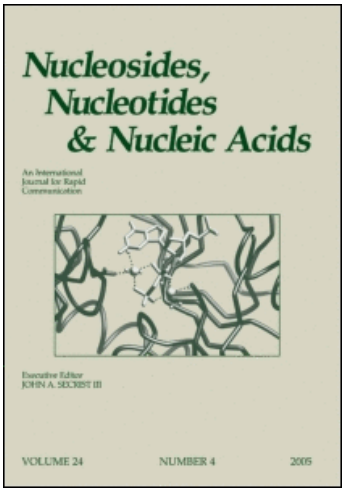

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IDENTIFICATION OF A GOOD C-*MYC* ANTISENSE OLIGODEOXYNUCLEOTIDE TARGET SITE AND THE INACTIVITY AT THIS SITE OF NOVEL NCH TRIPLET - TARGETING RIBOZYMES.

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ABSTRACT. A region of c-*myc* mRNA was identified which permitted very efficient antisense effects to be achieved in living cells using chimeric methylphosphonate – phosphodiester antisense effectors. Novel inosine – containing ribozymes (which cleave after NCH triplets) were directed to an ACA triplet within this region and delivered into living cells. No ribozyme intracellular activity could be identified. Very low ribozyme function was also observed in *in vitro* assays using a 1700nt substrate RNA.

There is great interest in developing new *ex vivo* purging strategies as potential treatments for Chronic Myeloid Leukaemia (CML). The oncogene c-*myc* may be a suitable target in such studies as it is thought to be required for BCR-ABL induced transformation[1,2] and MYC and BCR-ABL may co-operate to inhibit apoptosis by inducing expression of BCL2[3]. Furthermore, MYC protein has a short half-life[4] and antisense oligodeoxynucleotide - induced reduction of c-*myc* mRNA levels cause suppressed MYC protein expression which results in inhibition of CML cell proliferation[5].

We have previously shown that oligonucleotides targeting the translation initiation codon[6] and possessing chimeric methylphosphonate (PC) – phosphodiester (PO) structure induce only transient effects[7] even when introduced to cells by reversible cell membrane permeabilisation using streptolysin O[7] (SLO) from 20μM extracellular concentration. Consequently, a more efficient target site was sought. Two strategies were considered: empirical and theoretical.

Fig. 1 presents the results obtained from an *in vitro* ribonuclease H (RNase H) assay, wherein PO oligodeoxynucleotides, targeted to the c-*myc* translation initiation codon, were incubated with near full length (1700nt) c-*myc in vitro* transcript and low levels of *E. coli* RNase H. The ~1400nt and ~300nt RNA fragments, expected from oligodeoxynucleotide directed RNase H cleavage at the target site are indicated by the arrows to the right of the photograph. It may be seen from this figure that, in addition to the expected products, a number of unexpected cleavage fragments may also be observed (indicated by the arrows to the left of the photograph). The unexpected fragments result from RNase H scission of RNA sequences present in heteroduplexes with the antisense oligodeoxynucleotide which possessed only partial complementarity[8]. The c-*myc* mRNA sequence was scanned for regions of significant contiguous complementarity to the initiation codon – targeted oligodeoxynucleotide, selected results from this are presented in Fig. 2. Cleavage of the 1400nt expected product at the site marked as D would result in the upper and lower unexpected fragments of Fig. 1 (~800nt and 600nt respectively), whereas cleavage at the site marked as E would result in the middle unexpected fragment (~700nt). It was hypothesised that, because only partial heteroduplexes were able to support efficient cleavage by RNase H, these sites may possess a more accessible secondary structure and so would be better targets for antisense oligonucleotides.

The secondary structure of c-*myc* mRNA was also computer modelled using RNA structure 2.52[9] (IBM-PC program available from http://rna.chem.rochester.edu/) to calculate ten closely related most energetically favoured set of base interactions. The resultant structures were visualised using RNA draw 1.0[10] (IBM-PC program available from http://rnadraw.base8.se/). Fig. 3 presents a typical structure obtained from such calculations. Regions found to possess open – loop morphology in the majority of the modelled structures were considered to be potential antisense target sites. Fig. 3 is annotated with the translation initiation target site[6] (A), the two potential sites from the empirical data presented above (D and E) and four putative open – loop regions (F to I).

Oligodeoxynucleotides were synthesized complementary to the six potential target sites (D to I) marked on Fig. 3 and assayed for antisense efficacy in living cells following delivery by reversible cell membrane permeabilisation using streptolysin O[7] (SLO). One of these target sites (D) was found to support substantially more efficient antisense effects than the original (A) site. The data presented in Figure 4 shows that a

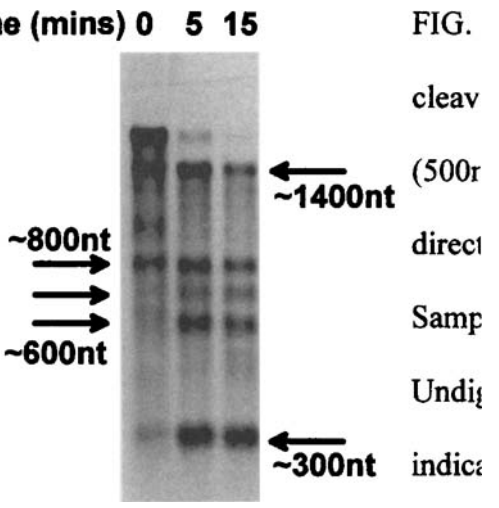

FIG. 1. Blot showing *E.coli* RNase H (0.025U/µl) cleavage of 1700nt *c-myc in vitro* transcribed RNA (500ng), directed by a 15-mer PO oligonucleotide directed to the translation initiation codon (1µM). Samples were removed at the indicated time. Undigested RNA is at the top of the gel, the arrows indicate cleavage products.


```
     559 ------> 573
5'...AUGCCCCUCAACGUU...3'   RNA
     ***************
A 3'...TACGGGGAGTTGCAA...5'   Oligo
Complementary at 15 sequential bases.
Complementary at 15 bases in total.

     1147 ----> 1161
5'...UACCCUCUCAACGAC...3'   RNA
        ** *******
D 3'...TACGGGGAGTTGCAA...5'   Oligo
Complementary at 7 sequential bases.
Complementary at 9 bases in total.

     1264 ----> 1278
5'...GAGCCCCUGGUGCUC...3'   RNA
       ******     *
E 3'...TACGGGGAGTTGCAA...5'   Oligo
Complementary at 6 sequential bases.
Complementary at 7 bases in total.
```

FIG. 2. Selected regions of complementarity between the c-*myc* translation codon targeted PO oligodeoxynucleotide and 1700nt c-*myc in vitro* transcribed RNA. Site A is the fully complementary target site. Sites D and E are regions of partial complementarity.

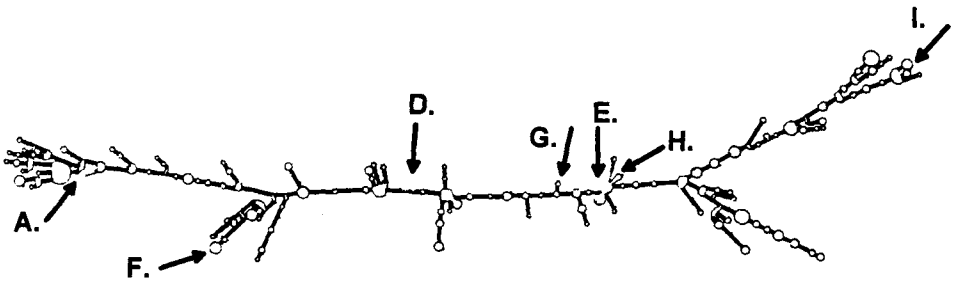

FIG. 3. Predicted c-*myc* 2121nt mRNA secondary structure. Indicated on the diagram are the two sites selected from empirical data (D and E) and the four sites selected from a number of closely related predicted minimum energy secondary structures (F to I).

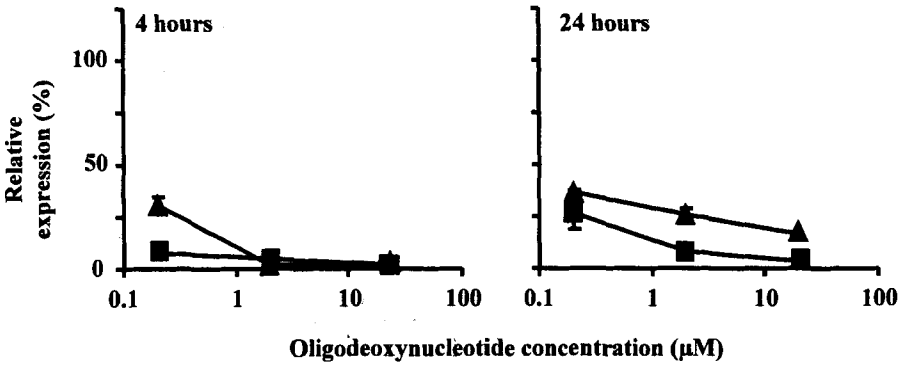

FIG. 4. Graphs showing the expression of c-*myc* mRNA (triangles) and MYC protein (squares) following SLO - mediated delivery of a chimeric PC - PO oligodeoxynucleotide targeted to the D site. Expression was normalised for cell number and expressed relative to that observed in SLO - permeabilised, No Oligonucleotide, controls. Efficient inhibition of gene expression lasting for at least 24 hours is obtained with 0.2μM of oligonucleotide.

fluorescently - labelled chimeric PC - PO oligonucleotide (5' Fluor...6PC:7PO:6PC...3') targeting the D site sequence (5'...UACCCUCUCAACGACAGCAG...3') induced antisense suppression of target c-*myc* mRNA and c-MYC protein expression for at least 24 hours following delivery from 0.2μM extracellular concentration. Control sense and nonsense chimeric oligodeoxynucleotides were found to not alter c-*myc* mRNA or protein expression, even when used at 20μM[5].

It was noticed that the newly selected D site contained an ACA triplet. Recently, a modified hammerhead ribozyme structure was described[11] which contains inosine in the active centre and cleaves NCH triplets (where N is any base and H is any base except G), hence expanding the range of sequences available to hammerhead ribozymes. The novel ribozyme structures were reported to have equivalent, or greater, single turnover rates than the classic form. For example, nuclease – resistant 2'-O-allyl - modified ribozymes possessed k_2 values of 0.30 and 0.17 (min^{-1}) for ACA and AUA containing oligoribonucleotide targets, respectively. We therefore wished to investigate the activity of such efficient ribozymes targeted to an apparently open loop region of c-*myc* mRNA, following delivery into cells.

Fig. 5. presents the inosine containing ribozyme sequence and structures used in this study. Pairs of active and inactive structures were obtained, which differed at the position marked on Fig. 5 with an asterisk. Active ribozymes (R1322, R1365 and R1397) contained ribo – G at this position whereas inactive control compounds (R1325, R1366 and R1398) contained 2' – O – allyl G. The three pairs of structures differed at the adjacent base, underlined in Fig 5, which was 2' – O – allyl U in R1322 / R1325, ribo – U in R1365 / R1366 and amino – U in R1397 / R1398.

The ribozymes and a D – site fluorescently labelled chimeric antisense oligodeoxynucleotide (AS ODN, 5' Fluor...5PC:9PO:5PC...3') were delivered into KYO1 cells using SLO reversible permeabilisation (SLO) and electroporation (EP)[12]. Duplicate treatments were performed as indicated on Fig. 6. Because of the larger volume required by the process of electroporation, relative to SLO permeabilisation, R1397 and R1398 were not delivered in this manner and only one AS ODN treatment could be performed due to limiting amounts of reagents. The effect on c-*myc* mRNA expression was examined by northern blotting 4 hours and 24 hours after delivery and photographs of the blots are presented in Fig. 6. The position of normal ~2400nt c-*myc* mRNA is

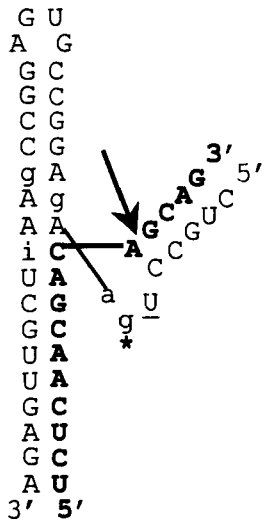

FIG. 5. Ribozyme structure shown hybridised to target c-*myc* mRNA. The characters in normal, upper case are 2'-O-allyl ribose residues in the ribozyme, the characters in normal lower case are ribose residues in the ribozyme. The "g" marked with an asterisk was replaced with a 2-O-allyl ribo G to generate inactive control compounds. Three structural variants of the ribozyme were obtained, differing at the underlined U position as described in the text. The characters in bold are mRNA sequence. The position of mRNA cleavage is indicated by the arrow.

indicated by the black arrows. The two 5' fragments resulting from cleavage of c-*myc* mRNA at target site D are indicated by the grey arrow. Two 5' cleavage fragments are observed because c-*myc* transcription is initiated from two promoters 174 base apart. It may be seen from this data that following neither delivery route nor at either time did any of the ribozymes, or inactive control compounds, reduce the expression of normal c-*myc* mRNA or induce the appearance of the expected cleavage fragments. In contrast, the AS ODN treated cells have substantially reduced c-*myc* mRNA expression at both time points (essentially ablated at 4 hours) following both delivery methods. Moreover, the expected cleavage products may be observed on the blots of RNA extracted from AS ODN treated cells 4 hours after both SLO and electroporative delivery.

It was found surprising that no ribozyme activity was observed following their delivery into cells: 2.5μM ribozyme was shown to induce scission of ~80% of 250nM

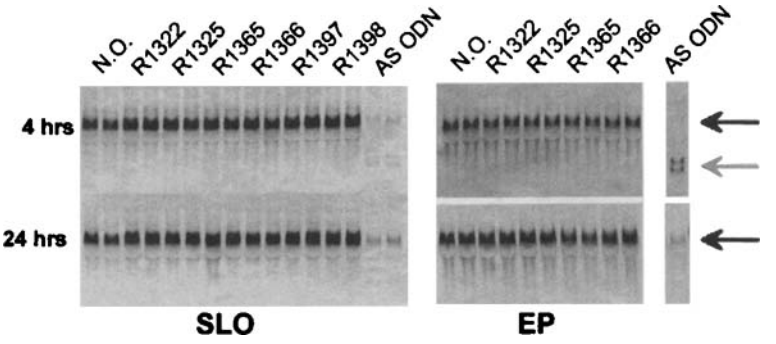

FIG. 6. Northern blots showing the effect on c-*myc* expression in KYO1 cells 4 hours and 24 hours after delivery of ribozymes (R1322, R1365 and R1397), inactive control ribozymes (R1325, R1366 and R1398) and an antisense oligodeoxynucleotide (AS ODN) using streptolysin O reversible permeabilisation (SLO) and electroporation (EP). No Oligonucleotide (N.O.) controls were included in both cases. Full length c-*myc* is indicated by the black arrows. RNA fragments resulting from cleavage of c-*myc* mRNA at the target site are indicated by the grey arrow. Only the AS ODN inhibited c-*myc* mRNA expression and produced the expected cleavage fragments.

oligoribonucleotide substrate within 20 minutes[11] and we reproducibly obtain intracellular oligonucleotide concentrations of this order following equivalent SLO delivery procedures. We therefore reproduced the experiments of Ludwig *et al*, except using *circa* 200nM of 1700nt *in vitro* transcribed c-*myc* RNA substrate, decreasing the concentration of ribozyme to 1μM and adding human placental ribonuclease inhibitor. The results of this experiment are presented in Fig. 7. It may be seen that each of the active ribozymes did cleave at the target site, scission products indicated with an arrow. However, the activity of these ribozymes was very low. Plots of ln[substrate concentration] against time were used to calculate the k_2 values to 0.013, 0.139 and 0.112 (hr^{-1}) for R1322, R1365 and R1397 respectively. Such values are less than 1% of the single turnover rates observed with oligoribonucleotide substrates[11].

CONCLUSIONS

C-*myc* mRNA has been the subject of previous attempts to identify optimal target sites from the predicted RNA secondary structure[13]. However, in that report no attempt was

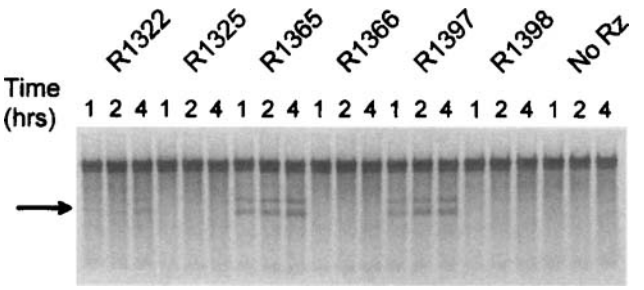

FIG. 7. Blot showing the time-course of *in vitro* scission of 1700nt *c-myc in vitro* transcribed RNA in the presence of 1μM ribozyme (R1322, R1365 and R1397) or inactive control compound (R1325, R1366 and R1398), or in the absence of added effector (No Rz). The position of the expected cleavage fragments are indicated by the arrow. Only the active ribozyme structures cleaved the substrate RNA to produce the expected fragments.

made to deliver the oligonucleotides into the cells and it was not clear that any correlation between activity and target secondary structure existed. In addition, because of the necessarily limited resolution at which RNA secondary structures are published, it is not possible to directly compare the structure obtained by Bacon and Wickstrom[13] to those which we obtained. Moreover, we had hoped that our more sophisticated approach of selecting target sites on the basis of the ten most favoured secondary structures would provide a method to identify accessible regions of RNA. However, empirical data, such as that presented in Fig. 1 appears to provide a better opportunity to identify accessible regions of mRNA than secondary structure calculations (Fig. 3).

Obtaining efficient antisense oligodeoxynucleotide suppression of gene expression using a particular target site does not imply that this site will also permit efficient ribozyme – mediated inhibition of gene expression, even if the target sequence contains a preferred triplet.

The last conclusion raises the question: "Why is a good antisense oligonucleotide target site not also a good ribozyme site?" We suggest that one reason may be the additional barrier to hybridisation caused by the extra bulk of ribozymes relative to antisense oligodeoxynucleotides. That is, ribozymes may require more accessible RNA

sequences than oligodeoxynucleotides. On this basis, we would predict that good ribozyme targets in full length mRNA would be good antisense oligodeoxynucleotide targets.

Another possible explanation for the lack of activity of these ribozymes within cells may be that certain ribozymes have been observed to display different activity *in vitro* and *in vivo*, possibly as the local conditions affect the structure of the ribozyme itself[14]. This explanation is not entirely in accord with the data we present here, in that the ribozymes we tested were also found to be relatively inactive *in vitro* with near full length target *in vitro* transcribed RNA.

EXPERIMENTAL

Ribozymes were generously supplied by Innovir GMBH (Rosdorf, Germany). Structures R1322, R1325, R1365 and R1366 were 5' fluorescently labelled using the FITC synthetic route. Phosphodiester and 5' fluorescein linked chimeric PC – PO oligodeoxynucleotides were synthesised and purified as previously described[15].

In vitro E. coli RNase H assays have been previously described[8]. *In vitro* ribozyme assays were performed as described[11] except using 1μM ribozyme, 200nM 1700nt *in vitro* transcribed c-*myc* RNA (transcribed using 9:1 ratio of UTP:fluos-UTP) and addition of Ribonuclease Block I (Stratagene, Cambridge, Cambridgeshire, UK) to 1U/μl.

Human CML KYO1 cells were maintained in exponential growth as previously described[5]. Intracellular delivery of oligo(deoxy)ribonucleotides into KYO1 cells was achieved by SLO reversible permeabilisation[7] or electroporation[12], as previously described.

Extraction of RNA from cells, formaldehyde gel electrophoresis, non – radioactive northern hybridisation and final chromogenic development of the blots was performed as described[5]. Extraction of protein from the cells, SDS-PAGE and western blot analysis has been similarly described[5].

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REFERENCES.

1. Sawyers, C.L.; Callahan, W.; Witte, O.N., *Cell*, **1992 70,** 901-910.

2. Afar, D.E.H.; Goga, A.; McLaughlin, J.; Witte, O.N.; Sawyers, C.L., *Science*, **1994 264**, 424-426.

3. Sanchez-Garcia, I.; Grutz, G. *Proc. Natl. Acad. Sci. USA*, **1995 92**, 5287-5291.

4. Spiller, D.G.; Giles, R.V.; Broughton, C.M.; Grzybowski, J.; Ruddell, C.J.; Tidd, D.M.; Clark, R.E., *Antisense & Nucl. Acid Drug Devel.*, **1998 8**, 281-293.

5. Giles, R.V.; Spiller, D.G.; Grzybowski, J.; Clark, R.E.; Nicklin, P.; Tidd, D.M., *Nucl. Acids Res.*, **1998 26**, 1567-1575.

6. Wickstrom, E.L.; Bacon, T.A.; Gonzalez, A.; Freeman, D.L.; Lyman, G.H.; Wickstrom, E., *Proc. Natl. Acad. Sci. USA*, **1988 85**, 1028-1032.

7. Giles, R.V.; Spiller, D.G.; Grzybowski, J.; Tidd, D.M., *Nucleosides & Nucleotides*, **1997 16**, 1155-1163.

8. Giles, R.V.; Tidd, D.M., *Nucl. Acids Res.*, **1992 20**, 763-770.

9. Mathews, D. H.; Andre, T. C.; Kim, J.; Turner, D. H.; Zuker M. In: *Molecular Modeling of Nucleic Acids. ACS Symposium Series 682.* Leontis, N.B.; SantaLucia, J.; (Eds.) American Chemical Society, Washington, D. C. **1998**, pp246-257.

10. Matzura, O.; Wennborg, A. *Computer Applications in the Biosciences (CABIOS)*, **1996 12**, 247-249.

11. Ludwig, J.; Blaschke, M.; Sproat, B.S., *Nucl. Acids Res.*, **1998 26**, 2279-2285.

12. Spiller, D.G.; Giles, R.V.; Grzybowski, J.; Tidd, D.M.; Clark, R.E., *Blood*, **1998 91**, 4738-4746.

13. Bacon, T.A.; Wickstrom, E. *Oncogene Res.*, **1991 6**, 13-19.

14. Domi, A.; Beaud, G.; Favre, A. *Biochimie*, **1996 78**, 654-662.

15. Spiller, D.G.; Tidd, D.M. *Anti-Cancer Drug Des.*, **1992 7**, 115-119.

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