Electronic Supporting Information for the paper

Luminescent Conjugates Between Dinuclear Rhenium(I) Complexes and Peptide Nucleic Acids (PNA) for Cell Imaging and DNA Targeting.

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General Materials and Methods

All the reactions were performed under N_2 and those involving the Rhenium complexes using Schlenk technique. All reagents were obtained from commercial suppliers and used without further purification and solvents were deoxygenated and dried by standard methods. Pyridazine-4-carboxylic acid was purchased from Aldrich, and used as received. The pentacarbonyl-rhenium chloride $Re(CO)_5Cl$ was prepared according to literature procedures.[1] The homothymine-PNA decamer labelled with fluorescein was prepared following a classical literature procedure.[2] Column chromatography was performed using Merck silica gel 60 (70-230 mesh).

^1H NMR spectra were acquired on a Bruker AVANCE DRX-400, Bruker AC300 and AMX at 300 MHz spectrometers; the chemical shifts (δ) are reported in parts per million relative to solvent peak. IR spectra were recorded on a Fourier Bruker Vector 22 FT, UV spectra were recorded by using a Jasco V-520 or Agilent 8453 UV/Vis spectrophotometer in a range of λ from 190 nm to 600 nm at room temperature. MS and HPLC-MS spectra were recorded on Advantage Thermofinnigan instruments (ESI source). ESI-HRMS spectra were recorded on Bruker Daltonics ICR-FTMS APEX II. EI-HRMS were acquired on a VG Autospec M246 spectrometer.

MALDI-TOF analysis were executed on a Bruker Omniflex spectrometer.

Vials with PTFE frits were used as reactor for solid phase synthesis. Automated solid phase syntheses were performed with peptide synthesizer "ABI433A", equipped with Synthassist 2.0 software for peptide synthesis, according to Applied Biosystems ABI 433A Peptide Synthesis User's Manual. MBHA (4-methylbenzhydrylamine hydrochloride salt) resin in 3 mL reaction vessel was used.

HPLC spectra of PNA oligomers were obtained with a HPLC AGILENT 1100 Series, using reverse-phase analytical column DISCOVERY® BIO WIDE PORE C18 (25 cm x 4.6 mm, 5 μm) and a semi-preparative column DISCOVERY® BIO WIDE PORE C18 (25 cm x 10 mm, 10 μm).

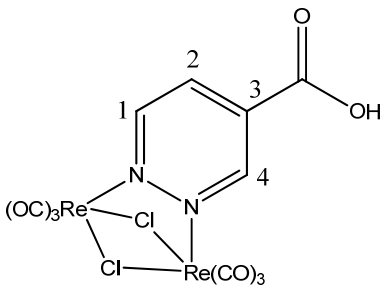
Elemental analyses were performed by the Department of Inorganic, Metallorganic and Analytical Chemistry of the University of Milan on a Perkin Elmer instrument. Melting points were obtained with a Büchi Melapparatus.

Acronyms of reagents and solvents (alphabetical order)

DIPEA	*N,N*-Diisopropylethylamine
DMEM	Dulbecco's Modified Eagle Medium
DMF	*N, N*- Dimethylformamide
DMSO	Dimethylsulfoxide
EDC.HCl	*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide HCI
HATU	*O*-(7-Azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate
HOBt	1- Hydroxybenzotriazole
NMP	1-Methyl-2-pyrrolidinone
TEA	Triethylamine
TFA	Trifluoroacetic acid
TFMSA	Trifluoromethanesulfonic acid
THF	Tetrahydrofuran

Synthesis of 4-(pyridazin-4-yl)-butanoic acid. The ligand was synthesized using a [4+2] Dies-Alder cycloaddition reaction between 1,2,4,5-tetrazine (prepared from hydrazine hydrate and formamidine acetate) and 5-hexynoic acid, according to a literature procedure for the synthesis of similar alkylated pyridazines.[3] An excess of 5-hexynoic acid (4.5 equiv) was added to a 0.2 M solution of 1,2,4,5-tetrazine in dioxane and the mixture was maintained for 30 days at room temperature. The completeness of the reaction was assessed by ^{1}H NMR. The ligand was then isolated from the alkyne excess by CH_2Cl_2/*n*-hexane precipitation. The purity of the crude product was proved by NMR spectroscopy and elemental analysis.Anal. calcd for $C_8H_{10}N_2O_2$: C 57.82, H 6.07, N, 16.86; found: C 57.68, H 6.17, N 16.39.^{1}H NMR (CD_2Cl_2):δ 2.04 (q, J = 7.5, 2H, CH_2-C*H*$_2$-CH_2-COOH), 2.46 (t, J = 7.5, 2H, CH_2-CH_2-C*H*$_2$-COOH), 2.75 (pt, J = 7.5 Hz, 2H, C*H*$_2$-CH_2-CH_2-COOH), 7.41 (dd, *J* = 2.6/5.2 Hz, 1H, Hc), 9.08 (m, 2H, Ha and Hb). ^{1}H NMR (DMSO): δ 1.84 (q, J = 7.5, 2H, CH_2-C*H*$_2$-CH_2-COOH), 2.25 (t, J = 7.5, 2H, CH_2-CH_2-C*H*$_2$-COOH), 2.65 (pt, J = 7.5 Hz, 2H, C*H*$_2$-CH_2-CH_2-COOH), 7.54 (dd, *J* = 2.0/5.2 Hz, 1H, Hc), 9.08 (d, *J* = 5.2 Hz, 1H, Ha), 9.12 (sb, 1H, Hb),12.13 (sb, 1H, OH).^{13}C NMR (DMSO): δ 24.9 (1C, CH_2-*C*H_2-CH_2-COOH), 31.4 (1C, *C*H_2-CH_2-CH_2-COOH), 33.4 (1C, CH_2-CH_2-*C*H_2-COOH) 126.7 (1C, *C*-Ha), 141.6 (1C, *C*q), 153.1 (1C, *C*-Hb), 151.6 (1C, *C*-Hc), 174.4 (1C, *C*OOH).

Synthesis of [$Re_2(\mu\text{-}Cl)_2(CO)_6(\mu\text{-}4\text{-}COOH\text{-}pdz)$] (1). The procedure used for the synthesis of all the previously reported [$Re_2(\mu\text{-}Cl)_2(CO)_6(\mu\text{-}diazine)$] complexes was employed.[4,5] A sample of $Re(CO)_5Cl$ (211 mg, 0.584 mmol) in 30 mL of toluene was treated with 37 mg (0.298 mmol) of 4-pyridazine-carboxylic acid and refluxed for 3 h, giving a red precipitate and an orange solution. The IR analysis showed that the cold solution, which contained several species, was very diluted and then it was discarded. The IR spectrum of the precipitate, on the contrary, showed mainly bands attributable to the expected complex **1**, contaminated by small amounts of by-products, constituted most likely by [$Re(CO)_3$(4-COOH-pydz)$_2$Cl] [6] and [$Re(CO)_3(\eta^6$-toluene)]$^+$ [7] (see Figure S1, red trace). Spectroscopically pure **1** was isolated by diethyl ether extraction from the precipitate and purified by CH_2Cl_2/*n*-hexane precipitation (118 mg, isolated yields 55%). IR (CH_2Cl_2) ν_{CO}: 2052 (mw), 2035 (s), 1951 (s), 1922 (s), 1761 (w) cm^{-1}. ^{1}H NMR (CD_2Cl_2): δ 8.52 (dd, *J* = 5.9/2.1 Hz, 1H, H2), 10.02 (dd, *J* = 1.1/5.9 Hz, 1H, H1), 10.19 (dd, *J* = 1.1/2.1 Hz, 1H, H4). HR-EI MS: m/z 735.846190, calcd for $C_{11}H_4N_2O_8Cl_2Re_2$: 735.846001. Satisfactory elemental analysis could not be obtained, the sample being probably contaminated by hydrogen-bonded water and traces of organic solvents, which could not be eliminated even after 24 h at 1.5×10^{-6} mbar. Anal. calcd for $C_{11}H_4Cl_2N_2O_8Re_2$: C 17.96, H 0.55, N, 3.81; found: C 19.63, H 1.13, N 3.56. For this reason the epsilon value reported in Table 1 for compound **1** should be considered somewhat underestimated.



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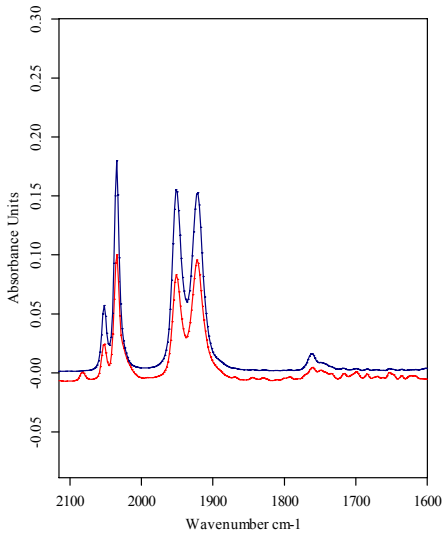
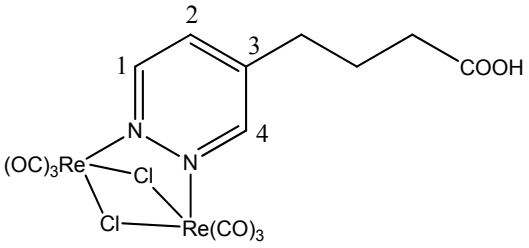


Figure S1. IR spectra (ν(CO) region, CH_2Cl_2) of the precipitate formed in the reaction (red trace) and of the isolated product **1** (blu trace)

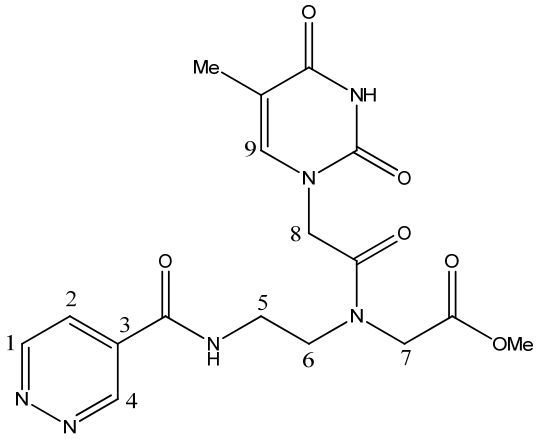
Synthesis of [Re_2(μ-Cl)$_2$(CO)$_6$(μ-4-(pyridazin-4-yl)-butanoic acid) (2). A sample of $Re(CO)_5Cl$ (37 mg, 0.102 mmol) in 5 mL of toluene was treated with 8.5 mg (0.051 mmol) of 4-(pyridazin-4-yl)-butanoic acid and refluxed for 2 h. The solution was evaporated to dryness and the residue dissolved in CH_2Cl_2 and precipitated with *n*-hexane, affording a microcrystalline sample of **2** whose purity was assessed by IR, NMR and mass-spectroscopy and by elemental analysis (36 mg, isolated yields 90%). Anal. calcd for $C_{14}H_{10}Cl_2N_2O_8Re_2$: C 21.63, H 1.30, N, 3.60; found: C 21.40, H 1.41, N 3.56. HR-EI MS: m/z 777.893620, calcd for $C_{14}H_{10}N_2O_8Cl_2Re_2$:777.892951. IR (CH_2Cl_2) ν_{CO}: 2050 (mw), 2033 (s), 1946 (s), 1918 (s), 1738 (w) cm^{-1}. ^1H NMR (CD_2Cl_2): δ 2.14 (pq, 2H, CH_2-C*H*$_2$-CH_2-COOH), 2.59 (t, J = 7.0, 2H, CH_2-CH_2-C*H*$_2$-COOH), 3.01 (pt, 2H, C*H*$_2$-CH_2-CH_2-COOH), 7.90 (m, 1H, H2), 9.69 (m, 2H, H1 and H4), 8.45 (s, broad, 1H, OH) ppm .



2

Synthesis of pyridazine-4-carboxylic acid PNA monomer conjugate 5. To a solution of pyridazine-4-carboxylic acid (50 mg; 0.403 mmol) in dry DMF (3.5 ml) HOBt (54.5 mg; 0.403 mmol) was added, then after cooling at 0 °C, a solution of EDC.HCl (77 mg, 0.403 mmol) in DMF (4.5 ml) was added. The reaction was allowed to warm at room temperature and then stirred for 30 min. PNA monomer **3**[8] (166 mg; 0.403 mmol) was added as a solid followed by slow addition of TEA (135 μl; 98 mg; 0.967 mmol). A slightly warming the mixture was observed. The reaction was followed by TLC (CH_2Cl_2/MeOH 9:1). After the solvent was evaporated under reduced pressure,

the oily yellow crude product was dissolved in CH_2Cl_2 (20 ml) and left to stir for 1h: the off-white solid thus precipitated was separated by centrifugation and crystallized from water (2 ml), obtaining **5** as a grey solid (81 mg , 50%). M.p.: 216-9 °C (dec.). For ^1H and ^{13}C NMR (DMSO) (2 rotamers: A/B in 2/1 ratio) see Table S1. ESI-MS: *m/z* 427.5 [M^+ + Na] (100%), 875.4 (90%), 1141 (60%), 1011 (35%); HR-ESI MS: *m/z* 427.13328 (+Na), 449.11640 (+2Na); calcd. 427.13365 for $C_{17}H_{20}N_6O_6Na$. Anal. Calcd. for $C_{17}H_{20}N_6O_6$: C 50.49, H 4.99, N 20.79; found: C 50.90, H 5.02, N 20.25.



5

	^{1}H	^{13}C
1	9.53 (m) A; 9.46 (m), B	152.6
4	9.43 (m) A; 9.41 (m), B	149.0
2	7.98 (dd *J* = 2.4/5.6 Hz) A; 7.90 (dd), B	124.5
3	-	131.7 A 132.0 B
C=O α to pydz	-	164.8 A 163.9 B
5	3.58 A; 3.43 B	38.2 A; 37.5 B
6	3.58 A; 3.50 B	46.7 A; 46.5 B
8	4.14 A; 4.39 B	47.9 A; 49.0 B
7	4.50 A; 4.71 B	48.3 A; 48.2 B
-OCH$_3$	3.71 A; 3.63 B	52.7 A; 52.3 B
C=O α to 8	-	167.9 A; 168.5 B
CO-OCH$_3$	-	170.5 A; 169.8 B
CH$_3$ thymine	1.73	12.3
CH thymine	7.30 A; 7.22 B	142.5
C$_q$ thymine		108.7 A; 108.6 B
C=O 2 thymine		151.4
C=O 4 thymine		164.3

Table S1. ^{1}H and ^{13}C attributions of **5** (300K, DMSO, 400 MHz). A and B indicate the major and minor rotamers of PNA.

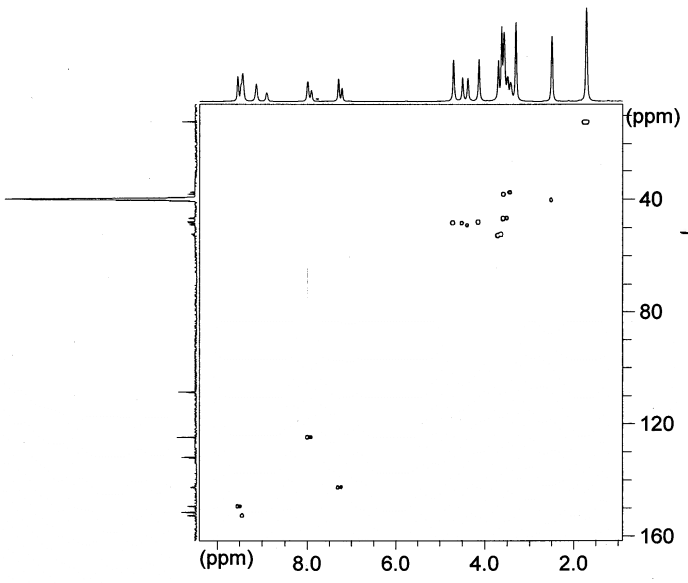


Figure S2a ^{1}H-^{13}C HSQC NMR (DMSO; 400MHz) of **5**

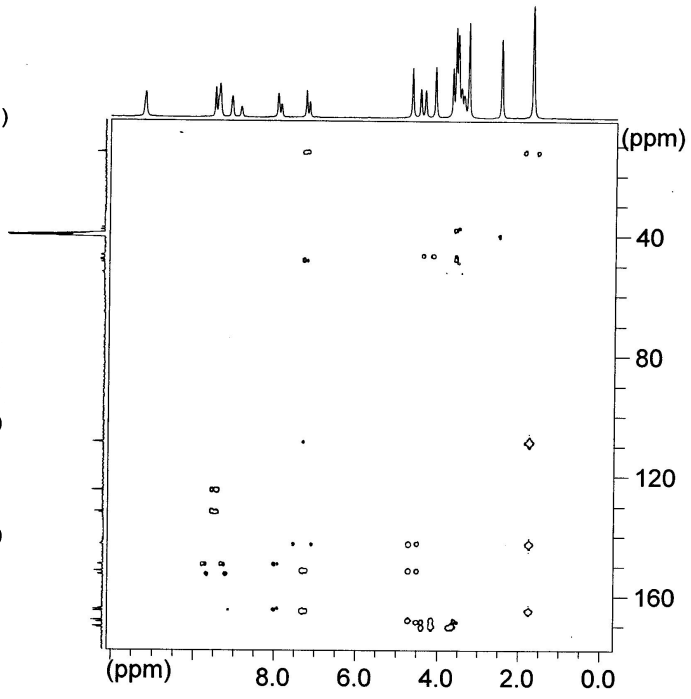
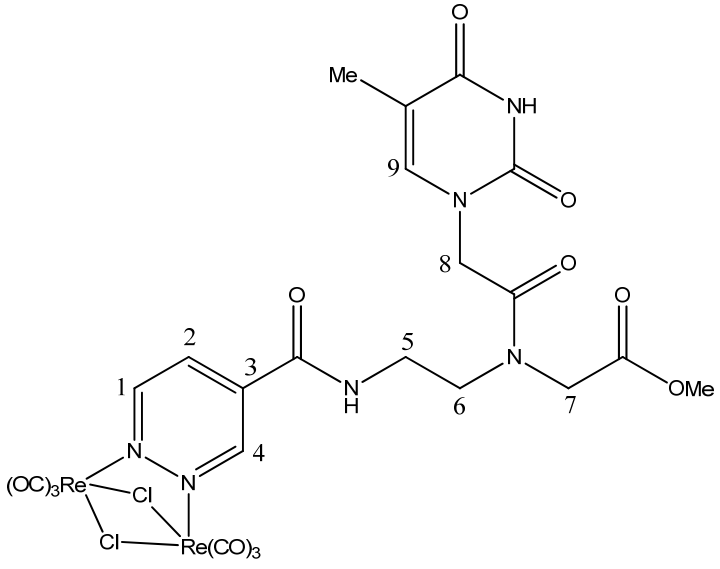


Figure S2b ^{1}H-^{13}C HMBC (DMSO; 400MHz) of **5**

Synthesis of 4 by complexation of 5 with $Re(CO)_5Cl$. $Re(CO)_5Cl$ (19.0 mg, 0.053 mmol) was added to a suspension of **5** (10.0 mg, 0.025 mmol) in 15 ml of freshly distilled toluene, and the mixture refluxed for 1.5 h, affording a light orange solution. After solvent evaporation the crude product was purified by column chromatography (AcOEt/EtOH 4:1) affording **4** as an orange solid in 60% yield. Elemental Anal. calcd for $C_{23}H_{20}Cl_2N_6O_{12}Re_2$: C 27.20, H 1.98, N 8.27; found: C 27.50, H 2.20, N 7.85. IR (CH_2Cl_2) ν_{CO}: 2050 (m), 2034 (s), 1947 (s), 1919 (s), 1685 (m) cm^{-1}. HR-ESI MS: *m/z* 1038.95208 (M^+ + Na), calcd. for $C_{23}H_{20}N_6O_{12}Cl_2Re_2Na$: 1038.95288 . For ^{1}H and ^{13}C NMR (CD_2Cl_2) (2 rotamers: A/B in 2/1 ratio) see Table S2.

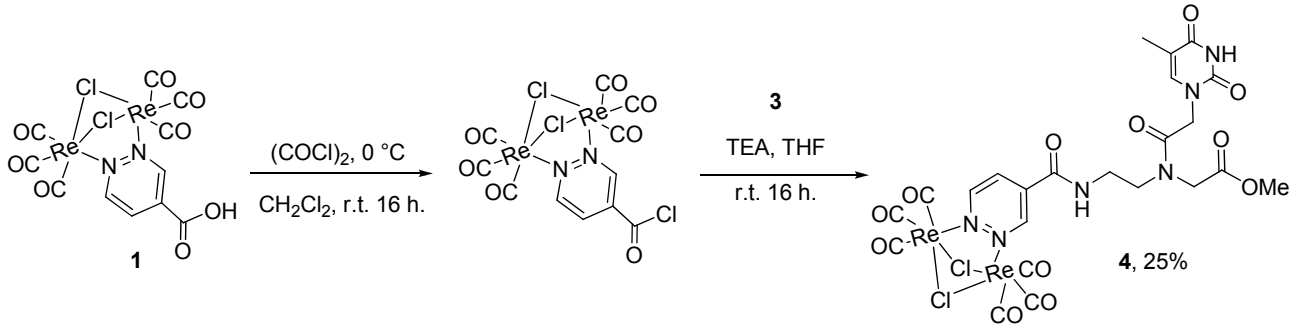


4

	^{1}H	^{13}C
1	10.14 (m)	161.4
4	9.9 (m)	162.2
2	8.38 A; 8.41 B	129.4
3	-	
C=O α to pydz	-	173.1
5	3.83 B	37.4
6	3.71 B	47.7
8	4.13 A; 4.23 B	48.6 A; 49.6 B
7	4.41 A; 4.37 B	48.8
-OCH$_3$	3.85 A 3.87 B	52.2
C=O α to 8'	-	167.4
CO-OCH$_3$	-	171.9
CH$_3$ thymine	1.89 A; 1.85 B	12.0
CH thymine	6.89 A; 6.94 B	140.9
C$_q$ thymine		110.7
C=O 2 thymine		150.6
C=O 4 thymine		163.4

Table S2. ^{1}H and ^{13}C attributions of **4** (300K, CD_2Cl_2, 400 MHz). A and B indicate the major and minor rotamers of PNA.

Synthesis of 4 by reaction of complex 1-acid chloride and monomer 3.



In a round bottom flask equipped with $CaCl_2$ valve, $(COCl)_2$ (150µl; 225 mg; 1.78 mmol) was slowly added at 0°C to a solution of **1** (50 mg; 0.068 mmol) in dry CH_2Cl_2 (5 ml). The reaction was allowed to warm at room temperature and left to stir overnight, then was heated to 35°C for 1 h. The excess of $(COCl)_2$ was evaporated under reduced pressure and the dark red solid was taken up with dry CH_2Cl_2 (5 ml) and evaporated (3 times). The resulting dark red solid was dissolved in dry THF (5 ml) and the resulting solution was added to a solution of **3** (25 mg; 0.061 mmol) in dry THF (1 ml) and TEA (3 drops),obtaining an orange suspension, that was stirred at room temperature. The reaction was followed by TLC (AcOEt/MeOH 9:1). After 16 h the solvent was evaporated, the crude solid was taken up with AcOEt (5 ml) and washed with H_2O (3 × 10 ml). The water phase is then extracted with AcOEt (3 × 5ml). The organic phases were dried over Na_2SO_4, and evaporated

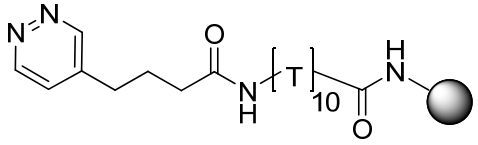
under reduced pressure. Purification through Biotage automated chromatography system (AcOEt/MeOH 9:1) gave **4** as an orange solid (15.4 mg; 25%).

Preparation of the resin-supported homo-thymine *aeg*PNA decamer 8. It was run following the procedure reported in: P. E. Nielsen (Ed.), *Peptide Nucleic Acids: Protocols and Applications.* Second ed., Horizon Bioscience, Wymondham, UK, 2004, using Applied Biosystems ABI 433A Peptide Synthesizer and according the procedure described in User's Manual. MBHA (4-methylbenzhydrylamine hydrochloride salt) resin in 3 mL reaction vessel on 20 μM scale using Boc strategy was used.

PNA cleavage from the resin. The resin was washed with TFA (2 × 200 μL) and then shaken for 1 h with a solution of TFA/TFMSA/thioanisole/*m*-cresol 6:2:1:1 (500 μL). The reaction mixture was then filtered and the resin washed with TFA (4 × 200 μL), collecting the filtrate which was concentrated under nitrogen flow. Et_2O (5 mL) was added to the residue to precipitate PNA. Centrifugation of the slurry gave a white solid, which was washed with Et_2O (8×5 mL) and dried to afford the PNA decamer.

The cleaved PNA was characterized by reverse phase HPLC (gradient from water: CH_3CN 95:5 to CH_3CN 100% in 60 min. t_R: 10 min) and MALDI-TOF: *m/z* 2679.41 (M^+); 2700.60 (M^+ + Na); 2717.80 (M^+ +K).

Coupling of diazine ligands to homothymine PNA decamer : general procedure.



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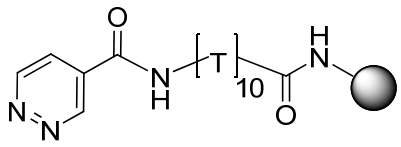


9

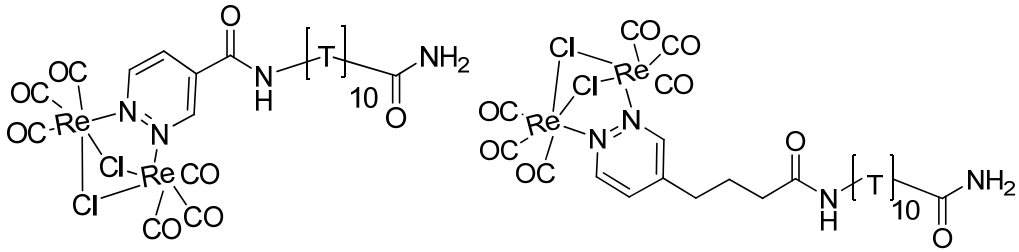
The resin bound-PNA **8** (0.2 mmol/g, 98 mg, 19.6 μmol) was swollen with CH_2Cl_2 (3 ml) for 1 h then filtered, treated with DIPEA 5% in CH_2Cl_2 (3 ml) for 2 min (the procedure is repeated twice) and washed again with CH_2Cl_2 (2 × 3 ml). At room temperature a solution of the appropriate diazine (0.10 mmol) and DIPEA (33 μl; 0.196 mmol) in NMP (1 ml) was added to a solution of HATU (37.3 mg; 0.098 mmol) in NMP (0.5 ml); the resulting mixture, after shaking for 2 min, was added to the resin and the mixture shaken for 2 h. The solution was filtered off and the resin washed with NMP (2 × 5 ml) and CH_2Cl_2 (2 × 5 ml). The coupling procedure was repeated a second time with the same amount of reagents. The resin thus obtained was washed with NMP (2 × 5 ml), CH_2Cl_2 (2 × 5 ml), MeOH/CH_2Cl_2 (2 × 5 ml), CH_2Cl_2 (2 × 5 ml).

The outcome of the reaction was checked through a cleavage of a little a sample of the resin as described above.

PNA from resin **9**: MALDI-TOF: *m/z* 2810 (M^+ +Na); HPLC (H_2O/CH_3CN gradient from 95:5 H_2O:CH_3CN to 100% CH_3CN in 60 min): t_R = 12 min.

PNA from resin **10**: MALDI-TOF: *m/z* 2829 (M^+), 2852 (M^+ +Na), 2867 (M^+ +K); HPLC (H_2O/CH_3CN gradient from 95:5 H_2O:CH_3CN to 100% CH_3CN in 60 min): t_R = 13 min.

Complexation of resins 9 and 10 with $Re(CO)_5Cl$ to give Re-PNA conjugates 6 and 7.



6 **7**

In a 10 ml schlenk tube, a sample of resin-bound homo-thymine decamer diazine **9** or **10** (0.2 mmol/g, 109 mg, 0.022 mmol) was swollen in 10 ml of freshly distilled toluene. A large excess of $Re(CO)_5Cl$ (75 mg, 0.207 mmol) was added and the suspension was refluxed for 3 h. The resin changed colour during the reaction from off-white to red. The course of the reaction was monitored by IR following the disappearance of the CO band of the reagent in the solution, the IR spectrum also showed ν_{co} bands of $Re_2(\mu\text{-}Cl_2)(CO)_8$ and $[Re(CO)_3Cl(diazine)_2]$. After 3h the reaction was allowed to cool at room temperature, the resin was filtered through a syringe, washed with toluene (2 × 5 ml), and dried under vacuum for 4 h. Re-PNA conjugates **6** and **7** were obtained by cleavage of the whole resin, and were purified by semi-preparative reverse phase HPLC (gradient in H_2O/CH_3CN from 50:50 to 100% CH_3CN in 60 min, t_R **6**: 28 min., t_R **7**: 29 min.). Compound **6:** MALDI-TOF: *m/z* 3399.4 (M^+), HR-ESI MS: *m/z* 1699.45369 (M^{2+}), calcd. for $C_{121}H_{147}N_{43}O_{47}Cl_2Re_2$: 1699.44617.

Compound **7**: MALDI-TOF: *m/z* 3437.9 (M^+); HR-ESI MS**:** *m/z* 1147.31610 (M^{3+}), calcd. for $C_{124}H_{154}N_{43}O_{47}Cl_2Re_2$: 1147.31555.

Photophysical characterization.

Steady state fluorescence measurements were performed with a Cary Eclipse (Varian Inc., Australia) spectrofluorometer, at constant temperature (293 K), controlled by a Peltier thermostatic system, and on a Jobin-Yvon Fluorolog-3 spectrofluorometer equipped with double monochromator and a Xe lamp. Both the instruments were equipped with a Hamamatsu R928P photomultiplier tube as detector. For the measurements on de-aerated solutions, the samples were prepared under N_2 by introducing the quartz cuvettes into suitable Schlenk tube, and were deoxygenated before measurements by bubbling N_2 for 20 minutes. Emission spectra were corrected for the spectral sensitivity of the detection system by standard correction curves. The emission intensities were normalized to a nominal absorption value of 0.1. Quantum yields were determined by comparison with the emission of $[Ru(bipy)_3]Cl_2$ in de-aerated acetonitrile ($\Phi = 0.062$)[9] and aerated water ($\Phi = 0.028$).[10] Absorption and emission spectra of **2** and **7** are shown in Figures S3 and S4.

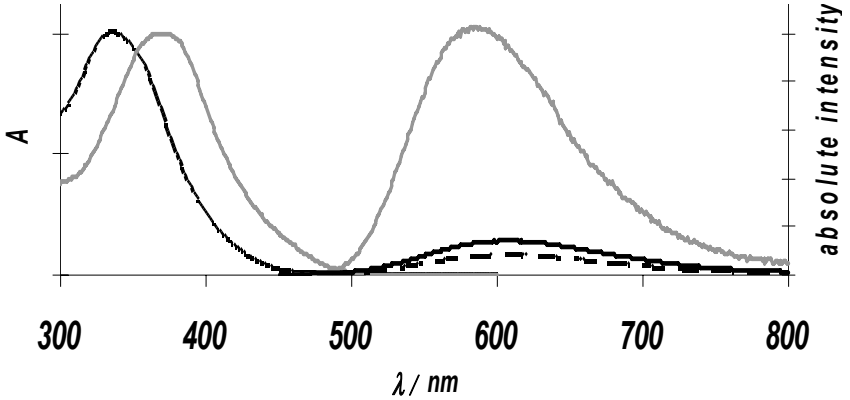


Figure S3. Absorption (left) and emission (right) spectra of **2** in de-aerated toluene (grey), de-aerated CH_3CN/H_2O (black) and aerated CH_3CN/H_2O 1:1 (dotted black).

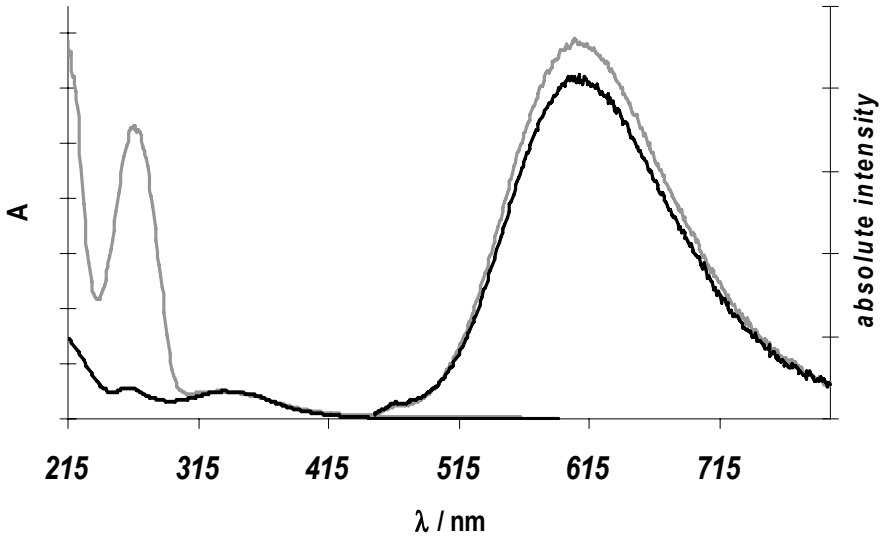


Figure S4. Comparison of the absorption (left) and emission (right) spectra of **2** (black) and **7** (grey) in de-aerated CH_3CN/H_2O (1:1).

Lifetimes were measured by frequency-domain methods. For complex **2** a frequency modulated phase fluorometer (Digital K2, I.S.S., Urbana IL) was employed, using the 454.5 nm line of an Argon ion laser for the excitation, as previously described.[11] At least fifteen data points have been acquired at logarithmically spaced frequencies in the range of 0.3-60 MHz with a cross correlation frequency of 400 Hz. Typical errors affecting the lifetime measurements are about 5%, with the exception of the longer lifetimes (i.e. >500ns), for which the error rises to about 10% due to the non optimal working frequency range available of the amplifiers. For compound **7**, which does not adsorb significantly at 454 nm, a laser diode at 370 nm has been used as an excitation source.

TPA measurements and cellular uptake. The laser source was a mode-locked Ti:sapphire laser (Mai Tai HP, Spectra Physics, CA) with pulses of 120 fs full width at half maximum and 80 MHz repetition frequency. The optical setup was built around a confocal scanning head (FV-300, Olympus, Japan) mounted on an upright optical microscope (BX51, Olympus, Japan) equipped with a high working distance objective (NA = 1.1, wd = 2 mm, 60X, water immersion, Olympus, Japan). Non-confocal TPE imaging was performed through the FV-300 scanning unit after removing the largest pinhole from the pinhole wheel. The objective simultaneously focused the laser beam on the sample and collected the signal in epi-fluorescence geometry through the non-descanned collection channels described hereafter. The non-descanned detection system (ND-unit), collected the emitted light right above the microscope objective lens, thereby avoiding the complex optical path back to the photomultipliers in the FV-300 scanning head. The signal reaching the ND-unit is fed to two Hamamatsu analog output photomultipliers (HC125-02, Hamammatsu, Japan) whose 21 mm (diameter) photocathode ensured the collection of most of the light during scanning. The ND unit has been designed to minimize the distance between the entrance pupil of the objective and the active area of the detector. The fluorescence signal was filtered by a 485/50, a 535/50 and a 600/40 band-pass filter in order to select the fluorescence light and remove either scattering or undesired auto-fluorescence from the sample and it has been processed by means of the Fluoview 5.0 software (Olympus, Japan). The two-photon excitation spectrum has been measured in solution at 10 nm interval steps from 720 to 940 nm with an excitation power on the sample of about 20 mW for **2** (in toluene) and 50 mW for the **7** (in CH_3CN/H_2O). The transmission efficiency of the optical setup has been taken into account in order to register the spectrum with constant exciting power. Wide field transmission images were recorded through a Photometrics Cascade II:512 EMCCD (512x512 pixel, 16x16 μm pixel size), mounted on top of the microscope through a 0.5x connector (273x273 μm^2 field of view).

Cellular uptake experiments have been performed on hek293 cells. 24 hours before the experiments, 1×10^6 cells per well have been plated on tissue 6 wells plates in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-Glutamine, Penicillin–Streptomycin (EuroClone), at 37 C in a 5% CO_2 atmosphere. The culture medium has been replaced with phosphate buffer solution (PBS) at pH 7.2-7.4 30 minutes before the start of the measurements. A small aliquot (50 μl) of a 0.12 mM DMSO solution of **7** was added to 2 mL of phosphate buffer solution (PBS) in different wells of the plate, giving a 3 μM concentration of **7** in the wells. Images have been recorded 10 min after the addition of the complex by exploiting two photon excitation at 770 nm (with an excitation power of 80 mW). The bleed through of autofluorescence has been verified on non stained samples by measuring the fluorescence emission in the presence and in the absence of the band pass filter selecting the emission of the complex (Figure S5). Images shown in the paper are the result of 5 kalman average scans with 10 μs of residence time per pixel. The field of view was variable between 235×235 μm^2 and 39×39 μm^2 depending on the experiment, and it is indicated in the figures captions.

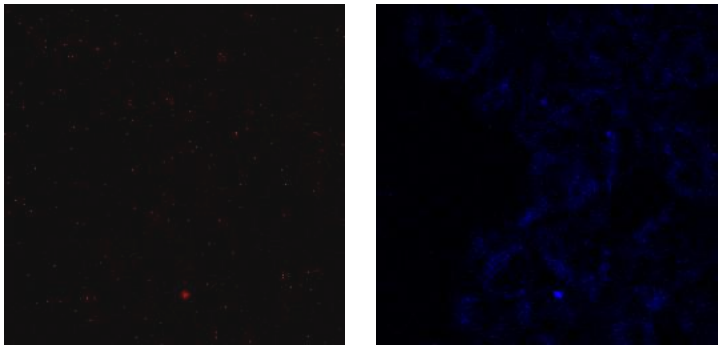


Figure S5: Cells autofluorescence, through the 600/40 (left) and 485/30 (right) band pass filters. Field of view 117×117 μm^2.

The effect of DMSO on the cell morphology and on its autofluorescence has been studied by adding the same volume of DMSO as in the experiments with the PNA complexes and by recording both the fluorescence emission and the wide field images before and after the addition of DMSO. As shown in the pictures below, no substantial effect on the cells can be observed on the time scale of the uptake experiments. A more detailed study on the effect of DMSO on cell viability has been carried out as described below.

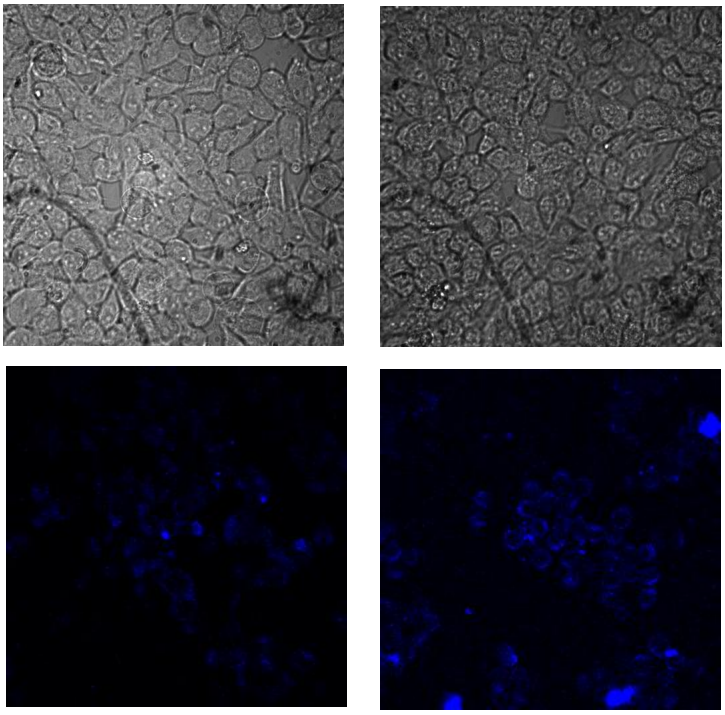


Figure S6: Upper panel: wide field transmission images recorded before (left) and after (right) the addition of DMSO. Lower panel: autofluorescence images recorded before (left) and after (right) the addition of DMSO, field of view 235×235 μm^2.

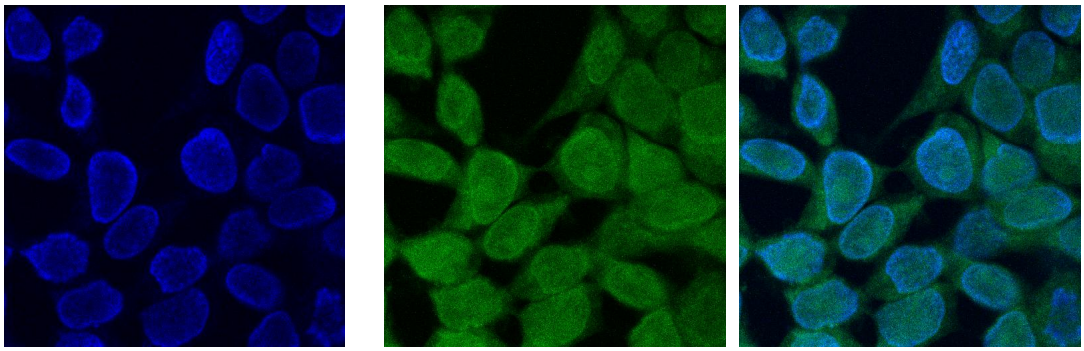


Figure S7: Images of HEK-293 cells stained with the Re-PNA conjugate **7**, recorded about 10 min after the addition of the complex, through a 485/30 (left) and a 535/50 (center) band pass filter and their superposition (final **7** concentration 3 μM; field of view: 78×78 μm^2).

The relevance of DMSO in improving cellular uptake of complex **7** has been verified by performing an experiment with an aqueous solution of **7**. Then 150 μl of a 40 μM solution of **7** in PBS buffer have been added to 1.9 mL of PBS buffer in a well of the plate (so obtaining the same 3 μM final concentration of **7**, as in the experiments with DMSO solutions of **7**). No uptake was detectable up to one hour after the addition of the sample, and the luminescence was concentrated in the spaces between the cells, as shown in Figure S8.

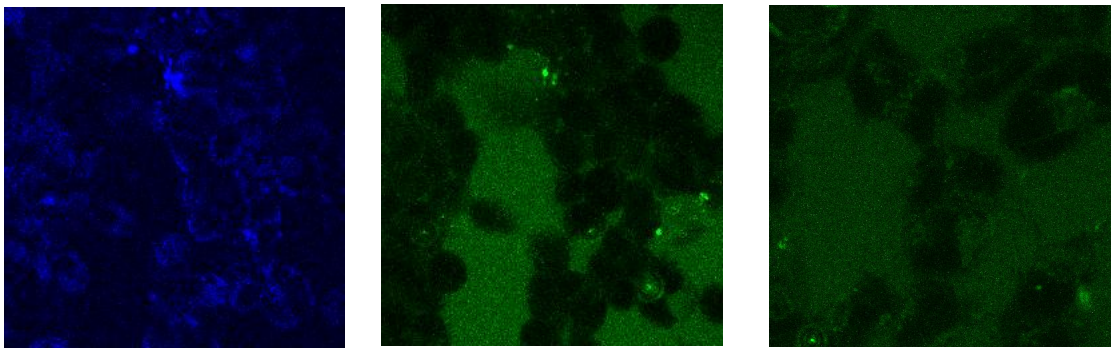


Figure S8: Images recorded about 10 minutes (left e middle, through the 485/30 and 535/50 band pass filters, respectively, field of view: 117×117 μm^2) and 1 hour (right, green filter, field of view: 78×78 μm^2) after the addition of **7** in water solution. The autofluorescence emission in the left image has been enhanced by adjusting the LUT to show the position of the cells.

In order to investigate the specificity of the uptake of **7**, we performed measurements in the same experimental conditions (laser power, DMSO and sample concentration) by adding to different wells of the plates the same amount (50 µl) of either a 0.13 mM DMSO solution of fluorescein-labelled PNA or a 0.13 mM DMSO solution of complex **2**. The uptake of complex **2** appears similar to that of its PNA conjugated **7** (Fig S9), though less pronounced in the green region. On the contrary, the fluorescein-labelled PNA sample, though extremely bright (the PMT gain had to be reduced by a factor of 3), appears to not permeate small vesicles inside the cytoplasm region of cells (figure S10), whose nature should be further investigated.

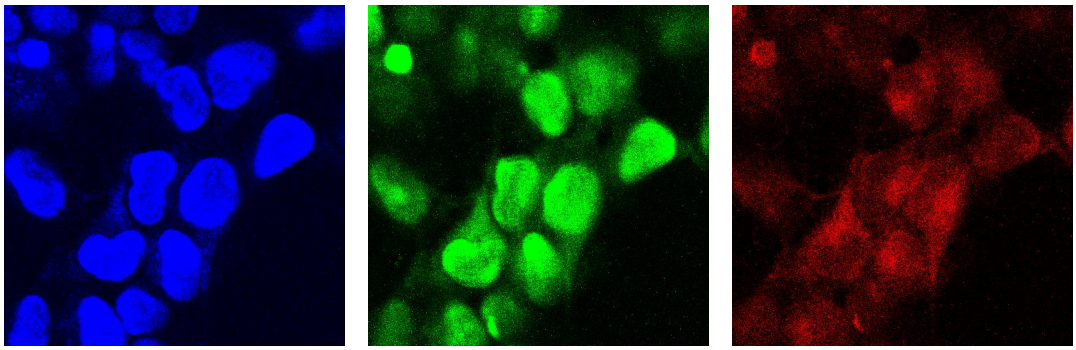


Figure S9. Emission of cells stained with DMSO solutions of **2** (3 µM) through the three different band pass filters; field of view 78×78 μm^2.

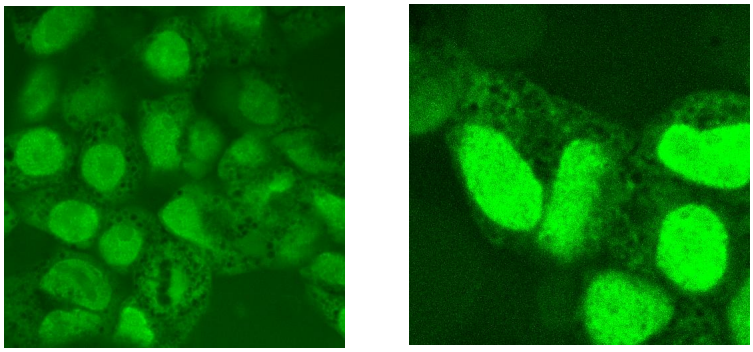


Figure S10. Cells stained with fluorescein-labeled PNA: left: not zoomed 78×78 μm^2, right: zoomed 39×39 μm^2. 535/50 nm band pass filter only.

In vitro toxicity assay

We tested the potential toxicity of the compounds of interest using the CellTiter–Blue Cell Viability Assay.

HEK-293 cells were seeded in 96-well plates at a concentration of $1x10^6$ cells/ml in 100 µl of complete DMEM (Dulbecco's Modified Eagle's Medium) additioned with 10% FBS, pen/strep and L-glu, in presence of the different molecules analyzed or DMSO alone. 30 minutes after the stimulation of the cells with the different compounds, the viability of the cells was measured using the CellTiter–Blue Cell Viability Assay (Promega, Madison, WI, USA) according to manufacturer's instructions. In brief, 50 µl of CellTiter-Blue solution were added to the cell culture and after 4 hours of incubation the plate was read at 570-600 nm using a plate-reader. Means were compared by unpaired t-test. Data are expressed and plotted in Figure S10 as means ± SD values.

The statistical analysis did not show any specific toxicity of conjugate **7** compared to DMSO alone (P value >0.5 in all the conditions tested). The absence of any toxicity due to **7** was further demonstrated when this conjugate was suspended in water and added to the cell culture (last bar in the histogram of Fig. S11). Furthermore, the analysis demonstrated that the amount of DMSO used to dissolve the fluorophores for cell imaging (DMSO 2.5%) did not show any relevant toxicity on HEK-293 cells. The only compound that showed a statistically significant toxic effect on HEK-293 cells was the Fluorescin-PNA compound: P value of Fluorescin-PNA 3µM (with DMSO 2.5%) < 0.05 compared to DMSO 2.5% alone, and P value of Fluorescin-PNA 0.6 µM (with DMSO 0.6%) < 0.005 compared to DMSO 0.6%.

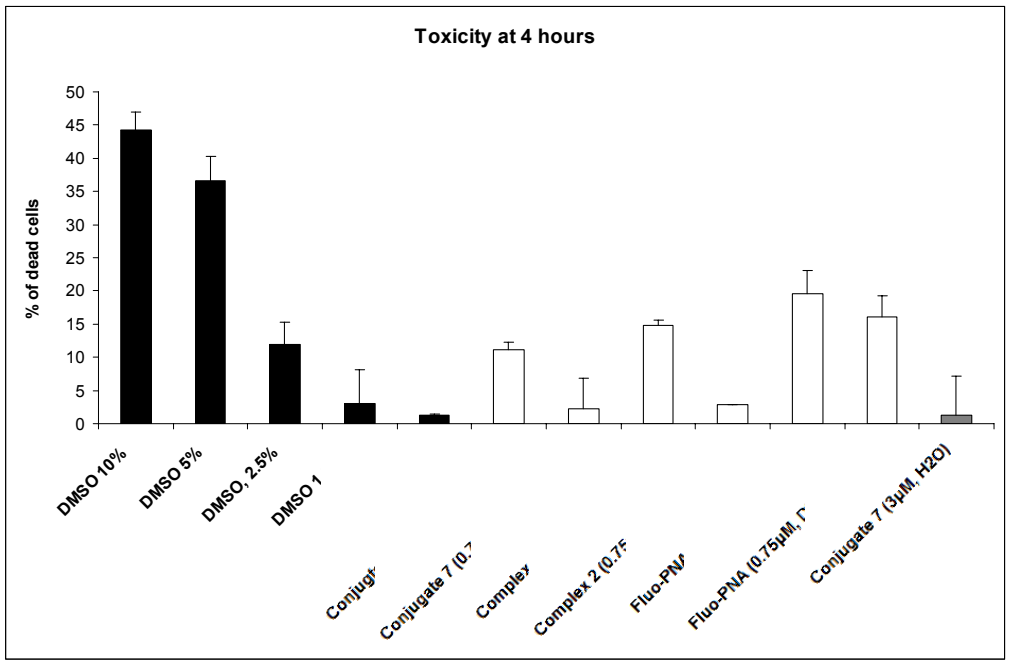


Figure S11. Dead cell-percentages of HEK-293 cells treated for 4 hours with the different compounds at the indicated concentration (Fluo-PNA: Fluorescin-PNA)

Thermal Melting Analysis

Binding affinity of the Re-PNA decamer complex **7** with complementary homo-adenine *ss*DNA decamer was evaluated. Owing to the fact that homo-thymine PNA decamer forms a triple helix with the complementary homo-adenine DNA, the sample was prepared by mixing **7** with the complementary *ss*DNA in a 2:1 ratio (final concentration of PNA: 5 μM) in 1:1 H_2O/phosphate buffer solution (pH 7.0, 100 mM NaCl, EDTA 0.1 mM). Hybridization was done by heating the solution at 90 °C for 5 min; the solution was then slowly cooled to 20 °C.

The resulting solution was subjected to UV measurements at 260 nm from 20 °C to 95 °C using a Agilent 8453 spectrometer equipped with an Agilent 89090A Peltier temperature controller. The UV absorbances were collected at 260 nm every 1 °C, with an equilibration time of 25 s for each measured point, and plotted to obtain the melting curve shown in Figure S12, which exhibited an inflection point at 73 °C.

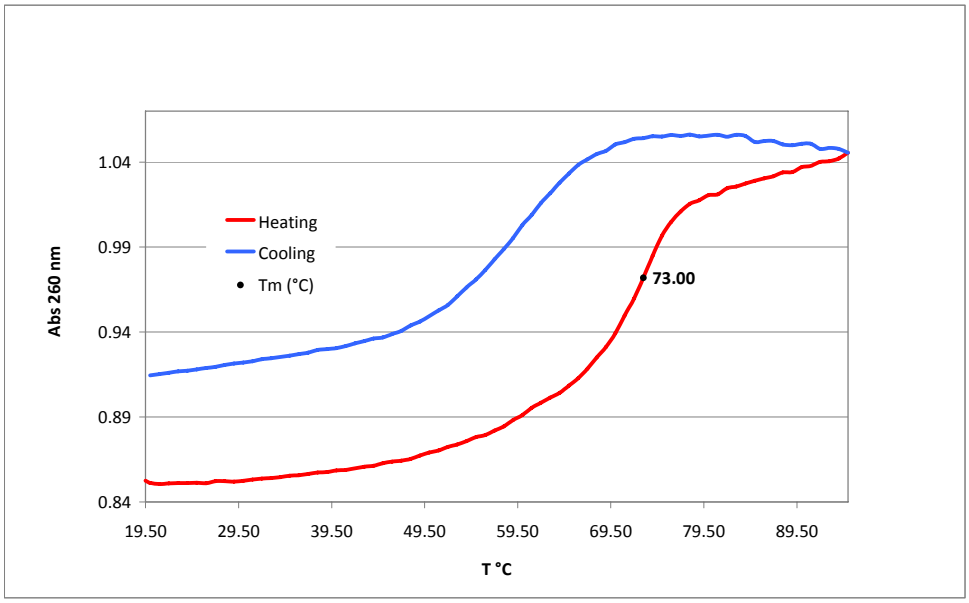


Figure S12. Heating (red) and cooling (blue) curves obtained in the thermal melting analysis of complex **7**.

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