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Rationally Induced RNA:DNA G-Quadruplex Structures Elicit an Anticancer Effect by Inhibiting Endogenous eIF-4E Expression

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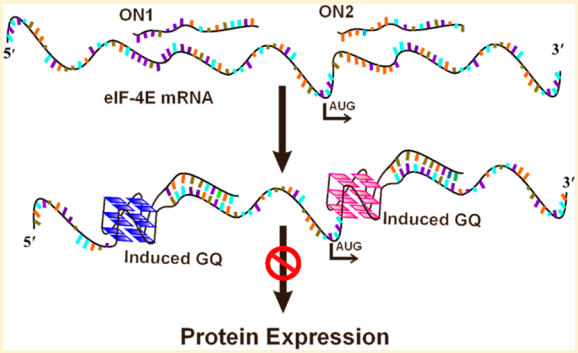
Supporting Information

ABSTRACT: RNA G-quadruplex (GQ) structures act as regulators of a diverse array of cellular processes including translation, pre-mRNA processing, and mRNA targeting. We report here a strategy of harnessing the natural ability of RNA GQs to inhibit translation by rationally inducing a GQ on a targeted mRNA to knockdown endogenous gene expression. We chose to target eIF-4E because of its key role in translation initiation and overexpression in multiple cancers and with the expectation that downregulation of eIF-4E would result in antiproliferation of cancer cells. Targeted hybrid (RNA:DNA) GQ structures were induced at the 5′-untranslated region (UTR) and the protein coding region of the eIF-4E mRNA by rationally designed and partially modified extraneous DNA sequences and their effect on eIF-4E expression was determined. The formation of a stable induced G-quadruplex was established by biophysical and biochemical methods. Thermodynamic parameters calculated from CD melting indicate formation of a stable induced GQ at a physiologically relevant salt concentration. We established the specificity and efficacy of the induced GQ formation by monitoring the targeted repression of a reporter gene. Most importantly we have demonstrated that inducing GQ in the 5′-UTR and the protein coding region of eIF-4E mRNA in human cancer cells results in 30% and 60% inhibition of the endogenous protein expression, respectively. Treating with the GQ inducing oligonucleotide sequences resulted in a decrease in the viability of human cancer cells in a dose-dependent manner. The above concept opens up a new strategy for targeted modulation of endogenous gene expression.



RNA G-quadruplexes (GQs) have been associated with several functional roles, such as telomere maintenance, transcription termination, pre-mRNA processing including splicing and polyadenylation, RNA turnover, and mRNA targeting.[1] RNA GQ structures in the 5′-UTR are functionally significant in repressing translation of several pathologically important genes such as NRAS, MT3-MMP, TRF2, ERS1, THRA, and BCl-2.[2−7] The role of GQ structures in the 5′-UTR of mRNA in regulation of translation generally varies with the position and the stability of the structures.[8−10] The presence of a GQ in the 5′-UTR has been mostly observed to repress translation with the exception of transforming growth factor β2 (TGFbeta2)[11] and when present in the IRES.[12,13] Recently it was reported that RNA GQ structures within the protein coding sequence of hERα repress translation which results in expression of a truncated protein that readily undergoes proteolysis.[14] Therefore, the wide range of functional roles of RNA GQ structures makes them attractive therapeutic targets, and indeed they have been targeted by several small molecule ligands. For example, small molecules have been used to modulate translation either by disrupting the RNA GQs to revert their repressive effects or stabilize the GQs to enhance repression.[3,15−18] However, the targeting of RNA GQs by small molecules for regulation of specific genes is rather challenging due to the low level of structural variation within RNA GQs, as they are known to almost exclusively adopt parallel conformation.[8] Hence additional novel and rational design approaches are crucial for targeting specific endogenous RNA GQs.

GQ structures are formed when two or more G-quartets stack upon each other and in the center the oxygen lone pair of the carbonyl groups coordinates with metal cations, typically K^+.[19] A G-quartet is formed by four guanine bases arranged in a square planar pattern with Hoogsteen hydrogen bonding. The formation of an intramolecular GQ requires at least four stretches of guanines with each containing two or more contiguous guanine residues. While unimolecular GQs require four stretches of Gs provided by a single strand, bimolecular GQs need only two stretches of guanines in a strand for interaction with a targeting strand, which would provide in trans two additional guanine stretches. Therefore, a bimolecular GQ structure could be selectively induced in a target mRNA sequence to repress translation if it is strategically located, such as within the 5′-UTR or the protein coding region of an mRNA (Figure 1) and contains two stretches of Gs. A previous report described the use of a short G-rich RNA (5′-GGGCCCGGG-3′

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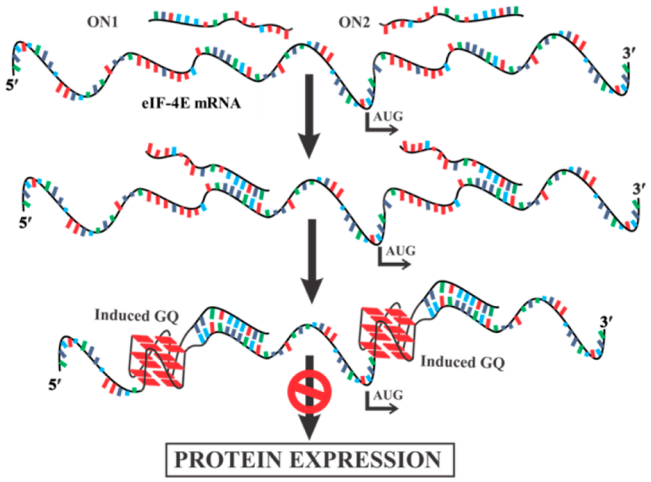

Figure 1. Schematic representation of targeted induction of GQ in eIF-4E mRNA in the 5′- UTR and the protein coding region.

and 5′-GGGUUAGGG-3′) to inhibit expression of an engineered reporter gene via formation of an intermolecular RNA GQ in the 5′-UTR and in the coding sequence.[20] However, such an approach potentially can be extremely nonspecific given the high propensity of occurrence of the sequence $G_{(3-4)}N_{(1-5)}G_{(3-4)}$ in the transcriptome where N can be any nucleotide. In another report inducing the formation of a GQ structure in a target RNA sequence with a guanine-tethered oligonucleotide has been observed to block the reverse transcriptase activity *in vitro*,[21] where the mechanism of action is rather unclear.

Human eukaryotic initiation factor-4E (eIF-4E) is an essential factor for translation initiation which often is designated as an oncogene and is overexpressed in several cancer cell types.[22−24] It is a component of the eIF-4F complex, and by binding to the 7-methyl guanosine cap structure at the 5′-end of mRNAs, it provides the critical interface between mRNA, recruitment of eIF-4A and eIF-4G, and the 40S ribosomal subunit.[25] The eIF-4E is the least abundant among translation initiation factors, thereby acting as the rate limiting agent in translation initiation. Additionally eIF-4E repression by drugs, antisense oligonucleotides, and siRNA inhibits growth in various cancer cells such as gastric, breast carcinomas, and mesothelioma cells, which makes it a good target for downregulation of its expression for therapeutic application.[26] The eIF-4E mRNA harbors sequences both in the 5′-UTR and in the coding sequence, which are ideal targets for rational induction of GQs. We designed a bifunctional oligonucleotide that contained (i) two G-stretches of three contiguous guanines each and (ii) a segment that would guide the G-stretches to its target mRNA.

Our studies on inducing GQ structures within the 5′-UTR and the coding region of eIF-4E mRNA showed repression of eIF-4E expression in human cervical cancer cells with a concomitant decrease in cancer cell proliferation. Targeted induction of GQ can be a powerful strategy for selective regulation of gene expression for therapeutic application.

EXPERIMENTAL PROCEDURES

In Vitro Transcription of a Segment of 5′-UTR of Human eIF4E mRNA. A 36-nucleotide fragment of the eIF-4E mRNA was *in vitro* transcribed and purified by 15% denaturing polyacrylamide gel electrophoresis (PAGE). The RNA band was harvested via the crush and soak method by tumbling the gel slices at 4 °C in elution buffer (300 mM NaCl, 10 mM Tris-HCl (pH 7.4), and 0.1 mM EDTA). The eluent was phase concentrated using 2-butanol and subsequently precipitated from the aqueous phase by ethanol. The RNA pellet was dissolved in 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA unless mentioned otherwise.

DNA Oligonucleotides. Modified and unmodified DNA oligonucleotides for in vitro characterization and cell studies were purchased from Integrated DNA Technologies, Inc.

Folding of the Oligonucleotides To Form Induced GQ. The induced GQ structure was formed by mixing equimolar amounts of RNA and ON1 in 150 mM KCl or LiCl, 10 mM Tris-HCl, and 0.1 mM EDTA (pH 7.5). The target RNA and ON1 were also folded individually in the same buffer and salt concentrations. The solutions were heated to 95 °C and cooled to 25 °C with a gradient of 15 °C/10 min in a thermocycler.

Circular Dichroism (CD) Spectroscopy. The CD spectra were obtained with a Jasco J-810 spectropolarimeter using a cell with 0.1 cm path length. The oligonucleotides and induced GQ were folded as mentioned above. The spectrum of only buffer was subtracted from the individual samples for background correction. For nuclease digestion assay induced GQ structures formed in 150 mM K^+ were treated with 3 units of DNase 1 and RNase T1, and the CD spectra were observed after every 5 min for 15 min at 37 °C. The melting curves indicate a change in molar ellipticity at 263 nm by varying the temperature from 25 to 97 °C at a rate of 20 °C per hour in the CD spectropolarimeter. Thermodynamic parameters and T_m values were calculated using the van't Hoff method.[27]

Radiolabeling of RNA and DNA Oligonucleotides. The RNA and DNA oligonucleotides were radiolabeled using the same method as described before.[4] In brief, the phosphate on the 5′- end of the *in vitro* transcribed RNA was enzymatically removed by calf-intestinal alkaline phosphatase (CIP, NEB). The CIP treated RNA and commercially obtained DNA were 5′-end radiolabeled by T4 polynucleotide kinase (PNK, NEB), [γ-^{32}P] ATP (PerkinElmer). The radiolabeled full-length RNA and ON1 was isolated by 15% denaturing PAGE and then extracted as described previously.

Native Gel Electrophoretic Mobility Shift Assay. The 5′-end radiolabeled RNA, DNA and RNA:DNA (10000 cpm) were mixed with 40 pmol of unlabeled samples in 150 mM KCl and 150 mM LiCl in 10 mM Tris-HCl pH 7.5 and 0.1 mM EDTA in a total volume of 10 μL. The oligonucleotides were folded and the induced GQ was formed using the same procedure as mentioned above. The complexes were resolved by 13% native polyacrylamide gel electrophoresis at 5−6 °C in Tris-borate-EDTA buffer. The gel was exposed to a phosphorimager screen and then visualized by Typhoon Phosphorimager FLA 9500 (GE Healthcare, Life Sciences).

RNase T1 Footprinting. The 5′-end radiolabeled RNA was dissolved in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA in the presence of 150 mM KCl or 150 mM LiCl and unlabeled ON1 in increasing concentrations (0−5 μM) were folded as mentioned above. Once reactions attained the appropriate temperature, the RNA was digested with 0.02 U of Ambion RNase T1 (Life Technologies) for 1 min at 37 °C. The reactions were terminated by using an equal volume of stop buffer (7 M urea, 10 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA). Treated RNA was electrophoresed on a 12% denaturing PAGE, dried on Whatman paper, and exposed to

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a phosphorimager screen and visualized by a Typhoon Phosphorimager FLA 9500 (GE Healthcare, Life Sciences).

Dimethyl Sulfate (DMS) Footprinting. The 5′-end radiolabeled ON1 was prepared in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA in the presence of 150 mM KCl with 5 μM unlabeled DNA. The induced GQ structure was formed as mentioned before with equimolar unlabeled RNA. The samples were treated with 1% DMS at room temperature for 2 min. The reactions were stopped by adding stop buffer (2 M β-mercaptoethanol, 300 mM sodium-acetate, 250 μg/mL salmon sperm DNA) in an 11:1 ratio and ethanol precipitated. The DNA pellets were then dried and cleaved by the addition of 70 μL of 10% piperidine and incubating at 90 °C followed by removal of the piperidine by vacuum drying. The pellets were dissolved in urea loading buffer, and the fragments were separated on a 15% denaturing polyacrylamide gel. The gel was dried and exposed to a phosphorimager screen and then visualized by scanning the screen on a Typhoon Phosphorimager FLA 9500 (GE Healthcare, Life Sciences).

Plasmid Construction. A 43-nucleotide segment of the 5′-UTR of eIF-4E mRNA was inserted into the psiCHECK-2 plasmid upstream of the Renilla luciferase gene (hRluc) by insertion mutagenesis, and the plasmid henceforth will be referred to as psiC2-4E-UTR (Figure 4a). Primers were designed and ordered from Integrated DNA Technologies (IDT). Insertion mutagenesis was performed by QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Tech) as per manufacturer's protocol, and successful insertion into the plasmid was confirmed by sequencing at The Plant Microbe Genomic Facility (Ohio State University). The hVEGF-Bicis plasmid was constructed as previously mentioned.[12]

Reporter Gene Assay. HeLa cells were grown in 96-well plates in Dulbecco's modified Eagle's medium (DMEM) with low glucose (HyClone) supplemented with 10% fetal bovine serum and 1% antibiotics streptomycin and penicillin at 37 °C in 5% CO_2 in a humidified incubator. The plasmids psiC2-4E-UTR and hVEGFBicis were transfected by Lipofectamine 2000 as per manufacturer's protocol for 8 h and then treated with 25 μM of ON1 and SW. After 24 h of treatment of the ON1 dual luciferase assay was performed with Dual-Glo Luciferase Assay system as per manufacturer's protocol, and the results were read by the SpectraMax M4 plate reader (Molecular Devices, LLC). In both cases, psiC2-4E-UTR and hVEGFBicis, the ratios of Renilla (L_R)/Firefly(L_F) luciferase expression were normalized with untreated expression levels.

Quantitative RT-PCR. HeLa cells were grown in six-well plates in DMEM as described above. DNA oligonucleotides (25 μM) were added while seeding the cells. Total cellular RNA was extracted from HeLa cells using a TriPure Isolation Reagent (Roche Applied Sciences) as per manufacturer's protocol. The cDNA was synthesized using qScript cDNA SuperMix (Quanta Biosciences). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and eIF-4E mRNA were subjected to qRT-PCR using a Perfecta SYBR Green Super Mix (Quanta Biosciences) on an Eppendorf Mastercycler RealPlex2 in the presence of appropriate set of primers. The relative mRNA levels were estimated by the comparative C_t method (Livak method).

Western Blotting. HeLa cells were grown as above and treated with 25 μM oligonucleotides for 24 h. Proteins were extracted from the cells with RIPA buffer (sc-24948, Santa Cruz), 50 μg of protein lysate was separated by 12% SDS–PAGE. The proteins were detected by mouse monoclonal eIF-4E antibody (P-2, sc-9976) at 1:200 dilution and GAPDH (G-9, sc-365062) antibody at 1:5000 dilution. Horseradish peroxidase-conjugated goat antimouse IgG (sc-2005) was used as secondary antibody at 1:1000 dilutions. Proteins were visualized by Western Blotting Luminol Reagent (sc-2048) in ChemiDoc-ItTS2 Imager (UVP, LLC)

Cell Viability Assay. HeLa cells were seeded in a 96-well plate at 10 000 cells per well and treated with increasing concentrations of the oligonucleotides and incubated for 24 h. CellTiter 96 AQueous Non-Radioactive Cell Assay (Promega) was used to assess the cell viability by measuring the absorbance at 490 nm, as per manufacturer's protocol. The cell viability percentages calculated were normalized with untreated controls. The data were fitted using a dose–response curve with variable Hill slope to determine the EC_{50} value in Origin 8.

RESULTS

RNA and DNA Oligonucleotides Associate To Form a Parallel Induced Hybrid GQ Structure. In order to determine the formation of induced GQ structures *in vitro*, we chose a fragment of the 5′-UTR of eIF-4E, which had the requisite characteristics to participate in induced GQ formation. A 36-nucleotide fragment from the human eIF-4E mRNA which included a GGGAGGG sequence was used for the initial biophysical and biochemical characterizations. The targeting DNA oligonucleotide ON1 (Table 1) and the target RNA were mixed in equimolar amounts (5 μM) in the presence of physiologically relevant K^+ ion concentration (150 mM), which was followed by heating and slow cooling to form possible higher order structures. The sharp increase in molar ellipticity (55%) at 263 nm and a significant decrease (50%) at 240 nm of the CD spectrum (Figure 2a) in the presence of an equimolar amount of DNA and RNA is indicative of the formation of an induced parallel hybrid (RNA:DNA) GQ. The formation of the induced GQ is further alluded to by the fact that when individual ellipticity values of 5 μM DNA and 5 μM RNA were added, it showed a considerably lower intensity than the highest observed intensity of the induced GQ spectrum at 263 nm (Figure 2a). Parallel RNA:DNA hybrid GQ structures have been observed before[21] and might be due to the involvement of the RNA strand, as RNA GQs always adopts parallel GQ structures.[28] The CD spectra also suggest that the target RNA alone forms some higher order structure, which can potentially be due to formation of an all RNA intermolecular GQ.

Table 1. DNA Oligonucleotide Sequences Used in the Study[a]

Modified DNA oligonucleotides used for cell studies	
ON1	C*G*TTTAGGCAATCAA GGGAG*G*G
Scrambled Quadruplex 1 (SQ1)	C*G*TTTAGGCAATCAA GACCG*A*C
ON2	A*C*CTTTCCTTGTATA GGGAG*G*G
Scrambled Quadruplex 2 (SQ2)	A*C*CTTTCCTTGTATA GACCG*A*C
Scrambled Duplex (SD)	N*N*NNNNNNNNNNNNN GGGAG*G*G
Scrambled Whole (SW)	N*N*NNNNNNNNNNNNNNNNNN*N*N

[a]Asterisk (*) denotes phosphorothioate modification which renders the oligonucleotides exonuclease resistant.

The formation of the bimolecular RNA:DNA hybrid GQ should result in a larger complex than either the unstructured RNA or the DNA individually and hence can be observed by native gel electrophoretic mobility shift in a gel. The electrophoretic mobility shift assay was performed with

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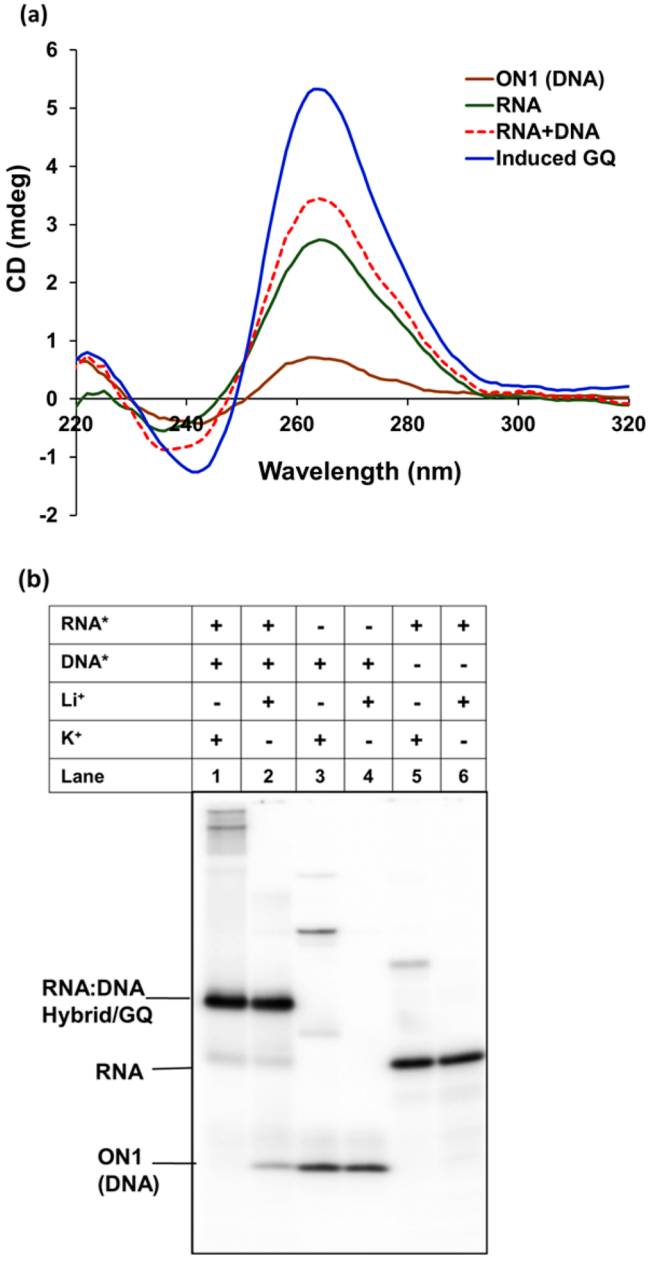

Figure 2. CD spectroscopy and native gel mobility shift assay to determine the formation of induced GQ. (a) CD spectra of induced GQ formation. The spectral patterns of 5 μM of DNA and RNA are shown in brown and green solid lines, respectively. The induced GQ formed by the 5 μM of RNA:DNA is shown in blue solid line, exhibits an increase in the 263 nm absorbance compared to the trace (red dotted line) of the added CD spectrum of individual oligonucleotides. (b) The native gel mobility shift assay shows the association of RNA and DNA in the presence of K^+ and Li^+ (lanes 1 and 2).

radiolabeled RNA and DNA in the presence of 150 mM of Li^+ or K^+. To maintain the oligonucleotide concentration at 5 μM, used during the CD experiments, the radiolabeled RNA and ON1 were mixed with their unlabeled versions.

The mobility shift assay results (Figure 2b, lanes 1 and 2) clearly indicate the association of RNA and DNA in the presence of K^+ and Li^+ respectively. The higher order structure formed by RNA in the presence of K^+ is probably a bimolecular GQ (lane 5), which corroborates with our CD data. Upon the basis of the observation of reduced mobility bands, the ON1 in the presence of K^+ (Lane 3) very likely forms various higher order species. The CD data in combination with the gel mobility shift data indicate association of ON1 with the target RNA to form a parallel induced GQ.

Enzymatic and Chemical Footprinting Data Indicate the Formation of an Induced GQ and the Duplex Structures in Tandem. The formation of the induced GQ structure was further confirmed by both enzymatic and chemical footprinting. RNase T1 cleaves single-stranded G-residues in RNA and protection of the G-residues from the cleavage suggests that they are very likely involved in duplex or quadruplex formation. The residues G 7−9 and G 11−13 of the target RNA are designed to be involved in induced GQ structure with the ON1, while the residues G 16 and G 20 would form the duplex (Figure 3a). The cleavage (Figure 3b) of all the G-residues in the 36-nucleotide 5′-radiolabeled RNA is observed in lane 2 in the presence of Li^+. The monovalent cation Li^+ was used as a control as it has been well established that Li^+ does not stabilize GQ structures. On addition of the DNA oligonucleotide, ON1 (1−5 μM) in the presence of K^+, complete protection was observed both in the putative quadruplex and duplex regions respectively (Figure 3b, lanes 4−6). The complete protection pattern persisted in the presence of K^+ even at the lowest ON1 concentration (1 μM). However, when the K^+ was swapped with Li^+, band protection in the GQ region (G 7−9) decreased even in the presence of highest used concentration of ON1 (5 μM). The protection of a subset of residues (G 11−13) persisted, which might be due to structural hindrance caused by the proximal duplex region. Thus, the RNase T1 footprinting results clearly indicate the formation of induced RNA:DNA hybrid GQ and the duplex structures in the presence of K^+, while in Li^+ the induced GQ structure is significantly destabilized. We did observe a few G-residues in the RNA only lane that showed slight protection in the GQ forming region in the presence of K^+ (lane 3), which can be explained by the formation of intermolecular GQ structures by the RNA as was previously suggested based upon the observations made during CD and gel mobility shift assays.

To further confirm the formation of induced GQ structure, we performed dimethyl sulfate (DMS) footprinting on the radiolabeled ON1 in presence and absence of the unlabeled target RNA. DMS methylates the N7 position of guanine residues and N3 position of adenine.[29] Since the N7 position of the G is involved in the Hoogsteen base-paring to form the quartets in a GQ their protection is an indication of formation of such structure. The DMS modified all of the G and A residues in the ON1 (Figure 3c, lane 2). However, when the induced GQ structure is formed in the presence of unlabeled target RNA, the A-residues in the ON1 are resistant to methylation, as the N3 in the Watson−Crick face of A is involved in duplex formation. The G-residues in the induced GQ forming region (G 16−18, G 20−21) are strongly protected due to formation of induced GQ structure in the presence of 150 mM K^+ (Figure 3c, lanes 3 and 4) compared to equimolar Li^+ (Figure 3c, lanes 6 and 7). Thus, RNase T1 and DMS footprinting confirm the formation of an induced RNA:DNA hybrid GQ structure in the presence of physiologically relevant concentration of K^+ ions.

The Induced GQ Structure Is Stable at Physiological Temperature and Is Resistant to Nuclease Digestion. Once formation of induced GQ structure was established by enzymatic and chemical footprinting, the stability of the

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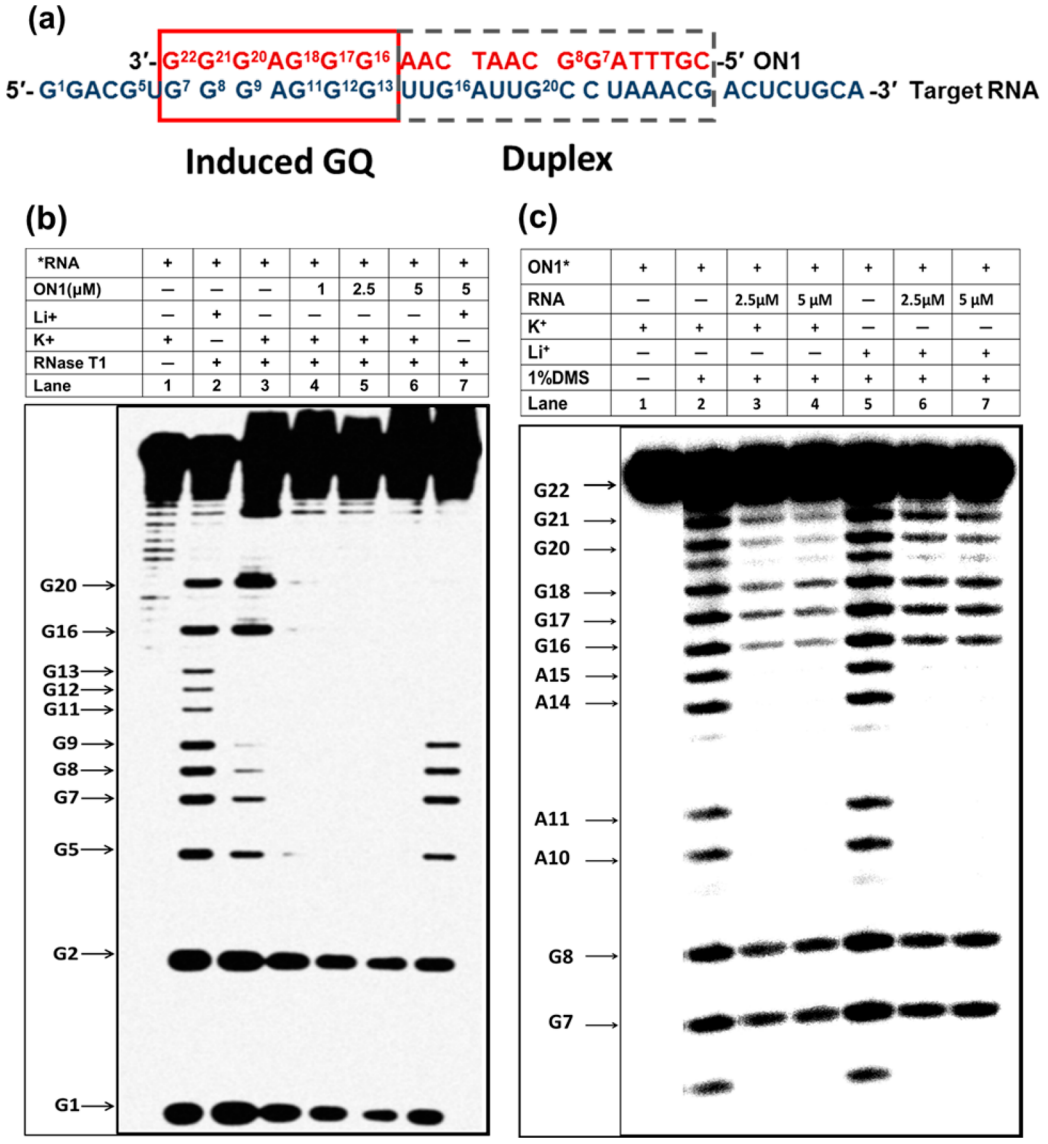

Figure 3. Images of gels showing enzymatic and chemical footprinting. (a) Schematic representation of the association of the target RNA sequence with the ON1. The G-residues are numbered for both the RNA and the DNA which are involved in the induced GQ and the duplex formation. The induced GQ and the duplex regions are indicated by separate boxes. (b) Image of a gel showing RNase T1 footprinting of the 5′-radiolabeled target RNA in presence and absence of targeting oligonucleotide ON1 in K^+ and Li^+. The formation of induced GQ is shown by the protection of the GQ forming residues (G-7, 8, 9, 11, 12, 13) in lanes 4, 5, and 6 in the presence of ON1 and K^+. In the presence of Li^+ (lane 7), the induced GQ is destabilized as observed by the reappearance (no protection) of the bands. (c) DMS footprinting of the 5′-radiolabeled ON1 in absence and presence of the target RNA and K^+ (lanes 3 and 4) clearly shows the increased protection compared to Li^+ ion (lanes 6 and 7) in the induced GQ forming region in the presence of both RNA and DNA.

Table 2. Thermodynamic Parameters Calculated from CD Melting Experiments by van't Hoff Plot, First Derivative Analysis and Isothermal Titration Calorimetry Data Represented as Mean ± Standard Error of the Mean (SEM)

thermodynamic parameters of induced GQ formation				
T_m (°C)	N (stoichiometry)	$\Delta H°$ (kJ/mol)	$\Delta S°$ (kJ mol^{-1} K^{-1})	$\Delta G°_{37}$ (kJ/mol)
59.6 ± 0.8	1.15 ± 0.03	−283.6 ± 24.8	−0.9 ± 0.1	−19.2 ± 1.4

structure was analyzed under the exact same conditions that are amenable to induced GQ formation, i.e., 150 mM K^+, 5 μM of each of RNA and ON1. CD melting experiments were performed by monitoring changes in CD intensity at 263 nm. The T_m of the structure was observed to be 59.6 ± 0.8 °C which is in agreement with the T_m of previous studies on similar structures.[21] The annealing and melting curves (Figure S1, Supporting Information) showed reversible characteristic, which suggests that the molecules were at thermodynamic equilibrium. The T_m values calculated from van't Hoff plots and by first-derivative analyses agreed with each other. Thermodynamic parameters from the melting curves were calculated on the basis of a two-state model that considers only the folded and unfolded states of the molecules. In the presence of 150 mM K^+, the standard Gibbs free energy ($\Delta G°$) at 37 °C was calculated to be −19.2 ± 1.4 kJ/mol, indicating a stable induced

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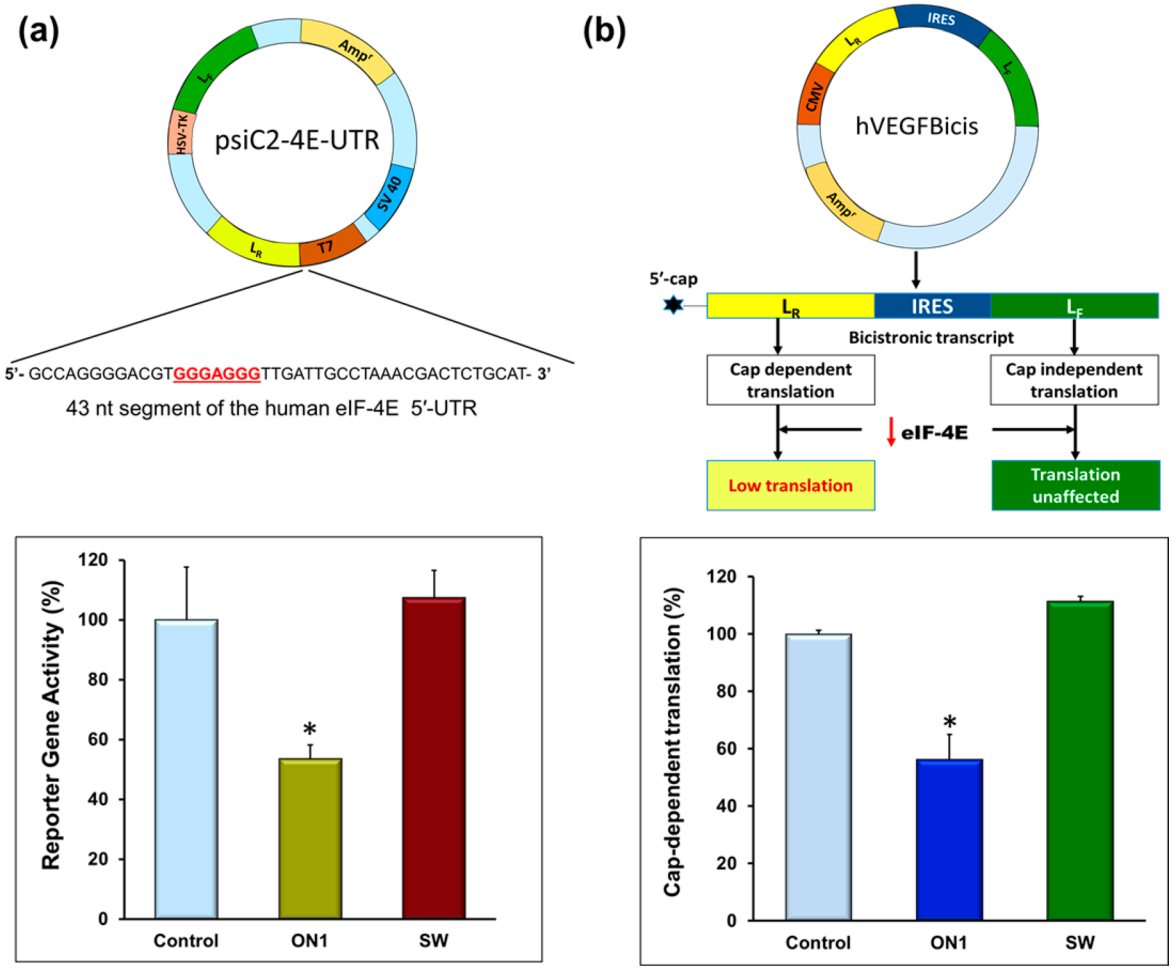

Figure 4. Histograms showing results from dual luciferase reporter plasmid assays. (a) The repression of reporter gene Renilla luciferase in psiC2-4E-UTR by formation of induced GQ structures by ON1 compared to the untreated and whole scrambled (SW) oligonucleotide (SW) treated cells (b) cells transfected with hVEGFBicis plasmids exhibited repression of cap-dependent translation initiation when treated with ON1. The ratios of Renilla (L_R)/Firefly(L_F) luciferase expression were normalized with untreated expression levels in both assays. Data represented as mean ± SEM, and the significance of the data was determined by *t* test analysis ($p < 0.0005$).

GQ. The calculated $\Delta H°$ of −283.6 ± 24.8 kJ/mol and $\Delta S°$ of −0.9 ± 0.1 kJ mol^{-1} K^{-1} values (Table 2) compare well with previously reported values for GQs.[30] Thus, the free energy of formation of induced GQ is solely driven by the favorable change in enthalpy. The stoichiometry (*N*) of the induced GQ formation by isothermal titration calorimetry (ITC) was measured to be 1.15 ± 0.026 (data not shown). The 1:1 association of the target RNA and ON1 oligonucleotide conforms well to the expected formation of a bimolecular induced GQ structure presumably with a duplex in tandem.

We also tested the induced GQ structure for its ability to resist nuclease digestion because nucleases will be less effective in cleaving a compact structure. The induced GQ structure formed in 150 mM of K^+ with 5 μM of RNA and ON1 each was treated successively with DNase 1 and RNase T1. There were no significant quantitative and qualitative changes of the CD spectrum even after 15 min of incubation at 37 °C (Figure S2, Supporting Information). The results described above suggest that the target RNA and ON1 associate in a 1:1 stoichiometry to form an induced GQ and the duplex which are inaccessible to nuclease at physiological temperature.

Induced GQ Structures Specifically Downregulate the Expression of a Targeted Gene in a Reporter Plasmid. The GQ structures when present within an mRNA modulate its translation. Thus, a GQ structure artificially induced on an mRNA in a targeted fashion might be able to downregulate its translation. The prerequisites for GQ induction on the target mRNA are the lack of endogenous PQS and an IRES but the presence of at least two G-stretches. Search for an optimum target resulted in human eIF-4E mRNA as it is embedded with all the needed characteristics for studying induced GQ formation. To test whether induced GQ structures can modulate gene expression in cells, a dual luciferase reporter plasmid was constructed with a 43-nucleotide fragment from the 5′-UTR of human eIF-4E mRNA inserted just upstream of the Renilla luciferase gene (Figure 4a). The plasmid was then transfected into HeLa cells, and the cells were treated with DNA oligonucleotides (Table 1) for 24 h following which the dual luciferase activities were measured. The DNA oligonucleotides contained phosphorothioate modification at two nucleotides on both ends to provide resistance against exonucleases.[31] The phosphorothioate modification is not known to interfere with the formation of GQ structures.[32] The ON1 reduced the expression of the reporter gene by ∼44% compared to the untreated cells (Figure 4a). The whole scrambled control (SW) had no significant change on the expression of the reporter gene. The results indicate that GQ structures repressed translation when induced in the 5′-UTR of reporter plasmids,

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and the repression level is in the range similar to what was observed previously for naturally occurring intramolecular RNA GQ structures.[8]

It is well established that IRES-dependent translation does not require eIF-4E for initiation.[33−37] Thus, the downregulation of eIF-4E which is known to affect the cap-dependent translation initiation should not affect IRES driven (cap-independent) translation initiation. The bicistronic reporter plasmid hVEGFBicis (Figure 4b) expresses the Renilla luciferase by cap-dependent translation initiation whereas the Firefly luciferase by cap independent translation under the influence of human vascular endothelial growth factor (hVEGF) IRES A.[12] The hVEGFBicis was used to observe the effect of the induced GQ structures on endogenous eIF-4E expression with the expectation that the lowering of eIF-4E level would negatively affect cap dependent translation initiation while the cap independent translation would remain unaffected (Figure 4b). As hypothesized, HeLa cells transfected with the plasmid when treated with ON1 exhibited down-regulation of cap-dependent translation as was measured by lowering of the Renilla/Firefly activity. However, Renilla/Firefly ratio did not show any change when the cells were treated with the control oligonucleotide SW most likely due to its lack of effect on eIF-4E expression. The inhibition of cap-dependent translation by induced GQ formation ON1 established the target specificity of the strategy.

Targeted Induction of GQ Structure Downregulate the Endogenous Human eIF-4E Expression by Translation Repression. A reporter construct with an insert derived from the naturally occurring eIF-4E transcript showed reduced reporter gene expression in cancer cells when treated with ON1, presumably via induced GQ formation which indicate that the region within eIF-4E is a viable target for induction of GQ. In a separate assay, reduction in expression of the transcript under cap-dependent translation initiation was observed in a dual luciferase reporter gene assay, while the cap-independent translation initiation remained unaltered, which provided strong indirect evidence that endogenous eIF-4E expression was inhibited. This prepared the deck for a direct measurement of the effect of induced GQ formation on endogenous eIF-4E expression level.

The human eIF-4E gene produces three transcripts wherein sequence amenable to induced GQ formation in the 5′-UTR region is present in two of the three transcripts, whereas a similar sequence was present in the coding region of all three transcripts (Table S1, Supporting Information). The targeting oligonucleotides ON1 and ON2 consists of two functional segments, a guiding sequence (GS) that is designed to hybridize to the target mRNA forming a duplex and two G-rich stretches to induce GQ by enlisting two G-stretches from the target RNA. The 15-nucleotide duplex region formed by ON1 and the target RNA can elicit an antisense mechanism potentially causing an inhibition of translation either by an RNase H mediated degradation of the targeted mRNA or a translation blockade.[31,38,39] Intramolecular GQ structures repress translation via a translation blockade and mostly with unaltered mRNA levels. It was therefore necessary to determine the effects of each of the functional regions in isolation and in combination and appropriate controls were designed to address both of those issues (Table 1). In one of the controls, the quadruplex forming region was scrambled (SQ1) keeping the duplex region unaltered, in the second the duplex forming region was scrambled (SD) keeping the segment that would participate in GQ formation unchanged, and finally, one was designed in which the entire sequence was scrambled (SW). HeLa cells were treated with partially modified ON1, ON2, and various control oligonucleotides without any delivery vehicle. The endogenous levels of eIF-4E was measured by Western Blotting (Figure 5a) using GAPDH as a control and showed a

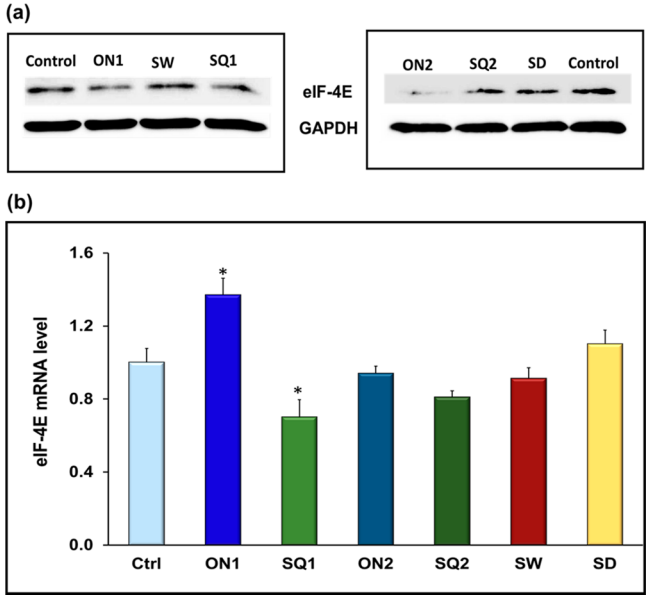

Figure 5. eIF-4E protein and mRNA expression levels in HeLa cells treated with ON1, ON2, and various control oligonucleotides. (a) Western blot of the eIF-4E protein expression levels when treated with various oligonucleotides. (b) Histogram of relative eIF-4E mRNA levels in HeLa cells when treated with oligonucleotides as measured by qRT-PCR. The mRNA levels were normalized to GAPDH mRNA levels. Data represented as mean ± SEM and the significance of the data was determined by *t* test analysis ($p < 0.025$).

30% reduction in the eIF-4E levels in ON1 treated samples and 60% reduction in ON2 treated samples based upon densitometric analysis by ImageJ software (Figure S3, Supporting Information). The effects of the duplex forming sequences (SQ1 and SQ2) on eIF-4E levels were not evident from the measured protein levels. The effect of control oligonucleotides SW and SD on protein levels were also insignificant (*t* test analyses). The targeted downregulation of the eIF-4E by induced GQ forming oligonucleotides and near absence of any effect in the control samples increased our confidence on the success of such targeted repression strategy.

To investigate the mechanism of an induced GQ mediated effect on protein production which can be either due to mRNA degradation or by translation arrest or a combination of both, eIF-4E mRNA levels were measured by qRT-PCR in ON1, ON2, and control oligonucleotides treated HeLa cells (Figure 5b). Although the protein level of the ON1 treated cells showed about 30% reduction, the mRNA level of ON1 treated cells was not reduced, and in fact it was 37% higher than the untreated. However, the SQ1 and SQ2 treated cells showed ∼30% and ∼20% reductions respectively in the mRNA levels. The ON2, SW and SD treated cells had no significant change in the eIF-4E mRNA levels compared to untreated cells. The ON1 sequence targets the 5′-UTR of eIF-4E mRNA, which leads to translation repression via formation of a stable secondary structure on the mRNA. It has been previously

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reported that formation of a stable secondary structure on the 5′-UTR of some AU-rich mRNAs result in stabilization of the mRNAs.[40] Given that eIF-4E mRNA harbors AU-rich elements in its 3′-UTR, it is likely that the GQ formation on its 5′-UTR stabilized the mRNA resulting in its higher expression level. Also, it is clear from the data that the repression in protein level has negligible if any contribution from the classic RNase H mediated antisense effect.

Targeting Endogenous eIF-4E mRNA by GQ Inducing Sequences Inhibits Proliferation of Human Cancer Cells. Eukaryotic initiation factor-4E (eIF-4E) is essential for initiation of cap-dependent translation and protein synthesis. Because of its least abundance among translation initiation factors, eIF-4E acts as the rate limiting agent in translation initiation.[24,26] The repression of endogenous eIF-4E levels by induced GQ structures led us to investigate their effects on cell proliferation. We used ON1 and ON2 to determine their antiproliferative activities on HeLa cells. Cells were grown in the presence of targeting and the control oligonucleotides, and cell viability was observed by MTS assay after 24 h of treatment. Induced GQ forming oligonucleotides ON1 and ON2 had a dose-dependent antiproliferative effect on the cancer cells with EC_{50} values of 15 μM and 5.4 μM respectively (Figure 6a). The control oligonucleotides SQ1, SQ2, SW, and SD did not exhibit any significant loss of cell viability (Figure 6b), suggesting the lack of any antisense mechanism in downregulation of eIF-4E expression. The antiproliferative action of GQ inducing ON1 and ON2 agrees well with their effect on the downregulation of endogenous eIF-4E levels. Interestingly targeted induction of GQ in the coding regions had a more robust effect in terms of both repression of protein expression and inhibition of cell proliferation. Thus, rationally targeted induced GQ formation not only downregulates a gene of choice but also affects antiproliferative activity on human cancer cells.

DISCUSSION

The purpose of this study is to establish that expression of an endogenous targeted gene can be modulated at the translation level by inducing RNA:DNA hybrid G-quadruplex structures. It is suggested that in the case of cap-dependent translation initiation, the formation of an RNA GQ in the 5′-UTR might act as a hindrance to the 5′-3′ scanning of the 43S preinitiation complex, which in turn downregulates the translation.[8] Additionally formation of a GQ within the coding region has been shown to result in aborted translation resulting in truncated protein products.[14] At least four G-stretches must be available for a GQ formation, and the G-stretches can be provided by one or more strands, which determine the molecularity of the GQ. The stability of the GQ structures depends on the number of quartets among other factors; for example, it is well-known that the stability of three-tiered GQ structure is more stable than the two-tiered GQs,[41] and a majority of the GQs discovered so far are three-tiered. The propensity of occurrence of potential RNA:DNA hybrid GQ enabling sequences are significantly higher compared to potential intramolecular GQ forming sequences, and such structures have been known to be formed naturally during transcription.[42,43] Hence we decided to induce a GQ structure that (i) would be stable under normal physiological conditions of temperature and K^+ concentrations and (ii) mimic naturally occurring structures and mechanisms in cells.

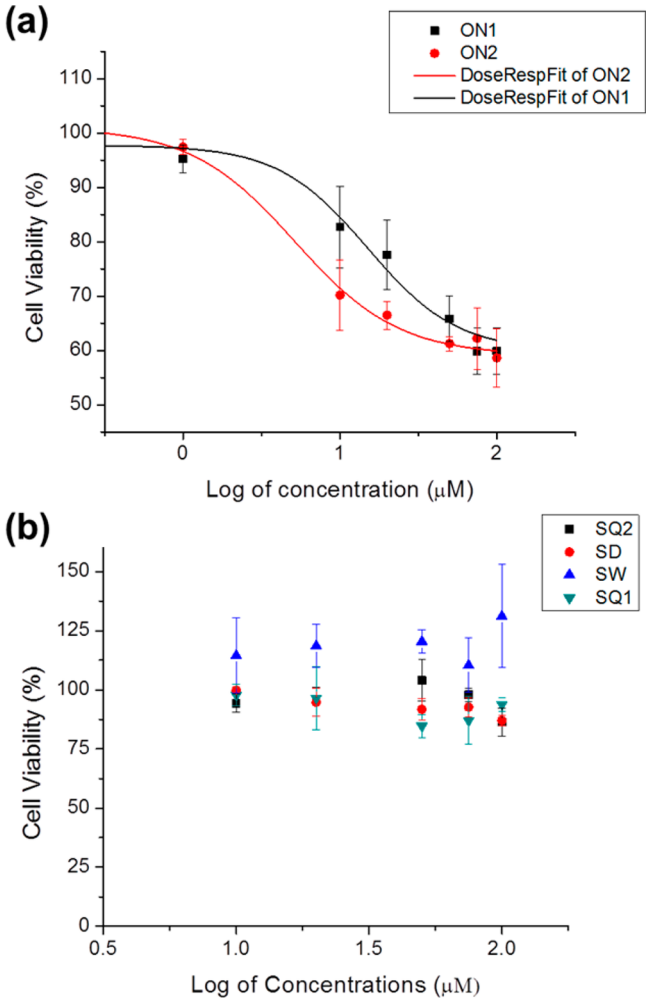

Figure 6. The dose response effect of increasing concentrations of ON1, ON2, and control oligonucleotides on Hela cell viability. (a) The EC_{50} value of ON1 was observed to be 15 μM and ON2 to be 5.4 μM when the data were fitted using dose−response curve with variable Hill slope. (b) The control oligonucleotides exerted no significant effect on the HeLa cell proliferation. Data represented as mean ± SEM.

The formation of a stable induced GQ structures was comprehensibly determined by a diverse set of biochemical and biophysical methods. Induction of the GQ would downregulate the target eIF-4E level which in turn would adversely affect canonical translation initiation. We first used a dual reporter based assay in which we introduced a segment from the 5′-UTR of the eIF-4E mRNA, and it harbored two G-stretches of three guanines each. An extraneous DNA sequence (ON1) rationally targeted to the introduced sequence was able to downregulate the reporter gene. Since the introduced reporter plasmid contained a sequence inherent to eIF-4E mRNA, when the cells were treated with ON1 sequence it also should downregulate the endogenous eIF-4E level. We assumed that under such conditions of lower eIF-4E level both the Renilla luciferase gene and the control Firefly luciferase gene were affected similarly. The downregulation of eIF-4E was further suggested by the observation of a decrease in the ratio of expression of two genes in a bicistronic construct where translation of the mRNA under canonical control of translation initiation was normalized to the IRES controlled one.

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The true test for any gene expression inhibition strategy is to be able to downregulate an endogenous gene *in cellulo*. We used human cervical cancer cells to test our strategy, and to our knowledge the effect of repression of eIF-4E in such cell line is not known. The eIF-4E sequence allowed us to target two different regions of the transcripts. ON1 that targeted the 5′-UTR of eIF-4E showed a little over 30% repression, while about 60% inhibition was observed in the presence of ON2 that targeted the coding region of the transcripts. A possible reason for the enhanced repressive effect of ON2 may be because there are three eIF-4E transcripts, and the target segment for ON1 only occurs in two of them, while the ON2 target exists in all three transcripts. We established that the repression of eIF-4E occurs at the translation level from the qRT-PCR results. We also observed that the levels of eIF-4E mRNA were increased with ON1 treatment. The eIF-4E mRNA harbors AU-rich (ARE) elements in its 3′-UTR.[44] Previous reports have suggested that translational repression of ARE embedded reporter RNAs containing stable secondary structures increased the half-life of those mRNAs, thus increasing the mRNA level.[40,45] Thus, the presence of the stable induced GQ structure may have caused translation blockade resulting in an increased eIF-4E mRNA level.

The differential effects of ON1 and ON2 on the translation repression of eIF-4E may also be due to the unrelated mechanism of inhibition. While the ON1 is expected to cause translation blockade by interfering with ribosome scanning that affects its ability to efficiently start protein synthesis, induced GQ formation in the protein coding region by ON2 might act by early termination of protein synthesis thereby leading to proteolysis of the truncated protein product and decrease in the eIF-4E expression. We are able to draw this inference based upon a previous report that has demonstrated that GQ structures when present in an open reading frame slow down or temporarily stall the translation elongation followed by proteolysis.[14] The data suggest a lack of any classic RNase H-dependent antisense mechanism. For example, the SQ1 and SQ2 control sequences showed a weak downregulation of mRNA, which did not translate into lowering of protein expression or cell growth inhibition. The ON2 showed ∼60% reduction of protein expression but SQ2 did not show any lowering of protein expression. Thus, the duplex alone failed to elicit any response at the protein level or on cell growth.

The oligonucleotide sequences used in the report showed low micromolar EC_{50} values. Since our primary goal was to establish a proof of concept of the strategy, we did not employ any external delivery vehicle for the oligonucleotides while treating the cells. We relied solely on the natural ability of the oligonuclotides to enter the cells and elicit their responses. We believe that use of a delivery method would lower the EC_{50} values of the oligonucleotides.

The strategy described in the work enables us to utilize multiple targets within an mRNA independently yet simultaneously. In fact our studies on targeting both the 5′-UTR and the protein coding region of eIF-4E mRNA simultaneously by ON1 and ON2 resulted in a moderate synergistic effect on antiproliferation of cancer cells (data not shown). Thus, targeting of multiple sites within transcripts of a gene using this approach may greatly enhance the efficacy of lowering of gene expression.

CONCLUSION

Since GQ structures are known to modulate several cellular processes, such as mRNA splicing, editing, and transcription termination, the formation of induced GQ structures can potentially modulate such processes in targeted fashion if appropriate target sites are available. The antiproliferative effects of induced GQs provide us with an additional tool for rational drug design against diseases such as cancer. It is widely accepted that there is no magic bullet for several diseases including cancer. Thus, novel approaches are needed to provide alternatives in conjunction with current strategies to fight such diseases. Future studies would include delivery of the oligonucleotides to enhance the efficacy of the oligonucleotides. The above concept opens up a new strategy for targeted modulation of endogenous gene expression. Additionally, our results show that we can harness and emulate the power of a naturally occurring mechanism for rational control of gene expression.

ASSOCIATED CONTENT

Supporting Information

CD melting profile, nuclease resistance of induced GQ, target sequence location in the eIF-4E mRNA, histogram of densitometry analysis of the Western blots. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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