Identification of a New G-Quadruplex Motif in the *KRAS* Promoter and Design of Pyrene-Modified G4-Decoys with Antiproliferative Activity in Pancreatic Cancer Cells

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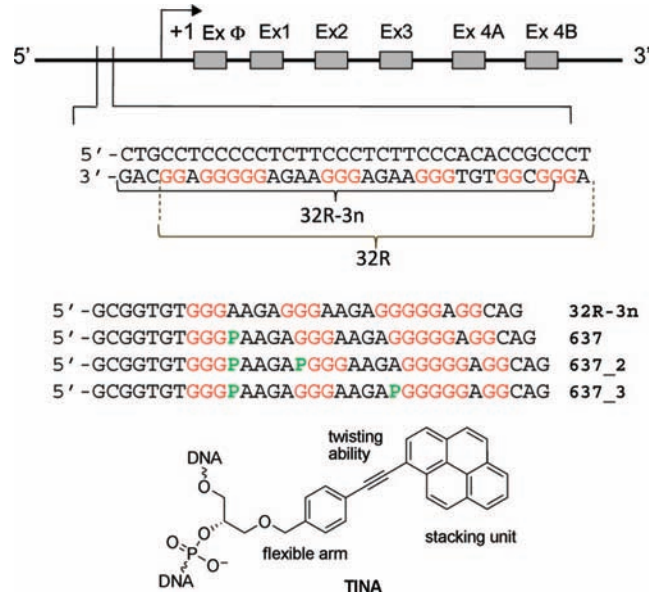
A new quadruplex motif located in the promoter of the human *KRAS* gene, within a nuclease hypersensitive element (NHE), has been characterized. Oligonucleotides mimicking this quadruplex are found to compete with a DNA−protein complex between NHE and a nuclear extract from pancreatic cancer cells. When modified with (*R*)-1-*O*-[4−1-(1-pyrenylethynyl) phenylmethyl]glycerol insertions (TINA), the quadruplex oligonucleotides showed a dramatic increase of the T_m (ΔT_m from 22 to 32 °C) and a strong antiproliferative effects in Panc-1 cells.

Introduction

The molecular pathogenesis of human pancreatic cancer involves the accumulation of genetic lesions in proto-oncogenes, tumor suppressor genes, and DNA mismatch repair genes.[1] Of particular importance are mutations in the *KRAS* gene, as they are sufficient to initiate the malignant transformation of the cells.[2] Because pancreatic cancer shows a poor response to conventional treatments,[3] one possible strategy to sensitize cancer cells to chemotherapy would be the targeting of *KRAS*.[4] We recently focused on a nuclease hypersensitive element (NHE) located in the promoter of *KRAS*, upstream of the transcription start site.[5] The G-rich strand of NHE was found to form a G-quadruplex (or G4-DNA) suspected to be involved in transcription regulation.[6,7] Further investigations suggested however that NHE is characterized by a complex structural polymorphism, going beyond the formation of only one G-quadruplex. In fact, we have identified within NHE a second G-rich motif (called **32R-3n**, Scheme 1) that forms a G-quadruplex different from the one previously described.[7] As blocks of guanines have been found to occur with a high frequency in the promoters of proto-oncogenes,[8,9] the hypothesis that G4-DNA structures might be involved in gene regulation is gaining wide support. We interestingly found that the G-quadruplexes of *KRAS* recognize nuclear proteins from pancreatic extracts which specifically bind to duplex NHE.[7] Considering that NHE is an essential element for transcription,[5] we reasoned that G-rich oligonucleotides mimicking the *KRAS* quadruplexes could be used as decoys to target the proteins that are associated to NHE and recognize G4-DNA (G4-decoys). We enhanced the stability of the G4-decoys by inserting in their sequence (**32R-3n**) one or two (*R*)-1-*O*-(4-(1-pyrenylethynyl)phenylmethyl]glycerol (TINA[a])[10] (Scheme 1). Tested in Panc-1 cells, the TINA G-decoys showed a strong and dose-dependent antiproliferative activity. Such activity correlates with the capacity of the decoy molecules to efficiently compete with a DNA−protein complex formed within NHE.

Scheme 1. G-Rich Sequence in the Human *KRAS* Promoter[a]



[a] The guanine motifs **32R-3n** and **32R** are indicated by parentheses. Oligonucleotides derived from **32R-3n** conjugated to one or two TINA units (P). The structure of TINA, (*R*)-1-*O*-[4-1-(1-pyrenylethynyl)phenylmethyl]glycerol, is reported.

Results and Discussion

In a previous study, we discovered that a guanine motif of NHE, called **32R** (Scheme 1), adopts a parallel G-quadruplex recognized by nuclear proteins.[6,7] However, further investigations showed that NHE exhibits a complex structural polymorphism. This was suggested by DNA polymerase stop assays (SI-1) and by the fact that, on shifting sequence **32R** by three nucleotides, a new guanine motif is obtained, **32R-3n**, which folds in a different way than **32R**, as indicated by CD spectroscopy (Figure 1a). Indeed, while the CD of **32R** is typical of a parallel quadruplex[11] (T_m = 72 °C), the CD of **32R-3n** with two positive ellipticities at 260 and 290 nm suggests that

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[a] Abbreviations: FAM, 6 carboxyfluorescein; TAMRA, tetramethylrhodamine; TINA, (*R*)-1-*O*-[4−1-(1-pyrenylethynyl)phenylmethyl]glycerol; PEI, polyethyleneimine.

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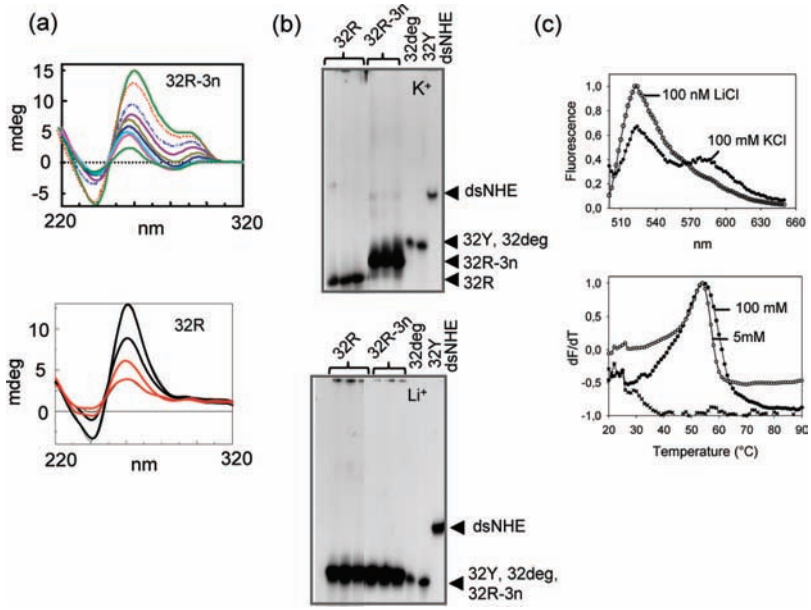


Figure 1. (a) Circular dichroism spectra at various temperatures (20−90 °C) of **32R-3n** and **32R** in 50 mM Tris-HCl pH 7.4, 100 mM KCl. (b) Native 18% PAGE of **32R**, **32R-3n** (from left: 20 nM heated 90 °C and incubated 1 h in buffer A/B; 20 and 1000 nM heated and incubated 24 h in buffer A/B) and reference oligonucleotides (20 nM in buffer A/B); 50 mM Tris-HCl pH 7.4, 50 mM KCl (buffer A) or LiCl (buffer B). (c) Fluorescence spectra of 200 nM **DL-32R-3n** in 50 mM Tris-HCl pH 7.4, 100 mM KCl or LiCl, obtained exciting FAM at 485 nm (top); d*F*/d*T* versus *T* curves of **DL-32R-3n** (Ex 485 nm; Em 530 nm) (bottom). Low curve was obtained with only buffer.

Table 1. Oligonucleotides Used in This Study (5′ → 3′)[a]

F-GCGGTGTGGGAAGAGGGAAGAGGGGGAGGCAG-T	**DL-32R-3n**
AGGGCGGTGTGGGAAGAGGGAAGAGGGGGAGGCAG	**32R**
AAApTGATpGGATpGC	**3P**
CCCCTTpTCTTTTTT	**Tc**
CCCCTTpTpCTTTTTT	**Tnc**
GCGGTGTCGCPAAGACGCAAGACGCGGAGG-CAG	**637_mut**
GCATTCTGATTACACGTATTACCTTCACTCCA	**32deg**
GCGGTGTGGGAAGAGGGAAGAGGGTGAGGCAG	**32R-3n-1T**
GCGGTGTGGGAATAGGGAATAGGGGGAGGCAG	**32R-3n-2T**
GCGGTGTGTGAAGAGTGAAGAGTGGGATGCAG	**32R-3n-4T**
CTCCGCCTCCCCCTCTTCTCTTCCACACCGC	**32Y**
AGCGCGGTGTGCGAAGAGCGAAGAGGCGGAGG	**M1**
AGCCCGGTGTGCCAAGAGCCAAGAGCCGGAGG	**M2**
ACCCCGGTGTCCCAAGACCCAAGACCCGGAGG	**M3**
TTApGGGTTAGGGTTAGGGTTAGGG	**Htelo**

[a] **P** = (*R*)-1-*O*-[4−1-(1-pyreneylethynyl)phenylmethyl]glycerol; **T** = TAMRA; **F** = FAM.

a mixed parallel/antiparallel quadruplex is formed.[12] The T_m of quadruplex **32R-3n** determined by UV and CD measurements is 49 °C (SI-2). That **32R-3n** folds into an *intra*molecular quadruplex was also seen by PAGE and fluorescence resonance energy transfer (FRET) experiments. Figure 1b shows that in 100 mM KCl **32R-3n** migrates faster than unstructured oligonucleotides of the same size (**32deg** and **32Y**) do, but slightly slower than quadruplex **32R** (Table 1). By contrast, in 100 mM LiCl, where quadruplex DNA is not stable, all 32mer oligonucleotides show the same mobility. FRET experiments were performed with **32R-3n** dual-labeled at the 5′ and 3′ ends with FAM and TAMRA (**DL-32R-3n**). In 100 mM LiCl, **DL-32R-3n** does not show any FRET between FAM and TAMRA because the two fluorophores are far from each other when the oligonucleotide is unstructured, in keeping with PAGE. Instead, in 100 mM KCl, **DL-32R-3n** folds into a quadruplex in which the two fluorophores are close to each other. Thus, upon excitation at 475 nm, emission at 525 and 585 nm is observed (Figure 1c). To see if **DL-32R-3n** folds into more than one structure, we measured FAM emission (525 nm) as a function of temperature and obtained melting curves at different oligonucleotide concentrations.[13] The fact that the curves obtained

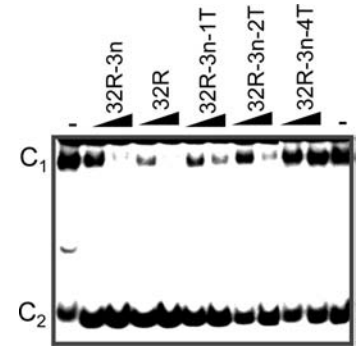


Figure 2. EMSA showing that duplex dsNHE forms with a Panc-1 extract two DNA−protein complexes: C_1 and C_2. Complex C_1 is competed by quadruplexes **32R** and **32R-3n** as well as by the mutants **32R-3n-1T**, **32R-3n-2T** that adopt a G-quadruplex, but not by mutant **32R-3n-4T** that does not. dsNHE was 20 nM, competitors in excess 50- and 100-fold.

at 0.1 and 5 μM gave one transition with a T_m = 54 °C (slightly higher than the T_m obtained by CD because of the stabilizing effect of the two fluorophores) indicates that **DL-32R-3n** folds into only one *intra*molecular structure, in 100 mM KCl. Previous studies have shown that duplex NHE, an essential element for transcription, forms with nuclear extracts from pancreatic cells two main DNA−protein complexes (C_1 and C_2).[6,7] We discovered that complex C_1 is competed specifically by **32R-3n** and **32R** in the G-quadruplex form (Figure 2). That the competition is mediated by the oligonucleotide secondary structure is demonstrated by the fact that mutants **32R-3n-1T** and **32R-3n-2T**, which maintain the quadruplex structure, competed with C_1, whereas **32R-3n-4T**, which is unable to form a G-quadruplex, did not (SI-3). This suggests that the proteins in C_1 recognize both the folded and duplex conformation of NHE. In the light of this finding, we asked if the *KRAS* quadruplex-forming oligonucleotides can behave as decoys and "sequester" proteins associated to NHE (G4-decoys). Because the protein−decoy interactions should be mediated by the three-dimensional

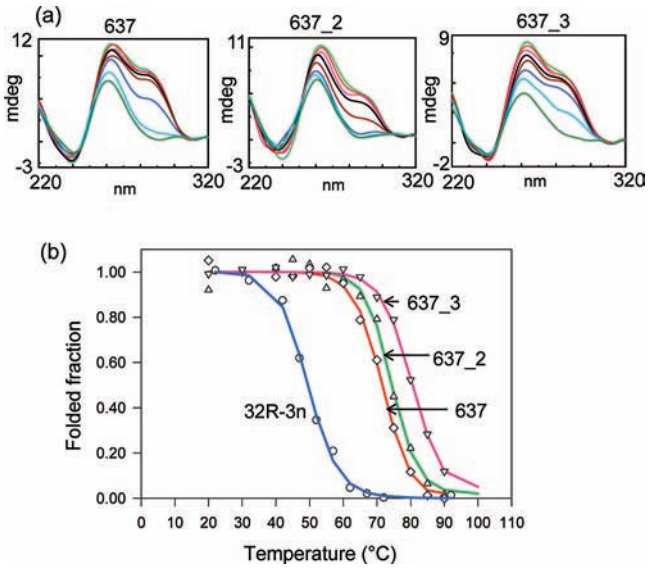


Figure 3. (a) CD spectra at various temperatures (20−90 °C) of 3 μM TINA conjugates in 50 mM Tris-HCl, pH 7.4, 100 mM KCl. (b) Fraction of folded structure versus T plots, obtained from the CD spectra recorded by renaturing the conjugates from 90 to 20 °C. Solid lines are the best-fits of the experimental points (SI-4).

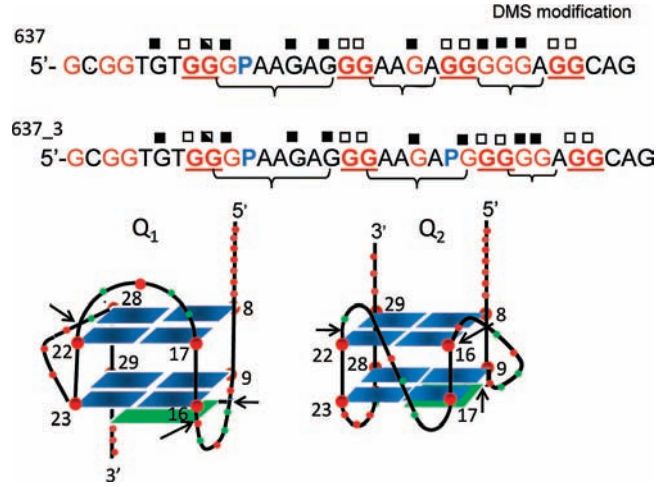
Table 2. Thermodynamic Parameters of Quadruplex Formation by **32R-3n** and TINA Conjugates in 50 mM Tris-HCl, pH 7.4, 100 mM KCl[a]

	no. TINA	T_m (°C)	ΔH (kcal/mol)	ΔS (e.u.)	ΔG^b (kcal/mol)
32R-3n		49	−45.2 ± 6	−141 ± 19	−1.8 ± 0.5
637	1	71	−53.7 ± 6	−155 ± 19	−5.6 ± 0.6
637_2	2	75	−61.9 ± 10	−179 ± 30	−6.7 ± 0.7
637_3	2	81	−60.6 ± 9	−174 ± 26	−6.8 ± 0.7

[a] Parameters obtained as described in Supporting Information. [b] ΔG calculated at 37 °C from $\Delta G = \Delta H - T\Delta S$.

structure assumed by the oligonucleotides, efficient G4-decoys should have a high stability in the cellular medium. Thus, we designed G4-decoys with the sequence of **32R-3n** and containing one or two insertions of (*R*)-1-*O*-(4-(1-pyrenylethynyl)phenylmethyl]glycerol units (TINA)[10] (Scheme 1). It is known that quadruplex DNA can be stabilized by free ligands added to the aqueous solution such as cationic porphyrins,[14] telomestatin,[15] perylene derivatives,[16] trisubstituted acridines,[17] and isoalloxazines.[18] In our study, TINA was covalently inserted in the sugar−phosphate backbone of **32R-3n**, in a position adjacent to the runs of guanines, so that the pyrene moiety could stack to the ends of the structure by means of its flexible phenylmethyl-glycerol arm (Scheme 1). We previously demonstrated that the covalent attachment of a pyrene unit either at the 5′ or 3′ ends of oligonucleotides increased the stability of the resulting duplexes by more than 17 °C.[19] In this study, we have hypothesized that a similar effect could occur with TINA stacking on the G-tetrads. To see if the TINA insertions influence the oligonucleotide folding, we analyzed by CD the conjugates in 100 mM KCl between 20 and 90 °C (Figure 3a). As they show the same type of signature as **32R-3n**, also the TINA conjugates are likely to form a mixed parallel/antiparallel G-quadruplex.[12] By plotting the 290 nm ellipticity as a function of temperature, we found that the T_m of **637**, **637_2** and **637_3** (71, 75, and 81 °C, respectively) are dramatically higher than the T_m of **32R-3n** (49 °C) (Figure 3b, Table 2). As the melting curves obtained by heating and cooling were reversible, for each quadruplex, we determined the fraction of folded species as a function of temperature. The best-fit of these data to an *all-or-none* model (SI-4) provided the thermodynamic parameters of quadruplex formation (Table 2). It can be seen that the ΔH's for **637**, **637_2**, and **637_3** (−54, −62, and −61 kcal/mol) are more negative than the ΔH of **32R-3n** (−45 kcal/mol). Moreover, the ΔS 's (−155, −178, −173 cal/mol K) are lower than the ΔS of **32R-3n** (−141 cal/mol K). These data are consistent with TINA stacking to the ends of the G-quadruplex.

Scheme 2. Summary of DMS-Footprinting Data of **637** and **637_3** (P = TINA) in 50 mM Tris-HCl pH 7.4, 100 mM KCl, and Structures of the G-Quadruplex Formed by **637**[a]



[a] The guanines underlined are involved in the G-tetrads. The loop bases are in parentheses. Guanines cleaved (full squares), uncleaved (open squares), and partly cleaved (semi-full squares) are indicated. Arrows indicate the TINA positions in the designed conjugates. Green square represents TINA in **637**. Oligonucleotide **637_3** should form similar structures, with two G-tetrads and three 6/5/3 loops.

To gain insight into the structure assumed by the TINA conjugates, DMS-footprinting experiments have been performed. The results of the cleavage patterns are summarized in Scheme 2. By combining the footprinting and CD data, we proposed a mixed parallel/antiparallel quadruplex with two G-tetrads and loop topologies as shown in Q_1 or in Q_2. As the couple of pyrenes in **637_2** generates a stronger excimer emission than the pyrenes in **637_3**, the loop topology of the quadruplexes is most likely that reported in Q_1 (SI-5). However, a conclusive structure assignment can be obtained only by NMR. As observed for **32R-3n**, TINA conjugates **637**, **637_2**, and **637_3** were also found to compete with the formation of the DNA−protein complex C_1, formed by dsNHE and Panc-1 extract (Figure 4a). Control oligonucleotides **Tc**, **Tnc**, and **3P**, containing up to three pyrenes, did not abrogate complex C_1. Direct evidence that quadruplex **32R-3n** binds to nuclear proteins was obtained by analyzing its mobility after 30 min incubation with the nuclear extract. EMSA shows that four DNA−protein complexes, B_1−B_4, are formed (Figure 4b). Interestingly, B_1 is due to a protein that specifically binds to quadruplex **32R-3n**, but not to quadruplex **32R**. In contrast, complexes B_2−B_4 are formed by both *KRAS* quadruplexes. These complexes are not competed by the unstructured oligonucleotides **M_2**, **M_3**, and **32R-3n-4T**. The fact that B_3 and B_4 are competed by dsNHE indicates that these proteins recognize both the folded and duplex conformation of NHE, in agreement with the data of Figure 2. Finally, the complex specific for quadruplex **32R-3n** (B_1) is competed by the TINA quadruplexes, but not by control **32Y** or **637_mut**, which do not assume a quadruplex conformation (Figure 4c). As **32R-3n** and TINA conjugates abrogate the formation of the DNA−protein complex C_1, which is formed in a promoter

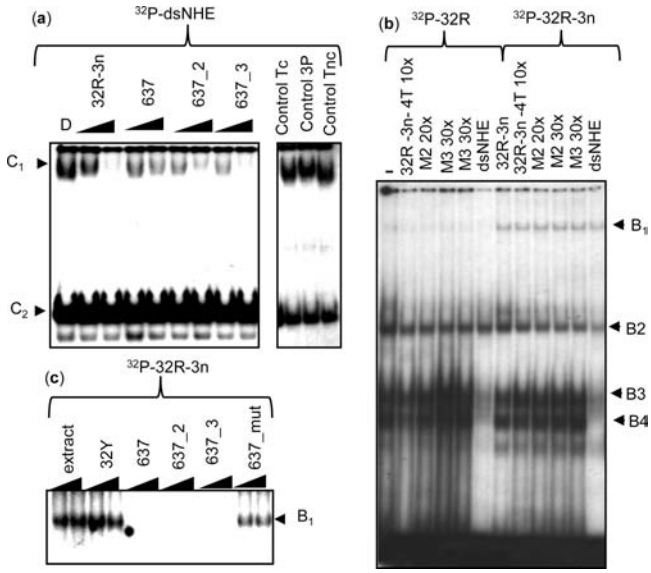


Figure 4. (a) Radiolabeled dsNHE (32R:32Y) and Panc-1 nuclear extract (3 μg) incubated for 30 min give two main complexes C_1 and C_2 by EMSA. Complex C_1 is competed by **32R-3n**, **637**, **637_2**, and **637_3**, added 50- or 100-fold in excess over dsNHE (20 nM). Control **3P**, **Tc**, and **Tnc** do not compete C_1. (b) Quadruplex **32R-3n** incubated with Panc-1 extract forms complexes B_1-B_4. Excess oligonucleotides **32R-3n-4T**, **M2**, and **M3** do not compete B_1-B_4, while dsNHE competes B_3 and B_4. (c) Oligonucleotides **637**, **637_2**, **637_3** (50- or 100-fold excess) compete complex B_1 formed by 20 nM **32R-3n** and 2 μg of Panc-1 nuclear extract.

region essential for transcription, we investigated if these G-rich oligonucleotides have antiproliferative activity in pancreatic cancer cells (Panc-1). The conjugates were delivered to Panc-1 cells by using polyethyleneimine (PEI). Flow cytometry showed that the conjugates mixed to PEI were efficiently taken up by Panc-1 cells (not shown). They were delivered to the cells in two doses of 625 nM each, one 48 h after the other, and the cell viability was measured by resazurin assay at intervals of two days (Figure 5a). The antiproliferative effect after 8 days of treatment is shown as a histogram in Figure 5b. It can be seen that all three TINA quadruplex oligonucleotides repress cell growth, compared to untreated or control treated cells. The TINA conjugates show growth inhibition between 30 and 75%. The most active compound is **637_3**: the one forming the most stable quadruplex. In a dose−response experiment, we found that the IC_{50} of **637_3** is ~500 nM (SI-6). As pancreatic cancer cells, including Panc-1, are refractory to chemotherapeutic agents, the results of this study have a therapeutic value. The activity of the TINA conjugates cannot be ascribed to pyrene moieties itself, as controls **Tc**, **B13**, and **Tnc** do not show any antiproliferative activity. There is a clear correlation between activity and oligonucleotide structure: in fact, control oligonucleotides $\mathbf{M_1}$, $\mathbf{M_2}$, $\mathbf{M_3}$, and **32R-3n-4T** that do not form a G-quadruplex are not bioactive. Moreover, **Htelo**, an oligonucleotide with the human telomeric sequence and one TINA insertion, forming according to CD spectroscopy an antiparallel G-quadruplex with $T_m = 77$ °C, does not show any antiproliferative activity. This suggests that the observed growth inhibition is due to a specific decoy effect of the oligonucleotides mimicking the *KRAS* G-quadruplex. Although the activity of the G4-decoys seems to depend on their stability (**32R-3n** forming the least stable quadruplex show little bioactivity, while **637_3** forming the highest stable quadruplex shows the highest bioactivity), further investigations are required to elucidate the structure/function relationship of these molecules.

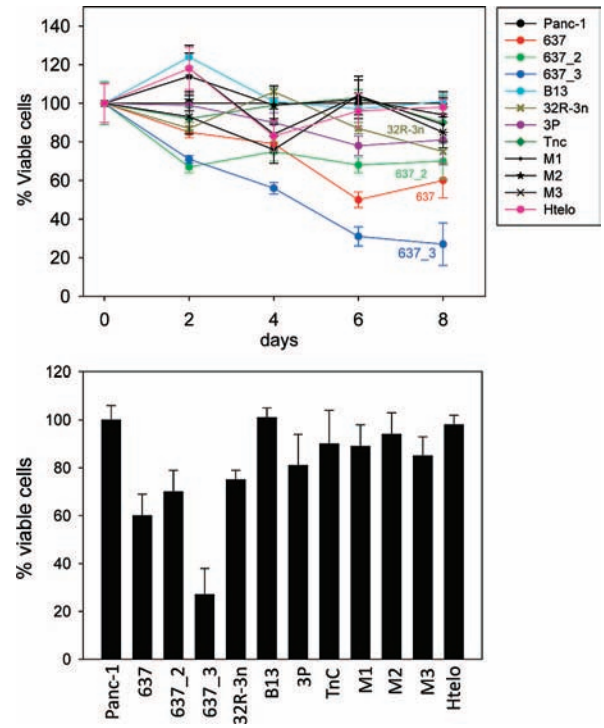


Figure 5. (top) Cell growth assay. Panc-1 cells have been twice transfected with the oligonucleotides (625 nM each treatment) in the presence of PEI. Cell viability was measured by the resazurin assay at intervals of two days. Each bar value (±SE) is the average of three independent experiments. Ordinate reports the % viable cells respect to untreated cells. (bottom) Percent viable Panc-1 cells 8 days after oligonucleotide delivery (two doses of 625 nM oligonucleotides).

Previous studies have shown that quadruplex oligonucleotides may represent a new class of antiproliferative compounds, whose biological activity results from their capacity to bind to specific cellular proteins such as nucleolin/NF-kB essential modulator[20−22] and Stat3 protein.[23] In this study, we have shown that TINA-modified oligonucleotides mimicking a quadruplex formed within a regulatory promoter element of *KRAS* inhibit proteins binding to the promoter and show a strong antiproliferative activity. The characterization of the proteins bound by the TINA-oligonucleotides is in progress.

Experimental Section

Synthesis and Purification of TINA Conjugates. TINA-modified oligo-deoxynucleotides were synthesized as previously described[10] on an Expedite nucleic acid synthesis system model 8909 from Applied Biosystems using 4,5-dicyanoimidazole as an activator. An increased deprotection time (100 s) and coupling time (2 min) for 0.075 M solution of TINA phosphoramidite in a 1:1 mixture of dry MeCN/CH_2Cl_2 were applied. The DNA synthesis was finished in a DMT-on mode. The oligonucleotides were cleaved off from the solid support (2 h, 25 °C) and deprotected (55 °C, overnight) using 32% aqueous ammonia. Purification of oligonucleotides was accomplished using a reverse-phase semipreparative HPLC on Waters Xterra MS C_{18} column. Buffer A [0.05 M triethylammonium acetate (TEAA), pH 7.4], buffer B [75% CH_3CN/25% 0.05 M TEAA, pH 7.4]. Gradients: 2 min 100% A, linear gradient to 70% B in 38 min, linear gradient to 100% B in 10 min, and then 100% A in 10 min (product peak at ~24−27 min with the maximum absorbance at 260 and 365 nm). After evaporation of the solvent, the oligonucleotides were diluted with 1.5 M aq NaOAc (150 μL) and precipitated from EtOH (550 μL). The purity of the final oligonucleotides was checked by ion-exchange chromatography using LaChrom system from Merck Hitachi on GenPak-Fax column (Waters). Basic buffer (gradient of 2 M aq

NaCl in 0.01 M aq NaOH, pH 12) was applied to disrupt secondary structures. The modified oligonucleotides were confirmed by MALDI-TOF analysis on a Voyager Elite biospectrometry research station from PerSeptive Biosystems. Calculated and experimental MW values and purity are: **637** (10744.9 [M + K^+ + Na^+]/10750.0, 100%); **637_2** (11149.3/11150.0, 100%); **637_3** (11149.3/11149.0, 100%); **637_mut** (10441.9/10440.0, 92%), **Htelo** (8042,2/8048,0, 100%). The purity of oligonucleotides **3P**, **Tc**, and **Tnc** was checked prior to their use and found to be 87, 96, and 90%, respectively. Unmodified oligonucleotides and **DL-32R-3n** have been purchased from MWG (Germany) and purified by HPLC or PAGE (7 M urea, 55 °C). Oligonucleotide concentrations were determined by UV-absorbance at 260 nm, 90 °C, and the calculated single-stranded extinction coefficients were based on a nearest neighbor model (extinction coefficient for p monomers is 22400 at 260 nm).

Preparation of Nuclear Protein Extract and EMSA. Nuclear extracts from Panc-1 cells were obtained as previously described.[7] Protein concentration was measured according to the Bradford method. Protein−DNA interactions were analyzed by electrophoresis mobility shift assays (EMSA). Five nM end-labeled duplex NHE or G-quadruplex (**32R** or **32R-3n**) were incubated in Tris-HCl 20 mM, pH 8, KCl 30 mM, $MgCl_2$ 1.5 nM, DTT 1 mM, glycerol 8%, Phosphatase Inhibitor Coktail I (Sigma) 1%, NaF 5 mM, Na_3VO_4 1 mM, poly [d*I* − d*C*] 2.5 ng/μL, nuclear extract 0.25 μg/μL, for 2 h at 37 °C. The analyses were carried out in 5% polyacrylamide gels in TBE at 20 °C.

Cell Culture and Proliferation Assay. The human cell line used is from pancreatic adenocarcinoma (Panc-1). The cells were maintained in exponential growth in DMEM medium containing 100 U/mL penicillin, 100 mg/mL streptomycin, 20 mM L-glutamine, and 10% fetal bovine serum (Celbio, Milan, Italy). Cell viability was measured using resazurin: 25 μM resazurin was added to the cell medium and the fluorescence measured after 1 h (Ex 535 nm; Em 590 nm; cut off 570 nm) with a spectrofluorometer (Spectramax, Gemini XS, Molecular Devices).

CD and Fluorescence (FRET) Experiments. CD measurements were obtained with a Jasco J-600 spectropolarimeter equipped with a thermostatted cell holder. Fluorescence measurements (Ex 485 nm; Em spectra 500−600 nm; cut off 515 nm) were carried out with a Spectramax spectrofluorometer, using a 96-well plate in which each well contained 50 μL of 200 nM dual-labeled **32R** in 50 mM Tris-HCl, pH 7.4, LiCl, or KCl. Fluorescence melting curves have been obtained with a real-time PCR apparatus (iQ5, BioRad), using 50 μL solutions of dual-labeled **DL-32R-3n** (Ex 485 nm; Em 530 nm).

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Supporting Information Available: Additional experimental details, polymerase stop assay, CD, UV and fluorescence spectra, best-fit analysis, dose−response antiproliferative data. This material is available free of charge via the Internet at http://pubs.acs.org.

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