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# Design, synthesis and biochemical investigation, by *in vitro* luciferase reporter system, of peptide nucleic acids as new inhibitors of miR-509-3p involved in the regulation of cystic fibrosis disease-gene expression

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## Synthesis of miR-509-3p and PNA1-3

The miR-509-3p mimic (2'-OMe modified) was purchased and purified by the oligonucleotide synthesis facility at CEINGE-Biotecnologie Avanzate.

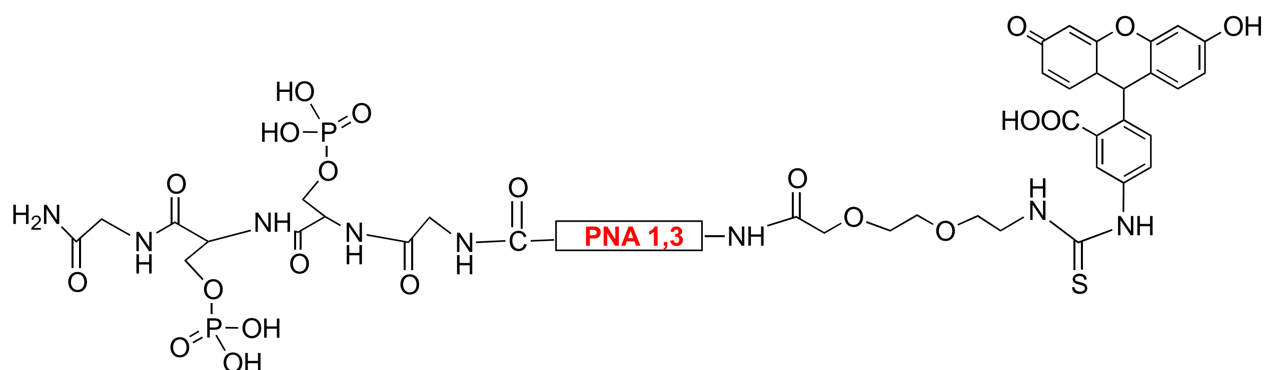
The sequences of PNAs used in this study are listed in Table 1. Trifluoroacetic acid (TFA), dimethylformamide (DMF), dichloromethane (DCM), N,N-diisopropylethylamine (DIPEA), N-methyl-pyrrolidone (NMP), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), 2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-isothiocyanate-benzoic acid (FITC), phosphoric acid $H_3PO_4$, 4-methyl-benzhydrylamine-resin (MBHA resin, 0.5 mmol/g), and all standard protected Fmoc-amino acid derivatives were purchased from Sigma-Aldrich. Fmoc-L-Ser[PO(OBzl)OH]-OH building block, 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) 2-(1-H-7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and 1-hydroxybenzotriazole (HOBt) were purchased from Novabiochem.

For PNA synthesis, all Fmoc/Boc protected monomers and the 2-[2-(fluorenylmethoxycarbonylamino)ethoxy]ethoxyacetyc (AEEA) spacer-linker were purchased from Link Technologies. Analytical and semipreparative RP-HPLC were performed at room temperature on a Jasco PU-2089 equipped with a Jasco UV-2070 UV/Vis detector.

A Thermo C18 column (4.6 $\times$ 250 mm, particle size 5 $\mu$m, particle pore diameter 100 Å) was used for analytical RP-HPLC. For semipreparative HPLC, a Merck C18 column (10$\times$ 250 mm, 5 $\mu$m, 100 Å) was used. The concentration of each PNA has been evaluated by UV measurement using the calculated molar extinction coefficient value at 260 nm (see Table 1).

**Table 1.** PNA and miR-509-3p sequences used in this study. PNA sequences are written from C to N terminus.

| Molecule | Sequence | Molar extinction coefficient ($M^{-1}$ * $cm^{-1}$) |
|---|---|---|
| **PNA-1** | G-S(P)-S(P)-G-actaaccatgcaga-Linker-FITC | 149,600 |
| **PNA-2** | K-K-actaaccatgcaga-Linker-FITC | 149,600 |
| **PNA-3** | G-S(P)-S(P)-G-tgccgagtaggcac-Linker-FITC | 143,600 |
| **miRNA** | UGAUUGGUACGUCUGUGGGUAG | 205,000 |




From C to N terminus

*Synthesis of the bis-phosphate-PNAs (PNA1 and PNA3)*

The bis-phosphate-PNA1,3 were prepared by solid-phase synthesis with Fmoc-strategy on a polystyrene-based MBHA resin. The MBHA resin (50 mg) was swollen in DCM for 30 min and washed four times with DMF. The resin was treated twice with a solution of 20% piperidine (2 mL) in DMF for 10 min, and subsequently washed four times with DMF.

The resin was then functionalized with a Fmoc-glycine monomer using the following coupling conditions: Fmoc-Gly (5 eq., in NMP 0.25M), HATU (3.6 eq. in DMF 0.2M) and DIPEA (5 eq.)/lutidine (6 eq.), 1 h at room temperature.

In the synthesis the Fmoc group was removed by a treatment with a 5% DBU in DMF solution (5 min). In the case of Fmoc-Ser amino acids the basic treatment was prolonged (20 min). After each coupling step a capping procedure was performed using acetic anhydride (5%)/lutidine (6%) in DMF solution.

Couplings of Fmoc-L-Ser[PO(OBzl)OH]-OH were achieved using the following conditions: Fmoc-Ser monomer (8 eq. in NMP 0.4M), HATU (8 eq. in DMF 0.4M) and DIPEA (8 eq.)/lutidine (12 eq.), 15 h at room temperature.

After the serine couplings, a further glycine residue was attached on the N-terminal of the serine tract following the previously described coupling with glycine monomer.

The couplings with the PNA monomers were carried out using the following conditions: Fmoc-PNA monomer (8 eq. in NMP 0.4M), HATU (8 eq. in DMF 0.4M) and DIPEA (8 eq.)/lutidine (12 eq.), 4 h at room temperature.

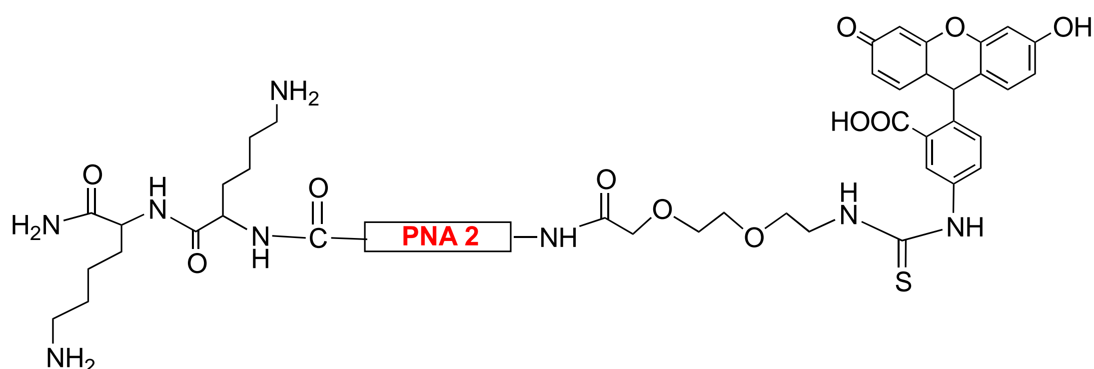
The coupling with AEEA-COOH monomer (8 eq.) was performed using the same conditions described for PNA couplings.

The FITC conjugation was performed using the following conditions: FITC monomer (5 eq., 0.2M) was dissolved in DMF/DIPEA (2.5:97.5 v/v) and the solution was added to the resin. The mixture was gently shaken in the dark for 15 h. The functionalized PNA-FITC oligomer was detached from the resin using a treatment with TFA/anisole/ethanedithiol (9:0.5:0.5; v/v/v) for 3.5 h and precipitated with cold diethyl ether. This treatment assures the complete deprotection of the phosphate groups. The precipitated was collected by centrifugation and resuspended in ether twice. The pellet was dissolved in 0.1% $H_3PO_4$ in water and lyophilized. The crude sample was purified by semipreparative RP-HPLC using a linear gradient from 10% to 90 % of eluent B in eluent A in 30 min. Eluent A: 0.05% $H_3PO_4$ in water; Eluent B: 0.05% $H_3PO_4$ in acetonitrile;

UV/VIS-detector was set at 495 nm, corresponding to the maximum of absorption of FITC. The collected yellow fractions were lyophilized and stored at -20°C in the dark.

The isolated PNA1 oligomer was characterized by MALDI-TOF spectrometry on a Bruker Autoflex I instrument using α-cyano-4-hydroxycinnamic acid, 10 mg/mL in acetonitrile-3% aqueous TFA (1:1, v/v) as the matrix. *m/z* found 4769.3.

The isolated PNA3 oligomer was characterized by MALDI-TOF spectrometry on a Bruker Autoflex I instrument using using α-cyano-4-hydroxycinnamic acid, 10 mg/mL in acetonitrile-3% aqueous TFA (1:1, v/v) as the matrix. *m/z* found 4818.2.




From C to N terminus

*Synthesis of the bis-lysine-PNA (PNA2)*

The polystyrene-based MBHA resin (50 mg) was swollen in DCM for 30 min and washed four times with DMF. The resin was treated twice with a solution of 20% piperidine (3 mL) in DMF for 10 min, and subsequently washed four times with DMF.

Couplings with Fmoc-L-Lysine(Boc)-OH amino acid were achieved using the following conditions: Fmoc-L-Lysine(Boc)-OH (4 eq.), HBTU (3.6 eq.), HOBt (3.6 eq) and DIEA (8 eq.) were dissolved in DMF (2 mL) and the resulting solution was added to the resin and allowed to react for 30 min.

The PNA tract was synthesized using the same procedure described for PNA1,3. The couplings with AEEA linker and FITC were performed using the same procedure described for PNA1,3.

After each coupling step a capping procedure was performed using acetic anhydride (5%)/lutidine (6%) in DMF solution. In the synthesis the Fmoc group was removed by treatment with 20% piperidine in DMF solution (10 min).

Successively, the synthesized Lys-Lys-PNA-AEEA-FITC was detached from the resin and precipitated in diethyl ether using the same conditions described for PNA1,3.

The crude sample was purified by semipreparative RP-HPLC using a linear gradient from 10% to 90 % of eluent B in eluent A in 30 min. Eluent A: 0.05% $H_3PO_4$ in water; Eluent B: 0.05% $H_3PO_4$ in acetonitrile.

The isolated PNA2 was characterized by MALDI-TOF spectrometry on a Bruker Autoflex I instrument using using α-cyano-4-hydroxycinnamic acid, 10 mg/mL in acetonitrile-3% aqueous TFA (1:1, v/v) as the matrix. *m/z* found 4577.9.

## Preparation of miRNA/PNA heteroduplexes (annealing)

The miR-509-3p/PNAs heteroduplexes (1:1.5 or 1:5) were formed by heating the mixture of the samples dissolved in 100 mM KCl, 10 mM $K_2HPO_4$, at 90°C for 5 min and slowly cooling at room temperature for 12 h.

## UV and UV melting studies

The UV spectra were recorded on a Jasco V-530 UV spectrophotometer equipped with a Peltier-type temperature control system (model PTC-348WI). Thermal denaturation experiments were carried out in the temperature range 25-90 °C by monitoring the absorbance at 260 nm at a heating rate of 0.5°C/min. The apparent Tm was estimated from the maximum in the first derivative of the melting profile.

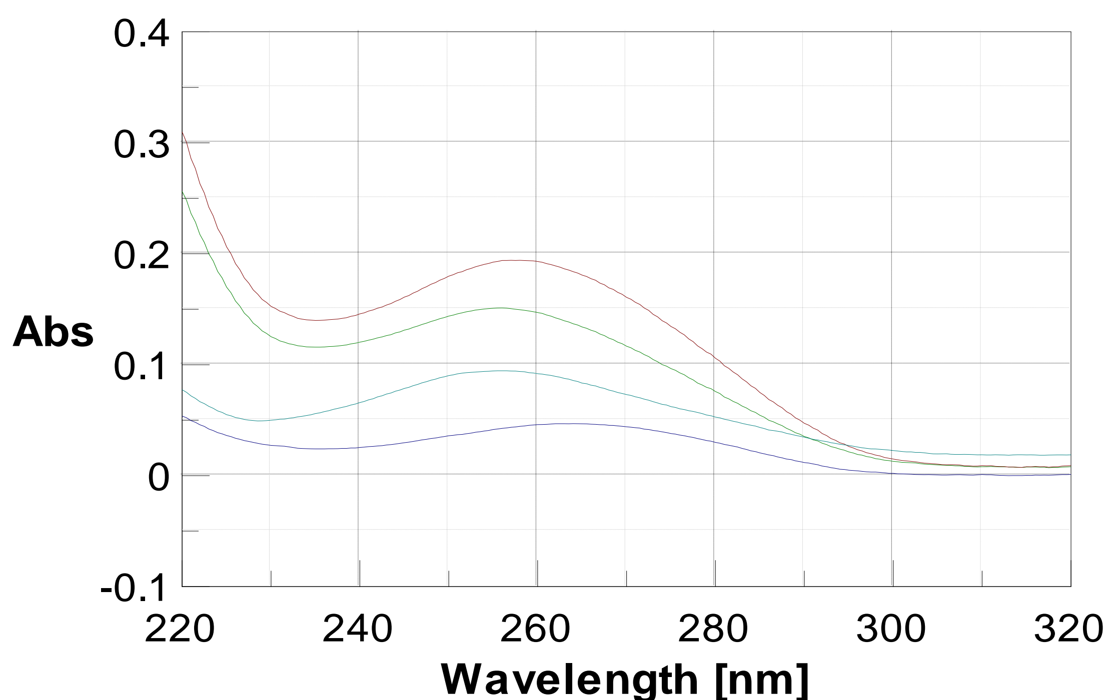



**Fig. S1** UV spectra of miR-509-3P (blue), PNA1 (green), miR-509-3P/PNA1 (1:1.5) (cyan) and the arithmetical sum of blue and green lines (red).

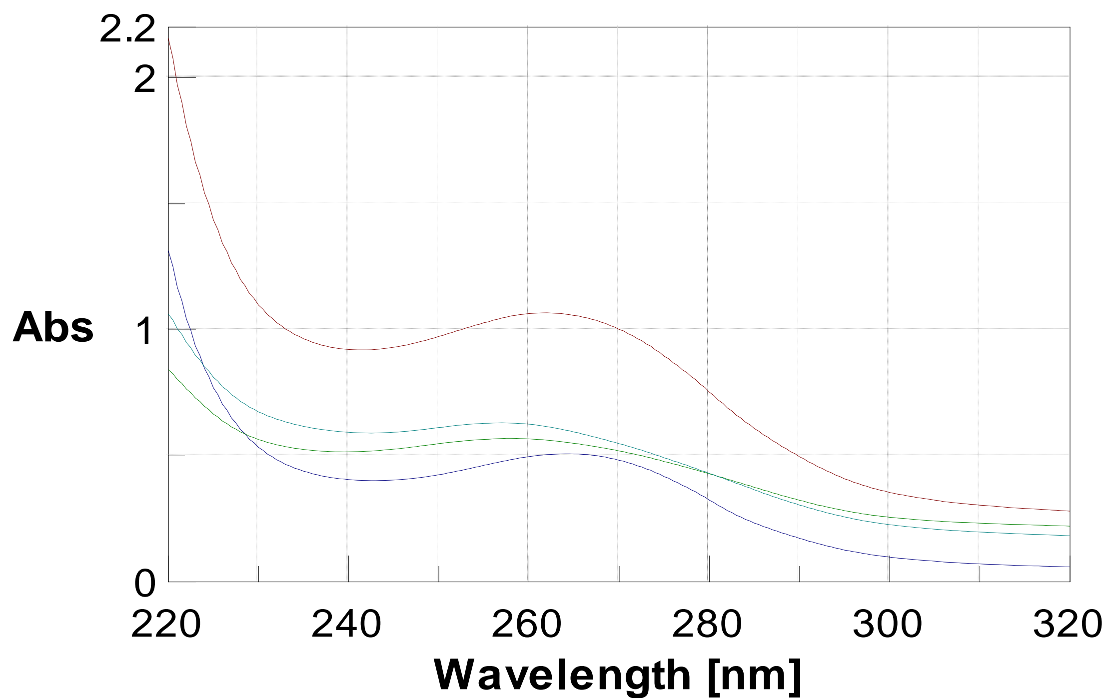



**Fig. S2** UV spectra of miR-509-3P (blue), PNA2 (green), miR-509-3P/PNA2 (1:1.5) (cyan) and arithmetic sum of blue and green lines (red).

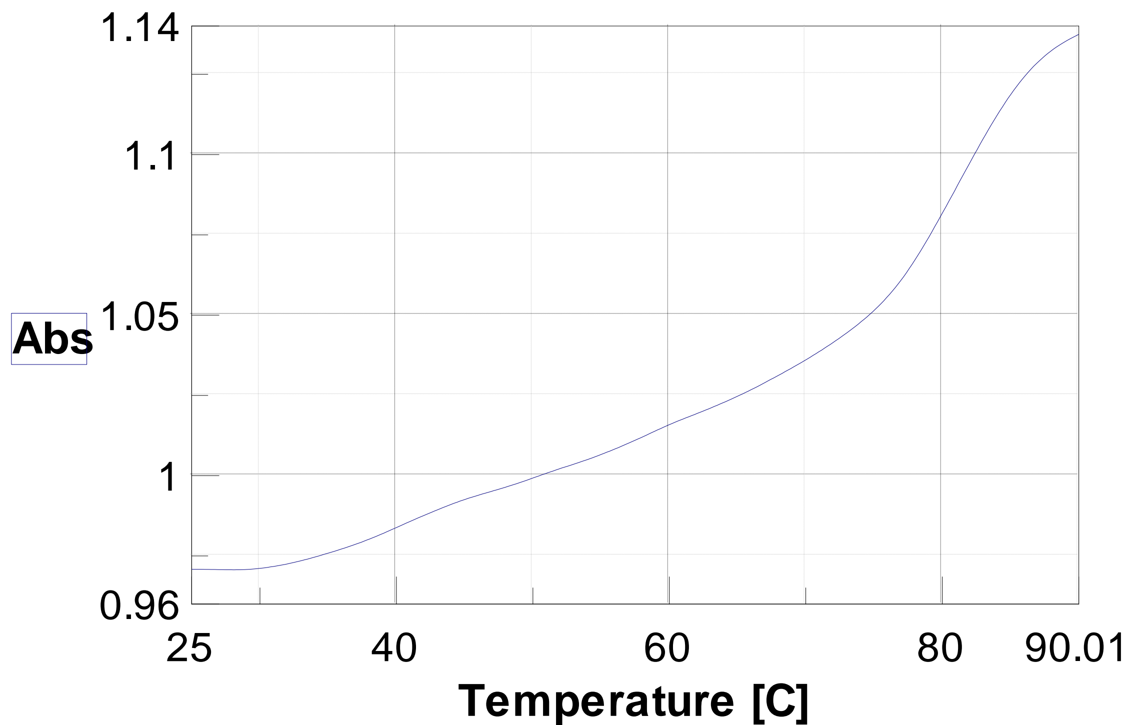



**Fig. S3** UV melting profile of miR-509-3P/PNA1 (1:1.5) heteroduplex.

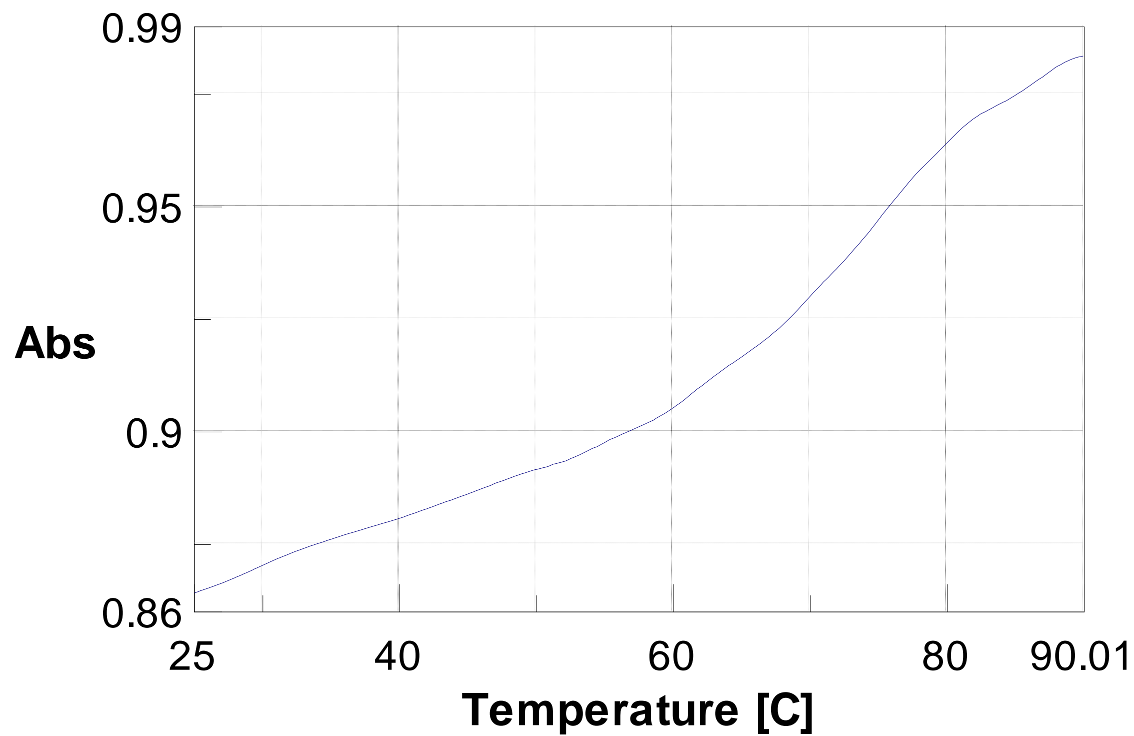



**Fig. S4** UV melting profile of miR-509-3P/PNA2 (1:1.5) heteroduplex.

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## CD studies

CD spectra were recorded with a Jasco J-715 spectropolarimeter equipped with a Peltier Thermostat Jasco ETC-505T using 0.1 cm path length cuvettes and calibrated with an aqueous solution of 0.06% d-10-(1)-camphorsulfonic acid at 290 nm. The molar ellipticity [□] (deg $cm^2$ $dmol^{-1}$) was calculated from the equation: [□] = [□]obs/10 $l$ $C$, where [□]obs is the ellipticity (mdeg), $C$ is the oligonucleotide molar concentration, and $l$ is the optical path length of the cell (cm). CD measurements (220-320 nm) were carried out at a scan rate of 100 nm/min with a 2 nm bandwidth. The concentration of the miRNA/PNA1 and miRNA/PNA2 complexes was $1\times10^{-5}$ M. The spectra were signal–averaged over at least three scans and baseline corrected by subtracting the buffer spectrum.

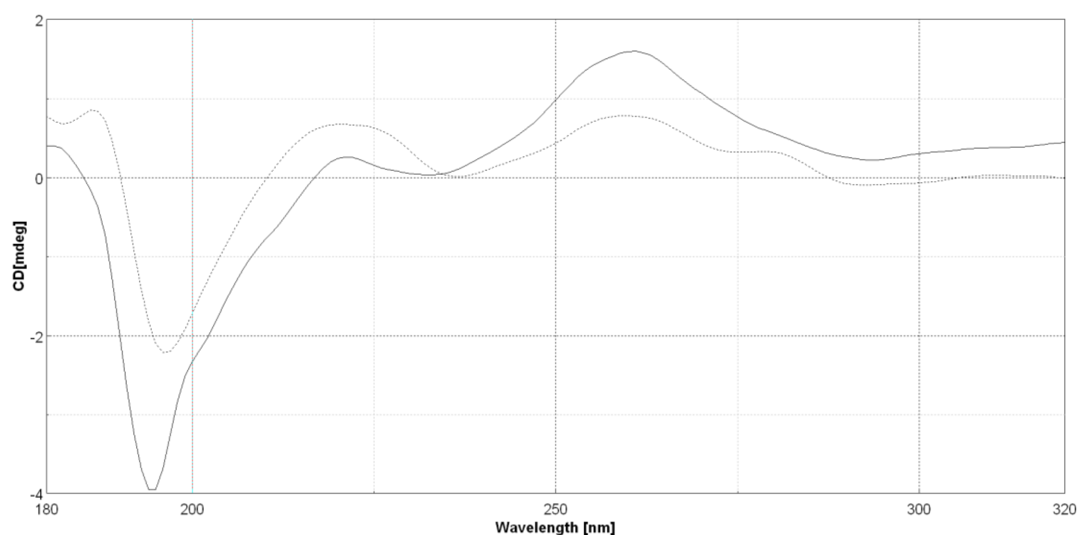


**Fig. S5** CD spectra recorded at 20 °C of miR-509-3p/PNA1 (solid line) and miR-509-3p/PNA2 (dotted line).

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## Cell line, Construct and Transfections

A549 cell line, human lung carcinoma cells, was purchased from ATCC (Manassas, USA). Cells were maintained in Dulbecco's modified Eagle's medium (Gibco Invitrogen, USA) with 10% heat-inactivated fetal bovine serum (HyClone, USA) without the addition of antibiotics. Luciferase construct bearing the 3'UTR of CFTR gene (8) was used as miR-509-3p sensitive. Transfection of A549 cells with miRNA-mimics (Qiagen, Germany, EU) or PNA was performed with Attractene Transfection Reagent (Qiagen). Cells were seeded in 96-well plates and were cotransfected 12 hrs after seeding with the firefly reporter constructs described above (20 ng), the Renilla reporter plasmid pCMV-RL (5 ng), and the miR-509-3p mimic. Twelve hours after transfection of cells with constructs and miR-509-3p mix, cells were transfected with PNA anti-miR-509-3p. The transfection efficiency (75% for PNA1 and 25% for PNA2) was assessed by measuring the percentage of fluorescent cells relative to the total numer of cells. Twenty-four hours after transfection, cells were lysed and Firefly and Renilla luciferase activities were determined using the Dual-Glo Luciferase Assay System (Promega Corporation). The relative reporter activity was obtained by normalization to the Renilla luciferase activity.

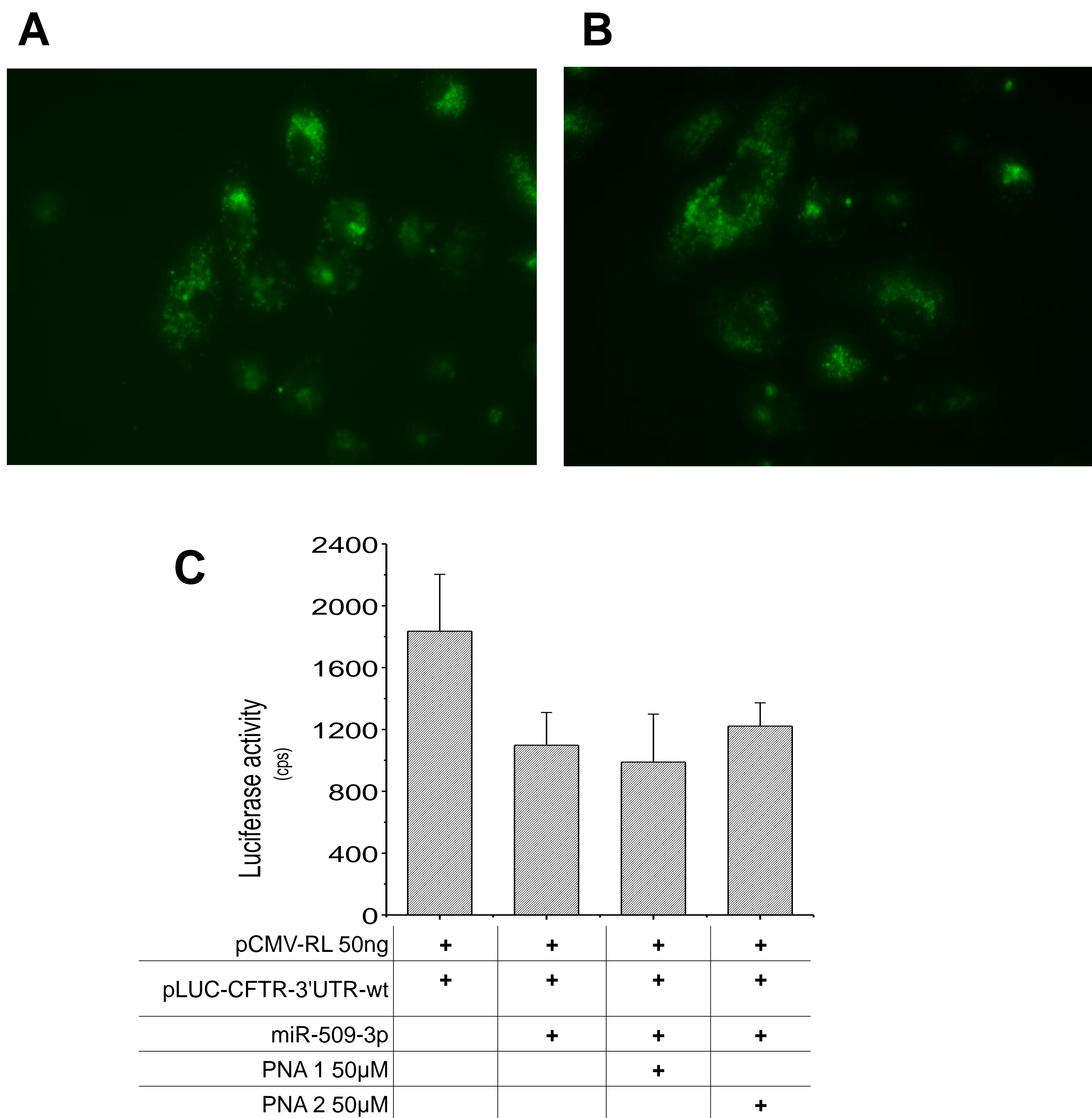
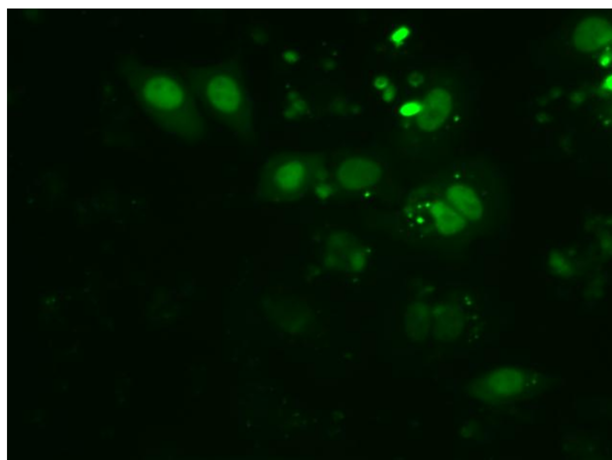



**Fig. S6** Representative uptake of FITC-labeled PNA1 (panel A) and PNA2 (panel B) by A549 cells and their effect as miRNA inhibitors (panel C) in the absence of any transfection reagent.

A



B

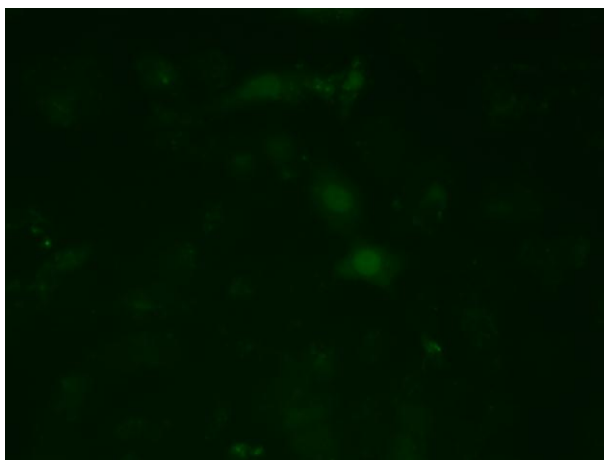


**Fig. S7** FITC-labeled PNA1 (panel A) and PNA2 (panel B) transfected in A549 cells.

## Electrophoretic Mobility Shift Assay

The miR-509-3p mimic (2'OMe–modified) was synthesized by the oligonucleotide synthesis facility at CEINGE-Biotecnologie Avanzate (Naples – Italy). The miRNA and PNA1 or PNA2 were annealed in 1X NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl2, 1 mM DTT, pH7.9 at 25 °C) for 2 hrs at room temperature. All the reactions were loaded into 20% polyacrylamide gels in 0.5X Tris-Borate-EDTA (TBE) buffer and run at 140 V for 3 hrs. The fluorescence signal was acquired placing the wet gel directly on the platen of Typhoon 8600 scanner. After fluorescence acquisition the gel was EtBr stained, destained in distilled water and the image acquired with a Gel Doc 2000 (Bio-Rad).

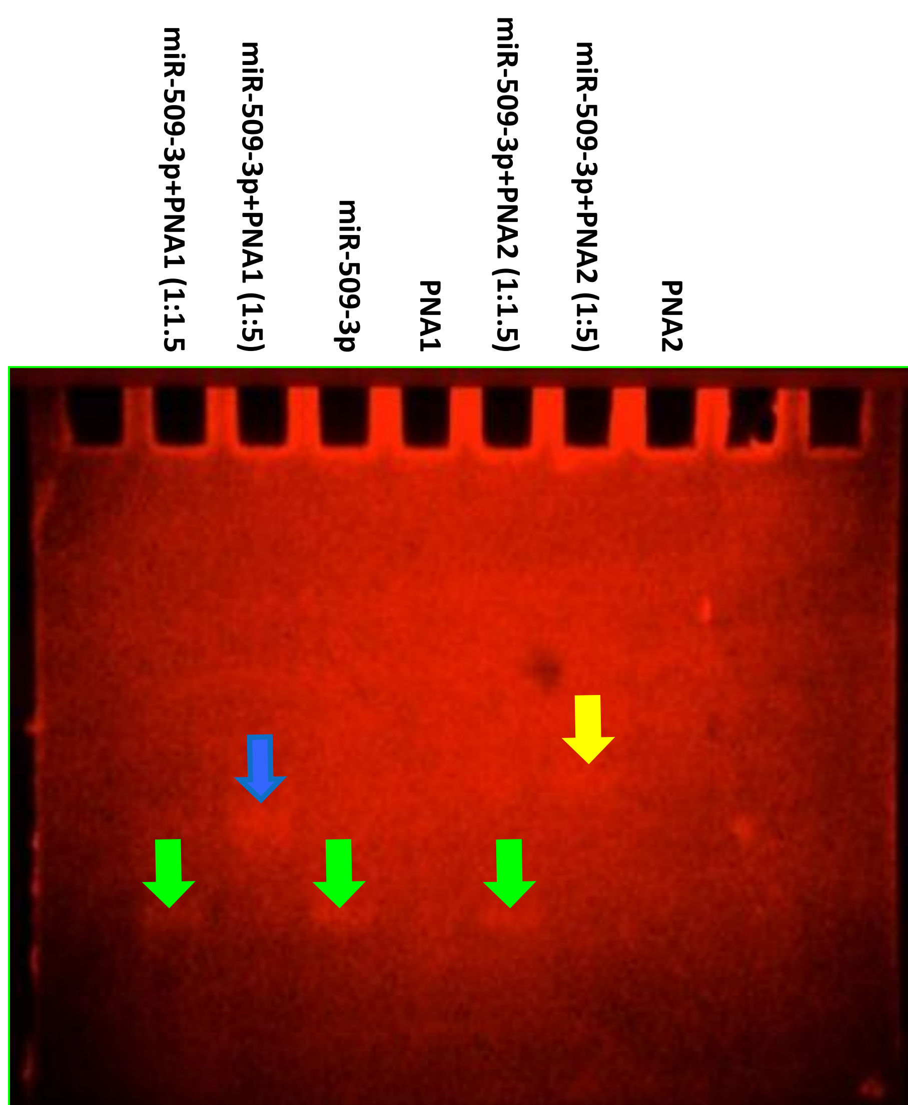



**Fig. S8** Ethidium Bromide staining of the EMSA. The green arrows show the position of miR-509-3p migration; the blue arrow shows the position of the miR-509-3p/PNA1 heteroduplex in a 1:5 ratio; the yellow arrow shows the position of the miR-509-3p/PNA2 heteroduplex in a 1:5 ratio.