



REVIEW

Antisense therapeutics in chronic myeloid leukaemia: the promise, the progress and the problems

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DNA sequences which are complementary or 'antisense' to a target mRNA can inhibit expression of that mRNA's protein product. Antisense therapeutics has therefore received attention for inhibiting oncogenes in haematological malignancy, in particular in chronic myeloid leukaemia. However, it is now becoming clear that antisense therapeutics is considerably more problematic than was naively initially assumed. In this article, some of these difficulties are discussed, together with the achievements in CML so far. Considerable further research is required in order to define an optimal antisense therapeutics strategy for clinical use. *Leukemia* (2000) 14, 347–355.

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Introduction

Over the past 15 years, haematological oncology has undergone a molecular revolution, driven by rapid technical progress in molecular biology.¹ The explosive growth in knowledge about the genetic mechanisms of neoplasia has undoubtedly led via the clinical laboratory to major improvements in patient care. Examples in present day practice include immunoglobulin and T cell receptor gene studies in the diagnosis of lymphoproliferative disorders, and the molecular monitoring of *BCR-ABL* transcript levels following allogeneic BMT for chronic myeloid leukaemia (CML). However, the impact of this new knowledge in designing novel therapeutic strategies has been less impressive. It is now clear that the therapeutic targeting of nucleic acids is considerably more problematic than was initially thought. At present it would not be difficult to argue that the therapeutic targeting of nucleic acids has yet to contribute anything to the outlook for patients with haematological malignancies. Yet behind the scenes, there has been considerable progress in understanding how nucleic acid therapeutics might work, as the technical difficulties involved are appreciated.

Several approaches are available for the therapeutic targeting of nucleic acids. Most current effort is focussing on targeting DNA, and this has become colloquially called 'gene therapy'. This can be achieved by the stable integration of new genetic material into the genome, which may then be passed on to the progeny of transected cells. This approach may be used not only to replace defective useful genes, but also to interfere with the effects of unwanted genes by replacing or augmenting a counteracting gene.^{2,3} DNA can also be targeted to inhibit gene expression at the transcriptional level by triple helix forming oligodeoxynucleotides and other techniques (reviewed in Ref. 4). In this setting there is no stable integration of genetic material into the genome, and thus no passing of effects on to progeny.

An alternative strategy is to use nucleic acids to modify gene expression at the translational level. By convention, this approach does not fall under the umbrella of 'gene therapy' since the target is messenger RNA (mRNA) rather than a gene. Short segments of DNA can be used to target the mRNA of a gene of interest; this is by convention referred to as 'antisense', because the base sequence of the DNA segment is complementary to the 'sense' target mRNA sequence. Clinical data are now beginning to emerge on the therapeutic application of antisense technology in the treatment of CML. The purpose of this review is to discuss these clinical data in the context of the underpinning science. An alternative mRNA targeting approach is the use of ribozymes, which are catalytic RNA molecules that cleave specific sequences in mRNA. The progress and problems facing ribozymes have been recently reviewed,^{5,6} and will not be discussed here.

Antisense theory

Zamecnik and Stephenson⁷ reported in 1978 that a 13-nucleotide strand of DNA that was complementary to an RNA sequence in the Rous sarcoma virus would bind to the viral RNA and inhibit replication. Since this initial report of synthetic oligodeoxyribonucleotides (ODN) blocking gene expression, it has become clear that antisense ODN can inhibit gene expression in a sequence-specific manner.⁸ The attraction of antisense therapeutics for altering gene expression is the high degree of specificity, without altering the expression of genes with closely related sequences. If leukaemic cells have a different pattern of gene expression to normal cells, antisense ODN might specifically inhibit expression of a leukaemia-specific gene, without any effect on wild type or other closely similar normal genes. Theoretical calculations indicate that a minimum of 12–15 bases is required to uniquely identify an RNA target within the mammalian genome.^{4,9}

The mechanisms whereby antisense DNA might perturb translation of its target mRNA have recently been reviewed.⁴ The inhibition arises not simply from physical blockage of ribosome access to the target mRNA, but because the DNA–RNA heteroduplex formed by hybridisation with antisense DNA is the substrate of the ubiquitous cellular enzyme ribonuclease H (RNase H). This enzyme cleaves only the RNA strand in DNA–RNA heteroduplexes, leaving the antisense DNA molecule intact, which can then proceed to target further RNA molecules.¹⁰

Many oncogenes relevant to haematological malignancy are created by translocation events, resulting in a mRNA unique to the malignant cell. Examples include *BCR-ABL* arising from the Philadelphia (Ph) t(9;22) translocation in CML, and *PML-RAR α* and other fusion genes which arise from specific translocations in acute leukaemias. A unique sequence

of bases is created across each of these transcript fusion junctions, which is not present in the wild-type counterpart of the contributing genes. ODN which is complementary to sequences spanning the fusion junction will hybridise over its full length, but only to a few bases of wild-type mRNA in normal cells. The DNA–RNA heteroduplex formed by full-length hybridisation in malignant cells is more stable than that formed in normal cells, and will target RNase H more efficiently. In theory, therefore, translation of the protein product of a fusion gene might be decreased to a greater degree than normal products, and thus the malignant phenotype might be modified.

Some translocations cause overexpression of a normal gene, rather than a novel fusion gene. Examples include *BCL-2* genes leading to overexpression of those proteins. Although it is theoretically possible to target the base sequence spanning the fusion junction in the same manner as for example *BCR-ABL*, this sequence is highly variable from one tumour to another. A more usual approach is therefore to target some portion of the overexpressed genes' mRNA, typically the initiation codon or critical sequences in the translated portion. A disadvantage of this approach is that complete ablation of genes such as *c-MYC* and *BCL-2* may have unpredictable unwanted effects on normal cells.

The problem of nucleases

A major problem for antisense ODN design is that all biological fluids contain substantial nuclease activity. The constituent nucleosides of 'natural' DNA and RNA are held together by phosphodiester (PO) linkages. Although PO-linked ODN will target RNase H well, they are rapidly degraded by nucleases to their constituent deoxynucleosides. Significant degradation of all PO-linked ODN may occur in a matter of a few minutes.¹¹ These deoxynucleoside degradation products may themselves be non-specifically toxic or growth inhibitory to a variety of haematological cells.^{12–15} 3' degradation products of *BCR-ABL* directed PS-linked and PO-linked ODN have been implicated as responsible for non-specific antiproliferative effects in CML cells.^{16,17} It is not clear if these observations apply to ODN with alternative linkage chemistry.¹⁸ ODN in which the constituent nucleosides are held together solely by PO linkages are therefore of no therapeutic potential.

Replacement of the oxygen atom of the acidic hydroxyl group of the ODN backbone yields structures that are more resistant to nuclease degradation. Substitution by a sulphur atom creates a phosphorothioate (PS) linkage, and many groups have extensively studied PS-linked ODN. An important advantage is that PS-linked ODN retain the ability to direct RNase H-mediated destruction of the target RNA. They may, however, bind non-specifically to a variety of intracellular molecules, including nucleic acid polymerases.¹⁹ In CML cells PS-linked ODN may modify expression of the *BCR-ABL* product, p210, by an aptameric effect.^{20,21} Furthermore, the stringency of hybridisation in living cells is difficult to control. Apparent positive results with PS-linked ODN may therefore be due to non-specific non-antisense effects.²² It is therefore possible that at least some of the biological effects claimed for PS-linked ODN in intact healthy cells were not achieved through a base-specific antisense mechanism.

Methylphosphonate (MP)-linked ODN analogues (in which the acidic hydroxyl of the PO linkage is replaced by a methyl group) were originally developed by Miller *et al.*²³ ODN in

which all internucleoside linkages are MP are totally resistant to both endo- and exonucleases. In contrast to PS-linked ODN, they do not bind non-specifically to intracellular protein. All MP-linked ODN have been used to target *BCR-ABL* mRNA, with a reported selective decrease in p210 protein levels and an antiproliferative effect.²⁴ However, they are very poorly soluble, exhibit low affinity for their target complementary RNA sequences, and target RNase H poorly.²⁵ The solubility of MP-linked ODN may be improved by the addition of a 3' tail of PO-linked nucleosides, but this may result in non-antisense inhibition of growth.²⁶

An alternative strategy for nuclease protection is to design so-called 'gapmers', in which a central PO-linked section is preserved, since this activates cellular RNase H particularly well. In order to confer nuclease resistance, this is flanked at both 3' and 5' ends by a section linked by nuclease-resistant internucleoside linkages. PS: PO chimaeric ODN are less likely to bind non-specifically to proteins, yet are more resistant to exonucleases than all-PO ODN. No studies of PS: PO chimaeric ODN have been carried out on CML cells, although we have reported that increasing the PS to PO ratio within 2'-ribose modified ODN markedly reduced antisense activity in the CML cell line KYO-1.²⁷ We have worked extensively with MP: PO chimaeric ODN structures, in which the MP-linked ends not only protect against exonuclease degradation but also modulate the stringency of hybridisation within cells (through the helix destabilising properties of MP linkages). The PO-linked central section directs RNase H destruction of the target mRNA, and also confers good solubility properties on the molecules.^{10,11,28}

Other non-antisense effects of ODN

Certain base sequences in ODN may produce cellular effects by a sequence specific but non-antisense mechanism. Four consecutive guanosine nucleosides may produce effects unrelated to the modulation of expression of any specific gene.^{29,30} If the dinucleoside motif CpG is present in the context of the hexameric palindromic sequence AACGTT, this may lead to interferon production and augmentation of natural killer function in T cells.³¹ Provided the cytosine residue is not methylated, this CpG motif may also lead to potent B cell proliferation and differentiation, and wholly PS-linked ODN elicit approximately 100-fold greater effect than all-PO-linked ODN, with MP: PO chimaeric ODN having very weak stimulatory effects.³² The mechanism whereby CpG ODN induces immune activation is not clear, but may be mediated via the transcription factor nuclear factor-kappa B, which subsequently leads to the induction of other genes involved in inflammation.³³ Immunostimulatory CpG motifs may enhance the efficacy of monoclonal antibody therapy of lymphoma.³⁴ A CpG motif within a MP: PO ODN of random sequence produces apoptosis of T cells though not other cell lineages, suggesting that some antisense effects reported on T cells may be non-specific.³⁵

Antisense effects are of limited duration

In the presence of cell extracts, wholly PO-linked ODN are substantially degraded after 5 min, and after 30 min no intact ODN is detectable by HPLC. In contrast, both PS-linked and chimaeric MP: PO-linked ODN remain intact after 30 min incubation.¹¹ However, effects are wearing off by 8–24 h after

introduction, and this is likely due to ODN degradation by intracellular nucleases.^{36,37} This time course may be adequate to inhibit the expression of proteins with a short half-life, and non-specific interactions with mRNA species whose protein products are more stable will be relatively unimportant. Conversely, for genes whose product has a long half-life, ODN of conventional structure may not exert an effect for sufficiently long enough to alter target protein levels.³⁷

The problem of cell uptake

ODN are very poorly transported into human cells. PO- and PS-linked ODN are polyanionic, and are unable to diffuse across the cell membrane. Uptake is by an energy-dependent process,³⁸ predominantly by fluid-phase pinocytosis.⁸ Internalised ODN therefore remain mostly confined within vacuoles, still separated by a membrane from their target mRNA in nucleus and cytoplasm.^{39,40} ODN may later be extruded intact from the cell, although some degradation and ODN chain length extension may also occur, the degree of which may vary between different cell types.^{19,41} It is not clear whether ODN uptake is receptor-mediated. Several cell-surface proteins have been identified which bind PO- and PS-linked ODN,⁴² although these may bind polyanions non-specifically. MP-linked ODN are not so strongly polyanionic, and there is evidence that they may enter by passive diffusion.²³ Subsequent work, however, suggests that uptake of MP: PO: MP chimaeric ODN is also largely energy-dependent, by adsorptive or fluid-phase pinocytosis.^{38,43}

Intact DNA molecules can undoubtedly achieve access to the cytosol following systemic administration, since small amounts of injected DNA may lead to specific expression of immunogenic proteins, an observation which underpins naked DNA vaccination strategies.⁴⁴ Some cell types may take up ODN more readily than others. Keratinocytes may take up ODN following simple topical application.^{45,46} ODN uptake into haematological cells is poor,⁴⁷ and leukaemic cells appear more resistant to uptake enhancement strategies.⁴⁸

Methods of enhancing cellular ODN uptake

Several modifications of ODN structure have been reported to enhance cellular uptake, with concomitant increase in antisense efficacy. These include introduction of a lipophilic dodecanol chain,⁴⁹ 5'-cholesterol,⁵⁰ and poly-L-lysine.⁵¹ However, the conjugation of certain lipophilic groups to the ODN backbone may result in increased cell membrane association without increasing the delivery of ODN to the site of target mRNA.⁵² ODN may be loaded into liposomal vesicles,^{53–55} or associated with cationic liposomes,⁴⁸ although not nanoparticles⁵⁶ or polyethylenimine.⁵⁷ ODN encapsulated in liposomes (although not unencapsulated ODN) will downregulate ICAM-1⁴⁸ and procollagen.⁵⁸ Liposomes have been used to deliver all MP-linked ODN targeting *BCR-ABL*²⁴ and alternative nuclease resistant structures targeting *BCL-2*.⁵⁹

Ex vivo electroporation of target cells has been reported to enhance ODN delivery, leading to a tumour-specific effect.⁶⁰ *BCR-ABL* directed ODN introduced into CML cells by electroporation downregulates *BCR-ABL* mRNA to a greater degree than in non-electroporated cells,⁵² and transient suppression of p210 expression has been reported.⁶¹ ODN have also been introduced into cells by retroviral strategies.^{62–65}

Our group has demonstrated that following the introduction

of ODN into leukaemic cells by reversible membrane permeabilisation with streptolysin O (SL-O), authentic antisense inhibition of gene expression can occur. SL-O will increase ODN uptake into CML cells by 50- to 100-fold, without any evidence of significant toxicity. On treatment with ODN directed against *BCR-ABL*, SL-O permeabilised CML cells showed significantly decreased *BCR-ABL* mRNA levels (unlike non-permeabilised cells). This antisense inhibition may be achieved by a variety of ODN structures, including all PO-linked, all PS-linked and chimaeric MP: PO structures.^{11,66,67} SL-O permeabilisation will also enhance ODN uptake into CML haemopoietic cell harvests, without undue toxicity.⁶⁸

In vitro data targeting specific genes

BCR-ABL

The Ph translocation juxtaposes the *ABL* gene on chromosome 9q34 *BCR* on chromosome 22q11. Although the genomic breakpoints on both chromosomes 9 and 22 may vary from patient to patient over several kilobases, they occur only in introns.⁶⁹ The abnormal 8.5 kb *BCR-ABL* transcript comprises a proximal *BCR*-derived portion ending with exon 2 (denoted by b2) or 3 (denoted by b3), fused to *ABL* exons 2 to 11 (denoted by a2).⁷⁰ Almost all Ph-positive CML patients express either b3a2 or b2a2 transcripts, depending on whether exon b3 is included. The great attraction of CML for antisense therapeutics is that only two alternative ODN sequences will target the *BCR-ABL* junction of almost all patients. There was therefore much initial optimism that antisense therapeutics might be of benefit in CML.⁷¹

ODN directed against the *BCR-ABL* junction were first reported in 1991 as inhibitory to the growth of CML cell lines.⁷² Several groups have subsequently reported that 18-26-mer ODN decrease *BCR-ABL* mRNA levels, p210 expression and the *in vitro* growth of CML cells and cell lines.^{22,73–76} Treatment of CML cell lines by ODN targeting *BCR-ABL* restores the sensitivity of CML cell lines to apoptosis inducing agents, and this has been taken as evidence that *BCR-ABL* may act at least in part by the suppression of normal apoptotic pathways.^{77,78}

However, most of these studies have used ODN linked by PS internucleoside linkages, and it is not clear if these ODN are acting by a sequence-specific antisense mechanism.^{22,26,74,79} Chimaeric MP: PO-linked ODN will specifically cleave a target mRNA sequence spanning the *BCR-ABL* junction in a cell-free system (where the problems of ODN uptake and intracellular localisation are removed).¹¹

The half-life of the p210 protein is in excess of 40 h.^{37,80} Whilst *BCR-ABL* mRNA can be effectively suppressed by antisense ODN, this effect wears off 8–24 h following exposure, and p210 levels are unaltered by a single ODN treatment analogous to the clinical *ex vivo* purging of CML cells.^{37,79} *BCR-ABL* may therefore be a particularly difficult oncogene to target with conventional ODN structures.

c-MYC

Translocations involving *c-MYC* are an important event in the malignant transformation of B cells. These juxtapose *c-MYC* to either the immunoglobulin heavy, or kappa or lambda light chain loci on chromosomes 14q32, 2p11 or 22p11, respectively. In T cell malignancy, translocations of *c-MYC* to the T

cell receptor α chain locus on 14q11 may occur. Each of these arrangements results in a deregulation of the transcription of *c-MYC* so that it is expressed constitutively at high levels.⁸¹ Gene re-arrangements affecting *c-MYC* have been identified in virtually all cases of Burkitts lymphoma,⁸² and the related L3 subtype of ALL. Translocations involving *c-MYC* are also present in many other high-grade lymphoproliferative disorders.^{83–87}

Although the *c-MYC* gene is not directly affected by the Ph translocation, there are several lines of evidence to suggest that *MYC* is of relevance to the maintenance of the leukaemic phenotype in CML cells. *MYC* function is required for *BCR-ABL*-induced transformation.^{88,89} *MYC* may cause activation of cyclinE/Cdk2 kinase, which may involve collaboration with *RAS*,⁹⁰ providing a molecular explanation for earlier experiments showing a co-operation between these two oncoproteins in malignant transformation.^{91,92} *RAS* in turn may be functionally overexpressed in CML, since the C-terminal of normal BCR binds GRB and SOS, and the resultant complex has *RAS* GAP activity, which is impaired in the *BCR-ABL* fusion protein. Thirdly, *MYC* and *BCR-ABL* may cooperate via the induction of BCL-2 to inhibit apoptosis and enhance tumorigenicity.⁹³ Therapeutic reduction in cellular *MYC* protein levels therefore has considerable potential.

MYC protein has a half-life of around 15 min,⁹⁴ and may therefore be more readily targetable than proteins with a long half-life such as *BCR-ABL*.^{37,80} ODN directed against the initiation codon of *c-MYC* will decrease expression of *c-MYC* mRNA and protein, and will inhibit the proliferation of cell lines overexpressing *MYC*.^{37,95–97} This effect is not durable, however, and control mRNA is also decreased,³⁷ possibly because the initiation codon antisense sequence contains both a G-tetrad sequence and also the hexamer palindromic sequence AACGTT, both of which may cause non-specific non-antisense effects.^{29,32} Targets elsewhere in the *c-MYC* coding sequence may be less susceptible to these non-specific effects, since chimaeric MP: PO ODN directed against bases 1147–1166 reduced *c-MYC* mRNA and protein expression to undetectable levels at 4 h, and less than 20% of control values even at 20 h.³⁷

In some Burkitts lymphoma cell lines, the chromosome 8 *c-MYC* breakpoint may lie downstream of the normally used *c-MYC* promoter sequence, and cause loss of the consensus splice sequence at the 5' end of the first intron. In these circumstances, intronic sequences are present in the mature mRNA transcript, and translation may be initiated from a cryptic promoter. ODN targeting this abnormal tumour-specific promoter lead to a profound drop in *MYC* protein levels, and inhibition of proliferation.⁹⁸

ODN as *ex vivo* purging agents

Autologous transplantation has become part of the routine management of many haematological malignancies. However, many patients relapse following the procedure. Gene marking studies suggest that contaminating tumour cells which are inadvertently re-infused with the graft may contribute to relapse in AML⁹⁹ and CML.¹⁰⁰ Accordingly, antisense ODN have been studied as agents for purging haematopoietic cell harvests prior to autologous transplantation. These studies have so far been confined to CML. *BCR-ABL* targeted ODN incubated with normal peripheral blood nucleated cell harvests do not affect the yield of CFU-GM.¹⁰¹ Of some concern for *ex vivo* antisense ODN purging strategies are reports

that primitive CML progenitors may possess the *BCR-ABL* translocation, yet not express *BCR-ABL* mRNA.^{102,103}

Four separate studies have examined *ex vivo* purging of CML grafts by antisense ODN, summarised in Table 1. None of these compared the clinical effects to those seen with unpurged grafts. Two of these are currently only reported in abstract form, and two used antisense ODN supplied by commercial organisations. Some studies treated entire harvests, while others treated CD34 selected harvests in order to limit reagent costs.

Eight patients (seven chronic, one advanced phase) have been transplanted using cells purged with PS-linked antisense ODN directed against *c-MYC*. The *MYC* product is a nuclear binding protein that controls the passage through G1/S phase of the cell cycle,¹⁰⁴ and it may play a critical role in haematopoietic cell development.^{105–107} Although expression is not restricted to leukaemic cells, leukaemic progenitors may be more susceptible to inhibition of *c-MYC* than normal progenitors *in vitro*,^{108,109} although normal progenitor growth is also suppressed.¹¹⁰ Four of six assessable patients had improvement in the proportion of Ph-positive metaphases following BMT.^{4,111}

Of eight cases of advanced disease autografted with cells purged by PS-linked 26-mer ODN directed against the *BCR-ABL* fusion junction, two cases showed a karyotypic response.¹¹² Three of four cases initially treated with harringtonine and ara-C and transplanted with ODN directed against *BCR-ABL* achieved some haematological benefit.¹¹³ We have reported three further cases (two in first chronic phase, one in accelerated phase) who underwent autografts with cells which had been purged with antisense ODN directed against *BCR-ABL*. Our purging technique used SL-O permeabilisation followed by chimaeric MP:PO ODN targeting the *BCR-ABL* junction. Two cases achieved lasting haematological benefit, although with cytogenetic evidence of persisting disease.¹¹⁴

A common theme of each of these reports is that engraftment may be delayed following antisense purged transplantation, although this does not appear to have resulted in significant adverse clinical problems over and above those expected following an unpurged autograft. Some patients, notably those with advanced disease, appear to have derived clinical benefit, though not cure, although it is very difficult to divorce the effect of purging from that achievable with an unpurged graft. It is not clear from the present data whether the ODN are exerting a true antisense effect. Parallel studies of *BCR-ABL* mRNA and protein levels on the present clinical purges would be helpful in resolving these questions, but are particularly difficult to perform because of the low numbers of spare purged cells available for scientific study. Favourable laboratory results following purging (for example on *in vitro* progenitor colony growth) may not correlate well with the clinical outcome.¹¹⁴ In view of the short duration of ODN effect, it may be over optimistic to expect a single ODN purge to produce long-term cures of CML following autografting.

In vivo ODN administration

There are as yet very few data on the *in vivo* effect of ODN in haematological malignancy. Skorski *et al*^{115,116} have investigated the effect of *in vivo* PS-linked ODN on the development of leukaemia in SCID mice injected with the CML cell line BV173, which expresses a b2a2 *BCR-ABL* transcript. Nine days of systemic b2a2-directed 26-mer delayed the development of fatal leukaemia by about 10 weeks, when compared

Table 1 Clinical results using antisense ODN purged autografts in CML

Ref.	Target	ODN linkages	Uptake enhancement	No. of CP:AP cases	Conditioning	Days to neutrophils >0.5: platelets >50 ×10 ⁹ /l	Cytogenetic outcome at day 90	Clinical outcome
4 and 111	c-MYB	PS	No	7:1	BuCy	Not reported: 7 of 8 cases engrafted	4 of 6 cases 85–100% Ph negative	?
112	BCR-ABL	PS	No	0:8	Bu/VP16	19–56: 21–105	2 of 8 cases Ph negative	7 in 2nd CP at 14+ months
113	BCR-ABL	PS	No	2:2	Cy/TBI	18–31: 24–68	2 of 3 cases >75% Ph negative	3 alive at 10+ months
114	BCR-ABL	MP:PO chimera	SL-O	2:1	Bu	25–51: 128–177	All Ph positive	2 of 3 in CP at 18+ months

Bu, busulphan; Cy, cyclophosphamide; TBI, total body irradiation; PS, phosphorothioate; MP, methylphosphonodiester; PO, phosphodiester; CP, chronic phase; AP, acute phase.

to a 6-base mismatched, sense or no ODN control. An 18-mer PS-linked ODN targeting the first 6 codons of *BCL-2* mRNA has been reported to specifically reduce *BCL-2* mRNA and protein expression, and to promote apoptosis and inhibit the growth of B cell lymphoma in a SCID mouse model.¹¹⁷ When administered intravenously to patients with low-grade non-Hodgkin lymphoma refractory to other therapy, reduction in lymph node size was seen in two of nine patients.¹¹⁸ A PS-linked 20-mer ODN targeting a part of exon 10 of p53 mRNA has been given to 16 patients with AML by continuous infusion without significant toxicity.¹¹⁹ The application of this approach to AML therapy is not immediately apparent, since although p53 levels initially fall, there is a subsequent four-fold increase in p53 level, and concern has been expressed about the specificity of this ODN.¹²⁰

The toxicity of ODN administered *in vivo* is not well studied at present. Inflammatory reactions at the site of infusion may occur,¹¹⁸ as may transient hyperglycaemia,^{118,119} and abnormalities of laboratory measures of liver function¹¹⁹ and blood clotting.¹²¹

Future developments

The targeting of multiple sites within an mRNA sequence may increase the antisense effect. Complete ablation of targeted mRNA could not be achieved by microinjection of a single antisense ODN species into *Xenopus* oocytes, and this may be because of 'resistant' mRNA molecules of differing secondary/tertiary structure.¹²² This resistance can be overcome by the introduction of ODN targeting different sites on the mRNA. It is therefore possible that greater antisense inhibition may be achieved by mixtures of ODN, complementary to different sites in the target mRNA. This is undoubtedly an important question if reducing gene expression below the threshold achievable with a single ODN would be required to elicit a cellular phenotypic response.

Helix stabilising chimaeric 2'-O-propyl PS oligoribonucleotide/PS oligodeoxynucleotide molecules introduced into cells by lipofection will remain intact for long periods, with prolonged antisense activity.^{123,124} Similarly, incorporation of helix stabilising C-5-propyne-pyrimidine analogues into PS-linked ODN enhances hybridisation potential and preserves RNase H targeting, with prolonged antisense activity in microinjected cells.¹²⁵ However, these chemically modified ODN are inefficient at entering cells.¹²⁶

Although high affinity ODN structures may have little to offer over conventional structures for the targeting of genes whose product has a short half-life,^{27,37} they could be of much greater relevance in improving the molecular effect of ODN targeting mRNA whose product has a long half-life, such as *BCR-ABL*.

The nucleic acid sugar-phosphate backbone can be completely replaced by a polyamide chain, to create polyamide nucleic acids (PNA), in which only the bases from the conventional nucleic acid prototype are preserved.^{127,128} PNA exhibit excellent resistance to nucleases, and hybridise strongly with complementary nucleic acid. Although PNA will not target RNase H, a gapmer containing an RNase H targeting section might be superior to conventional ODN structures for targeting genes with a long product half-life.

High affinity ODN may lead to some unexpected effects on mRNA splicing, and thus on the protein product. Morpholino oligonucleotide analogues possess an unusual structure, wherein the deoxyribose moiety of DNA is replaced with a 6-membered morpholino ring, and the charged PO internucleoside linkage is replaced by an uncharged phosphoramidate linkage.¹²⁹ These structures show excellent nuclease resistance, though do not recruit RNase H;¹³⁰ to be effective they need to target the translation initiation site. CML cells treated with morpholino analogues targeting the translation initiation site of *c-MYC* mRNA produced a 47 kDa truncated protein product, comprising the carboxyterminal portion of the intact *c-MYC* protein.¹³¹ It is likely that this effect arises because of recruitment of more distal cryptic start sites within mRNA. This effect might have clinical relevance if the resultant truncated protein product has altered biological effects or stability. An analogous effect may also be useful for correcting the abnormal splicing of beta-globin mRNA in beta-thalassaemia.^{132,133}

The fate of antisense ODN-treated haematopoietic cells after *in vivo* re-infusion is completely unknown. Cells in which there has been substantial antisense-mediated mRNA destruction may be more vulnerable to apoptotic and extracellular controls. If so, antisense ODN may not need to appreciably alter target protein expression; sudden cleavage of a target mRNA may be sufficient to ablate tumour cells, thus modifying the idea of antisense ODN as 'magic bullets'. Marking experiments to follow the fate of antisense purged transplants *in vivo* may therefore be of considerable help in developing antisense purging technology.

Conclusions

It is now becoming clear that antisense therapeutics is considerably more problematic than was naively initially assumed. The high expectations of the approach have yet to have a substantial impact on clinical practice, and this has led to a certain waning of clinical interest in antisense strategies. At the same time, there is abundant evidence that antisense ODN are effective *in vitro*, and may act in a highly specific way. Critical analysis of the molecular and cellular behaviour of antisense ODN indicates that the clinical strategies studied so far are sub-optimal, either because of unfavourable antisense chemistries, the wrong or kinetically unfavourable target, or the failure to achieve intracellular access. Considerable further basic research is required; an optimal antisense strategy is therefore some years away.

Does antisense therapeutics have a role in the current management of CML? The observation that patients with advanced CML or low-grade lymphoma may derive some clinical benefit is encouraging if scientifically surprising, and underlines our lack of knowledge about the way in which antisense ODN may be acting *in vivo*. The sparse clinical data currently available do not suggest that antisense ODN cause harm. At present, further observational clinical studies appear justified if conventional approaches are ineffective or inappropriate. The results of such studies may then in due course lead on to prospective randomised clinical trials to evaluate the role of antisense ODN in the management of a variety of haematological malignancies.

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