protein it encodes (p27) plays a key role in maintaining cellular homeostasis. Therefore, γ -ACA-modified PNAs may be used in therapeutic strategies targeting miRNA dysregulation. Furthermore, compared to conventional PNAs, which often require additional conjugation for enhanced efficacy, strategic modifications aimed at strengthening PNA–miRNA interactions may be a viable alternative approach for optimizing therapeutic outcomes.

γ -PNAs have been investigated for their potential applications in various biological contexts. A chemically modified γ -PNA encapsulated in PLGA nanoparticles was developed to inhibit miR-210, a key regulator in cervical cancer and other solid tumors [20]. Treatment with the modified γ -PNA significantly reduced tumor growth and improved tumor morphology in a HeLa xenograft model. Similarly, a previous study using a tail-clamp γ -PNA targeting miR-155 in lymphoma cells and xenograft mouse model reported a 90–95% reduction in miR-155 levels, increased apoptosis, and significant tumor growth suppression [41]. In addition to miRNA targeting, γ -PNAs have been used to target genomic DNA, demonstrating efficacy in silencing oncogenes, such as c-Myc, in multiple cancer models [42]. These findings highlight the broad applicability of γ -PNA modifications beyond miRNA inhibition, suggesting their potential for use in gene regulation and cancer therapy. In contrast to these earlier approaches, the present study focuses on backbone-level optimization rather than on delivery-dependent strategies, highlighting γ -ACA modification as an intrinsic determinant of miRNA inhibition efficiency.

In addition to their use in cancer therapy, γ -PNAs have shown promise in the field of gene editing. γ -PNAs with polyethylene glycol substitutions have been used to enhance DNA binding, facilitating efficient gene editing in hematopoietic stem cells both ex vivo and

in vivo, with clinically relevant levels of correction observed in models of β -thalassemia [43]. Furthermore, γ -PNAs with hydroxymethyl- γ -substituents have demonstrated superior gene editing efficiency over conventional PNAs, further emphasizing their potential for therapeutic applications [44]. More recently, γ -substituted PNAs have been shown to enable efficient and precise in vivo gene correction in disease-relevant models without inducing double-strand breaks, highlighting their translational potential as a therapeutic genome-editing platform [45].

Our study demonstrates that γ -ACA modification enhances the hybridization stability of PNA–miRNA interactions while maintaining biological efficacy. This enhancement is likely attributable to structural stabilization and improved molecular interactions with the target miRNA. γ -position modifications in the PNA backbone are known to induce helical preorganization, transforming randomly folded structures into more stable helices, which may contribute to increased duplex stability.

Previous structural investigations have demonstrated that PNA can engage RNA targets through interactions that extend beyond canonical base pairing. Notably, NMR-based structural analyses of PNA–dsRNA triplexes revealed hydrogen bonding interactions between the amide proton of the PNA backbone and oxygen atoms of the RNA phosphate group, highlighting a direct contribution of the PNA backbone to complex stabilization [46]. In this context, the γ -ACA modification may promote additional hydrogen bonding or alter the conformational rigidity of the PNA backbone, thereby facilitating more stable hybridization with its target, as previously reported for γ -modified PNAs [9,12,18,23]. These structural adaptations may collectively contribute to the enhanced hybridization stability and miRNA inhibition observed in this study, underscoring the significance of γ -ACA modification in optimizing PNA function.

Despite these promising findings, certain limitations of this study should be acknowledged. First, this study was conducted in an in vitro model using lung cancer cell lines, and further in vivo validation will be necessary to comprehensively assess the therapeutic potential of the modified PNA. Moreover, advanced delivery systems need to be explored to optimize biodistribution and intracellular delivery. Considering that γ -ACA modification primarily enhances hybridization stability, complementary delivery strategies—such as nanoparticle-based carriers or lipid conjugation—may further improve therapeutic performance. The exact molecular mechanisms underlying γ -ACA modification-induced functional enhancement of PNAs also warrant further investigation. Structural analysis and biophysical characterization of binding dynamics using techniques such as isothermal titration calorimetry or molecular dynamics simulations will be required to better understand these mechanisms. In addition, it is crucial to evaluate the potential off-target effects of γ -ACA-modified PNAs to ensure their specificity and minimize unintended interactions with non-target sequences.

5. Conclusions

Our study provides novel insights into the potential of γ -ACA-modified PNAs as miRNA inhibitors. We demonstrate that γ -ACA modification is associated with increased hybridization stability and improved functional inhibition of miR-221-3p in lung cancer cell lines, which may contribute to enhanced regulation of miRNA targets such as CDKN1B. Although these findings are based on in vitro models, they suggest that rational backbone engineering—rather than delivery enhancement alone—can be a promising strategy to optimize PNA performance. Further in vivo studies, advanced delivery approaches, and detailed biophysical analyses will be required to fully elucidate the mechanisms underlying γ -ACA-mediated effects and to assess their broader therapeutic applicability. Overall, our

results support the concept that backbone-level modification of PNAs can be a valuable route toward expanding their utility in gene modulation.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cimb48020197/s1>.

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Abbreviations

The following abbreviations are used in this manuscript:

PNAs	Peptide nucleic acids
γ -ACA	γ -amino carboxylic acid
γ -PNAs	γ -Modified PNAs
γ -GPNAs	γ -guanidinyI-modified PNAs
PLGA	Poly(lactic-co-glycolic acid)
CPP	Cell-penetrating peptide
T _m	Melting temperature
qPCR	Quantitative polymerase chain reaction
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
PBS	Phosphate-buffered saline
HBSS	Hanks' balanced salt solution
RPMI	Roswell Park Memorial Institute

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