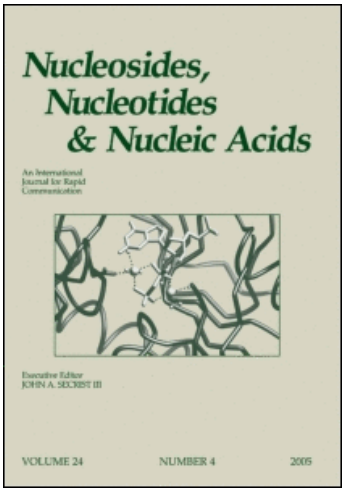

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ENHANCED ANTISENSE EFFECTS RESULTING FROM AN IMPROVED STREPTOLYSIN-O PROTOCOL FOR OLIGODEOXYNUCLEOTIDE DELIVERY INTO HUMAN LEUKAEMIA CELLS.

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Abstract. An improved protocol for delivering oligodeoxynucleotides into the cytoplasm and nucleus of human cells in culture using streptolysin O is described. The new procedure permitted reduced target protein expression to result from antisense suppression of target mRNA levels.

One of the fundamental problems associated with antisense technology is that of the introduction of oligodeoxynucleotides into the intracellular compartments of intact cells. The problem is particularly acute for human leukaemia cells which may not respond to cationic lipid delivery methods[1].

It has been reported that streptolysin O may be used to reversibly permeabilise human leukaemia cells to antisense effectors[2-5]. However, cells permeabilised to oligodeoxynucleotides in this way did not demonstrate reduced levels of the target protein following antisense inhibition of target mRNA expression. Furthermore, cells treated with streptolysin O under these conditions failed to respond normally to subsequent addition of dimethylsulfoxide (DMSO), which selectively inhibits the synthesis of c-*myc* mRNA and MYC protein[6,7], indicating a significant metabolic abnormality.

Here we report a significant improvement to the published streptolysin O "biochemical microinjection" protocol which allows antisense oligodeoxy-

nucleotide - dependent reduction in mRNA expression to result in reduced target protein levels.

RESULTS AND DISCUSSION

To investigate whether the reported antisense reduction of mRNA levels[3-5] resulted in suppressed protein expression, anti - c-*myc* oligodeoxynucleotides (Fig. 1) were introduced into human acute lymphoblastic leukaemia MOLT-4 cells using streptolysin O as previously described[2-5]. Briefly, exponentially growing MOLT-4 cells were washed and resuspended in permeabilisation buffer[9] ("PB", 137mM NaCl, 100mM piperazine-*N,N'*-bis[2-ethanesulfonic acid]; 1,4-piperazine diethanesulfonic acid, pH 7.4, 5.6mM glucose, 2.7mM KCl, 2.7mM EGTA, 0.05% BSA,) and oligodeoxynucleotides were added to the desired concentrations. Permeabilisation was achieved by addition of streptolysin O (3.5U/10^6 cells, dissolved in PB at 500U/ml) to the 100µl cell suspensions and incubation at 37°C for 20 minutes. Resealing was initiated by addition of 1ml of normal growth media (90% RPMI-1640, 10% foetal calf serum heat inactivated by treatment at 56°C for 30 minutes) and further incubation at 37°C.

Fig. 2 presents the results obtained when cultures of MOLT-4 cells, resuspended in PB, were permeabilised in the absence of oligodeoxynucleotide (3) or in the presence of 20 µM 5'F-MA383AS (3), MA14pAS-3'F (3), MD14pAS-3'F (5), ME14pAS-3'F (1) or 20 µM MA14pAS-3'F + 20 µM MD14pAS-3'F + 20 µM ME14pAS-3'F "Mixture 60 µM" (3), the number in brackets indicates the number of replicates. In an attempt to minimise unaffected cells and thereby maximise the antisense suppression of c-*myc* mRNA and MYC protein, relatively harsh permeabilisation conditions were selected: cells permeabilised and resealed 56.7% ± 3.6%, cells killed 35.5% ± 4.3%. Samples were taken 4 hours after the start of the experiment for analysis of c-*myc* mRNA by northern blotting and MYC protein by western blotting. Densitometrically determined RNA and protein levels are expressed as a percentage of their respective mean "No Oligo" values.

Relative to control cells permeabilised and resealed in the absence of oligodeoxynucleotide, all cells biochemically microinjected with antisense

Oligodeoxynucleotide structure and sequence	Code
3'.....T/A/C / G-G-G - G-A-G - T-T-G / C/A/A~F...5'	5'F-MA383AS
3'...F~T-A-C - G-G-G - G-A-G - T-T-G - C-A-A.....5'	MA14pAS-3'F
3'...F~A-T-G - G-G-A - G-A-G - T-T-G - C-T-G.....5'	MD14pAS-3'F
3'...F~C-T-C - G-G-G - G-A-C - C-A-C - G-A-G.....5'	ME14pAS-3'F
5'...F~A/T/G / C-C-C - C-T-C - A-A-C / G/T/T.....3'	5'F-MA383S
5'...F~T/A/C / G-G-G - G-A-G - T-T-G / C/A/A.....3'	5'F-MA383iAS
5'...F~A/C/A / G-G-T - A-G-T - G-G-G / A/T/C.....3'	5'F-MA383cNS

FIG. 1. Human c-*myc* oligodeoxynucleotide structures and sequences. A dash (-) between bases indicates the presence of a normal phosphodiester residue, whereas a slash (/) indicates a methylphosphonodiester internucleoside linkage. Fluorescein attachment to the 5' or 3' termini is indicated by F~ or ~F. The name of each oligodeoxynucleotide is shown to the right of the sequence and is derived from the structure and sequence as follows. The series is shown by the first two alphanumerics "MA", "MD" and "ME" indicating c-*myc* site A, D and E (positions 559 to 573, 1147 to 1161 and 1264 to 1278 respectively of HSMYC1, GenBank accession V00568). The next three alphanumerics indicate structure in the conventional 5' to 3' direction, "14p" has all 14 internucleoside bonds of the normal phosphodiester type, "383" has three methylphosphonates at each end and eight central phosphodiesters; the next one to three characters indicate actual sequence, "S" being sense "AS" antisense and "iAS" and "cNS" being inverse antisense and nonsense respectively. Finally, "5'F-" and "-3'F" denote fluorescein attachment to the 5' or 3' termini. The oligodeoxynucleotides were synthesised as previously described[8]. Fluorescein tagging of the 3' end was achieved by initiating synthesis from fluorescein derivatized CPG support.

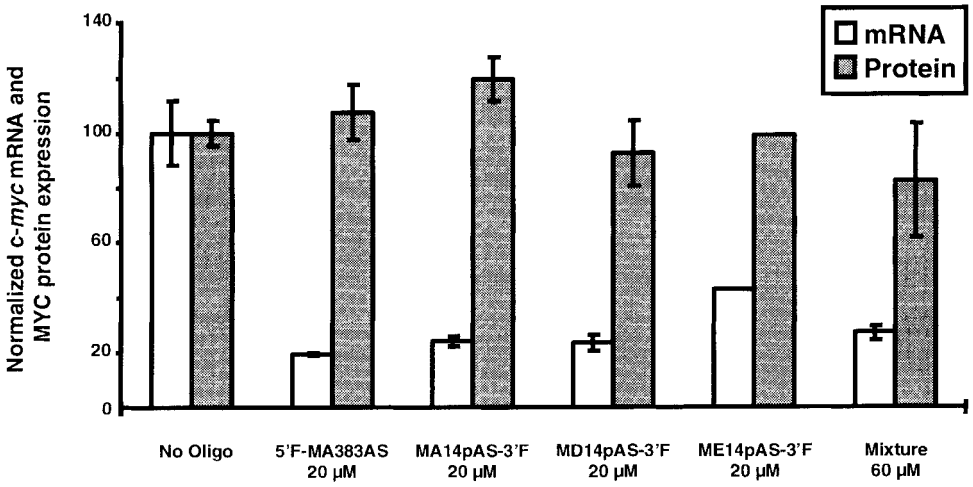

FIG. 2. Histogram showing mean ± standard deviation of c-*myc* mRNA and MYC protein expression in MOLT-4 cells permeabilised with streptolysin O in the absence and presence of antisense oligodeoxynucleotides.

oligodeoxynucleotides showed significantly reduced c-*myc* mRNA expression. No substantial difference was evident between cells treated with the A site chimeric "383" (5'F-MA383AS), the A site phosphodiester (MA14pAS-3'F) and the D (MD14pAS-3'F) site phosphodiester oligodeoxynucleotides (20% to 25% of control "No Oligo" cells), but the site E phosphodiester (ME14pAS-3'F) was less effective (43% of control "No Oligo" cells). Notably, a mixture of the three all - phosphodiester antisense effectors at 20 μM each (final oligomer concentration 60μM) was no more effective at reducing c-*myc* mRNA expression (27% of control "No Oligo" cells). than either 5'F-MA383AS, MA14pAS-3'F or MD14pAS-3'F alone at 20 μM.

The relative expression of MYC protein in these experimental cells was also determined and presented in Fig. 2. In contrast to the results obtained with c-*myc* mRNA, it may be clearly seen that in no case did streptolysin O-dependent introduction of antisense effectors result in MYC protein levels significantly lower than that found in control cells permeabilised in the absence of oligodeoxynucleotide. This was an unexpected finding because the expression of mRNA is rapidly reduced in experiments under these conditions (less than 2 hours to reach maximal antisense effect, data not shown). The c-*myc* target was selected for this study because MYC protein has been reported to have a half-life of *circa* 15 minutes[10]. It was confirmed that the MYC protein half-life in MOLT-4 cells was *circa* 15 to 30 minutes by independent treatments with 2% DMSO (inhibits transcriptional elongation of the c-*myc*[6,7]) and 20μg/ml cycloheximide (non-specific inhibitor of protein synthesis), data not shown. Thus, for samples removed 4 hours after the start of the experiment the level of c-*myc* message would have been reduced to *circa* 20% of that of the "No Oligo" control for at least 2 hours, which represents an estimated 4 MYC protein half-lives, without a concomitant reduction in MYC protein level.

In the light of these results it appeared likely that the process of streptolysin O permeabilisation was disturbing the cells physiology to the extent that protein turnover was halted for a period of time. This possibility was tested by administering 2% DMSO to MOLT-4 cells after streptolysin O reversible

permeabilisation. Control cells not treated with streptolysin O responded fully to the addition of DMSO: c-*myc* mRNA and MYC protein expression levels of 7.9% and 17.9%, respectively, of that found in the DMSO untreated cells. In contrast, MOLT-4 cells treated with streptolysin O showed no response to DMSO either in the expression of c-*myc* mRNA ("No DMSO" 110.0% and "DMSO" 126.3% of the control "no streptolysin O no DMSO" value) or MYC protein ("No DMSO" 52.1% and "DMSO" 47.7% of control). These results were taken to indicate that normal mRNA and protein turnover was interrupted in cells treated with streptolysin O. A degree of reduced MYC protein expression resulted from the streptolysin O treatment itself.

It was noticed that the permeabilisation buffer described by Barry *et al.*[9] and subsequently selected by us[2-5] was hypertonic compared to the intracellular environment. We therefore investigated whether permeabilisation in an isotonic buffer would compromise the cells physiology to a lesser extent and permit antisense inhibition of protein expression. MOLT-4 cells were washed and resuspended in serum free RPMI-1640 cell growth medium, then streptolysin O permeabilised (6.25U/10^6 cells, dissolved at 1000U/ml in Mg^{2+} / Ca^{2+} - free phosphate buffered saline, 10 minutes at 37°C) in the absence of oligodeoxynucleotide or in the presence of 20μM chimeric antisense (5'F-MA383AS) or 20μM chimeric control (5'F-MA383S, 5'F-MA383cNS, 5'F-MA383iAS) methylphosphonodiester - phosphodiester analogue structures. Resealing was achieved as before. Samples taken 2 hours after the start of the experiment and analysed by dual parameter flow cytometry demonstrated that the percentage of cells permeabilised / resealed and the percentage killed under the new conditions compared favourably with the optimum previously reported[2] (*ca.* 80% permeabilised and resealed, 10% dead). The antisense oligodeoxy-nucleotide treated cells were 92.6% ± 0.4% permeabilised and resealed with 1.9% ± 0.2% of the cells dead, similar values were obtained for control oligonucleotide treated cells. Cells treated with streptolysin O in the absence of oligodeoxynucleotide were found to be 97.2% ± 0.1% viable. The expression of c-*myc* mRNA and MYC protein two hours after the start of this experiment was analysed and the data presented in Fig. 3. Densitometrically determined RNA

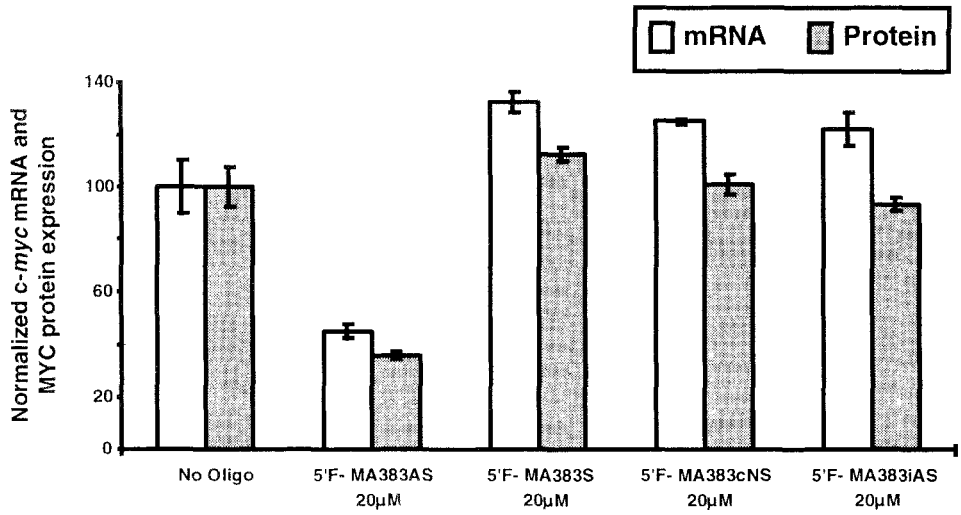

FIG. 3. Histogram showing mean ± standard deviation of c-*myc* mRNA and MYC protein expression in MOLT-4 cells resuspended in serum free RPMI-1640 permeabilised with streptolysin O in the absence and presence of antisense oligodeoxynucleotides.

and protein levels were corrected for loading by reference to the absolute number of viable cells in the flow cytometry sample and are expressed as a percentage of their respective mean "No Oligo" values. It may be seen that the expression of c-*myc* mRNA in MOLT-4 cells biochemically microinjected with 20μM 5'F-MA383AS was significantly reduced (45.2% ± 2.5% relative to the "No Oligo" control). Furthermore, in this experiment the reduction in mRNA correlated with significant reduction of MYC protein levels (36.2% ± 1.2% relative to control cells). Neither the sense, inverted antisense nor the nonsense control oligodeoxynucleotide analogues reduced the abundance of c-*myc* mRNA or MYC protein relative to the "No Oligo" samples. Not one of the methylphosphonodiester - phosphodiester "383" structure chimeric oligodeoxynucleotide analogues induced detectable inhibition of the expression of p53 or GAPDH mRNA or p53 protein controls (data not shown).

The proliferation of the cells in the experiment of Fig. 3 was assessed. The doubling times varied from *ca.* 21 hours (5'F-MA383S, 5'F-MA383iAS) through

22 hours (5'F-MA383AS, 5'F-MA383cNS) to *ca.* 23 hours (No Oligo), which are not significantly different from the typical exponential growth doubling times obtained with un - manipulated MOLT-4 cells (~22 hours). In addition, human leukaemia cells resuspended in RPMI-1640 prior to streptolysin O permeabilisation responded to subsequent treatment with DMSO or cycloheximide in an identical manner to control cells which were not permeabilised. Such data underlines the non - toxic nature of streptolysin O "biochemical microinjection" in serum free RPMI-1640 medium.

The duration of antisense inhibition of c-*myc* mRNA and MYC protein expression in MOLT-4 cells following streptolysin O - dependent introduction of oligodeoxynucleotide was investigated. MOLT-4 cells were washed and resuspended in RPMI-1640 and reversibly permeabilised with streptolysin O in the presence of 5'F-MA383AS or in the absence of oligodeoxynucleotide. Samples were removed at intervals for analysis by northern and western blotting and the densitometrically determined mRNA and protein expression data was processed as described for Fig 3. The time course presented in Fig. 4 shows that the chimeric methylphosphonodiester - phosphodiester antisense analogue, 5'F-MA383AS, induced maximal inhibition of c-*myc* mRNA expression at the one hour time point (c-*myc* mRNA levels 19.4% ± 1.8% of control "No Oligo" values) and maintained inhibition for up to seven hours after the start of the experiment (c-*myc* mRNA levels 74.4% ± 5.1% of control). Significant inhibition of MYC protein expression may again be seen to follow the introduction of 5'F-MA383AS, maximal inhibition occurred in the two hour time point, one hour after maximal mRNA inhibition, (MYC protein expression 16.6% ± 15.7% of control values) and inhibition of protein expression continued for a comparable time to suppression of mRNA level (7 hours, MYC protein expression 79.6% ± 20.6% of control values).

The apparent intracellular concentrations of 5'F-MA383AS were calculated from the flow cytometrically determined mean green fluorescence associated with the live cells for each of the time points in this experiment, as previously described[8]. We have experimentally determined that this measure correlates

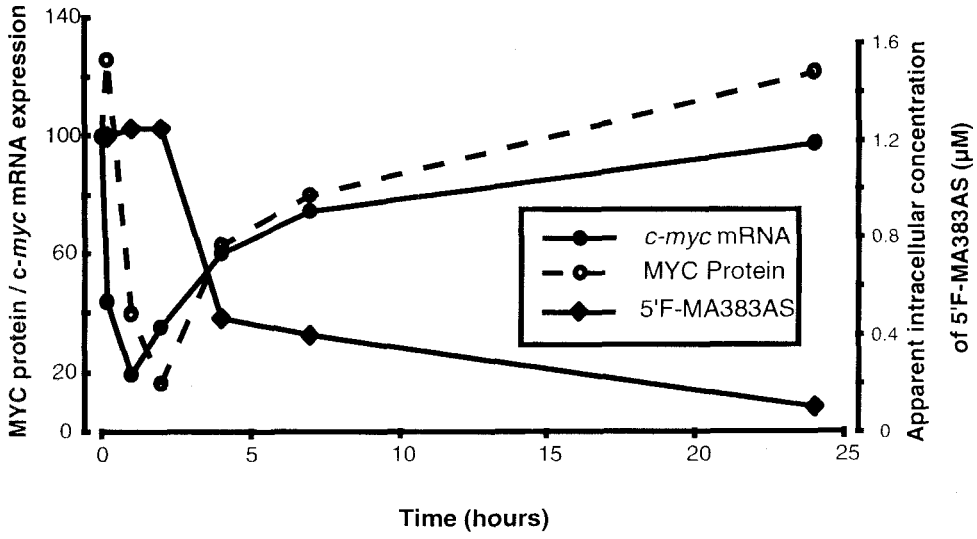

FIG. 4. Graph showing time course of c-*myc* mRNA and MYC protein expression in MOLT-4 cells following reversible permeabilisation by streptolysin O in the presence of 20μM 5'F-MA383AS (left hand ordinate) and flow - cytometrically determined apparent intracellular concentration of 5'F-MA383AS (right hand ordinate).

well with the amounts of intact oligodeoxynucleotide which may be extracted from the cells (assayed by gel electrophoresis, data not shown). It may be seen from Fig. 4 that loss of antisense suppression of c-*myc* mRNA and MYC protein coincides with a significant reduction in the apparent intracellular concentration of the antisense effector. Chimeric oligodeoxynucleotides with smaller central phosphodiester sections and phosphorothioate derived oligodeoxynucleotide analogues possess greater resistance to human cellular nucleases[4] and show much enhanced persistence under the assay conditions of Fig. 4 (data not shown). Taken together, these data suggest that the main reason for loss of antisense effects in cells permeabilised with chimeric "383" structure oligodeoxynucleotides was nucleolytic degradation of the antisense effector.

In summary, this report presents:

(i) an improved, non - toxic, protocol for introducing oligodeoxynucleotides into cells using streptolysin O, which may provide a means to manipulate gene expression in cell lines and in *ex vivo* bone marrow purges;

(ii) evidence that chimeric methylphosphonodiester - phosphodiester oligodeoxynucleotides act as highly specific antisense effectors;
(iii) evidence that oligodeoxynucleotides with greater resistance to cellular nucleases may be required for extended durations of antisense inhibition of gene expression.

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