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European School of Oncology Task Force Papers: Gene Therapy—a Future in Cancer Management?

Antisense Therapy for Malignant Disease

S.G. O'Brien, M.A. Kirkland and J.M. Goldman

INTRODUCTION

THE POTENTIAL to 'switch off' the genes that are implicated in oncogenesis is clearly very attractive. This goal has been the incentive for many academic and commercial research groups to apply antisense technology to various malignant diseases. Over a period of at least 15 years of antisense experimentation, many successes have been claimed, although very few clinical applications have yet been developed. This, to some extent, reflects the many difficulties that are associated with the investigation of antisense oligomers in biological systems as well as the synthesis problems that have, until recently, limited the quantity of oligomer than can be synthesised. Advances in biotechnology have now enabled the synthesis of clinically useful quantities of various oligomers, and fuelled the hope that these molecules may have a future, not only in the investigation of gene expression, but also as therapeutic agents in malignancy, viral infection, vascular disease and inflammatory processes. However, the pharmacodynamics, cellular uptake, intracellular

transport and molecular interactions of synthetic oligonucleotides are complex and many aspects of these mechanisms are poorly understood. The purpose of this review is to convey a realistic and current appraisal of antisense technology as applied to malignant disease, exploring some of the exciting achievements to date as well as a number of problematic areas.

PRINCIPLES OF THE ANTISENSE APPROACH

Antisense (AS) oligomers are short synthetic sequences of DNA or RNA, usually between 15 to 30 bases. By designing an oligomer to be complementary or 'antisense' to a specific gene target sequence, it appears to be possible to inhibit transcription if DNA is targeted, or translation if mRNA is the target. The minimum number of bases that should theoretically define a unique human mRNA sequence is, on average, 13; for DNA the figure is 17 [1]. In principle, by designing AS oligomers that are longer than this, the genetic machinery of a cell can be specifically disrupted at various levels: transcription (so-called 'triple helix' interactions), pre-mRNA splicing, nuclear and cytoplasmic mRNA 'capping' and/or degradation and cytoplasmic translation. In order to be effective, AS oligomers need to remain stable both extra- and intracellularly, cross cell membranes, and be localised to their site of action.

Stability

Naturally occurring DNA, with a phosphodiester (PO)-linked 'backbone', is particularly sensitive to enzymatic degradation by the ubiquitous exonucleases found *in vivo* and in most *in vitro* model systems that contain serum. In order to overcome this problem of rapid degradation, a variety of synthetic nucleic acid modifications have been devised. Although most of these modified molecules do indeed exhibit enhanced nuclease resistance, other important features of the molecule, such as its Tm, solubility, structure and functional operating conditions, may be compromised.

Uptake

It now seems clear that AS oligomers enter eukaryotic cells in conditions of simple co-incubation, but at relatively low levels. The exact mechanisms by which oligomers gain entry to cells have yet to be elucidated in detail, but there is good evidence that both fluid phase pinocytosis and receptor-mediated endocytosis play a part at different concentrations. Data from an HL60 cell system [2] demonstrate that oligomers bind to specific receptors on these cells. Interestingly, there appear to be a range of different cell surface proteins that bind to different oligomers with phosphorothioate (PS) oligomers binding far more avidly than PO. Only a limited proportion of available extracellular oligomer will reach its intended site of action within the cell (the cytoplasm and nucleus) because the absorptive endocytic process is rapidly saturable, internalised oligomers are trapped and degraded within lysosomes [3], and there is a considerable efflux rate [2]. Efforts to enhance uptake have included conjugation with poly-L-lysine [4] and the use of various lipophilic compounds as carriers [5]. Liposomal packaging improves nuclease resistance as well as improving uptake, and this method of oligomer delivery may have an important role in the development of antisense therapeutics. However, the ideal oligomer design and method of delivery are still elusive.

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Another method to introduce longer RNA antisense sequences (and ribozymes – catalytic RNA molecules [6]) into cells has been the use of retroviral expression vectors. These vectors enable the integration of DNA into the host genome that encodes the continuous production of RNA antisense (or ribozyme) molecules. Whilst effective in those cells that are successfully transduced [7], the low efficiency of transduction limits the potential of this approach at the present time.

Mechanisms of action (*Figure* 1)

The simplest concept of antisense inhibition of gene expression involves the blocking of a cytoplasmic target mRNA sequence by an antisense oligomer. The interaction could simply block the normal action of the translation machinery, but there is also evidence that the RNA molecule of the RNA/DNA heteroduplex is cleaved by the RNAseH group of intracellular enzymes [8]. In this way, a single AS oligomer molecule may be able to act in a catalytic manner, directing the cleavage of several mRNA molecules. In the 'antigene' strategy, oligomers are designed to bind to a specific nuclear DNA sequence, forming a triple helix that may inhibit transcription of the target gene [1, 9]. However, the target sites are limited to homopurine/homopyrimidine sequences and the triplice helical structures can be unstable in certain conditions. Transcription may also be inhibited by synthetic DNA sequences corresponding to the transcription factor binding sites of specific promoters. It is not clear at this time which of these strategies—targeting mRNA, DNA or transcription factor binding sites—will prove to be the most successful, and, indeed, it is likely that different strategies will be required for different targets.

It is now evident that AS oligomers do not simply interact with other nucleic acid molecules. Non-specific interactions of polyanionic oligomers (such as PO and PS oligomers) with other cellular components, such as protein kinase C β1, CD4 and a lysosomal proton pump, as well as elements of HIV, such as gp 120, HIV-1 reverse transcriptase and other proteins [10], are also known to occur. Methylphosphonates do not display such non-specific binding characteristics to the same degree and are better taken up by cells, but are less soluble in physiological conditions than other forms.

Certain oligomer motifs are also known to be associated with non-sequence-specific interactions [11,12]. This potential for AS oligomers to induce biological effects which are not due to true AS interactions means that it is essential that AS experiments are rigorously designed and conducted before mechanistic claims can be made. This is an issue that is currently being actively debated amongst antisense researchers.

MOLECULAR TARGETS FOR ANTISENSE OLIGOMERS

Many targets have been evaluated in malignant cells [13]. Because of the operating limitations of the triple helix approach, most investigators are targeting mRNA in an attempt to inhibit translation. The usual target site is the AUG initiation codon of the target mRNA, but in some systems this has proved not to be the ideal site. Sometimes targeting the 5′ cap region, for example, has proved more fruitful.

Ideally, an antisense target gene should be one that is expressed only in malignant cells. Such a stringent requirement is, with a few notable exceptions, a luxury that cannot be fulfilled, and one is obliged to target genes which a malignant cell relies on for survival/proliferation more than a normal cell. Potential tumour-specific targets include fusion genes, such as

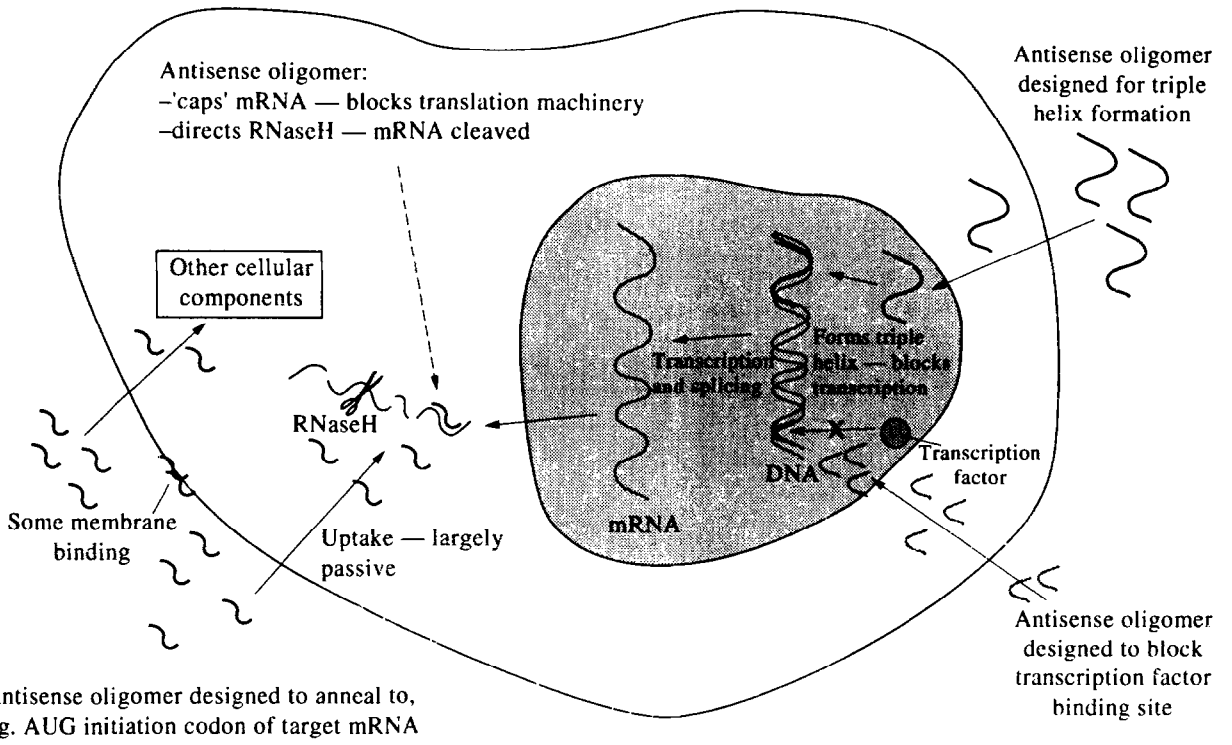

Figure 1. Schematic diagram of antisense mechanisms of action. Antisense oligomers can be designed to favour the type of interactions shown (see text for detail). The strategy in most common usage is to target mRNA—usually the initiation codon, but other sites within an mRNA molecule are also used.

BCR-ABL in CML and PML/RARα in acute promyelocytic leukaemia, and point mutations within genes, such as H-RAS and p53. In the latter case, however, it is necessary that the antisense oligomer be able to reliably distinguish mRNA species differing by a single base. This degree of spcificity has yet to be achieved in most experimental systems.

Other potential targets in malignant cells include amplified genes (such as ERB B-2/NEU in breast cancer), oncogenes which are overexpressed as a result of translocations (such as BCL-2 in follicular lymphomas or MYC in Burkitt's lymphoma), growth factor receptor genes or drug resistance genes (such as MDR-1). In addition, some normal genes may prove to be more essential for the survival of malignant cells than normal cells, as appears to be the case with MYB in acute and chronic myeloid leukaemia [14, 4].

It is important to bear in mind that the successful inhibition of a single gene using an antisense approach may still not be sufficient to make a major impact on a malignant cell. It is likely that in many malignant diseases more than one gene is important in conferring a survival/proliferation advantage. This may be a limitation of the antisense approach, but at the present time it is not possible to predict with confidence whether this will, in reality, be a major stumbling block.

EXPERIENCE WITH ANTISENSE OLIGOMERS IN MALIGNANT DISEASE

In vitro *studies*

At least 30 genes involved in oncogenesis have been the subject of antisense experiments using various cellular and cell-free *in vitro* experimental systems (for review, see [13, 15]). The chemistry, length and target sites of the oligomers used in these studies have varied considerable, and it is not always possible to safely conclude that the observed effects were true antisense effects. In our laboratory, we have, for the last 4 years, been investigating the use of AS oligomers in CML. The unique BCR-ABL fusion gene (formed as a result of t(9;22)), found in 95% of patients with this disease, provides a very attractive target for the use of AS oligomers, to specifically inhibit or possibly kill leukaemic cells. Following splicing, only two possible mRNA products are usually present—either B2A2 or B3A2. The junctional region of these two forms of mRNA constitutes the leukaemia-specific targets. However, we began this work by investigating the effect of antisense oligomers directed against the BCR initiation codon region [16], on the basis that this region of mRNA is often the most sensitive to antisense effects. It was found that 18mer PO oligomers complementary to the first coding exon, when introduced into K562 cells by electroporation, appeared to down regulate BCR-ABL and induce cell death, while an antisense directed against BCR codons 5 to 11 was ineffective. Martiat and colleagues [7] have extended these studies using retrovirally transduced antisense sequences directed against both upstream BCR and the BCR-ABL junction. Again using K562 as the target cell line, they found inhibition of cell growth with both of these antisense sequences, though cell death was not seen.

A number of other groups have targeted BCR-ABL junctional sequences using antisense oligomers. Szczylik and colleagues [17] reported inhibition of CML blast crisis cells by 18mer PO oligomers, and two groups have reported modest inhibition of CFU-GM from patients in chronic phase [18, 19]. Some CML cell lines, notably BV173 [20], have also shown sensitivity to AS. However, our own results, are less encouraging. We have been unable to demonstrate sequence-specific inhibition of chronic phase CFU-GM using either PO or PS oligomers [21]. We have also found that, while a number of CML cell lines are inhibited by AS oligomers, this inhibition is also not sequence-specific, in

that both B3A2 and B2A2 antisense oligomers are equally inhibitory in all lines, irrespective of the junctional sequence expressed by the cells. These results again emphasise that oligomers can cause biological effects which appear to be true AS effects but, on closer inspection, prove to be due to some other mechanism.

ANIMAL MODELS

In vivo test systems have been used to assess the biological effects of AS oligomers. These have mostly been murine models of haematological malignancies, although there is some experience with solid tumour models. An HTLV-1 encoded gene, *tax*, can be expressed in transgenic mice and causes the development of fibrosarcomas that express NF-κB-inducible early genes. Cell lines have been derived from such tumours and injected into syngeneic mice [22]. A 3′ PS modified PO AS oligomer directed against the initiation codon of NF-κB mRNA has brought about complete regression of the tumours that develop in such animals in all cases. Untreated mice, or those treated with sense control oligomers, died by 12 weeks in comparison to treated animals which had no recurrence for at least 5 months.

Gray and colleagues [23] transformed NIH-3T3 mouse fibroblasts with the activated c-Ha-*ras* oncogene from the T24 human bladder carcinoma cell line. They then pre-treated these cells with an AS oligomer complementary to a target in the 5′ flanking region of the c-Ha-*ras* RNA transcript before injecting them subcutaneously into athymic nude mice. Growth of tumours derived from these cells was significantly reduced for up to 14 days, when compared to control oligomer, and levels of cellular RAS p21 were reduced by more than 90% of the control.

In addition, an antisense K-*ras* retroviral construct has also been employed to prevent the growth of human lung cancer cells implanted orthotopically in nu/nu mice [24], and regional instillations of antisense N-*myc* have been observed to reduce neuroectodermal tumour mass in athymic mice by 50% [25].

Pocock and colleagues [26] have developed a SCID-hu B-cell lymphoma mouse model by the intravenous inoculation of the cell line DoHH2, derived from a patient with B-cell lymphoma bearing the t(14;18) translocation. They have employed a basic PO antisense molecule with one PS linkage at either end (to confer nuclease resistance), which was designed to be complementary to the first open reading frame of the BCL-2 gene. Their later work has also involved giving continuous infusions of oligomer over a 14-day period. By day 28, untreated and sense and non-sense treated mice had all developed lymphoma histologically, but the antisense treated group had not. BCL-2 is a very attractive model for an antisense approach. There is now good evidence that overexpression of this gene in t(14;18) inhibits the normal pathways of apoptosis, so prolonging the life of the malignant cell. Antisense inhibition of BCL-2 is, therefore, one of the situations where one might expect antisense treatment to exert a cytotoxic rather than cytostatic effect.

In other haematological malignancies, some interesting effects have been seen. Skorski and colleagues [27] have injected a mixture of normal bone marrow cells and CML cells into irradiated mice, following pre treatment with an AS oligomer directed against the BCR-ABL breakpoint, with or without mafosfamide. They detected normal but not leukaemic haemopoietic colonies of human origin in the marrow of immunodeficient mice 1 month after treatment. Another CML mouse model has been made by the inoculation of K562 cells into SCID mice [28]. In this system, it has been observed that mice treated with antisense oligomers directed against MYB survived approximately 3.5 times longer than controls. Interestingly, in this context, the addition of BCR-ABL AS oligomers did not confer any additional survival advantage.

CLINICAL TRIALS

Clinical investigation with human subjects using AS oligomers has only started in the last 18 months or so. As well as approved trials investigating the potential of MYB AS oligomers to prevent restenosis after angioplasty [29], trials have recently begun in malignant disease.

Initial clinical trials have concentrated on the treatment of haematological malignancies, in part because these have been most intensively investigated as targets for antisense therapy, and also because bone marrow is ideally suited for *ex vivo* manipulation. Trials of marrow purging in patients with CML prior to autologous transplantation have commenced in Rome (targeting BCR-ABL) and in Philadelphia (targeting MYB). Preliminary results show that patients treated with MYB-purged marrow have engrafted rapidly (A. Gewirtz, University of Pennsylvania), suggesting that toxicity may not prove to be a major limiting factor.

Workers in Omaha, Nebraska have been conducting a phase I clinical trial of systemic PS AS oligomers to p53 in relapsed and refractory AML [30]. This is still at an early stage of evaluation, but so far no adverse effects of *in vivo* oligomer administration have been encountered. It is probably too early to draw conclusions regarding clinical efficacy.

The treatment of solid tumours by AS will require *in vivo* delivery systems, and work remains to be done in this area. To our knowledge, no trials of AS oligomers in solid tumours have yet been started.

CONCLUSIONS

The modern revolution in molecular biology has allowed us to define numerous genetic defects in specific disease processes, both malignant and non-malignant. Conceptually, it would seem that the next generation of medical therapeutics should be designed to correct those specific defects, either by replacing missing genes or specifically altering the expression of defective genes. The current intense interest in 'gene therapy' broadly represents the replacement aspects of this therapeutic concept and antisense technology (which has also been labelled 'antigene therapy') offers the possibility of being able to specifically alter the expression of defective genes [31].

If one considers that the molecular genetic revolution, arguably started in 1953 with the first description of the structure of DNA, it is salutary to consider how few therapeutic advances have evolved from our vastly expanded understanding of biological molecular processes, in particular the molecular basis of oncogenesis. To date, neither gene therapy nor antisense therapy has fulfilled their clinical promise as methods of specifically inhibiting or killing cancer cells whilst leaving normal cells relatively unharmed. However, it is too early to dismiss these therapeutic approaches, as appropriate technology is advancing rapidly. There are currently several strands of ongoing research which may converge to allow these genetic therapeutic approaches to blossom. We must not deceive ourselves that effective gene-targeted therapies are imminent, but there is room for guarded optimism that antisense technology may enable significant advances in the development of more specific antineoplastic agents.

ANTISENSE USERS GROUP

The authors have recently founded an 'Antisense Users Group' as an open forum for discussion of the basic science, applications and problems of antisense technology. The intention is to hold 9–12-monthly meetings, the first meeting having been held in April 1994 near London. If any readers are interested in supporting this group please contact Dr O'Brien.

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