Supporting Information

Photoactivated cationic alkyl-substituted porphyrin binding to g4-RNA in the 5'-UTR of *KRAS* oncogene represses translation

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Experimental Section

Porphyrins and Oligodeoxynucleotides

Porphyrin meso-tetrakis (mono-N-tetradecyl, N-methyl-4-pyridyl) porphine (TMPyP4-C14) was obtained from Frontier Scientific Inc, Logan, UT, U.S.A, while meso-tetrakis (N-methyl-4-pyridyl) porphine (TMPyP4) and meso-tetrakis (N-methyl-2-pyridyl) porphine (TMPyP2) were purchased from Porphyrins Systems (Lubeck, Germany). They were dissolved in water and conserved in aliquots of 0.5 mM at -80°C. The stability in aqueous solution of the porphyrins was checked by measuring its UV-Vis spectrum at intervals of weeks. Oligoribonucleotides, unmodified or labeled with Fam or Tamra at the 5' and 3' ends, respectively, were synthesized and HPLC purified by Microsynth (CH). The samples were conserved in 100 μM aliquots in water at -80 ° C.

FRET experiments

Fluorescence measurements were carried out with a Microplate Spectrofluorometer System (Perkin Elmer 2300 Enspire, USA) using a 96-well black plate, in which each well contained 50 μl of 200 nM dual-labelled oligoribonucleotide in 50 mM phosphate buffer, pH 7 and potassium chloride as specified in the figure captions. The emission spectra were obtained setting the excitation wavelength at 475 nm, the cut-off at 515 nm and recording the emission from 500 to 650 nm. Fluorescence melting experiments were performed on a real-time PCR apparatus (iQ5, BioRad, Hercules, CA), using a 96-well plate filled with 50 μl solutions of dual-labelled oligoribonucleotides, in 50 mM phosphate buffer, pH 7 and potassium chloride at different concentrations as specified on the figure. The protocol used for the melting experiments is the following: (i) equilibration step of 5 min at low temperature (20°C); (ii) stepwise increase of the temperature of 1°C/min for 76 cycles to reach 95°C. All the samples in the wells were melted in 76 min. After melting the samples were re-annealed and melted again, showing the same melting curves.

Cell proliferation assay

Panc-1 cells were seeded in a 96-well plate at a density of 3 x 10^3 cells/well. The following day they have been treated with porphyrin in the dark for 24 h, then irradiated with metal halogen lamp at a fluence of 8 mW/cm^2 for 15 min (14 J/cm^2). Cell proliferation, in terms of metabolic activity, was determined by the resazurin assay 24, 48 and 72 h following irradiation (Sigma-Aldrich, Milan, Italy). An aliquot of resazurin solution (2.5 mg/ml) in DMSO was added directly to each plate well, to a final concentration of 6.25 μg/μl, which contained the cells in 100 μl of growth medium. The cells were incubated for 1 hour and then the fluorescence (Ex 535 nm; Em 590 nm) was read on an automated spectrofluorimeter plate reader (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, California). Data are presented with graph reporting the percentage of metabolic activity compared to untreated cells. The data are the average of at least three independent experiments.

Western blot

Total protein lysates (30 µg) were electrophoresed on 12 % SDS-PAGE and transferred to a nitrocellulose membrane at 70 V for 2 h. The filter was blocked for 1 h with 5% BSA solution in TBST (10 mM Tris pH 7.9, 150 mM NaCl and 0.05 % Tween) (Sigma-Aldrich, Milan, Italy) at room temperature. The primary antibodies (mouse monoclonal anti-actin, Oncogene, diluted 1:10000; mouse anti-nucleoporin p62, BD Biosciences, diluted 1:2000; mouse monoclonal c-KRAS Oncogene, diluted 1:40), were overnight incubated with the samples at 4 °C. The expressions of β-actin and nucleporin were used as an internal control. The filters were washed with a 0.05 % Tween in PBS and subsequently incubated for 1h with the secondary antibody anti-mouse IgG for nucleoporin and KRAS, diluited 1:10000 (Calbiochem). For β-actin we used an anti-mouse IgM, diluted 1:10000 (Calbiochem). Each secondary antibody was coupled to horseradish peroxidase. For the detection of the proteins we used Super Signal®West PICO, and Super Signal®West FEMTO (Thermo Fisher Scientific Pierce). Exposure length depends on the antibodies used and was usually between 30 seconds to 5 min. The protein levels were quantified by Quantity ONE 4.6.5 software with Chemidoc (Bio Rad).

Cell culture

The human cell line used in this study is from pancreatic adenocarcinoma (Panc-1). The cells were maintained in exponential growth in Dulbecco's modified eagle's medium (DMEM) medium containing 100 U/ml penicillin, 100 mg/ml streptomycin, 20mM L-glutamine and 10% foetal bovine serum (Euroclone, Milano, Italy).

Confocal Microscopy

Panc-1 cells were plated (2.5 x 10^5) on coverslips (diameter 24 mm) and after 24 h treated with porphyrin for 24 h. The cells were washed twice with PBS, fixed with 3 % paraformaldehyde (PFA) in PBS for 20 min. After washing with 0.1 M glycine, containing 0.02 % sodium azide in PBS to remove PFA and Triton X-100 (0.1 % in PBS), the cells were incubated with Hoechst to stain the nuclei. The cells were analysed using a Leica TCS SP1 confocal imaging system.

FACS analysis

Panc-1 cells were plated in a 6-well plate at density of 2 x 10^5 cells/well. After one day, the cells were treated with 10 µM porphyrin for 2, 4, 6 and 8 h before being washed with PBS, trypsinized and pelleted. The pellets were suspended in 500 µl PBS and immediately analyzed by FACScan Flow Cytometer (Becton-Dickinson, San Jose, USA) equipped with a single 488 nm argon laser. A minimum of 10000 cells for each sample were acquired in list mode and analyzed using Cell Quest software. The cell population was analyzed by FSC light and SSC light. The signal was detected by FL3 (680 nm) channel in log scale.

RNA extraction, RT and real-time PCR quantification of KRAS mRNA

RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA), following manufacter instruction.
cDNA synthesis: 10µl of RNA in diethyl pyrocarbonate (DEPC) treated water (extracted from about 100000 cells) was heated at 55°C and placed in ice. The solution was added with 15 µl of mix, containing (final concentration) 1x First strand buffer (Invitrogen), 0.01 M DTT (Invitrogen), 1.6 µM primer dT (MWG Biotech, Ebersberg, Germany), 1.6 µM Random hexamer (Microsynth AG, Switzerland), 0.4 mM dNTPs solution containing equimolar amounts of dATP, dCTP, dGTP and dTTP (Euroclone, Milano, Italy), 0.8 U/µl RNAse OUT, 8 U/µl of M-MLV reverse transcriptase (Invitrogen). The reaction were incubated for 1h at 37°C and stopped with heating at 95°C for 5 min. As a negative control the reverse transcription reaction was performed with 10 µl of DEPC water.
Real-time reaction was performed with 1x iQ™ SYBR Green Supermix (Bio-Rad Laboratories, CA, USA), 300nM of each primer, 1/10 of RT reaction. KRAS expression are normalized with *HPRT*, β2-Microglobulin. The sequences of the primers used for amplification are: HPRT for 5' AGA CTT TGC TTT CCT TGG TCA GG 3'; *HPRT* rev 5' GTCTGGCTTATATCCAACACTTCG 3'; β2-Microglobulin for 5' TGCTGGATG ACGTGAGTAAACC 3', rev 5' CATTCCTGAAGCTGACAGCATTC 3'; *KRAS* for 5' CAGGAAGCA AGTAGTAATTGATGG 3', rev 5' TTAATGGCAAATACACAAAGAAAGC 3'. The PCR cycle was: 3 min at 95°C, 40 cycles 10s at 95°C, 30s at 56°C with thermocycler CFX96 Real-Time PCR detection system (Bio-Rad).

Circular dichroism

CD spectra have been collected with a JASCO J600 spectropolarimeter equipped with a thermostatted cell holder. RNA samples in 50 mM Tris-HCl, pH 7.4, 100 mM KCl were 6 µM. The spectra were recorded in 0.5 cm quartz cuvette at increasing temperature. Ordinate is expressed in mdeg.

ROS measurement

ROS level in Panc-1 cells were determined 24 h after irradiation by incubating the cells in PBS for 30 min at 37°C containing 5 µM of 5-(and 6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate, acetyl ester CM-H2DCFDA (C6827, Molecular Probes, Invitrogen, Milan, Italy). CM-H2DCFDA was metabolized by non-specific esterases to the non-fluorescence product of 5-(and 6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate, acetyl ester, which was oxidized to the fluorescent product by ROS. Then, the cells were washed twice in PBS, trypsinized, resuspended in PBS and measured for the ROS content by FACS (FACScan, Becton Dickinson, San Josè, USA).

Table S1. Conservation of g4 motifs in *KRAS* mRNA 5'UTR.

SPECIES	SEQUENCES	POSITION[A]
HUMAN	----GC**GG**C**GG**C**GG**A**GG**C----------GU**GG**C**GG**C**GG**CGAA**GG**UGGC----	-184/-154
CHIMPANZEE	----GC**GG**C**GG**C**GG**A**GG**C----------GU**GG**C**GG**C**GG**CGAC**GG**UGGC----	-177/-147
COW	---------- GC**GG**C**GG**C**GG**TGGC**GG**UGGC----	-145
MOUSE	----GC**GG**C**GG**CUGA**GG**C**GG**C----GU**GG**C**GG**C**GG**CUGAGAC**GG**C----	-183/-160
PIG	----GC**GG**C**GG**C**GG**A**GG**C----------GC**GG**C**GG**C**GG**TGGC**GG**CGGC----	-183/-160

(A) positions of the first G of the g4-RNA motif relative to the translation start site;

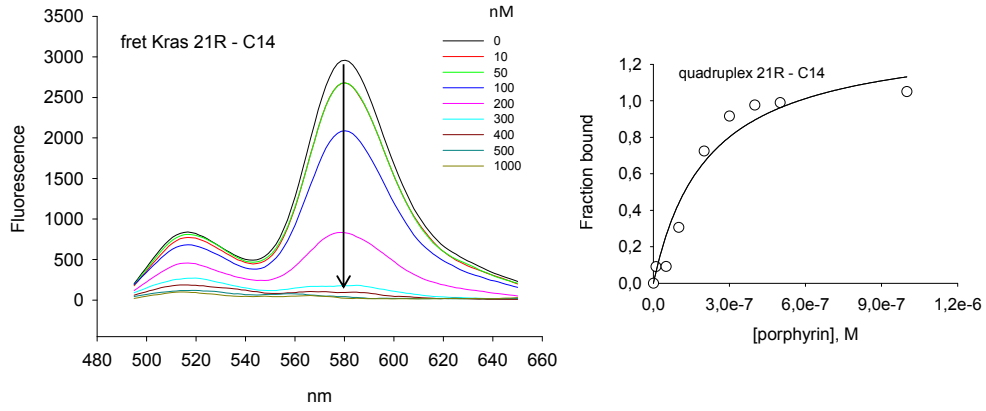


Figure S2: FRET titration in 50 mM phosphate buffer, pH 7, 140 mM KCl, showing the interaction between a quadruplex formed by a G-rich sequence (5'-AGGGCGGTGTGGGAAGAGGGA) located within a nuclease hypersensitive element of the human *KRAS* promoter (Chem Comm., 2011, 47, 4965-4967). C14 binding to the DNA quadruplex quenches the emission between 520 and 580 nm. From the titration we obtained a binding curve that gave a K_D of 2.1 x 10^{-7} M. A similar experiment was performed with TMPyP4. Table S2 report the binding data.

Table S2: Dissociation constants (K_D) for the interaction between the RNA quadruplexes with the porphyrins C14 and TMPyP4 in 50 mM phosphate buffer, pH 7, 140 mM KCl.

Porphyrins	utr-1 G4-RNA K_D, M	utr-2 G4-RNA K_D, M	*NRAS* G4-RNA[1], K_D, M	*KRAS* G4-DNA[2], K_D, M
C14	1.5 (±0.5) x 10^{-7}	2.8 (±0.5) x 10^{-7}	1.0 (±0.2) x 10^{-7}	2.1 (±0.8) x 10^{-7}
TMPyP4	1.6 (±0.6) x 10^{-7}	1.3 (±0.3) x 10^{-7}	1.3 (±0.3) x 10^{-7}	1.1 (±0.1) x 10^{-7}
TMPyP2	No binding	No binding	No binding	No binding

[1]*NRAS* G4-RNA is a G-rich sequence located in the 5'-UTR of *NRAS* mRNA that forms a quadruplex [5'-UGUGGGAGGGGCGGGUCUGGG] (Nat Chem Biol 2007, 3 218-221);

[2]*KRAS* G4-DNA is a G-rich sequence locate in a critical nuclease hypersensitive site of the KRAS promoter [5'-AGGGCGGTGTGGGAAGAGGGA] (Chem Comm., 2011, 47, 4965-4967]

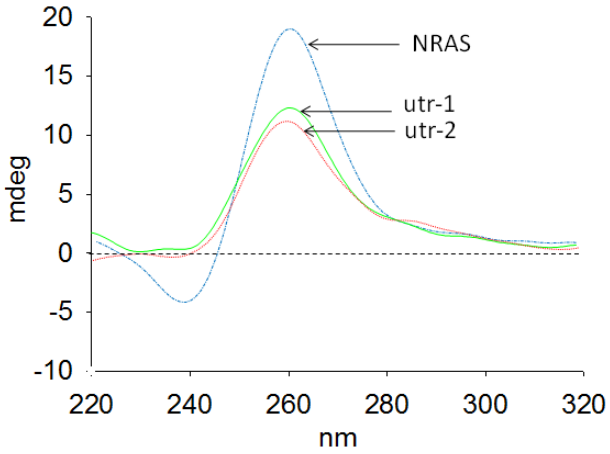


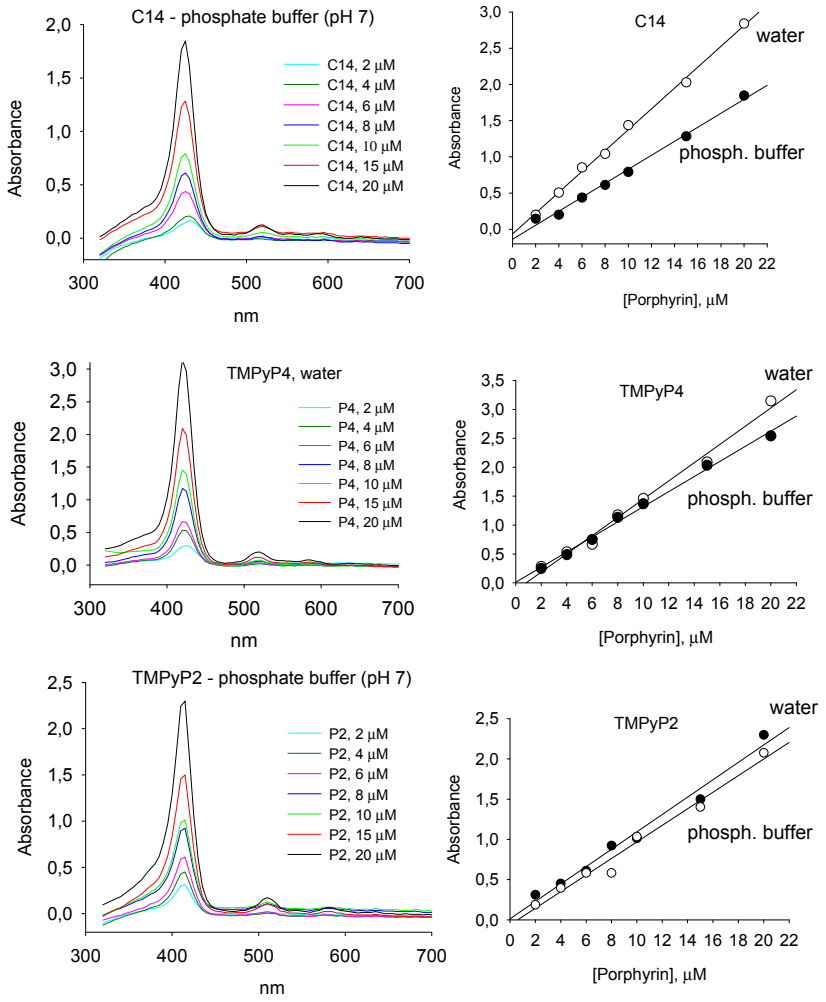
Figure S3. CD spectra of utr-1 r(GCGGCGGCGGAGG), utr-2 r(UGGCGGCGGCGAAGGU) and of the NRAS r(UGUGGGAGGGGCGGGUCUGGG) sequences located in the NRAS 5'UTR region. While NRAS forms a g4-RNA with three G-tetrads, utr-1 and utr-2 form a g4-RNA with two G-tetrads. CD spectra have been obtained in 50 mM Tris-HCl, pH 7.4, 100 mM KCl, oligonucleotide concentration of 6 µM, cuvette 0.5 cm pathlength.

Table S4: Melting temperatures of g4-RNA utr-1 and utr-2 in 50 mM phosphate buffer, pH 7, containing increasing amounts of KCl, determined by FRET-melting experiments with oligonucleotides labeled with FAM and TAMRA.

	utr-1[1]	utr-2[2]	NRAS[3]
KCl (mM)	T_M (°C)	T_M (°C)	T_M (°C)
10	59	48	72
20	61	48,5	
50	63,5	49,5	85
100	66	56	
140	67	58	>90

[1]utr-1 sequence: r(GCGGCGGCGGAGG)
[2]utr-2 sequence: r(UGGCGGCGGCGAAGGU)
[3]NRAS sequence: r(UGUGGGAGGGCGGGUCUGGG)

(a)



(b)

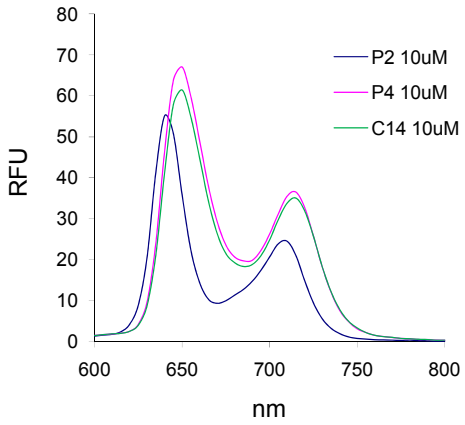


Figure S5. (a) UV-vis spectra of C14, TMPyP4 and TMPyP2 at increasing concentrations. Inset shows the Abs at 420 nm *versus* porphyrin concentration; (b) Fluorescence spectra of TMPyP2, TMPyP4 and C14. Ex 420 nm.

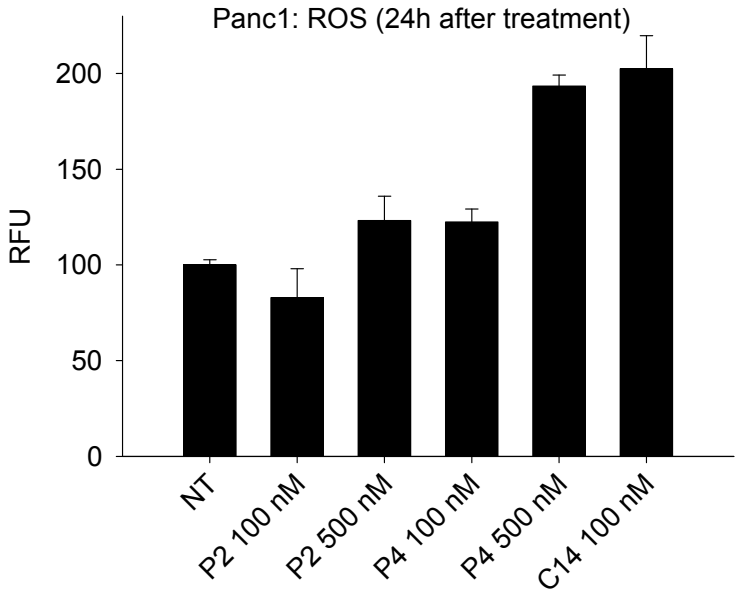


Figure S6. Levels of ROS generated in Panc-1 cells treated with porphyrins TMPyP2, TMPyP4 and C14.

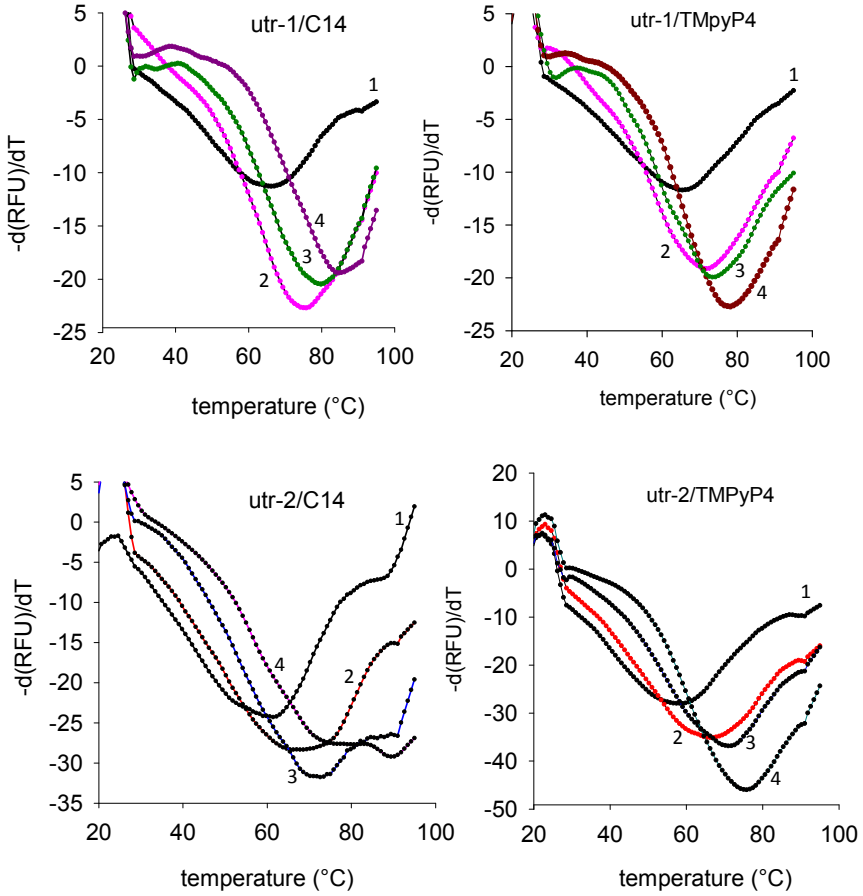


Figure S7: g4-RNA quadruplexes *utr*-1 and *utr*-2 doubly labeled with FAM and TAMRA have been analyzed by FRET-melting in 50 mM phosphate buffer, pH 7, 140 mM KCl. Curve 1= 200 nM utr-1 or utr-2 without ligand; curve 2= 200 nM utr-1 or utr-2 plus 200 nM porphyrin; curve 3= 200 nM utr-1 or utr-2 plus 400 nM porphyrin; curve 4= 200 nM utr-1 or utr-2 plus 1000 nM porphyrin.

Table S7: T_M of g4-RNA quadruplexes utr-1[1] and utr-2[1] in 50 mM phosphate buffer, pH 7, 140 mM KCl and increasing amounts of porphyrins[2] C14 and TMPyP4

C14 (nM)	G4-RNA, utr-1, T_M (°C)	G4-RNA, utr-2, T_M (°C)
0	64	56
200	74	66
400	79	71
1000	83	>75 (biphasic)
TMPyP4 (nM)	G4-RNA, utr-1, T_M (°C)	G4-RNA, utr-2, T_M (°C)
0	64	56
200	72	66
400	74	71
1000	79	76

[1]The concentration of utr-1 [r(GCGGCGGCGGAGG utr-1] and utr-2 [r(UGGCGGCGGCGAAGGU)] was 200 nM;
[2]Porphyrin TMPyP2 does not bind to the RNA quadruplexes

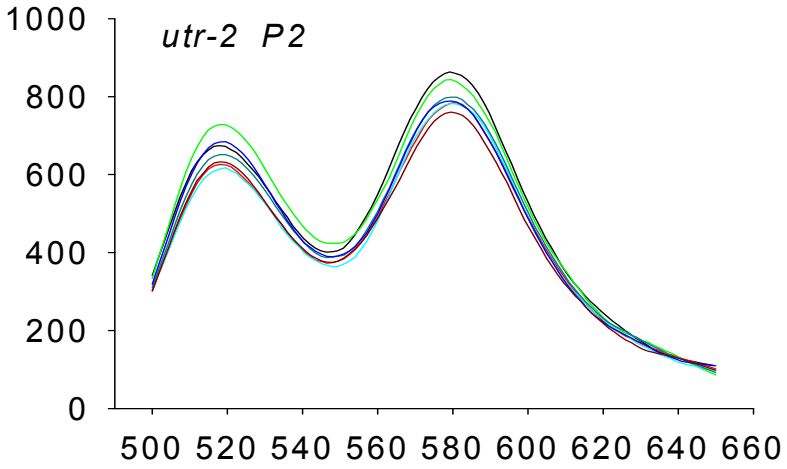


Figure S8. FRET titration of 200 nM quadruplex utr-2 in the presence of increasing amounts of porphyrin TMPyP2. (200, 400, 600, 800, 1000, 1200 and 1600 nM). Experiment carried out in 50 mM PBS, 140 mM KCl.

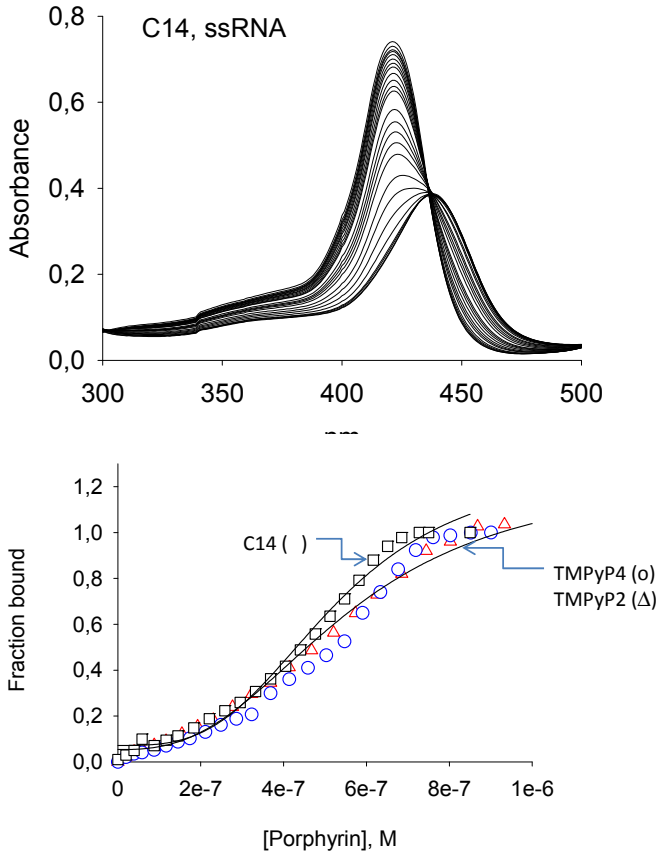


Figure S9: UV-vis titrations showing the binding between ssRNA (5'-GUUGGAGCUGAUG-GCGUAG) and the cationic porphyrins. TMPyP2 binds to ssRNA with an affinity similar to that of C14 and TMPyP4. The K_D values obtained are 5.5 x 10^{-7}, 6.3 x 10^{-7} M for C14 and P2/P4 respectively, i.e about 2-4 times higher than the K_D for quadruplex *utr*-1 or *utr*-2.

For an RNA duplex formed by 5'-GUUGGAGCUGAUGGCGUAG and 5'-CUACGCCAUCAGCUCCAAC, we obtained K_D values for C14 and TMPyP4 of 0.32 x 10^{-6}, 1.0 x 10^{-6} M, respectively (TMPyP2 shows a very low affinity for duplex DNA).

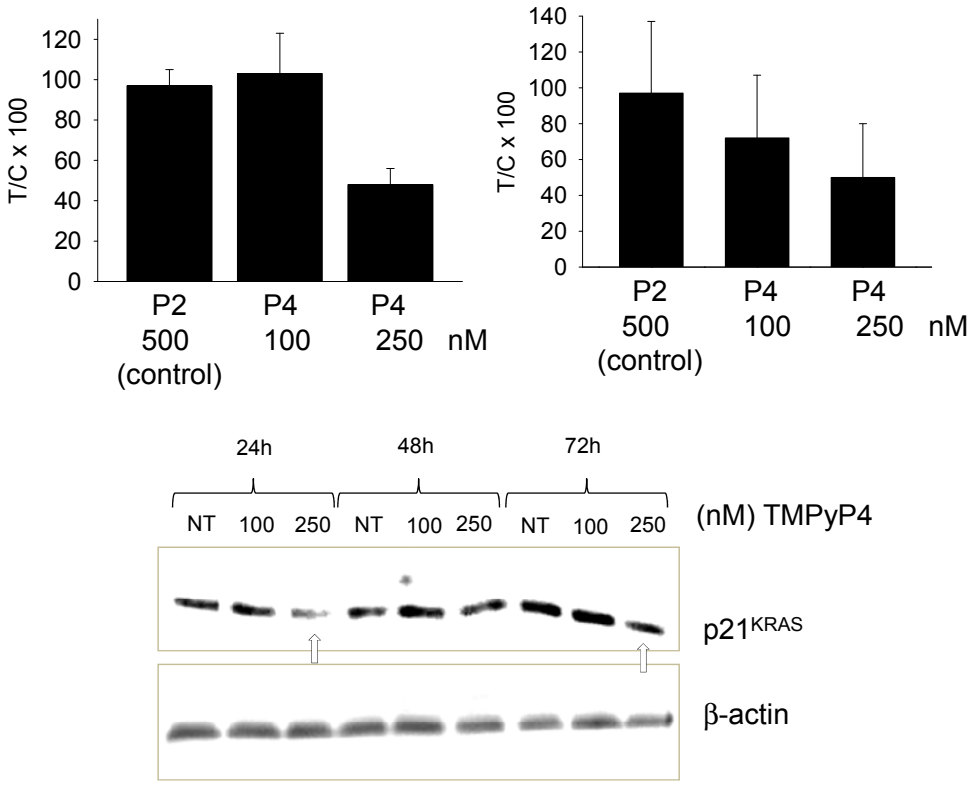


Figure S10. (Top panels) RT-PCR quantification of *KRAS* transcripts, compared to HPRT and β2-microglobulin, in Panc-1 cells treated with porphyrin (P2, P4) and light; (Left panel) analysis performed 1h after cell irradiation, right panel 6h after irradiation; (Bottom panel) Western immunoblots showing the level of $p21^{RAS}$ in Panc-1 cells untreated (NT) or treated with 100 or 250 mM TMPyP4 and light (14 J/cm^2). Compared to β-actin, the level of ras protein $p21^{KRAS}$ is reduced at 24 and also 72 h.