MAZ-binding G4-decoy with locked nucleic acid and twisted intercalating nucleic acid modifications suppresses KRAS in pancreatic cancer cells and delays tumor growth in mice

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ABSTRACT

KRAS mutations are primary genetic lesions leading to pancreatic cancer. The promoter of human KRAS contains a nuclease-hypersensitive element (NHE) that can fold in G4-DNA structures binding to nuclear proteins, including MAZ (myc-associated zinc-finger). Here, we report that MAZ activates KRAS transcription. To knockdown oncogenic KRAS in pancreatic cancer cells, we designed oligonucleotides that mimic one of the G-quadruplexes formed by NHE (G4-decoys). To increase their nuclease resistance, two locked nucleic acid (LNA) modifications were introduced at the 3'-end, whereas to enhance the folding and stability, two polycyclic aromatic hydrocarbon units (TINA or AMANY) were inserted internally, to cap the quadruplex. The most active G4-decoy (2998), which had two para-TINAs, strongly suppressed KRAS expression in Panc-1 cells. It also repressed their metabolic activity (IC50 = 520 nM), and it inhibited cell growth and colony formation by activating apoptosis. We finally injected 2998 and control oligonucleotides 5153, 5154 (2 nmol/mouse) intratumorally in SCID mice bearing a Panc-1 xenograft. After three treatments, 2998 reduced tumor xenograft growth by 64% compared with control and increased the Kaplan–Meier median survival time by 70%. Together, our data show that MAZ-specific G4-decoys mimicking a KRAS quadruplex are promising for pancreatic cancer therapy.

INTRODUCTION

A large body of data obtained during the past 20 years shows that the double helix is not the only structure formed by DNA under physiological conditions. DNA is also able to assume alternative structures, in particular within sequences rich in guanine (1). One unusual structure consisting in quartets of guanines stacked on each other, called G-quadruplex or G4-DNA, has drawn the attention of several researchers, and an increasing number of studies indicate that G4-DNA acts as a transcription regulator for certain genes (2–16). A number of studies have been devoted to the human telomeric repeat (TTAGGG)n: the 3'-overhang sequence of the chromosome ends forming G4-DNA structures that stabilize the chromosome against endogenous nucleases and represent a target for anticancer drugs (17–20).

Recent bioinformatic analyses have revealed that G-rich quadruplex-forming sequences occur with a high frequency in genome regions immediately upstream of the transcription start site. This raises the hypothesis that G4-DNA may be involved in transcription regulation (21–24). The seminal study of Hurley and co-workers (3) on c-MYC provided the first piece of evidence supporting the role of G4-DNA in transcription, and this stimulated many other investigators to explore functions and properties of G4-DNA. Against this background, our laboratory has focused on the genes of the ras family, in particular KRAS and HRAS, as their mutant alleles are involved in the pathogenesis of different types of cancers, and their promoters contain G-rich elements potentially capable to fold in G4-DNA (4–7).

The promoter of the human KRAS gene contains a nuclease-hypersensitive element (NHE), which is essential for transcription (25–27). Previous studies from our group...
have shown that in the presence of potassium, the purine strand of NHE is able to fold into different G4-DNA structures recognized by several nuclear proteins, including hnRNP A1 and PARP-1 (4,5,7,10). We also found that murine analog of NHE binds to MAZ (myc-associated zinc-finger), a zinc-finger factor that activates transcription (8). We, therefore, hypothesized a decoy strategy to inhibit oncogenic KRAS in human pancreatic cancer cells. Our approach is based on the rationale that the introduction in the cells of short DNA fragments harboring the binding site of a transcription factor should compete with the binding of the transcription factor to its natural target in the promoter, with the effect of inhibiting transcription. When a decoy strategy was applied against NF-kB and STAT3, the oligonucleotides strongly inhibited transcription. When a decoy strategy was applied against NF-kB and STAT3, the oligonucleotides strongly inhibited the binding of NF-kB or STAT3 to the corresponding cis-elements (28–31).

In this study, we have hypothesized that oligonucleotides mimicking KRAS quadruplexes should sequester essential proteins and block transcription. To enhance their activity, the anti-KRAS decoy oligonucleotides should maintain the 3D structure recognized by the cognate transcription factor and be resistant to the nucleases. We, therefore, designed decoy oligonucleotide variants with terminal locked nucleic acid modifications and polycyclic aromatic hydrocarbon (PAH) insertions such as para-TINA (P), ortho-TINA (O) and AMANY (Y) (32). Moreover, we synthesized decoys where one or two loops were replaced with a MADS (M) linker (see later in the text). A previous study by Pedersen and co-workers (34) demonstrated that para-TINA stabilizes Hoogsteen-type parallel triplexes and exhibited an excellent discrimination between single- and double-stranded DNA. Later, we demonstrated that P, placed at the top of a G4-DNA, brought about a strong stabilization of the structure (10).

In an attempt to design G-quadruplexes with chemical modifications to be used as decoy molecules against the KRAS gene, we investigated the impact of PAHs on the folding, stability and potency of the designed oligonucleotides. We found that a G4-decoy with two para TINA insertions and two LNA modifications at the 3'-end strongly inhibited KRAS expression, cell growth and colony formation in pancreatic cancer cells. Moreover, 1998 delivered intratumorally in SCID mice bearing a Panc-1 tumor xenograft strongly delayed tumor growth and increased the median survival time compared with mice untreated or treated with control oligonucleotides.

MATERIALS AND METHODS

Oligonucleotides

All unmodified oligonucleotides and dual-labeled polymerase chain reaction (PCR) probes have been purchased from Microsynth (Balgach, Switzerland). The modified oligonucleotides were synthesized on a G-locked nucleic acid (LNA) support as described in Supplementary Data. Their molecular weight was confirmed by MALDI-TOF analysis on an Ultraflex II TOF/TOF system from Bruker (a MALDI-LIFT system) with HPA-matrix (10 mg of 3-hydroxyxipicolinic acid in 50 mM ammonium citrate/70% acetonitrile) (Table 1). The purities of the final oligonucleotides were found to be >85% by analytical ion-exchange chromatography with a Merck Hitachi LaChrom system on a GenPak-Fax column (Waters).

Circular dichroism

Circular dichroism (CD) spectra have been collected with a JASCO J600 spectropolarimeter equipped with a thermostatted cell holder. Oligonucleotides (3 or 6 μM) have been dissolved in 50 mM Tris–HCl, pH 7.4, 100 mM KCl, and the spectra have been recorded in 0.2- or 0.5-cm quartz cuvette, at increasing temperatures. The ordinate is expressed in millidegrees.

Cell culture, transfection and proliferation assay

Human pancreatic cancer cells (Panc-1) were maintained in exponential growth in Dulbecco’s modified Eagle’s medium (DMEM) containing 100 U/ml of penicillin, 100 mg/ml of streptomycin, 20 mM L-glutamine and 10% fetal bovine serum (Euroclone, Milano, Italy). Before oligonucleotide transfection, the cells were maintained for 48 h in DMEM with 0.25% fetal bovine serum, then they were plated and transfected by using jetPEI (Polyplus Transfection, Illkirch, France) according to the manufacturer’s in vitro protocol for DNA oligonucleotides transfection with N/P = 3 (N = nitrogen residues in the jetPEI, P = phosphate groups in the DNA). Transfection of plasmids pCMV-MAZ and pCDNA-3 (control vector) was performed by plating 10^5 cells in each well of a 24-well plate. After 24 h, the cells were transfected with 500 ng of plasmid by using JetPEI (N/P = 5). Panc-1 cells (4 × 10^4 in each well of a 24-well plate) were transfected with 6 μl of MAZ-specific siRNA (10 μM) (sc-38035 Santa Cruz) or control siRNA (sc-44230, Santa Cruz) with Metafectene SI (Biontex Laboratories GmbH, Martinsried, DE, USA) following the manufacturer’s instructions.

The metabolic activity (MA) of Panc-1 cells was measured by plating on a 96-well plate, with 4000 cells in each well, and transfecting them with 72 pmol of oligonucleotide and jetPEI (N/P = 3). MA was measured after 24, 48 or 72 h by a resazurin assay: 25 μM resazurin was added to the cell medium, and the fluorescence was measured after 1 h (Ex 535 nm; Em 590 nm) with a spectrofluorometer EnSpire 2300 Multilabel Reader (Perkin Elmer).

Recombinant MAZ, nuclear extracts and electrophoretic mobility shift assays

The preparation of recombinant MAZ and nuclear extracts was carried out as described in Supplementary Data. Protein–DNA interactions were analyzed by electrophoretic mobility shift assays (EMSA). Radiolabeled

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quadruplex 32R-3n (8 or 10 nM) was incubated with 4 µg of MAZ protein or 3 µg of nuclear extract, as indicated in figure captions, for 30 min at room temperature in 20 mM Tris–HCl, pH 8, 30 mM KCl, 1.5 nM MgCl₂, 1 mM DTT, 8% glycerol, 1% Phosphatase Inhibitor Cocktail I (Sigma), 5 mM NaF, 1 mM Na₃VO₄ and 2.5 ng/µl of poly [dI-dC] (binding buffer). The analyses were carried out in 5% polyacrylamide gels in 1× Tris–borate (TB) at 20°C.

**Western blot assays**

A total of 2.25 × 10⁵ Panc-1 cells were transfected with 595 pmol of decoys (350 nM); cells were collected in sodium dodecyl sulfate (SDS) buffer (4% SDS, 20% glycerol and 0.125 M Tris–HCl, pH6.8), and proteins were quantified. Equal amounts of proteins were separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and blotted for 2 h at 70 V in 25 mM Tris, 192 mM glycine and 20% methanol at 4°C on a nitrocellulose membrane. The membranes were incubated with a primary anti-KRAS antibody (diluted 1:250) (Abcam, ab102007) and a secondary rabbit IgG peroxidase-conjugated antibody (1:10,000) (Calbiochem). The β-actin level in each sample was measured with anti-β-actin antibody (1:10,000, CP01, Calbiochem) and a mouse IgM peroxidase-conjugated antibody (1:10,000) (Calbiochem). The antibodies were diluted in 10 mM Tris, pH 7.9, 150 mM NaCl, 0.05% Tween and 5% bovine serum albumin. The signal was developed with Super-Signal West Pico or Femto (Pierce) and detected with ChemiDOC XRS, Quantity One 4.6.5 software (Bio-Rad Laboratories, CA, USA).

**RNA extraction and real-time PCR**

RNA extraction was performed as described in Supplementary Data. Real-time PCR multiplex reactions were performed with 1× Kapa Probe fast qPCR kit for KRAS and housekeeping genes hypoxanthine–guanine phosphoribosyltransferase (HPRT) and β2-microglobulin, 200 nM of each primer, 100 nM of each probe and ~0.7 µl of reverse transcription. The PCR cycle was 3 min at 95°C, 50 cycles 10 s at 95°C and 60 s at 58°C. MAZ reactions were performed with 1× Kapa SYBR fast qPCR kit, 300 nM of each primer, 0.7 µl of reverse transcription primer. Primers are hmafoward 5’-CTCCAGTCCC GCTTCT and hma reverse 5’-GGGAGCAAGTCCAC CT. The PCR cycle was 3 min at 95°C, 40 cycles of 10 s at 95°C and 30 s at 58°C. PCR reactions were performed with a CFX 96 real-time PCR controlled by Bio-Rad CFX Manager V1.5 (Bio-Rad Laboratories, CA, USA). All expressions were normalized with housekeeping genes. The sequences of the primers and probes used for the amplifications are as follows: for KRAS, the probe is FAM-TAC TCTCTTTGACCTTGCTTG-BHQ1 (accession No. NM_033360, from 352 to 372), the sense primer is 5’-CCATGATGCTGCTTACAT (from 415 to 432, NM_033360), the antisense primer is 5’-TATATCCAACATAGAG (from 271 to 292, NM_033360) and the antisense primer is 5’-ATGTACT GGTCCTCATT (from 379 to 396, NM_033360). For β2-microglobulin, the sense primer is ROX-TATGCCTGCC TGTGAACC-BHQ2 (from 352 to 370, NM_004048), the antisense primer is 5’-CCCCACTGAAAAAGATGATA (from 333 to 350 NM_004048), the sense primer is 5’-CCCACTGAAAGATTGATA (from 379 to 396, NM_004048) and the antisense primer is 5’-CCATGATGCTGCTTACAT (from 415 to 432, NM_004048). For HPRT1, the probe is Cy5-CTTGCG TGTGAACC-BHQ2 (from 352 to 370, NM_004048) and the antisense primer is 5’-CCCACTGAAAGATTGATA (from 379 to 396, NM_004048) and the antisense primer is 5’-CCATGATGCTGCTTACAT (from 415 to 432, NM_004048). For HPRT1, the probe is Cy5-CTTGCG TGTGAACC-BHQ2 (from 352 to 370, NM_004048) and the antisense primer is 5’-CCCACTGAAAGATTGATA (from 379 to 396, NM_004048) and the antisense primer is 5’-CCATGATGCTGCTTACAT (from 415 to 432, NM_004048).

**Apoptosis assays**

Caspase activity assay was performed with Apo-ONE™ Homogeneous Caspase-3/7 Assay (Promega), according to the manufacturer’s protocol. Annexin V–propidium iodide assay was performed with Annexin V Apoptosis Detection Kit (Santa Cruz), following the manufacturer’s instructions. Cell cycle analysis was carried out in the cells (2.5 × 10⁵) treated with 360 pmol oligonucleotide. The cells have been harvested 24 h after oligonucleotide transfection and treated for 1 h at 4°C with a solution containing 70% ethanol and 30% phosphate-buffered saline (PBS). The cells were then washed with PBS and stained with propidium iodide (50 ng/µl) in the presence of RNase.
A (100 ng/μl) in PBS for 1 h. Flow cytometry measurements were performed with FACSscan (Becton Dickinson).

**Colony-forming assay**

Panc-1 cells were transfected with the G4-decoys as described earlier in the text for proliferation assays. After 18 h, the wells were treated with trypsin, and one-third of the volume of each well was seeded on 100-mm diameter plates and grown under normal culture conditions. After 1 week, the cells were stained with 2.5% methylene blue in 50% ethanol for 10 min. Colonies of >50 cells were counted.

**Double-filter DNA–protein binding assay**

This assay was performed as described previously (35). A concise description is provided in Supplementary Data.

**Human tumor xenograft model in SCID mice**

SCID mice of ~20 g were implanted subcutaneously with human Panc-1 cells (15 x 10⁶ cells) in 100 μl of physiological solution using a 23-gauge needle. Each mouse received one injection in the right flank for the development of a tumor xenograft in 6–7 weeks. Treatment with decoy and control oligonucleotides started when the tumor had a size between 30 and 50 mm³ corresponding to 30–50 mg. The mice were randomized in groups of four animals and injected intratumorally with a solution containing 2 nmol oligonucleotide, in vivo-jetPEI (Polyplus Transfection) at N/P ratio 6 and 5% glucose. Each mouse, except those of the untreated group, received three doses of oligonucleotides at Days 1, 6 and 11. The tumor growth was measured with a caliper twice a week. Tumor volume was calculated according to V = [(small diameter)² x (large diameter)]π/6 every 4 days up to 44 days since the first treatment. The tumor volume (cm³) was transformed in mass (mg), assuming a density of 1. All procedures involving animals have been approved (N. 42) by the Ethical Committee of the University of Trieste.

The data were statistically analyzed and subjected to factorial ANOVAs. Statistical significance level was set at P < 0.05. When the individual effect of the treatments and the interactions between the independent variables in a 2 x 2 ANOVA were significant, a post hoc Tukey–Kramer test for significance of the differences in the mean values was performed. For all analyses, we used the Systat package (SYSTAT Inc., Evanston, IL, USA). For survival analyses, we obtained the Kaplan–Meier curves (SPSS 11); the P values were calculated by a logrank test.

**RESULTS**

MAZ binds to G4-DNA of the KRAS promoter and activates transcription

The promoter of the human KRAS oncogene contains an NHE, which is essential for transcription (25–27). NHE is composed of six runs of guanines (G-runs 1–6 from the 5’-end) separated by ‘AAGA’, ‘TGT’, ‘A’ and ‘C’ and exhibits a complex structural polymorphism. Within the purine strand of NHE, we identified three quadruplex-forming motifs, namely, 32R, 21R and 32R-3n, each forming a stable G-quadruplex (4,5,7,10) (Figure 1 A and B). We proposed for each quadruplex a putative structure deduced from DMS-footprinting and CD data (4,5,7,10). We previously had found by chromatin immunoprecipitation that MAZ, a transcription factor recognizing blocks of guanines, whose consensus sequence is GGG(A/C)GG (36), binds to the murine analog of NHE (8). As the murine and human NHE sequences show a high homology, MAZ should also bind to human NHE, which contains two MAZ-binding sites, GGGCGG and GGGAGG, respectively, at the 5’- and 3’-ends (Figure 1C). Mobility shift experiments showed that recombinant MAZ (Supplementary Figure S1) recognizes all three G4-DNA structures of NHE, in particular the one formed by 32R-3n with which it forms a retarded band of high intensity (Figure 2A). The radiolabeled KRAS quadruplexes were also incubated with nuclear extracts from pancreatic (Panc-1) and cervical (HeLa) cancer cells. It can be seen that with both extracts, the three KRAS quadruplexes form at least two DNA–protein complexes, in keeping with the fact that several proteins were pulled down by the purine strand of NHE in the folded conformation (5).

To explore the functional role played by MAZ within the KRAS transcription, we measured KRAS mRNA in Panc-1 cells where the MAZ gene was either silenced with siRNA or overexpressed with pCMV-MAZ. The results showed that when MAZ was overexpressed, KRAS transcription was 3.5-fold upregulated compared with the control (cells treated with a non-specific plasmid) (Figure 2B). In contrast, when MAZ was silenced (residual MAZ was 10% of control), KRAS transcription was downregulated to 40% of the control (Figure 2C). Together, the data suggest that MAZ is a transcription factor that activates human KRAS transcription. This is consistent with previous data from our laboratory showing that MAZ activates the murine KRAS gene (8).

A decoy strategy to inhibit KRAS in pancreatic cancer cells

Hingorani and co-workers (37) have demonstrated that the expression of mutant KRAS in mouse pancreas is sufficient to initiate cancer. KRAS encodes for a GTP-binding protein that activates the MAPK/ERK pathway controlling cell proliferation. When protein p21RAS is mutated, it loses its capacity to hydrolyze GTP to GDP, thus remaining locked into an active state that stimulates constitutively cell growth. Increasing evidence supports the notion that KRAS is a key element in the pathogenesis of several types of cancer and a primary target for anticancer drugs. KRAS is mutated in >90% of pancreatic adenocarcinomas and in ~50% of colorectal carcinomas (38–40).

In the light of our findings, we hypothesized that the expression of KRAS in pancreatic cancer cells can be downregulated by using decoy oligonucleotides mimicking one of the potential NHE quadruplexes. The G4-decoys
given to the cells should compete with the binding of MAZ to the KRAS promoter, inhibit KRAS expression and, given the mitogenic character of the gene, also cell growth (6,10).

To sort out which NHE quadruplex the G4-decoys should mimic, we transfected Panc-1 cells with oligonucleotides 32R, 32R-3n and 21R (100, 200, 400, 800 and 1200 nM) in the presence of jetPEI as a transfection reagent (Polyplus, Transfection) and measured by a resazurin assay the MA of the cells 48 h after treatment. We found that oligonucleotide 32R-3n caused the strongest inhibition of the MA, with an IC50 of 700 ± 49 nM (data not shown). We, therefore, focused our study on the quadruplex-forming motif 32R-3n.

The affinity between quadruplex 32R-3n and recombinant MAZ was assessed by a double-filter–binding assay, as previously described (35) (Figure 2D). We incubated the radiolabeled 32R-3n quadruplex (5 nM, 100% in the folded conformation) for 1 h at room temperature with increasing amounts of MAZ in a buffer added with 2 mg/ml of poly d(I-C) (unspecific competitor). As a control, quadruplex 32R-3n was incubated with increasing amounts of two unspecific proteins: trypsinogen and ov-albumin. The percentage of quadruplex bound to MAZ was plotted as a function of protein concentration, and the curve was best-fitted to a standard binding equation (Sigma Plot 10). A KD of 320 ± 2 nM was obtained.

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The KD of recombinant MAZ for quadruplex 32R-3n was 320 ± 2 nM. The Kd of recombinant MAZ for quadruplex 32R-3n

Figure 1. (A) Sequence of the NHE present in the human KRAS promoter upstream of the transcription start site. NHE contains six guanine repeats and three quadruplex-forming motifs, named 32R (repeats 1–6), 32R-3n (repeats 2–6) and 21R (repeats 1–4); (B) CD of the quadruplex-forming sequences at 20°C and 90°C; (C) sequence homology between human and murine NHEs. Human NHE contains two binding sites [[GGG(A/C)GG] for the transcription factor MAZ at the 5'- and 3'-ends.

[Figure 1A: Diagram of the NHE sequence with MAZ binding sites highlighted.]
Figure 2. (A) Mobility shift assay showing the binding of recombinant MAZ to KRAS quadruplexes of NHE. Increasing amounts of MAZ (1, 2 and 4 μg) were incubated for 30 min in binding buffer with 10 nM radiolabeled G-quadruplex and run in 5% PAGE in TB buffer (500 V, 3 h, 20°C). The white arrows indicate the MAZ-G4 complexes. The radiolabeled quadruplexes have also been incubated with nuclear extract from Panc-1 and HeLa cells (3 μg). They form two main G4-protein complexes (P:G4). Bovine serum albumin (4 μg) was used as a control. (B) Left panel shows the level of MAZ mRNA in Panc-1 cells transfected with pCMV-MAZ; right panel shows KRAS mRNA level in the same cells. C = cells treated with an empty plasmid, T = treated cells. (C) Left panel shows the level of MAZ mRNA in Panc-1 cells in which MAZ was silenced with siRNA; right panel shows KRAS mRNA level in the same cells. C = cells treated with control siRNA, T = treated cells. Student’s t-test, **P < 0.01. (D) Filter-binding assay showing the binding of recombinant MAZ to quadruplex 32R-3n, in 50 and 100 mM KCl. Top membrane (nitrocellulose) shows MAZ [or control proteins, trypsinogen (T), ovalbumin (O)] bound to the quadruplex; bottom membrane (nylon+) shows unbound radiolabeled quadruplex. The fraction of quadruplex 32R-3n bound to MAZ is plotted against the MAZ concentration. The binding curve relative to MAZ was best-fitted to a standard binding equation. A K_D of 320 ± 2 nM was obtained.
Design of anti-KRAS G4-DNA decoys with chemical insertions

A critical issue about the use of decoy molecules is their stability in the serum and cellular environment. Indeed, when oligonucleotides with a natural sugar–phosphate backbone, as 32R-3n or TINA-modified analogs, are incubated in serum, they are degraded in few hours. We, therefore, introduced at the 3'-end of the G4-decoys two LNA modifications to make them resistant against the nuclease (Supplementary Figure S2). In addition, we increased their thermostability by introducing between G10-A11 and A21-G22 the following PAH insertions (10) (Figure 3): para-TINA (P) in decoy 2998; ortho-TINA (O) in decoy 3014; AMANY (Y) in decoy 3044. We designed decoys also with (i) P and MADS (M), a phenyl-enbis-naphthalene linker replacing either the first (decoy 3015) or the second (decoy 3043) ‘AAGA’ loop and (ii) two M linkers replacing both loops (decoy 3069). As a control, we designed two oligonucleotides with five G → C mutations that prevent them from folding into a quadruplex: the first with one P unit (5153), the second with both P and LNA modifications (5154) (Table 1).

PAGE shows that P and O insertions promote the intramolecular folding of 32R-3n (Figure 4A). Indeed, after an overnight incubation in 100 mM KCl, although 32R-3n migrates as a mixture of mono and bimolecular quadruplexes, its para- or ortho-TINA analogs (2998 and 3014) migrate only as a folded species, but characterized by different CD spectra, with ellipticity R_{290}/265 ratios of 0.83 and 1.3, respectively (Figure 4B). In contrast, compound 3044 with Y insertions migrated about half as folded and half as multimolecular G4-DNA species, mostly in the antiparallel conformation (R_{290}/265 = 1.52). When one lateral loop of 2998 was replaced with M (3015 or 3043), the resulting oligonucleotides migrated as mono- and multi-molecular species. Instead, when both loops were replaced with M (3069), the sequence migrated as bi- and multi-molecular parallel (p) quadruplexes (R_{290}/265 = 0.3).

The active G4-DNA decoys compete with the binding of MAZ to NHE

As a first screening aimed to find out which of the designed G4-decoys was biologically active, we measured their capacity to lower the MA in Panc-1 cells. The cells were treated with 600 nM G4-decoys in the presence of jetPEI, and after an incubation of 48 h, a resazurin assay was performed. The compound that mostly reduced the MA (~70% compared with control) was 2998 with P/LNA modifications. Sequence 3014 with O/LNA and unmodified 32R-3n caused a weaker decrease of the MA (~50%). Instead, a modest effect on the MA (<40%) was observed with all the other modified sequences (3044, 3015, 3043 and 3069), as well as control oligonucleotides 5153 and 5154 (Figure 4C). We then asked whether there is any relationship between CD/PAGE data and the biological activity. The following observations can be made: (i) the most active sequences are those folding into a monomolecular quadruplex (2998 and 3014) whose CD shows two positive peaks at 265 and 290 nm and a negative peak at 240 nm, typical of a quadruplex topology (45); (ii) the sequences forming a quadruplex with one or two loops replaced by M (3015, 3043 and 3069) exhibit low activity; and (iii) the sequences (3044 and 3043) folding into a quadruplex presumably in the ap conformation (R_{290}/265 > 2.9) also show low activity.

According to our hypothesis, the active decoys should mimic the quadruplex formed by 32R-3n and sequester the transcription factor MAZ. We, therefore, determined their affinity for MAZ by a PAGE competition assay (Figure 4D). It can be seen that 2998 and 3014 strongly compete with the binding of MAZ to quadruplex 32R-3n. A 10-fold excess reduces the binding, but a 50-fold excess completely eliminates it. A similar result was obtained with unmodified 32R-3n and 3044. Oligonucleotide 3044 behaved in a somewhat surprising manner because it competed with the binding of MAZ to 32R-3n without showing any impact on the MA in Panc-1 cells. As we will argue further on, this might be due to the fact that 3044 aggregates in the cell medium (Supplementary Figure S2). Control oligonucleotides 5153 and 5154 did not compete with the binding of MAZ to quadruplex 32R-3n because of their being unstructured. Interestingly, the oligonucleotides 3043, 3015 and 3069, lacking either one or two loops, were also unable to bind to MAZ, suggesting the importance of the loops in the MAZ–quadruplex interaction.

To further investigate the activity of the designed decoys, we focused on the most active compound 2998 and the least active 3044, as well as on control sequences 5153 and 5154.
Putative structure of the lead G4-decoy 2998 containing two \textit{para}-TINA insertions

Sequence 32R-3n exists as a monomer $\leftrightarrow$ dimer equilibrium and shows a CD with ellipticities at 265 and 290 nm, in 100 mM KCl (Figures 1B and 4A) (Supplementary Figure S3). When the ‘GCGG’ repeat at the 5′-end is cut-off or replaced with ‘GCAA’, 32R-3n forms only a dimeric quadruplex, whose CD is characterized by a 265 nm ellipticity (Figure 5A and B). We, therefore, attributed the 290 nm ellipticity to the monomeric quadruplex formed by 32R-3n. The intramolecular quadruplex showing ellipticities at 265 and 290 nm is that with a mixed \textit{p}/ap folded topology.

The DMS-footprinting of the monomolecular form of 32R-3n, obtained at 10 nM, shows that guanines G4, G8–G10, G15–G17, G22–G26 are protected from DMS methylation in 100 mM KCl: a typical signature of quadruplex formation (Supplementary Figure S4).

The methylation pattern is reported in Figure 5A. As the first ‘GCGG’ repeat is interrupted by a cytosine, 32R-3n should fold into a quadruplex where one base is looped out from the G-tetrad core [the nuclear magnetic resonance of a quadruplex with a bulged topology has been recently reported (46,47)]. The fact that the G22–G26 repeat is protected from DMS suggests that 32R-3n should form more than one conformer, involving G22–G23–G24, G23–G24–G25 and G24–G25–G26. However, a TMPyP4-photocleavage assay of quadruplex 32R-3n revealed a strong reactivity of G22. As TMPyP4 stacks on the G-tetrad besides binding to the loops (48,49), the G22–G23–G24 conformer is probably more abundant (Supplementary Figure S4).

When two \textit{P} insertions were introduced in 32R-3n, adjacent to a guanine repeat, i.e., between G10–A11 and A21–G22, the modified strand (2998) showed a dramatic increase of the $T_M$ from 50°C to 79°C, in 100 mM KCl.
This suggests that the pyrene chromophores stack on the external G-tetrads, thus stabilizing the quadruplex. As 2998 migrates with one sharp band and shows a CD with two ellipticities at 265 and 290 nm ($R_{290/265} = 0.83$), it should assume a mixed $p$/$ap$ conformation (Figure 5C). To corroborate the proposed structure, we synthesized a sequence variant where $P$ between G10 and A11 was shifted inside the loop (5763). Expectedly, the $T_M$ of the resulting quadruplex dropped to 63°C because one $P$ stacking was lost. In the same way, when we disrupted the guanine repeat G8–G10, either by replacing G10 with A (5765) or by shifting $P$ next to G9 (5764), the $T_M$ dropped to 63°C and 64°C, respectively, because the two variants could form only two G-tetrad quadruplexes (PAGE and CD spectra of these decoy variants are reported in Supplementary Figure S5).

Taken together, the data suggest that 2998 should form a highly stable mixed $p$/$ap$ quadruplex with two para-TINA insertions stacking on the external G-tetrads.

**G4-decoy with para-TINA insertions represses KRAS**

The level of $KRAS$ mRNA in Panc-1 cells treated with 600 nM 2998, 32R-3n, 3044 and controls 5153, 5154, was determined by quantitative real-time PCR (Figure 6A). The amount of $KRAS$ transcript was referred to the housekeeping genes $HPRT$ and $b_2$-microglobulin. It can be seen that 24 h after treatment, $KRAS$ mRNA in the cells treated with 32R-3n and 2998 was reduced to 20 and 10%, respectively, of the control (untreated cells). In contrast, the cells treated with decoys 3044, 5143 and 5154 showed $KRAS$ mRNA expression levels similar to that of the untreated cells. This result correlates nicely with the inhibition of the MA caused by the oligonucleotides. Next, we measured by a western blot analysis the level of the KRAS protein in the treated Panc-1 cells, to rule out the possibility that the observed mRNA repression was the result of a temporary fluctuation unable to effectively impact on translation. As the half-life of human p21 RAS proteins has been found to vary...
from 20 (50) to 36 h (51), the western blots were carried out at 48 and 72 h after treatment. Panc-1 cells, 48 and 72 h after transfection with decoys 32R-3n, 2998, 3044, 5153 and 5154, were lysed, and the protein extract was analyzed by immunoblotting with a KRAS-specific antibody. Figure 6B shows the results of a typical experiment. We performed three independent experiments, the average data of which are reported in a histogram. It can be seen that 2998 and 32R-3n suppress protein p21RAS to 19 ± 6 and 46 ± 21% of the control at 48 h and to 9 ± 8 and 34 ± 14% at 72 h after treatments. In accord with the MA and real-time mRNA data, 3044, 5153 and 5154 did not show any activity.

The higher inhibitory activity of 2998 compared with 32R-3n can be rationalized in terms of its stronger capacity to fold into a specific intramolecular quadruplex ($R_{290/265} = 0.9$) and better resistance against the nucleases.

**G4-DNA decoy 2998 inhibits the clonogenic capacity of Panc-1 cells**

As mutant KRAS constitutively stimulates cell growth in Panc-1 cancer cells, a reduced level of protein p21RAS is expected to inhibit cell growth and colony formation in the treated cells (10). To test this hypothesis, Panc-1 cells, treated with G4-decoys 2998, 3044, 32R-3n and control oligonucleotides 5153 and 5154, have been seeded in plates (Materials and Methods’ section). After an incubation of 7 days, the colonies were stained with methylene blue (visible colonies have at least 50 cells) (Figure 7). The data show that untreated cells form colonies that are visible with methylene blue and uniformly distributed in the plate, whereas the cells treated with 2998 form a dramatically lower number of colonies. Instead, 3044, 5153 and 5154 did not produce any significant inhibitory effect on colony formation, thanks to their incapacity to block the MA of the cells and knockout KRAS. The colonies were counted, and the results were reported in a histogram. It was found that the percentage of clonogenicity of Panc-1 cells treated with 2998 was reduced to 6 ± 1% of the control, whereas oligonucleotides 3044, 5153 and 5154 did not produce any significant inhibitory effect on colony formation, thanks to their incapacity to block the MA of the cells and knockout KRAS. The colonies were counted, and the results were reported in a histogram. It was found that the percentage of clonogenicity of Panc-1 cells treated with 2998 was reduced to 6 ± 1% of the control, whereas oligonucleotides 3044, 5153 and 5154 did not produce any significant inhibitory effect on colony formation, thanks to their incapacity to block the MA of the cells and knockout KRAS.

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G4-DNA decoy 2998 inhibits the clonogenic capacity of Panc-1 cells
assay, 2998 and, to a lower extent, 32R-3n promoted apoptosis, whereas 5153 and 5154 did not. The percentages of early and late apoptotic cells induced by the oligonucleotides 2998, 32R-3n, 5153 and 5154 are 36.1 ± 7, 30.5 ± 9, 8.4 ± 4 and 6.8 ± 2, respectively. All data are summarized in Supplementary Table S6.

**G4-decoy 2998 reduces tumor growth and increases the median survival time in SCID mice bearing a Panc-1 xenograft**

The activity of decoy 2998, which seemed to be strong in cell-based in vitro experiments, was also assessed in vivo, in SCID mice bearing a Panc-1 tumor xenograft. It should be remembered that the growth of Panc-1 cells strongly depends on KRAS expression because this gene is mutated in exon 1; thus, it produces a constitutively active p21Kras protein that stimulates cell growth (37,38). SCID mice bearing a subcutaneous Panc-1 tumor xenograft of ~30–50 mm³ were randomized into four groups of four mice each. They were intratumorally injected with a 50-μl solution containing 2 nmol oligonucleotide/mouse and in vivo-jetPEI. This treatment was repeated three times (at Days 1, 6 and 11), and the tumor size was measured every 4 days with a caliper. Apart from the untreated group (Group 1, treated only with 5% glucose), the other groups were treated with 2998 (Group 2), control oligonucleotides 5153 (Group 3) and 5154 (Group 4), which both are not able to fold into a quadruplex thanks to their five G → C mutations. The data of Figure 9A show that from Day 13 after the first treatment, the G4-decoy 2998 strongly reduced the growth of the tumor xenograft as compared with the non-treated or control-treated groups (*P < 0.001). Typical images of an untreated and a 2998-treated mice are reported in Supplementary Figure S7. In Figure 9B, we report the Kaplan–Meier survival curves showing that the median survival times of the various groups was Group 1 (untreated) 60.5 days, Group 2 (treated with 2998) 103.5 days, Group 3 (treated with 5153) 71 days and Group 4 (treated with 5154) 71.5 days. The median survival time of the mice treated with 2998 increased by 70% compared with the control groups (*P < 0.006).

Together, our data indicate that oligonucleotide 2998 is an effective transcription factor G4-decoy that shows in vitro a marked capacity to repress KRAS and cell growth in Panc-1 cells. Furthermore, in vivo, it strongly delays the growth of a Panc-1 xenograft in SCID mice and significantly increased the median survival time of the mice treated with 2998.

**DISCUSSION**

In this study, we have tested the capacity of chemically modified G4-DNA decoy oligonucleotides to knockout the KRAS oncogene in Panc-1 cells—the primary genetic lesion responsible for the malignant transformation of pancreatic cells (38–40)—and to delay in vivo the growth of a Panc-1 tumor xenograft in SCID mice. The decoy approach, which has been introduced 2 decades ago, provides a powerful tool to target the genetic roots of a disease. The term decoy is often confounded with
Figure 8. (A) Apo ONE assay showing caspase 3/7 activation in Panc-1 cells 30 h after treatment with decoy and control oligonucleotides. (B) Cell cycle analysis of Panc-1 cells 24 h after treatment with decoy and control oligonucleotides. The cells were stained with propidium iodide and analyzed by flow cytometry. (C) Propidium iodide/annexin V assay for the detection of early and late apoptotic Panc-1 cells, 40 h after transfection with the control and decoy oligonucleotides.
their action is associated to protein binding has already been provided. For instance, nucleolin plays a role in mediating the effect of the well-known quadruplex-forming aptamer AS1411 (68). In the present study, we demonstrate that the designed G4-decoys exhibiting an anti-proliferative effect in pancreatic cancer cells (10) bind to transcription factor MAZ, thus subtracting from the Kras promoter a protein essential for transcription activation.

MAZ was first identified as a GA-box–binding transcription factor in the c-Myc promoter that controls transcription initiation and termination (69). Its functional role in the context of transcription is complex because some genes are activated by MAZ (36,70–73), and others are repressed (74,11). Here, we provide evidence that MAZ activates transcription of the human Kras gene. A similar behavior was already observed with murine Kras (8).

To inhibit the Kras oncogene in pancreatic cancer cells, we designed anti-MAZ decoys with the sequence of 32R-3n, but containing two types of chemical modifications: LNA modifications at the 3′-end and PAH insertions at specific positions. LNA nucleotides have an extra connection between the 2′ oxygen and 4′ carbon, thus forming a bridge that ‘locks’ the ribose in the 3′-endo conformation. This makes the quadruplex-forming oligonucleotides resistant against nucleases. Indeed, although quadruplex 32R-3n or 32R-3n with P insertions is degraded in serum, their analogs with two LNA at the 3′ show a fairly good resistance. Previous studies demonstrated that the introduction of P at the ends of duplex or quadruplex DNA strongly increases the Tm (10,75). An interesting effect of TINA (P or O) is its capacity to favor an intramolecular folding of the strand in which they have been inserted. Indeed, we found that decoy 2998 with P and decoy 3014 with O migrate in a polyacrylamide gel as monomolecular species only, whereas the other decoys with Y or M modifications form mono- or multimolecular species. In addition, 3044 with Y insertions showed a high propensity to aggregate in vitro. It is possible that in the crowding intracellular conditions, this decoy aggregates even further, with the result that its decoy potency is severely compromised.

Although the PAH units introduced in sequence 32R-3n enhanced the thermostability of the resulting quadruplex (PAH-containing quadruplexes have Tm > 70°C; data not shown), only 2998 with two P insertions showed strong activity, in vitro and in vivo. DMS-footprinting, CD and a sequence variant analysis suggest that 2998 should adopt a 3 G-tetrad mixed p/ap quadruplex with P stacked on the external G-tetrads. In a previous work, we proposed for an analog of 2998, devoid of LNA modifications at 3′-end, a mixed p/ap quadruplex with 2 G-tetrads. The apparent incongruence is probably because of the fact that the TINA insertions interfere with DMS-footprinting (10).

The Ras family of oncogenes is mutated in ~30% of all human cancers (76). Kras is mutated at codon 12 in ~80% of pancreatic adenocarcinomas, 50% of colorectal carcinomas and 40% of non-small cell lung cancer (38–40). As previous studies have shown that mutant

aptamers, but the two molecules are conceptually different. Aptamers are DNA or RNA oligonucleotides that bind specifically to a given target and are usually obtained from a synthetic combinatorial library by exponential enrichment iterative methodology (SELEX) (54). Instead, decoys are DNA or RNA fragments with a naturally occurring sequence—normally a protein-binding site—whereas the other decoys with Y or M modifications form mono- or multimolecular species. In addition, 3044 with Y insertions showed a high propensity to aggregate in vitro. It is possible that in the crowding intracellular conditions, this decoy aggregates even further, with the result that its decoy potency is severely compromised.

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KRAS is sufficient to initiate cancer in rat pancreas (37). KRAS represents an important target for anticancer drugs. Pancreatic cancer cells are dependent on mutant ras functions, and the downregulation of KRAS has been demonstrated to affect cell growth (52,77). This notion is supported by previous studies from our laboratories (78) and also comes from a recent study where mutant KRAS expression was knocked down by adenovirus-mediated siRNA (52). All these studies demonstrate that the inhibition of activated RAS protein increases apoptosis. The results of the present study are consistent with the aforementioned observations. In particular, we found that KRAS suppression mediated by the G4-decoy 2998 strongly decreases cell growth and colony formation of Panc-1 cells. As already found in non-small lung cancer cells, the knockdown of ras in Panc-1 cells promotes apoptosis, as indicated by annexin V and propidium iodide, caspase 3/7 and cell cytomtery assays. Our para-TINA and LNA-modified G4-decoy 2998 shows a remarkable capacity to slow down the growth of a Panc-1 tumor xenograft in nude mice. After three treatments, tumor growth was completely arrested up to Day 25. At longer time points, the tumor xenograft initiated a steady growth but at a lower rate than the controls did. This suggests that on increasing the number of treatments, it should be possible to arrest completely tumor growth over a longer period. As we expected, control 5153 and 5154 showed an unspecific antitumor effect that vanished at longer time points. This is likely because of the presence of guanines in these oligonucleotides (65,66).

In summary, we have developed an anti-MAZ G4-decoy oligonucleotide with locked nucleic acid and twisted-intercalating nucleic acid modifications that mimics a quadruplex structure formed by a critical KRAS G-element. When this engineered oligonucleotide is transferred into the cells, it knocks out KRAS by a putative mechanism based on the sequestration of MAZ. Thanks to its chemical modifications, the decoy is rather resistant to the nucleases and produces a strong effect in pancreatic cancer cells, as well as in a tumor xenograft. Caspase 3/7, FACS and annexin V–propidium iodide assays showed that the inhibitory effect promoted by decoy 2998 is mediated by apoptosis. The results of our study suggest that the G4-decoy 2998, engineered with LNA and TINA modifications, may have potential in treating pancreatic cancer or sensitizing tumor cells to chemotherapy.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Materials and Methods, Supplementary Table 1 and Supplementary Figures 1–7.

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