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Cytotoxic G-rich oligodeoxynucleotides: putative protein targets and required sequence motif

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ABSTRACT

It has recently been shown that certain oligodeoxynucleotides (ODNs) designed as catalytic DNA molecules (DNAzymes) exhibit potent cytotoxicity independent of RNA-cleavage activity in a number of cell lines. These cytotoxic ODNs all featured a 5′ G-rich sequence and induced cell death by a TLR9-independent mechanism. In this study, we examined the sequence and length dependence of ODNs for cytotoxicity. A G-rich sequence at the 5′ terminus of the molecule was necessary for cytotoxicity and the potency of ODNs with active 5′ sequences was length dependent. Cytotoxicity appeared to be generally independent of 3′ sequence composition, although 3′ sequences totally lacking G-nucleotides were mostly inactive. Nucleolin, elongation factor 1-alpha (eEF1A) and vimentin were identified as binding to a cytotoxic ODN (Dz13) using protein pull-down assays and LC-MS/MS. Although these proteins have previously been described to bind G-rich ODNs, the binding of eEF1A correlated with cytotoxicity, whereas binding of nucleolin and vimentin did not. Quiescent non-proliferating cells were resistant to cytotoxicity, indicating cytotoxicity may be cell cycle dependent. Although the exact mechanism of cytotoxicity remains unknown, marked potency of the longer (⩾25 nt) ODNs in particular, indicates the potential of these molecules for treatment of diseases associated with abnormal cell proliferation.

INTRODUCTION

Single-stranded oligodeoxynucleotides (ODNs) and in particular certain G-rich ODNs have been widely reported to have effects on cells ranging from the induction of senescence and aging (1) to inhibition of proliferation (2–4). In some cases, secondary structure formation of ODNs (e.g. G-quadruplexes) facilitates the recognition by cellular protein(s) thus leading to cytotoxicity (5). In other cases, anti-proliferative activity of ODNs is related to the ability of the ODN to bind specific cellular proteins independent of secondary structure (6,7). Known targets of G-rich oligodeoxynucleotides include the human ribosomal protein L7a (8), nucleolin (9,10), elongation factor 1A (7,11), STAT3 (12) and growth factors (13).

DNAzymes are single-stranded DNA molecules that are able to cleave RNA in a site-specific manner (14). The molecules consist of a 5′ and 3′ binding arm and a catalytically active core region. The stability and activity of the DNAzymes *in vitro* make them a useful tool for gene silencing, and a number of different therapeutic applications have been proposed (14). We recently showed that the cellular effects of some ODNs, originally designed as DNAzymes against the transcription factors *c-jun* and *c-myc*, were not due to the cleavage of the target mRNA (15). Indeed, DNAzymes such as Dz13 (*c-jun*) and Rs6 (*c-myc*) and their catalytically inactive controls were cytotoxic when transfected into several cultured cell lines at concentrations as low as 10–50 nM (15). These cytotoxic ODNs all featured G-rich regions at the 5′ extremity suggesting that this sequence element is required for cytotoxicity. Although this cytotoxicity did not appear to be dependent on the formation of G-quadruplexes and other secondary structures (15), replacement of any of the three contiguous guanosines of Dz13 with 7-deaza-guanosine abolished cytotoxicity. We therefore postulated that the 5′ extremity sequence element is necessary for cytotoxicity and may be involved in mediating specific interactions with cellular proteins.

The aims of the present article were to clarify the sequence elements or 'motifs' required for cytotoxicity, to investigate the activity of various cytotoxic ODNs on different cell types, to determine the culture conditions required for cytotoxicity, and finally, to identify proteins that selectively bind to these cytotoxic molecules.

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MATERIALS AND METHODS

Oligodeoxynucleotides

HPLC-purified oligodeoxynucleotides were purchased from Sigma and Trilink Biotechnologies. Stock solutions were made up to a concentration of 50 μM in nuclease-free water and stored at −20°C. The oligodeoxynucleotides used are listed in Table 1. The 3′ biotinylated and 5′ Oregon-Green labeled congeners of Dz13 had the identical deoxyribonucleotide sequence to DT1549 (Table 1) in which the 3′-3′T inversion of Dz13 is missing.

Cell culture

Human dermal microvascular endothelial (HMEC-1) cells were maintained in MCDB131 medium (Gibco) containing 10% foetal bovine serum (FBS), 2 mM L-glutamine, 1 μg/ml hydrocortisone (Sigma) and 10 ng/ml epidermal growth factor (EGF; Sigma) with or without 5 U/ml penicillin–streptomycin. Rat vascular smooth muscle (RSMC), human embryonic kidney (HEK-293), HCT116 human colon cancer, NIH3T3 mouse fibroblast and human retinal pigmented epithelium (ARPE-19) cells were cultured in DMEM F12 medium (Gibco)

Table 1. Oligodeoxynucleotides investigated

Name	Sequence	Comments*
Dz13	CGGGAGGAAGGCTAGCTACAACGAGAGGCGTTG(3′-3′T)	Dz13 (19)
DT1549	CGGGAGGAAGGCTAGCTACAACGAGAGGCGTTG	Dz13 without 3′ inversion
DT1501	CTGGAGGAAGGCTAGCTACAACGAGAGGCGTTG(3′-3′T)	Dz13 with T in 5′ arm instead of G
Dz13scr	GCGACGTGAGGCTAGCTACAACGAGTGGAGGAG(3′-3′T)	Dz13 scrambled
DT1309h	CGGGAGGAAGGCTAGCTACAACGAGAGGCGTTG-**B**	DT1549 with 3′ biotin
DT1310c	GCGACGTGAGGCTAGCTACAACGAGTGGAGGAG-**B**	Dz13scr with 3′ biotin instead of inversion
DT1530	CGGGAGGAA (3′-3′T)	5′ sequence of Dz13
DT1531	CGGGAGGAAAGCAACATCGATCGG (3′-3′T)	DT1530 + inverted catalytic core
DT1532	CGGGAGGAAGGCTAGCTACAACGA (3′-3′T)	DT1530 + catalytic core
DT1536	CGGGAGGAAGGCTAGCTACAA**GC**AGAGG**GC**TTG (3′-3′T)	Dz13 with CG in tail changed to GC
DT1537	CGGGAGGAAGGCTAGCACACAGAGGGTCATGGT (3′-3′T)	Dz13 with scrambled tail
DT1538	CGGGAGGAAG**N23** (3′-3′T)	Dz13 5′ sequence + random mix of nucleotides
DT1539	CGGGAGGAAG**A23** (3′-3′T)	Dz13 5′ sequence plus polyA tail
DT1544	CGGGAGGAAG**N5**	Dz13 5′ sequence + 5 random bases
DT1545	CGGGAGGAAG**N10**	Dz13 5′ sequence + 10 random bases
DT1546	CGGGAGGAAG**N15**	Dz13 5′ sequence + 15 random bases
DT1547	CGGGAGGAAG**N20**	Dz13 5′ sequence + 20 random bases
DT1548	CGGGAGGAAG**N25**	Dz13 5′ sequence + 25 random bases
DT1552	CGGGAGGAAG**H25**	Dz13 5′ sequence + 25 A, C or T
DT1553	CGGGAGGA **N27**	Reduced 5′ Dz13 + 27 random bases
DT1554	CGGGAG **N29**	Reduced 5′ Dz13 + 29 random bases
DT1555	CGGG **N31**	Reduced 5′ Dz13 + 31 random bases
DT1570	CGGGAGGAAGTAGTAGTAGTAGTAGTAGTAG	Dz13 5′ sequence + (TAG)8
DT1571	CGGGAGGAAGTAGGATTAGGATTAGGATTAGGAT	Dz13 5′ sequence + (TAGGAT)4
DT1572	GGGAGGAAAG**N5**	Modified 5′ sequence + 5 random bases
DT1573	GGGAGGAAAG**N10**	” + 10 random bases
DT1574	GGGAGGAAAG**N15**	” + 15 random bases
DT1575	GGGAGGAAAG**N20**	” + 20 random bases
DT1576	GGGAGGAAAG**N25**	” + 25 random bases
Rs6 (DT1556)	TGAGGGGCAGGCTAGCTACAACGACGTCGTGA(3′-3′C)	*c-myc* DNAzyme (29)
DT1533	TGAGGGGCA(3′-3′T)	5′ extremity of Rs6 plus terminal inverted T
DT1534	TGAGGGGCAAGCAACATCGATCGG (3′-3′T)	5′ extremity of Rs6 and inverted catalytic core
DT1535	TGAGGGGCAGGCTAGCTACAACGA (3′-3′T)	5′ extremity of Rs6 and catalytic core
DT1600	**N12**CGGGAGGAAG**N13**	Dz13 5′ motif in middle of random mix
DT1601	**N25**CGGGAGGAAG	Dz13 5′ motif at 3′ end of random mix
DT1602	**N25**GAAGGAGGGC	Dz13 5′ motif inverted at 3′ end of random mix
DT1557	TGAGGGGCAG**N25**	Rs6 5′ sequence + 25 random bases
DT1558	TGAGGGGC**N27**	Reduced 5′ Rs6 + 27 random bases
DT1559	TGAGGG**N29**	Reduced 5′ Rs6 + 29 random bases
DT1560	TGAG**N31**	Reduced 5′ Rs6 + 31 random bases
DT1565	**OG**-CGGGAGGAAGGCTAGCTACAACGAGAGGCGTTG	Oregon-Green labelled DT1549
GT(DT1605)	TGTTTGTTTGTTTGTTTGTTTGTTTGT	GT-ODN (11)
GRO29A (DT1563)	TTTGGTGGTGGTGGTTGTGGTGGTGGTGG	Nucleolin-binding ODN (24)
20AG	AGGGAGGGAGGAAGGGAGGG	G-rich ODN (17)
DT1591	AGGGAGGGAGGAAGGG	Length variant (16-mer) of 20AG
DT1592	AGGGAGGGAGGAAGGGAGGGAGGG	Length variant (24-mer) of 20AG
DT1593	AGGGAGGGAGGAAGGGAGGGAGGGAGGG	Length variant (28-mer) of 20AG
DT1594	AGGGAGGGAGGAAGGGAGGGAGGGAGGGAGGG	Length variant (32-mer) of 20AG
DT1595	AGGGAGGGAGGGAGGGAGGGAGGG	AGGG repeat ODN
NT36 (DT1577)	AAGAGGTGGTGGAGGAGGTGGTGGAGGAGGTGGAGG	ATM-inducing ODN (23)

*number in brackets indicates reference number.

supplemented with 10% FBS and 2 mM L-glutamine with 5 U/ml penicillin–streptomycin.

Transfection and cellular assays

Cells were seeded in 96-well black MicroClear plates (Greiner) (4×10^3 cells/well) or 6-well plates (1.2×10^5 cells/well) in growth medium containing 5% FBS for HMEC-1, HCT116, HEK-293, NIH3T3 and ARPE-19 cells or 10% FBS for RSMC cells. Cells were transfected 24 h after seeding with different concentrations of ODNs in triplicate using FuGene6 (Roche) as previously described (15). Cell survival was assessed 48 h post-transfection in the 96-well plate format using the Cell Titer^{TM}-Blue cell viability assay (Promega) as previously described (15). Briefly, culture medium was replaced with 100 µl OptiMEM and 20 µl of Cell-titer^{TM} blue reagent and incubated for 2 h at 37°C. Fluorescence was measured at $544_{EX}/590_{EM}$ using FLUOstar OPTIMA (BMG Labtechnologies). For the cell density experiments, ARPE-19, NIH3T3 or HMEC-1 cells were seeded at densities of 4000 and 50 000 cells per well in 96-well plates and transfected with 0–200 nM ODN in triplicate. Transfection efficiencies at the 2 cell densities were determined in duplicate in 60 mm dishes using a 5′-oregon green ODN (DT1565; Table 1) in a single experiment. The 60 mm dishes were seeded with a similar number of cells/cm^2 as for the 96-well plates for both seeding densities. For low density transfection, 2.6×10^5 cells/dish were seeded while for high density transfection 3.25×10^6 cells/dish were seeded. Fluorescence of mock and DT1565-transfected cells was measured 48 h post-transfection using fluorescence-activated cell sorting (FACS).

Protein extraction

Cells were washed twice with PBS and total proteins extracted using MPER (Pierce) or RIPA (150 mM NaCl, 0.1% w/v sodium dodecyl sulphate, 1% v/v Nonidet P-40, 0.5% w/v sodium deoxycholate, 50 mM Tris-HCl pH 8). Subcellular proteins were extracted using the ProteoExtract kit (Calbiochem). All extractions were performed in the presence of EDTA-free protease inhibitors (Roche). Cellular debris was removed by centrifugation at 10 000*g* for 20 min at 4°C.

Protein pull-down assays

Protein pull-down assays were performed using oligodeoxynucleotides as 'bait' in order to identify Dz13-binding proteins. Dynabeads-Streptavidin (Dynal) were washed twice in 2× Buffer A (10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl, pH 7.4). Beads (0.5 ml) were resuspended in 1 ml 2× Buffer A to a final concentration of 5 µg/µl beads. For *in vitro* assays, beads were incubated in an equal volume of 2 µM 3′ biotinylated congeners of Dz13 or Dz13scr (DT1309h and DT1310c respectively, Table 1) made up in Buffer B (20 mM HEPES, 100 mM KCl, 0.2 mM EDTA, 0.01% v/v NP-40, 10% v/v glycerol, pH 7.5) and the mixture incubated at room temperature for 10 min with gentle mixing. The beads were washed with 3 × 1 ml Buffer B prior to addition of protein. Protein extract (up to 1 mg) was incubated with the bead–DNA mixture for 10 min at room temperature with shaking. For the majority of protein pull-downs, the beads were washed with 20 × 1 ml Buffer B following incubation with protein extract and proteins were eluted by two washes with 1 µM Dz13 or Dz13scr at room temperature. In one experiment, the elutions were performed with 10 µM Dz13 or Dz13scr. In between each elution, the beads were washed with 3 × 1 ml Buffer B.

To test whether the same proteins eluted with other ODNs of interest an experiment was undertaken in which the bead preparation, protein incubation and washing steps were performed as described above. Following washing of the beads, non-specific proteins were eluted by 2 × 5 min incubation with 1 µM non-biotinylated Dz13scr and the beads washed again with 3 × 1 ml Buffer B. This bead preparation was split into six identical samples and proteins eluted by 2 × 5 min incubations with 1 µM ODN (Dz13, NT36, DT1501, Rs6, DT1595 or GT). The resulting sample supernatants were collected and analysed and each of the bead samples was again washed with 3 × 1 ml Buffer B and proteins eluted with 1 µM Dz13. This experiment was performed twice.

For *in situ* protein pull-down assays, cells were transfected with 100 nM biotinylated congeners of Dz13 or Dz13scr (i.e. DT1309h and DT1310c) for 24 h. Beads were washed three times with 1 ml Buffer B and resuspended at 5 µg/µl prior to incubation for 10 min at room temperature with protein extracts prepared from these cells using MPER. Following incubation, beads were washed with 20 × 1 ml Buffer B and resuspended in 20 µl Buffer B. Washing and elution steps were then performed as described above.

Protein analysis

Fractions generated from pull-down assays were concentrated in a Centricon 10000 Mwt cutoff 0.5 ml centrifugal device (Millipore) at 13 800*g* for 70 min at 15°C. Proteins (from cell extracts or concentrated pull down fractions) were denatured at 70°C for 10 min in 4× Loading Buffer (Invitrogen), loaded onto NuPAGE 4–12% bis-tris acrylamide gels (Invitrogen) and electrophoresed at 140 V in MOPS-SDS running buffer. Following electrophoresis proteins were either silver-stained as described by Rabilloud *et al.* (16) or transferred to a PVDF membrane at 30 V for 2 h. Membranes were blocked in 3% BSA in TBST (10 mM Tris pH 8, 30 mM NaCl, 0.05% v/v Tween-20) for 1 h at room temperature. Primary antibodies were incubated at concentrations recommended by the manufacturers for 1 h at room temperature or overnight at 4°C in 5% skim milk-TBST. Membranes were washed 3 × 5 min in TBST and incubated for 1 h at room temperature with horseradish peroxidase conjugated antibodies (1:2000 in 5% skim milk-TBST; DakoCytomation). Membranes were washed 3 × 5 min in TBST and visualized by chemiluminescence using ECL (Amersham) and Hypersensitive film (Amersham). The following commercially available antibodies were used: EF1A (CBK-KK1; Upstate Biotechnology), vimentin (V9; Sigma) and STAT3 (F-2; Santa-Cruz Biotechnology).

Liquid chromatography and tandem mass spectrometry

Total protein was digested by incubating 100 µl of sample, 25 µl of 10 mM NH_4HCO_3 and 1 µg trypsin at 37°C for 16 h. The digested peptides were loaded onto a C18 precolumn (500 µm × 2 mm, Michrom Bioresources) using H_2O:CH_3CN (98:2, 0.1% formic acid, buffer A) at 20 µl/min. After a 10 min wash, the pre-column was switched (Switchos) in-line with an analytical column containing C18 RP silica (PEPMAP, 75 µm × 15 cm, LC-Packings) or a fritless C18 column (75 µm × ~12 cm). Peptides were eluted using a linear gradient of buffer A to H_2O:CH_3CN (40:60, 0.1% formic acid-buffer B) at 200 nl/min over 60 min. The column was connected via a fused silica capillary to a low volume tee (Upchurch Scientific) where high voltage (2300 V) was applied and a nano electrospray needle (New Objective) or fritless column outlet was positioned ~1 cm from the orifice of an API QStar Pulsar i hybrid tandem mass spectrometer (Applied Biosystems). The QStar was operated in an information-dependent acquisition mode. A TOF-MS survey scan was acquired (m/z 350–1700, 0.5 s) and the two largest precursors (counts > 10) sequentially selected by Q1 for MS/MS analysis (m/z 50–2000, 2.5 s). A processing script generated data suitable for submission to the database search programs. CID spectra were analysed using Mascot MS/MS ion search (Matrix Science). The criteria were: trypsin digestion allowing up to 1 missed cleavage, oxidation of methionine, peptide tolerance of 1.0 Da and MS/MS tolerance of 0.8 Da. A Mascot score >18 indicated identity.

RESULTS

Motif experiments

Variants of Dz13 and other selected DNAzymes, which reduce proliferation in HMEC-1 cells but do not act through cleavage of the RNA substrate, were designed in order to elucidate the sequence requirements for cytotoxicity. First, a set of oligodeoxynucleotides containing regions of the Dz13 sequence was designed in order to identify any requirement for an active motif. ODNs corresponding to the 5′ sequence of the first 9 bases of Dz13 (DT1530), this 5′sequence plus the 10–23 catalytic core (15 bp) in the reverse orientation (DT1531) and the 5′sequence plus the catalytic core in the correct orientation (DT1532) were tested. All of these were substantially less active than Dz13 (data not shown), suggesting that either length or some other sequence requirement had not been met. Similar results were obtained with the corresponding ODN based on the 5′ Rs6 sequence (DT1533-5, Table 1; Figure S1a).

In order to elucidate the requirements of the tail for cytotoxicity, Dz13 analogues of the same length but with modified 3′ tail sequences were tested (Table 1). These included both CG dinucleotides in the 3′ tail changed to GC (DT1536), the first 16 bases of Dz13 plus scrambled tail (DT1537), a pool of ODNs all consisting of the first 10 bases of Dz13 followed by a random mix of nucleotides in tail sequence (DT1538) and the first 10 bases of Dz13 followed by a polyA tail (DT1539). The polyA-tailed Dz13 analogue (DT1539) exhibited no cytotoxicity in HMEC-1 cells whereas DT1536 demonstrated intermediate cytotoxicity (Figure 1A). Both the scrambled tail analogue (designed to remove the tail hairpin structure) and the mixture of random-tailed oligodeoxynucleotides had activity that was indistinguishable from Dz13, indicating minimal sequence requirements for the 3′ tail in these 33-mer oligodeoxynucleotides.

To elucidate the positional requirement for a 5′ G-rich element, three pools of ODNs featuring the 5′ motif from Dz13 (first 10 bases) were constructed with either the 5′ motif in the middle of a random sequence pool, at the 3′ end or at the 3′ end in reverse order (DT1600-1602, Table 1). None were significantly cytotoxic, although some activity was noted for the 3′ reverse motif ODN (Figure 1B). This suggests that the motif may also be recognized in the 3′ to 5′ direction or that part of the activity relates to the rest of the molecule. In any case, this experiment confirmed that the optimal placement for the G-rich sequence of Dz13 is at the 5′ end.

Given the lack of activity of the shorter variants (DT1530–DT1532), the next experiment sought to clarify the length requirement for the observed cytotoxicity. This was tested by using ODN pools containing the first 10 bases of Dz13 and 5, 10, 15, 20 or 25 random bases (DT1544-48, Table 1). Cytotoxicity was concentration and length dependent with only the 35-mer ODN pool (DT1548) matching the potency of Dz13 (Figure 1C), thus confirming the role of the tail length. A similar result was obtained with DT1572-6, which are based on a slightly different 5′ sequence (data not shown).

Given previous results indicating that potency was dependent on a relatively unbiased nucleotide composition of the 3′ tail, a new 35-mer ODN was tested in which the random tail was composed only of A, C and T bases (DT1552). This ODN was essentially devoid of cytotoxic activity over the concentration range tested (data not shown). These results indicate that the tail sequence has no particular sequence requirements but that potency is dependent on a relatively unbiased nucleotide representation, including some G content.

A set of 35-mer oligodeoxynucleotides were designed and synthesized in order to identify the minimal 5′ motif required for activity. This set comprised pools of oligodeoxynucleotides for which the first 10 bases of the Dz13 5′ G-rich sequence (plus a 25 random base tail; DT1548) was progressively reduced (DT1553–DT1554) to the first 4 bases of this sequence plus a 31 base tail (DT1555). Only DT1553 (first 8 + 27 random) and DT1548 (first 10 + 25 random) retained full activity (Figure 1D). The corresponding experiment with the Rs6 sequence (DT1557–60) produced gradated cytotoxicity profiles with activity increasing with increased retention of the 5' Rs6 sequence (Figure S1b).

The Dz13 5′ G-rich sequence, when extended with tail sequences of TAG and TAGGAT repeat elements (DT1570 and DT1571, respectively) had reduced activity relative to Dz13 (data not shown). These tail sequences were specifically free of C to prevent the formation of GC clamps and possibly more complex secondary structures.

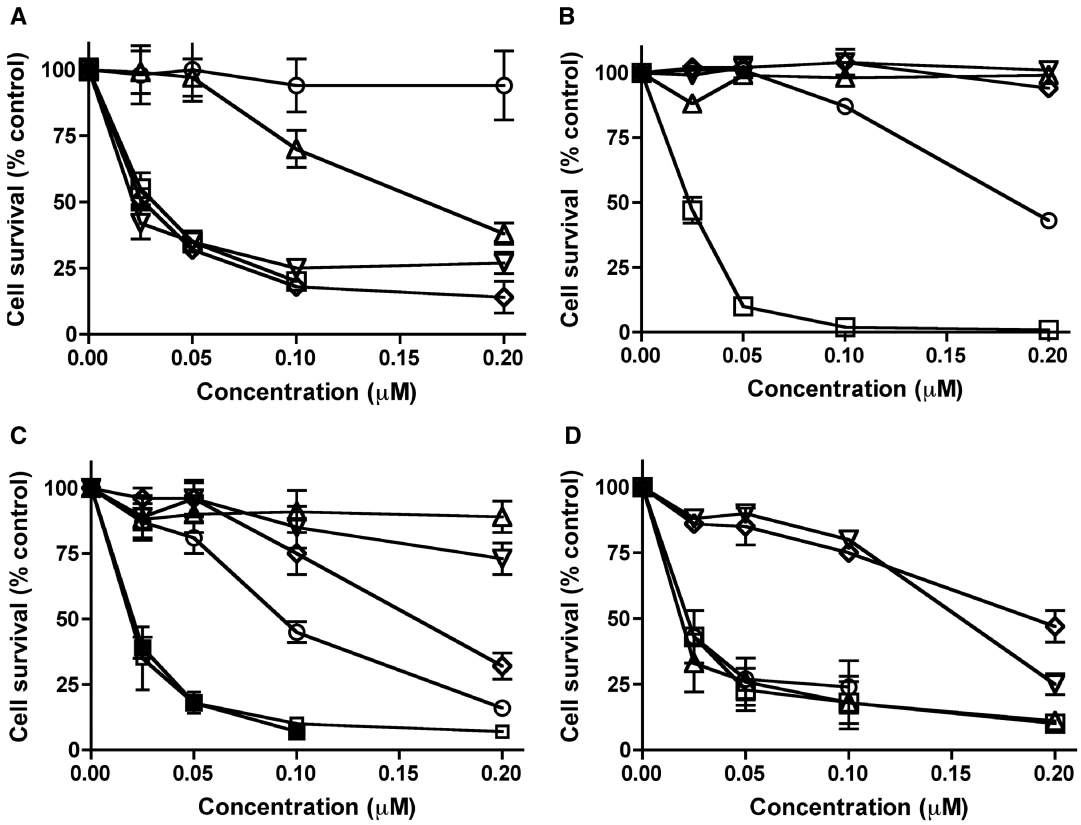

Figure 1. Cytotoxic activity of Dz13 analogues in HMEC-1 cells. The oligodeoxynucleotides (Table 1) were tested in HMEC cells and transfected at a range of concentrations and cell survival measured 48 h later. Cell survival as a percentage of untransfected cells is shown +/− SD from two independent experiments. (**A**) Investigation into the 3′ tail sequence required for cytotoxicity: Dz13 (square); DT1536 (triangle); DT1537 (inverted triangle); DT1538 (diamond) and DT1539 (circle). (**B**) Effect of Dz13 3′ sequence positioning: first 10 bases of Dz13 positioned in the middle (DT1600; inverted triangle), at the 3′ end (DT1601; diamond) or at the 3′ end in reverse (DT1602; circle) were transfected along with Dz13 (square) and Dz13scr (triangle). (**C**) Effect of 3′ tail length: first 10 bases of Dz13 and an additional 5 (DT1544; triangle), 10 (DT1545; inverted triangle), 15 (DT1546; diamond), 20 (DT1547; circle) or 25 (DT1548; open square) random bases were transfected along with Dz13 (solid square). (**D**) Effect of decreasing retention of 5′ terminal sequence: first 10 (DT1548; square), 8 (DT1553; triangle), 6 (DT1554; inverted triangle) and 4 (DT1555; diamond) bases of the Dz13 5′ G-rich sequence with the balance made up with random nucleotides were transfected along with Dz13 (circle).

As reported earlier (15), other published G-rich oligodeoxynucleotides are capable of inducing cytotoxicity under the same conditions in HMEC-1 cells including the 20AG ODN described by Cogoi *et al.* (17). We examined whether extension of this sequence in a manner analogous to the Dz13 experiments would influence cytotoxic activity. Consistent with the Dz13 results, 16- (DT1591), 24- (DT1592), 28- (DT1593) and 32-mer (DT1594) variants of the 20-mer 20AG yielded length-dependent cytotoxic activities with the longest two molecules having similar profiles (data not shown).

Activity of Dz13 in contact-inhibited cells

Given their potent cytotoxic activity against proliferating cells, Dz13 and the other cytotoxic oligodeoxynucleotides could be of use in several disease states. Dz13 has been reported as being active in several preclinical models of disease including ocular angiogenesis (18), vascular intimal proliferation (19) and cancer (20), which are all diseases where inappropriate proliferation is present. To investigate any possible differential activity relative to non-proliferating cells, we examined the activity in two additional cell lines that display contact inhibition, namely ARPE-19 and NIH-3T3. In particular, ARPE-19 cells form differentiated, polarized monolayers *in vitro* (21) similar to that present in normal retinal epithelium. As shown in Figure 2A, proliferating ARPE-19 cells were sensitive to Dz13-induced cytotoxicity with an IC_{50} between 50 and 100 nM, whereas Dz13scr was inactive. Dz13 cytotoxicity in ARPE-19 cells was abrogated over that concentration range by increasing the cell seeding density to 50 000 cells/well, at which seeding density the cells grew to a uniform dense monolayer. Similar results were obtained for the murine fibroblast 3T3 cells, which are also contact-inhibited and for HMEC-1 cells, which form multilayered sheets at maximal confluence (data not shown). To rule out reduced transfection as the cause for this effect, repeat experiments were scaled up and transfection efficiency was determined using a fluorescent 5′-OregonGreen-488 congener of Dz13 (DT1565; Table 1), which was complexed with Fugene6 and transfected at a final concentration of 100 nM. DT1565 had reduced cytotoxicity compared to Dz13 (data not shown), which reduced the confounding effects of toxicity on analysis, and the transfection efficiency as determined by FACS

analysis showed higher transfection at the higher cell density (Figure 2B). These preliminary experiments indicate selective cytotoxicity of Dz13 to proliferating cells as opposed to those that are contact-inhibited and non-proliferating.

Identification of proteins binding to Dz13

In order to identify whether proteins bind the cytotoxic ODNs and if binding correlated with cytotoxic potency, protein pull-downs were performed using 3′ biotinylated congeners of Dz13 and Dz13scr (DT1309h and DT1310c, respectively; Table 1) as 'bait'. In HMEC-1 cytotoxicity assays, these 3′-biotinylated ODNs had very similar cytotoxic properties to their untagged counterparts (data not shown). For the pull-down experiments, the biotinylated ODN was coupled to magnetic streptavidin beads and a crossover design employed in which beads coupled to the biotinylated ODN of interest were incubated with HMEC-1 protein, washed with the opposite ODN and eluted with the non-biotinylated bait ODN. That is, when the beads were prepared with the biotinylated congener of Dz13scr and loaded with cell protein extracts, they were subsequently washed with a Dz13 solution and proteins eluted with a Dz13scr solution. For the Dz13-coupled beads, a large number of non-specific proteins were washed off with Dz13scr and a number of strong staining bands were obtained by competitive elution with Dz13,

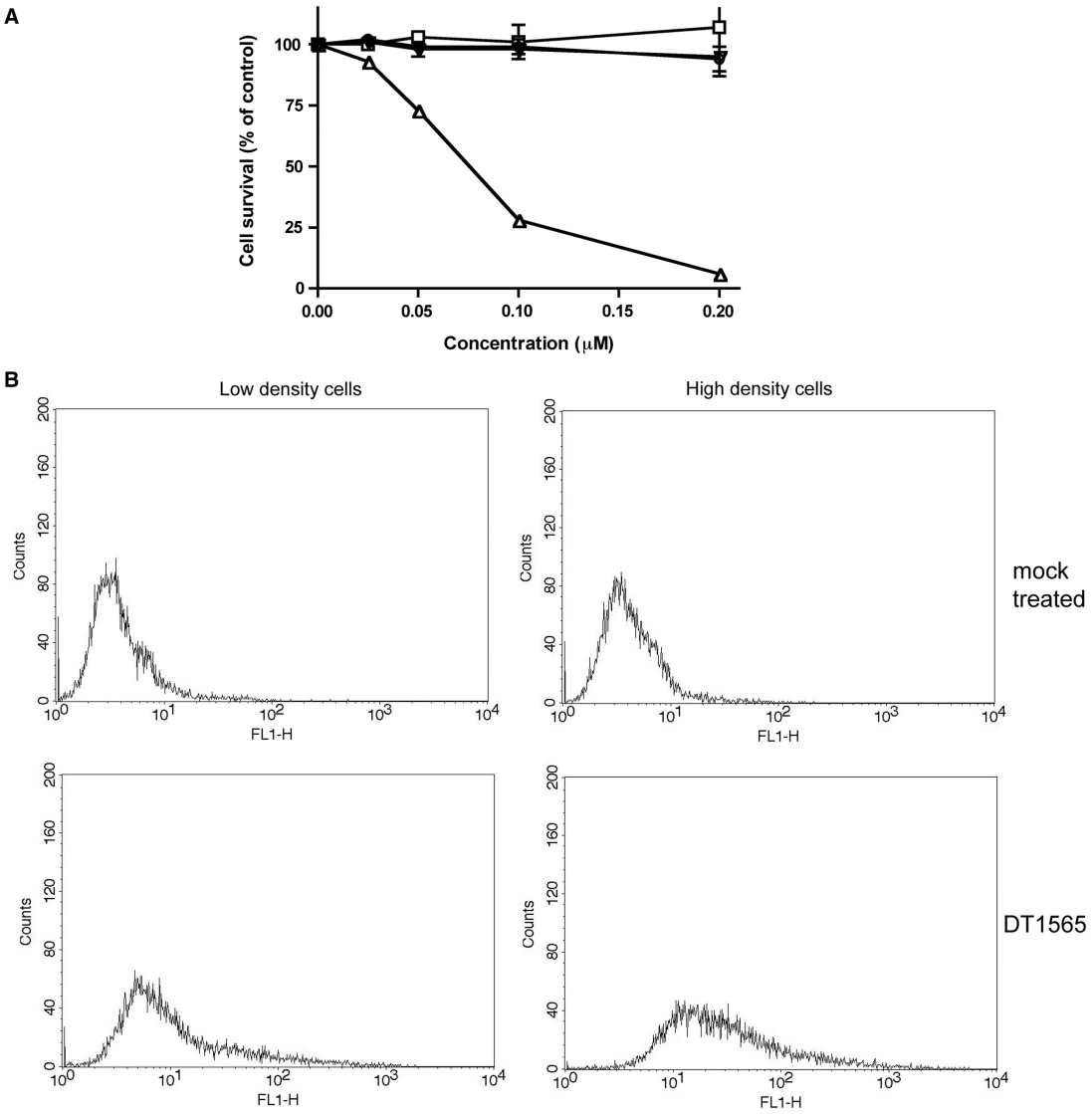

Figure 2. Effect of seeding density on cytotoxicity of Dz13. (**A**) A total of 50 000 ARPE-19 cells per well were plated for high density and transfected with 0–200 nM Dz13 (circle) or Dz13scr (inverted triangle). A total of 4000 cells per well were plated for low density and transfected with Dz13 (triangle) or Dz13scr (square). Transfections were performed in triplicate and cellular proliferation measured 48 h post-transfection. The data from two independent experiments represent the mean survival as a percentage of untransfected cells from each of the densities +/− SD. (**B**) Examination of transfection efficiency of DT1565 in low density and high density seeded HMEC-1 cells. Also, 2.6×10^5 (low density) or 3.25×10^6 (high density) cells were seeded in 60 mm dishes and mock-transfected or transfected with 100 nM DT1565 (3′ Oregon Green). Fluorescence was measured 24 h later in duplicate using fluorescence-assisted cell sorting analysis (FACS).

the major one being a ~51 kDa protein (Figure 3A). Elution with the non-cytotoxic Dz13scr yielded a large number of bands including a predominant band at ~39 kDa, however unlike Dz13, none of these were specifically eluted by Dz13scr. Silver staining revealed a lack of protein remaining bound to unconjugated beads following extensive washing (data not shown).

In order to generate enough material to identify proteins eluting from the beads, the experiment was scaled up 5-fold and 1 mg of protein was loaded onto beads coupled to the biotinylated ODNs. In these experiments, the number of washes was increased to 30 while the elution volume was kept constant. Following concentration of the eluted fractions, the proteins were digested with trypsin and analysed by LC-MS/MS, with liquid chromatography used to separate the reasonably complex mixture of peptides prior to mass spectrometric analysis. A number of proteins eluting with both Dz13 and Dz13scr were identified (Table 2). Proteins identified in the Dz13-eluting fraction (from Dz13-coupled beads) included microtubule-associated protein 4 (MAP4), nucleolin, vimentin, elongation factor 1A (eEF1A), plasminogen activator inhibitor 1 (PAI-1) RNA-binding protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and signal recognition particle 14 kDa. Proteins identified in the Dz13scr-eluting fraction (from Dz13-coupled beads) included annexin II, GAPDH, heterogeneous nuclear ribonuclear proteins A1, Lamin A/C isoform 1 and nucleolin. The 51 kDa band was competitively eluted by Dz13 in three independent experiments and eEF1A was identified in these fractions by LC-MS/MS each time. Overall, a total of 10 peptides from this protein were identified that together spanned most of the protein sequence (Table 2). Western blotting of pull-down proteins with an antibody directed to eEF1A confirmed that it was the major band identified by silver staining at 51 kDa and that the protein bound to both Dz13 and Dz13scr beads but was only eluted by Dz13 (Figure 3B). The LC-MS/MS identification of vimentin was also confirmed by western blots in which vimentin was shown to bind to Dz-13 coupled beads as well as to Dz13scr-coupled beads (data not shown). Elution of vimentin from the beads with Dz13 or Dz13scr was not detected by western (data not shown). This may reflect the fact that more protein (5-fold) was loaded onto the beads for LC-MS/MS analysis. Vimentin distribution within the various cell compartments was assessed using cell fractionation and western blotting 24 h post-transfection with Dz13 and Dz13scr. Vimentin was mostly localized to the cytoskeletal and nuclear fractions and this distribution did not appear to be modified 6 or 24 h following transfection with 200 nM Dz13 (data not shown).

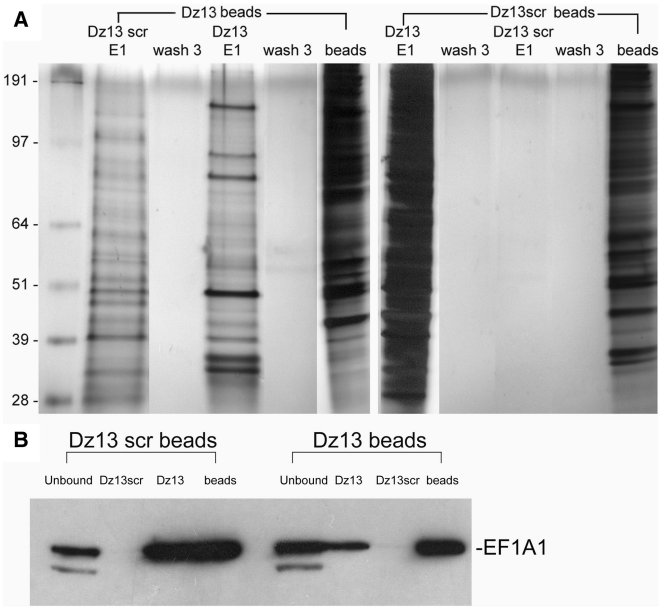

Figure 3. Pull-down experiments using total protein lysates from HMEC-1 cells. HMEC-1 protein extract was incubated with biotinylated Dz13 (left lanes) or Dz13scr (right lanes) coupled to streptavidin beads and proteins eluted with either 1 μM Dz13scr or 1 μM Dz13 as indicated. The beads were washed three times with binding buffer between ODN elutions. Protein fractions were concentrated, electrophoresed and bands revealed by silver staining (**A**) or by eEF1A western (**B**). E1 indicates elution #1 and unbound refers to residual proteins in flow-through following capture of proteins to beads. The approximate molecular weight (kDa) is indicated.

Table 2. Proteins binding to Dz13 and Dz13scr identified by LC-MS/MS

Ensembl Gene ID	Protein	Number of peptides matched	Predicted MW (kDa)
	Dz13-eluted proteins		
ensg00000140319	Signal recognition particle 14 kDa	2	14
ensg00000156508*	Elongation factor 1A	10	51
ensg00000101210*			
ensg00000026025	Vimentin	2	54
ensg00000047849	Microtubule associated protein 4	2	121
ensg00000142864	Plasminogen activator inhibitor -1 RNA-binding protein	1	42
ensg00000111640	Glyceraldehyde 3 phosphate dehydrogenase	1	36
ensg00000115053	Nucleolin	1	77
	Dz13scr-eluted proteins		
ensg00000182718	Annexin II	2	39
ensg00000111640	Glyceraldehyde 3 phosphate dehydrogenase	3	36
ensg00000135486	Heterogeneous nuclear ribonuclear protein A1	3	22
ensg00000160789	Lamin A/C isoform 1	1	80
ensg00000115053	Nucleolin	1	77

*Peptides identified by MS are found in both eEF1A1 and eEF1A2.

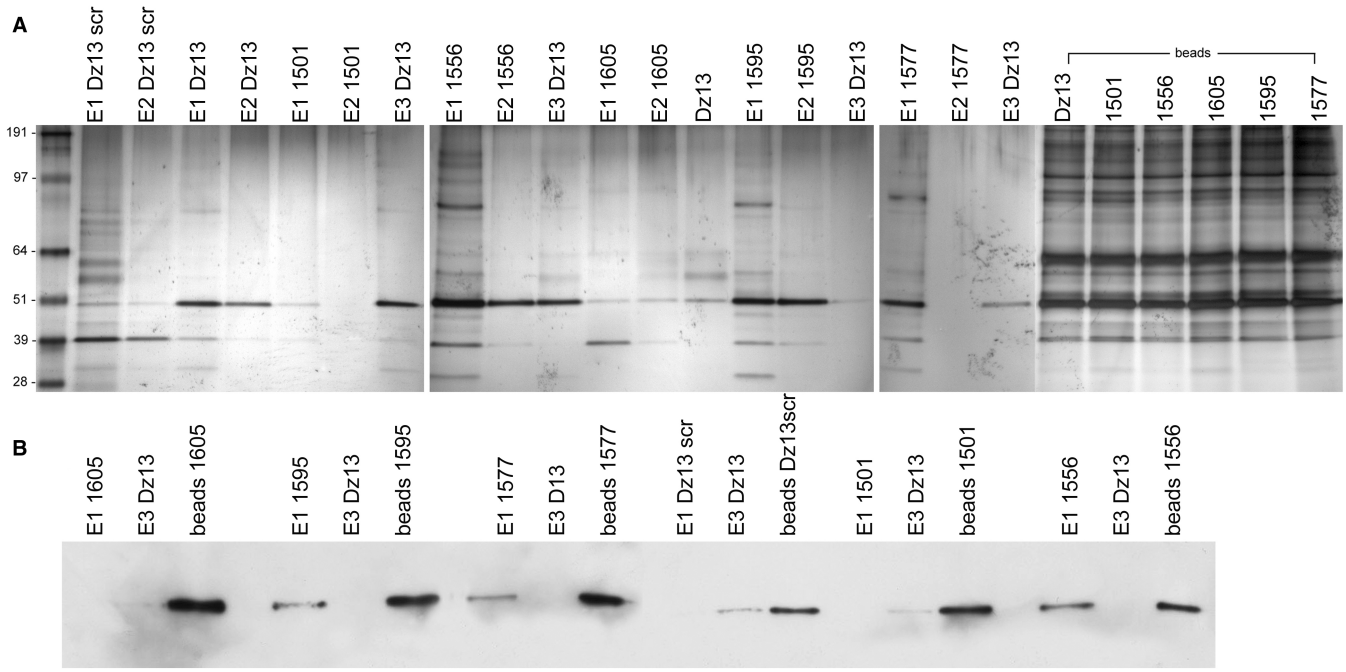

Figure 4. Elution of eEF1A from Dz13-beads using cytotoxic and non-cytotoxic ODNs. Following binding of HMEC-1 proteins to Dz13-beads, proteins were eluted twice using 1 μM of the indicated ODN. Before secondary elution with 1 μM Dz13 the beads were washed three times with binding buffer. Proteins were electrophoresed and the gels (**A**) silver-stained or (**B**) transferred to PVDF membrane and probed for eEF1A. Lanes labelled 'beads' refers to proteins left bound to beads after wash and elutions steps. The experiment was performed twice and the data presented have been compiled from separate gels but are representative results from a single experiment. E refers to 'Elution' and the approximate molecular weight (kDa) is indicated.

Role of eEF1A

In order to further define the role of eEF1A in the cytotoxic mechanism of Dz13 and to determine whether cytotoxicity correlates with eEF1A binding, pull-down assays were performed where active and inactive oligodeoxynucleotides were used to elute proteins bound to Dz13. DT1501 (rat *c-jun* Dz13 homologue) and Dz13scr are non-cytotoxic to HMEC-1 cells under the conditions used here (15). Cytotoxicity experiments were performed with a GT-rich oligodeoxynucleotide (DT1605; Table 1) previously reported as binding to eEF1A and inducing cytotoxicity in human T-lymphoblasts (11). However, this GT ODN was not cytotoxic to HMEC-1 cells at concentrations of up to 200 nM (data not shown). The oligodeoxynucleotides DT1595 (AGGG repeat), Rs6 (DT1556) and NT36 (DT1577; ATM-inducing) all resulted in significant cytotoxicity to HMEC-1 cells (15). Elution of eEF1A from Dz13-coupled beads occurred for all the cytotoxic oligodeoxynucleotides tested (Figure 4). In contrast, elution of eEF1A using non-cytotoxic oligodeoxynucleotides was very poor (Figure 4). Nevertheless, when Dz13 was subsequently used as an eluting solution, eEF1A was strongly released from beads that had been first eluted with the non-cytotoxic oligodeoxynucleotides Dz13scr and DT1501, and to a lesser extent with DT1605. eEF1A was only weakly released by Dz13 from beads where eEF1A had been strongly eluted using the cytotoxic oligodeoxynucleotides DT1595, DT1577 and DT1556 (Figure 4). This demonstrates that eEF1A initially captured by the biotinylated Dz13 beads and only weakly released by the non-cytotoxic ODN could still be released with Dz13 solutions.

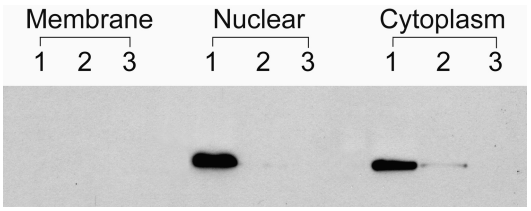

Figure 5. Binding of eEF1A from different cellular fractions of HMEC-1 cells. Protein pull-downs using Dz13-beads were performed on cytoplasmic, nuclear and membrane/organelle fractions. 1, eEF1A bound to Dz13-beads; 2, eEF1A eluting from Dz13 beads with 1 μM Dz13; 3, eEF1A eluting from Dz13-beads protein with 1 μM Dz13scr.

To determine the cellular localization of the eluting eEF1A, the pull-down procedure was performed on the cytoplasmic, nuclear, membrane and cytoskeletal fractions of HMEC-1 cells. Whereas eEF1A from the cytoplasmic, nuclear and membrane/organelle fractions bound to Dz13 beads, the major source of eEF1A eluted by Dz13 was from the cytoplasm (Figure 5). Pull-downs were also performed with total protein lysates from a number of other cell lines including RSMC, HEK-293 and HCT-116, to which Dz13 is cytotoxic (15). Predominant elution by Dz13 of the 51 kDa band was observed in all cases and this band was confirmed as being eEF1A by western blotting (data not shown). The results indicate that eEF1A binding also occurs in other cell lines in which Dz13 is cytotoxic. When examined directly by western blotting, the

abundance of eEF1A was not dependent on the cell type and was not affected by treatment with Dz13 for 24 h (data not shown).

The amount of eEF1A eluted with Dz13 was concentration dependent in that a concentration of 10 μM Dz13 eluted more eEF1A than a concentration of 1 μM (data not shown). Nevertheless, in all experiments elution was only partial, and residual eEF1A was found associated with beads post elution (Figures 3 and 4). This suggests that the interaction might not be canonical or that eEF1A undergoes a conformational change upon binding that inhibits its release. In addition to *in vitro* binding experiments, cells were transfected with 100 nM biotinylated Dz13 and Dz13scr and pull-downs performed directly on cell extracts. There was binding of eEF1A to both Dz13 and Dz13scr beads (data not shown), confirming the presence of the interaction of both oligodeoxynucleotides when the binding occurs within the cells. Furthermore, despite the high abundance of eEF1A, a possible interaction between Dz13 and eEF1A directly in cells was demonstrated using fluorescence colocalization experiments (Figure S2).

DISCUSSION

Whilst there are a number of reports describing the cytotoxic nature of certain oligodeoxynucleotides, in particular G-rich oligodeoxynucleotides (see Introduction Section), there is little known about the motif or sequence requirements for cytotoxicity. The current study attempted to define further the exact requirements for cytotoxicity and identify proteins involved in binding to these oligodeoxynucleotides, thereby unravelling the mechanisms involved in the eventual cytotoxicity of the molecules.

We determined that the 5′ sequence, the core or the tail of Dz13 alone are insufficient to regenerate the cytotoxic activity of Dz13 against HMEC-1 cells, thereby indicating a length and/or sequence requirement. We used the novel strategy of testing pools of oligodeoxynucleotides comprised of random sequences along with the 5′ G-rich elements of Dz13 and Rs6 in various position and length contexts to demonstrate that the G-rich element needs to be present at the 5′ extremity and be followed by a tail component that is preferably composed of mixed nucleotides. A comparison of the active oligodeoxynucleotides, in combination with the 5′-motif reduction experiment, leads us to conclude that the required 5′ sequence is G-rich, composed of 6–9 nt with at least four consecutive purines. The presence of a triple G motif (G-G-G) provides for the greatest potency and the 5′ extremity of the triple G motif needs to be positioned no more than 3 nt from the 5′ end of the ODN. We have previously shown by CD that Dz13 does not assemble into stable secondary structures *in vitro* (15). Nevertheless, substitution of any of the guanosines in the triple G motif of Dz13 abrogated its cytotoxic activity (15). This suggests that the hydrogen bonding activity of these guanosines does not lead to G-quadruplex formation, but is nevertheless required for cytotoxicity, perhaps by enhancing binding of the oligodeoxynucleotides to intracellular proteins. The tail sequence requirements were intriguing in that most sequences with some degree of 'complexity' supported cytotoxic activity, but some individual tail sequences, in particular those free of guanosines, had reduced activity. It is possible that the less complex tail sequences such as the polydA tail are being sequestered by polydA-binding proteins thereby abrogating cytotoxicity. Wu *et al.* (22) have previously demonstrated that tail sequence and complexity encourages the multimeric aggregation of the oligodeoxynucleotides, thereby activating TLR9 and cell uptake. However, we have previously demonstrated a need for transfection and ruled out endosomal TLR engagement in the mechanism of action of Dz13 and Rs6 against HMEC-1 cells (15). Therefore, although multimeric assembly remains a possibility, it is unlikely to relate to uptake and TLR engagement.

We previously compared the activity of Dz13, a prototypic G-rich oligodeoxynucleotide, with that of other published sequences including NT36, an ATM-inducing oligodeoxynucleotide (23) and 20AG, an oligodeoxynucleotide originally designed to be a triplex-forming inhibitor of K-ras (17). Both of these oligodeoxynucleotides have purine tracts in the 5′ sequence and NT36 is of comparable length to the active ODNs described in the present study. As shown for Dz13, the potency of the shorter 20AG ODN was length dependent. That is, the cytotoxicity of the AG molecule increased as the length of the molecule increased.

ODNs have been proposed to exert their cytotoxicity through specific interaction with cellular proteins such as nucleolin (24) and eEF1A (7). Nucleolin was identified as one of the proteins that bound to Dz13 and Dz13scr. The absence of an effect on HMEC-1 proliferation by the nucleolin-binding oligodeoxynucleotide GRO29A provided further evidence for the lack of direct involvement of nucleolin in Dz13-mediated cytotoxicity. Likewise, a representative from a class of GT-rich ODNs, which reportedly binds eEF1A and is cytotoxic to human T-lymphoblasts (11), was not cytotoxic to HMEC-1 cells. Furthermore in our experiments, the GT oligodeoxynucleotide did not displace eEF1A from Dz13 capture beads as potently as Dz13 or the other cytotoxic ODNs tested, indicating that it has less binding affinity to the eEF1A found in this cell line. The ODNs described in the present study therefore represent a novel class of potently cytotoxic molecules.

The ability of several of the proteins, including eEF1A to bind competitively to the cytotoxic oligodeoxynucleotides and not be eluted by the non-cytotoxic ODNs *in vitro* suggests that the ODNs may competitively bind to proteins within the cells and that this is mechanistically relevant to the cytotoxicity of the molecule. eEF1A is an extremely abundant protein with a multitude of roles including protein synthesis, stress-sensing, apoptosis and cellular proliferation (25,26). Scaggiante *et al.* (7) recently reported a correlation between eEF1A binding and cytotoxicity of G-rich oligodeoxynucleotides that is independent of secondary structure formation. They also reported that some non-cytotoxic G-rich oligodeoxynucleotides bind eEF1A to a small degree in the absence

of competitor (7). The fact that both Dz13 and Dz13scr were able to bind eEF1A *in vitro* and in cells indicates that some of the captured eEF1A is also binding via a non-specific mechanism in HMEC-1 cells. However, the selective release of eEF1A from Dz13 capture-beads with those ODNs that are cytotoxic suggests that there is a relationship between eEF1A binding and cytotoxicity.

eEF1A has been proposed to act as a signalling molecule in proliferating cells through the binding of other nucleic-acid-binding proteins such as ZPR1 (27) followed by translocation to the nucleus. Once inside the nucleus eEF1A has the capacity to bind DNA, RNA and RNA polymerase and therefore potentially plays a role in transcriptional regulation. Binding of eEF1A to Dz13 was observed in nuclear, cytoplasmic, membrane/organelle and cytoskeletal fractions; cytoplasmic eEF1A eluted most strongly from Dz13 beads. This could reflect a greater concentration of DNA-binding proteins in the nuclear fraction and consequently a greater number of proteins eluting in general or that nuclear eEF1A has a higher affinity for Dz13. It is possible that the higher affinity cytotoxic oligodeoxynucleotides displace eEF1A from nuclear or cytoplasmic protein complexes such as the nuclear cytotoxicity-related complex (CRC; 7), thereby inhibiting the normal functioning of eEF1A in the cell, resulting in a reduction in cellular proliferation and eventually cell death.

Vimentin was also identified in the pull-down assay. Vimentin has been shown to bind to G-rich ODNs, causing the translocation of the vimentin–DNA complex to the nucleus (28). However, this was not evident in HMEC-1 cells transfected with Dz13. Collectively the protein pull-down experiments indicate that binding of cytotoxic ODNs to eEF1A and other proteins is potentially a key event in the mechanism of action of the molecules.

In conclusion, we have determined that ODNs such as Dz13 and Rs6 which were initially designed as catalytic DNAzymes, belong to a group of cytotoxic G-rich ODNs with novel sequence and length requirements. Although the mechanism is unclear, it is likely to be mediated through binding to eEF1A. These molecules are preferentially active against proliferating cells and therefore constitute part of a new class of potentially useful molecules for the treatment or investigation of diseases characterized by abnormal cell proliferation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Riou,J.F., Guittat,L., Mailliet,P., Laoui,A., Renou,E., Petitgenet,O., Megnin-Chanet,F., Helene,C. and Mergny,J.L. (2002) Cell senescence and telomere shortening induced by a new series of specific G-quadruplex DNA ligands. *Proc. Natl Acad. Sci.*, **99**, 2672–2677.
2. Scaggiante,B., Morassutti,C., Dapas,B., Tolazzi,G., Ustulin,F. and Quadrifoglio,F. (1998) Human cancer cell lines growth inhibition by GTn oligodeoxyribonucleotides recognizing single-stranded DNA-binding proteins. *Eur. J. Biochem.*, **252**, 207–215.
3. Cogoi,S., Ballico,M., Bonora,G.M. and Xodo,L.E. (2004) Antiproliferative activity of a triplex-forming oligonucleotide recognizing a Ki-ras polypurine/polypyrimidine motif correlates with protein binding. *Cancer Gene Ther.*, **11**, 465–476.
4. Cogoi,S., Quadrifoglio,F. and Xodo,L.E. (2004) G-rich oligonucleotide inhibits the binding of a nuclear protein to the Ki-ras promoter and strongly reduces cell growth in human carcinoma pancreatic cells. *Biochemistry*, **43**, 2512–2523.
5. Dapic,V., Bates,P.J., Trent,J.O., Rodger,A., Thomas,S.D. and Miller,D.M. (2002) Antiproliferative activity of G-quartet-forming oligonucleotides with backbone and sugar modifications. *Biochemistry*, **41**, 3676–3685.
6. Dapic,V., Abdomerovic,V., Marrington,R., Peberdy,J., Rodger,A., Trent,J.O. and Bates,P.J. (2003) Biophysical and biological properties of quadruplex oligodeoxyribonucleotides. *Nucleic Acids Res.*, **31**, 2097–2107.
7. Scaggiante,B., Dapas,B., Grassi,G. and Manzini,G. (2006) Interaction of G-rich GT oligonucleotides with nuclear-associated eEF1A is correlated with their antiproliferative effect in haematopoietic human cancer cell lines. *FEBS J.*, **273**, 1350–1361.
8. Russo,G., Cuccurese,M., Monti,G., Russo,A., Amoresano,A., Pucci,P. and Pietropaolo,C. (2005) Ribosomal protein L7a binds RNA through two distinct RNA-binding domains. *Biochem. J.*, **385**, 289–299.
9. Hanakahi,L.A., Sun,H. and Maizels,N. (1999) High affinity interaction of nucleolin with G-G-paired rDNA. *J. Biol. Chem.*, **274**, 15906–15912.
10. Dempsey,L.A., Sun,H., Hanakahi,L.A. and Maizels,N. (1999) G4 DNA binding by LR1 and its subunits, nucleolin and hnRNP D, a role for G-G pairing in immunoglobulin switch recombination. *J. Biol. Chem.*, **274**, 1066–1071.
11. Dapas,B., Tell,G., Scaloni,A., Pines,A., Ferrara,L., Quadrifoglio,F. and Scaggiante,B. (2003) Identification of different isoforms of eEF1A in the nuclear fraction of human T-lymphoblastic cancer cell line specifically binding to aptameric cytotoxic GT oligomers. *Eur. J. Biochem.*, **270**, 3251–3262.
12. Jing,N., Li,Y., Xu,X., Sha,W., Li,P., Feng,L. and Tweardy,D.J. (2003) Targeting Stat3 with G-quartet oligodeoxynucleotides in human cancer cells. *DNA Cell. Biol.*, **22**, 685–696.
13. Benimetskaya,L., Tonkinson,J.L., Koziolkiewicz,M., Karwowski,B., Guga,P., Zeltser,R., Stec,W. and Stein,C.A. (1995) Binding of phosphorothioate oligodeoxynucleotides to basic fibroblast growth factor, recombinant soluble CD4, laminin and fibronectin is P-chirality independent. *Nucleic Acids Res.*, **23**, 4239–4245.
14. Cairns,M., Saravolac,E. and Sun,L. (2002) Catalytic DNA: a novel tool for gene suppression. *Current Drug Targets*, **3**, 269–279.
15. Rivory,L., Tucker,C., King,A., Lai,A., Goodchild,A., Witherington,C., Gozar,M.M. and Birkett,D. (2006) The DNAzymes Rs6, Dz13 and DzF have potent biological effects independent of catalytic activity. *Oligonucleotides*, **16**, 297–312.
16. Rabilloud,T., Vuillard,L., Gilly,C. and Lawrence,J.J. (1994) Silver-staining of proteins in polyacrylamide gels: a general overview. *Cell. Mol. Biol.*, (Noisy-le-grand), **40**, 1135–1143.
17. Cogoi,S., Rapozzi,V., Quadrifoglio,F. and Xodo,L. (2001) Anti-gene effect in live cells of AG motif triplex-forming oligonucleotides containing an increasing number of phosphorothioate linkages. *Biochemistry*, **40**, 1135–1143.
18. Fahmy,R.G., Waldman,A., Zhang,G., Mitchell,A., Tedla,N., Cai,H., Geczy,C.R., Chesterman,C.N., Perry,M. *et al.* (2006) Suppression of vascular permeability and inflammation by targeting of the transcription factor c-Jun. *Nat. Biotechnol.*, **24**, 856–863.
19. Khachigian,L.M., Fahmy,R.G., Zhang,G., Bobryshev,Y.V. and Kaniaros,A. (2002) c-Jun regulates vascular smooth muscle cell

growth and neointima formation after arterial injury: inhibition by a novel DNA enzyme targeting c-Jun. *J. Biol. Chem.*, **277**, 22985–22991.
20. Zhang,G., Dass,C.R., Sumithran,E., Di Girolamo,N., Sun,L.Q. and Khachigian,L.M. (2004) Effect of deoxyribozymes targeting c-Jun on solid tumor growth and angiogenesis in rodents. *J. Natl Cancer Inst.*, **96**, 683–696.
21. Dunn,K.C., Aotaki-Keen,A.E., Putkey,F.R. and Hjelmeland,L.M. (1996) ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. *Exp. Eye Res.*, **62**, 155–169.
22. Wu,C.C., Lee,J., Raz,E., Corr,M. and Carson,D.A. (2004) Necessity of oligonucleotide aggregation for toll-like receptor 9 activation. *J. Biol. Chem.*, **279**, 33071–33078.
23. Nur-E-Kamal,A., Li,T.K., Zhang,A., Qi,H., Hars,E.S. and Liu,L.F. (2003) Single-stranded DNA induces ataxia telangiectasia mutant (ATM)/p53-dependent DNA damage and apoptotic signals. *J. Biol. Chem.*, **278**, 12475–12481.
24. Bates,P.J., Kahlon,J.B., Thomas,S.D., Trent,J.O. and Miller,D.M. (1999) Antiproliferative activity of G-rich oligodeoxynucleotides correlates with protein binding. *J. Biol. Chem.*, **274**, 26369–26377.
25. Duttaroy,A., Bourbeau,D., Wang,X.L. and Wang,E. (1998) Apoptosis rate can be accelerated or decelerated by overexpression or reduction of the level of elongation factor-1 alpha. *Exp. Cell Res.*, **238**, 168–176.
26. Lamberti,A., Caraglia,M., Longo,O., Marra,M., Abbruzzese,A. and Arcari,P. (2004) The translation elongation factor 1A in tumorigenesis, signal transduction and apoptosis: review article. *Amino Acids*, **26**, 443–448.
27. Gangwani,L., Mikrut,M., Galcheva-Gargova,Z. and Davis,R.J. (1998) Interaction of ZPR1 with translation elongation factor-1alpha in proliferating cells. *J. Cell. Biol.*, **143**, 1471–1484.
28. Hartig,R., Shoeman,R.L., Janetzko,A., Tolstonog,G. and Traub,P. (1998) DNA-mediated transport of the intermediate filament protein vimentin into the nucleus of cultured cells. *J. Cell Sci.*, **111**, 3573–3584.
29. Sun,L.Q., Cairns,M.J., Gerlach,W.L., Witherington,C., Wang,L. and King,A. (1999) Suppression of smooth muscle cell proliferation by a c-myc RNA-cleaving deoxyribozyme. *J. Biol. Chem.*, **274**, 17236–17241.