Antisense oligonucleotides directed against p53 have antiproliferative effects unrelated to effects on p53 expression

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Summary: Antisense oligonucleotides targeting p53 have been hailed as a potentially new technique for treating patients with cancer, and there have been encouraging reports of good patient tolerance in vitro and of antiproliferative effects in vivo. However, evidence is lacking that these oligonucleotides are acting via an antisense interaction to modulate p53 expression. We examined a phosphorothioate antisense oligonucleotide, directed against exon 10 of the TP53 gene, and a chimaeric phosphorothioate–phosphodiester oligonucleotide directed against the p53 translation initiation codon. Both failed to specifically suppress p53 gene product in a cell-free assay system or to have any effect on mutant p53 expression by human pancreatic cancer cell lines. Antiproliferative effects were apparent, especially with the phosphorothioate antisense oligonucleotide, but this was independent of the p53 status of the cells (mutant, wild-type or absent) and also occurred with the control (sense and randomised) oligonucleotides. The most dramatic antiproliferative effects were seen with the ‘control’ phosphorothioate oligonucleotides. These findings suggest that the antiproliferative effects of some antisense oligonucleotides may be unrelated to expression of the gene they have been designed to target.

Keywords: antisense; oligonucleotide; p53; cancer therapy

Modulation of gene expression by naturally occurring antisense interactions is well documented in prokaryotes and may occur naturally in eukaryotic cells (reviewed in Murray and Crockett, 1992; Thomas, 1992; Nellen and Lichtenstein, 1993). Antisense techniques have also been devised to selectively reduce gene expression by the sequence-specific binding of complementary nucleic acids. Such techniques have become powerful tools for selectively reducing the expression of target genes in vitro, and there is increasing interest in the possibility of using the same technology in vivo for therapeutic purposes.

The antisense oligonucleotide approach involves the exogenous administration of short, synthetic, single-stranded oligonucleotide sequences, generally DNA based, which are taken up relatively inefficiently by the cell and released into the cytoplasm. They are thought to act predominantly by blocking translation of mRNA (reviewed in Murray and Crockett, 1992; Toulmé, 1992; Carter and Lemoine, 1993; Nellen and Lichtenstein, 1993; Prins et al., 1993). Sequences complementary to the protein-coding part of the mRNA are thought to act mainly by causing cleavage by RNase H, which specifically degrades the RNA component of RNA–DNA hybrids. Sequences which target the 5′ untranslated region are thought to prevent attachment and sliding of the 40S ribosomal subunit by steric hindrance, and those which bind close to the AUG initiation codon may prevent further assembly of the translation initiation complex.

Most work has been done with oligonucleotides modified to increase their stability and lipid solubility, for example by replacement of the phosphodiester linkage in the sugar–phosphate backbone to increase nuclease resistance. A phosphorothioate linkage (in which the oxygen atom is replaced by a sulphur atom) is a commonly used modification. Chimaeric oligonucleotides have the modified linkage for only part of the molecule, usually the 5′ and 3′ ends, since intracellular degradation is mainly due to exonucleases (Giles and Tidd, 1992; Ortigao et al., 1992; Toulmé, 1992).

TP53 is an attractive target for an antisense approach in human cancer for several reasons. Although classified as a tumour-suppressor gene, the mutant forms commonly found in human tumours have many of the features associated with a dominant oncogene, including transforming activity (Parada et al., 1984). TP53 is the most frequent gene to be mutated in human cancer, and expression of mutant p53 is thought to be important in a wide range of tumours, including many that are primarily resistant to conventional forms of therapy (Nigro et al., 1989; Levine, 1992; Levine et al., 1994). Wild-type p53 is dispensable for normal cell growth and metabolism, albeit at the cost of an increased susceptibility to malignant change (Malink et al., 1990; Srivastava et al., 1990; Donehower et al., 1992; Harvey et al., 1993). There is also evidence for a gene dosage effect so that complete suppression of mutant p53 expression may not be necessary for a significant anti-tumour effect, especially if there is some residual wild-type gene expression (Schafer et al., 1994). Lastly, there is evidence that p53 expression may be modulated naturally by the endogenous production of antisense RNA transcripts (Khochbin et al., 1992).

Phosphorothioate oligonucleotides have now been given systematically to animals and humans (Iversen et al., 1992; Spinolo et al., 1992a). In a phase I clinical trial a phosphorothioate oligonucleotide targeting p53 exon 10, OL(1) p53, was given by continuous infusion for up to 10 days and was well tolerated with few adverse effects (Spinolo et al., 1992a; Bishop et al., 1993; Bayever et al., 1993, 1994). This oligonucleotide has been reported to have a significant antiproliferative effect on AML (Bayever et al., 1994) and pancreatic cancer cells (Bayever and Haines, 1993) in vitro, and, when given by systemic infusion to patients, to inhibit the growth of their leukaemic blasts in vitro (Spinolo et al., 1992b; Bishop et al., 1993; Bayever et al., 1993, 1994). Exciting as these reports are, no evidence has been provided that the oligonucleotide is acting via an antisense interaction or that it modulates p53 expression. We have investigated this oligonucleotide in pancreatic cancer cell lines, since expression of mutant p53 is thought to be an important early event in pancreatic carcinogenesis (Barton et al., 1991; Wylie et al., 1993) and because pancreatic cancer is notoriously resistant to currently available cancer therapies (Alanen and Joensuu, 1993; Ellis and Cunningham, 1994). For comparison, we have also investigated a chimaeric phosphorothioate–phosphodiester antisense oligonucleotide which targets a different region of p53 mRNA, the initiation codon.

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This oligonucleotide has been reported to reduce p53 expression and alter proliferation in vitro in chronic myeloid leukaemia (CML) cells (Bi et al., 1994).

**Materials and methods**

**Cells**

Six pancreatic cell lines were studied, three of which (PANC-1, AsPC-1, CaPan-2) were obtained from the American Type Culture Collection (ATCC) and three from original sources. PT45 and 818.4 were gifts from Dr H Kalithoff and Dr W Schmiegel (Department of Immunology, University Hospital Eppendorf, Hamburg, Germany) and Colo-357 from Dr G Morgan (Surgical Division, Denver, CO, USA). Cells were cultured in RPMI-1640 or Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated (35°C for 30 min) fetal calf serum (FCS) and antibiotics. Media were supplied by ICRF Media Production and serum obtained from Life Technologies.

**Antisense oligonucleotides**

Six antisense oligonucleotides were synthesised and purified by high-performance liquid chromatography at the ICRF Oligonucleotide Synthesis Laboratory, Clare Hall. On receipt, they were double washed with 70% ethanol, briefly air dried and resuspended in sterile RPMI-1640 to a final concentration of 100 μM. After checking the concentration by spectrophotometry, they were stored in aliquots at −20°C.

The antisense oligonucleotide OLl p53as (corresponding to A-OdN or OLl p53 in other reports) is complementary to a region within exon 10 of the TP53 gene, and the phosphodiester backbone is replaced throughout with a phosphorothioate linkage (Spinolo et al., 1992b; Bayever and Haines 1993; Bishop et al., 1993; Bayever et al., 1993, 1994).

Two control oligonucleotides were selected for OLl p53as: OLl p53s, which is complementary to OLl p53as, and OLl p53rand, in which the OLl p53as sequence is randomised. Both control oligonucleotides were synthesised with phosphorothioate linkages throughout (indicated by underlining).

OLl p53as CCCTGCTCCCCCTTGTCCTC
OLl p53s GGGAGCAGGGGGGAGCAGGG
OLl p53rand GGGCGCTTCCTCCTGCGCCC

The antisense oligonucleotide Bip53as is complementary to the 18 nucleotides flanking and including the TP53 ATG initiation codon and the phosphodiester backbone is replaced by phosphorothioate links between the first four and last four nucleotides to improve stability (Bi et al., 1994). Two control oligonucleotides were selected for Bip53as: Bip53s, which is complementary to Bip53as, and Bip53rand, in which the sequence Bip53as is randomised. Both control oligonucleotides have the same chimaeric structure with three phosphothioate links between the first four and last four nucleotides (indicated by underlining).

Bip53as CCGCTCTCCATGGCAGT
Bip53s ACTGCGATAGAGAGCAGC
Bip53rand GTCCTCTGGATCG

The oligonucleotide sequences were checked for predicted secondary structure by direct scrutiny and by computer simulation. Bip53as, Bip53s, OLl p53as and OLl p53s were all predicted to form stems of two nucleotides and Bip53rand and OLl p53rand were predicted to form a stem of three nucleotides (CGG). All were checked for matches and complementarity to other human genetic sequences. Significant complementarity was found with several other proliferation and differentiation-related genes (for example, OLl p53as and granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor β-chain mRNA; OLl p53s and human platelet-derived growth factor A; and integrin α-3 chain mRNA). No significant complementarity was detected between the control oligonucleotides Bip53s, Bip53rand, OLl p53s and OLl p53rand and TP53, TP53 cDNA or the transcription factor AP2 CDNA. However, when the sequence of firefly luciferase was checked for fortuitous complementarity with the oligonucleotides used, significant complementarity was found in some instances. Bip53as had no significant complementarity with firefly luciferase, Bip53s had one region of complementarity starting at codon 1946 (11–18 bp) and Bip53rand had five regions of complementarity starting at codons 17 (10–18 bp), 1481 (12–18 bp), 1658 (12–18 bp), 1745 (12–18 bp) and 2129 (11–18 bp). OLl p53s was complementary to one region starting at codon 1423 (12–20 bp), OLl p53s was complementary to one region (11–20 bp) starting at codon 401 and OLl p53s was complementary to four regions starting at codons 355 (9–20 bp), 1785 (11–20 bp), 1952 (10–20 bp) and 2134 (10–20 bp). The sequences of the SP6 and T7 promoters were also checked for complementarity with the six oligonucleotides and no significant complementarity was found.

To verify that the antisense oligonucleotides selected were capable of binding their putative target sequences, at least in DNA form, PCR amplifications were performed using the pSP65p53 plasmid (kindly supplied by Dr T Crooke, Ludwig Institute for Cancer Research, St Mary's Hospital Medical School, London, UK) as template. Polymerase chain reaction (PCR) with primer pairs Bip53s and OLl p53as or Bip53as and OLl p53s reliably produced the 1007 bp predicted product using a variety of PCR conditions (data not shown).

**The CellTiter 96 non-radioactive cell proliferation/cytotoxicity assay (MTT assay)**

The Promega CellTiter 96 non-radioactive cell proliferation/cytotoxicity assay (MTT assay), was performed according to the manufacturer's instructions, as follows. A cell suspension was prepared from cells growing in standard tissue culture dishes by trypsin/ versene treatment. Cells were counted and the suspension adjusted to give 1–2 x 10^5 cells ml \(^{-1}\) then 100 μl was placed in each well of replicate 96-well culture dishes in the presence of different concentrations (0.5 μM, 1 μM, 5 μM and 10 μM) of antisense and control oligonucleotides. MTT assays were performed after 24, 48 and 72 h growth. Fifty microlitres of MTT dye solution was added to each well and the plates returned to the 37°C incubator for 4 h. The 100 μl of solubilisation solution was added to each well and incubation continued for another hour. The contents of each well were then mixed briefly using a multichannel pipette and the absorbance measured on an enzyme-linked immunosorbent assay (ELISA) plate reader (Titertek Multiskan MCC/340) at a wavelength of 540 nm. Each assay was performed in a multiple of 8 and the results averaged.

**p53 ELISA assay**

A quantitative measure of p53 expression was obtained using a recently developed ELISA assay (Vojtesek et al., 1993). Initially cells were made quiescent by growing in serum-deficient (0.5% FCS) medium overnight. Then the medium was changed to incorporate 10% FCS and different concentrations of oligonucleotide. Later experiments were performed with cells plated out directly into standard (10% FCS) medium containing different concentrations of oligonucleotides. After various periods of the medium was changed to ice in a buffer containing 150 mM sodium chloride, 50 mM Tris pH 7.4, 5 mM EDTA, 1% NP-40, 1 mM phenylmethyl-sulphonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), aprotinin 10 ng ml \(^{-1}\) and leupeptin 10 ng ml \(^{-1}\). Cell extracts were centrifuged at 100000 g for 30 min and the pellets discarded. The protein content of the supernatants was measured using the BCA protein assay kit (Fierce) according to the manufacturer's instructions. Supernatants were stored at −20°C before use.

Meanwhile, Falcon 96-well microtitre plates were incubated overnight at room temperature in a humid chamber with 50 μl per well of antibody DO-7 (Novocastra), diluted 1:500 in phosphate-buffered saline (PBS). Plates were washed with PBS and blocked for 2 h with 200 μl of PBS/3% bovine serum albumin (BSA) at room temperature, then rinsed again.
with PBS. Fifty microliters of cytosol extract was added to each antibody-coated well and incubated for 3 h at 4°C. Plates were washed with PBS and 50 μl of the second antibody, CM-1 (Novocastra) diluted 1:1000 in PBS/1% BSA, was added to each well. Plates were incubated for 2 h at 4°C, washed with PBS and peroxidase-conjugated swine anti-rabbit antiserum (Dako), diluted 1:500 in PBS/1% BSA, was added, 50 μl per well. After 2 h incubation at 4°C and a final wash with PBS, bound enzyme activity was detected as follows. A fresh solution of o-phenylenediamine and hydrogen peroxide was made up in 50 mM sodium phosphate buffer, pH 6.0 (2 mg of o-phenylenediamine and 1 μl of hydrogen peroxide per ml of sodium phosphate buffer). One hundred microliters of this was added to each well and the reaction monitored for 5–20 min at room temperature then stopped with the addition of 100 μl of 1 m M hydrochloric acid. The absorbance was measured on an ELISA plate reader at a wavelength of 492 nm. All assays were performed in quadruplicate.

To calibrate, 12 serial dilutions (ranging from 1 to 1000 ng ml⁻¹) of purified soluble recombinant p53 protein (kindly supplied by Dr A Coffer, Protein Isolation and Cloning Laboratory, ICRF, Lincoln’s Inn Fields, London, UK) were run with every experiment. Control assays were also performed on each occasion with no p53 protein (lysis buffer only), no DO-7 antibody, no CM-1 antibody and no peroxidase-conjugated swine antirabbit antiserum so that background non-specific reactivity could be accounted for.

In vitro transcription/translation assay

In vitro transcription/translation assays were performed using a Promega TNT coupled reticulocyte lysate system according to the manufacturer’s instructions. Briefly a master mix was made up on ice containing TNT rabbit reticulocyte lysate (12.5 μl per sample), TNT reaction buffer (1 μl), TNT SP6 RNA polymerase or TNT T7 RNA polymerase (0.5 μl), 1 mM amino acid mixture minus methionine (0.5 μl), RNasin ribonuclease inhibitor (0.5 μl) and [α-35S]l-methionine (2 μl). The mixture was added to 500 ng of DNA template with or without the appropriate oligonucleotide and diethylpyrocarbonate (DEPC)-treated water to a final volume of 25 μl. The reaction mix was then incubated at 30°C for 1–2 h. An aliquot was mixed with an equal volume of 2× sample loading buffer and boiled for 2 min to denature the protein. Six microliters of each sample was electrophoresed on a 10% denaturing polyacrylamide (acylamide–bisacrylamide, 29:1) gel overnight at a constant current of 3.5 mA. The gel was to cut to size, fixed, soaked in Amplify (Amersham International), dried and exposed to radiographic film for 2–6 h.

Three plasmids were used as template. The plasmid pSP65p53 has full-length wild-type TP53 cDNA cloned downstream to an SP6 RNA polymerase promoter. In addition to full-length p53 protein, in vitro transcription/translation is known to produce a 46 kDa protein as a result of translation initiation at the methionine residue at nucleotide 332 and other smaller internally initiated peptides (Harlow et al., 1985). The plasmid T7AP2, kindly supplied by Dr Julia Bosher (ICRF Oncology Unit, Hammersmith Hospital, London, UK), has the cDNA for transcription factor AP2 cloned downstream of a T7 promoter. In vitro transcription/translation of this construct produces a 46 kDa protein. In vitro transcription/translation of luciferase template DNA, supplied with the kit, was used as a positive control and produces a 61 kDa protein. Every experiment included a negative control without template DNA.

Results

Morphological effects of oligonucleotide treatment

Six pancreatic cancer cell lines were selected for analysis. Two express mutant p53 (PANC-1, PT45), two express wild-type p53 (Colo-357 and 818.4) and two express no p53 (AsPC-1 and CaPan-2) (Barton et al., 1991; Ruggeri et al., 1992; Kalthoff et al., 1993; Simon et al., 1994; Berrozpe et al., 1994). Each was treated with two antisense oligonucleotides, Bisp53as and OL1p53as, which target the AUG initiation codon and exon 10 of p53 mRNA respectively. Each cell line was also treated with sense and sequence-randomised controls (Bisp53s, Bisp53rand, OL1p53s and OL1p53rand respectively). Four different oligonucleotide concentrations (0.5 μM, 1 μM, 5 μM, 10 μM) were used.

Within 24 h of exposure to the oligonucleotides profound morphological changes were noted in cells growing in OL1p53s (Figure 1). To a lesser extent the same morphological changes were seen in cells growing in the presence of OL1p53rand, although at 0.5 μM concentration of OL1p53rand the cells appeared relatively unharmed. All cell lines were affected regardless of TP53 status, although to slightly different extents, and the effects were obviously dose related and became more noticeable as time went on. The cells clumped together, rounded up and lost attachment to the tissue culture dishes and there were fewer cells present (as
reflected in the MTT findings). However, many of the 'rounded-up' cells appeared still to be viable since they could be re-established in culture after removal from the oligonucleotide solution and washing.

These morphological effects were consistently seen in all the cell lines being prepared for MTT assay but not in cells being prepared for ELISAs. Cells for ELISAs were grown for 16 h in serum-deficient (0.5% FCS) culture medium and were fully attached to the culture dishes before the oligonucleotide was added, whereas the cells for MTT assay were plated out directly into medium containing the oligonucleotides, having been removed from large culture dishes by standard versene/trypsin treatment. Others have observed that the effects of oligonucleotide administration may vary depending on how

![Graphs showing effect of antisense and control oligonucleotides on cell proliferation/cytotoxicity in a cell line expressing mutant p53 (PT45).](image)

**Figure 2** Effect of antisense and control oligonucleotides on cell proliferation/cytotoxicity in a cell line expressing mutant p53 (PT45). Cells were grown continuously in the presence of oligonucleotide and MTT assays were performed at 24, 48 and 72 h. For this cell line six graphs are given showing the effects of four different concentrations of oligonucleotide on cell growth. For clarity, error bars are omitted on all but the 'no oligonucleotide' control curves. Very similar results were obtained with five other cell lines: PANC-1 (mutant p53), Colo-357 and 818.4 (wild-type p53) as well as AsPC-1 and CaPan-2 (no p53).
long after cell passage the oligonucleotides are added (H Kalthoff, personal communication).

Effects of oligonucleotides on cell proliferation/cytotoxicity

Cell proliferation/cytotoxicity was measured using the MTT assay for each cell line after 24, 48 and 72 h growth in four different concentrations (0.5 μM, 1 μM, 5 μM, 10 μM) of each oligonucleotide. To a large extent the MTT assays reflected the microscopic appearances of the oligonucleotide-treated cells. The OL1p53 oligonucleotides were non-specifically toxic to all the cell lines regardless of TP53 status. The toxicity was dose and duration dependent, with OL1p53s generally being more toxic than OL1p53rand, which was more toxic than OL1p53as. There was some variation in this pattern from one cell line to another. For example, OL1p53s and OL1p53as seemed equally toxic to the cell lines Colo-357, and CaPan-2 and OL1p53rand seemed more toxic than OL1p53s and OL1p53as to the cell line AsPC-1. All the OL1p53 oligonucleotides were considerably more toxic than the Bip53 oligonucleotides.

The Bip53 oligonucleotides had a mild antiproliferative effect at the highest concentration (10 μM), but this was non-specific with respect to the oligonucleotide (Bip53as, Bip53s and Bip53rand were equally toxic) and the cell line, affecting them to approximately the same extent regardless of TP53 status. Representative MTT results are shown in Figure 2.

Effect of oligonucleotides on p53 expression in pancreatic cell lines

Using the ELISA described, we were unable to detect p53 protein in the cell lines previously documented to lack p53 expression and also in the cell lines with only wild-type p53 expression (Barton et al., 1991; Ruggeri et al., 1992; Kalthoff et al., 1993; Berrozpe et al. 1994). However, p53 was readily detectable in cell lines expressing mutant p53 (PANC-1, FT45 and others), so all the ELISAs were performed using cell lines of this type. FT45, p53 protein levels increased after 12, 24 and 48 h growth in the absence or presence of the antisense and control oligonucleotides, at four different concentrations (0.5 μM, 1 μM, 5 μM, 10 μM) for the OL1p53 oligonucleotides, and at seven different concentrations (0.5 μM, 1 μM, 5 μM, 10 μM, 20 μM, 50 μM and 100 μM) for the Bip53 oligonucleotides. The calibration curve is shown in Figure 3 and representative results in Figure 4.

No difference in p53 level was detectable 12, 24 or 48 h after oligonucleotide was added, regardless of the oligonucleotide added or the final oligonucleotide concentration, even at high concentrations of Bip53 oligonucleotides. Since the morphological effects of the OL1p53 oligonucleotides were only apparent when cells were plated out directly into the oligonucleotide-containing medium after trypsin/verase treatment, we repeated the ELISA measurements on cells passaged in this manner and still observed no apparent effect on p53 protein levels.

Effects of oligonucleotides on in vitro transcription/translation

Using the plasmid p5P65p53 as template we analysed the effects of different concentrations of oligonucleotide on in vitro transcription/translation using the TNT coupled reticulocyte lysate system (Promega). In the absence of oligonucleotide, p53 protein was readily produced, in addition to a 46 kDa and other smaller protein products from alternative internal initiation sites. Slightly less protein was produced overall when any oligonucleotide was present in the reaction mix. In addition, a dose-related inhibition of p53 protein production (and smaller internally initiated proteins) was apparent with the antisense oligonucleotide Bip53as but not for its sense and randomised controls, Bip53s and Bip53rand. Suppression was detectable at 0.5 μM concentration and was almost complete at a concentration of 4 μM (Figure 5). However, at 4 μM concentration Bip53as also significantly inhibited luciferase protein production (Figure 5) and slightly inhibited AP2 protein production (Figure 6). Both these proteins are encoded by genes to which Bip53as has no significant complementarity.

The antisense oligonucleotide OL1p53as and its controls OL1p53s and OL1p53rand all appeared to suppress p53 protein (and smaller internally initiated proteins) production to some extent. At a concentration of 0.5 μM, OL1p53as significantly suppressed and OL1p53rand completely suppressed p53 protein production. At 1 μM concentration p53 protein production was also markedly suppressed by OL1p53s (Figure 7). However, the same effects were apparent when luciferase or AP2 DNA was transcribed and translated in the presence of these oligonucleotides (Figures 6 and 7).

Discussion

The phosphorothioate antisense oligonucleotide OL1p53as, directed against exon 10 of p53 mRNA, failed to specifically suppress p53 protein production in a cell-free assay system or mutant p53 expression by pancreatic cancer cell lines growing in vitro. In six different pancreatic cell lines, antiproliferative effects were apparent at higher doses when the cells were pretreated with versene/trypsin, but this was independent of


p53 status (mutant, wild-type or absent), and in most cell lines more dramatic antiproliferative effects were seen with the control oligonucleotides OL1p53s and OL1p53rand. These control oligonucleotides also appeared to affect cell–cell or cell–substratum interactions after trypsin/versene treatment at relatively low concentrations, causing the cells to round up, clump together and lose attachment to the culture dish.

These results, and particularly the results of the in vitro transcription/translation experiments, strongly suggest that the encouraging antiproliferative effects observed with OL1p53as by other investigators are not due to a specific antisense interaction leading to modulation of p53 expression. Fully phosphorothioate-substituted oligonucleotides are now known to have undesirable features, notably a tendency to non-specific toxicity owing to non-sequence-specific protein binding (including various growth factors, protein kinase C and transcription factors, slow cellular uptake and activation of RNAse H at sites other than the main target sequence (Stein and Krieg, 1994). Runs of four or more Gs in phosphorothioate oligonucleotides have also been documented to produce non-specific growth inhibition independent of any antisense effect (Cohen, 1993; Stein and Krieg, 1994). For this reason the OL1p53s control oligonucleotide could be predicted to have more non-specific toxicity than OL1p53as, but similar effects were observed with OL1p53rand, which does not have runs of Gs.

Interestingly, a morphological phenomenon similar to that seen with OL1p53s and OL1p53rand has been reported with another unrelated phosphorothioate oligonucleotide (Narayanan et al., 1992). Narayanan and co-workers used a phosphorothioate 21-mer targeting the AUG initiation codon of the DCC gene and found that the antisense oligonucleotide but not the sense control resulted in a loss of adhesion to the substratum. The cells remained viable but appeared rounded up and detached from the substratum, and this
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the cDNA for transcription factor AP2 is cloned downstream
of a T7 promoter. All three OLlp53 oligonucleotides significantly
suppress protein production from this template and Bip53as
slightly suppresses protein production despite there being no
significant complementarity between any of these oligonucleotides
and the AP2 gene or T7 promoter. N, No DNA control; 0, no
oligonucleotide; S, sense control; A, antisense; R, randomer
control; B, Bip53 oligonucleotides. O, OLlp53 oligonucleotides.

Figure 5 Effects of Bip53 antisense oligonucleotide and controls on p53 protein production by in vitro transcription/translation. The antisense oligonucleotide Bip53as suppresses p53 production in a dose-related manner but also suppresses luciferase production. Slight inhibition of p53 and luciferase protein synthesis is seen at higher doses with both control oligonucleotides Bip53s and Bip53rand. N, no DNA control; 0, no oligonucleotide; S, Bip53s; A, Bip53as; R, Bip53rand.

Figure 6 Effects of Bip53 and OLlp53 antisense oligonucleotides and controls on AP2 protein production by in vitro transcription/translation of the T7AP2 expression plasmid. This plasmid has the cDNA for transcription factor AP2 is cloned downstream of a T7 promoter. All three OLlp53 oligonucleotides significantly suppress protein production from this template and Bip53as slightly suppresses protein production despite there being no significant complementarity between any of these oligonucleotides and the AP2 gene or T7 promoter. N, No DNA control; 0, no oligonucleotide; S, sense control; A, antisense; R, randomer control; B, Bip53 oligonucleotides. O, OLlp53 oligonucleotides.

RNase H. by the time exon 10 is reached translation of the p53 message is almost complete. It is possible that minimally truncated p53 proteins which still contain the important highly conserved regions and which retain significant wild-type p53 activity might be produced. Furthermore, p53 protein detected in acute myeloid leukaemia (AML) blast cells is usually wild type not mutant (Fenaux et al., 1991; Slingerland et al., 1991; Sugimoto et al., 1991; Hu et al., 1992). According to current models of p53 activity, a reduction in expression of wild-type p53 would be predicted to result in loss of growth control not growth suppression, as reported in these experiments with haematopoietic cells.

The results of experiments using the chimaeric phosphorothioate - phosphodiester antisense oligonucleotide Bip53as, directed against the p53 translation initiation codon, support the contention that great care must be exercised in designing and interpreting the results of antisense experiments. We found that Bip53as did suppress p53 protein production by in vitro transcription/translation but also suppressed protein production from unrelated control luciferase and AP2 genes. However, in a pancreatic cell line expressing mutant p53 there was no demonstrable suppression of p53 protein production even at very high concentrations (100 μM). A mild non-specific antiproliferative effect was seen at higher concentrations similar to that seen with the control oligonucleotides Bip53s and Bip53rand and regardless of the p53 status of the cells. Although Bip53as has been reported to down-regulate p53 expression in CML cells (Bi et al., 1994), our results suggest that the expression of other genes might also be modulated and (as for the OLlp53 oligonucleotides) that these are not necessarily predictable on the basis of sequence complementarity or homology with the oligonucleotide. Interestingly, in the CML cells, suppression of p53 expression using OLlp53as stimulated colony formation and promoted proliferation, supporting the notion that suppression of wild-type p53 expression might be growth stimulatory in certain circumstances (Bi et al., 1994).

Even if a perfect antisense oligonucleotide could be found which only affected p53 expression, problems would remain before antisense oligonucleotides against p53 could be considered a realistic option for patients with cancer. Even in malignancies in which expression of mutant p53 with loss of the wild-type allele predominates, effective suppression of mutant p53 expression might be hazardous, for some mutant
proteins retain important wild-type functions. Reduction in expression of a mutant p53 protein by antisense interaction has already been reported to enhance tumour cell proliferation and tumorigenecity of lung cancer cell lines (Mukhopadhyay and Roth, 1993).

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