

# Selecting optimal oligonucleotide composition for maximal antisense effect following streptolysin O-mediated delivery into human leukaemia cells

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## ABSTRACT

It is widely accepted that most cell types efficiently exclude oligonucleotides *in vitro* and require specific delivery systems, such as cationic lipids, to enhance uptake and subsequent antisense effects. Oligonucleotides are not readily transfected into leukaemia cell lines using cationic lipid systems and streptolysin O (SLO) is used to effect their delivery. We wished to investigate the optimal oligonucleotide composition for antisense efficacy and specificity following delivery into leukaemia cells using SLO. For this study the well characterised chronic myeloid leukaemia cell line KYO-1 was selected and oligonucleotides (20mers) were targeted to an empirically identified accessible site of *c-myc* mRNA. The efficiency and specificity of antisense effect was measured 4 and 24 h after SLO-mediated delivery of the oligonucleotides. C5-propyne phosphodiester and phosphorothioate compounds were found to present substantial non-specific effects at 20  $\mu$ M but were inactive at 0.2  $\mu$ M. Indeed, no antisense-specific effect was noted at any concentration at either time. All of the other oligonucleotides tested induced some measurable antisense effect, except 7 (chimeric, all-phosphorothioate, 2'-methoxyethoxy termini) which was essentially inactive at 20  $\mu$ M. The rank efficiency order of the remaining antisense compounds was  $4 = 3 \gg 9 \gg 10 = 8 = 5 = 6 > 11$ . The efficient antisense effects induced by the chimeric methylphosphonate-phosphodiester compounds were found to be highly specific. Increased phosphorothioate content in the oligonucleotide backbone correlated with reduced antisense activity (efficacy: 2'-methoxyethoxy series  $9 \gg 8 \gg 7$ , 2'-methoxytriethoxy series  $10 > 11$ ). No consistent evidence was obtained for increased activity correlating with increased oligonucleotide-mRNA heteroduplex thermal stability. In conclusion, the chimeric methylphosphonate-phosphodiester oligodeoxynucleotides present the most favourable characteristics of the compounds tested, for efficient and specific antisense suppression of gene expression following SLO-mediated delivery.

## INTRODUCTION

Chronic myeloid leukaemia (CML) is a proliferative disorder of the haematopoietic stem cell, which results in the accumulation of myeloid cells and their progenitors (1). A specific chromosomal translocation t(9;22)(q34;11), termed the Philadelphia (Ph) chromosome, is found in the great majority of patients with CML. This translocation fuses the *c-abl* and major breakpoint cluster (*bcr*) genes (2). The aberrant *bcr-abl* transcript derived from this region is translated to produce BCR-ABL (p210) tyrosine kinase which displays enhanced activity over wild-type *c-ABL* (p145; 3) and is believed to be central to the pathogenesis of CML.

CML cell growth in culture has been reported to be arrested following introduction to the growth medium of antisense oligodeoxynucleotides targeting *bcr-abl* (4,5). Antisense oligodeoxynucleotide-dependent suppression of *bcr-abl* mRNA expression may be readily obtained (6,7) but p210 BCR-ABL protein levels may not be significantly lowered (8,9), due to the very long half life of BCR-ABL protein (10). Consequently, BCR-ABL may be a poor target for antisense oligodeoxynucleotide mediated inhibition of expression (9). The observed inhibition of cell proliferation following use of *bcr-abl* targeted oligodeoxynucleotides may, therefore, result from 'cross-over' antisense activity at non-target mRNA sites (11) or from non-antisense mechanisms (8,12) which may have a sequence-specific component (13).

On the other hand, MYC function is required for BCR-ABL induced transformation (14,15) and MYC and BCR-ABL may co-operate, via the induction of BCL2, to inhibit apoptosis and enhance tumorigenicity (16). Moreover, MYC protein may provide a better target for antisense inhibition than BCR-ABL, as MYC protein displays a short half life (17) (~15 min) and antisense induced suppression of *c-myc* mRNA has been shown to result in suppressed MYC protein expression (9,18).

One of the fundamental problems associated with antisense technology is that of delivery of oligonucleotides into the intracellular compartments of intact cells *in vitro*. Uptake generally proceeds by incorporation of exogenously applied antisense effectors into endocytotic vesicles, which results in the oligodeoxynucleotides remaining separated from their mRNA molecular targets by a lipid membrane (19–23). Consequently, a range of methods has been examined for their ability to achieve pharmacologically relevant oligodeoxynucleotide concentrations in the cytoplasm and nucleus of target cells. One approach was conjugation of

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the antisense effector to lipophilic molecules (24) (for example cholesterol; 24,25) or compounds previously described as drug carriers (for example, poly-L-lysine; reviewed in 26). Transfection methods have been tried, such as encapsulation into liposomes (reviewed in 26), association with cationic liposomes (27), polyalkylcyanoacrylate nanoparticles (28) or polyethylenimine (29) and reversible cell permeabilization by electroporation (30) or treatment with SLO (18,31,32).

Different cell types display significantly different responses to the various delivery methods. Human keratinocytes appear to take up oligodeoxynucleotides following simple topical application (22,33). A range of cells take up oligonucleotides following cationic lipid delivery, the most widely used *in vitro* transfection technique. However, cationic lipids may not enhance the uptake of antisense compounds into leukaemia cells (27). In contrast, human chronic myeloid leukaemia cells from culture lines (7,32,34) and of patient origin (35) respond well to reversible permeabilization with SLO. Indeed, SLO delivery is used in an on-going clinical trial of *ex vivo* bone marrow purging with oligonucleotides targeted to *bcr-abl* mRNA (36).

In the present study, congeners of a *c-myc* phosphorothioate 20mer oligonucleotide were investigated for antisense efficacy and specificity in KYO-1 chronic myeloid leukaemia cells following SLO-mediated delivery. The experimental design was selected, in part, for relevance to future therapeutic *ex vivo* purging of CML marrows, an interest of the groups working in Liverpool. The oligonucleotides were targeted to bases 1147–1166 of *c-myc* mRNA (GenBank, 'HSMYC1'), a position which appears to be accessible for oligonucleotide hybridisation. The oligonucleotides investigated were phosphorothioate, 5-methylcytosine phosphorothioate, phosphodiester and phosphorothioate C5-propyne pyrimidine structures and chimeric molecules composed of a central ribonuclease H (RNase H) activating region (phosphodiester or phosphorothioate) and termini composed either of methylphosphonate residues or 2'-ribose modified phosphodiester or phosphorothioate residues. Here we present results that show that chimeric methylphosphonate-phosphodiester oligodeoxynucleotides were the most active at suppressing *c-myc* expression, following intracellular delivery with SLO. The high efficacy was not obtained at the expense of non-specific activity.

## MATERIALS AND METHODS

### Oligonucleotide synthesis

The 20mer oligonucleotides used in this study are presented in Table 1. Details of the chemical modifications are presented in Figure 1. The antisense sequence is complementary to bases 1147–1166 of 'HSMYC1' (GenBank accession number V00568). The phosphorothioate oligodeoxynucleotide 5, was purchased from Oswel DNA (Southampton, Hampshire). Oligonucleotides 6–11 were supplied by Novartis (Basle, Switzerland).

Chimeric methylphosphonate-phosphodiester oligodeoxynucleotides were synthesised as previously described (21) using phosphoramidites and methylphosphoramidites from Glen Research Inc. (UK Supplier, Cambio, Cambridge) and the slow 1  $\mu\text{mol}$  cycle (version 1.23) on an Applied Biosystems (Warrington, Cheshire) 381A DNA synthesizer, with the exception that the base deprotection procedure recommended by Agrawal and Goodchild (37) was substituted for that used by Miller (38).

A 2 h ammonia treatment was used to ensure complete deprotection of the 5'-Amino-Modifier C6-TFA (Glen Research) group and permit efficient subsequent attachment of fluorescein. Treatment with ethylenediamine/ethanol (1:1 by volume) was for 18 h.

C5-propyne pyrimidine oligonucleotides were synthesized from propanediol derivitized CPG support in like manner using C5-propyne pyrimidine phosphoramidites and purine phosphoramidites (Glen Research Inc.). Phosphorothioate internucleoside linkages were obtained using tetraethylthiuram disulphide (TETD) as the sulphurizing reagent in place of the iodine oxidising reagent on the synthesizer (39). Deprotection was by concentrated ammonia treatment at 55°C overnight.

### Cell culture

The acute lymphoblastic leukaemia MOLT4 [European Collection of Animal Cell Cultures (ECACC), Porton Down, Wiltshire] and chronic myeloid leukaemia KYO-1 (generous gift of Drs M.Kirkland and S.O'Brien, LRF Leukaemia Unit, Hammersmith Hospital, London) cell lines were maintained in exponential growth in RPMI-1640 medium (GIBCO, Paisley, Renfrewshire) supplemented with heat inactivated (65°C, 1 h) foetal calf serum (SeraLab, Crawley Down, Sussex) to 10%. All procedures were performed with cultures assayed to be >95% viable by trypan blue exclusion. The hybridoma lines Mycl-9E10 and Mycl-3C7 (which express antibodies to human MYC protein, ECACC) were maintained in exponential growth in RPMI-1640 medium supplemented with 10% heat inactivated foetal calf serum. Supernatant was collected by centrifugal removal of cells and stored at 4°C until required.

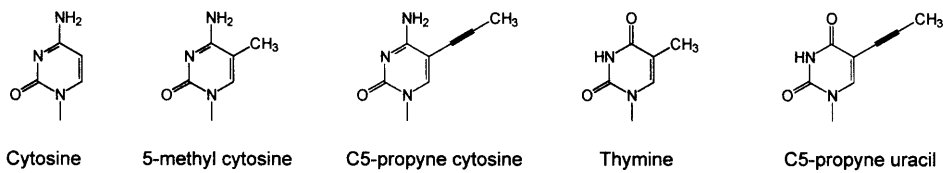
### Reversible cell permeabilisation

SLO was used to reversibly permeabilize cells towards oligonucleotides (7,31,32) according to a recently revised protocol (18). SLO was obtained from Sigma (Poole, Dorset), suspended at 1000 U/ml in  $\text{Mg}^{2+}/\text{Ca}^{2+}$ -free phosphate buffered saline and activated by addition of dithiothreitol to 5 mmol/l followed by incubation at 37°C for 2 h. Small aliquots of activated SLO were stored at -20°C until required. Cells to be permeabilised ( $5 \times 10^6$  per treatment) were washed and resuspended in 200  $\mu\text{l}$  serum-free RPMI-1640 medium, then permeabilized by addition of 30–50 U SLO and incubation at 37°C for 10 min, in the presence or absence of oligonucleotide. The precise amounts of SLO required for maximal permeabilization and resealing were identified by a dose-response optimisation procedure prior to each experiment. Resealing was achieved by addition of 1 ml of pre-warmed and gassed normal growth media (90% RPMI-1640, 10% heat inactivated foetal calf serum) and a further 20 min incubation at 37°C. Cells were transferred to culture flasks containing 9 ml of pre-warmed and gassed normal growth media and incubation continued at 37°C. Samples of 1 ml ( $\sim 5 \times 10^5$  cells) were taken 30 min, 4 and 24 h after the initiation of permeabilization for flow cytometric analysis. Samples of 2 ml ( $\sim 10^6$  cells) were removed 4 and 24 h after the start of permeabilization for mRNA and protein analysis.

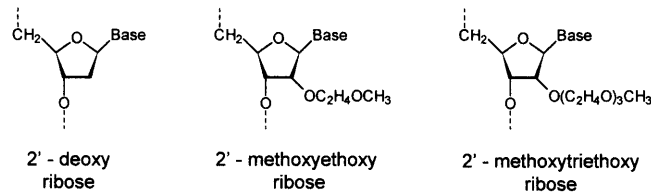
### Flow cytometry

Dead cells in the samples were stained by addition of 10  $\mu\text{g}/\text{ml}$  propidium iodide and incubation for 10 min on ice. Cells were washed, resuspended in 1 ml ice-cold normal growth medium and

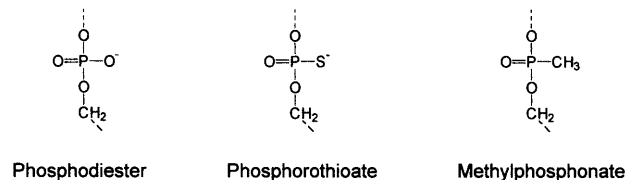
### A. Base Modifications



### B. Ribose Modifications



### C. Internucleoside Linkage Modifications



**Figure 1.** Chemical structures of the oligonucleotides used in this study. (A) Natural Cytosine, 5-methyl cytosine and thymine are depicted with the unnatural bases C5-propyne cytosine and C5-propyne uracil. (B) Natural 2'-deoxy ribose is depicted with synthetic 2'-methoxyethoxy ribose and 2'-methoxytriethoxy ribose. (C) Natural phosphodiester internucleoside linkage is depicted alongside synthetic phosphorothioate and methylphosphonate linkages.

assayed for red and green fluorescence on a Cytoron Absolute bench top flow cytometer system (Ortho Diagnostic Systems, Loudwater, Buckinghamshire) using a protocol which samples a precisely known volume. This procedure provides information on the total number of cells per ml in the original culture, the % viability of those cells and, when a green fluorescent compound has been introduced by SLO, the % permeabilized and resealed (32).

#### mRNA analysis

Total RNA was extracted from cell pellets using a guanidinium thiocyanate/acid phenol method (40). Formaldehyde gel electrophoresis, capillary blotting to nylon hybridisation membrane, northern hybridisation and non-radioactive detection of mRNA species were performed according to standard techniques as previously described (23), except that digoxigenin-UTP labelled antisense *in vitro* transcripts were used as probe (41) rather than digoxigenin-dUTP labelled random primed DNA fragments.

#### Protein analysis

Cells were washed, pelleted and lysed by addition of 100 or 200  $\mu$ l warmed lysis buffer (0.04 mol/l Tris, pH 6.8, 10% glycerol, 1% 2-mercaptoethanol, 1% sodium dodecylsulfate, 0.001% bromophenol blue) followed by incubation at 95 °C for 10 min. Samples were fractionated by SDS-PAGE (42) through a 6% stacking gel and a 10% separation gel. Proteins present in the gels were

electroblotted onto reinforced nitrocellulose membranes ('Optitran', Schleicher and Schuell, UK distributor Anderman and Co. Ltd, Kingston-upon-Thames, Surrey). MYC protein was detected using a 1:1 mixture of Mycl-9E10 and Mycl-3C7 hybridoma supernatants as primary antibody, the secondary antibody was an alkaline phosphatase-conjugated sheep anti-mouse IgG (Sigma). Final chromogenic development was achieved as for northern blotting.

#### RNA folding prediction

The secondary structure of *c-myc* mRNA was estimated using the Wisconsin GCG package. *FOLDRNA* and *MFOLD* routines were used to calculate structure minimum energies. The results were visualised using the *SQUIGGLES* routine.

#### Densitometry and presentation of data

A Shimadzu CS9000 flying spot densitometer (Shimadzu, UK distributor Howe & Co. Ltd, Banbury, Oxfordshire) was used as previously described (43). The densitometrically determined expression of *c-myc*, p53 and GAPDH mRNA and MYC protein was corrected for the number of viable cells present in the sample from which RNA and protein was extracted [mRNA or protein expression = (densitometric area of band)/(number of viable cells in flow cytometry sample)]. The mean of the three SLO-only control values, present in every experiment, was set to 100% and

**Table 1.** Oligonucleotides used in this study

Number	Structure and sequence (3' → 5')
<b>Antisense</b>	
1	Pr~A <sub>5</sub> U <sup>*</sup> <sub>5</sub> G <sub>5</sub> G <sub>5</sub> A <sub>5</sub> G <sub>5</sub> A <sub>5</sub> G <sub>5</sub> U <sup>*</sup> <sub>5</sub> U <sup>*</sup> <sub>5</sub> G <sub>5</sub> C <sup>*</sup> <sub>5</sub> U <sup>*</sup> <sub>5</sub> G <sup>*</sup> <sub>5</sub> U <sup>*</sup> <sub>5</sub> C <sup>*</sup> <sub>5</sub> G <sub>5</sub> U <sup>*</sup> <sub>5</sub> C <sup>*</sup> ~F
2	Pr~A <sub>0</sub> U <sup>*</sup> <sub>0</sub> G <sub>0</sub> G <sub>0</sub> A <sub>0</sub> G <sub>0</sub> A <sub>0</sub> G <sub>0</sub> U <sup>*</sup> <sub>0</sub> U <sup>*</sup> <sub>0</sub> G <sub>0</sub> C <sup>*</sup> <sub>0</sub> U <sup>*</sup> <sub>0</sub> G <sub>0</sub> U <sup>*</sup> <sub>0</sub> C <sup>*</sup> <sub>0</sub> G <sub>0</sub> U <sup>*</sup> <sub>0</sub> C <sup>*</sup> ~F
3	A <sub>mp</sub> T <sub>mp</sub> G <sub>mp</sub> G <sub>mp</sub> G <sub>mp</sub> A <sub>0</sub> G <sub>0</sub> A <sub>0</sub> G <sub>0</sub> T <sub>0</sub> G <sub>0</sub> C <sub>0</sub> T <sub>mp</sub> G <sub>mp</sub> T <sub>mp</sub> C <sub>mp</sub> G <sub>mp</sub> T <sub>mp</sub> C~F
4	A <sub>mp</sub> T <sub>mp</sub> G <sub>mp</sub> G <sub>mp</sub> G <sub>mp</sub> A <sub>mp</sub> G <sub>0</sub> A <sub>0</sub> G <sub>0</sub> T <sub>0</sub> G <sub>0</sub> C <sub>0</sub> T <sub>mp</sub> G <sub>mp</sub> T <sub>mp</sub> C <sub>mp</sub> G <sub>mp</sub> T <sub>mp</sub> C~F
5	A <sub>5</sub> T <sub>5</sub> G <sub>5</sub> G <sub>5</sub> A <sub>5</sub> G <sub>5</sub> A <sub>5</sub> G <sub>5</sub> T <sub>5</sub> G <sub>5</sub> C <sub>5</sub> T <sub>5</sub> G <sub>5</sub> T <sub>5</sub> C <sub>5</sub> G <sub>5</sub> T <sub>5</sub> C
6	A <sub>5</sub> T <sub>5</sub> G <sub>5</sub> G <sub>5</sub> A <sub>5</sub> G <sub>5</sub> A <sub>5</sub> G <sub>5</sub> T <sub>5</sub> T <sub>5</sub> G <sub>5</sub> C <sup>†</sup> <sub>5</sub> T <sub>5</sub> G <sub>5</sub> T <sub>5</sub> C <sup>†</sup> <sub>5</sub> G <sub>5</sub> T <sub>5</sub> C <sup>†</sup>
7	A <sub>me</sub> <sub>5</sub> T <sub>me</sub> <sub>5</sub> G <sub>me</sub> <sub>5</sub> G <sub>me</sub> <sub>5</sub> A <sub>me</sub> <sub>5</sub> G <sub>5</sub> A <sub>5</sub> G <sub>5</sub> T <sub>5</sub> T <sub>5</sub> G <sub>5</sub> C <sup>†</sup> <sub>5</sub> T <sub>5</sub> G <sub>me</sub> <sub>5</sub> T <sub>me</sub> <sub>5</sub> C <sup>†</sup> <sub>me</sub> G <sub>me</sub> <sub>5</sub> T <sub>me</sub> <sub>5</sub> C <sup>†</sup> <sub>me</sub>
8	A <sub>me</sub> <sub>0</sub> T <sub>me</sub> <sub>0</sub> G <sub>me</sub> <sub>0</sub> G <sub>me</sub> <sub>0</sub> A <sub>me</sub> <sub>5</sub> G <sub>5</sub> A <sub>5</sub> G <sub>5</sub> T <sub>5</sub> T <sub>5</sub> G <sub>5</sub> C <sup>†</sup> <sub>5</sub> T <sub>5</sub> G <sub>me</sub> <sub>0</sub> T <sub>me</sub> <sub>0</sub> C <sup>†</sup> <sub>me</sub> G <sub>me</sub> <sub>0</sub> T <sub>me</sub> <sub>0</sub> C <sup>†</sup> <sub>me</sub>
9	A <sub>me</sub> <sub>0</sub> T <sub>me</sub> <sub>0</sub> G <sub>me</sub> <sub>0</sub> G <sub>me</sub> <sub>0</sub> A <sub>me</sub> <sub>0</sub> G <sub>0</sub> A <sub>0</sub> G <sub>0</sub> T <sub>0</sub> G <sub>0</sub> C <sup>†</sup> <sub>0</sub> T <sub>0</sub> G <sub>me</sub> <sub>0</sub> T <sub>me</sub> <sub>0</sub> C <sup>†</sup> <sub>me</sub> G <sub>me</sub> <sub>0</sub> T <sub>me</sub> <sub>0</sub> C <sup>†</sup> <sub>me</sub>
10	A <sup>mt</sup> <sub>0</sub> T <sup>mt</sup> <sub>0</sub> G <sup>mt</sup> <sub>0</sub> G <sup>mt</sup> <sub>0</sub> A <sup>mt</sup> <sub>5</sub> G <sub>5</sub> A <sub>5</sub> G <sub>5</sub> T <sub>5</sub> T <sub>5</sub> G <sub>5</sub> C <sup>†</sup> <sub>5</sub> T <sub>5</sub> G <sup>mt</sup> <sub>0</sub> T <sup>mt</sup> <sub>0</sub> C <sup>†</sup> <sub>mt</sub> G <sup>mt</sup> <sub>0</sub> T <sup>mt</sup> <sub>0</sub> C <sup>†</sup> <sub>mt</sub>
11	A <sup>mt</sup> <sub>5</sub> T <sup>mt</sup> <sub>5</sub> G <sup>mt</sup> <sub>5</sub> G <sup>mt</sup> <sub>5</sub> A <sup>mt</sup> <sub>5</sub> G <sub>5</sub> A <sub>5</sub> G <sub>5</sub> T <sub>5</sub> T <sub>5</sub> G <sub>5</sub> C <sup>†</sup> <sub>5</sub> T <sub>5</sub> G <sup>mt</sup> <sub>5</sub> T <sup>mt</sup> <sub>5</sub> C <sup>†</sup> <sub>mt</sub> G <sup>mt</sup> <sub>5</sub> T <sup>mt</sup> <sub>5</sub> C <sup>†</sup> <sub>mt</sub>
<b>Sense</b>	
12	Pr~G <sub>5</sub> A <sub>5</sub> C <sup>*</sup> <sub>5</sub> G <sub>5</sub> A <sub>5</sub> C <sup>*</sup> <sub>5</sub> A <sub>5</sub> G <sub>5</sub> C <sup>*</sup> <sub>5</sub> A <sub>5</sub> A <sub>5</sub> C <sup>*</sup> <sub>5</sub> U <sup>*</sup> <sub>5</sub> C <sup>*</sup> <sub>5</sub> U <sup>*</sup> <sub>5</sub> C <sup>*</sup> <sub>5</sub> C <sup>*</sup> <sub>5</sub> C <sup>*</sup> <sub>5</sub> A <sub>5</sub> U <sup>*</sup> ~F
13	G <sub>mp</sub> A <sub>mp</sub> C <sub>mp</sub> G <sub>mp</sub> A <sub>mp</sub> C <sub>mp</sub> A <sub>0</sub> G <sub>0</sub> C <sub>0</sub> A <sub>0</sub> A <sub>0</sub> C <sub>0</sub> T <sub>0</sub> C <sub>0</sub> T <sub>mp</sub> C <sub>mp</sub> C <sub>mp</sub> C <sub>mp</sub> A <sub>mp</sub> T~F
<b>Inverse Antisense</b>	
14	Pr~C <sup>*</sup> <sub>5</sub> U <sup>*</sup> <sub>5</sub> G <sub>5</sub> C <sup>*</sup> <sub>5</sub> U <sup>*</sup> <sub>5</sub> G <sub>5</sub> U <sup>*</sup> <sub>5</sub> C <sup>*</sup> <sub>5</sub> G <sub>5</sub> U <sup>*</sup> <sub>5</sub> U <sup>*</sup> <sub>5</sub> G <sub>5</sub> A <sub>5</sub> G <sub>5</sub> A <sub>5</sub> G <sub>5</sub> G <sub>5</sub> U <sup>*</sup> <sub>5</sub> A~F
15	C <sub>mp</sub> T <sub>mp</sub> G <sub>mp</sub> C <sub>mp</sub> T <sub>mp</sub> G <sub>mp</sub> T <sub>0</sub> C <sub>0</sub> G <sub>0</sub> T <sub>0</sub> T <sub>0</sub> G <sub>0</sub> A <sub>0</sub> G <sub>0</sub> A <sub>mp</sub> G <sub>mp</sub> G <sub>mp</sub> G <sub>mp</sub> T <sub>mp</sub> A~F

Internucleoside linkages and termini: o, phosphodiester; s, phosphorothioate; mp, methylphosphonate; F~, 5(6)carboxy-fluorescein-aminohexanol linker; Pr~, propane diol protecting group. Sugar and base: N, unmodified 2'-deoxy ribose; me, 2'-methoxyethoxy; mt, 2'-methoxytriethoxy, †, 5-methyl cytosine, \*, C5 propyne pyrimidine.

the data points plotted relative to this ± standard deviation. This procedure was preferred to normalising relative to the expression of a control gene because of the potential for non-targeted activity in antisense experiments.

### RESULTS AND DISCUSSION

The oligonucleotides used in this investigation are detailed in Table 1 and their chemical modifications in Figure 1. All antisense compounds are complementary to bases 1147–1166 of *c-myc* mRNA ('HSMYC1', GenBank Accession number V00568). This site was initially noted as one at which oligomers targeted to the *c-myc* translation initiation codon (559–573) induced undesired cleavage *in vitro* (43), non-target sites 1147–1161 and 1264–1278. Pilot experiments were then performed wherein phosphodiester 15mer oligodeoxynucleotides targeted to positions 559–573, 1147–1161 and 1264–1278 of *c-myc* mRNA were delivered into MOLT4 and KYO-1 leukaemia cells, using SLO, and the resultant *c-myc* mRNA expression evaluated. Subsequently, 20mer phosphodiester oligodeoxynucleotides directed to the two most active of these regions (557–576 and 1147–1166) were compared to 20mer phosphodiester effectors targeted to sites which were predicted by computer to possess open loop secondary structures (378–397, 1244–1263, 1287–1306 and 1575–1595) for inhibition of *c-myc* mRNA expression, following SLO delivery. These experiments indicated that the region 1147–1166 was a good target for antisense oligonucleotides.

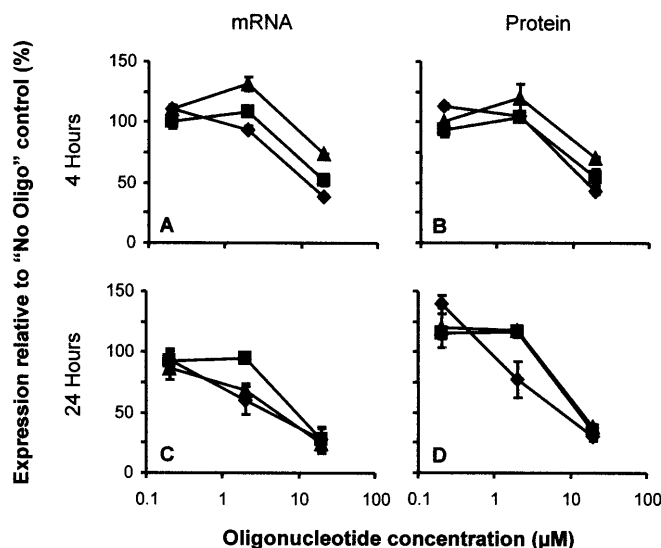
#### C5-propyne pyrimidine oligomers

C5-propyne pyrimidine modified oligomers (Fig. 1; 44–46) have very high RNA heteroduplex *T<sub>m</sub>s*, are active at sub-micromolar concentrations *in vitro* and can elicit specific antisense effects.

Antisense, control sense and inverse antisense (reverse polarity antisense) C5-propyne pyrimidine modified phosphorothioate oligodeoxynucleotides were introduced into the cytoplasm and nucleus of the CML cell line KYO-1 using SLO as described in Materials and Methods from extracellular oligomer concentrations of 0.2, 2 and 20 μM. The resultant expression of *c-myc* mRNA and protein 4 and 24 h afterwards is presented in Figure 2. Figure 2A shows that 4 h after delivery, the 20 μM antisense treatment inhibited *c-myc* mRNA expression to 37.8 ± 1.2% of SLO-only control. Below this concentration the antisense oligomer was essentially without effect on *c-myc* mRNA at 4 h. This suppression of *c-myc* mRNA expression is reflected in reduced MYC protein levels at 4 h (Fig. 2B). It is clear from Figure 2A and B that 20 μM control oligonucleotides suppressed *c-myc* gene expression to similar extents as was observed with the antisense sequence. Figure 2C and D show that 24 h after treatment with 20 μM antisense, sense or inverse antisense C5-propyne phosphorothioate oligomer, all samples show essentially equivalent suppression of *c-myc* mRNA or protein expression. Samples treated with 0.2 μM oligodeoxynucleotide show no significant reduction in *c-myc* mRNA or protein level, whereas samples treated with 2 μM effector present results intermediate to that obtained with 0.2 and 20 μM.

Cell growth was adversely affected in samples treated with high concentrations of C5-propyne phosphorothioate compounds. In the experiment detailed in Figure 2 control cells SLO, reversibly permeabilized without oligonucleotide in the extracellular medium, proliferated normally and increased in number by 72% over the 24 h analysis period. In contrast, cells permeabilized in the presence of 20 μM antisense or control sense or control inverse antisense decreased in number over this time by 20, 3 and 21%, respectively.





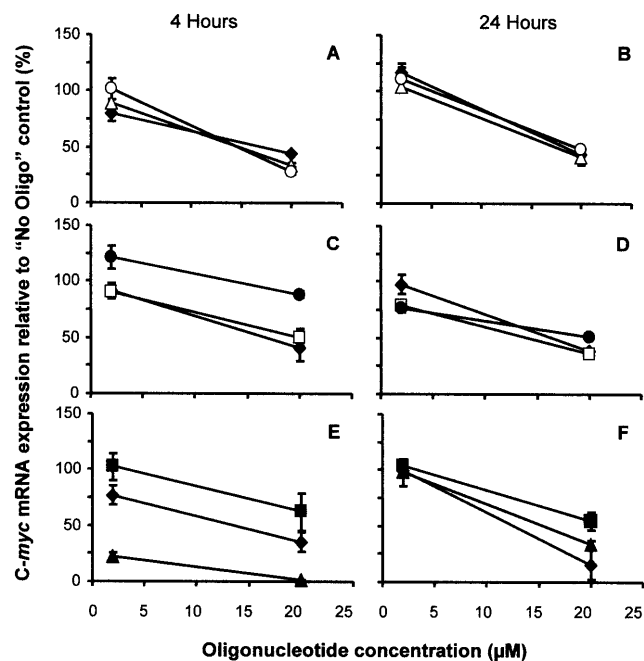
**Figure 2.** Graphs showing the expression of *c-myc* mRNA and protein, relative to SLO-only control, in human leukaemia KYO-1 cells following delivery of antisense (closed diamond, **I**) and control inverse antisense (closed square, **14**) and control sense (closed triangle, **12**) 20mer C5-propyne phosphorothioate oligodeoxynucleotides. KYO-1 cells were suspended in RPMI-1640 growth media containing 0.2, 2 or 20  $\mu\text{M}$  oligodeoxynucleotide and reversibly permeabilised by SLO as described in the Materials and Methods. Samples were removed 4 (**A** and **B**) and 24 h (**C** and **D**) after the start of the experiment and analysed by northern blotting for *c-myc* mRNA expression (**A** and **C**) and by western blotting for MYC protein expression (**B** and **D**). No difference in activity at suppressing the expression of *c-myc* mRNA or protein at either 4 or 24 h may be distinguished between the antisense and two control oligomers. The number, structure and sequence of the compounds is detailed in Table 1 and Figure 1.

C5-propyne oligodeoxynucleotides were examined in a system relevant to future *ex vivo* purging of CML bone marrow. In contrast to the highly specific results published elsewhere (44–46), we found substantial non-specific activity. Indeed, the data presented in Figure 2 shows that no antisense-specific component could be distinguished for the suppression of *c-myc* mRNA and protein expression either 4 or 24 h after treatment with 20  $\mu\text{M}$  C5-propyne oligodeoxynucleotide. This concentration of oligonucleotide was also found to be non-specifically growth inhibitory.

Specific antisense effects were not observed when C5-propyne oligodeoxynucleotides were used at concentrations below 20  $\mu\text{M}$ . At 0.2  $\mu\text{M}$ , *c-myc* mRNA and protein levels were not reduced by either of the control sequences or the antisense oligomer. Intermediate effects of target expression were observed when using 2  $\mu\text{M}$  oligodeoxynucleotide, but again no difference could be discerned between the control and antisense sequence compounds. We conclude that C5-propyne modified oligodeoxynucleotides may not be suitable for suppression of gene expression in leukaemia cells following SLO-mediated delivery to the intracellular compartments.

### Chimeric 2'-methoxyalkoxy-2'-deoxy structures

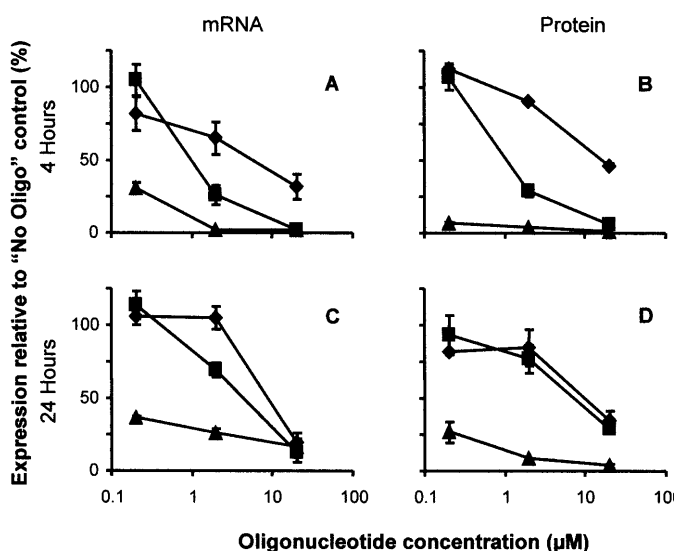
Another class of antisense effector receiving much attention is the so called 'second generation' chimeric molecules (47) which possess 2'-modified RNA termini and a central deoxynucleotide core capable of supporting ribonuclease H (RNase H) activity. We were particularly interested in 2'-methoxyalkoxy structures, such



**Figure 3.** Graphs showing the expression of *c-myc* mRNA, relative to SLO-only control, in human leukaemia KYO-1 cells 4 and 24 h following delivery of 20mer antisense oligonucleotides. KYO-1 cells were suspended in RPMI-1640 growth media containing 2 or 20  $\mu\text{M}$  oligonucleotide and reversibly permeabilised by SLO as described in the Materials and Methods. Samples were removed 4 (**A**, **C** and **E**) and 24 h (**B**, **D** and **F**) after the start of the experiment and analysed by northern blotting for *c-myc* mRNA expression. Data from three experiments are presented (**A+B**, **C+D** and **E+F**), each using **10** (closed diamond, chimeric, phosphodiester 2'-methoxytriethoxy termini, phosphorothioate 2'-deoxy ribose centre) as the common compound. (**A**) and (**B**) The results obtained from **10**, **5** (open circle, all-phosphorothioate) and **6** (open triangle, all-phosphorothioate). (**C**) and (**D**) The results obtained from **10**, **7** (closed circle, all-phosphorothioate, chimeric, 2'-methoxyethoxy termini, 2'-deoxy ribose centre) and **8** (open square, chimeric, phosphodiester 2'-methoxyethoxy termini, phosphorothioate 2'-deoxy ribose centre). (**E**) and (**F**) The results obtained from **10**, **9** (closed triangle, all-phosphodiester, chimeric, 2'-methoxyethoxy termini, 2'-deoxy ribose centre) and **11** (closed square, all-phosphorothioate, chimeric 2'-methoxytriethoxy termini, 2'-deoxy ribose centre). Note that oligomers **6–11** contain 5-methyl cytosine in place of cytosine. Only **9** displayed appreciable activity at 2  $\mu\text{M}$ . The number, structure and sequence of the compounds is detailed in Table 1 and Figure 1.

as 2'-methoxyethoxy (47–50) and 2'-methoxytriethoxy (47,48). Oligonucleotides constructed with these modifications not only display excellent nuclease resistance, characteristic of bulky 2'-substitutions (51,52), but also retain oligonucleotide–RNA duplex  $T_m$ s greater than that resulting from 2'-O-methyl RNA–RNA duplexes, which is unusual for large 2'-substituents (53,54).

Figure 3 presents the *c-myc* mRNA expression 4 and 24 h after SLO-mediated delivery of 2 and 20  $\mu\text{M}$  antisense 20mer phosphorothioate and chimeric 2'-methoxyalkoxy oligodeoxynucleotides. Three separate experiments were performed, each containing three oligonucleotide species, compound **10** (5-methylcytosine, 2'-methoxytriethoxy phosphodiester termini and phosphorothioate 2'-deoxy ribose gap) was included in all three experiments to permit meaningful comparison. The three experiments appear to be directly comparable, 4 h after introduction of 20  $\mu\text{M}$  of oligonucleotide **10** into KYO-1 cells *c-myc* mRNA expression was reduced to ~40%, relative to control, while 2  $\mu\text{M}$  of this compound resulted in expression of ~83% of the SLO-only



**Figure 4.** Graphs showing the expression of *c-myc* mRNA and protein, relative to SLO-only control, in human leukaemia KYO-1 cells following delivery of **4** (closed triangle, chimeric, methylphosphonate termini, phosphodiester 2'-deoxy ribose centre), **9** (closed square, all-phosphodiester, chimeric, 2'-methoxyethoxy termini, 2'-deoxy ribose centre) and **10** (closed diamond, chimeric, phosphodiester 2'-methoxytriethoxy termini, phosphorothioate 2'-deoxy ribose centre) 20mer antisense oligonucleotides. KYO-1 cells were suspended in RPMI-1640 growth media containing 0.2, 2 or 20  $\mu\text{M}$  oligonucleotide and reversibly permeabilised by SLO as described in the Materials and Methods. Samples were removed 4 (A and B) and 24 h (C and D) after the start of the experiment and analysed by northern blotting for *c-myc* mRNA expression (A and C) and by western blotting for MYC protein expression (B and D). Only **4** displayed appreciable activity at 0.2  $\mu\text{M}$ . The number, structure and sequence of the compounds is detailed in Table 1 and Figure 1.

control respectively. Figure 3A and B compare an all-phosphorothioate (**5**) and an all-phosphorothioate which contains 5-methylcytosine in place of cytosine (**6**) to the common oligomer (**10**). It may be seen that all three compounds present essentially equivalent results at both 4 and 24 h time points. The chimeric oligonucleotide with 5-methylcytosine, 2'-methoxyethoxy phosphodiester termini and a phosphorothioate DNA gap (**8**) also displayed activity similar to **10** (Fig. 3C and D). An all-phosphorothioate version of **8**, oligomer **7** (5-methylcytosine, 2'-methoxyethoxy phosphorothioate termini and a phosphorothioate DNA gap), appeared to be significantly less active than **10** at 4 h (Fig. 3C). Similarly, the all-phosphorothioate version of **10**, compound **11** (5-methylcytosine, 2'-methoxytriethoxy phosphorothioate termini and a phosphorothioate DNA gap), was also substantially less active than the common oligomer (Fig. 3E and F). In contrast, the only all-phosphodiester compound in these tests, structure **9** (5-methylcytosine, 2'-methoxyethoxy phosphodiester termini and a phosphodiester DNA gap), was substantially more active than **10**. *c-myc* mRNA was almost undetectable at 4 h in samples which were treated with 20  $\mu\text{M}$  oligomer and reduced to  $21.5 \pm 3.9\%$  following treatment with 2  $\mu\text{M}$  effector.

These results clearly demonstrate that incremental replacement of phosphodiester internucleoside linkages with phosphorothioate residues in otherwise identical structures, results in incrementally reduced antisense potency, following SLO-dependent delivery of the oligomers into human leukaemia cells. Compounds from both the chimeric 2'-methoxyethoxy-2'-deoxy (**7**, **8** and **9**) and chimeric 2'-methoxytriethoxy-2'-deoxy (**10** and **11**) series display this trend.

The fully phosphorothioate analogues were less active than those with phosphodiester termini and phosphorothioate central sections (activity: **11** < **10**, Fig. 3E and F and **7** < **8**, Fig. 3C and D). The one all-phosphodiester oligonucleotide examined in these experiments was the most potent (**9**, Fig. 3E). It was the only one to induce substantial antisense inhibition of *c-myc* mRNA expression at 2  $\mu\text{M}$ . Furthermore, these data do not support the argument that the lower activities observed with highly phosphorothioate substituted oligomers resulted from reduced oligonucleotide-mRNA heteroduplex  $T_m$ s, which would be expected from increasing phosphorothioate content (**55**). Both of the chimeric 2'-methoxyalkoxy-2'-deoxy all phosphorothioate oligomers (**11** and **7**) were less potent than the fully 2'-deoxy phosphorothioate congener (**6**), despite the presence of helix stabilising 2'-ribose modifications (**47,48**) in the termini. Indeed, in these experiments, no evidence was obtained for increased activity correlating with increased target mRNA affinity. The two all-2'-deoxy phosphorothioate oligomers (**5** and **6**) displayed essentially identical antisense activity despite the replacement of cytosine in one (**6**) with helix stabilising 5-methyl cytosine (**56**).

### Chimeric methylphosphonate-phosphodiester structures

In the light of the above observations it was considered appropriate to examine methylphosphonate-phosphodiester chimeric structures in the same experimental system. It has previously been demonstrated that the methylphosphonate internucleoside linkage is helix-destabilising, particularly for oligonucleotide-RNA heteroduplexes, and that chimeric compounds with incremental replacement of phosphodiester internucleoside linkages with methylphosphonate residues display incrementally reduced RNA heteroduplex  $T_m$ s (**57**). Notwithstanding the reduced heteroduplex  $T_m$ s, such compounds have been demonstrated to provide efficient and specific antisense effects when introduced into cultured cells (**7,58**).

Figure 4 presents the data obtained when an antisense chimeric methylphosphonate-phosphodiester with six methylphosphonate residues at each end and a central region of seven contiguous phosphodiester linkages, (**4**) was compared to the 'common' oligomer from Figure 3 (**10**) and the most active of the 2'-ribose modified chimeric compounds (**9**). Human CML KYO-1 cells were reversibly permeabilized with SLO in the presence of 20, 2 or 0.2  $\mu\text{M}$  oligonucleotide, or in the absence of oligomer and samples taken for analysis after 4 and 24 h. The data in this figure demonstrates that MYC protein expression in cells 4 and 24 h after treatment with oligonucleotides (Fig. 4B and D, respectively) essentially reflects the *c-myc* mRNA expression at these time points (Fig. 4A and C, respectively). It is also readily apparent that the *c-myc* mRNA expression (Fig. 4A and C) resulting from treatment with 2 and 20  $\mu\text{M}$  **9** and **10** is consistent with that presented in Figure 3. Lower concentrations of these two oligomers (0.2  $\mu\text{M}$ ) resulted in no observable suppression of *c-myc* gene expression. In comparison, **4** may be seen to be ~10-fold more active than **9**, with expression reduced to  $1.7 \pm 0.1\%$ ,  $1.7 \pm 0.1\%$  and  $30.0 \pm 4.0\%$  of mean SLO-only control in samples treated with 20, 2 and 0.2  $\mu\text{M}$  of this methylphosphonate-phosphodiester chimeric oligodeoxynucleotide, respectively.

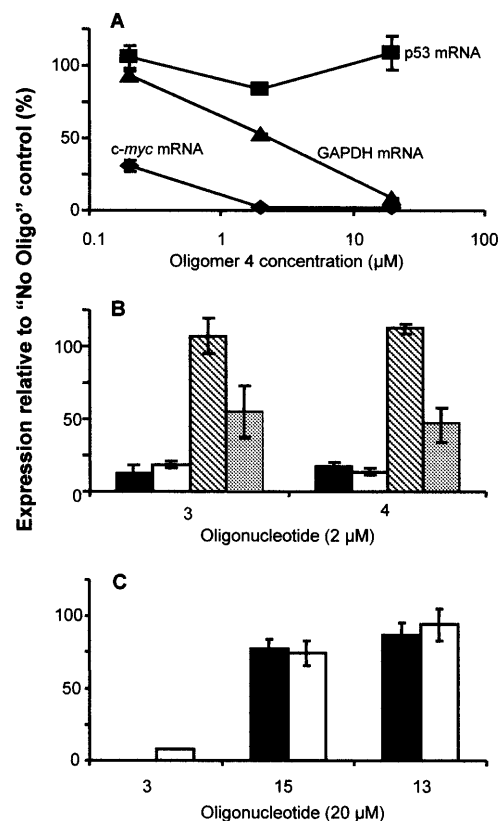
The antisense inhibition of *c-myc* mRNA expression resulting from SLO-mediated introduction of 20, 2 and 0.2  $\mu\text{M}$  **4** can be seen to last for at least 24 h (Fig. 4C). The antisense suppression of *c-myc* mRNA in cells 24 h after introduction of 2 and 0.2  $\mu\text{M}$

compound **9** may be seen to be rather less efficient than those induced by oligomer **4**.

Cells in which the *c-myc* mRNA and protein expression had been inhibited for 24 h by oligomer **4** displayed abnormal proliferation. A dose dependent inhibition of cell growth was observed. Cells treated with 20  $\mu\text{M}$  of this chimeric methylphosphonate-phosphodiester antisense oligodeoxynucleotide decreased in number by  $12 \pm 9\%$  by the 24 h point. Cultures from cells treated with 0.2  $\mu\text{M}$  of compound **4** proliferated, but at only half the rate of SLO-only control cells. Intermediate effects were obtained with cells SLO-permeabilized in the presence of 2  $\mu\text{M}$  **4**, neither proliferation nor decrease in number between the 4 and 24 h points was observed.

To ensure that the suppression of *c-myc* mRNA and protein evident in Figure 4 and the inhibition of proliferation were sequence-specific antisense effects a series of controls were performed. These data are presented in Figure 5. The samples used to provide the 4 h mRNA data of Figure 4 were also examined for non-target mRNA expression. Figure 5A presents the expression of target *c-myc* mRNA and non-target p53 and GAPDH mRNAs 4 h after SLO-mediated delivery of 0.2, 2 and 20  $\mu\text{M}$  structure **4**, relative to control SLO-only treated samples. The *c-myc* mRNA expression in this figure represent the same data as presented in Figure 4A and are included for comparative reasons. No significant reduction of p53 mRNA expression was obtained at any concentration tested. However, a dose-dependent reduction in GAPDH mRNA was observed. Examination of the GAPDH mRNA sequence ('HSGAPDR' GenBank accession number X01677) revealed a substantial partial complementarity to compound **4** (over bases 965–975, ten base pairs in total and including nine contiguous base pairs) which had the potential to direct the activity of RNase H. Lanes in northern blots probed for GAPDH, from samples treated with 20  $\mu\text{M}$  **4**, demonstrated the existence of a band with approximately the predicted size of the putative 5' fragment generated by RNase H cleavage of GAPDH mRNA at the site of partial complementarity with the antisense oligomer (data not shown). The antisense effectors **5–11** also induced modest decreases in GAPDH mRNA expression which were broadly in proportion to their targeted reduction of *c-myc* mRNA level, when delivered from 20  $\mu\text{M}$  extracellular concentration (data not shown). Nevertheless, no non-targeted cleavage of GAPDH mRNA, nor evidence of reduced GAPDH mRNA expression, could be detected when 0.2  $\mu\text{M}$  of the antisense effector **4** was used. Moreover, no reduction in cellular GAPDH protein levels were observed either 4 or 24 h after SLO-mediated delivery of 0.2, 2 or 20  $\mu\text{M}$  oligomer **4**. Thus, this interesting non-targeted antisense effect is most unlikely to induce any physiological response over the period investigated.

Figure 5B compares the potency of 2  $\mu\text{M}$  of **4** and a very similar methylphosphonate-phosphodiester chimeric oligodeoxynucleotide, structure **3**, at reducing the expression of target *c-myc* mRNA and protein, and non-target p53 and GAPDH mRNA, 4 h after SLO-mediated delivery. Oligomer **3** was identified in a separate study as an efficient inhibitor of *c-myc* expression (**9**) and differs from **4** at precisely one internucleoside linkage, which is phosphodiester in **3** and methylphosphonate in **4** (see Table 1). This test was performed using 2  $\mu\text{M}$  oligonucleotide as it would more sensitively reveal differences between the two oligonucleotides than similar tests using either 0.2 or 20  $\mu\text{M}$  of the antisense effectors. It may be seen that the profile of these two oligodeoxynucleotides at inhibiting target and non-target gene expression was essentially identical.



**Figure 5.** Graph and histograms presenting the expression of *c-myc*, p53 and GAPDH mRNAs and MYC protein, relative to SLO-only control, in human leukaemia KYO-1 cells 4 h after SLO-dependent delivery of 20mer oligodeoxynucleotides. (A) The concentration dependence of undesired effects. Cells were exposed to 0.2, 2 or 20  $\mu\text{M}$  **4** (chimeric, methylphosphonate termini, phosphodiester 2'-deoxy ribose centre) and analysed by northern blotting for *c-myc* (closed diamond), p53 (closed square) and GAPDH (closed triangle) mRNA expression. Efficient and specific antisense effects were obtained with 0.2  $\mu\text{M}$  oligomer. (B) Cells were exposed to 2  $\mu\text{M}$  **4** or **3** (chimeric, methylphosphonate termini, phosphodiester 2'-deoxy ribose centre) and analysed by northern blotting for *c-myc* (closed box), p53 (striped box) and GAPDH (shaded box) mRNA expression and by western blotting for MYC (open box) protein expression. The two antisense oligonucleotides produced essentially identical results. (C) The specificity of chimeric methylphosphonate-phosphodiester oligonucleotide effect. Cells were exposed to 20  $\mu\text{M}$  antisense (**3**), control inverse antisense (**15**) or control sense (**13**) oligomers and *c-myc* mRNA (closed box) and protein (open box) expression assayed by northern and western blotting respectively. Neither *c-myc* mRNA nor protein expression is significantly reduced by 20  $\mu\text{M}$  of either of the control methylphosphonate-phosphodiester oligodeoxynucleotides. The number, structure and sequence of the compounds is detailed in Table 1 and Figure 1.

Control inverse antisense (reverse polarity antisense, **15**) and sense (**13**) oligomers were compared to the antisense congener (**3**) for their capacity to reduce *c-myc* mRNA and protein expression. High concentrations of oligomer (20  $\mu\text{M}$ ) were delivered using SLO-mediated transfection as a stringent test of sequence specificity. These data are presented in Figure 5C and show that *c-myc* mRNA was undetectable and protein expression reduced to  $7.6 \pm 0.2\%$  of control cells following treatment with the antisense effector. In contrast, neither of the control oligodeoxynucleotides induced substantial reduction of *c-myc* mRNA or protein expression. In this experiment control, SLO-only treated cells increased in number by  $108 \pm 34\%$  over a 24 h assay period. Cells treated with 20  $\mu\text{M}$  of the control oligomers



proliferated to a similar extent ( $91 \pm 11$  and  $97 \pm 8\%$  for the inverse antisense and sense, respectively) whereas treatment with the antisense effector again resulted in a proliferation block (increase in number:  $4 \pm 7\%$ ).

Taken together, data presented in Figures 4 and 5 show that chimeric methylphosphonate–phosphodiester oligodeoxynucleotides are highly efficient and specific antisense effectors. Efficient inhibition of *c-myc* gene expression was obtained in CML KYO-1 cells following SLO-reversible permeabilization in the presence of just  $0.2 \mu\text{M}$  antisense effector and the inhibition was maintained for a duration of at least 24 h. The inhibition of *c-myc* expression was sequence specific as determined by the absence of inhibition of non-target gene expression when  $0.2 \mu\text{M}$  antisense effector was used. Furthermore, even after cells were SLO-reversibly permeabilized to a 100-fold higher concentration than would be required for efficient antisense effects, control sequence chimeric methylphosphonate–phosphodiester oligodeoxynucleotides did not substantially reduce *c-myc* gene expression or inhibit cell proliferation.

## CONCLUSIONS

We have investigated an antisense cell culture system which is highly relevant to potential *ex vivo* purging of CML marrows. SLO transfection has previously been demonstrated to efficiently deliver oligonucleotides into CML cell lines (7,18) and primary cultures (35). In this work we have investigated the ability of different oligonucleotide compositions to efficiently and specifically suppress *c-myc* mRNA and protein expression following SLO-mediated delivery into the KYO-1 CML cell line.

Oligonucleotides composed of terminal methylphosphonate ‘wings’ and a central phosphodiester ‘gap’ were found to provide the most efficient and specific results. When chimeric molecules with this composition were used, antisense inhibition of *c-myc* gene expression was maintained for at least 24 h and this resulted in a sequence-specific inhibition of proliferation. Molecules with this general structure and targeted to *c-myc* may provide for a future *ex vivo* purging agent.

Chimeric molecules with 2′-methoxyethoxy or 2′-methoxy-triethoxy wings and a 2′-deoxy gap were less active in this SLO-delivery cell culture assay. The reduced activity may be related to the lesser ability of 2′-ribose modified chimeric molecules to direct the activity of RNase H (59), relative to parent 2′-deoxy compounds. Interestingly, increased phosphorothioate content in such compounds was found to correlate with markedly reduced antisense activity. This phenomenon may reflect the widely reported propensity of phosphorothioate oligonucleotides to interact with protein.

In contrast to the published results, we found that no sequence-specific antisense effect could be obtained with C5-propyne pyrimidine phosphorothioate oligodeoxynucleotides when delivered using SLO.

There is a substantial contrast between the documented efficiency of phosphorothioate 2′-ribose modified chimeric molecules and C5-propyne pyrimidine oligonucleotides, and what we report here. On the basis of the work presented here, we are unable to exclude the possibilities that (i) different target sites within the *c-myc* mRNA would demonstrate different intrinsic sensitivities to oligonucleotides with the various chemistries that were used. For example, poor antisense target sites involved in mRNA secondary and tertiary structure might be more amenable

to attack by 2′-methoxyethoxy chimeras than methylphosphonate chimeras as a result of the greater hybridisation potential of the former. In this case the order of relative efficacy that we have observed might well be reversed. Or that (ii) alternative delivery methods could result in a changed rank potency. For example, the oligonucleotide efficacy order that we obtained using SLO reversible permeabilisation would not be duplicated if cationic lipid delivery vehicles were used, with an appropriate cell line. We have observed aberrantly low cellular uptake of methylphosphonate–phosphodiester chimeric molecules when using a cationic lipid preparation, relative to phosphorothioate analogues with the same length and sequence, due to the reduced net negative charge on the chimeric oligonucleotide backbone (unpublished observations). On the other hand, identical rank potencies to those described here are obtained for phosphorothioate and chimeric methylphosphonodiester–phosphodiester oligodeoxynucleotides delivered by electroporation (unpublished observations). Furthermore, it is possible that different delivery methods may result in altered subcellular distribution and/or bioavailability of the oligonucleotides. This point has not been adequately addressed in the literature. We are performing an investigation into these factors following SLO, electroporation and cationic lipid-mediated delivery of fluorescein-tagged phosphorothioate oligomers into cells of leukaemic and non-leukaemic origin.

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